Abstracts of papers presented
at the 2010 meeting on

GENE EXPRESSION & SIGNALING IN THE IMMUNE SYSTEM

April 21–April 25, 2010

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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Arranged by

Doreen Cantrell, *University of Dundee, UK*
Sankar Ghosh, *Columbia University, College of P&S*
Dan Littman, *HHMI/Skirball Institute, NYU School of Medicine*
Mark Schlissel, *University of California, Berkeley*

Cold Spring Harbor Laboratory
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GENE EXPRESSION & SIGNALING IN THE IMMUNE SYSTEM
Wednesday, April 21 – Sunday, April 25, 2010

Wednesday	7:30 pm	**1** Stem Cells and Early Developmental Decisions
Thursday	9:00 am	**2** Regulation of Immune Cell Development
Thursday	2:00 pm	**3** Poster Session I
Thursday	4:30 pm	Wine and Cheese Party *
Thursday	7:30 pm	**4** Chromatin Structure and Epigenetics
Friday	9:00 am	**5** Antigen Receptor Gene Assembly and Modification
Friday	2:00 pm	**6** Poster Session II
Friday	7:30 pm	**7** Signaling
Saturday	9:00 am	**8** Regulation of Immune Cell Function I
Saturday	2:00 pm	**9** Regulation of Immune Cell Function II
Saturday	6:00 pm	Concert
	7:00 pm	Banquet
Sunday	9:00 am	**10** Innate Immunity

Poster sessions are located in *Bush Lecture Hall*
* *Airslie Lawn*, weather permitting

Mealtimes at Blackford Hall are as follows:
Breakfast 7:30 am-9:00 am
Lunch 11:30 am-1:30 pm
Dinner 5:30 pm-7:00 pm

Bar is open from 5:00 pm until late

Printed on 100% recycled paper.

PROGRAM

WEDNESDAY, April 21—7:30 PM

SESSION 1 STEM CELLS AND EARLY DEVELOPMENTAL DECISIONS

Chairperson: **S. Smale,** UCLA School of Medicine, Los Angeles, California

THURSDAY, April 22—9:00 AM

SESSION 2 REGULATION OF IMMUNE CELL DEVELOPMENT

Chairperson: **M. Schlissel,** University of California, Berkeley

SESSION 3 POSTER SESSION I

THURSDAY, April 22—4:30 PM

Wine and Cheese Party

THURSDAY, April 22—7:30 PM

SESSION 4 CHROMATIN STRUCTURE AND EPIGENETICS

Chairperson: **H. Singh,** Howard Hughes Medical Institute, University of Chicago, Illinois

FRIDAY, April 23—9:00 AM

SESSION 5 ANTIGEN RECEPTOR GENE ASSEMBLY AND MODIFICATION

Chairperson: **F. Alt,** Howard Hughes Medical Institute, Children's Hospital, Boston, Massachusetts

SESSION 6 POSTER SESSION II

SESSION 7 SIGNALING

Chairperson: **A. Weiss,** Howard Hughes Medical Institute, University of California, San Francisco

SATURDAY, April 24—9:00 AM

SESSION 8 REGULATION OF IMMUNE CELL FUNCTION I

Chairperson: **D. Littman,** Howard Hughes Medical Institute, New York University School of Medicine, New York

SATURDAY, April 24—2:00 PM

SESSION 9 REGULATION OF IMMUNE CELL FUNCTION II

Chairperson: **D. Cantrell,** University of Dundee, United Kingdom

Foxo control of T lymphocyte homeostasis and tolerance
Weiming Ouyang, Omar Beckett, Qian Ma, Ji-hye Paik, Ronald A. DePinho, Ming O. Li.
Presenter affiliation: Memorial Sloan-Kettering Cancer Center, New York, New York. 262

Alexander Rudensky.
Presenter affiliation: Memorial Sloan-Kettering Cancer Center, New York, New York.

The transcription factor Blimp1 regulates differentiation and function of effector regulatory T cells
Erika Cretney, Annie Xin, Frederick Masson, Gabrielle Belz, Stephen L. Nutt, Axel Kallies.
Presenter affiliation: The Walter and Eliza Hall Insitute, Parkville, Australia. 263

Mechanisms controlling Th2 conversion of Foxp3-expressing Treg cells *in vivo*
Yisong Wan, Yunqi Wang, Richard Flavell, Meinrad Busslinger.
Presenter affiliation: University of North Carolina at Chapel Hill, Chapel Hill, North Carolina. 264

SATURDAY, April 24—6:00 PM

CONCERT
Grace Auditorium

The Carducci String Quartet
Michelle Fleming, violin / **Matthew Denton,** violin / **Emma Denton,** cello / **Eoin Schmidt-Martin,** viola

The Carducci Quartet is recognised as one of today's most exciting young string quartets. Based in the UK, the quartet holds residencies at Trinity College of Music in London, Cardiff University and at the Cork School of Music in Ireland. The 2009-2010 season will see the quartet perform over 90 concerts worldwide, including performances at London's Wigmore Hall and Washington's Library of Congress. European tours include multiple stops in Spain, Portugal, Denmark, France and throughout the UK, including their own festival in Highnam. In 2009 the quartet will also embark on debut tours of South America and Australia as well as engagements in North America including New York, Philadelphia, Santa Rosa, Los Angeles and Eureka.

Winners of the Concert Artists Guild Competition in New York, the Kuhmo International Chamber Music Competition and major prizes at the Bordeaux, London and Osaka competitions, the Anglo-Irish Carducci quartet has established an enthusiastic international following. The Quartet was short-listed for the 2008 Royal Philharmonic Society Chamber Music Award and has recently collaborated with such internationally renowned musicians as Nicholas Daniel, Julius Drake, Graham Oppenheimer, Charles Owen, Kazuki Sawa, Julian Bliss, Simon Rowland-Jones, James Gilchrist and Patricia Rosario. Other highlights include appearances at Carnegie Hall, the Verbier, West Cork and Wratislavia Cantans Festivals, an Aldeburgh residency, broadcasts for BBC Radio 3, RTE Lyric FM and BBC television, a cycle of the complete Bartok quartets and a Naxos recording of the Philip Glass quartets.

They have received wide critical acclaim for recordings on their own record label 'Carducci Classics', launched with a CD of Haydn String Quartets. Two world premiere recordings featuring C20th works by G. Whettam ('recording of the month' MusicWeb International) and J. Horovitz ('beautifully crafted works…excellent performances', BBC Music Magazine) were released in 2008. The complete quartets by Irish composer Brian Boydell was released this year, presented with funding from the Music Network/ Arts Council of Ireland Recording Scheme. The Carduccis have also recorded (Vivaldi and Piazzolla) with the Katona Twins Guitar Duo for Channel Classics.

The quartet has gained an enviable reputation for their performances of contemporary works and this season, the quartet will premiere new commissions from David Matthews, Adrian Williams, Simon Rowland Jones and Huang Ruo and will perform in the 'New Music, New Places' series, New York.They have also performed to great acclaim the works of Peter Maxwell-Davies, Anthony Powers, Michael Zev-Gordon and Joe Duddell, amongst others. The quartet has embraced collaborations with other disciplines, working alongside the Henri Oguike and Random Dance companies and with the renowned Sarod player Wajahat Kahn.

The Carducci Quartet's international engagements have taken them to the USA, Japan and throughout Europe, including Italy, where after performing numerous concerts at the Castagnetto-Carducci Festival in 1997 the quartet adopted the name "Carducci" with the blessing of the Mayor. The quartet studied with members of the Amadeus, Alban Berg, Chilingirian, Takacs and Vanbrugh quartets and, as part of the ProQuartet professional training programme in France, studied with Gyorgy Kurtag, Walter Levin and Paul Katz.

The quartet are passionate about taking classical music to the next generation and run chamber music courses for young musicians in the UK, France and Ireland. Their educational work continues with performances for school children supported by their own trust, The Carducci Music Trust, the Cavatina Chamber Music Trust and West Cork Music. They also regularly coach on the National Youth String Quartet Weekend, run by the London String Quartet Foundation at Chetham's School of Music. The Carducci Quartet gratefully acknowledges the support of the Coln Trust.

SATURDAY, April 24

BANQUET

Cocktails 7:00 PM Dinner 7:45 PM

SESSION 10 INNATE IMMUNITY

Chairperson: **S. Ghosh,** Columbia University College of Physicians & Surgeons, New York, New York

REGULATION OF HEMATOPOIETIC STEM CELL DIFFERENTIATION AND TRANSFORMATION BY THE UBIQUITIN SYSTEM

Linsey Reavie, Jie Gao, Shannon Buckley, Kelly Crusio, Iannis Aifantis

Howard Hughes Medical Institute and NYU School of Medicine, Department of Pathology, 550, 1st Avenue, New York, NY, 10016

Differentiation and self-renewal of hematopoietic stem cells (HSC) is controlled by an orchestrated integration of bone marrow environmental cues. Although much is known about the transcriptional control of this process, recent reports propose that post-translational regulation, including ubiquitination could also control stem cell differentiation. To test this hypothesis we visualize in vivo, using a novel reporter mouse strain, the ubiquitin-regulated protein stability of a single transcription factor, c-Myc. We demonstrate that the relative stability of c-Myc protein is instructive of HSC quiescence and self-renewal. These thresholds of c-Myc protein abundance are controlled by the E3 ubiquitin ligase Fbw7. We have previously shown that Fbw7 is a potent tumor suppressor acting on hematopoietic progenitors in acute lymphoblastic leukemia. Moreover, we demonstrate that the HSC "gene expression signature" is controlled by fine changes in relative stability of the c-Myc protein controlled by the activity of Fbw7. Furthermore, using whole genome genomic approaches, we pinpoint specific genes-regulators of HSC function that are directly controlled by c-Myc promoter binding. Finally, we demonstrate that regulation of self-renewal by c-Myc protein availability is a specific feature of adult HSC. Indeed, self-renewing embryonic stem cells express high levels of c-Myc protein that is only destabilized as cells differentiate due to the activity of the Fbw7 ligase. These studies offer the first example of a ubiquitin ligase:substrate pair able to orchestrate the molecular program of adult stem cell differentiation.

P53-MEDIATED HEMATOPOIETIC STEM CELL COMPETITION

Ruslan Medzhitov

HHMI, Yale University Medical School, Immunobiology, 300 Cedar Street, New Haven, CT, 06510

Cell competition was originally described in Drosophila as a process for selection of the fittest cells. It is likely to play an important role in tissue homeostasis in all metazoans, but little is known about its role and regulation in mammals. Using genetic mosaic mouse models and bone marrow chimeras, we describe here a form of cell competition in the hematopietic stem cell compartment. This competition is controlled by p53 but is distinct from the classical p53-mediated DNA damage response: it persists for months, is specific to the hematopoietic stem and progenitor cells, and depends on the relative rather than absolute level of p53 in competing cells. The competition appears to be mediated by a non-cell autonomous induction of growth arrest and senescence-related gene expression in outcompeted cells with higher p53 activity. p53-mediated cell competition of this type could potentially contribute to the clonal expansion of incipient cancer cells

NKAP IS REQUIRED FOR ADULT HSC MAINTENANCE AND SURVIVAL

Anthony Pajerowski[1,2], Michael Shapiro[1], Kim Gwin[1], Rhianna Sundsbak[1], Molly Nelson-Holte[1], Kay Medina[1], Virginia Shapiro[1]

[1]University of Pennsylvania, Pathology and Laboratory Medicine, 3600 Spruce Street, Philadelphia, PA, 19104, [2]Mayo Clinic, Immunology, 200 First St SW, Rochester, MN, 55905

Steady state hematopoiesis is sustained through differentiation balanced with proliferation and self-renewal of hematopoietic stem cells (HSCs). Disruption this balance can lead to the loss of HSCs and hematopoietic failure. NKAP is a transcriptional repressor, and conditional ablation of NKAP in the T cell lineage using lck-cre demonstrated that NKAP expression is absolutely required for early T cell development. We now show that NKAP is also absolutely required for the maintenance and survival of adult HSCs. Using vav-cre NKAP conditional knockout mice, which express cre in HSCs, we found that loss of NKAP leads to perinatal lethality and abrogation of hematopoiesis. Mx1-cre NKAP conditional knockout mice die 9-14 days after induction of the cre transgene with poly-IC, at which point hematopoiesis in the bone marrow has halted and HSCs (defined by lin-sca+c-kit+) have disappeared. The hematopoietic failure and lethality is cell intrinsic, as irradiated wild-type recipients reconstituted with Mx1-cre NKAP conditional knockout bone marrow also succumb with a similar time course. Even in the context of a completely normal bone marrow environment, using mixed bone marrow chimeras in which irradiated wild-type recipients were reconstituted with a 50/50 mix of wild-type and Mx1-cre NKAP conditional knockout bone marrow, Mx1-cre NKAP conditional knockout HSCs fail to sustain granulopoiesis 6 days after poly-IC induction. To understand the basis of the HSC defect, mixed chimeras were induced with a single injection of poly-IC and examined four days later. Mx1-cre NKAP conditional knockout HSCs exhibited defects in proliferation as measured by BrdU incorporation, and survival measured by Annexin V staining. Using Q-PCR, NKAP-deficient HSCs upregulate the cyclin-dependent kinase inhibitors p21 Cip1/Waf1 and p19 Ink4d, which may account for the decreased proliferation that was observed. Therefore, NKAP is one of a very small number of transcriptional regulators that is absolutely required for adult HSC maintenance and survival.

REPROGRAMMING OF T CELLS TO NATURAL KILLER-LIKE CELLS UPON BCL11B DELETION

Peng Li[1], Shannon A Burke[1,2], Juexuan Wang[1], Xiongfeng Chen[3], Dong Lu[1], Gordon Dougan[1], Brian Huntly[4], Bertie Gottgens[4], Nancy A Jenkins[5], Neal G Copeland[5], Francesco Colucci[2], Pentao Liu[1]
[1]Wellcome Trust Sanger Institute, Mouse Cancer Genetics, Hinxton, Cambridge, CB10 1HH, United Kingdom, [2]The Babraham Institute, Laboratory of Lymphocyte Signalling and DevelopmentThe Babraham Institute, Babraham, Cambridge, CB22 3AT, United Kingdom, [3]National Cancer Institute-Frederick, SAIC-Frederick, Frederick, Frederick, MD, 21701, [4] Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Hills Road, Cambridge, CB2 0XY, United Kingdom, [5]Institute of Molecular and Cell Biology, Institute of Molecular and Cell Biology, Biopolis Drive, Singapore , 138673, Singapore

T cells develop in the thymus and play critical roles in immunity. Interestingly, no single gene has been identified as essential for the maintenance of thymocytes and mature T cells. In mice, the transcription factor BCL11B plays important roles in fetal thymocytes, double-positive thymocyte selection, and it is dramatically upregulated in early T cells. Using a Bcl11b reporter mouse, we show that Bcl11b is expressed in T cells from DN1 thymocytes to mature T cells and absent from other leukocytes. To examine the function of Bcl11b in maintaining T cell identity, we generated conditional Bcl11b KO (flox) mice which, when crossed to the Cre-ERT2 strain, allows deletion of Bcl11b upon Tomoxifen (OHT) exposure. Strikingly, treatment of flox/flox mice with OHT reduced thymocyte cellularity 5-10-fold compared to flox/+ controls, coincident with an increase in cells expressing NK cell markers, including NKp46 and NK1.1, in the thymus and spleen. When Bcl11b was deleted in vitro from sorted DN1, DN2 or T cell-committed DN3 thymocytes and exposed to T cell differentiation conditions in the OP9-DL1 system, T cell development failed and CD3-/TCRβ- NKp46+ and NK1.1+ cells grew out. Interestingly, these cells were highly cytotoxic towards the underlying OP9 stroma and NK cell tumor target lines, suggesting a reprogramming from T cell developmental pathways to that of NK cells. In single-cell assays with DN3 thymocytes, reprogramming was 100% efficient upon removal of both Bcl11b alleles. Microarray analysis showed these 'Induced T-to-Natural Killer' (ITNK) cells had an NK cell genetic profile and decreased T cell gene expression. They were not simply the outgrowth of NK cells as they exhibited TCRβ V(D)J recombination, confirming a thymocyte origin. Bcl11b was also required for identity maintenance in mature T cells, as NKp46+ ITNKs grew out from sorted DP thymocytes and mature CD8+ splenocytes T cells following OHT treatment. These populations retained TCRβ expression, suggesting 'transdifferentiation' to ITNKs. Reprogramming is cell intrinsic not only in vitro but also in vivo as ITNKs were present in the spleen 6 weeks after transfer of Bcl11b-deleted DP thymocytes to alymphoid mice. Remarkably, these ITNKs were able to prevent the outgrowth of B16 melanoma in vivo compared to controls. ChIP analysis using antibodies to CSL/RBP-Jκ confirmed that the canonical Notch signalling directly regulates Bcl11b transcription level. In summary, we show here that Bcl11b is essential for T cell development and currently the only known transcription factor for the maintenance of T cell identity. Moreover, ITNK may prove to be beneficial in therapeutic cancer treatment.

SELECTIVE FUNCTIONS OF THE MODULATORY DNA-BINDING ZINC FINGERS OF IKAROS

Hilde Schjerven[1], Sarah Wadsworth[1], Steven Bensinger[2], Stephen T Smale[1]

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The C2H2 zinc finger is one of the most prevalent protein motifs in the mammalian proteome, with many C2H2 fingers involved in sequence-specific DNA binding. Most zinc finger proteins contain multiple tandem finger motifs, with each motif recognizing 2, 3, or 4 base-pairs. The multiple fingers within a given protein must therefore exist either for the purpose of highly specific recognition of long DNA sequences, or to allow recognition of a variety of shorter DNA sequences using distinct subsets of the zinc fingers. Biochemical studies of protein-DNA interactions by zinc finger proteins have been consistent with both possibilities. We have explored this issue in a biological context through targeted mutagenesis of the Ikzf1 gene, which encodes the Ikaros DNA-binding protein. Ikaros is a critical regulator of hematopoiesis and functions as a potent tumor suppressor in both mice and humans. Full-length Ikaros contains four zinc fingers near its N-terminus that are involved in sequence-specific DNA-binding and two additional fingers at its C-terminus that support dimerization and multimerization. Within the DNA-binding domain, fingers 2 and 3 are essential for all DNA-binding activity through their recognition of the core Ikaros consensus sequence, GGGAA. In contrast, fingers 1 and 4 do not appear to recognize the core consensus, but instead are important for binding to specific subsets of DNA motifs, presumably through contacts with base-pairs that flank the core consensus. To examine the mechanistic and biological significance of these modulatory zinc fingers, we have created mouse strains that lack the exons encoding either finger 1 or finger 4. Strikingly, the two mutant strains exhibit distinct subsets of the phenotypes observed with Ikaros null mice, suggesting that the two modulatory fingers support the regulation of distinct sets of target genes and even distinct biological functions. These findings and a detailed analysis of putative target genes help explain why hundreds of C2H2 zinc finger proteins may contain multiple tandem finger motifs. Moreover, the results suggest a refined strategy for uncovering the key targets of Ikaros that are responsible for its important biological functions.

GENETIC RELATIONSHIP BETWEEN THE CLASSICAL AND PLASMACYTOID DENDRITIC CELL LINEAGES

Boris Reizis[1], Hiyaa S Ghosh, Anna Bunin, Kanako L Lewis, Babacar Cisse

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Dendritic cells (DC) are key pathogen sensors that establish the link between innate and adaptive immune systems. The DC are comprised of two major types, classical or conventional antigen-presenting DC (cDC) and interferon-producing plasmacytoid DC (pDC). Although both DC types arise from the same progenitors, PDC are very distinct from cDC and instead show many features of lymphocytes. Thus, the lineage identity of PDC and their genetic relationship to cDC remain unclear. We have shown that bHLH transcription factor (E protein) E2-2 is an essential and specific regulator of PDC development in mice and in humans. Our recent genome-wide analysis of E2-2 targets in PDC demonstartes that it directly binds to many genes expressed preferentially in PDC and/or shared between PDC and lymphocytes. We also found that the inducible deletion of E2-2 from mature peripheral PDC leads to their spontaneous differentiation into cells with the typical cDC phenotype. The acquisition of cDC properties was confirmed by functional assays and genome-wide gene expression profiling. These data reveal a close genetic relationship between the two DC types, and suggest that E2-2 overrides the “default” cDC differentiation to both specify and actively maintain the unique PDC cell fate.

IDENTIFICATION OF HOXA9 TARGET GENES IN LYMPHO-HEMATOPOIETIC PROGENITORS.

Kay L Medina, Kimberly Gwin

Mayo Clinic, Immunology, 200 First Street SW, Rochester, MN, 55905

The generation of B cell precursors from hematopoietic multipotential progenitors (MPP) is a highly regulated process dependent on the temporal activation and coordinate function of multiple regulatory proteins. EBF and Pax5 are transcription factors essential for specification and commitment to the B cell fate, respectively. In this study we sought to identify novel developmental events that accompany specification of the B cell fate through comparative analysis of multipotent EBF-/- and Pax5-/- cell lines. Flow cytometric analysis of the cell lines revealed differential expression of several MPP-associated antigens that tracked with Hoxa9 transcription/function. Realtime PCR confirmed differential expression of Hoxa9 in the cell lines and during B cell differentiation. Hoxa9 is a key regulator of MPPs and Flt3 surface levels fractionate this subset. ChIP analysis revealed binding of Hoxa9 and co-factors Meis1 and Pbx1, to the Flt3 promoter in EBF-/- cells. Flow cytometric analysis of bone marrow hematopoietic progenitors from wildtype, Hoxa9-/-, and Hoxa7-/- mice revealed significant alterations in expression of three MPP-associated cell surface markers including Flt3, CD34, and CD27. These findings suggest a non-redundant, developmental stage specific requirement for Hoxa9 in the induction of an MPP genetic program during the pluripotent hematopoietic stem cell to lymphohematopoietic progenitor transition. Finally, flow cytometric analysis of ex vivo isolated B cell precursor subsets revealed downregulation of these antigens between the Pre-Pro-B and Pro-B stages that tracked with Hoxa9 transcription, validating the cell line data. Taken together, these data provide new insight into Hoxa9 regulated networks in primitive hematopoietic progenitors and B cell precursors.

PU.1 CONTROLS DENDRITIC CELL AND PLASMA CELL DEVELOPMENT THROUGH DISTINCT MECHANISMS

Sebastian Carotta, Jhagvaral Hasbold, Aleksander Dakic, Angela D'Amico, Phil Hodgkin, Lynn Corcoran, Li Wu, Stephen Nutt

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The transcription factor PU.1 plays multiple context and concentration dependent roles in lymphoid and myeloid cell development. Here we have investigated the function of PU.1 in the differentiation of dendritic cells (DC) and mature B cells. We found that PU.1 is essential for DC development *in vivo* and that conditional ablation of PU.1 in defined precursors, including the common DC progenitor, blocks Flt3 ligand-induced DC generation *in vitro*. PU.1 is also required for the parallel GM-CSF-induced DC pathway from early hematopoietic progenitors, but surprisingly is not required for monocyte-derived DC formation. Molecular studies demonstrated that PU.1 directly regulates the important cytokine receptor *Flt3* in a concentration-dependent manner, with $PU.1^{+/-}$ cells displaying reduced expression of Flt3 and impaired DC formation. These studies identify PU.1 as a central-regulator of DC development and provide one mechanism how altered PU.1 concentration can have profound functional consequences for hematopoietic cell development.
In contrast, inactivation of PU.1 specifically in committed B cells, allows relatively normal lymphopoiesis. However, the removal of both PU.1 and one of its interaction partners, IRF8, reveals a redundancy between these factors and results in increased immunoglobulin class switch recombination and a marked overproduction of plasma cells. Molecular analysis revealed that key target genes of the PU.1-IRF8 complex are *Pax5, Bcl6* and *Blimp-1* indicating that PU.1-IRF8 functions to maintain the transcriptional program of activated B cells, while concurrently repressing the plasma cell program. We propose that PU.1 and IRF8 function together as the genetic switch that controls the division-linked differentiation of B cells into plasma cells.

THE MOLECULAR MECHANISM OF AIRE

Jakub Abramson, Matthieu Giraud, Christophe Benoist, Diane Mathis

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Mutations in the transcriptional regulator, AIRE, cause APECED, a polyglandular autoimmune disease with monogenic transmission. Animal models of APECED have revealed that AIRE plays an important role in T cell tolerance induction in the thymus, mainly by promoting ectopic expression of a large repertoire of transcripts encoding proteins normally restricted to differentiated organs residing in the periphery. The absence of Aire results in impaired clonal deletion of self-reactive thymocytes, which escape into the periphery and attack a variety of organs. In addition, Aire is a proapoptotic factor, expressed at the final maturation stage of thymic medullary epithelial cells, a function that may promote cross-presentation of the antigens encoded by Aire-induced transcripts in these cells. Transcriptional regulation by Aire is unusual in being very broad, context-dependent, probabilistic and noisy. Structure/function analyses and identification of its interaction partners suggest a scenario in which Aire impacts transcription at several levels, including promotion of chromatin remodeling via induction of DNA double-stranded breaks, nucleosome displacement during elongation, and transcript splicing or other aspects of maturation. A broad siRNA screen of Aire-dependent transcription in a simplified cell system confirmed and extended these functional impacts.

PROMISCUOUS EXPRESSION OF TISSUE-RESTRICTED ANTIGENS IN THYMIC MEDULLARY EPITHELIAL CELLS: SELECTION AND REGULATION

Bruno Kyewski[1], Benedikt Brors[2], Sheena Pinto[1]

[1]German Cancer Research Center, Developmental Immunology, Im Neuenheimer Feld 280, Heidelberg, 69120, Germany, [2]German Cancer Research Center, Theoretical Bioinformatics, Im Neuenheimer Feld 280, Heidelberg, 69120, Germany

In the thymus a specific subset of thymic stromal cells - medullary thymic epithelial cells (mTECs) - express a highly diverse set of tissue-restricted antigens (TRAs) representing essentially all tissues of the body, which is known as promiscuous gene expression (pGE). This allows self-antigens, which otherwise are expressed in a spatially or temporarily restricted manner to become continuously accessible to developing T cells. The scope of central tolerance is to a large measure dictated by the pool of promiscuously expressed genes. Thus, even lack of a single TRA can result in spontaneous organ-specific autoimmunity. Promiscuously expressed gene, which have no structural or functional commonality, display two prominent features, they are highly clustered in the genome and show a preference for TRAs. In order to better understand these features, we set out to more precisely define the genomic organization of this gene pool. In particular, we probed to which extent and according to which rules predefined genomic clusters of TRAs are transcribed in mTECs. Our analysis proceeded from the bio-informatic definition of TRA clusters via gene expression analysis in mTECs using whole genome arrays to the in depth analysis of selected TRA clusters by RT- PCR at the population and single cell level. Patterns emerging from these studies will hopefully yield insight into molecular and evolutionary mechanisms responsible for selecting this gene pool. Conceivably, positional cues in the genome and/or particular properties of self-antigens (e.g. immunogenicity) could have been driving forces during the co-evolution of pGE and adaptive immunity.

CIS- AND *TRANS*-ACTING FACTORS INVOLVED IN INITIATING ThPOK EXPRESSION

Sawako Muroi, Hirokazu Tanaka, Chizuko Miyamoto, Ichiro Taniuchi

RIKEN, RCAI, Lab. Transcriptional Regulation, 1-7-22, Suehirocho, Turumiku, Yokohama, 230-0045, Japan

Induction of the *ThPOK* gene expression upon receiving TCR signals is crucial to activate genetic programming towards the $CD4^+$ helper-lineage in $CD4^+CD8^+$ double-positive (DP) thymocytes, as otherwise they commit to the $CD8^+$ cytotoxic-lineage. We have shown that de-repression of the *ThPOK* gene takes place in entire $CD4^+CD8^+$ DP thymocyte population at low extent upon removal of the *ThPOK* silencer. This observation indicates that both activation of enhancer(s) and reversal of the silencer activity are necessary for efficient induction of the *ThPOK* gene expression. By using reporter transgenic assay, we have recently mapped the enhancer activity at the 5' close proximity to the *ThPOK* silencer. Removal of this enhancer from the *ThPOK* locus impaired ThPOK expression only at initial phase, resulting in a partial redirection of class II-selected thymocytes even when they express monogenic class II specific TCRs. Furthermore, upcoming ThPOK expression at the later developmental stage, presumably by activating the proximal enhancer, induceed t*rans*-differentiation in some class II-selected thymocytes, which are once specified toward CD8-lineage and turn on mature CD8 enhancer, the *E8I* enhancer.
On the other hand, binding of Runx complexes to the *ThPOK* silencer in ThPOK-expressing cells indicates that reversal of the silencer involves other mechanisms beyond Runx binding. To identify *trans*-acting factors regulating the silencer function, we are characterizing functional properties of several silencer-binding proteins identified by biochemical purification. These results will be discussed in the context of how TCR signals are integrated into the regulatory pathway that initiates *ThPOK* gene expression.

BATF FAMILY AP-1 TRANSCRIPTION FACTORS REGULATE IMMUNE LINEAGE DECISIONS

Kenneth M Murphy, Kai Hildner, Barbara Schraml, Brian Edelson, Wataru Ise, Masako Kohyama-Ise, Jorn Albring, Wumesh KC, Ansu Satpathy, Wan-Lin Lee, Mona Mashayehki, Michelle Sandau, Theresa Murphy

Washington University School of Medicine, Department of Pathology and Immunology, Howard Hughes Medical Institute, 660 S. Euclid, Campus Box 8118, St. Louis, MO, 63110

We found that the AP-1 factors *Batf3* and *Batf* were expressed in an unusually restricted pattern of lineages and tissues of the immune system. Literature had suggested these factors were inhibitors of AP-1-dependent transcription, competing with Fos for Jun binding and forming transcriptionally inactive heterodimers. To test this idea, we generated mice deficient for members of the BATF family.

Batf-/- mice lacked development of T_H17 cells, which are induced by IL-6 and TGF-beta, produce IL-17, and coordinate acute inflammatory responses. While *Batf* is expressed in all T helper subsets, *Batf-/-* T cells show a selective block in T_H17 development, have normal T_H1 and T_H2 development, and are resistant to EAE, an indicator of T_H17 mediated autoimmune disease. Moreover, Batf is required for expression of ROR?T, a T_H17 transcription factor, and binds directly to putative regulatory elements in the IL-17 cytokine locus. Thus Batf appears to be necessary for the unfolding of the T_H17 developmental program, similar to IRF4. Expression analysis identified a number of genes that may be direct targets of Batf/Jun transactivation.

Batf3-/- mice selectively lacked development of the subset $CD8a^+$ conventional dendritic cells, in the lymphoid tissues, and of a related $CD103^+$ subset of cDC present in many peripheral tissues. These cells were thought to be specialized for antigen cross-presentation to CD8 T cells. Consistently, *Batf3-/-* mice are defective in cross-presentation and have reduced virus-specific $CD8^+$ T cell responses to West Nile and Sendai virus infections. They also are unable to reject syngeneic tumors that can be rejected robustly by wild type mice, indicating a role for cross-presentation in tumor immunity. Thus, Batf3 appears to be necessary for the development of a specific functional subset of dendritic cells, similar to IRF8. It would seem that Batf and Batf3 are not simply inhibitors of AP-1, since each seems necessary for expression of certain tissue specific target genes.

We are pursuing the mechanism by which these BATF factors control the development of these specific immune lineage programs.

GFI-1B NEGATIVELY REGULATES RAG EXPRESSION IN V-ABL TRANSFORMED PRO-B CELLS

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Using an AMuLV transformed pro-B cell line from a RAG-GFP knock-in mouse, we screened a bone marrow cDNA library for genes that could negatively regulate RAG expression. This screen revealed the proto-oncogene Gfi-1b. Gfi-1b is a sequence specific DNA binding protein found in various chromatin remodeling complexes and is associated with both epigenetic activation and inactivation of target gene expression. We showed that the inactivation of RAG expression by Gfi-1b requires DNA replication and that Gfi-1b functions both by binding directly to a site near the RAG locus enhancer and by down-regulating expression of FoxO1, a positive regulator of RAG expression. We generated AMuLV transformed cell lines from Gfi-1b and Gfi-1 (a closely related factor) mutant mouse bone marrow. The former, but not the latter, displayed greatly exaggerated levels of RAG expression as well as DNA damage as evidenced by increased numbers of histone phospho-H2AX foci. Gene expression microarray studies revealed that Gfi-1b regulates additional markers of B cell development including germline kappa transcripts and the transcription factor SpiB. Experiments currently in progress are aimed at determining the role of Gfi-1b during normal B cell development.

REGULATION OF B LYMPHOPOIESIS BY TRANSCRIPTION FACTOR EBF

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EBF1, a transcription factor, expressed in lymphoid progenitors, pro-B and mature B lymphocytes, participates in a regulatory network that determines the specification of the B cell lineage and repression of alternative cell fates. With the aim of understanding the molecular mechanisms underlying the function of EBF1 in pro-B cells, we performed a genome-wide ChIP sequencing analysis to identify direct EBF1 target genes. Comparison of the ChIP data with an analysis of gene expression in loss-and gain-of-function experiments allowed for the identification of three classes of direct targets that are poised for expression, activated or repressed by EBF1. In target genes that are poised for expression at a later stage of differentiation, EBF1 induces changes in chromatin structure that are distinct from changes detected in target genes that are transcriptionally activated by EBF1. To assess the role of EBF1 at the pro-B cell stage and at later stages of B cell differentiation, we generated and analyzed mice carrying a conditional allele of *Ebf1*. Induced loss of EBF1 resulted in multiple defects that will be discussed.

THE BTB/POZ DOMAIN PROTEIN MIZ-1 (ZBTB17) CONTROLS IL-7 RECEPTOR SIGNALING AT COMMITMENT STAGES OF B CELL DIFFERENTIATION BY REGULATING EXPRESSION OF BCL-2 AND THE JAK INHIBITOR SOCS1

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B cells are generated from hematopoietic stem cells (HSC) in the bone marrow through a number of well defined, developmental steps that include the up-regulation of CD19 and the rearrangement of immunoglobulin heavy and light chain genes. The early steps are regulated by cytokine signaling through Flt3 and IL-7R and the hierarchical expression of transcription factors, among them E2A, EBF and Pax5. Here we present evidence that the BTB/POZ domain transcription factor Miz-1 is an important novel regulator in this process with a critical role in early steps of B-cell development. Miz-1 deficiency correlated with an almost complete block of B-cell development in murine bone marrow precisely at the transition of the CLP/pre-pro-B cell stage to the pro-B cell stage. Although CLPs were present in Miz-1 deficient mice, they showed decreased expression of E2A, EBF and Pax5 but a high level of the JAK inhibitor Socs1. Consequently, Miz-1 deficient progenitors fail to activate the JAK/STAT5 pathway upon IL-7 stimulation. Our findings suggest that in normal CLPs Miz-1 dampens the expression of SOCS1 through direct binding to its transcription initiator site and thereby allows the activation of STAT5 and the subsequent up-regulation of EBF by IL-7 and indirectly of Pax5 and Rag1/2. In addition, we show that Miz-1 deficient pro-B cells and CLPs have an increased propensity to undergo cell death in the presence of IL-7. Surprisingly, ectopic expression of EBF (or Pax5) in Miz-1 deficient progenitor cells did not rescue this phenotype, although EBF has been shown to overcome IL-7-deficency, but the co-expression of a Bcl-2 transgene partly restored the development of committed B cells in Miz-1 deficient mice. Our expression analysis confirmed that in the absence of Miz-1, Bcl-2 couldn't be upregulated upon IL-7 stimulation in CLPs and ChIP data demonstrated a direct binding of Miz-1 to the Bcl-2 promoter, suggesting that Miz-1 exerts a second function in the survival axis of the IL-7R pathway by acting as an IL-7 dependent transcriptional activator of Bcl-2. The introduction of both EBF and Bcl-2 in Miz-1 deficient progenitors finally enabled us to fully reconstitute of our experimental system and to rescue B cell development in Miz-1 deficient precursors cultured on OP9 stroma in vitro. This experiment supports a model, in which Miz-1 exerts two functions in the IL-7R signaling pathway: one in the regulation of the IL-7/STAT5 dependent EBF-Pax5-Rag1/2 axis responsible for B-cell differentiation, and another role in IL-7 dependent up regulation of Bcl-2 that ensures survival of B-cell progenitors. Grants: CIHR MOP - 84526

CONTROL OF B CELL DEVELOPMENT AND *IGH* RECOMBINATION BY PAX5

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Hematopoietic stem cells (HSCs) develop into B cells by first differentiating to multipotent progenitors and common lymphoid progenitors (CLPs). Entry of the CLPs into the B cell lineage depends on the transcription factors E2A, EBF1 and Pax5. E2A and EBF1 are essential for early specification of the B cell lineage by activating the expression of several B-cell-specific genes including *Pax5*. Pax5 in turn restricts the broad developmental potential of lymphoid progenitors to the B cell pathway by repressing B-lineage-inappropriate genes and simultaneously activating B-cell-specific genes (Nutt et al., Nature 401, 556-562; Cobaleda et al., Nat. Immunol. 8, 463-370). Consistent with its B cell commitment function, *Pax5* is exclusively expressed in the B-lymphoid lineage, where its function is continuously required throughout B cell development. Pax5 furthermore regulates the contraction of the immunoglobulin heavy-chain (*IgH*) locus, which facilitates distal VH-DJH recombination in pro-B cells (Fuxa et al., Gene Dev. 18, 411-422). We have recently performed chromatin profiling of Pax5-regulated genes by ChIP-chip analysis, demonstrating that Pax5 regulates its target genes by inducing active chromatin at promoters and enhancers. These studies furthermore resulted in the identification of novel regulatory elements in the distal VH gene cluster, which are likely implicated in *IgH* locus control. In my presentation, I will report recent highlights of these studies.

GENETIC AND EPIGENETIC REPRESSION OF THE IGK INTRONIC ENHANCER BY STAT 5

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During B lymphopoiesis, the interplay of signals propagated through the pre-B cell antigen receptor (pre-BCR) and the interleukin 7 receptor (IL-7R) coordinate pre-B cell expansion with subsequent Ig*κ* recombination. Recently, we have demonstrated that pre-BCR mediated Ras-MEK-Erk activation coordinates cell cycle exit with *Igκ* recombination by both repressing *Ccnd3* transcription (encoding cyclin D3) and by upregulating free E2A which enhances *Igκ* transcription (Nat Immunol 2009, 10:1110). IL-7R-mediated STAT5 activation opposes this pathway by promoting *Ccnd3* transcription and by binding directly to the *Igκ* intronic enhancer (Eκi) and preventing E2A recruitment. These data revealed a novel role for STAT 5 as an important transcriptional repressor. We now demonstrate that STAT 5 inhibits *Igκ* transcription by binding to a site (iκS2) adjacent to the κE1 E-box site and preventing E2A binding. *In vitro*, introducing spacers between iκS2 and κE1 enables both transcription factors to bind and abrogates *Igκ* repression. *In vivo*, STAT 5 binding maintains Eκi histone 3 K27 trimethylation and prevents the acquisition of the markers of open chromatin (H3K4me1 and H4Ac) associated with entry into the small pre-B cell pool. These data demonstrate that STAT 5 mediates a comprehensive program of repression at the Eκi.

E2A AND CBP/P300 ACT IN SYNERGY TO PROMOTE CHROMATIN ACCESSIBILITY OF THE IMMUNOGLOBULIN K LOCUS IN NONLYMPHOID CELLS

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V(D)J recombination of immunoglobulin (Ig) and T cell receptor (TCR) genes is strictly regulated in a lineage- and stage-specific manner by the accessibility of target gene chromatin to the recombinase, RAG1 and RAG2 proteins. It has been shown that enforced expression of the basic helix-loop-helix protein, E2A, together with RAG1/2 in a nonlymphoid cell line BOSC23 can induce V(D)J recombination in endogenous Igκ and TCR loci by increasing chromatin accessibility of target gene segments. In this study, we demonstrate that ectopically expressed E2A proteins in BOSC23 cells have the ability to bind directly to the promoter and recombination signal sequence of Vκ genes and to recruit histone acetyltransferase CBP/p300. Overexpression of CBP/p300 in conjunction with E2A results in enhancement of E2A-induced histone acetylation, germline transcription and Igκ rearrangement. Conversely, knockdown of endogenous CBP/p300 expression by small interfering RNA leads to a decrease in histone acetylation, germline transcription and Igκ rearrangement. These observations indicate that E2A plays critical roles in inducing Igκ rearrangement by directly binding to and increasing chromatin accessibility at target gene segments.

MODULATION OF FASL AND ACTIVATION-INDUCED CELL DEATH (AICD) OF T CELLS BY IMIQUIMOD

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Introduction and Objectives: Activation of T cells results in antigen-specific clonal expansion and also differentiation to effector and/or memory cells. To maintain homeostasis, the majority of activated T cells must be removed after elimination of the invading pathogen or during chronic stimulation. Two main mechanisms control the contraction of these cells, namely activation-induced cell death (AICD) and activated T cell autonomous death (ACAD). Toll-like receptors (TLRs) present on the surface of antigen-presenting cells are potent modulators of both innate and adaptive immune responses. As TLRs can also be expressed by T cells it became reasonable to assume that TLRs may directly regulate activation, differentiation, proliferation and death of these cells. Thus, the main goal of this research is to investigate the influence of TLR ligands on AICD of T lymphocytes. **Methods and Results**: DO11.10 T cell hybridoma was stimulated with anti-CD3 antibodies (AICD), in the presence or not different TLR agonists, namely Pam3CSK4 (TLR1/2), Poly(I:C) (TLR3), LPS (TLR4), Flagellin (TLR5) and Imiquimod – R837 (TLR7). Apoptosis was measured by flow cytometry analysis of DNA content and/or phosphatidylserine externalization. Among all TLR ligands used, imiquimod significantly reduced cell death rates when added during anti-CD3 stimulation of T cells (AICD). Decreased AICD was correlated with a de-regulated FASL expression. This result was confirmed in mouse CD4 T cell blasts. **Conclusions**: Our results suggest that TLR7 stimulation in T lymphocytes may directly modulate FASL expression and AICD. **Financial Support**: CNPq, FAPESP.

DUAL FUNCTION OF THE POLYCOMB GROUP PROTEINS IN DIFFERENTIATED T HELPER CELLS

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In our search for factors that propagate the transcriptional programs of T helper (Th) lineages, we found that the Polycomb group (PcG) proteins, which are known as transcriptional silencers, function unconventionally and differentially, in primary Th1, Th2, and Th17 cells and in established Th cell lines, as transcriptional activators of the hallmark cytokine genes. Moreover, the PcG protein Mel-18 was necessary to recruit NFAT1 and T-bet to the Ifng promoter in Th1 cells. However, our results also indicate that the PcG proteins can act as transcriptional silencers, for example, of Tbx21 in Th17 cells. This dual function of the PcG proteins may participate in the mechanisms underlying the commitment and plasticity of the Th phenotypes.

DUAL ROLE OF BCL11B IN INKT DEVELOPMENT IN CONTROLLING GLYCOLIPID PRESENTATION BY DP THYMOCYTES AND EARLY INKT PROGENITORS

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iNKT cells are innate-like T lymphocytes that upon activation respond rapidly by cytokine production. They play increasing importance in immune regulation, antimicrobial protection and allergy. iNKT cells bear semi-invariant TCRs that recognize glycolipid antigens in the context of the non-classical MHC class I-like molecule CD1d. Thymic selection of iNKT cells is accomplished by cortical DP thymocytes which present endogenous glycolipids, as opposed to the selection of conventional thymocytes mediated by cortical thymic epithelial cells which present endogenous peptides. BCL11B is a transcription factor expressed in T lymphocytes, including iNKT cells. Here we provide genetic and biochemical evidence that BCL11B deficiency leads to an early block in iNKT cell development caused by an intrinsic defect in glycolipid presentation by cortical DP thymocytes, as well as by an inherent defect in early thymic iNKT precursors. The glycolipid presentation defect is associated with enlarged late endosomal/lysosomal compartment and accumulation of several glycolipid species, suggesting that the presentation defect could be attributed to a glycolipid storage disorder. Mechanisms underlying the failure of BCL11B-deficient DP thymocytes to efficiently present glycolipid are shown, together with the mechanisms responsible for the inherent defects in iNKT cells. These findings identify BCL11B as one of the major transcription factors for iNKT cell lineage development, not only by controlling early iNKT precursors, but also being intrinsically required for glycolipid presentation by cortical DP thymocytes, a process which is poorly understood.

Mycobacterium bovis BCG INDUCED EXPRESSION OF CYCLOOXYGENASE-2 INVOLVES NITRIC OXIDE DEPENDENT AND INDEPENDENT SIGNALING PATHWAYS

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Modulation of the host signaling events at the level of initiation as well as during course of infection represents a critical factor for the suppression of a generalized inflammatory response and possible persistence of mycobacteria within the host. In this intricate process, modulation of cyclooxygenase-2 (COX-2) levels, a key enzyme catalyzing the rate-limiting step in the inducible production of Prostaglandin E_2 (PGE_2) by mycobacteria like *Mycobacterium bovis* BCG act as an important factor influencing the overall host immune response. PGE_2 is known to exert immunosuppressive functions as inhibition of human T lymphocyte activation and proliferation, production of Th1 cytokines like IL-2 & IFN-γ but not of Th2 cytokines IL-4 & IL-5, induction of IL-10 production etc. Current study demonstrates that *M. bovis* BCG triggered TLR2-dependent signaling leads to COX-2 expression and PGE_2 secretion *in vitro* in macrophages and *in vivo* in mice. Further, the presence of PGE_2 could be demonstrated in sera or CSF of tuberculosis patients. Additionally, TLR2 agonist like Phosphatidyl-myo-inositol dimannosides (PIM2), an integral component of the mycobacterial envelope, could drive COX-2 expression. The induced COX-2 expression is dependent on NF-κB activation, which is mediated by iNOS/NO dependent participation of the members of Notch1-PI3K pathway or iNOS/NO independent signaling events. Importantly, loss of iNOS activity either in iNOS null macrophages or by pharmacological intervention in wild type macrophages severely abrogated *M. bovis* BCG ability to trigger Notch1-PI3K signaling axis as evidenced by deficiency in generation of Notch1 intracellular domain (NICD) as well as activation of PI3K signaling cascade. Notably, pharmacological inhibition of the biosynthesis of TLR2 ligand PIM2, markedly compromised *M. bovis* BCG ability to trigger Notch1 activation and COX-2 expression. Overall, we propose the involvement of iNOS/NO as one of the obligatory early proximal signaling signature in mycobacteria induced COX-2 expression in macrophages.

CLASS SWITCH RECOMBINATION REQUIRES HISTONE CHAPERONE FACT ASSISTED CHROMATIN ASSEMBLY OF IGH LOCUS

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Class switch recombination is a transcription coupled DNA cleavage and religation process, which begins with germline transcription through donor and acceptor switch (S) regions. Here we show that knocking down of histone chaperon FACT (facilitates chromatin transcription), comprised of SSRP1 and SPT16, completely inhibited CSR, although transcription of AID and germline transcripts remained intact. Knock down of FACT components drastically reduced γH2AX formation and DNA cleavage in switch regions. Depletion of FACT dramatically altered the chromatin structure of S-region by changing the nucleosome occupancy and histone ratio, leading to a defect in trans-histone modification cascade and enhancement of cryptic germline transcripts. Decreased nucleosomal barrier facilitated transcription, but the altered state of histone modification halted recombination. We propose FACT is essential for continued reassembly of nucleosomes during transcription through S-region, thereby preserving a dynamic structural integrity of the S-region chromatin, which is required for AID induced DNA cleavage.

REDUNDANT AND SPECIFIC ROLES OF NFAT TRANSCRIPTION FACTORS IN GENERATION AND FUNCTION OF DENDRITIC CELLS

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Nuclear factor of activated T cells (NFAT) was first described as a transcription factor involved in the activation and differentiation of T lymphocytes. The NFAT proteins are primarily regulated upon T cell receptor engagement at the level of their subcellular localization through the calcium/calmodulin-dependent phosphatase calcineurin. In addition, recent studies suggest different roles for NFAT family members in the regulation of proliferation and differentiation or apoptosis. Moreover, NFAT transcription factors appear to be involved in cell cycle control, thereby influencing tumor growth and survival. To address the role of NFAT in dendritic cells (DCs), we took advantage of mice having individual NFAT family members deleted or knocked down NFAT by lentivirally transduced shRNA. Here, we demonstrate that NFATc1 and NFATc2 exert contrary functions on dendritic cell (DC) generation, proliferation and maturation. Thus, during GM-CSF induced bone marrow differentiation towards myeloid dendritic cells (BMDCs), NFATc1 supports cell cycle progression and maturation, while NFATc2 inhibits proliferation. Different from T and B cells, in BMDCs only promoter 2 of NFATc1 is transcriptionally active and no positive feed-back to promoter 1 could be observed. When BMDCs are activated though, e.g. through Toll-like receptors, both NFATc1 and NFATc2 are silenced. At that point and in response to the β-Glucan receptor Dectin-1 another NFAT family member, namely NFATc3, is essential for the expression of pro-inflammatory IL2, IL12, IL13 and TNFα. Furthermore, NFATc3 counteracts the production of immune-suppressive IL10. Taken together, these results suggest opposing roles of NFATc1 and NFATc2 in the development of myeloid dendritic cells, while NFATc3 plays a crucial role in the cytokine expression pattern of activated cells.

A ROLE FOR E-BOX BINDING TRANSCRIPTIONAL REPRESSOR ZEB2 IN THE CD8$^+$ T-CELL RESPONSE TO INFECTION

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Zeb2 (also known as Zfhx1b and Sip1) is a DNA-binding multi-zinc-finger transcription factor found to play a significant role in the development of cells both early in neuronal differentiation and in cancer metastasis. By binding dual E2 box enhancer elements, Zeb2 acts primarily as a transcriptional repressor and may inhibit E-protein DNA binding, although it is known to act also as a transcriptional activator. While the closely related family member, dEG1/Zfhx1a/Zeb1, is known to plays an early role in lymphocyte development, the function of Zeb2 in T lymphocytes remains unknown. We find that Zeb2 is upregulated in activated T cells and hypothesize that it influences differentiation of mature cytotoxic T lymphocytes during an immune response. We are currently assessing the interplay between Zeb2 and E-proteins in differentiation of naïve CD8 T cells to cytotoxic effector cells.

HIGH AFFINITY CARBOHYDRATE AND NON-CARBOHYDRATE LIGANDS FOR LECTIN-TYPE ACTIVATION RECEPTORS OF NATURAL KILLER CELLS REGULATE EFFECTOR FUNCTION THROUGH PI3K PATHWAY, AND GENERATE PERMANENT IMMUNE PROTECTION AGAINST MELANOMAS

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Our laboratories are interested in understanding of complex interactions between activation receptors of natural killer (NK) cells, their target structures at tumor cell surface, and intracellular activation pathways resulting in the activation of NK cell effector functions at molecular and cellular level. To identify high afinity ligands, we produce stable recombinant soluble forms of NK cell receptors such as NKR-P1, CD69, and NKG2D (1,2), and use them in binding, inhibition and precipitation studies based on standard biochemical assays and oligosaccharide arrays. High affinity ligand mimetics are constructed by attachment of the active compounds to polyamidoamine or calix[4]arene cores, or by dimerization of the ligand through a defined chemical linker (3,4). GlcNAc-coated polyamidoamine dendrimers induce upregulation of antibody formation that triggered by their interaction with mNKR-P1C (5). GlcNAc-coated calix[4]arene downregulated the expression of GlcNAc transferases MGAT3 and MGAT 5, increased the susceptibility of tumor cells to natural killing, and increased the expression of mNKG2D through the activation of PI3K–ERK but not phospholipase C-g -JNK pathway (6). GlcNAc dimers can provide permanent protection in 70 % of mice bearing syngeneic B16S melanomas. This is due to activation of NKT lymphocytes, and subsequent infiltration of tumors by CD8+ cytotoxic lymphocytes. The exceptional signaling efficiency of GlcNAc dimers is explaned by sequential cooperative engagement of mNKR-P1A leading to the formation of large signaling complexes of about 20 MDa containing G proteins, b-arrestin, phosphorylated dynamin, Src kinase, Vav, Rac1, Grb2, and Ras (4). Use of combined ligand mimetics results in engagement of several target receptors, and efficient activation of NK cell effector functions due to effective receptor cross-talk.

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THE PPE PROTEIN RV1168C OF *MYCOBACTERIUM TUBERCULOSIS* ACTIVATES TRANSCRIPTION OF HIV-1 LTR PROMOTER VIA BINDING TO TOLL LIKE RECEPTOR 2

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There exists a synergistic relationship between HIV and *Mycobacterium tuberculosis*. Tuberculosis develops in an HIV patient as a result of exposure to the pathogen or reactivation of latent TB due to decrease in the strength of immune system. HIV is one of the most important factors for susceptibility and reactivation of tuberculosis. Once TB takes over, it results in the enhanced replication of HIV either as a result of immune response of the host to the *Mycobacterium tuberculosis* infection by production of proinflammatory cytokines or directly as a result of interaction of mycobacterial components with the host immune cells.
In the current study we show the up-regulation of HIV transcription by a member of PPE protein family (Rv1168c) of *Mycobacterium tuberculosis*. The protein is expressed on the surface of *Mycobacterium tuberculosis*. We show that this protein specifically interacts with Toll Like Receptor 2 (TLR2) on the macrophage surface resulting in the activation of NF-κB transcription factor. The latter then rapidly translocates to the nucleus thereby binding to the specific NF-κB binding sites in the HIV-1 LTR promoter element resulting in the activation of transcription (independent of Tat transactivator protein). Blocking of the TLR2 using the blocking antibody resulted in the abrogation of Rv1168c mediated HIV-1 LTR activation. Furthermore, infection of macrophage having stably integrated HIV-1 LTR linked to CAT reporter gene with *Mycobacterium smegmatis* transformed with Rv1168c protein resulted in up-regulation of HIV-1 LTR activity. Rv1168c interacts with the LRR domain of TLR2 (also recognized by Pam3CSK4) activating NF-κB but not to the LRR 10~15 known to induce anti-inflammatory signaling. Our results suggest that binding of some of the PPE proteins to the different domains of TLR2 results in the differential activation of NF-κB signaling cascade resulting in the activation of HIV-1 LTR. Furthermore, we first time show the involvement of a PPE protein in the activation of HIV-1 LTR. This study may be helpful in understanding the molecular mechanism of pathogenesis of HIV-1 during *Mycobacterium tuberculosis* co-infection.

IRF-5 REGULATES PLASMACYTOID DENDRITIC CELL COLONIZATION OF PERIPHERAL TISSUES.

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Plasmacytoid dendritic cells (pDCs) mature in the bone marrow and migrate to peripheral organs and lymphoid tissues, from where they can migrate to sites of infection or inflammation and locally produce type 1 interferons. Here we demonstrate that the transcription factor interferon regulatory factor 5 (IRF-5) is required for proper pDC colonization of these peripheral sites. IRF5 expression is first induced in the dendritic cell lineage as common myeloid progenitors differentiate into common dendritic cell progenitors. Although progenitor frequencies are normal, mature IRF5-/- pDC numbers are slightly expanded in the bone marrow relative to wild type controls and are reduced by nearly 90% in the spleen, lymph node, and blood. Competitive bone marrow reconstitutions of wild type and IRF5-/- recipients have demonstrated that this defect is intrinsic to IRF5-/- pDCs. Chemotaxis assays showed that IRF5-/- pDCs not only failed to migrate toward CXCL12 and CCL3, but also showed no background levels of migration in the absence of added chemokines, suggesting a defect in cellular motility. Microarray comparisons of IRF5-/- pDCs and wild type pDCs are consistent with this interpretation, as a number of genes associated with cytoskeletal rearrangement are dysregulated in IRF5-/- pDCs. Thus, the pDC and IRF-5, a cell type and a gene whose functions have independently been associated with systemic lupus erythematosus, are linked at the level of cellular migration and egress from the bone marrow.

ALTERNATIVE END-JOINING CATALYZES CLASS SWITCH RECOMBINATION, INTRA-SWITCH DELETIONS AND CHROMOSOMAL TRANSLOCATIONS IN THE ABSENCE OF BOTH KU70 AND DNA LIGASE 4

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Class Switch Recombination (CSR) in B lymphocytes is initiated by introduction of multiple DNA double-strand breaks (DSBs) into switch (S) regions that flank immunoglobulin heavy chain (IgH) constant region exons. CSR is completed by joining a DSB in the donor Sμ to a DSB in a downstream acceptor S region (e.g. Sγ1) by end-joining. In normal cells, many CSR junctions are mediated by classical non-homologous end-joining (C-NHEJ), which employs the Ku70/80 complex (Ku) for DSB recognition and the XRCC4/DNA ligase 4 (Lig4) complex for ligation. During IgH class switch recombination (CSR) in B lymphocytes, switch (S) region DSBs are joined by C-NHEJ to form junctions either with short micro-homologies ("MH-mediated" joins) or no homologies ("direct" joins). Multiple DSBs are introduced into Sμ during CSR, with some being re-joined or joined to each other to generate internal switch deletions (ISDs). In addition, S region DSBs can be joined to other chromosomes to generate translocations, the level of which is increased by absence of a single C-NHEJ component (e.g. XRCC4). In the absence of XRCC4 or Lig4, substantial CSR occurs via "alternative" end-joining (A-EJ) that generates largely MH-mediated joins. Because upstream C-NHEJ components remain in XRCC4- or Lig4-deficient B cells, residual CSR might be catalyzed by C-NHEJ using a different ligase. To address this, we have assayed for CSR in B cells deficient for Ku70, Ku80, or both Ku70 and Lig4. Ku70- or Ku80-deficient B cells have reduced, but still substantial, CSR. Strikingly, B cells deficient for both Ku plus Lig4 undergo CSR similarly to Ku-deficient B cells, firmly demonstrating that an A-EJ pathway distinct from C-NHEJ can catalyze CSR end-joining. Ku-deficient or Ku- plus Lig4-deficient B cells are also biased towards MH-mediated CSR joins; but, in contrast to XRCC4- or Lig4-deficient B cells, generate substantial numbers of direct CSR joins. In addition, we find, unexpectedly, that B cell activation for CSR generates substantial ISD in both Sμ and Sγ1 and that ISD in both is greatly increased by absence of C-NHEJ. IgH chromosomal translocations to the c-myc oncogene are also augmented in the combined absence of Ku70 and Ligase 4. Our findings suggest that more than one form of A-EJ can function in CSR and we are currently screening for A-EJ factors using an “in vitro” system.

FLT3L MEDIATED DENDRITIC CELL HOMEOSTASIS DURING STEADY STATE AND IN IMMUNOLOGICAL STRESS CONDITIONS

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Dendritic Cells (DCs) have a short life-span and need to be continuously replenished from hematopoietic stem and progenitor cells. Previously we found that Flt3 receptor expression on early dendritic cell progenitors and Flt3 tyrosine kinase signalling efficiently supports and is indispensable for DC development in mice and men. Flt3 is also expressed on mature steady-state lymphoid organ DCs; however its function on mature cells remains to be determined. Interestingly we found that depletion of DCs in CD11c-DTR mice by administration of diphtheria toxin lead to elevated levels of Flt3L in the serum without a change in expression of Flt3L mRNA, which strongly indicates that Flt3 expressed on DCs might be acting as a sink for Flt3L. Therefore DCs might play a regulatory role in the maintenance of Flt3L milieu. Flt3 ligand (Flt3L) is produced by stromal cells and some hemato-lymphoid cells; however, the relative contribution of stroma versus hemato-lymphoid cells to Flt3L levels in steady-state and in ongoing immune reactions is unknown. Our recent findings suggest that stromal cell produced Flt3L helps to maintain steady state DC homeostasis, while Flt3L produced by the hematopoietic cells helps in faster replenishment of DCs during an ongoing immune response and in combating foreign pathogens by enhancing the antigen presentation process. Further more, enhanced cDC development upon Treg depletion involves Flt3L produced by activated CD4 T cells. These key observations provide insight into regulation of DC homeostasis and function via tailored adaptation of the Flt3L cytokine milieu during steady state and under pathogenic stress.

HISTONE DEMETHYLASE KDM5A IS AN INTEGRAL PART OF THE CORE NOTCH-RBP-J REPRESSOR COMPLEX

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Timely acquisition of cell-fates and the elaborate control of growth in numerous organs depend on Notch signaling1. RBP-J transcription factor plays a dual role in regulating Notch target genes2. RBP-J actively represses target genes in the non-Notch receiving cells while it induces expression of target genes in cells receiving the Notch signal. Therefore, Notch signaling acts as a switch that reverses RBP-J-mediated repression of target genes. We hypothesize that histone methylation plays a key role in this dynamic regulation of Notch target genes.

Here, we show that the histone demethylase KDM5A is an integral, conserved component of Notch/RBP-J gene silencing. Methylation of histone H3 lysine 4 is dynamically erased and reestablished at RBP-J-sites upon inhibition and re-activation of Notch signaling. KDM5A physically interacts with RBP-J, this interaction is conserved in Drosophila and crucial for Notch-induced growth and tumorigenesis responses.

Together these findings indicate that dynamic regulation of histone methylation plays a key in the Notch target gene expression. With the in vivo evidence of Drosophila lid/KDM5A in Notch-induced tumorigenesis, this study suggests a pathway-specific tumour-suppressor role of KDM5A in cancer and provides the basis for studies in novel strategies to manipulate Notch-mediated carcinogenesis.

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CELL INTRINSIC E47 IS REQUIRED FOR STEM CELL SELF-RENEWAL AND DIFFERENTIATION BUT IS DISPENSABLE FOR SHORT-TERM MYELOID DEVELOPMENT

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The immune system is constantly replenished by a rare population of hematopoietic stem cells (HSC) residing in the bone marrow of adult organisms. E-proteins, the widely expressed basic helix-loop-helix (bHLH) transcription factors, appear to contribute to HSC activity, but their specific cell intrinsic functions remain undefined. In contrast to a recent report, we show that E47 is dispensable for the short-term myeloid differentiation of HSCs. In our quantitative assays, E47 deficient progenitors show competent myeloid production in vitro and in vivo as well as under burden of a pathogen mimic. We also show that long-term myeloid and lymphoid differentiation is compromised due to the progressive loss of the self-renewal potential of HSCs in vivo. Compromised self-renewal of E47 null HSCs is associated with over-proliferation and premature exhaustion under replication stress. These observations suggest that cell-intrinsic E47 is dispensable for the short-term myeloid repopulation activity of HSCs and, by contrast, that E47 plays an essential cell-autonomous role in the self-renewal of HSCs by preventing hyperproliferation-associated exhaustion following replication stress.

VITAMIN D RECEPTOR GENE 3' METHYLATION IS INFLUENCED BY ETHNICITY AND *TAQ*I GENOTYPE: POSSIBLE IMPACT ON TUBERCULOSIS SUSCEPTIBILITY.

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The vitamin D receptor gene (*VDR*) is one of the candidate genes implicated in genetic susceptibility to tuberculosis (TB). Genetic variants in the primary promoter, in exon 2 and the 3' end of the *VDR* were found to be associated with TB. The alleles or haplotypes of SNPs reported to be linked to TB predisposition are inconsistent between populations. The objective of the current study was to investigate the role of epigenetics in this phenomenon. The methylation status of the 3' *VDR* CpG island (CGI 1060) was characterized considering ethnicity, sex, the geno- or haplotype of SNPs it contain and TB status. DNA from EBV-transformed B lymphocytes originating from Caucasians (n=30) and Yoruba (YRI; n=30) as well as DNA from whole blood collected from TB cases (n=32) and matched controls (n=29) from the Venda Population, Limpopo Province, South Africa, were used. Bisulfite conversion and/or pyrosequencing facilitated quantification of CpG methylation and genotyping of SNPs in CGI 1060, including *Apa*I (rs7975232) and *Taq*I (rs731236). The significance of methylation differences was analysed using SPSS parametric and non-parametric tests. *Taq*I coincided with the methylatable 'C' of a CpG dinucleotide in CGI 1060 and directly influenced its methylation by introducing or removing a CpG 'C' that appeared to be always methylated when present (TC 50%, CC 100%). Presence of *Taq*I 'C' and coincident site-specific methylation increased regional methylation of CGI 1060 in African populations to levels significantly above levels in the absence of 'C' ($P < 0.001$, One-way ANOVA and Mann-Whitney U), but not significantly so in Caucasians. Despite a notably higher *Taq*I 'C' allele frequency in Caucasians (38% 'C') compared to YRI (21% 'C') and consequent higher site-specific methylation of the *Taq*I CpG (Caucasians 35% methylation vs. YRI 20% methylation, $P \leq 0.05$, Mann-Whitney U), CGI 1060 regional methylation was significantly higher in YRI than Caucasians ($P < 0.05$, One-way ANOVA). This difference was driven by higher site-specific methylation at 6/17 CpGs ($P < 0.05$, One-way ANOVA and Mann-Whitney U). Two of these CpG sites, immediately downstream of the *Taq*I CpG, were also significantly more methylated in Venda TB cases compared to controls ($P < 0.03$, One-way ANOVA and Mann-Whitney U). Epigenetics, specifically DNA methylation, may confound comparisons of alleles/haplotypes of 3' *VDR* SNPs found associated with TB in different populations. Hepitype analysis, combining haplotype and epitype, may reveal more powerfully genetic and environmetal factors contributing to disease predisposition.

CTCF AND COHESIN ORGANIZE THE MHC-II LOCUS AND REGULATE THE EXPRESSION OF HUMAN MHC-II GENES

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The MHC-II locus spans nearly 700 kb and encodes a dense cluster of genes, the majority of which function to present antigens to CD4+ T cells and initiate adaptive immune responses. MHC-II and the accessory genes encoded within the locus are regulated in a cell type dependent manner; while other genes within the locus are constitutively expressed in many cell types. Ten chromatin insulator CTCF binding sites were identified that divide the MHC-II locus into apparent evolutionary domains such that pairs of MHC-II genes are separated from each other by surrounding CTCF sites. Using RNAi, CTCF was found to be required for the expression of all MHC-II and MHC-II related genes but not the other genes within the locus. Knockdown of Rad21, a cohesin protein found at all 10 CTCF sites, diminished MHC-II gene expression. 3C assays were conducted across the MHC-II locus and defined two CTCF-dependent chromatin architectural states, each with multiple configurations and interactions. Independent of MHC-II gene expression, the 10 CTCF sites interacted with each other in a distance dependent manner. The CTCF sites also interacted with the promoter proximal regions of the MHC-II genes themselves. This interaction was only associated with MHC-II gene expression and dependent on the presence of CIITA, the MHC-II transactivator, bound to the proximal regulatory region of the MHC-II genes. CIITA-dependent, CTCF-site selection was also distance dependent, but it was found that CIITA-bound regions could interact with multiple CTCF sites. Disruption of the CTCF-dependent rearranged states eradicated expression. Using homologous recombination strategies, CTCF sites in an MHC-II locus encoding BAC were mutated and assessed for their activity. Preferences for interactions among the sites and promoters were observed in the minimal system, suggesting that such preferences may occur normally. Thus, CTCF and cohesin organize the MHC-II locus into a novel basal architecture of interacting foci and loop structures that dynamically rearrange in the presence of the transcriptional regulator CIITA to activate MHC-II gene expression. These results therefore define a non-traditional role for CTCF as an organizer of gene expression and display a unique dynamic set of chromatin organizational structures.

CONTRIBUTIONS OF THE TRANSCRIPTION FACTORS GATA3 AND THPOK TO THE ESTABLISHMENT AND MAINTENANCE OF CD4-LINEAGE DIFFERENTIATION.

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The transcription factors Gata3 and Thpok, together with Runx-family members Runx1 and Runx3, are required for the proper differentiation of CD4 and CD8 lineages, respectively, in the thymus. We and others previously proposed that Thpok serves as a ‘commitment’ factor and is not actually required to induce gene expression programs specific of CD4-lineage cells. However, this hypothesis had not yet been tested in cells with intact Runx activity. Here, we used a combination of genetic analyses and assessments of gene expression and histone modifications to analyze the role of Thpok in CD4-lineage ‘commitment’, understood as the repression or termination of CD8-specific gene expression programs, *vs.* ‘specification’, i.e. the onset of CD4-lineage specific gene expression. Our results indicate that Thpok is not required for 'CD4 specification' in MHC II-restricted thymocytes with an otherwise intact transcriptional circuitry, and we will discuss new observations delineating the respective contributions of Gata3 and Thpok to CD4-lineage differentiation. In addition, we will present experiments assessing the respective roles of Thpok and epigenetic DNA marking in repressing CD8-lineage gene expression in post-thymic CD4 T cells. Similarities and differences with *Cd4* silencing in CD8 cells will be discussed.

INVESTIGATING DICER FUNCTIONS IN VDJ RECOMBINATION AND REPAIR

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As well as being pervasive in regulating gene expression, the RNase III enzyme Dicer functions in establishing and maintaining transcription dependent barrier activity and controlling site specific recombination events. Lymphocytes exhibit differentiation blocks in the absence of Dicer; these blocks occur at developmental transitions dependent upon successful VDJ recombination and/or antigen receptor induced cellular proliferation. Lck-Cre:Dicer$^{flox/flox}$ mice exhibit reduced thymic cellularity and a partial block in development between the DN and DP stages. This phenotype is consistent with a role for dicer in TCRβ recombination, proliferation, and/or survival. To distinguish amongst these possibilities, we generated and analyzed Lck-Cre:Dicer$^{flox/flox}$ mice containing a pre-assembled endogenous Vβ1DJβ1.4 rearrangement (the Vβ1^{NT} allele). Repetitive DNA sequences upstream of the Vβ1DJβ1.4 exon mark a boundary between accessible (Vβ10) and inaccessible (Vβ4, Vβ16) germline Vβ segments. Vβ accessibility is characterized by cell surface expression, rearrangement over the pre-assembled Vβ1DJβ1.4 join, bi-directional transcription, and low CpG methylation of the promoter and coding sequences. Dicer deletion in Vβ1^{NT} thymocytes provides a defined system that allows us to assess potential functions of Dicer in boundary activity, accessibility, and recombination at the TCRβ locus. Vβ1^{NT} expression in Dicer$^{-/-}$ thymocytes leads to increased thymic cellularity compared to Dicer$^{-/-}$ cells; Dicer deletion in Vβ1^{NT} thymocytes results in partial loss of the accelerated DN3/DN4 transition driven by expression of pre-assembled TCRβ genes. Dicer inactivation does not affect Vβ4/Vβ16 accessibility, indicating that Dicer is not required for barrier activity of Vβ16-Vβ10 intergenic sequences. Dicer deletion does result in decreased expression, transcription, and increased CpG methylation of Vβ10, all of which are consistent with reduced Vβ10 accessibility and/or loss of cells attempting Vβ10 rearrangements, possibly due to incomplete or aberrant DNA repair in the absence of Dicer. To begin to distinguish between these two possibilities, we have generated and begun to analyze Lck-Cre:Dicer$^{flox/flox}$:Vβ1$^{NT/NT}$:Rag1$^{-/-}$ mice. Initial experiments reveal that Rag1 deletion leads to reemergence of the accelerated DN3/DN4 transition driven by Vβ1^{NT} expression. Collectively, our data suggest that Dicer function is required for normal activation of cellular checkpoint(s) upon RAG mediated DNA cleavage in developing thymocytes.

CYCLIN D2 INCOMPLETELY OVERLAPS WITH CYCLIN D3 IN THE ANTIBODY RESPONSE AND IS ESSENTIAL FOR SYNERGISTIC CELL CYCLE CONTROL BY BAFF AND IL-4

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Cell cycle control is critical for homeostasis in an antibody response. Upon stimulation by antigen and cytokines, resting mature B cells enter the cell cycle from the G_0/G_1 boundary, replicate and undergo clonal expansion. The D-type cyclins are known to promote cell cycle reentry and progression in cooperation with cyclin-dependent kinases (Cdk) 4 and 6, but their function in the antibody response is unknown. We now show that cyclin D2 is dispensable for the T-independent (TI) antibody response due to functional redundancy with cyclin D1 and cyclin D3. By contrast, ablation of cyclin D2 compromises the expansion of germinal center B cells and the generation of low affinity plasma cells in the T-dependent (TD) response because of incomplete compensation by cyclin D3 activation. Cyclin D1 apparently plays no role in the TD response, since cyclin D1 protein is not expressed despite the synthesis of cyclin D1 mRNA. Together, these data suggest that cyclin D2 is uniquely required to mediate specific physiologic signals for cell cycle activation in the TD response. In search of such signals, we have discovered that BAFF and IL-4 synergistically induce resting B cells to enter the cell cycle, leading to DNA replication and cell division, while IL-4 alone can not activate the cell cycle and BAFF induces only partial G1 progression. BAFF and IL-4 both are known to promote B cell survival *in vivo* and *ex vivo*. Induction of cell cycle entry by BAFF and IL-4 requires cyclin D2, through rapidly and selectively activation of cyclin D2 transcription that requires STAT6, but not cyclin D3 or D1 transcription. Our findings provide the first direct evidence that the D-type cyclins are differentially required for TI and TD antibody responses, and demonstrate an extraordinary specificity with which physiologic signals such as BAFF and IL-4 regulate D-type cyclins for homeostatic cell cycle control of the antibody response.

A NOVEL ROLE FOR THE TRAFS AS CO-ACTIVATORS AND CO-REPRESSORS OF TRANSCRIPTIONAL ACTIVITY

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The tumor necrosis factor receptor-associated factors (TRAFs) have been found to function as signaling intermediates for the TNF receptor superfamily, and are believed to orchestrate cytoplasmic signaling events downstream of receptor activation. In this study, we show that TRAFs 1-7 are present within the nucleus of Neuro2a cells, and that TRAF2 and TRAF3 translocate into the nucleus within minutes of CD40L-stimulation. Once in the nucleus, TRAF2 and TRAF3 very rapidly bind to chromatin, and more specifically to the nuclear factor-kappa B consensus element. Analysis of the transcriptional regulatory potential of TRAFs 1-7 by luciferase assay revealed that each of the TRAFs functions as a co-activator, co-repressor, or even as a transcription factor, in a cell- and target protein-specific manner. Our findings demonstrate for the first time that the TRAF proteins are not only intermediate signaling molecules, but also direct effectors by modulating transcriptional activity.

REGULATION OF BASAL AND INDUCIBLE EXPRESSION OF THE TRANSCRIPTIONAL CO-ACTIVATOR BOB.1/OBF.1 IN B VERSUS T CELLS

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The transcriptional co-activator BOB.1/OBF.1 is recruited to the DNA via protein–protein interaction with Oct1 or Oct2 and thereby enhances octamer-dependent transcription. BOB.1/OBF.1-deficient mice show severe defects at several stages of B cell development in maturation and function. The main characteristic of these mice is the complete failure to form germinal centers. In T cells, BOB.1/OBF.1 contributes to the maintenance of the balance between TH1 and TH2 cells. BOB.1/OBF.1 is continuously expressed throughout B cell development albeit at different levels. In addition, the expression of BOB.1/OBF.1 can be further upregulated by stimuli like αCD40+IL-4 or LPS. In T cells no basal expression of BOB.1/OBF.1 could be detected. However, it is inducible expressed by treatment of T cells with PMA/Ionomycin or antigen receptor engagement. The aim of the present study is to identify regulatory elements within the BOB.1/OBF.1 promoter as well as transcription factors involved in the basal an inducible BOB.1/OBF.1 expression in B versus T cells.
In silico analyses revealed, beside the known transcription factor binding site, a combined NF-AT/NF-κB site as well as an additional TATA-box within the BOB.1/OBF.1 promoter. Interestingly, we have also identified a combined NF-AT/NF-κB motif within the potential Oct2 promoter. Moreover, by using several different inducers and inhibitors, a co-regulation of Oct2 and BOB.1/OBF.1 could be observed. An array of genetic as well as biochemical analyses revealed an involvement of NF-κB and NF-AT transcrption factors in the transcriptional regulation of the BOB1/OBF.1 promoter activity.
Insights into the regulation of BOB.1/OBF.1 expression enable us to learn more about the transcriptional network necessary for proper B and T cell function.

TNFR:TNFR-LIGAND INTERACTIONS DRIVE CD8 T CELL CLONAL COMPETITION

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The response to self and foreign antigens requires a network of signals between antigen presenting cells and responding T lymphocytes. In our studies, we demonstrate that the ability to access the TNFR-ligand CD70 confers a competitive advantage to CD8+ T cells in response to foreign antigen. Specifically, adoptive transfer studies have demonstrated that CD8+ T cells sufficient for CD27 are able to suppress the expansion of endogenous antigen-specific CD8+ T cells to a greater extent than CD27-defficient CD8+ T cells. To dissect the mechanism by which the expression of CD27 confers a competitive advantage to CD8+ T cells, we utilized in vitro and in vivo assays in which we were able to demonstrate that dendritic cell surface expression of CD70 is reduced in the presence of CD27+ T cells, but not CD27-deficient T cells. Furthermore, preliminary studies indicate that upon engagement of CD27 by CD70, CD27 is proteolytically cleaved from the surface of CD8+ T cells and captured by CD70 expressing dendritic cells, effectively masking CD70 from being recognized by neighboring responding T cells. These data indicate that CD8+ T cells of the same or different antigen specificities can compete against one another by directly regulating the access of T cells to CD70. Our data predicts that if access to TNFR-ligands were limited, then the responding population of antigen-specific T cells would be selected for high affinity clones. Consistent with this hypothesis, antigen-specific CD8+ T cells from immunized CD27-/- mice have an increased affinity for peptide:MHC complexes compared to WT T cells. Collectively, our data indicate that T cells compete against each other for access to the TNF ligand CD70 on the surface of antigen presenting cells, and that regulation of interactions between CD27 and CD70 shapes not only the magnitude, but also the repertoire of responding CD8+ T cells.

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NF-KB SIGNALING COOPERATES WITH CMYC TO INDUCE B-CELL TRANSFORMATION AND MULTIPLE MYELOMA.

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Mature B-cell lymphomas represent the major class of human lymphomas overall, and the treatment of these diseases is a serious challenge in medicine. Biochemical and genetic analysis have firmly established that constitutive NF-κB signaling and deregulation of cMyc expression are recurrent events in the ABC subgroup of Diffuse Large B-cell Lymphoma (ABC□DLBCL), and in Multiple Myeloma (MM). However, it remains to be determined whether constitutive NF-κB signaling and deregulated cMyc expression cooperate to induce B-cell transformation and consequently are at the origin of ABC-BLBCL and/or MM.

To test this hypothesis we used conditional targeted mutagenesis in the mouse, to express a constitutively active IKK2 molecule (IKK2ca) and/or cMyc (MYC) in $CD19^{pos}$ B-cells through tamoxifen-induced Cre-mediated elimination of a STOP cassette. The genetic system of conditional targeted mutagenesis used in this work is unique in two ways. First, transgene activation occurs only in a small fraction of $CD19^{pos}$ B-cells (3-5%) through transient Cre expression, mimicking the sporadic nature of oncogenic events. Second, due to incomplete Cre mediated recombination in individual cells, this $CD19^{pos}$ B-cell population is constituted by $IKK2ca^{pos}/MYC^{pos}$, $IKK2ca^{pos}/MYC^{neg}$ and $IKK2ca^{neg}/MYC^{pos}$ expressing cells, providing all possible "oncogene" combinations in the same mouse at the same time.

While the $KK2ca^{neg}/MYC^{pos}$ B-cells decayed over time, $IKK2ca^{pos}/MYC^{neg}$ B-cells remained constant over time, in agreement with our previous results showing that constitutive NF-kB signaling per se, promotes B-cell survival but not proliferation. By contrast, there was a steady increase of the $IKK2ca^{pos}/MYC^{pos}$ population, and eventually, 100% of the mice succumbed to a plasma cell neoplasia derived from this population, with a median survival of 192 days. Tumor cells were present in bone marrow, spleen, and 75% of the mice exhibited liver metastasis.

Thus, using a conditional targeted mutagenesis system that recapitulates the sporadic nature of oncogenic events, we demonstrate that constitutive NF-κB signaling cooperates with deregulated cMyc expression to induce B-cell transformation and MM in the mouse. These results suggest that this oncogene combination represents a primary event in MM rather than ABC–DLBCL.

ROLE OF MIR-142 FUNCTION IN THE HEMATOPOIETIC SYSTEM

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Among the most prominent microRNAs in the hematopoietic system are two microRNAs generated from a single microRNA precursor, namely miR-142-5p and -3p. These two microRNAs are highly abundantly and almost exclusively expressed in the hematopoietic lineage. As their seed sequences differ from each other, they are predicted to target different groups of molecules. To investigate the functional role of these microRNAs, we generated a conditional allele to be able to delete miR-142 in a cell type specific manner. So far, we have analyzed mice in which miR-142-5p and -3p have been deleted in the germline. This resulted in splenomegaly, a dramatic reduction of T cells in spleen and blood, impeded B cell maturation in bone marrow and spleen, loss of B1 cells, and an increased proportion of granulocytes in spleen. These results and ongoing experiments will be discussed.

DE NOVO SYNTHESIS OF TRUNCATED NOTCH1 RECEPTOR PROTEINS IN IKAROS-DEFICIENT LEUKEMIC T CELLS USING A CRYPTIC INTRAGENIC PROMOTER.

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Mice bearing a knockdown mutation in the Ikaros gene ($Ik^{L/L}$) develop spontaneous T-cell lymphomas/leukemias that exhibit strong activation of the Notch pathway. How and which members of the Notch pathway contribute to T-cell transformation in vivo remain unclear. We investigated the role of Notch1, Notch3 and RBP-Jk in the progression of T-cell leukemias in $Ik^{L/L}$ mice by a genetic approach. Deletion of RBP-Jk markedly delays tumor initiation, indicating that the Notch pathway is essential for the development of Ikaros-deficient T-cell tumors. Deletion of Notch3 did not affect survival, suggesting that Notch3 is not required for tumor progression. Unexpectedly, deletion of Notch1 via its 5' promoter/exon1 significantly accelerated tumor progression. These Notch1-deleted tumors were inhibited in their growth by gamma-secretase inhibitors, showed strong activation of Notch target genes, and accumulated cleaved Notch1 proteins containing 3' PEST mutations. In addition, the tumor cells contained Notch1 transcripts that were initiated from a cryptic intragenic promoter and encode proteins lacking the extracellular domain. These results suggest that the combined loss of Ikaros and the conserved Notch1 promoter results in the synthesis of novel Notch1 oncogenic proteins.

REPRESSION OF *TCRB* RECOMBINATION AT THE NUCLEAR LAMINA

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Allelic exclusion allows only one functional *Tcrb* V-to-DJ rearrangement per thymocyte. This process is initiated by asynchronous recombination and maintained by feedback inhibition. Asynchronous recombination could result from either strictly ordered recombination of alleles, or by infrequent recombination of both alleles. Association with repressive nuclear compartments could potentially suppress *Tcrb* recombination efficiency. Two well characterized repressive compartments are the nuclear lamina and pericentromeric heterochromatin. Previously we used 3D immunoFISH to localize TCR genes and demonstrated that *Tcrb* alleles frequently and stochastically colocalize with the lamina. V-to-DJ recombined *Tcrb* alleles were more often located centrally, which could result either from infrequent V-to-DJ recombination of lamin-associated alleles or recombination of lamin-associated alleles followed by their dissociation from the lamina. We used 3D immunoFISH to label *Tcrb* alleles, the lamina, and DSB repair protein 53BP1 to analyze the location of recently rearranged *Tcrb* genes. We found that recently recombined alleles are underrepresented at the lamina. Therefore *Tcrb* loci do not substantially relocalize following recombination, and association with the lamina reduces recombination efficiency. Because some recombination events do occur at the lamina, we further investigated the conformation of lamin-associated alleles using a program capable of measuring the distance between the centers of *Tcrb* and 53BP1 foci and the surface of the lamina. We found that the 5' end and the 3' end of most *Tcrb* alleles were equidistant from the inner edge of the lamina. However *Tcrb* 53BP1 foci were clearly farther from the lamina than 5' *Tcrb* foci. This indicates that recombination of peripheral *Tcrb* alleles may occur on a subset of alleles that adopt a conformation in which a portion of the locus preferentially dissociates from the lamina. Therefore both the location and orientation of the *Tcrb* locus relative to the lamina likely play roles in asynchronous V-to-DJ recombination.

INTERFERON REGULATORY FACTOR-5 ACTIVATION IN INNATE IMMUNE SIGNALING

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The interferon regulatory factor 5 (IRF5) transcription factor is activated in response to pathogens that activate Tank-binding kinase-1 (TBK-1), Tumor necrosis factor receptor-associated factor-6 (TRAF6), or Receptor interacting protein-2 (RIP2). We compared the individual effects of these activators on the ability of IRF-5 to induce the interleukin-12 (IL-12) p40 promoter and interferon promoters. Compared with TRAF6 or TBK1, RIP2 was the most effective activator of IRF-5 transcriptional activation. The role of RIP2 in signaling by nucleotide-binding oligomerization domain (NOD)-like-receptors suggests that IRF5 may play a preferred role in NOD-like-receptor signaling under physiological conditions. Mass spectrometry was used to identify two specific serine residues phosphorylated on IRF-5 by TBK-1. Introducing phosphomimetic Asp substitutions for these residues confers constitutively active IRF5, suggesting phosphorylation on these residues facilitates TBK-1-mediated IRF5 function. TRAF6, an E3 ubiquitin ligase, stimulates the ubiquitination of IRF-5 and IRF-5 mediated induction of the IL-12 promoter. This induction is senstitive to the deubiquitinating activity of A20 as is the induction by RIP2. Our studies indicate that IRF-5 plays a primary role in innate immune responses to pathogens that activate the NOD-like receptor inflammasome, specific cytokine gene induction, and subsequently cell death.

INTERLEUKIN-23 PRODUCTION IN DENDRITIC CELLS IS NEGATIVELY REGULATED BY PROTEIN PHOSPHATASE 2A

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IL-12 and IL-23 are produced by activated antigen-presenting cells but the two induce distinct immune responses by promoting Th1 and Th17 cell differentiation, respectively. IL-23 is a heterodimeric cytokine consisting of two subunits: p40 that is shared with IL-12 and p19 unique to IL-23. In this study, we showed that the production of IL-23 but not IL-12 was negatively regulated by protein phosphatase 2A (PP2A) in dendritic cells (DC). PP2A inhibits IL-23 production by suppressing the expression of the IL-23p19 gene. Treating DC with okadaic acid that inhibits the PP2A activity or knocking down the catalytic subunit of PP2A with siRNA enhanced IL-23 but not IL-12 production. Unlike PP2A, MAP kinase phosphatase-1 or CYLD did not show an effect on IL-23 production supporting the specificity of PP2A. PP2A-mediated inhibition requires a newly made protein that is likely responsible for bringing PP2A and IKKβ together upon LPS stimulation which then results in the termination of IKK phosphorylation. Thus, our results uncovered an important role of the protein phosphatase in the regulation of IL-23 production and identified PP2A as a novel inhibitor of IL-23p19 expression in DC.

SELECTIVE GENE ACTIVATION BY THE NF-KB FAMILY OF TRANSCRIPTION FACTORS

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The NF-κB signaling pathway in the cytoplasm, the release of NF-kB dimers from IkB complexes, and the subsequent translocation into the nucleus and gene activation by NF-kB have been well studied. However, one central issue that remains unresolved is the precise mechanism by which each member of the NF-kB family differentially regulates the expression of a diverse array of genes in different cell types and in response to different stimuli. The five evolutionarily conserved NF-kB proteins (named RelA [p65], RelB, c-Rel, p50, and p52) contain an N-terminal 300-amino acid Rel homology region (RHR) that is responsible for DNA binding and dimerization. The NF-kB family members p65 and c-Rel recognize similar DNA sequences through identical DNA contact residues, yet the phenotypes of mutant mice suggest that these proteins regulate distinct sets of genes.

Previously, we showed that c-Rel is required for the induction of Il12b transcription upon lipopolysaccharide (LPS) stimulation of bone marrow-derived macrophages (S. Sanjabi et al. PNAS, 2000). We demonstrated that 46 unique residues within an 86-residue segment of the RHR of c-Rel are responsible for the c-Rel requirement for Il12b induction (S. Sanjabi et al., Genes and Dev. 2005). Although the residues of c-Rel and p65 that contact specific bases and the DNA backbone within the NF-kB recognition sequences are identical, homodimers of c-Rel and of a chimeric p65 protein containing the critical c-Rel residues bound with high affinity to a broader range of NF-kB recognition sequences than did wild-type p65 homodimers. These results demonstrate that the unique functions of closely related transcription factor family members can be dictated by differences in the range of DNA sequences recognized at high affinity, despite having similar binding site consensus sequences and DNA contact residues.

These findings highlighted our limited knowledge of the unique DNA-binding properties of each homodimeric and heterodimeric NF-kB species. To better define the similarities and differences in DNA-binding properties, we have critically analyzed binding kinetics of eight dimeric species through the use of surface plasmon resonance. Combined with detailed studies of consensus recognition sequences performed in collaboration with the lab of Martha Bulyk, our results have provided important insights that are likely to help explain the different physiological roles and mechanisms of action of the individual NF-kB family members and dimers.

DYNAMIC CHANGES IN TRANSCRIPTION FACTOR BINDING AT 3' REGULATORY REGION (3'RR) OF IMMUNOGLOBULIN HEAVY CHAIN LOCUS DURING B CELL ACTIVATION

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The immunoglobulin heavy (IgH) chain locus is subject to several tightly regulated processes, such as VDJ joining, somatic hypermutation and class switching (CSR). One of two key cis regulatory elements in the IgH locus, the 3' regulatory region (3'RR) contains multiple enhancers (hs3a, hs1,2, hs3b and hs4) and is required for IgH expression and class switching. Additional DNase I hypersensitive sites are located downstream of hs4. The chromosome conformation capture (3C) assay has revealed that the 3'RR physically interacts with the VH promoter region and with I regions, forming a scaffold on which IgH expression and CSR can occur. My experiments focus on examining the regulation of the 3'RR by various binding factors: these include CTCF, a protein that binds to hs4 downstream sequences and is associated with all mammalian insulators; cohesin, which colocalizes with most of the CTCF binding sites in the genome and thereby contributes to insulator activity; and transcription factors YY1 and Pax5, which are known to influence IgH-specific processes. Chromatin immuno-precipitation (ChIP) assays revealed dynamic binding of these factors to 3'RR elements during B cell development and during LPS stimulation for CSR. My experiments show that YY1 acquires binding to hs1,2 after 48 hrs of LPS stimulation. Pax5 binds to hs4 in resting and 96 hr LPS-activated B cells but not at 48 hrs when germ line transcripts are expressed. In addition, stimulation of normal B cells, while adding anti-IgM (which prevents switching), changes Pax5 binding, further suggesting a role for Pax5 in CSR. Binding of CTCF to hs3b and hs4 of the 3'RR is modest throughout LPS stimulation. However, cohesin binds to hs3b and hs4 at 0 hrs and 96 hrs but not at 48 hrs. Hence, cohesin binding is independent of CTCF. Such regulated binding of these factors at hs3b and hs4 indicate their potential roles in switching and germ line transcription as hs3b and 4 are critical for these events. As an approach to identify the function of CTCF in the regulation of IgH, we knocked down CTCF with shRNA in pre-B, B and plasma cell lines. Initial experiments showed that CTCF was not essential for IgH loop formation in plasma cells. Interestingly, CTCF appears to contribute to the regulation of Pax5 binding in the 3'RR. In CTCF-depleted cell lines, Pax5 acquired binding to specific 3'RR sites. In addition, during B cell development, Pax5 binding to hs1.2 decreases while CTCF binding to hs1.2 increases. In further support of this reciprocity, EMSA shows that CTCF can compete with Pax5 binding in vitro. The mechanisms by which transcription factor binding to the 3'RR is regulated are under study. (NIH AI13509)

SUSTAINED EXPRESSION OF MIR-155 IN HEMATOPOIETIC PROGENITOR CELLS CAUSES A MYELOPROLIFERATIVE DISORDER IN MICE VIA REPRESSION OF THE SHIP1 PHOSPHATASE

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Mammalian microRNAs (miRNAs) are emerging as key regulators of the immune system. Here we report a strong but transient induction of miR-155 in mouse bone marrow after injection of bacterial lipopolysaccharide (LPS) correlated with granulocyte/monocyte (GM) expansion. Demonstrating the sufficiency of miR-155 to drive GM expansion, enforced expression in mouse bone marrow cells caused GM proliferation in a manner reminiscent of LPS treatment. MiR-155 expressing mice also exhibited marked splenomegaly and extramedullary hematopoiesis. Extending relevance to human disease, mir-155 levels were 4.5 times higher in the bone marrow of acute myeloid leukemia (AML) patients as compared to normal, and 8 times higher in M4/M5 AML patients as compared to normal. To determine which genes mir-155 regulates, Raw 264.7 cells were stably transfected with a mir-155 expressing vector, and a microarray showed downregulation of 1080 genes. Of these, 89 contained conserved mir-155 binding sites as determined by Targetscan 4.0. Among these were eleven genes relevant to hematopoiesis and myeloproliferation. We knocked each of these genes down in the mouse hematopoietic system using retroviral vectors expressing small interfering RNAs (siRNAs), and found that specific knockdown of SHIP1 results in a myeloproliferative disorder (MPD) that is strikingly similar to that observed in miR-155 expressing mice. We used gain and loss of function approaches to show that miR-155 represses SHIP1 through direct 3'UTR interactions that have been conserved throughout evolution. Repression of endogenous SHIP1 by miR-155 occurred following sustained overexpression of either mouse or human miR-155 in hematopoietic cells both in vitro and in vivo, and resulted in increased activation of the kinase AKT during the cellular response to LPS. Furthermore, SHIP1 was also repressed by physiologically regulated miR-155, which was observed in LPS treated wild type versus miR-155$^{-/-}$ primary macrophages. Taken together, our study unveils a molecular link between miR-155 and SHIP1, and provides evidence that miR-155 repression of SHIP1 contributes to the pathogenesis of the myeloid diseases MPD and AML.

EPIGENETIC REGULATION OF *TCRA* GENE RECOMBINATION IN DEVELOPING T CELLS.

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Lymphocyte development is driven by V(D)J recombination, a process by which antigen receptor gene segments are rearranged to create a vast repertoire of antigen receptor specificity. Because V(D)J recombination entails the cleavage and joining of widely dispersed gene segments many millions of times each day, the antigen receptor loci are particularly dynamic regions of the genome, and even a miniscule error rate would still carry considerable risk of translocation. Indeed, leukemias and lymphomas are among the most common malignancies in humans, and chromosomal translocations involving the antigen receptor loci are a common underlying mechanism. Multiple mechanisms regulate sequential accessibility of the loci to the recombinase machinery including changes in histone modifications and in nuclear compartmentalization. Our lab is focused on investigating the mechanisms by which recombination is coordinated on the individual alleles of antigen-receptor loci during B and T cell development. Our recent analyses of the *Immunoglobulin* loci suggest a new model for recombination whereby the V(D)J recombinase and DNA damage response machinery coordinate DNA cleavage and nuclear positioning to initiate allelic exclusion and preserve genomic integrity during recombination (Hewitt et al, 2009). Homologous *Ig* alleles physically pair up in a stage-specific manner that parallels the sequential stages of their recombination. We also found, surprisingly, that this interallelic association is mediated by the V(D)J recombinase and the introduction of a double-strand break at one *Ig* allele induces ATM-dependent repositioning of the other allele to pericentromeric heterochromatin to prevent further cleavage. An absence of ATM leads to biallelic RAG-mediated cleavage, which results in breaks and translocations.We are now investigating how the recombination process of the *Tcra* locus is regulated in developing thymocytes. This locus is of interest because: i) the *Tcrd* locus is embedded within *Tcra*, and is recombined in early double-negative T cells, whereas *Tcra* is recombined later at the double-positive T cell stage, and ii) *Tcrd* is the only antigen receptor locus that is not allelically excluded. Using 3D-DNA in *situ* hybridization and immunofluorescence, we focused on understanding how the recombinase machinery, ATM and other components of the DNA double stranded break repair pathway, and environmental signaling pathway, coordinate the regulation of *Tcra* recombination. Our data suggest that a dialog between recombination and repair pathways, as well as environmental signals influence accessibility of the *Tcra* locus.

NUCLEAR TRANSPORT DYNAMICS OF STAT6

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Signal transducers and activators of transcription (STATs) are latent proteins that are activated in response to different cytokines and hormones and transduce signals from the cytoplasm to induce gene expression in the nucleus. There are seven members in this family in mammals and STAT6 transcription factor is essential for the development of protective immunity, however the consequences of its activity can also contribute to the pathogenesis of autoimmune disease. It is activated by tyrosine phosphorylation in response to cytokine stimulation, but the mechanisms by which it enters the nucleus have not been defined. We used live cell imaging techniques (fluorescence recovery after photobleaching and fluorescence loss in photobleaching) to demonstrate the continual nuclear import and export of STAT6 is independent of tyrosine phosphorylation. The protein domain required for nuclear entry includes the coiled coil region of STAT6 and was found to function similarly before or after cytokine stimulation. The dynamic nuclear shuttling of STAT6 appears to be mediated by the classical importin-α-importin-β1 system. Although STAT6 is imported to the nucleus continually, it accumulates in the nucleus following tyrosine phosphorylation due to its ability to bind DNA. These findings will impact both diagnostic strategies and strategies to block the deleterious effects of STAT6 in autoimmunity.

AKT IS REQUIRED FOR NF-KB-DEPENDENT TNF-A PRODUCTION

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NF-κB family transcription factors are critical regulators of gene transcription. Akt is known to contribute to the activation of numerous downstream pathways including NF-κB. Using an NF-κB enriched microarray we found that many NF-κB regulated genes were upregulated after T cell activation of which only a subset were affected by Akt inhibition. These genes include TNF-α, Tnfsf14, Csf2, Il6, Il10 etc. In this study we further investigated the role of Akt in NF-κB-mediated TNF-α gene expression. The chromatin immuno-precipitation assay showed that recruitment of the NF-κB p65 subunit to the promoter of TNF-α was reduced by inhibition of Akt. The DNA binding activity of p65 was also decreased by Akt inhibition. Similar results were demonstrated on TNF-α production in a Th2 cell line, bone marrow derived macrophages and primary Th1 cells and human whole blood in the presence of Akt inhibitor. Taken together, these data provide compelling evidence for the role of Akt in NF-κB mediated TNF-α gene expression.

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P18^{INK4C} ORCHESTRATES HOMEOSTATIC CELL CYCLE CONTROL OF PLASMA CELL DIFFERENTIATION IN RAPIDLY CYCLING AND APOPTOTIC PRECURSORS

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Induction of G_1 cell cycle arrest by the Cdk4/6 inhibitor p18^{INK4c} (p18) is critical for the generation of non-cycling end-stage plasma cells. In the absence of p18, CD138$^+$ plasmacytoid cells continue to cycle but are rapidly eliminated by apoptosis *in situ*. These findings provide the first direct evidence for cell cycle control of plasma cell differentiation *in vivo*, and suggest that cell cycle attenuation by p18 imposes a final homeostatic checkpoint in plasma cell differentiation. We now show that p18 selectively acts in a rare population of rapidly cycling and apoptotic CD138^{hi}/B220^{hi} **i**ntermediate **p**lasma **c**ells (iPCs). iPCs are poised to differentiate to IgG-secreting plasma cells, while retaining activated B cell signatures such as CD40, CD22 and GL7. p18 is required for the generation or maintenance of the iPC pool and promotes timely differentiation to plasma cells. Conversely, CD40 signaling accelerates the cell cycle in opposition to p18 in iPCs and enhances apoptosis. Consistent with cell cycle control of apoptosis, the iPC pool is expanded by the loss of the pro-apoptotic Bim or overexpression of the anti-apoptotic Bcl-xL. p18 is dispensable for the expression of transcription factors that are essential for germinal center formation or plasma cell differentiation. Blimp-1 and Bcl-6 are expressed fully and mutually exclusively in individual iPCs, except for a minor proportion expressing both. These Blimp-1$^+$/Bcl-6$^+$ iPCs are preferentially protected by p18 and Bcl-xL. On this basis, we propose that p18 targets iPCs, which are dynamic and transient intermediates between activated B cells and plasma cells, for homeostatic control of terminal plasma cell differentiation by cell cycle-coupled apoptosis.

DEFINING PRINCIPLES OF CHROMOSOMAL TRANSLOCATIONS IN LYMPHOID CELLS

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Translocations in tumors are considered to represent very low frequency events that are strongly selected at the cellular level. However, we have recently demonstrated that mechanistic factors have the ability to substantially influence the frequency of recurrent translocations. Such mechanistic factors include the frequency of DSBs at two loci, the spatial proximity of the two loci, and the repair pathways functionally available to carry out the actual joining. To gain further insights into mechanisms that promote translocations, we are developing high-throughput methods to identify translocation partners from fixed DNA DSBs in a genome-wide fashion. By targeting I-SceI recognition sequences into the γ1 switch region or into the c-myc locus we were able to ask for translocations appearing in cycling primary B cells from a defined DSB site.
Our preliminary analysis showed that in WT or H2AX deficient cells a defined DSB does not translocate to a very limited number of chromosomal loci, as suggested from some tumor studies, but rather has a relatively broad distribution to many sites on most chromosomes. On the other hand, there are areas that appear to be targeted more frequently for translocations, including sites along the same chromosome. Ongoing analyses which seek to saturate translocation maps and compare translocations frequencies in different genetic backgrounds (e.g. deficient in particular DSB response or repair factors) should help to more clearly define the role in primary B cells of various factors, including nuclear organization, DNA sequence, and repair pathways in suppressing and/or enhancing recurrent translocations.

THE ADP-RIBOSYLTRANSFERASE PARP-14 MEDIATES IL-4-INDUCED SURVIVAL SIGNALING IN B CELLS BY ORCHESTRATION OF A COORDINATE METABOLIC RESPONSE.

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Interleukin (IL)-4 is an important survival factor for hematopoietic cells such as lymphocytes. Mechanisms of anti-apoptotic signaling induced by IL-4 apart from PI3K-Akt activation are not clear. Previous work identified PARP-14 as a protein with intrinsic ADP-ribosyltransferase activity that is highly expressed in B cells and interacts with the IL-4-induced transcription factor Stat6. ADP-ribosyltransferases and PARPs catalyze mono- and poly-ADP-ribosylation, transferring ADP from NAD+ to target proteins. ADP-ribosylation is a post-translational modification which influences gene transcription, DNA repair and apoptosis. Although there are 17 PARP family members, almost nothing is known about biological roles or mechanisms of mammalian PARPs other than PARP-1. We now show that roles of PARP-14 in mediating IL-4 induction of metabolic functions of B lymphocytes are central to a requirement for PARP-14 in IL-4-induced survival signaling. The evidence indicates that a coordinate enhancement of glycolysis and mitochondrial function is orchestrated by PARP-14 in B cells and is crucial for the protective role of IL-4. In addition, AMP-activated protein kinase (AMPK) activity regulated by IL-4 and metabolic fitness are pre-conditions for PARP-14-dependent survival signaling. Consistent with a vital biological role, PARP-14 regulates energy metabolism in B cells from Eμ-Myc transgenic mice and contributes to pathogenesis of Eμ-Myc-induced lymphoma. Together, these results reveal a novel function of a mammalian ADP-ribosyltransferase in mediating cytokine-regulated metabolic activity and survival of lymphocytes.

BERBERINE INHIBITS LPS-INDUCED NO PRODUCTION POSSIBLY DUE TO THE ACTIVATION OF AKT PATHWAY IN RAW 264.7 CELLS

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The berberine (BBR), an isoquinoline alkaloidal component of Coptidis Rhizoma, became known to have anti-inflammatory, anti-oxidative and anti-diabetic effects. The molecular mechanism, however, involved in the anti-inflammatory effect of BBR is not fully understood. In this study, we found that berberine suppressed the prostaglandin E2 (PGE2) and nitric oxide (NO) production in LPS-activated RAW 264.7 cells. Interestingly, BBR decreased the enzyme activity of COX-2 but failed to inhibit the COX-2 expression. Meanwhile, NO reduction by BBR was due to the inhibition of inducible nitric oxide (iNOS). Moreover, our results revealed that the repression of iNOS but not COX-2 expression may come from the activation of Akt, downstream of the PI3K signal cascade. The treatment wortmannin, PI3K inhibitor, protected the BBR-induced reduction of iNOS expression. In summary, our data indicated that BBR exhibits the anti-inflammatory properties via differential inhibitory effect on COX-2 and iNOS activities in LPS-activated RAW 264.7 cells.

GENETIC REGULATION OF HUMAN EFFECTOR VS CENTRAL MEMORY CYTOTOXIC LYMPHOCYTE GENERATION BY IL-12 AND IFN-A

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$CD8^+$ cytotoxic T lymphocytes (CTLs) play a major role in defense against intracellular pathogens. While effector CTLs eliminate the infection, a small population of memory cells are retained that yields more rapid and robust response upon re-infection. Antigen presenting cells secrete an array of innate cytokines including IL-12 and IFN-α after recognition of pathogens. Both IL-12 and IFN-α have been shown to act as the third signal regulating the development of CTLs. We have shown that these two cytokines have a non-redundant effect in generation of human effector vs memory CTL. IL-12 alone is sufficient for effector CTL genesis marked by IFN-γ and TNF-α production, as well as increased cytolytic activity. In contrast, human CTLs activated in the presence of IFN-α developed central memory phenotype. Using microarray analysis we have investigated how IL-12 and IFN-α differentially regulate the genetic programming pathways that give rise to effector vs central memory among multiple human donors. We have also analyzed the gene expression patterns of cells sorted out of peripheral blood that display surface markers of effector and memory CTL. With this approach, we will determine the unique pathways that are upregulated in response to either IL-12 or IFN-α, which eventually leads to stably expressed gene expression in memory cells.

THE ACTIVATED RAS/MEK/ERK PATHWAY REDUCES THE ANTI-VIRAL INTERFERON-A RESPONSE BY INHIBITING GENE TRANSCRIPTION OF MULTIPLE INTERFERON-A-RESPONSIVE GENES

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Interferons (IFN) are produced by most cells in the body in response to viral infection and induce the expression of many diverse genes with anti-viral, anti-tumor and immunoregulatory functions. Because IFNs have such diverse physiological roles they have been used clinically for the treatment of Hepatitis C virus infection, cancer and Multiple Sclerosis. However, there are many cellular suppressors that can reduce the efficacy of IFN therapy. Our previous work has shown that activation of the Ras/MEK pathway allows viral replication even in the presence of IFN-α. In addition, we have recently shown that activation of the Ras/MEK pathway in NIH 3T3 mouse fibroblast cells inhibits transcription of the STAT2 gene, an essential mediator of the IFN-α pathway, to significantly, but partially, reduce the IFN-α response. Here we sought to identify additional genes, which are necessary for an effective IFN-α response, whose expression is repressed by the Ras/MEK pathway. Using global gene expression analysis we identified IFN-inducible genes that are upregulated by treatment with MEK inhibitor U0126 (MEK-downregulated IFN-inducible (MDII) genes). The expression levels of five anti-viral genes (STAT2, GBP2, Ptx3, IRF7 and Iigp2) in response to U0126 and/or IFN treatment were confirmed by quantitative RT-PCR. We hypothesize that a common transcriptional regulator controls expression of many of the MDII genes to globally reduce IFN-α signaling. To identify this unknown transcriptional regulator, we have analyzed the promoter regions of a number of these genes using luciferase reporter constructs and identified potential regulatory regions that respond to U0126 treatment. Further *in silico* analysis of the promoter regions followed by ChIP experiments to analyze promoter occupancy by putative transcription factors will be performed to identify the transcription factor responsible for regulating expression of the MDII genes. Identification of a transcriptional regulator used by cellular suppressors of the IFN pathway may reveal additional therapeutic strategies to restore IFN-responsiveness to cell types that are resistant to IFN therapy.

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STOMATIN-LIKE PROTEIN 2 RECRUITS PROHIBITINS TO CARDIOLIPIN-ENRICHED MICRODOMAINS AND REGULATES MITOCHONDRIAL BIOGENESIS DURING T CELL ACTIVATION

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The demands for energy substrates in a eukaryotic cell rise as the cell transitions from a resting state to an activated state. This process is well documented in lymphocytes undergoing mitogenic responses. In these cells, the increasing energy requirements are met by increasing production of ATP either by glycolysis or by oxidative phosphorylation (OXPHOS), both processes being regulated by signaling from antigen receptors and costimulatory molecules. Scattered evidence suggests that these demands on cellular bioenergetics correlate with mitochondrial biogenesis. However, the molecules that regulate mitochondrial biogenesis in response to lymphocyte activation remain unknown.

We have reported that stomatin-like protein 2 (SLP-2), a mitochondrial inner membrane protein, is up-regulated in human T lymphocytes during activation and this correlates with enhanced T cell function. The mechanism by which SLP-2 modulates T cell activation is currently unknown. Our work indicates that SLP-2 interacts with prohibitin-1 and 2 and binds to the mitochondrial inner membrane phospholipid cardiolipin. Up-regulation of SLP-2 expression increases de novo synthesis of cardiolipin and the cellular cardiolipin content as well as the formation of metabolically active mitochondrial membranes. Up-regulation of SLP-2 is also associated with an increase in the expression of the nuclear transcriptional co-activator PGC-1α (required for mitochondrial biogenesis) and in mitochondrial DNA replication. Altogether, these events translate into increased intracellular ATP stores, increased mitochondrial uptake of calcium, and enhanced resistance to apoptosis through the mitochondria-dependent intrinsic pathway, the end result being increased T lymphocyte effector responses. We have generated a conditional T cell knockout mouse for SLP-2 and preliminary data corroborate the functional phenotype *in vivo*.

Based on these results, we propose that SLP-2 modulates T cell activation by recruiting prohibitins to cardiolipin-enriched microdomains in the mitochondrial inner membrane and regulating mitochondrial biogenesis.

PAX5 AND ITS DOWNSTREAM EFFECTOR CD19 ARE MAJOR POSITIVE REGULATORS OF C-MYC PROTEIN LEVELS.

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Pax5, a B-cell specific transcription factor, is crucial for normal B-cell development and is also overexpressed through chromosomal translocations in a subset of B-cell neoplasms. Previously, we had shown that activation of ITAM proteins and B-cell receptor signaling by Pax5 contributes to B-lymphomagenesis (Cozma et al, J Clin Invest, 2007). However, the molecular basis of its transforming activity is not fully understood. In particular, it is not known whether and how Pax5 interacts with other oncogenic transcription factors. Our new data demonstrate that overexpression of Pax5 increases c-Myc protein stability and steady-state levels. This was discovered using Pax5-negative plasmacytoma cell lines and Myc5, a c-Myc-induced murine lymphoma with spontaneous silencing of Pax5 (Yu et al, Blood, 2003; Johnson et al, Nat Immunol, 2004). Consequently, the ability of c-Myc to bind to and inhibit the promoter of the tumor suppressor miR15a/16-1 (Chang et al, Nat Genet, 2008) was markedly enhanced in presence of Pax5. Conversely, Pax5 knockdown decreased c-Myc protein expression in Myc5 and P493-6 cells, where c-Myc expression is driven by the retroviral LTR and the synthetic tetO7-TP promoter, respectively. This suggested a promoter-independent, post-translational mechanism of c-Myc regulation. After analyzing several Pax5 effectors in Pax5-negative cells, we found that constitutive ITAM signaling does not rescue c-Myc levels, while CD19, a B cell-specific co-receptor, was a potent inducer of c-Myc protein. This effect was due at least in part to the restoration of AKT signaling and inhibition of GSK3-beta function. Moreover, ectopic CD19 expression in murine plasmacytoma and Myc5 cells was sufficient to boost cell expansion both in vitro and in vivo. Thus, Pax5 and its downstream effector CD19 could be considered major regulators of c-Myc oncogenic activity in B-cell neoplasms. This work was supported by the Leukemia & Lymphoma Society fellowship #5259-09 to EYC and the National Institutes of Health grant R01CA102709 to A.T.-T.

TRANSCRIPTION FACTOR NETWORK REGULATING CD4+ T HELPER 17 LINEAGE DIFFERENTIATION

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IL-17-producing T lymphocytes have recently been shown to comprise a discrete lineage of proinflammatory helper T cells, termed Th17 cells. While important for the clearance of a variety of pathogens at mucosal surfaces, Th17 cells have also been implicated as major contributors to tissue inflammation and autoimmunity. Our group has identified the orphan nuclear receptor RORγt as a key transcription factor (TF) regulating Th17 development, being both required and sufficient for the generation of IL-17-producing cells. Other nuclear factors, namely STAT3 and IRF-4, have also been demonstrated to be essential for this process. The objective of this study is to delineate the otherwise obscure mechanisms by which RORγt regulates transcription, and how RORγt collaborates with STAT3 and IRF-4 to mediate changes in chromatin structure and gene expression at target loci. To this end, we will employ chromatin immunoprecipitation followed by high throughput sequencing (ChIP-Seq) to compile genome-wide maps of direct TF binding and of histone modifications in naive T cells activated under Th17-polarizing conditions. This strategy will be coupled with transcriptome analysis of wild-type and TF-deficient Th17 cells to define factor-dependent expression profiles, and distinguish activated versus repressed targets. Preliminary analyses reveal a high degree of overlap in Th17-associated TF binding, suggesting coordinate regulation of gene expression. This work will provide mechanistic insight into the function of RORγt, STAT3, and IRF-4 in Th17 cells and, moreover, delineate the transcriptional regulatory network that underlies Th17 lineage specification and function.

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EPIGENETIC REGULATION OF THE HUMAN IL-3/GM-CSF LOCUS DURING T CELL DEVELPMENT

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The closely linked IL-3 and GM-CSF genes are located within a cluster of cytokine genes co expressed in activated T cells. Their activation in response to TCR signaling pathways is controlled by specific inducible upstream enhancers.
To study the developmental regulation of this locus in T lineage cells we created a transgenic mouse model encompassing 130 kb of the human IL-3 and GM CSF genes plus the known enhancers. We demonstrated that the IL 3/GM-CSF locus undergoes progressive stages of activation. Looking first at immature T cells, we found that the IL-3/GM-CSF locus was epigenetically and transcriptionally silent in CD4/CD8 double positive thymocytes, thereby minimizing the potential for inappropriate activation during the course of TCR selection. Thymocytes lacked all of the tissue-specific and inducible DNaseI Hypersensitive Sites (DHSs) associated with regulatory elements, with the exception of the insulator that segregates the two genes. Intra-cellular FACS staining of mature T cells revealed that the IL-3 and GM-CSF genes are differentially regulated with stepwise increases in active modifications and the proportion of cytokine-expressing cells, throughout the course of T cell differentiation. Freshly isolated spleen T cells exhibited essentially no inducible IL-3 expression and only 6% of cells could express GM-CSF. The locus did not reach its maximal transcriptional potential until after T cells had undergone blast cell transformation to become fully activated proliferating T cells. In stimulated T blast cell cultures, 24% of cells expressed both IL-3 and GM-CSF, 37% expressed just GM-CSF, almost no cells expressed IL-3 alone, and 40% of cells expressed neither gene. Hence, there appears to be stochastic activation of this locus, as has been observed for the IL-4 and IL-13 genes. The transition from thymocytes to T blast cells was accompanied by the acquisition of active histone modifications within tissue-specific DHSs located far upstream and downstream of the locus, and the ability to induce DHSs at enhancers. The ability to express IL-3 was dependent not on the previously defined proximal IL-3 enhancer, but on a novel powerful enhancer located 37 kb upstream. We also found that memory CD4 positive T cells, but not naïve T cells, maintain a remodelled chromatin structure resembling that seen in T blast cells.

HETEROGENEITY OF HUMAN IL-22-PRODUCING CD4+ T CELLS

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In addition to IL-17A and IL-17F, mouse Th17 cells also produce the IL-10-related cytokine IL-22. IL-22 plays a non-redundant role in immunity to pathogenic bacteria such as *Klebsiella pneumoniae* and *Citrobacter rodentium*. Similar to IL-10, both pro and anti-inflammatory effects have been described for IL-22. Th17 cell-derived IL-22 has been shown to mediate IL-23-induced skin inflammation in models of psoriasis, indicating a proinflammatory function of IL-22. However, IL-22 has also potent anti-inflammatory activities in models of acute hepatitis and inflammatory bowel disease. These studies showed that, although IL-17 and IL-22 are coexpressed by inflammatory Th17 cells in the mouse, these cytokines could have diverging activities on inflammatory responses depending on the tissue and the model studied. Recent reports have suggested that human IL-22 producing $CD4^+$ T cells may represent a separate T helper cell subset. We have determined which human $CD4^+$ T cell subsets secrete IL-22 and how IL-22 expression is regulated during $CD4^+$ T cell differentiation. We found that in peripheral blood, similar to IL-17, most of the IL-22-secreting T cells were memory cells expressing the chemokine receptor CCR6. Analysis of cytokine production at the single cell level revealed than only one third of Th17 cells also produced IL-22 whereas the major IL-22-secreting cell populations were a subset of Th1 cells and “Th22” cells that produce neither IL-17 nor IFN-γ. Finally, we identified a small population of $CD4^+$ T cell producing the 3 cytokines. Thus, our results indicate that IL-22-producing $CD4^+$ T cells represent a heterogeneous cell population.
We were then interested in the molecular mechanisms involved in the differentiation of IL-22 producing T cells. We found that in cord blood-derived naïve $CD4^+$ T cells, T cell receptor signaling was sufficient to induce IL-22 expression, and this could be inhibited by cyclosporine A. Addition of pro-inflammatory cytokines increased IL-22 expression, while TGF-β and IL-4 strongly decreased IL-22 production.
In conclusion, we found that only a minority of Th17 cells secrete this cytokine suggesting that IL-22 is not a Th17 “signature” cytokine in humans. Our results indicate that different human $CD4^+$ T cell subsets can produce IL-22. This heterogeneity could explain the dual role observed for IL-22-producing cells, which may depend on the additional cytokines they produce. Our current work addresses the epigenetic modifications at the IFNG/IL26/IL22 locus that could determine this heterogeneity in cytokine gene expression.

MODULAR REGULATION OF TRANSCRIPTIONAL CHANGES INDUCED BY ALVEOLAR TRANSMIGRATION OF HUMAN NEUTROPHILS

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The accumulation of large numbers of neutrophils in the lungs and airways is common to many forms of inflammatory lung disease, and large numbers of neutrophils accumulate in the lungs and airways during acute lung injury. Phenotypic changes and gene expression changes that neutrophils undergo as they leave the peripheral circulation and migrate into the airspaces have not been explored in the context of chemotactic signaling, and in relation to transmigration in other immune cell types. In experimental models, neutrophils rapidly enter the pulmonary parenchyma and then migrate into the alveolar space after systemic or pulmonary administration of bacteria or bacterial products, such as endotoxin (i.e., lipopolysaccharide or LPS). The importance of neutrophils in contributing to the development and severity of ALI is demonstrated by studies in which lung vascular permeability and other indices of lung injury are attenuated following endotoxin challenge or other pathophysiologic insult in neutropenic animals.

We employed a human model of Acute Lung Injury based on bronchoscopic instillation of endotoxin in a lung subsegment followed 16 hours later by repeat bronchoscopy and lavage, yielding from 35 to 500 million neutrophils. Functional assays including survival, cytokine release, respiratory burst, and chemotaxis were performed, and this data and the gene expression data were contrasted with peripheral blood neutrophils isolated from the same individual in order to isolate population variability.

Overall, these experiments demonstrate that neutrophils undergo profound alterations in gene expression and functional properties when they migrate from the peripheral circulation into the lung in response to an endotoxin challenge. Statistically significant changes in gene expression occur in approximately 15% of the neutrophil transcriptome. Pulmonary neutrophils have greatly diminished chemotactic abilities, a reduced priming index, and enhanced superoxide anion release in response to fMLP stimulation. These alterations are unlikely to have been acquired through direct exposure of the neutrophils to endotoxin, as endotoxin is rapidly cleared from the lungs, but rather as a result of contact with pulmonary cell populations, such as endothelial and epithelial cells, encountered during migration from the blood to the lung as well as through exposure to the proinflammatory milieu induced in the alveoli by endotoxin stimulated macrophages and epithelial cells.

FUNCTIONAL ANALYSIS OF PP2A INHIBITOR PROTEIN CIP2A IN LYMPHOCYTE DEVELOPMENT AND IMMUNE SYSTEM RESPONSE

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Our laboratory has recently identified a novel human oncoprotein, designated as Cancerous Inhibitor of PP2A (CIP2A). CIP2A is overexpressed in common human malignancies and promotes breast cancer aggressiveness (Junttila et al :, Cell 2007 ; Come et al., Cancer Research 2009). In cancer cells, CIP2A promotes malignant growth but its physiological role remains to be determined. The work presented in this abstract suggests that CIP2A is involved in the development and responses of the immune system.

To address the physiological role of CIP2A, we have generated a CIP2A deficient and a transgenic mouse model constitutively expressing the protein. In addition to testis, bone marrow, spleen, lymph nodes and thymus are among the very few organs expressing CIP2A in human or mouse tissues. Moreover, CIP2A is expressed at significant levels in germinal center cells, both in the spleen and lymph nodes, indicating a role for CIP2A in lymphocyte activation. This idea is reinforced by the fact that ex-vivo activated B and T cells strongly upregulate CIP2A.

Under SPF conditions, CIP2A deficient mice present a normal apparent phenotype and lifespan. However, loss of CIP2A alters B cell numbers, suggesting a role for CIP2A in lymphocyte homeostasis. In order to study this possible link between CIP2A and lymphocyte activation, we are planning to immunize mouse models deficient or constitutively expressing CIP2A and compare their immune response to the one of normal mice. Finally, to investigate whether CIP2A is involved in lymphomagenesis, we have crossed our knock-out mice with the Eμ-Myc lymphoma model. Results of these in vivo studies will be presented.

The selective profiles of CIP2A in the immune system and lymphocytes suggests it to be an important regulator of the immune system. We aim at determining the precise role of this recently discovered protein in the development of immune system and response.

CONTROL OF IL-6 EXPRESSION IN B CELLS BY OCTAMER BINDING FACTORS

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The pro inflammatory cytokine IL-6 has been shown to promote the development of activated CD4+ T cells into T follicular helper cells (T_{FH}) during an immune response. T_{FH} cells secrete IL-21, and collaborate with B cells in the formation of germinal centres (GC) during T-cell-dependent antibody responses. Dendritic cells and macrophages have been implicated in supplying IL-6 to the activated CD4+ T cells. Here, we demonstrate that B cells are also a source of IL-6. We observed that IL-6 expression in B cells was dependent on the transcription factor Oct2 and it's co-activator OBF-1 in toll-like-receptor ligand stimulated in vitro cultures, as well as in B cells derived from influenza infected mice. IL-6 expression in follicular B cells in the draining lymph node was an early event during an influenza response, peaking on day two of infection.

To study the molecular regulation of IL-6 expression by Oct2 or OBF-1 we analyzed the IL-6 locus for putative binding sites (octamer motifs). We identified several octamer sites within the IL-6 promoter region in silicio and were able to show direct interaction of the transcription factors with a subset of the sites.

Finally, the IL-6 secreted by activated B cells was sufficient to promote the differentiation of IL-21-secreting CD4+ T cells in an in vitro co-culture system. The induction of IL-21-secreting CD4+ T cells required OBF-1 in the activated B cells. This study demonstrates the dependence of IL-6 expression in B cells on the transcription factors Oct2 and OBF-1, and suggests a role for B cell-derived IL-6 in the formation of IL-21 producing T helper cell subsets.

THE SRF NETWORK IN THYMOCYTE SELECTION

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The SRF cofactor SAP-1, a member of the Ternary Complex Factor (TCF) subfamily of Ets domain proteins, is an ERK target required for thymocyte positive selection. Although SAP-1 is the predominant TCF in thymocytes, the other family members, Elk-1 and Net, are also expressed, and we therefore tested their role in selection. Elk-1 deletion did not impair positive selection, but exacerbated the SAP-1 defect. In contrast, the Netδ mutation, a putative null, had no deleterious effect either alone or in combination with SAP-1 deficiency, and triply deficient thymocytes were no more defective than SAP-1-/- Elk-1-/- cells. Inactivation of the other TCFs did not affect SAP-1-independent processes, including β-selection, Treg selection and negative selection, although reduced marginal-zone B cells were observed in SAP-1-/- Elk-1-/- animals. SAP-1 target genes in TCR-stimulated DP cells whose transcription was further impaired by Elk-1 inactivation, including Egr-1 and Egr-2, were identified by array analysis as probable SAP-1 effectors in positive selection. Although chromatin immunoprecipitation revealed subtle differences in promoter targeting specificity between the TCFs, ectopic expression of Elk-1 could rescue the SAP-1 selection defect, indicating that it is the expression level of SAP-1 in thymus relative to Elk-1, rather than any specific functionality, that underlies its critical role in thymocyte development. In contrast, ectopic Net expression did not rescue the SAP-1 null selection defect, which may reflect differences in target gene selection by this TCF.
Inactivation of SRF itself results in a complete block in thymocyte positive selection. We used retroviral expression of SRF mutants in an SRF null background to investigate this phenomenon in more detail. Wildtype SRF rescued the positive selection defect. In contrast, a SRF V194E, a point mutant that is incapable of interaction both with the TCFs and a second family of SRF co-activators, the MRTFs, failed to rescue the selection defect, indicating that cofactor interaction is essential for SRF activity in this context. Fusion of the TCF regulatory domain to SRF SRF V194E restored its activity, and this required the integrity of its ERK phosphorylation sites. The major role of SRF in thymocyte development is thus to provide a platform for TCF recruitment, enabling ERK mediated activation of target genes essential for the selection process.

AUTOREACTIVE T CELL RESPONSES AGAINST OXIDATIVE STRESS-INDUCED MODIFICATIONS OF SELF ANTIGENS

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The immune system relies on a tightly regulated balance to ensure proper activation against pathogens while minimizing the risks of an autoimmune attack that could result in disease. While many aspects of central and peripheral tolerance mechanisms have been elucidated, important questions related to adaptive immunity to self antigens remain to be evaluated. One area that remains poorly studied is the effect of oxidative damage in altering self proteins, how these modified epitopes can be recognized by the immune system and what are the outcomes of this recognition. Oxidative stress induced by aging, smoking, pathogens, tumors and inflammatory responses can lead to oxidative modifications of proteins and peptides and therefore tilt the balance within the immune system. Our lab has developed an *in vivo* mouse model to study tissue-specific autoimmunity by analyzing the immune response against modified antigens coming from the retina. Carboxyethylpyrrole (CEP) is an oxidation product of docosahexaenoic acid (DHA), a fatty acid highly concentrated in the outer retina, and has been implicated in the pathogenesis of age-related macular degeneration (AMD): CEP-adducted proteins and anti-CEP autoantibodies are elevated in AMD patients. Similar to the human disease, immunization of mice with CEP-MSA (mouse serum albumin) results in autoantibodies and AMD-like pathology. *Ex vivo* restimulation of splenocytes results in the activation of CD4+ T cells (associated with IL-17 and IFN-gamma production) as well as CD8+ T cells, suggesting the generation of immunological memory against these antigens. In addition, we have observed immunogenicity against other lipid oxidative modifications, such as carboxyalkylpyrroles (CAP). Our results provide proof of principle that oxidative stress-induced modification of self antigens can produce an adaptive immune response and establish our system as an ideal model to investigate this underestimated type of immunity. This work will lead to a better understanding of the basic signaling events that are required for maintenance of tolerance and could have important clinical implications for age-related disorders, immunotherapy and cancer.

ONTOGENY AND EPIGENETIC REGULATION OF PROMISCUOUS GENE EXPRESSION: EVIDENCE FOR FLUCTUATING GENE EXPRESSION

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Promiscuous expression of tissue-restricted antigens (TRAs) in medullary thymic epithelial cells (mTECs) is essential for central T cell tolerance. The molecular regulation of this unusual gene expression, in particular the interplay between genetic and epigenetic mechanisms, is only poorly understood. By studying promiscuous expression of the mouse casein locus, we report that transcription of this locus develops gradually and starts with the casein beta and gamma genes during ontogeny. With increasing age of the embryo we observe an increase in the percentage of mature TECs, first expressing single and subsequently multiple TRAs. Transcription is preceded by promoter demethylation in immature mTECs followed by local locus decontraction and introduction of active histone marks upon mTEC maturation. Promiscuous transcription of the casein cluster however does not correlate with locus-wide epigenetic marks. Moreover, analysis of an additional locus showed that promiscuous expression is transient in single mTECs and we observed mono- and biallelic expression patterns. Transient or fluctuating gene expression could conceivably add to the diversity of self-antigen display in a confined microenvironment and thus enhances the efficacy of central tolerance.

CRITICAL ROLE FOR RAG2 IN SUPPRESSING V(D)J RECOMBINATION-DRIVEN THYMIC LYMPHOMA

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V(D)J recombination in lymphocytes proceed via double-strand breaks generated by the RAG1/2 endonuclease. This recombination mechanism has long been thought to be involved in genomic instability leading to cancer. In fact, because antigen-receptor gene rearrangement entails breaking and rejoining the chromosome several times before a complete immunoglobulin or TCR molecule can be expressed at the cell surface, V(D)J recombination has been termed a "disaster waiting to happen."
Analysis of RAG1 and RAG2 mutants has demonstrated that the RAG post-cleavage complex normally prevents V(D)J recombination-associated DSBs from accessing alternative end joining pathways and promoting aberrant rearrangements.
To test the consequences of a mutant RAG complex *in vivo*, we generated a knock-in mouse model harboring a RAG2 truncation (RAG2tr) and examined lymphocyte differentiation, genomic stability and tumorigenesis in this setting. Significant mature B and T cell numbers are generated in $RAG2^{tr/tr}$ knock-in mice, showing that the mutant RAG recombinase retains *in vivo* function. We found that, as compared with *p53 null* mice, $RAG2^{tr/tr}/p53^{-/-}$ animals more rapidly develop tumors (mean survival: $RAG2^{tr/tr}/p53^{-/-}$ = 14 weeks as compared with $p53^{-/-}$ = 23 weeks; $p < 5.8e-7$). Also, in contrast to $p53^{-/-}$ animals, $RAG2^{tr/tr}/p53^{-/-}$ mice virtually all die of thymic lymphomas associated with recurrent translocations frequently involving chromosomes that harbor antigen receptor loci. These results indicate that the tumorigenic events were initiated by the mutant RAG1/RAG2tr endonuclease. To test whether recombination defects exist in $RAG2^{tr/tr}$ thymocytes, we assayed for Tcrα locus integrity by three-dimensional interphase DNA-fluorescent in-situ hybridization (FISH). We found that $RAG2^{tr/tr}$ thymocytes have a three- to six-fold increase in the number of cells harboring aberrant Tcrα locus as compare with wild type thymocytes, indicating that RAG2 is required for the stability of the Tcrα locus.
Thus, our study provides the first *in vivo* evidence for a role of RAG2 in maintenance of genome stability and tumor suppression during V(D)J recombination.

MONOCYTE HETERGENEITY AND c-MAF DEPENDENT *M. TUBERCULOSIS* GROWTH IN HUMAN *MYCOBACTERIUM TUBERCULOSIS*-INFECTION

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We studied monocyte heterogeneity and the factors that regulate mycobacterial growth in human *Mycobacterium tuberculosis* (*M. tb*) infection. In 5 healthy donors, *M. tb* H37Rv grew 5.6-fold higher in human CD14+CD16- macrophages compared to CD14-CD16+ macrophages (7.9 ± 2.6 x 10^6 vs 1.4 ± 0.7 x 10^6 CFU per well, p=0.002). CD14+CD16- monocytes from 13 healthy donors produced higher amounts of IL-10, compared to CD14-CD16+ monocytes upon culturing with γ-irradiated *M. tb* (329.8 ± 61.0 vs 1.8 ± 0.9 pg/ml, p=0.0002). Expression of mRNA for c-maf, a transcription factor, was upregulated in *M. tb*-stimulated CD14+CD16- monocytes, compared to CD14-CD16+ monocytes, as measured by real time PCR (3.1 ± 0.8 vs 0.7 ± 0.07 arbitrary units, p=0.03). c-maf siRNA inhibited IL-10 production by CD14+CD16- monocytes, compared to control siRNA (134.5 ± 76.7 vs 532.2 ± 190.3 pg/ml, p=0.02). Similarly, c-maf siRNA inhibited *M. tb* H37Rv growth in CD14+CD16- macrophages compared to control siRNA (2.6 ± 1.0 x 10^6 vs 10.33 ± 1.5 x 10^6 CFU per well, p=0.0005). In 5 healthy donors, recombinant IL-10 marginally enhanced *M. tb* growth in CD14-CD16+ macrophages (5.9 ± 0.8 x 10^6 vs 2.5 ± 0.3 x 10^6 CFU per well, p=0.01) and had no effect on *M. tb* growth in CD14+CD16- macrophages (13.6 ± 2.5 x 10^6 vs 20.0 ± 3.2 x 10^6 CFU per well, p=0.13) suggesting that c-maf dependent *M. tb* growth is not dependent on IL-10. To determine the c-maf dependent mechanisms that regulate *M. tb* growth, we compared gene expression of γ-irradiated *M. tb*-cultured CD14+CD16- and CD14-CD16+ monocytes, using microarray analysis. In CD14+CD16- cells, mRNA expression for 230 genes was >1-fold higher than in CD14-CD16+ cells. Of these genes, 22 have a c-maf binding site in their promoter region. Among these genes, we first selected hyaluronan synthase1 (HAS1) that was previously shown to regulate extracellular *M. tb* growth in lungs. Expression of HAS1 mRNA was up regulated in CD14+CD16- monocytes compared to CD14-CD16+ monocytes upon culturing with γ-irradiated *M. tb*, as measured by real time PCR (8.7 ± 2.1 vs 1.5 ± 0.13 arbitrary units, p=0.02). c-maf siRNA inhibited HAS1 expression in *M. tb*-stimulated CD14+CD16- monocytes, compared to control siRNA (1.1 ± 0.3 vs 4.8 ± 1.2, p =0.02). Studies are underway to determine the effect of HAS1 siRNA on intracellular survival of *M. tuberculosis* in CD14+CD16- macrophages.

THE NOVEL SIGNALING MECHANISM OF TCR INDUCED T CELL ACTIVATION

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Ezh2 and Ezh1 lysine methyltransferases are the key enzymatic components of the PRC2 complex, an essential epigenetic regulator of gene expression during development, conserved from fly to man. Our earlier findings revealed an unexpected role of Ezh2 in T cell antigen receptor (TCR) signaling. We found that Ezh2 is present in the T cell cytosol where it interacts with Vav, a potent signal transducer downstream of the TCR and co-stimulatory receptors. Here we report an extension of these studies where we show an essential and selective role of Ezh2 and Ezh1 in TCR induced T cell activation. We will present data showing a nucleus independent function of Ezh2/Ezh1 in T cell signaling and immunity. We will also report on the role of nuclear Ezh2 in the regulation of T cell differentiation and maintenance of T cell identity.

ALTERED METABOLIC FLUX AND T CELL SURVIVAL

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Cell survival decisions are fundamental to immune homeostasis and regulation. Cell metabolism is becoming increasing recognized as an important input to cell survival and T cell memory differentiation. Glycolysis can be upregulated and oxidative phosphorylation suppressed by active hypoxia-inducible factors (HIFs). We used a genetic approach to modify T cell metabolism by deleting VHL, the major negative regulator of the HIFs. We observe perturbations in cell survival, proliferation, and response to infection of peripheral T cells null for VHL. Further study of the mechanisms and signaling pathways underlying these perturbations may yield insights into how metabolism affects T cell survival decisions and memory differentiation.

FLT3 SIGNALING REGULATES THE DIFFERENTIATION AND SURVIVAL OF MULTIPOTENT LYMPHOHEMATOPOIETIC PROGENITORS

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Signals from the bone marrow microenvironment are critical for the development of lymphohematopoietic progenitors (LHP). Previous studies established that the receptor tyrosine kinase, Flt3 and its ligand, Flt3-ligand (FL) regulate LHP development. Mice deficient in FL exhibit striking reductions in Flt3+/hi LHP and B cell precursors (BCP), and evidence decreased lymphoid lineage priming. Currently, the mechanism by which Flt3 signaling regulates lymphocyte development is unknown. In this study, we investigated the role of Flt3 signaling in the proliferation, differentiation, and survival of LHP and common lymphoid progenitors (CLP). Unexpectedly, we found that mono-allelic expression of FL (*FL+/-*) resulted in a dose-dependent reduction in LHP and CLP. Thus, Flt3 signaling sets a threshold for bone marrow lymphopoiesis. This finding is further supported by limiting dilution analysis, which revealed that LHP from *FL+/-* mice have reduced B cell precursor frequency. *In vivo* bromodeoxyuridine incorporation studies established that Flt3 signaling is not essential for the proliferation of LHP. However, Annexin V staining showed that Flt3 signaling is important for the survival of LHP. Additional evidence supportive of the role of Flt3 signaling in the survival of LHP was obtained by FL withdrawal from FL-dependent *EBF-/-* and *E2A-/-* cell lines, which revealed increased *Bim* transcripts. To examine the role of Flt3 signaling in differentiation, we sorted GMLP and LMPP from *FL+/-* mice. Real-time PCR showed reduced *rag1* and *ebf1* transcripts and increased *id1* and *id2* transcripts in these subsets. The increases in *id1* and *id2* transcripts were recapitulated in the cell line FL withdrawal experiments. Id1 and Id2 are E2A antagonists and inhibitors of B lymphopoiesis. Id1 is expressed in primitive hematopoietic progenitors and downregulated in LHP and flow cytometric analysis of *Id1-/-* mice revealed increased frequencies of CLP. To determine if Flt3 signaling regulates LHP by limiting Id1 expression, we generated *FL-/- x Id1-/-* mice. However, analysis of compound knockouts revealed that loss of Id1 did not restore the deficiency in LHP in *FL-/-* mice, suggesting downregulation of Id2 may be involved. Taken together, these data provide the first mechanistic insight into the roles of Flt3 signaling in LHP and B cell development: survival and differentiation.

USP8 CONTROLS T-CELL DEVELOPMENT AND IMMUNE HOMEOSTASIS

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Ubiquitin modification represents a versatile mechanism to control cellular functions. It is a reversible process that is counteracted by the activity of ubiquitin deconjugating enzymes (DUBs). More than 90 different DUBs are encoded in the human genome including around 50 members that belong to the family of the ubiquitin specific proteases (USPs). Despite their high number and diversity, the physiological function and biological relevance of individual DUBs in discrete cell types is poorly defined.
Using cre-loxP-mediated gene targeting, we have generated mice with a T-cell-specific deletion of USP8 (ΔT-USP8). In T-cells many key signalling molecules are controlled by ubiquitin-mediated mechanisms. For instance, the stability of the E3-Ligase "gene related to anergy in lymphocytes" (GRAIL) has been shown to be controlled by USP8. Deletion of USP8 in T-cells caused severe autoimmune inflammatory bowel disease leading to premature death of the animals. CD4+ cells in the periphery are highly activated demonstrating an essential function of USP8 in the prevention of aberrant T-cell responses in vivo. In addition, the numbers of CD4+ and CD8+ single positive cells in spleen, lymphnodes and thymus of ΔT-USP8 mice are strongly reduced due to an impaired transition from the CD4+CD8+ double positive to the CD8+ and CD4+ single positive stage of thymocyte development.
Using yeast two-hybrid screening, we have identified USP8-interacting molecules including potential substrates. The molecular mechanisms that underlie USP8 function are currently under investigation and will be discussed.

THE TRANSCRIPTION FACTOR AP4 MODULATES EPIGENETIC *CD4* SILENCING

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CD4 co-receptor expression is negatively regulated through activity of the *Cd4* silencer in $CD4^{-}CD8^{-}$ DN thymocytes and $CD8^{+}$ cytotoxic lineage T cells. *Cd4* silencing in committed $CD8^{+}$ T cells is maintained through epigenetic processes once it is established by the silencer. We previously demonstrated that members of the Runx family of transcription factors are required for *Cd4* silencing both in DN thymocytes and $CD8^{+}$ T cells. However, additional factors cooperating with Runx proteins for establishment of *Cd4* silencing remain unknown. To identify such factors contributing to *Cd4* silencing, we used microarray and RNAi-based approaches and identified a bHLH-ZIP transcription factor, AP4, as a molecule contributing to *Cd4* gene regulation. In AP4-deficient mice, DN thymocytes de-repressed CD4 expression. In contrast, AP4-deficient $CD8^{+}$ T cells appeared to normally downregulate CD4 expression. However, AP4 deficiency resulted in a significant increase in the frequency of CD4-expressing $CD8^{+}$ T cells in mice harboring point mutations in the *Cd4* silencer. These observations suggest that AP4 modulates *Cd4* silencing both in DN and $CD8^{+}$ cytotoxic lineage T cells.

THE ZINC FINGER PROTEIN MAZR IS PART OF THE TRANSCRIPTION FACTOR NETWORK THAT REGULATES CD4/CD8 CELL FATE DECISION OF DP THYMOCYTES

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CD4/CD8 lineage specification of thymocytes is linked with coreceptor expression. Previously, the transcription factor MAZR was identified as an important regulator of *Cd8* gene expression. To further investigate the physiological function of MAZR during T cell development, *MAZR–/–* mice were generated. Here we demonstrate that MAZR functions at two distinct stages of T cell development. We show that variegated CD8 expression by loss of *Cd8* enhancers is reverted in MAZR-deficient mice, confirming that MAZR negatively regulates the *Cd8* loci during the DN to DP transition. Moreover, loss of MAZR led to a partial redirection of MHC class I-restricted thymocytes into $CD4^+$ helper-like T cells. By using either MHC class I-restricted TCR transgenic mice or *MAZR–/–* BM chimeric MHC class II-deficient mice, we demonstrate that MAZR-deficiency led to a partial redirection of MHC class I-restricted thymocytes into $CD4^+$ helper-like T cells. This correlated with derepression of ThPOK, an essential molecule for helper lineage differentiation, in MHC class I-signaled DP and $CD4^+CD8^{lo}$ *MAZR–/–* thymocytes. MAZR bound the ThPOK locus, suggesting a direct control of ThPOK expression. Thus, our data provide genetic evidence that MAZR is a part of the transcription factor network that controls CD4/CD8 cell fate decision of DP thymocyte by regulating ThPOK expression.

CONTROL OF INTERFERON EXPRESSION AND ANTIVIRAL RESPONSE BY DYNAMIC HISTONE H3 METHYLATION

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Antiviral immunity depends largely on the ability of infected cells to activate expression of type I interferon (IFN) and interferon inducible genes. Strict regulation of virus-dependent IFN expression is essential for immune system homeostasis, as attenuated or excessive IFN production is associated with immune deficiencies or autoimmunity, respectively. IFN genes are dormant in non-infected cells, but become transcriptionally active upon encounter with the virus. Although, many of the events and factors that lead to activation of IFNs have been identified, mechanisms of silencing IFNs have not been characterized. Here, we identify di-methylation of histone H3 lysine 9 (H3K9me2) as a potent regulator of Ifnβ expression. We found that the repressed state of Ifnβ depends on H3K9me2, which is catalyzed by the histone methyltransferase G9a. In turn, activation of Ifnβ expression in response to viruses is associated with rapid demethylation of H3K9me2. We found that the deficiency in G9a or overexpression of Jumonji family demethylases resulted in more rapid and robust Ifnβ transcription and greatly enhanced cell resistance to viral infection. Therefore, the Ifnβ promoter exists in a repressed chromatin state prior to virus-induced activation and demethylation of H3K9me2 is an important step towards transcriptional activation. In summary, we identified a novel control mechanism of Ifnβ expression that may facilitate the treatment of immune disorders associated with aberrant Ifnβ production.

ROLE OF THE CTCF/COHESIN COMPLEX IN V(D)J REARRANGEMENT

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The creation of a diverse antibody repertoire requires efficient utilization of the large number of V_H and Vκ genes. The >100 functional V_H genes are spread over 2.5 Mb region and need to have access to the single DJ_H rearrangement. Likewise, ~100 functional Vκ genes are spread over 3.1 Mb, and all need to efficiently recombine to one of 4 Jκ genes within a 1.5 kb region. In order for V_H genes and for Vκ genes throughout the loci to rearrange efficiently, the large V regions need to contract so that all of the V genes can obtain access to the small D-J portion of the locus. 3D-FISH studies have shown that this contraction occurs via the formation of multiple loops, forming a rosette-like structure within the V_H locus. CTCF is a transcription factor associated with insulators and boundary elements that regulates gene expression by long-range chromosomal looping. We therefore hypothesized that CTCF may be crucial for the contraction of the Ig loci.

We performed ChIP-chip, and more recently ChIP-seq, to determine if CTCF was a candidate for being involved in creating the loops at the Ig loci, and demonstrated many CTCF sites in the V_H and Vκ loci (and also in the TCRβ and TCRα/δ V loci). However, CTCF binding in the *Igh* locus was largely unchanged throughout differentiation, suggesting that CTCF binding alone cannot be responsible for stage-specific locus compaction. Because cohesin can co-localize with CTCF, we performed ChIP/qPCR for the cohesin subunit Rad21 and found lineage and stage-specific higher level recruitment of Rad21 to CTCF. Interestingly, although we found many CTCF/cohesin sites within the V_H portion of the *Igh* locus, there were only two other locations of CTCF binding within the rest of the *Igh* locus: one large region in the 3' enhancer which had been previously described, and, importantly, 2 close sites just 5' of the end of the D_H gene cluster. This is the ideal location to have a boundary element separating the V_H portion of the locus from the D_H and J_H portion, and thus we propose that these CTCF sites may act as a boundary/insulator preventing premature accessibility of the V_H locus at the time when D-J rearrangement is occurring. This is being tested by 3C analysis. We propose that these DFL-flanking sites begin to bind to CTCF/cohesin sites located throughout the V_H locus during the stage of V_H to DJ_H rearrangement, thus resulting in the formation of multiple loops. Knockdown of CTCF in pro-B cells followed by 3D-FISH is being done to demonstrate whether CTCF plays a functional role in locus compaction. In addition, due to its boundary function, CTCF may control the spread of epigenetic changes within the large Ig loci.

CO-EXPRESSION OF THE EPSTEIN-BARR VIRAL PROTEINS LMP1 AND LMP2A, MIMICKING CONSTITUTIVELY ACTIVE FORMS OF CD40 AND BCR, DOES NOT LEAD TO B CELL TRANSFORMATION

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Epstein-Barr virus (EBV) preferentially infects human B cells and establishes a lifelong persistence in long-living memory B cells. The virus is associated with different lymphoproliferative malignancies, like Burkitt´s lymphoma and Hodgkin lymphoma. During primary infection the virus expresses nine viral proteins, referred to as latency III, leading to the immortalization of B cells. These viral proteins code for six nuclear antigens (EBNA 1-6) and three membrane proteins (LMP1, LMP2A and LMP2B). However in vivo cytotoxic T cells recognize infected cells and eliminate these cells. Hence, expression of the viral proteins is downregulated and viral persistence is established in the memory B cell compartment. In the quiescent state no viral protein or only EBNA1, which is not recognized by the immune system, is expressed (latency I/0). In most cases of EBV-associated tumors the virus does not exist in latency III but in latency I or in latency II, which is characterized by the expression of LMP1, LMP2A and EBNA1. Thus the latency II expression profile was found in EBV positive Hodgkin tumor cells (HRS). To study the influence of LMP1 and LMP2A on tumor development, we transfected expression vectors for LMP1, LMP2A and LMP1/LMP2A into an EBV conditionally immortalized human B cell line (EREB2-5), in which EBNA2, the main regulator of latency III, can be regulated by estrogen. After estrogen withdrawal we investigated the ability of LMP1 and LMP2A to immortalize B cells as well as the signaling pathways activated by both proteins. The analysis revealed that in the absence of EBNA2 the co-expression of LMP1 and LMP2A induces limited proliferation and improved survival of EREB cells but is not sufficient to maintain B cell immortalization.

TEMPORAL DIFFERENCES IN THE DEPENDENCY ON PHOSPHOINOSITIDE DEPENDENT KINASE 1 DISTINGUISH THE DEVELOPMENT OF VA14 INKT CELLS, REGULATORY T CELLS AND CONVENTIONAL T CELLS

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The present study uses two independent genetic strategies to explore the requirement for Phosphoinositide dependent kinase-1 (PDK1) in the development of T cell subpopulations from CD4/8 double positive thymocytes. The data show that there is a selective requirement for PDK1 catalytic activity for the development of NKT cells in the thymus. These cells use a Vα14 invariant T cell antigen receptor to recognise glycolipids presented by CD1d molecules and play a role in immune surveillance and immune homeostasis. CD4/8 double positive thymocytes that do not express PDK1 or express a catalytically inactive PDK1 mutant lack even the earliest Vα14 NKT progenitors, although these cells can differentiate into conventional CD4, CD8 or regulatory T cell subsets. PDK1 phosphorylates the activation loop of numerous AGC kinases, including Protein Kinase B (PKB, also called Akt). The PDK1 requirement for iNKT cell development reflects that these cells require PKB for proliferative expansion in response to Interleukin 15 or antigen receptor stimulation. These data identify PDK1 as a kinase that integrates cytokine and antigen receptor signaling to support the proliferative expansion of iNKT cells in the thymus. The data also reveal that there is constitutive PDK1 signaling in conventional SP α/β T cells that fine-tunes expression of coreceptors and adhesion molecules and is essential to allow SP thymocytes to populate the periphery.

MZB1 REGULATES INNATE B CELL FUNCTIONS BY MODULATING CALCIUM HOMEOSTASIS AND INTEGRIN ACTIVATION

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Marginal zone (MZ) B cells of the spleen and peritoneal B1 cells are specialized B cell subsets implicated in bridging the innate and adaptive immune responses, categorizing these cells as “innate-like B cells”. Besides their separate localization in distinct regions of secondary lymphoid organs and the peritoneal as well as the pleural body cavities, these cells differ from conventional, recirculating follicular (Fo) B cells by their attenuated Ca^{2+} mobilization, fast kinetics of antibody secretion and increased cell adhesion.
Here we identify a gene, termed *Mzb1*, as a determinant of the specialized functions of MZ B and B1 cells. In these B cell subsets, *Mzb1* is expressed at high levels, and we show that Mzb1 protein is localized in the lumen of the endoplasmatic reticulum (ER). Proteomic analysis revealed an association of Mzb1 with ER-localized chaperones and oxidoreductases being important regulators of the oxidative folding of integrins and the ER Ca^{2+} store content, respectively. Knockdown of Mzb1 in a B cell line and in primary MZ B cells results in increased Ca^{2+} mobilization and cell proliferation. Moreover, Mzb1 knockdown in MZ B cells decreases LPS-induced antibody secretion and integrin-mediated adhesiveness by a defect in generating an active conformation of integrins. Taken together, these data suggest that Mzb1 is a determinant for the diversification of the immune functions of MZ B and B1 cells by regulating key features such as ER Ca^{2+} stores, antibody secretion and surface expression of integrins.

EXTRACELLULAR AUTOREGULATION BY IL-2 IN T-CELL ACTIVATION: A TIME TO COOPERATE, A TIME TO COMPETE

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Cells of the immune system communicate and transfer information among them to coordinate immune responses. Cytokines are important mediators of this complex intercellular communication network. Understanding such complex interactive networks may be facilitated by studying simpler modules which occur abundantly throughout the network. This approach was found highly useful in studying intracellular molecular networks. Here, we present an extension of those methods to study modules of intercellular communication networks.

Autoregulation, or autocrine feedback, occurs when a cell expresses receptors to a cytokine that it secretes. This is the simplest module of the intercellular cytokine communication network, and is abundantly found throughout the network. We study extracellular feedback in the context of T-cell activation, where IL-2 is secreted by T-cells upon stimulation of their T-cell receptor (TCR), and expression of the alpha subunit of the IL-2 receptor (IL-2R) is up-regulated upon IL-2 induced signaling. We present here a mathematical model which describes this extracellular feedback, and study its behavior. Unknown model parameters are determined from quantitative measurements of expression levels of key proteins involved, under well defined inputs to TCR and IL-2R.

We apply the model to analyze a system of T-cells that are activated and are interacting with each other via IL-2, for example two T-cells of different TCR specificities entering a lymph node and being activated. The model shows a variety of dynamic behaviors including cooperation between the two clones under some conditions, and clonal competition under others. Our modeling results suggest another, yet un-described, role for cytokines in T cell activation dynamics, introducing temporal considerations into the process of T cell activation and inter-clonal interactions. Specifically, a cell with higher TCR signal wins only if it enters the competition in an early stage, but looses if it arrives too late. Therefore, we hypothesize that one of the roles of cytokine mediated extracellular positive feedback might be to serve as a time window, limiting the time available for clones to become activated. Limitation by cytokines of the time for response might accelerate the immune response, even if the most avid clone is not selected, thus balancing response accuracy and time to initiation. Our model suggests a unified explanation for a variety of experimentally observed phenomena such as clonal dominance and bystander effects, and may have implications for vaccine design and autoimmunity.

TARGETING OF AID-MEDIATED SEQUENCE DIVERSIFICATION TO IMMUNOGLOBULIN GENE LOCI

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Activation-induced cytidine deaminase (AID) is a DNA mutator that is essential for somatic hypermutation (SHM), immunoglobulin gene conversion (GCV), and class switch recombination in activated B cells. High levels of mutagenic events occur predominantly in immunoglobulin genes, while the remainder of the genome remains protected.
Using chicken DT40 cells as our model system, we previously identified a 4 kb regulatory region (named 3′RR for its location) in the Ig light chain locus that contains a targeting element for AID-mediated sequence diversification and a transcriptional enhancer. Using gene targeting and a systematic deletion strategy a distinct mutation enhancer element (MEE), 1.3 kb in size, was found within the 3′RR that when deleted completely abrogates SHM and GCV, while transcription remains largely unaffected. Cross-complementation approaches are currently ongoing to identify such *cis*-acting elements in the murine Ig loci. Preliminary evidence suggests that the two major regulatory elements in the murine Igκ locus are not sufficient for targeting, while strongly supporting transcription. Overall, this finding suggests that two distinct types of regulatory sequences exist in Ig loci: transcriptional enhancers and mutational enhancers. The identification of specific binding sites and the corresponding *trans*-acting factors are in progress.

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ESSENTIAL ROLES FOR THE RNA BINDING PROTEINS TIS11B AND TIS11D IN B-LYMPHOCYTE DEVELOPMENT.

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Eukaryotic cells have been shown to regulate gene expression post-transcriptionally through the binding of proteins and small RNA species to sequences within the 3' untranslated region (UTR) of mRNAs. In the cytoplasm RNA binding proteins may act to confer stability to the transcript, to destabilise the mRNA or to affect translation and there is evidence that they may also have a role in the nucleus and affect RNA processing events such as polyadenylation site choice. The Tis11 family of proteins consists of Tis11, Tis11B, and Tis11D all of which have a tandem CCCH zinc finger structure that has been shown to bind to AU-rich elements (ARE) of mRNAs.

In order to study the role of Tis11 family proteins in lymphocyte development our lab developed transgenic mouse strains with floxed alleles of Tis11B and Tis11D for deletion by cell type specific Cre recombinases. Simultaneous deletion of Tis11B and Tis11D in lymphocyte progenitors in mice using CD2 Cre gives rise to a block in B-cell development around the pro-B cell stage and very few pre-B cells are formed in the bone marrow of these mice. The mice also develop acute T-cell lymphoblastic leukaemia due to expansion of the CD8+ immature single positive T-cell population. A microarray of the thymus of these mice showed an increase in Notch-1 mRNA which may be responsible for T-cell leukaemia and the reduction in number of B-cells that develop.

To assess the role of Tis11B and Tis11D in B-cell development I have carried out FACS sorts to collect pre-pro-B cells and pro-B cells from the Tis11Bfl/fl, Tis11Dfl/fl CD2 Cre and wild-type mice and compared the abundance of potential target transcripts by quantitative RT-PCR. The results suggest that there may be a reduction in rag-1 and rag-2 expression in the Tis11Bfl/fl, Tis11Dfl/fl CD2 Cre pre-pro-B cells and pro-B cells compared to wild-type mice. I plan to continue to analyse the phenotype of Tis11Bfl/fl, Tis11Dfl/fl CD2 Cre mice and identify Tis11B and Tis11D target mRNAs in B-cell progenitors.

ROLE OF *MLL* DURING LYMPHOCYTE DEVELOPMENT

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Chromosome translocations at the 11q23 locus occur in a majority of infant acute lymphocytic leukemia (ALL) cases as well as a significant percentage of adult acute leukemia cases. These translocations disrupt the Mixed Lineage Leukemia (MLL) gene and frequently result in the production of chimeric fusion oncoproteins, most commonly MLL-AF4 in the infant ALL cases. Gene expression and immunophenotype data support the concept that MLL-AF4 immortalizes an early B-cell precursor with myeloid stem cell features. To dissect the pathways leading to the development of B-cells that may be under wild-type *Mll* regulation, we performed the following studies using conditional mutagenesis and developmentally regulated Cre expressing mouse models.

We have previously reported that *Mll* supports steady-state hematopoiesis through its role in maintaining hematopoietic stem cells (HSCs) and multiple progenitors in the bone marrow. By using the developmentally-regulated pan-hematopoietic Vav-Cre transgene to excise *Mll*, we demonstrate that the developmentally regulated excision of *Mll* results in defective B lymphopoiesis in addition to other multi-lineage defects. To more precisely assess the role of *Mll* during commitment to the lymphocyte lineages, we excised *Mll* using the Rag1-Cre knock-in model. Despite efficient excision of *Mll* in all early lymphocyte progenitors, we again found a selective B-cell reduction in peripheral and bone marrow populations of Rag1-Cre; *Mll F/F* animals. The reduction in peripheral B-cells in these animals was preceded by a significant and age-dependent reduction in developing B-cells in the bone marrow. In particular, a block in the transition from the pro-B to pre-B stage represented the largest fold difference between control and Rag1-Cre; *MllF/F* animals. Dysregulated surrogate light chain and μ chain expression in the Mll-deficient B cells suggest the pre-B cell receptor checkpoint as the major Mll-dependent step during B-cell differentiation. Further analysis of *Mll-/-* B-cells revealed a defective IgH rearrangement and reduction of intracellular heavy chain proteins. In periphery, the reduced B-cell output from the bone marrow resulted in a parallel reduction in B-cells at all stages of maturation. In contrast, peripheral and thymic T-cell numbers were largely normal at all ages tested, although a partial block at the DN3-DN4 stage was observed in two week-old animals. These results elucidate an important role for *Mll* in B-cell development and suggest a possible mechanism for the proclivity of MLL-drive ALL for the B-cell lineage.

IDENTIFICATION OF MIRNAS INVOLVED IN LEUKEMIC TRANSFORMATION AND/OR B CELL DEVELOPMENT

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miRNAs regulate numerous physiologic processes and also contribute to diseases such as cancer. Our goal is to identify miRNAs involved in the regulation of B cell development or that contribute to the oncogenic transformation of B cells by the Abelson Murine Leukemia Virus (AMuLV) and to identify the targets and processes they regulate. The chemotherapeutic agent STI-571 (Gleevec) specifically inhibits the oncogenic tyrosine kinase expressed by AMuLV. We observed previously that STI-571 treatment of Abelson pro-B leukemia cells results in their exit from the cell cycle and differentiation to a pre-B cell-like state. We used this system to conduct a microarray-based screen for miRNAs regulated by v-Abl activity.

We observed the upregulation of a group of miRNAs once v-Abl was inactivated and B cell progression was allowed; a smaller subset of miRNAs were downregulated with this treatment. When individually overexpressed in Abelson pro-B cells, two of these regulated miRNAs result in the induction of germline kappa transcription, a key aspect of the normal pro-to-pre-B cell transition. We are in the process of examining potential miRNA targets that may result in the observed regulation of germline kappa transcription. Our goal is to elucidate the pathway through which these miRNAs regulate germline kappa transcription, and to test whether such a pathway is similarly regulated during primary B cell development.

THREE DIMENSIONAL CONFORMATION OF THE MOUSE CD8 GENE LOCUS IN DIFFERENT CELL POPULATIONS

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Nuclear architecture and chromatin reorganisation have recently been shown to orchestrate gene expression and act as key players in developmental pathways. The formation of chromatin loops has been shown to involve direct interactions between distant regulatory elements such as LCRs, enhancers and promoters. Direct in vivo evidence to support the looping model has come from studies in the β-globin and other loci. In order to investigate how regulatory elements in the mouse CD8 gene locus are arranged in space and in relation to each other, the 3C technique was employed to identify long range interactions between the different elements during T cell development. In order to compare the spatial organisation of the CD8 locus in cells where the CD8α and CD8β genes are expressed (CD8+), with cells in which they are silenced (CD4+) or have never been transcribed (B cells), lymphocytes from spleens of wild type C57Bl/6 mice were sorted and used for 3C analysis. Our data are consistent with an interpretation that the cis-regulatory elements of the CD8α and CD8β genes, as well as the two genes, spatially cluster together in CD8 expressing cells, where the locus is active, to form an ACH. In contrast, in cells that have silenced (CD4+ cells) or have never activated the locus (B cells) the interactions are much weaker, indicating a more linear, non structured chromatin conformation in these cells. These observations confirm the dynamic state of chromatin during developmental state decisions.

THE TRANSCRIPTION FACTOR ETS-1, BUT NOT THE CLOSELY RELATED FACTOR ETS-2, INHIBITS ANTIBODY-SECRETING CELL DIFFERENTIATION

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B cell differentiation into antibody-secreting plasma cells (ASCs) is a tightly-regulated process under the control of multiple transcription factors. We have previously shown that one such transcription factor, Ets-1, blocks the transition of B cells to ASCs via two separate activities: (1) stimulation of target genes that promote B cell identity such as Pax-5 and (2) interference with the functional activity of the transcription factor Blimp-1, which is required for ASC formation. Ets-1 is a member of a multigene family, several of whose members are expressed within the B cell lineage, including the closely-related protein Ets-2. We demonstrate that Ets-1, but not Ets-2, can block ASC formation. This is despite the fact that Ets-1 and Ets-2 are highly homologous, bind to identical DNA sequence motifs and are thought to regulate overlapping sets of target genes *in vivo*. We further demonstrate that several regions of the Ets-1 protein are required for maximal activity in inhibiting ASC formation including the highly-conserved Ets DNA binding domain as well as less conserved regions within the N-terminus of the protein. However, known post-translational modifications at the N-terminus of Ets-1 are not required for its activity. Mutation of key arginine residues in the DNA binding domain of Ets-1 abolish Ets-1 DNA binding, but result in proteins with partial activity in blocking ASC formation. This suggests that DNA binding contributes to Ets-1 function, but is not the exclusive mechanism by which this protein functions. Current experiments are geared towards identifying a mutation of Ets-1 that can disrupt its physical interaction with Blimp-1 so that we can further validate the relevance of this pathway in regulating ASC differentiation. Together, our results support a model in which Ets-1 had unique effects on ASC differentiation both by regulating target genes and by interactions with the master regulator of ASC formation, Blimp-1.

AMINO TERMINAL PHOSPHORYLATION OF ACTIVATION-INDUCED CYTIDINE DEAMINASE SUPPRESSES C-MYC-IGH TRANSLOCATION

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Activation-induced cytidine deaminase (AID) is a mutator enzyme that initiates class-switch recombination and somatic hypermutation of immunoglobulin genes (Ig) in B lymphocytes. However, AID also produces off-target DNA damage, including mutations in oncogenes and double stranded breaks that can serve as substrates for oncogenic chromosomal translocations. AID is strictly regulated by a number of mechanisms, including phosphorylation at serine 38 and threonine 140, which increase activity. Here we show that phosphorylation can also suppress AID activity in vivo. Serine 3 is a novel phospho-acceptor which, when mutated to alanine, leads to increased class switching and c-myc/Igh translocations without affecting AID levels or catalytic activity. Conversely, increasing AID phosphorylation specifically on serine-3 by interfering with serine/threonine protein phosphatase 2A (PP2A) leads to decreased class switching. We conclude that AID activity and its oncogenic potential can be down-regulated by phosphorylation of serine 3, and that this process is controlled by PP2A.

AID GENERATES URACILS IN VARIABLE AND SWITCH REGION DNA FROM B CELLS

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Activation-induced deaminase (AID) is a B cell enzyme that initiates somatic hypermutation in variable and switch regions of immunoglobulin genes. Two contrasting mechanisms have been proposed for AID to act on either RNA or DNA. Biochemical and genetic data strongly support deamination of cytosine to uracil in DNA, but no physical evidence of increased uracils in B cell DNA has been reported. To find the rogue uracil, we used three techniques: Southern, PCR, and ligation-mediated PCR assays. Activated B cells were collected from mice deficient for uracil-DNA glycosylase (UNG), which would retain any uracils in the DNA. DNA was prepared and digested in vitro with UNG and abasic endonuclease. If uracils are present, the DNA would be cleaved and produce a loss of signal in Southern and PCR assays. Our results show a decrease of intact DNA from the variable and switch regions but not the constant regions of immunoglobulin genes. This mechanism is specific to AID as there was no decrease in variable or switch region DNA from *Ung*$^{-/-}$ *Aid*$^{-/-}$ B cells. Furthermore, we found that deamination in the switch region occurs early after activation, prior to cell division. After division, there were fewer uracils, which had presumably been repaired during replication. Finally, we mapped the position of uracils in the variable region by ligation-mediated PCR and sequencing. DNA was digested with UNG and endonuclease, and linkers were ligated to the breaks and amplified. The majority of linkers were found at cytosine residues in hotspot motifs, in accord with the DNA deamination theory.

ROQUIN IS AN RNA-BINDING PROTEIN THAT FORMS COMPLEXES WITH P BODY COMPONENTS TO POST-TRANSCRIPTIONALLY REPRESS *ICOS* IN A MIRNA-INDEPENDENT MANNER

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Mice with a homozygous point mutation in the *rc3h1* gene that encodes the Roquin protein develop severe autoimmune disease (Vinuesa et al., 2005, Nature 435, 452-8). It has been shown that Roquin represses ICOS by promoting mRNA decay and increased ICOS expression in the mutant mice contributes significantly to pathology (Yu et al., 2007, Nature, 450, 299-303).
We investigated the molecular mechanism by which Roquin represses ICOS. Knockdown approaches revealed that ICOS expression is placed under the control of Roquin in Th1 and Th2 cells. In a deletion mutagenesis we identified a carboxy-terminal sequence in Roquin with a high content of asparagine and glutamine residues that is functionally important and specifies localization of Roquin to P bodies, but is not required for enrichment of Roquin in stress granules. Similarly, we determined that Roquin-mediated ICOS repression does not require the essential stress granule protein TIA-1, instead functionally depends on the P body component Rck. We genetically tested a requirement for cellular RNAi by using Dicer-deficient mouse embryonic fibroblasts clones, which are incapable of microRNA biogenesis, and by using mouse embryonic stem cells with gene deletion of argonaute 1-4, which are unable to form microRNA-induced silencing complexes (miRISC). Our data show no requirement for microRNAs and miRISC formation in Roquin-mediated ICOS repression. Instead, we demonstrate direct physical interaction of Roquin protein with *ICOS* mRNA *in vitro* and binding of Roquin to *ICOS* mRNA in primary T cells. Our experiments uncover a novel mode of RNA-protein interaction, in which the amino-terminal ROQ domain cooperates with its adjacent zinc finger. Analyzing Roquin-interacting proteins we find specific and RNase-insensitive interactions of Roquin with Rck and Edc4 proteins in mouse T cells, which are critical factors in P body-associated mRNA-decay. We therefore propose that Roquin prevents autoimmunity through interaction with *ICOS* mRNA and by coordinating assembly of functional messenger ribonucleoprotein complexes that downregulate ICOS expression posttranscriptionally.

REORGANIZATION OF MACROPHAGE MEMBRANE PROTEINS TO FORM A "PHAGOCYTIC SYNAPSE" IS REQUIRED FOR DECTIN-1 ACTIVATION

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Macrophages and dendritic cells use a panoply of receptors to detect microbial pathogens. These receptors generate diverse intracellular signals that combine to define the nature of the net inflammatory response. Some receptors, such as Toll-like receptors, primarily trigger inflammatory cytokine and chemokine production, while other receptors additionally trigger direct anti-microbial responses such as phagocytosis and a respiratory burst. While the former are useful even if a cell is not in direct contact with a microbe, the latter require contact to be effective. Dectin-1 detects β-glucans in fungal cell walls and is a central player in the anti-fungal response. Detection of β-glucan-containing particles (e.g. yeast) by Dectin-1, which signals via an ITAM-like motif, triggers phagocytosis and an oxidative burst, in addition to the production of inflammatory mediators. In contrast, soluble β-glucans, which are too small to trigger phagocytosis, fail to activate Dectin-1 signaling. Using confocal microscopy we found that upon β-glucan particle binding by Dectin-1, membrane tyrosine phosphatases are segregated away from the receptor. Our data indicate that "phagocytic synapse" formation is required to initiate Dectin-1 signaling, and that the inability of soluble β-glucans to trigger such reorganization accounts for their failure to activate Dectin-1.

DIFFERENTIAL REGULATION OF INTERLEUKIN-12 FAMILY MEMBERS : CRITICAL ROLE OF INTERFERON REGULATORY FACTORS

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Interleukin(IL)-12 plays a key role in the polarization of CD4 T cells into Th1 effector cells producing interferon (IFN)-γ. A closely related cytokine, IL-23, was shown to promote the activity of Th17 cells, which are responsible for auto-immune inflammation in several experimental models. IL-27, a third member of the IL-12 family emerged as an important regulator of innate and adaptive immune responses. As these cytokines dramatically influence the outcome of the immune response, it is crucial to identify the early regulatory mechanisms that control the IL-23/IL-12/IL-27 balance. We analysed transcriptional regulation of the genes encoding the different subunits in response to TLR agonists. We assessed the role of the MyD88-, TRIF- and IFN-dependent pathways. We found that expression of both IL-12p35 and IL-27p28, but not of IL-12/23p40, IL-23p19 or EBI3 requires the TRIF/IRF3 pathway. Transcriptional activation of these 2 genes also depends on MyD88-dependent activation and IFN-dependent upregulation of IRF1. Furthermore, we demonstrated that expression of the IL-27p28 gene is dependent on a secondary recruitment of the ISGF3 complex to a proximal ISRE site. This event was not involved in IL-12p35 gene activation. We confirmed this result on splenic dendritic cells in vivo in the course of experimental endotoxemia. We also observed that both IRF3-/- and IRF9-/- mice developed an exacerbated form of experimental autoimmune encephalomyelitis (EAE) associated with increased antigen-specific Th17 responses. We conclude that besides their role in the induction of type I IFNs and anti-viral responses, IRF3 and ISGF3 also modulate the balance between IL-12 family members and thereby could influence autoimmune inflammation.

T CELL SPECIFIC ADAPTER PROTEIN (TSAD) MODULATES LCK-SH2 DOMAIN LIGAND PREFERENCE

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The eight Src kinases harbor a conserved tyrosine in the SH2 domain that is not found in other SH2 domains. Phosphorylation of this residue in Lck and Src (Lck Y192 and Src Y215) has previously been shown to alter the peptide binding specificity of these SH2 domains. Here we report that the T cell specific adapter protein (TSAd) promotes phosphorylation of Lck Y192 in Jurkat T cells transiently expressing TSAd. Moreover, co-expression of TSAd and the inducible Tec kinase Itk further increased the Lck Y192 phosphorylation, whereas expression of Itk alone was not sufficient. Since phosphorylation of Lck Y192 seems to be associated to the expression of TSAd, and the SH2 domain of kinases influences the substrate of choice, we propose that TSAd promotes alteration of the Lck substrate preference. As TSAd displays three interaction sites for the Lck-SH2 domain, we first examined whether the affinity of Lck-SH2 for TSAd phosphopeptides was affected by phosphorylation of Lck Y192. The tyrosine phosphorylation of Lck Y192 was mimicked by an acidic residue (E) at the tyrosine position. We observed that isolated intact Lck-SH2 domains with the Y192E substitution led to reduced binding to the classic Lck-SH2 domain motives TSAd Y305 and the HMT peptide. In contrast, the TSAd pY290 peptide which is not a typical Lck-SH2 motif, had a 15 fold higher affinity for the Lck-SH2-Y192E domain than to the Lck-SH2 domain. Furthermore we wanted to explore whether the affinity of the Lck-SH2 domain to other Lck ligands were positively affected by phosphorylation of Lck Y192. A custom made peptide spot array including 431 phosphopeptides, representing previously reported phosphorylated tyrosines (PhosphoSite) in Lck interacting molecules (Human Protein Reference Database), were tested for binding to the intact or mutated Lck-SH2 domain. Several of the phosphopeptides displayed altered binding specificity of the Lck-SH2 and Lck-SH2-Y192E. One of these proteins was Itk that showed higher binding of Lck-SH2-Y192E to both pY180 and pY511, both of which are sites known to regulate Itk activity. We have recently shown that TSAd promotes Lck-mediated phosphorylation of Y511 in Itk. Whether activated Itk may phosphorylate either TSAd or Lck is under investigation in our lab. Our current working hypothesis is that TSAd functions as a scaffold for Lck and Itk, allowing Lck and Itk to reciprocally transphosphorylate each other. Since Lck Y192 is a predicted Itk phosphorylation site we propose that TSAd ensures that Lck alters its ligand preference through transient phosphorylation of its SH2 domain by Itk.

NEGATIVE FEEDBACK REGULATION OF ANTIGEN RECEPTORS THROUGH CALMODULIN INHIBITION OF E2A

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During B lymphocyte development, the cells have to pass several checkpoints verifying the functionality of their antigen receptors. Expression of a pre-B-cell receptor (pre-BCR) with membrane-bound Ig heavy chain protein associated with surrogate light chain (SLC) proteins is a critical checkpoint that monitors for functional heavy chain rearrangement. Signaling from the pre-BCR induces survival and clonal expansion, thereby driving the preferential expansion and differentiation of cells with high quality heavy chains. The proliferation has of course to be limited, and pre-BCR signaling turns also off transcription for the SLC proteins and the co-receptor CD19. Later in the development, the BCR is used to select cells for receptor editing, select cells allowed to leave the bone marrow and select against autoreactive cells. To optimize the initial wave of antibody defense against infections, the B-cells with highest antigen affinity are selected to generate the extrafollicular plasmablast responses. B-cells with less strong antigen binding are selected for the germinal center (GC) where they undergo somatic hypermutation (SH) and the GC B-cells with highest affinity are selected for plasma cell differentiation. Such cells typically have 5-10 mutations in their Ig genes. They have increased their antigen binding strength with many orders of magnitude through several rounds of SH. Each round leads to one or a few mutations, followed by selection for increased affinity. After a number of cycles, the B-cells with highest affinity are selected for plasma cell differentiation and thereby antibody production. BCR signaling has therefore to be able to detect improvements in antigen affinity, even if relatively small, over an extremely broad range of affinities. Here we have examined whether this ability could be due to an effect of BCR signaling on the BCR signalosome itself. We show that both the pre-BCR and the BCR are subject to general negative feedback regulation. Activation of signaling leads to down-regulation of the receptor proteins as well as many co-receptors and proteins participating in signal pathways from the receptors. Thus, the down-regulation of the pre-BCR is much broader than previously anticipated, and the BCR can down-regulate itself to enable sensitive detection of successive improvements in antigen affinity of the receptor over a very large span of affinities during somatic hypermutation. We show also that the mechanism of the feedback inhibition for most of the proteins of the BCR signalosome, and most other up- and down-regulations of genes upon BCR stimulation, is through inhibition of the transcription factor E2A by Ca^{2+}/calmodulin.

ATM AND ITS SUBSTRATES ARE ESSENTIAL FOR LYMPHOCYTE DEVELOPMENT AND CHROMOSOMAL V(D)J RECOMBINATION IN THE ABSENCE OF XLF

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The Ataxia Telangiectasia-mutated protein (ATM) detects DSBs, stabilizes broken ends, and activates repair pathways and checkpoints and is also involved in DSB joining during V(D)J recombination; however, in the absence of ATM, V(D)J recombination still occurs at substantial levels and lymphocyte development is only modestly affected. The XRCC4-like factor (XLF, also called Cernunnos) was isolated based on its mutation in human immune-deficient patients. Mice that lack XLF show general genomic instability associated with a DNA repair defect, but in contrast to deficiency for classical non-homologous end joining (C-NHEJ) proteins, like XRCC4, XLF deficient mice show very modest defects in V(D)J recombination and lymphocyte development. However, we now show that mice with a dual deficiency for both ATM ($ATM^{-/-}$) and XLF ($XLF^{\Delta/\Delta}$) have a block of lymphocyte development at the progenitor B and T cell stages similar to that observed in C-NHEJ deficient backgrounds. Moreover, we show that $ATM^{-/-}XLF^{\Delta/\Delta}$ A-MuLV transformed pro-B lines, reminiscent of XRCC4-deficient pro-B lines, appear nearly completely deficient for ability to join ends during attempted V(D)J recombination of chromosomal substrates, apparently due to defective V(D)J recombination associated with severely compromised NHEJ. We further show that the synergy between ATM and XLF in end joining during V(D)J recombination involves the ATM kinase activity and implicate several ATM substrates (e.g. H2AX and 53BP1) as also having synergistic functions with XLF. Thus, our findings reveal a much more major end-joining role for ATM in V(D)J recombination, and potentially general DSB repair, than previously anticipated because the role was masked by synergistic functions of XLF. Conversely, by studying the requirement for XLF in ATM deficient cells, we have now also demonstrated a major role for XLF in end-joining during V(D)J recombination. Consistent with the synergy between ATM and XLF likely being required to allow C-NHEJ, $ATM^{-/-}XLF^{\Delta/\Delta}$ mature B cells (generated via IgH and IgL knock-in alleles) are not substantially more impaired for IgH class switch recombination than either deficiency alone, apparently due to the ability of alternative end-joining to still catalyze joining of broken ends during CSR. New studies on overlapping functions of ATM substrates with XLF and potential differential requirements for XLF plus ATM on chromosomal versus extra-chromosomal V(D)J recombination will also be presented.

ANALYSIS OF FUNCTION OF INTRONIC EM ENHANCER IN THE IGH LOCUS

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The functional immunoglobulin heavy chain genes are generated by regulated recombination of variable ($V_{H)}$, diversity (D_H) and joining (J_H) gene segments. Prior to initiation of recombination activation-specific histone modification are largely restricted to the D_H-Cμ domain, providing a plausible explanation for D_H recombination occurring before V_H recombination. We have previously shown that deletion of the intronic enhancer, Eμ, results in a partially active locus that is characterized by loss of histone acetylation but less effect on other form of epigenetic activation. Here we demonstrate that Eμ is also involved in nuclear location and chromosome conformation of the IgH.

NOTCH2 SIGNALING IS INSTRUCTIVE IN MARGINAL ZONE B CELL DIFFERENTIATION IN VIVO

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Notch1 signaling is essential for the development of T cells and blocks B cell development in the bone marrow when deregulated. Notch2 is dispensable for T cell development, but has a pivotal role in marginal zone (MZ) B cell lineage decision. However, the contribution of Notch2 to MZ B cell development has still not been completely elucidated. In this work, we have characterized a conditional transgenic mouse strain that expresses a constitutive active Notch2 receptor (Notch2IC) specifically in B cells. Notch2IC expression from the pro-B cell stage dependent on mb1-*Cre* blocked early B cell development, and led to the formation of ectopic T cells in the bone marrow. Activation of Notch2IC dependent on CD19-*Cre* strongly shifted B2 cells to the MZ B cell compartment at the expense of follicular (Fo) B cells. Thereby, most MZ B cells appeared to branch from the T1 transitional B cell stage, and displayed enhanced PI3K and MAPK signaling, but reduced NF-κB activation. Favoured MZ B cell generation was achieved even in the absence of CD19, which has been shown to be essential for MZ B cell development. Our findings not only underline the differences in the outcome of Notch2 signaling depending on the B cellular differential stage, but point to an instructive role of Notch2 during MZ B cell lineage decision. Moreover, our data indicate that Notch2 is sufficient to induce MZ B cell development.

BIOCHEMICAL EVIDENCE FOR SPECIFIC PAIRWISE INTERACTIONS OF MOUSE NKR-P1B/D:CLRB AND NKR-P1F:CLRG RECEPTORS ENGAGED IN LECTIN – LECTIN INTERACTIONS

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Mouse NKR-P1B/D:Clrb and NKR-P1F:Clrg receptor : ligand pairs are important components of the receptor "zipper" that occurs at the contact between natural killer cells and its target cell [1], and represent a recently discovered example of lectin – lectin interactions important for recognition of immune cell subsets. Previously, immunologists have brought evidence for the involvement of the NKR-P1F:Clrg pair in cellular activation such as T cell proliferaction [2], while the NKR-P1B/D:Clrb pair has been shown to inhibit natural killing [3]. In order to study the above interactions by biochemical techniques, we have amplified the individual cDNA clones for the above receptors by RT-PCR using spleens from B6/BL mice as the starting material. DNA fragments coding for the extracellular ligand binding domains of the two receptors were transferred into pET-30 bacterial expression vectors. After induction of protein production with IPTG, the protein precipitated into inclusion bodies, from which they could be refolded in vitro using standard refolding protocols. The quality of the refolding for both receptors was confirmed checking the disulfide bonding which fitted into the pattern expected for C-type lectins. Thus, using ion cyclotron resonance mass spectrometry with both MALDI and LC-ESI ionization [4], we have found that in NKR-P1F, the first cysteine is paired with the second one, the third with the sixths, and the fourth with the fifth. In Clrb the cysteine pairing was proved to be identical except the fourth and fifth cysteine, which were both missing in this receptor. In order to increase the stability of NKR-P1D, the third cysteine which does not fit into the pattern usual for this family of receptors was substituted for serine. The resulting C118S NKR-P1D, just as the Clrb, was shown to be monomeric in solution. Using gel filtration and analytical ultracentrifuge we were unable to prove the interaction between Clrb and NKR-P1D in these monomeric forms. Experiments to prove this interaction by SPR are underway. Efforts to prepare the receptors in dimeric forms in which they appear on the membrane, and experiments to see if these forms interact will follow.
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SATB1 REGULATES TCRA RECOMBINATION AND THE RE-INDUCTION OF RAG GENE EXPRESSION IN DOUBLE POSITIVE THYMOCYTES

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SATB1 is a nuclear matrix/scaffold- associated DNA binding protein that participates in the maintenance of chromatin architecture and regulates expression of many genes. SATB1 is predominantly expressed in thymocytes and SATB1 mRNA expression peaks at the DP stage. In order to investigate whether SATB1 is involved in TCRα rearrangement we generated SATB1 conditional knockout mice in which SATB1 is deleted at the long term HSC stage by a vav-cre transgene. These mice have a reduced number of total thymocytes but a relatively normal DN to DP transition. We analyzed TCRα recombination by assessing the distribution of Jα usage in SATB1-deficient thymocytes and found that usage of 3' Jα segments decreases dramatically compared with wild type thymocytes. Therefore secondary rearrangement is impaired in SATB1-deficient thymocytes. Short DP life-span or failure of Rag re-induction in DP thymocytes can impair secondary rearrangement. Expression of anti-apoptotic genes Bcl2 and Bcl-xl are comparable in wild-type and SATB1-deficient DP thymocytes. Moreover Bcl2 transgenic mice did not eliminate the difference in Jα usage between wild-type and SATB1-deficient mice. However we did detect substantial reductions in the expression of Rag1 and Rag2 in SATB1-deficient DP thymocytes. Rag re-induction in DP thymocytes depends on the ability of a distant anti-silencer to counteract the effects of the Rag silencer. In order to investigate the reason for failed re-induction of Rag in SATB1-deficient DP thymocytes, we analyzed histone modifications and RNA Pol II occupancy across the Rag locus. Consistent with reduced transcription, H4 acetylation and H3K4 di/trimethylation of Rag1 and Rag2 are diminished in SATB1-deficient thymocytes. Pol II occupancy was high at four sites in the wild type locus: Rag1 promoter, Rag2 promoter, silencer and anti-silencer. SATB1-deficient DP thymocytes show reduced Pol II occupancy at the Rag1, Rag2 promoter and silencer, but normal occupancy at the anti-silencer. We hypothesize that SATB1 deficiency may regulate chromatin conformation and Pol II access to Rag1 and Rag2. 3C analyses of long distance interactions in the Rag locus are currently underway.

ACTIVATION OF THE *TCRA* LOCUS GERMINAL TRANSCRIPTION AT B-SELECTION BY AN INDUCIBLE ENHANCEOSOME

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The T-cell receptor α locus (*Tcra*) enhancer (Eα) is essential to activate germinal transcription at the *Tcra* locus during β-selection and V(D)J recombination in double-positive (DP) thymocytes. We have studied the molecular requirements for induction of these transcripts in cell models for pre-TCR signaling and primary mouse thymocytes. Concordantly with an essential role for pre-TCR-induced signaling for this activation, *Tcra* germinal transcription was dependent on constant phospholipase Cγ signaling that suggests a crucial role for binding of inducible transcription factors to Eα. Furthermore, *Tcra* transcription was strongly dependent on calcineurin-dependent calcium and MAPK/ERK signaling indicating that NFAT factors, among other inducible factors, are crucially involved in this function. We found that both NFAT and AP-1 can bind to multiple sites within Eα. Furthermore, NFAT factors are part of the Eα enhanceosome assembled in vivo during β-selection. Transient transfection experiments indicate that Eα is responsible for activation by calcium signaling through calcineurin. All these experiments together indicate that binding of inducible factors, such as NFAT and AP-1 factors, are part of an active Eα enhanceosome that it is essential for *Tcra* transcriptional activation during β-selection. Future efforts will address the study of Eα activity control by signaling transduced by pre-TCR versus TCR along thymocyte maturation and in peripheral Tαβ cells.

ASSOCIATION OF *CD4* AND *CD8* THROUGH RUNX ENABLES LONG-RANGE EPIGENETIC GENE REGULATION *IN TRANS*

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T cell fate is associated with mutually exclusive expression of CD4 or CD8 in helper and cytotoxic T cells, respectively. RUNX transcription factors simultaneously activate the *Cd8* locus, silence the *Cd4* locus, and repress the *ThPOK* locus, which encodes a transcription factor required for CD4 expression, but coordination of these disparate activities has been difficult to unravel. Here we find that interplay among members of this transcription factor circuitry alters nuclear organization; RUNX mediates association of *Cd4* and *Cd8* while Th-POK keeps the loci apart. Moreover, targeted deletions within *Cd4* modulate CD8 expression and pericentromeric repositioning of *Cd8*. We propose that communication between *Cd4* and *Cd8* enables long-range epigenetic regulation *in trans* so that expression of one excludes the other in mature SP cells.

GSK3-MEDIATED PHOSPHORYLATION OF PSF CONTROLS CD45 ACTIVATION-INDUCED EXON SKIPPING

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Signal-induced alternative splicing is a primary, but poorly understood, mechanism for regulating protein isoform expression in response to changing cellular environments. In T cells, signal-induced alternative splicing controls the relative expression of the five isoforms of the transmembrane tyrosine phosphatase CD45 in response to cellular activation. CD45 is essential for TCR signaling and exon skipping upon activation leads to reduced CD45 activity and T cell attenuation. However, the signaling pathway leading to activation-induced exon exclusion remains elusive. Here we show that in resting T cells the splicing regulatory protein PSF is held in an inactive complex with TRAP150, which is regulated by direct GSK3-mediated phosphorylation. Reduced GSK3 activity upon T cell activation releases PSF from TRAP150, thereby allowing it to bind splicing regulatory elements in CD45 and repress variable exon inclusion. Pharmacological inhibition or knock-down of GSK3 in resting T cells both induce a CD45 splicing pattern resembling that of activated T cells, clearly implicating GSK3 as a new signal responsive regulator of alternative splicing in T cells. This was observed in the JSL1 T cell line and in primary human T cells, confirming in vivo relevance. We propose a model in which de novo synthesis of PSF in the absence of GSK3 activity is required to induce CD45 exon skipping. Our data provide evidence for a new signaling pathway leading from TCR engagement to reduced GSK3 activity, RNA binding of PSF, CD45 exon exclusion and finally T cell attenuation.

IL-17RC IS REQUIRED FOR IMMUNE SIGNALING VIA AN EXTENDED SEFIR DOMAIN IN THE CYTOPLASMIC TAIL

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IL-17 functions at the interface of the adaptive and innate arms of immunity, as it is produced by T helper 17 (Th17) cells but drives inflammatory responses in mesenchymal and epithelial cell types. This functional coordination is crucial for host defense against extracellular pathogens such as *Candida albicans* and, conversely, for the pathogenic effects of autoimmunity. The inflammatory program initiated by IL-17 signaling occurs downstream of the IL-17 receptor (IL-17R), which is comprised of two subunits: IL-17RA and IL-17RC. While IL-17RA signals as part of a pre-assembled multi-subunit complex, IL-17RA homodimers are not sufficient for functional IL-17 signaling. Rather, IL-17RC, a distinct member of the IL-17R family, is an essential component of the IL-17R complex. However, the specific molecular and cellular contributions of the IL-17RC subunit to IL-17 signaling and immunity remain unclear. Therefore, we aimed: (1) to define regions within the IL-17RC cytoplasmic tail that are integral to IL-17 signaling and (2) to define contributions of IL-17RC to IL-17-dependent immune responses *in vivo*. To delineate specific regions of the IL-17RC cytoplasmic tail required for proper IL-17 signal transduction, IL-17RC receptor truncation mutants were created and evaluated for downstream signaling activities. We found that functional IL-17 signaling requires the IL-17RC SEFIR domain, a conserved signaling motif found on all IL-17R family members. However, an additional downstream sequence is also necessary for target gene expression. We show further that IL-17RC interacts with the adaptor/E3 ubiquitin ligase Act1, and that the functional IL-17RC mutants containing the extended SEFIR region interact specifically with a phosphorylated isoform of Act1. The IL-17RC cytoplasmic tail, however, is not required for co-association with its partner, IL-17RA. Finally, we found that IL-17RC is required for IL-17-dependent responses during oral mucosal infections caused by the commensal fungus *Candida albicans*. These results show that IL-17RC is critical for IL-17-dependent responses both *in vitro* and *in vivo*. Further insight into the functional contribution of IL-17RC will shed light on its role in modulating IL-17-dependent inflammatory responses and may provide an avenue for therapeutic intervention in IL-17 dependent diseases and host defense.

USING FLUORESCENCE *IN SITU* HYBRIDIZATION TO EXAMINE CLASS SWITCHING TO IGE IN HUMAN B CELLS

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Isotype determination by class switch recombination (CSR) is an integral step in the development of an immune response. The mechanism of CSR is still not well understood. A different recombination event in B cells, VDJ recombination, has been examined by fluorescence in situ hybridisation (FISH) and has revealed the events occurring at both alleles. We have undertaken a study using FISH to analyse the events involved in CSR in human B cells.

The heavy chain locus is a highly repetitive region of DNA, making the individual heavy chain genes difficult to identify using FISH. We have developed a novel approach using bioinformatics to analyse this region. Utilising this technique we have created unique probes that can identify CSR from μ to γ/ε or γ to ε heavy chain genes in a single human B cell. The combination of immunocytochemistry and FISH provides a powerful tool to examine the nuclear architecture and microenvironment in which the heavy chain locus resides. It has allowed us to differentiate between the two alleles and the events that are occurring independently on each allele.

We have examined the colocalisation of the heavy chain locus with proteins involved in CSR including AID, UNG and Ku70. We have also identified the chromatin marks at the different alleles using a panel of antibodies against histone markers, including acetylated H3 and H3K4 trimethylation (markers of active gene transcription), as well as H3K9 trimethylation and H3K27 trimethylation (markers of active gene repression). Utilising these techniques we are dissecting the mechanism of CSR switching to IgE in human B cells.

DELETION OF THE RNA-BINDING PROTEINS TIS11B AND TIS11D IN MICE LEADS TO DEREGULATED EXPRESSION OF NOTCH-1, ARRESTED B CELL DEVELOPMENT AND T LYMPHOBLASTIC LEUKAEMIA

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TIS11b and TIS11d are RNA-Binding proteins that regulate the expression of target mRNA transcripts. They interact with AU-rich sequences in the 3'untranslated region (3'UTR) of target transcripts and induce mRNA deadenylation, destabilization and translational inhibition. We have generated conditional knockouts of both these genes and crossed them to CD2Cre mice which effect deletion early in T and B cell development. Single knockout mice show interesting but modest defects in B cell development. In anticipation of functional redundancy between TIS11b and TIS11d we intercrossed the single knockout mice. Remarkably these double knockout mice develop T lymphoblastic Leukaemia (T-ALL) and die around the age of 3-5 months. B cell development is arrested at the pro-B stage. Haemaopoietic stem cells purified from bone marrow and cultured on the OP9-DL1 stromal line show increased proliferation and differentiation to double positive T cells. Microarray performed on thymus from 5 week old mice (prior to development of leukaemia) revealed elevation of several mRNAs including Notch-1. This was confirmed by RT-PCR and elevated Notch-1 proteins was demonstrated by FACS. The B cell arrest and leukaemic phenotype in the TIS11b/d double knockout mice is almost identical to that seen in previously published Notch-1-over-expressing mice. Furthermore, the Notch-1 3'UTR contains a region of high interspecies conservation within which sit three predicted binding sites for TIS11b/d. Luciferase reporter assays confirm repressive activity of TIS11b/d upon these regions. Furthermore, this repression is abolished by point mutation within the RNA-binding domain. When cultured in vitro, proliferation and survival of tumour cells is Notch dependent and is suppressed by a gamma secretase inhibitor.

This suggests a novel mode of regulation of Notch-1 expression at the post-transcriptional level mediated by RNA binding proteins and is the first direct evidence of a role for RNA-binding proteins in the development of malignancy. Ongoing work aims to identify corresponding mutations in human cases of T-ALL and to attempt to rescue the murine phenotype using in vivo notch1 inhibition.

PAX5 CONTROLS THE B LINEAGE SPECIFIC GENE EXPRESSION PROGRAM THROUGH ASSOCIATION WITH THE NUCLEAR MATRIX

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The nuclear matrix refers to the highly branched non-chromatin network and granules inside of the nuclear lamina, which has long been proposed as the centers for various biological reactions, such as DNA replication, RNA transcription, and RNA splicing. Pax5 is an important regulator for B lineage cell development, which controls the B lineage gene expression program involving hundreds of positive and negative target genes. Here, we show that majority of the endogenous Pax5 proteins in human and murine B lineage cells are associated with the nuclear matrix; where they occupy the centers for transcription as indicated by co-localization with the RNA polymerase II and the TATA box binding protein (TBP). Detailed analyses identified that the partial homeodomain (HD) and lysine 67, 87, and 89 residues within the PRD domain of Pax5 are required for its association with the nuclear matrix. Mutation of these lysine residues disrupts Pax5 mediated activation and repression of hundreds target genes. Based on these results, we propose an experimental model that Pax5 controls the B lineage gene expression through recruitment of the target genes to the transcription center associated with the nuclear matrix.

CHEMICAL AND GENETIC STUDIES OF RORΓT, A CRITICAL REGULATOR OF TH17 CELL DIFFERENTIATION

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The mouse nuclear hormone receptor retinoic acid-related orphan receptor-γt (RORγt) is necessary and sufficient for the differentiation of interleukin 17 (IL-17)-producing T helper (Th17) cells, which comprise one of the distinct effector T cell lineages involved in the regulation of host defenses, particularly against extra-cellular bacteria and fungi. Recent studies from numerous laboratories indicate that the Th17 cells have key pro-inflammatory roles in various autoimmune diseases and in cancer. For example, mice lacking IL-17 or the p19 subunit of IL-23, which is required for the expansion of Th17 cells in vivo, were resistant to various animal models of diseases, including experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (IBD). In accordance with these observations, previous work in our laboratory has also shown that interrupting RORγt function prevents progression of autoimmune diseases in mice, suggesting that it may be a good therapeutic target for these diseases.

Despite the potential of RORγt as a target for anti-inflammatory drugs, ligands and other factors that regulate its activity have yet to be identified. We have developed an activity-based assay system in *Drosophila* S2 cells to perform genome-wide loss-of-function and small-molecule chemical compound screens, which may allow us to identify chemical/genetic modulators, including components involved in the ligand biosynthetic pathway. At least two different classes of small molecule compounds identified from the screens function as specific inhibitors for RORγt to selectively block both mouse and human Th17 cell differentiation *in vitro*. Moreover, treatment of animals with these compounds not only delays onset, but also reduces severity of EAE progression in both active and passive EAE induction models. We also identified multiple *Drosophila* genes involved in several interesting biological pathways that positively or negatively regulate the function of RORγt. Regulatory roles of their mammalian orthologues in Th17 cell differentiation are currently being tested and ensuing results will be presented.

TRANSCRIPTION FACTOR ETS1 REGULATES DEVELOPMENT AND PROGRESSION OF ATOPIC DERMATITIS

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Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic and eczematous skin lesions. AD is caused by complex pathogenic factors including gene susceptibility, skin barrier dysfunction, bacterial infection and immunological factors. Eosinophils and Th2 type cytokines including IL-4, IL-5, IL-10, IL-13 and IL-31 play important role in the development and progression of AD. Although many studies elucidated the pathogenesis of AD, little information is available for the genetic factors associated with AD development and pathogenic processes. Recently, we have found that Ets1 knock out (KO) mice developed spontaneous AD and were more susceptible to induce experimental AD. To elucidate underlying mechanisms of exuberant AD symptoms in Ets1 KO mice, we investigated functional characteristics of T and B cells related with AD pathogenesis. The alterative genetic program in Ets1 KO mice resulted in upregulation of IL-5, IL-9, IL-10 and IL-31 expression, hyper-plasmacytosis and hyper-production of IgE levels, which contributed to AD pathogenesis. In T cells, Ets1 negatively regulated a set of AD pathogenic genes including IL-5, IL-10 and IL-31 by direct binding on each promoter region. Moreover, Ets1 concomitantly acted as a transcriptional activator of Pax5 gene but as a transcriptional repressor of Blimp1 gene through direct binding on each promoter region of each target genes. Conclusively, our findings suggest that Ets1 acts as the master regulator in AD pathogenesis in which transcription factor Ets1 modulates effector function of T and B cells by regulating multiple target genes resulting in prevention of AD development and progression. This research was supported by grants from the regional technology innovation program of the MOCIE (No. RTI05-01-01), the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare and Family Affairs (A080588-5) and by systems biology infrastructure establishment grant provided by GIST.

AN ESSENTIAL DEVELOPMENTAL CHECKPOINT FOR EARLY T CELL DEVELOPMENT

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T cells, the major player of adaptive immunity, are generated from hematopoitic stem cells (HSCs) through a line of differentiation steps. Whereas later steps serving as checkpoints for the quality of T cell receptor have been well defined, mechanisms of earlier steps where pluripotent HSCs are restricted to unipotent T cell progenitors are poorly understood. We have previously shown that progenitors developing in the thymus are determined to T cell lineage by shutting-off the potential to produce myeloid, dendritic and NK cells, at the midst of c-kit+CD25+ (DN2) stage, termed “DN2-determination step”. Here we show that this DN2-determination step is serving as a critical developmental checkpoint. We firstly demonstrate that, by culturing murine prethymic progenitors on immobilized DLL4 protein in the presence of cytokines including IL-7, cells differentiated towards T cell lineage were arrested just before the DN2-determination step, and the arrested cells undergo self-renewal cycle keeping non-T lineage potentials. These arrested DN2 cells were induced to be determined to T cell lineage by simply reducing the IL-7 concentration, eventually giving rise to $CD4^{+}CD8^{+}$ αβT cells. We further show that, a very similar arrest and self-renewal of progenitors were observed in thymocytes of Bcl11b-/- mice, and the DN2-arrest in the DLL4/IL-7 culture did not occur in progenitors in which Bcl11b is enforced to express. Our study thus revealed a novel Bcl11b-dependent checkpoint of T cell development, the blockage of which induces self-renewal of arrested progenitors, providing a clue to elucidate the molecular mechanisms establishing T cell lineage identity.

THE ROLE OF ARGONAUTE2 IN ANTIVIRAL DEFENSE

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Recent studies suggested a role of miRNAs in regulation of the pathogen-host interaction. The pathogens, including some viruses, were found to produce miRNAs. Conversely, host response to pathogens is associated with changes in the pattern and levels of miRNA expression. The execution of the miRNA function is mediated by the Ago family of proteins. We argue that multiplicity of the Ago proteins in mammalian cells as well as in cells of other organisms may reflect the differential ability of individual Ago's to regulate response to distinct pathogens. To test this hypothesis we studied the antiviral response of mouse cells deficient in Ago1, Ago2, 3 or 4 proteins. At this point, we found that deficiency in Ago2 boosts the levels of virus or synthetic nucleic acid-induced IFNβ production. An increase expression of IFNβ by Ago2 deficient cells is associated with higher, as compared to wild-type cells, resistance to the cytopathic effect of RNA viruses such as Influenza A and Vesicular Stomatitis Virus (VSV). These data revealed a previously unknown negative role of Ago2 in regulation of antiviral response. We will present the data that address the mechanism of Ago2 involvement in antiviral response.

IL-7 SIGNALING RESTRICTS ANTIGEN RECEPTOR REARRANGEMENT TO NON-CYCLING CELLS

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V(D)J recombination is a somatic recombination event that enables enormous diversity to be created from a limited number of gene segments. It is critical that this process is restricted to non-cycling cells to prevent replication from occurring over broken or unrepaired DNA. IL-7, a cytokine that is secreted within the local environment of developing B and T cells, provides essential proliferation and survival signals to lymphocytes while also guiding their development. IL-7 is required for promoting the accessibility of select antigen receptor loci, thereby promoting their recombination. In apparent contradiction, recent data demonstrates that IL-7 represses the expression of the Rag genes, essential components of the recombination process. It has remained unclear how IL-7 can both promote and inhibit recombination within a single cell population such as pro-B cells. We provide evidence that pro-B cells are heterogeneous in terms of IL-7R expression in a manner that is directly related to cell cycle. In addition, cycling cells have lower levels of Rag transcripts than cells in G1/GO. Decreased Rag transcription correlates with increased association with pericentromeric heterochromatin (PCH), a repressive compartment within the nucleus, providing a potential mechanism for repression. Interestingly, the Igh locus (a target whose accessibility has been positively linked to IL-7) does not alter its association with PCH according to cell cycle status. We propose a model in which IL-7 acts as a “gate-keeper” to restrict recombination to non-cycling cells by actively inhibiting RAG expression via altered nuclear compartmentalization.

E-PROTEINS ARE REQUIRED FOR DEVELOPING THYMOCYTES TO ENTER THE CD4 LINEAGE

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Development of thymocytes from the $CD4^+CD8^+$ double positive (DP) to the $CD4^+$ or $CD8^+$ single positive (SP) stage is a tightly regulated and obligatory step for the production of mature αβ T cells. This developmental transition is initiated upon proper expression of a T cell receptor (TCR) and proceeds through a variety of differentiation events, including the decision for cells to enter either the CD4 or CD8 T cell lineage. Current understanding of the underlying transcriptional regulation directing DP to SP development reveals a complex network of factors required for interpreting TCR selection signals, controlling cell survival, and determining lineage fate. Among the known players in these processes, the E-protein transcription factors HEB and E2A have been shown to be critical for both positive selection of DP thymocytes and DP cell survival. However, data from these studies suggested that the extent of E-protein function during the DP to SP transition does not end there. Here we investigate an additional role for E-proteins in directing CD4 vs. CD8 lineage choice. To analyze the function of E-proteins during DP to SP development, we utilize a double conditional knockout mouse model that results in deletion of the genes encoding HEB and E2A at the DP stage. T cells generated in these mice are predominantly CD8 lineage with only a small proportion of CD4 lineage cells. Elimination of the pro-apoptotic factor Bim, critical for negative selection of thymocytes, was unable to rescue this CD4 lineage defect. Furthermore, CD8 lineage T cells continued to be generated even in the presence of a major histocompatibility complex (MHC) class II restricted TCR transgene (which usually drives CD4 development). These results suggest that CD4 lineage cells fail to develop in the absence of E-proteins and instead are diverted to the CD8 lineage. The resulting mature, E-protein deficient T cells were able to proliferate and produce cytokines upon stimulation, demonstrating that the functions of E-proteins downstream of TCR signaling may be unique and specific to thymocyte development during maturation from the DP to SP stage.

CONSERVED NON-CODING DNA ELEMENTS IN THE FOXP3 GENE CONTROL THE REGULATORY T CELL LINEAGE

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Immune homeostasis is dependent upon tight control over the size of a population of regulatory T (Treg) cells capable of suppressing over-exuberant immune responses. The Treg cell subset is comprised of cells that commit to the Treg lineage by up-regulating the transcription factor (TF) Foxp3 either in the thymus (tTreg) or in the periphery (iTreg). Considering a central role for Foxp3 in Treg differentiation and function, we hypothesized that conserved non-coding DNA sequence (CNS) elements at the Foxp3 locus encode information defining the size, composition and stability of the Treg population. Here, we demonstrate that each of three Foxp3 CNSs (CNS1-3) has a distinct non-redundant function in Treg cell fate determination. We demonstrate a critical cell-intrinsic role for c-Rel in Foxp3 induction. The pioneer element CNS3, which acts to potently increase the frequency of Treg cells generated in the thymus and the periphery, binds cRel in in vitro assays. In contrast, CNS1, which contains a TGF-β/NFAT response element, is superfluous for tTreg differentiation, but plays a prominent role in iTreg generation in gut-associated lymphoid tissues (GALT). Finally, CNS2, while dispensable for Foxp3 induction, is required for continuous Foxp3 expression in the progeny of dividing mature Treg cells. Intriguingly, Foxp3-containing protein complexes bind to CNS2 in a CBFβ/Runx1 and CpG DNA demethylation-dependent manner, suggesting that Foxp3 recruitment to this “cellular memory module” facilitates the heritable maintenance of the active state of the Foxp3 locus and, therefore, Treg lineage stability. Together, our studies demonstrate that the composition, size, and maintenance of the Treg cell population are controlled by Foxp3 CNS elements engaged in response to distinct cell-extrinsic or -intrinsic cues.

RECRUITMENT OF THE IKK COMPLEX TO TCR MICROCLUSTERS DURING T CELL ACTIVATION

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NF-κB family transcription factors are critical for the regulation of many genes important for both innate and adaptive immunity. Common to all receptors that induce the classical NF-κB pathway is the activation of the trimeric inhibitor of κB kinase (IKK) complex. This complex consists of two catalytic subunits (IKK1/IKK2 or IKKα/IKKβ) and a scaffolding or adaptor subunit known as IKKγ or NF-κB essential modulator (NEMO). Here we demonstrate, using live cell confocal microscopy with fine spatial and temporal resolution, that the IKK complex is recruited to early sites of T cell receptor for antigen (TCR) signaling known as microclusters. Furthermore, we show that IKK is specifically recruited to microclusters containing the tyrosine kinase ZAP-70, but not those with the adaptor SLP-76. Using a panel of Jurkat T cell variants lacking specific signaling proteins, we have determined which elements of the TCR signaling machinery are required for IKK localization to the microclusters. Finally, we have performed structure/function analysis of IKKγ itself to determine which domains within IKKγ mediate its recruitment to TCR microclusters, and will present the results of this analysis.
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PIM2 INDUCED MMP-9 EXPRESSION IN MACROPHAGES REQUIRES PI3K AND NOTCH1 SIGNALING

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Activation of inflammatory immune responses during granuloma formation by the host upon infection of mycobacteria is one of the crucial steps that is often associated with tissue remodeling and breakdown of the extracellular matrix. In these complex processes, matrix metalloproteinase-9 (MMP-9) plays significant role in tissue remodeling. In this study, we investigated the molecular mechanisms underlying Phosphatidyl-myo-inositol dimannosides (PIM2), an integral component of the mycobacterial envelope, triggered MMP-9 expression in macrophages. PIM2 triggers the activation of Phosphoinositide-3 Kinase (PI3K) and Notch1 signaling leading to MMP-9 expression in a Toll-like receptor 2 (TLR2)-MyD88 dependent manner. Notch1 signaling perturbations data demonstrate the involvement of the cross-talk with members of PI3K and Mitogen activated protein kinase pathway. Enforced expression of the cleaved Notch1 in macrophages induces the expression of MMP-9. PIM2 triggered significant p65 nuclear factor -kB (NF-kB) nuclear translocation that was dependent on activation of PI3K or Notch1 signaling. Furthermore, MMP-9 expression requires Notch1 mediated recruitment of Suppressor of Hairless (CSL) and NF-kB to respective promoters. Taken together, these data implicate PI3K and Notch1 signaling as obligatory early proximal signaling events during PIM2 induced MMP-9 expression in macrophages.

53BP1 FACILITATES CRUCIAL LONG RANGE INTERACTIONS IN THE IGH LOCUS DURING CLASS SWITCH RECOMBINATION

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Immunoglobulin (Ig) class switch recombination (CSR) uses a distinct nonhomologous end joining (NHEJ) pathway to repair DNA double strand break (DSB) intermediates. During CSR the Igh locus is dependent on 3D chromatin architecture mediated by long range intra-chromosomal interactions between distantly located enhancers to create a looped structure. 53BP1 is a DNA damage response protein which is rapidly recruited to sites of chromosomal DNA double strand breaks (DSBs) and is essential for CSR. Based on two key observations it has been suggested that within the Igh locus, 53BP1 facilitates synapsis of distant broken DNA ends that are located in switch (S) regions. First, in 53BP1 deficient- but not WT B cells enhanced intra-S region rearrangement is detected. Second, 53BP1 deficiency is associated with genome instability at the Igh locus upon induction of DSBs implying that 53BP1 is involved in tethering distantly located DSBs. We have used the chromosome conformation capture (3C) technique to assess the contribution of 53BP1 to the spatial organization of the Igh locus in the presence and absence of DSBs. In B cells deficient for 53BP1 or AID there was modest impact on multiple long range interactions during CSR. Unexpectedly, intra-chromosomal interactions were profoundly reduced in B cells doubly deficient for 53BP1 and AID, suggesting that prior to the induction of DSBs, 53BP1 is a crucial contributor to the spatial organization of the Igh locus. Chromatin immunoprecipitation (ChIP) assays using two independent antisera, indicate that 53BP1 is resident on S regions in resting B lymphocytes from WT and AID deficient B cells indicating that 53BP1 occupancy is independent of DSB induction. ChIP analyses also demonstrate the presence of H4K20me2 modifications in S region of resting B cells. The H4K20me2 mark is recognized by the 53BP1 tudor domain and functions to anchor the protein to chromatin. Others have shown that in mice doubly deficient for Suv4-20h1 and Suv4-20h2, the absence of H4K20me modifications is correlated with delayed recruitment of 53BP1 to DSBs, reduced CSR and instability of chromosome 12 at the Igh locus. Replication protein A (RPA) is also found in S regions and is an interaction partner with 53BP1 suggesting that the combined presence of H4K20me2 and RPA might target 53BP1 to S regions in resting B cells. Our findings provide evidence that 53BP1 is specifically recruited to the Igh locus independent of the presence of DSBs and is a key organizer of chromatin architecture during CSR. This work was supported by the National Institutes of Health, USA (RO1AI052400 to A. L. K.)

ROLE OF SHORT NON-CODING RNAS IN B CELL DEVELOPMENT

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In order to explore the role of miRNAs and other small interfering RNAs in B cell survival and orchestration of the B lymphocyte developmental program we undertook a global approach, ablating all Dicer dependent short RNAs by conditionally deleting Dicer at the earliest stage of B cell development. We found that B cell specific ablation of Dicer resulted in an essentially complete developmental block in the bone marrow at the pro-B to pre-B cell transition. By introducing a pro-survival EμBcl2 transgene or by knocking out one of the genes targeted by miRNAs throughout B cell development, Bim (a pro-apoptotic BH3 family member), we achieved considerable rescue of Dicer null B cells. Rescue of B cell development in the absence of short non-coding RNAs allowed us to investigate the role of these short RNAs in lymphocyte proliferation, V(D)J rearrangement and B cell receptor expression. While we found the basic mechanism of V(D)J recombination was intact in the absence of short non-coding RNAs, there was a discernable impact on Ig repertoire because of a shift in DH element accessibility at the heavy chain locus and aberrant N nucleotide addition among Ig light chain joints (Koralov, Muljo et al Cell 2008).
We are now comparing Dicer, Drosha and DGCR8 deficient B cells, rescued in a similar manner as above with EμBcl2 transgene, to distinguish the role of miRNAs from other short non-coding RNAs throughout B cell development. In particular we are investigating the role of local Ig transcripts in DH element accessibility. We also uncovered a role of short non-coding RNAs in Ig expression in mature cells. Dicer null B cells exhibit a surface Ig low phenotype and appear to have undergone repetitive gene rearrangement at their kappa loci, characterized by elevated Jκ4/5 usage, increased IRS/RS recombination and striking skewing towards 5' Vκ gene usage. We are currently examining the possible role of short non-coding RNAs in Ig locus accessibility and downstream BCR signaling to identify what pathway may be responsible for the Ig low phenotype in Dicer null B cells. Combining an Ig transgene with transgenic Bcl2 leads to complete rescue of B cell development, allowing us to study the role of short non-coding RNAs on other B cell intrinsic processes that may involve this element of regulation – somatic hypermutation and class switch recombination.

IDENTIFICATION OF CIS-REGULATORY ELEMENTS TARGETING AID-MEDIATED SEQUENCE DIVERSIFICATION TO THE CHICKEN IMMUNOGLOBULIN LIGHT CHAIN GENE

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Somatic hypermutation (SHM) and gene conversion (GCV) are two closely related processes that increase the diversity of the primary immunoglobulin (Ig) repertoire. Both processes are initiated by the activation-induced cytidine deaminase (AID) that converts cytosine residues to uracils in a transcription-dependent manner and processed by direct replication and error-prone DNA repair. It is unknown how this mutagenic activity is targeted almost exclusively to Ig loci.
To identify cis-acting elements involved in targeting SHM and GCV to the chicken Ig light chain (IgL), we used DT40 cells and systematically deleted non-coding sequences from the IgL locus using standard gene targeting strategies. We identified a novel as of yet uncharacterized regulatory region (named 3′RR) in the chicken IgL gene, containing a classical transcriptional enhancer and also cis-acting DNA elements essential for targeting AID-mediated sequence diversification. We are continuing our systematic deletion strategy to identify the minimal sequence required for targeting and to determine the trans-acting factors that bind to it. To identify similar targeting elements in the murine Igκ locus, we use a cross-complementation approach replacing the 3′RR in DT40 cells with murine Igκ sequences. Previous mouse transgenic studies indicated that both iEκ and 3'Eκ were necessary for the SHM of Igκ transgenes, and we are currently testing the ability of these enhancers to restore SHM/GCV in the chicken locus.

DISTINCT, NON-REDUNDANT FUNCTIONS OF TNF/LT EXPRESSED BY LTICS IN MUCOSAL IMMUNITY

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Intestinal mucosal surface is constantly exposed to a complex and dynamic community of microorganisms. Therefore, it is crucial that host organism monitors and regulates microbial interactions with intestinal epithelial surfaces using gut-associated immune system (GALT). GALT represents complex network of various lymphoid structures, such as Peyer's Patches (PPs), isolated lymphoid follicles (ILFs)and lamina propria lymphocytes. Recently, lymphoid tissue-inducer cells are reported as crucial cell type for development of PPs and ILFs. Previously, TNF and lymphotoxin α (LTα) and beta (β) were shown as critical factors for development of these structures, although cellular sources of these cytokines remain largely unknown. To study role of TNF, LTα and LTβ expressed by LTICs, mice with floxed TNF, LTα and LTα genes were crossed with mice bearing Cre-recombinase under Rorγt-promoter. LTα and LTβ produced by LTICs are crucial for PPs and LN development, while TNF expressed by LTICs has critical, non-redundant functions in PPs development. Analysis gut IgA production revealed no role of LTICs – derived LTβ in IgA production, while ablation of LTα in LTICs resulted in complete absence of IgA from feces, implying critical role of soluble LTα (sLTα) in the control of T-cell independent IgA production in the gut. In addition, gut IgA levels were only impaired in TNFR1 KO mice, suggesting that sLTα mediates its effects via both TNFR1 and TNFR2. Taken together, our data revealed distinct, non-redundant functions of TNF/LT expressed by LTICs in mucosal immunity.

CD8 GENE REPOSITIONING IN THE NUCLEUS DURING THYMOCYTE DEVELOPMENT

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The functional architecture of the interphase nucleus has recently become a focus of interest. Chromatin modifications and nuclear spatial organisation seem to function in concert to co-ordinate the responses of a cell to environmental and intracellular signals that lead to the establishment of a gene expression program. The positioning of a gene in relation to its chromosome territory (CT) has been studied extensively in recent years. In our study, we followed the repositioning of the CD8α gene in relation to its chromosomal backbone during the developmental program of T cells, from DN thymocytes to DP and then CD4 SP and CD8 SP in the thymus and eventually to mature CD4+ and CD8+ cells in the spleen, and compared it to B cells where the gene has never been expressed. As the CD8 gene is located on mouse chromosome 6, its repositioning was studied in relation to its sub-chromosomal territory (sCT), defined by specific mcb fragments that were generated by micro-dissection of chromosome 6 resulting in a library of 8 overlapping fragments. The distance of the gene was measured both from the centre and from the nearest edge of its sCT. Our results suggest that during T cell development the CD8 gene in CD8 expressing cells relocates from an inner core of its chromosomal domain to a nuclear location where transcription becomes possible. As this is also true for the CD4 gene, this gene motility appears to be a more general feature of gene regulation during T cell development.

PLASTICITY OF TH17 AND TH1 CELLS IN EAE

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Th17 cells are a potentially pathogenic T helper subset in autoimmunity. Th17 cells are characterized by secretion of IL-17A, IL-17F and IL-22 and are largely dependent of the transcription factor RORγt and the cytokine IL-23. Although Th17 cells were shown to be highly pathogenic in animal autoimmune models, our recent data show that for CNS autoimmunity the major cytokines produced by Th17 cells such as IL-17A, IL-17F and IL-22 are dispensable. Recent data showed that Tregs as well as Th17 cells share flexibility in response to the surrounding cytokine milieu. To investigate the stability and the fate of differentiated Th17 cells we created a new mouse strain that allows for cell fate experiments. In these mice the IL-17F expressing cells are irreversibly marked by Cre mediated excision of a loxP-flanked-STOP cassette of the Rosa-EYFP transgene. We found that sorted EYFP positive Th17 cells are highly plastic whether generated in vitro or in vivo. Adoptively transferred Th17 cells largely lost IL-17 expression in a lymphopenic environment and expressed IFNγ. In contrast, encephalitogenic Th1 cells stayed stable IFNγ expressers although some Th1 cells of lymph nodes became double positive for IL-17A and IFNγ. Additionally our data show that Th17 cells do not become longterm bone marrow resident memory cells. This flexibility questions whether Th17 cells can be put into one line of stable T helper subsets as Th1 or Th2 cells. Th17 might rather constitute a state of T cells under the influence of a pro-inflammatory milieu.

DNA BASED ADDRESS SIGNALS THAT REGULATE THE INTERACTION OF THE IGH LOCUS WITH THE NUCLEAR LAMINA

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Compartmentalization of the mammalian genome within the nucleus is considered to regulate various processes such as transcription, RNA processing, DNA repair and recombination. The murine IgH locus has been shown to be associated with the nuclear lamina in multi-potential hematopoietic progenitors, T-lineage cells and fibroblasts and repositioned within the nucleoplasm during early B cell development. We have proposed that such compartmentalization may restrict access of the IgH locus to the transcriptional and recombination machinery in T-lineage cells. Consistent with this proposal, repositioning of active genes to the lamina can result in their transcriptional repression. Recently, targeted DNA methylation (DamID) has been used to map lamina-associated domains (LADs) in human fibroblasts that span nearly forty percent of the genome and preferentially contain inactive chromatin. We have used high resolution DamID and chromatin crosslinking to analyze the interaction of the germ-line IgH locus with the lamina and to delineate and characterize LADs in chromosomes of mouse fibroblasts. Such analysis reveals that the entire IgH locus (~3Mb) constitutes a LAD. Using DNA sequences from the IgH locus and another developmentally regulated LAD (Cyp3a) we demonstrate the presence of lamina-associating sequences (LASs) that, upon ectopic integration in fibroblasts, reposition loci to the nuclear lamina. The interaction of LASs as well as the endogenous IgH and Cyp3a loci with the lamina is disrupted by inhibition of histone deacetylase (HDAC) activity. Unlike detachment, re-association of LASs with the nuclear lamina is dependent on cell division. These results suggest that both DNA sequence elements and chromatin structure are required for cell cycle dependent LAD assembly and maintenance. Thus as is the case for proteins, chromosomal loci contain nuclear address signals that are important for gene location and activity. Future experiments are focused on delineating the nature of the sequence elements and factors that target the IgH locus to the nuclear lamina.

LEUKEMIA-ASSOCIATED MUTATIONS IN TET2 DIMINISH CATALYTIC ACTIVITY

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TET2 is a close relative of TET1, an enzyme that we recently showed converts 5-methylcytosine to 5-hydroxymethylcytosine in DNA. *TET2* mutations and chromosomal deletions spanning the *TET2* locus are frequently found associated with myelodysplastic syndromes (MDS), myeloproliferative disorders (MPD) and frank myeloid malignancies (acute myeloid leukemia, AML, and chronic myelomonocytic leukemia, CMML). The mutations have been assumed to be loss-of-function mutations since both alleles of TET2 are generally affected. Here we show that TET2 expression in cells causes loss of 5-methylcytosine staining in parallel with increased staining with an antibody to 5-hydroxymethylcytosine and an increased level of 5-hydroxymethylcytosine in genomic DNA. This activity is abolished or severely impaired by engineered mutations predicted to impair catalytic activity, as well as by leukemia-associated mutations in residues predicted to play a role in catalysis. The mutational status of the *TET2* locus, determined by SNP analysis and sequencing, correlated well with the level of 5-hmC in genomic DNA from patients with MDS, MPD and other myeloid malignancies. These findings indicate that appropriate hydroxymethylation of DNA is critical for suppressing myeloid tumorigenesis.

COHESINS AND GENE EXPRESSION: FROM PROOF-OF-PRINCIPLE TOWARDS CELL LINEAGE- AND DEVELOPMENTAL STAGE-SPECIFIC REGULATION.

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Cohesin-mediated sister chromatid cohesion is essential for chromosome segregation and post-replicative DNA repair. An additional role for cohesin in the regulation of gene expression has recently been recognized based on findings in model organisms and in patients with Cornelia de Lange syndrome. This non-canonical role of cohesin in gene regulation has been rationalized, at least in part, by findings that cohesin is recruited to mammalian chromosomes by the insulator protein CTCF and that cohesin knockdown perturbs long-range chromosomal interactions. Here we take a genetic approach to determine the impact of cohesin on cell lineage-specific and developmental stage-specific gene expression in developing T cells.

THE IMMUNOLOGICAL GENOME PROJECT

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ImmGen is a collaborative group of Immunology and Computational Biology labs which aims to determine, on a broad scale, the patterns of gene expression and genetic regulatory networks of immune system cells in the mouse. We will illustrate some of the results and computational predictions, which arrive at an unusual perspective on transcriptional regulation.

In the first phase, which is well under way, ImmGen participants are generating under carefully standardized conditions a "complete" compendium of whole-genome microarray datasets. This will encompass most defined cell populations of the adaptive and innate immune systems, at major stages of their differentiation and activation (lymphoid, myeloid, stroma; ~250 populations overall). Each participating lab is addressing questions relevant to particular lineages of interest (relationships between cells populations, transcript flow through differentiation or cell migration, regulatory structures). The global data are also used for computational reconstruction of regulatory networks, grouping genes into modules of genes that are coregulated by the same regulatory factors (transcription or signaling factors), generating predictions of their regulatory elements, some of which are shared across the entire system, others being lineage-specific.

In the second phase, we are beginning to use "perturbations" (natural or induced genetic variation, drug treatment, TF transfections) to test the variability/robustness of transcriptional control, and to validate and refine the computational predictions of regulatory relationships.

ImmGen data are made publicly available through usual public databases and through a project-specific website (www.immgen.org), for which we are exploring novel ways to display expression data and its organization in gene families and modules, or throughout the physical genome. In addition, ImmGen is open to suggestions from the community, either for defined cell types not previously included, or as add-on experiments that might benefit from ImmGen's standardized processing.

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CONTROL OF GENE TRANSCRIPTION BY NATURAL AND SYNTHETIC HISTONE MIMICS

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Histone mimics are short stretches of amino acid residues that are homologous or identical to the N-terminal portion of histone H3. Histone mimics are present in numerous proteins of human, mouse, viral or bacterial origin. However, function of these mimics remains unknown. We have identified the presence of a histone mimic within the functionally important influenza protein NS1. We show that similar to histones, the NS1 histone mimic could be modified by acetylation, methylation and phosphorylation. Furthermore, similar to the modified histones, the NS1 histone mimic controls expression of genes in mammalian cells. These findings suggest a possible role of the virally encoded histone mimic in regulation of the host gene expression during influenza infection. I will present the data that address the structural and functional features of the influenza histone mimic. I will also discuss the results of the experiments that utilize synthetic histone mimics to control gene expression in immune cells.

EPIGENETIC REGULATION OF T CELL DIFFERENTIATION

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Each cell type is associated with a distinct epigenome that is characterized by a set of distinct chromatin modifications. We previously showed that while TCR-inducible genes are poised by chromatin modification in resting T cells, significant changes in histone modifications accompany T helper cell differentiation. Signature cytokine genes exhibit either active or repressive chromatin modifications as expected for their expression status; however, key transcription factors such as Tbx21 and Gata3 associate with bivalent modifications in non-expressing lineages. The epigenetic mechanisms underlie the T cell specificity and plasticity. To understand how the epigenetic patterns are established, we analyzed the genome-wide distribution of GATA3, a key transcription factor required for double positive cell and Th2 differentiation, in all well-defined T cell lineages. Our data indicate that GATA3 regulates critical components of multiple signaling and transcription pathways by mediating epigenetic modifications.
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THE YY1 REPO DOMAIN NEEDED FOR PCG FUNCTION IS NECESSARY FOR B CELL DEVELOPMENT AND INTERACTS WITH CONDENSIN PROTEINS TO REGULATE IG REARRANGEMENT.

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Conditional knock-out of transcription factor YY1 in the B cell lineage results in pro-B cell arrest. The PcG protein EZH2 shows a similar phenotype. We deleted a 25 amino acid YY1 domain (the REPO domain) that is necessary and sufficient for YY1 PcG function. We used this mutant, and wild-type YY1 retroviral constructs, to transduce bone marrow from YY1 conditional knock-out mice and transferred these cells into irradiated recipient mice. While wild type YY1 rescued B cell development, the REPO deletion mutant failed to rescue the B cell lineage with dramatic reduction in immature B cells. We also found that Ig kappa rearrangement was reduced in the REPO mutant background. We used the YY1 REPO domain to isolate interacting proteins and found that the REPO domain interacts with proteins from the condensin complex. We found that YY1 and condensin proteins co-localize at numerous sites across the Ig kappa locus and that RNAi knock-down of condensin subunit SMC4 inhibits Ig kappa rearrangement. Our results suggest that YY1 and condensin proteins are involved in Ig locus contraction and rearrangement.

THE MOUSE IMMUNOGLOBULIN HEAVY CHAIN V-D INTERGENIC SEQUENCE CONTAINS INSULATORS THAT MAY REGULATE ORDERED V(D)J RECOMBINATION

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During immunoglobulin heavy chain (Igh) V(D)J recombination, D-to-J precedes V-to-DJ recombination in an ordered manner, controlled by differential chromatin accessibility of the V and DJ regions, and essential for correct antibody assembly. However, with the exception of the intronic enhancer Eμ, which regulates D to J recombination, cis-acting regulatory elements have not been identified. We have assembled the sequence of a strategically located 96kb V-D intergenic region in the mouse Igh, and analysed its activity during lymphocyte development. We show that Eμ-dependent D antisense transcription, proposed to open chromatin before D to J recombination, extends into the V-D region for more than 30 kb in B cells before, during and after V(D)J recombination and in T cells, but terminates 40kb from the first V gene. Thus, subsequent V antisense transcription before V to DJ recombination is actively prevented and must be independently activated. To find cis-acting elements that regulate this differential chromatin opening, we identified six DNase I hypersensitive sites (HS) in the V-D region. One conserved HS upstream of the first D gene locally regulates D genes. Two further conserved HSs near the D region mark a sharp decrease in antisense transcription, and both HSs bind CTCF in vivo. Further, they both possess enhancer-blocking activity in vivo. We propose they are enhancer-blocking insulators preventing Eμ-dependent chromatin opening extending into the V region. Thus they are the first elements identified that may control ordered V(D)J recombination and correct assembly of antibody genes.

CHROMATIN ARCHITECTURE AND THE GENERATION OF ANTIGEN RECEPTOR DIVERSITY

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The immunoglobulin heavy-chain (Igh) locus is organized in distinct regions that contain multiple variable (VH), diversity (DH), joining (JH) and constant (CH) coding elements. In previous studies we have determined the 3D-structure of the Igh locus in pre-pro-B and pro-B cells. These findings indicated that the Igh locus undergoes large-scale conformational changes upon developmental progression. We have extended these findings and examined how Igh locus contraction is established in developing B cells. As a first approach to resolve this questions, we have isolated hematopoietic progenitor cells that carry an inducible Id2 construct. These cells upon withdrawal of Id2 these progenitor cells readily differentiate to become committed to the B cell lineage. We observed in this in vitro system that Igh locus contraction occurs prior to Igh VDJ gene rearrangement. Additionally, we observed that the Igh locus consists of three compartments at the pre-pro-B cell stage but that the compartments merge within a five days period upon withdrawal of Id2. Collectively, these data indicate that the Igh locus undergoes distinct topologies during the development of B-lineage cells, to ultimately establish distinct topology that allows long-range genomic interactions to occur with relatively high frequency. We have used multicolor 3D-FISH to confirm the 3D-topology as resolved by trilateration. Based on the observations obtained by others as well as our own, we propose that Igh locus topology as observed in pre-pro-B cells is a general feature of the eukaryotic chromatin fiber.

AID PRODUCES WIDESPREAD DNA DAMAGE AND MATURE B CELL LYMPHOMAS WITH RECIPROCAL CHROMOSOME TRANSLOCATIONS *IN VIVO*

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Cancer initiating translocations such as those associated with lymphomas require formation of paired double strand breaks (DSBs). Activation-induced cytidine deaminase (AID) produces widespread mutation in mature B cells by introducing U:G mismatches in target DNA. These lesions can be processed to produce DSBs in *IgH* and more rarely in *c-myc* but the extent of genome instability produced by AID and whether this leads to translocation associated malignancy is unknown. We have shown that de-regulated expression of AID causes widespread chromosomal damage, which alone is insufficient to induce B cell lymphoma; transformation requires concomitant loss of the tumor suppressor p53. Mature B cell lymphomas arising as a result of de-regulated AID expression harbor clonal reciprocal translocations involving a diverse group of *Ig* and *non-Ig* genes that are direct targets of AID, including miRNA142, a previously unknown target, which is translocated in human B cell malignancy. We conclude that AID produces DSBs throughout the genome, some of which lead to lymphoma associated chromosome translocations in mature B cells.

THE ANTIBODY DIVERSIFICATION ENZYME AID IS TARGETED TO SWITCH REGIONS BY KAP1 AND HP1 DURING IMMUNOGLOBULIN CLASS SWITCH RECOMBINATION.

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Immunoglobulin class switch recombination (CSR) is initiated by the specific targeting of activation induced cytidine deaminase (AID) to transcribed switch (S) regions and the subsequent generation of double stranded DNA breaks (DSBs). AID targeting during CSR correlates with the induction of epigenetic modifications at S regions, including trimethylation of lysine 9 of histone H3 (H3K9me3), and has been suggested to be mediated by the C-terminus of AID. Although this domain is required for the specific function of AID in CSR, its actual role and the interactions it mediates are poorly understood. Here we show that the C-terminus of AID associates with KAP1 (KRAB-associated protein 1)) and that AID can form a complex with KAP1 and HP1 (heterochromatin protein 1), which is recruited to transcribed S regions in vivo during CSR. Furthermore, we show that conditional inactivation of KAP1 in B cells does not affect somatic hypermutation but results in defective CSR, a defect due to reduced levels of DSB formation at transcribed S regions. Finally we show that CSR requires the in vivo association between KAP1 and HP1. We propose that AID associates with KAP1 and HP1 and that this complex is tethered to transcribed S regions bearing the H3K9me3 mark, thus providing a targeting mechanism that links for the first time the C-terminus of AID to epigenetic modifications induced during CSR.

PTIP PROMOTES CHROMATIN CHANGES CRITICAL FOR IMMUNOGLOBULIN SWITCH RECOMBINATION

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Histone H3K4 trimethylation is a chromatin mark of transcription initiation and is mediated by a number of MLL-like complexes; however, to what extent these complexes promote gene expression important for immune function is unknown. The BRCT domain-containing PTIP protein is a component of the MLL3/MLL4 methyltransferase complex and, interestingly, also accumulates at sites of DNA damage. Using Ptip conditional deletion mice, we investigated PTIP function in B lymphocytes, whose programmed DNA rearrangements require transcription and DNA repair. Here we show that activated B cells deficient for PTIP proliferate and survive normally but display impaired H3K4me3 most significantly at the immunoglobulin heavy-chain (Igh) switch regions, with very few other changes genome-wide. Further experiments reveal that B cells lacking PTIP have severe immunoglobulin class-switching defects because of impaired transcription initiation through downstream Igh switch regions, marked by loss of histone acetylation and RNA Polymerase II (Pol II) association. As a separate function, we show that PTIP accumulation at DNA breaks also contributes to class-switch recombination (CSR) and genome stability independently from Igh switch transcription. Our experiments demonstrate that PTIP promotes histone modifications and Pol II association critical for initiation of Igh switch region transcription and AID accessibility while also having a minor function in the repair of CSR-associated DNA breaks, suggesting a non-redundant role for the MLL3/MLL4 complex in altering antibody effector function.

REGULATION OF IMMUNOGLOBULIN CLASS SWITCH RECOMBINATION

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Mature B cells upon encountering antigens undergo class switch recombination (CSR), a deletional recombination reaction that replaces the constant region of the expressed immunoglobulin molecule so that the B cell changes from expressing IgM to IgG, IgE or IgA. CSR requires the generation of DNA double-strand breaks (DSBs) at repetitive DNA elements, called switch (S) regions in the immunoglobulin locus, followed by ligation of DSBs between two different S regions. It is now clearly established that the activity of the DNA deaminase AID at S regions leads to the generation of DSBs. However, the mechanism that targets AID activity specifically to the S regions and the process by which the DSBs thus generated are ligated to complete CSR are outstanding unresolved questions. Using a combination of biochemical screens for AID interactors and short-hairpin-mediated knock-down in B cells, we have identified the splicing regulator polypyrimidine tract binding protein 2 (PTBP2) as a factor that promotes binding of AID to S regions to activate CSR. We also show that DNA-end processing factor CtIP plays a significant role in the joining of DSBs during the completion phase of CSR. Our results have implications on how AID activity is targeted to the immunoglobulin locus and how the classical non-homologous end-joining and the “alternative” end-joining pathways mediate ligation of DSBs at S regions during CSR.

MECHANISMS OF CHROMOSOMAL TRANSLOCATIONS IDENTIFIED BY LARGE-SCALE LIBRARIES

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Recurrent translocations in tumors are often considered to represent very low frequency events that are strongly selected at the cellular level. However, we have recently demonstrated that mechanistic factors have the ability to substantially influence the frequency of recurrent translocations. Such mechanistic factors include the frequency of DSBs at two loci, the spatial proximity of the two loci, and the repair pathways functionally available to carry out the actual joining. To gain further insights into mechanisms that promote translocations, we are developing high-throughput methods to identify translocation partners from fixed DNA DSBs in a genome-wide fashion. One of the systems employs RAG1/2 or I-SceI endonuclease generated DSBs introduced into the genome of Abelson Murine Leukemia virus-transformed pro-B cell lines arrested in the G1 phase of the cell cycle. The other system employs cycling primary B cells in which I-SceI mediated DSBs were generated either at the site of the γ1 switch region or into the c-myc locus. While we are still relatively early in our analysis, several preliminary conclusions have been suggested. First , analyses of independent starting DSBs in different chromosomes of ATM or H2AX deficient cells show that a defined DSB does not translocate to a very limited number of chromosomal loci, as suggested from some tumor studies, but rather has a relatively broad distribution to many sites on most chromosomes. On the other hand, there are areas that appear to be targeted more frequently for translocations, including sites along the same chromosome, as well as regions of the same or different chromosomes that undergo high frequency breaks. Ongoing analyses which seek to saturate translocation maps and compare translocations frequencies in different genetic backgrounds (e.g. deficient in particular DSB response or repair factors) should help to more clearly define the role of various factors, including nuclear organization, DNA sequence, and repair pathways in suppressing and/or enhancing recurrent translocations. The implications of our findings in the context of DSB repair mechanisms and models for factors that promote translocations will be discussed.

RECOMBINATION CENTERS AND THE INITIATION OF V(D)J RECOMBINATION

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We are interested in the mechanism and regulation of V(D)J recombination, the reaction that assembles and diversifies immunoglobulin and T cell receptor genes. We have developed chromatin immunoprecipitation (ChIP) methodology to study the binding of the RAG1 and RAG2 proteins to antigen receptor loci during lymphocyte development. Using this methodology and mice expressing an active site mutant of RAG1 (D708A RAG1), we find that RAG protein binding occurs in a highly focal manner to a small region of active chromatin encompassing Igκ and Tcrα J gene segments and Igh and Tcrβ J and J-proximal D gene segments. Formation of these small RAG-bound regions, which we refer to as recombination centers, occurs in a developmental stage- and lineage-specific manner. Interestingly, each RAG protein is independently capable of specific binding within recombination centers. While RAG1 binding is restricted to regions containing recombination signal sequences (RSSs), RAG2 binds to thousands of sites in the genome in a pattern that mirrors that of trimethylated lysine 4 of histone 3 (H3K4me3). In the Tcrα locus, RAG1 binding requires the presence of the Tcrα enhancer and is strongly influenced by the TEA promoter and transcription elongation. We propose that V(D)J recombination is initiated within recombination centers in the following steps: 1) accessibility control elements such as enhancers and germline promoters create small regions of highly active chromatin spanning J (and where present, proximal D) gene segments; 2) the RAG proteins are recruited into these regions via RAG-RSS, RAG2-H3K4me3, and RAG1-RAG2 interactions, leading to the formation of a stable RAG1/RAG2/RSS complex; 3) partner RSSs compete for capture by the RAG proteins in the recombination center, with successful capture being a low probability event that leads to formation of a 12RSS-23RSS synaptic complex and DNA cleavage. Recombination centers provide an attractive means of regulating the initiation of V(D)J recombination, enforcing allelic exclusion, and facilitating DNA repair and reaction fidelity.

REGULATION OF IMMUNOGLOBULIN GENE RECOMBINATION THROUGH HOMOLOGOUS PAIRING OF ALLELES

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During their development, B lymphocytes undergo several rounds of V(D)J recombination to form a novel antigen receptor gene. The uniqueness of the antigen receptor is crucial: each lymphocyte must express only one antigen receptor specificity so as to avoid cross-reactivity and autoimmunity. To ensure that only one antibody specificity is produced, recombination is completed at only one allele per locus. This “allelic exclusion” ensures monoallelic gene expression, much as X chromosome inactivation ensures that only one X chromosome is active in females. Because D-J rearrangement takes place first, on both Igh alleles, allelic exclusion established at the V to DJ stage clearly relies on some sort of feedback mechanism. But how it is established has remained a major enigma. Another major question has been how genomic stability is maintained during rearrangement, given the multiple double-strand breaks produced at each chromosomal locus.

We recently found that the answer to both questions involves pairing of homologous alleles. Multiple mechanisms allow alignment and pairing of chromosomes, but they can be divided into those that require double strand break formation and those that do not—the preferred mechanism varies with the organism. In the case of V(D)J recombination, RAG expression initiates a phase of Igh pairing, while RAG cleavage triggers the DNA damage response, recruiting ATM to the site of the break. ATM then mediates relocation of the uncleaved allele to pericentromeric heterochromatin, likely to halt further recombination attempts while the first allele is undergoing repair. In the absence of ATM, cleavage (as indicated by the formation of γH2AX foci) occurs at significantly higher frequencies on both Igh alleles. We conclude that pairing of homologous alleles during V(D)J recombination not only helps establish allelic exclusion but serves to stabilize the chromosome being repaired and reduce the chance of translocations by halting RAG cleavage on the second allele.

H2AX REGULATES DNA END-PROCESSING DURING V(D)J RECOMBINATION

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Developing lymphocytes generate millions of RAG-mediated DNA double strand breaks (DSBs) each day as they attempt to assemble antigen receptor genes. These breaks are generated in G1-phase cells and are normally repaired by the non-homologous end joining (NHEJ) pathway of DNA DSB repair. Cells with un-repaired RAG DSBs are channeled into cell death pathways preventing these breaks from being resolved as potentially dangerous lesions such as chromosomal translocations. In response to DNA DSBs, Atm phosphorylates the histone variant H2AX, forming γ-H2AX, in chromatin flanking the breaks with γ-H2AX functioning to recruit many DNA damage response proteins to DNA break sites. Mice with combined deficiencies of H2AX and p53 have an increased incidence of lymphoid tumors with translocations involving antigen receptor loci consistent with the notion that H2AX functions in the repair of RAG-mediated DNA DSBs. However, developing H2AX-deficient lymphocytes do not exhibit overt defects in V(D)J recombination. We demonstrate that H2AX, and specifically γ-H2AX, regulates the endonucleolytic and exonucleolytic processing of un-repaired broken DNA ends generated by RAG cleavage. In this regard, hairpin-sealed coding ends in H2AX-deficient cells are efficiently opened and resected in the absence of Artemis, the nuclease normally required for opening hairpin-sealed coding ends. This Artemis-independent processing of coding ends is dependent on Atm and CtIP and is inhibited by γ-H2AX and MDC-1, which normally binds to γ-H2AX in chromatin at the DSB site. Although some of the hairpin-opened coding ends in Artemis$^{-/-}$:H2AX$^{-/-}$ cells form coding joints, these joints have significant deletions that would preclude the generation of a functional antigen receptor gene. Moreover, in contrast to normal coding joints, they exhibit a more extensive use of micro-homologies. Strikingly, a large fraction of coding ends persist un-repaired in Artemis$^{-/-}$:H2AX$^{-/-}$ cells even though the hairpin-sealed coding ends have been opened. Moreover, these coding ends engage pathways normally activated during homologous recombination (HR) in cells at post-replicative stages of the cell cycle. We propose that H2AX prevents un-repaired RAG DSBs generated in G1-phase cells from being processed in ways that preclude their repair by NHEJ and promote their access to alternative repair pathways that could lead to their aberrant resolution as potentially dangerous chromosomal translocations rather than pathways that would promote cell death.

NOVEL INSIGHTS INTO THE GENE REGULATORY NETWORKS REGULATING HUMAN T HELPER (TH) CELL DIFFERENTIATION

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Selective activation of lymphocyte subsets plays an important role in the pathogenesis of human allergy and chronic inflammatory and autoimmune diseases. Immune response is also crucial in host defense against cancer. We aim at understanding the molecular mechanisms of signaling pathways and transcriptional and epigenetic regulation that lead to the differentiation of distinct lymphocyte subsets. Moreover, we aim at revealing the key points of convergence and divergence between these pathways, thereby identifying new targets through which cell differentiation can be appropriately manipulated.

We have used systematically the state-of-the art genome-scale measurement technologies together with effective computational methods to investigate the complicated networks and the molecular mechanisms involved in human Th cell differentiation. Our genome wide analysis of regulation during the early stages of
human Th cell differentiation provides an insight into a dynamic network that regulates and gives rise to the complex Th cell selective phenotypes. Our data shows differential regulation of both known genes coding for components of certain pathways and novel genes with unknown functions. In addition, we have exploited siRNA-mediated knockdown experiments followed by genome-wide transcriptome analysis combined with ChIP-chip studies to reveal potential upstream key factors involved in the regulation of Th differentiation.The data indicating a role of SATB1 and ATF3 in regulating human Th cell differentiation will be presented in detail.

BRUTON'S TYROSINE KINASE IS A CRITICAL MEDIATOR OF TRIF-DEPENDING SIGNALING

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Toll-like receptors (TLRs) are innate immune receptors that recognize pathogen-associated molecular patterns found on microbes. In particular, TLR3 mediates immune response to viruses by recognizing double-stranded RNA and initiating signaling via the adaptor TRIF. We demonstrate that Bruton's tyrosine kinase (BTK) plays a critical role in TRIF-dependent signaling and acts downstream of TRIF but at a nodal point before the bifurcating of the NFkB and IRF3 pathways. In the absence of BTK, TLR3-stimulated macrophages failed to secrete IFN-β and other inflammatory cytokines such as IL-6 and TNF-α. BTK-deficient mice were also less susceptible to poly(I:C)-induced septic shock and death. Biochemically, BTK signaling is required for TLR3-induced RIP1 ubiquitination that leads to NFkB activation as well as for TBK-1 induction that is important for IRF3 phosphorylation. Thus, BTK is an essential mediator of TRIF-dependent signaling downstream of TLR3.

MYD88-SIGNALLING IN B CELLS SUPPRESSES T CELL-MEDIATED INFLAMMATION

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Toll like receptors mediate recognition of conserved microbial structures. Engagement of TLR has been shown to initiate an inflammatory cascade in innate immune cells that in turn orchestrate and instruct effector adaptive immune responses. B cells hold a particular position in the immune system displaying characteristics of both adaptive and innate immune cells. On one hand they elicit antigen-specific responses and produce high affinity antibodies, whereas by virtue of their expression of pattern recognition receptors, including TLR, they are equipped to sense and rapidly respond to microbes. Here we show that MyD88, a major signaling adaptor downstream most TLR, can also have anti-inflammatory functions through B cells. We found that mice lacking MyD88 selectively in B cells suffered a more severe chronic form of experimental autoimmune encephalomyelitis (EAE) - a mouse model of multiple sclerosis. In contrast, MyD88 deficient mice were resistant to disease. The exacerbated disease observed in mice with MyD88-deficient B cells correlated with increased autoreactive Th1 and Th17 cells, suggesting that MyD88 in B cells promoted disease resolution by limiting inflammatory T cell responses. The suppressive function of B cells required TLR2/4 but not TLR9, indicating that distinct TLR differentially contribute to disease development and resolution. The agonists triggering the protective TLR on B cells were likely derived from Mycobacterium tuberculosis present in the adjuvant used to induce disease, rather than commensal flora. Absence of MyD88 in B cells was dispensable for germinal centre formation and antigen-induced antibody production, arguing against a requirement for MyD88 in B cell activation. However it resulted in strikingly reduced titers of natural IgM. Using mice deficient for secreted IgM, we found that IgM had a protective function in EAE that interfered with the development of autoreactive Th1 and Th17 responses and controlled disease onset and severity. Altogether, these findings demonstrated a dual role for MyD88 in immune responses: MyD88 signaling in cells other than B cells, presumably DC and macrophages, is critical for the initiation of inflammation and T cell responses, whereas MyD88-signalling in B cells antagonizes this effect, thereby controlling overt inflammatory responses.

ROLE OF BCL-6 IN PLASMA CELL DIFFERENTIATION

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Bcl-6 (B cell lymphoma 6) regulates germinal center formation and differentiation of B cells into high affinity antibody-producing plasma cells. The double negative regulatory circuit between Bcl-6 and Blimp-1 has been well established. We now reveal new mechanisms how Bcl-6 prevents plasma cell differentiation. We show that Bcl6 directly promotes the expression of Bach2, a known suppressor of Blimp-1. Moreover, Bcl-6 suppresses Blimp-1 expression through direct binding to IRF4 gene, as well as by promoting the expression of MITF, a known suppressor of IRF4. We also provide evidence that Bcl6 is needed for the expression of AID and UNG, the indispensable proteins for somatic hypermutation and class-switch recombination, and UNG appears to be a direct target of Bcl-6 regulation. Together our findings reveal a complex regulatory network, in which Bcl-6 acts as a key element dictating the transition from B cells to plasma cells.

HYDROGEN PEROXIDE AS A REGULATOR OF GENE EXPRESSION AND SIGNALING IN THE IMMUNE SYTEM AND ITS POTENTIAL PROTECTION AGAINST THE RADIOBIOLOGICAL EFFECTS OF A TERRORIST NUCLEAR ATTACK

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The ability of the first primordial cell, endowed with innate immunity, to survive and proliferate its background of high ionizing radiation, raises the question of what evolutionary mechanism increased humankind's sensitivity to radiation. Perhaps it was the development of the adaptive immune system. A sudden exposure to high dose radiation results in the production of reactive oxygen species (ROS) from radiolysis of the 70-80% body water content. Damaging radicals destroy biomolecules giving strong signals to Toll-like receptors (TLR). Mediated by the TLR activation of those mitogen-associated protein kinases (MAPKs) that cause pro-inflammatory cytokine secretion, and generate an exacerbated adaptive immune response, the level of oxidative stress and damage to body tissues is significantly greater. Innate cell responders (neutrophils and macrophages) to these detected damage-associated molecular patterns, recruit adaptive immune cells (lymphocytes), all secreting ROS, and conferring a self-repetitive pro-inflammatory status to the affected tissues. Among the ROS, is the intermediate, hydrogen peroxide, H_2O_2 , that impacts on cell signaling pathways in a highly concentration-dependent manner. It contributes to biomolecular damage by oxidizing certain highly susceptible amino acids on proteins causing their fragmentation. The TLR would detect these fragments as threats. H_2O_2 is a regulator of the inflammatory process. Together with TNF-α, and NF-κB, it interacts synergistically creating a pro-inflammatory loop; absent NF-κB and TNF-α, H_2O_2 restores homeostasis to the affected tissues by signaling the gene expression of heme oxygenase (HO) that catabolizes heme, with carbon monoxide (CO) as a second messenger. Cells experiencing low CO concentrations secrete ROS, termed 'oxidative conditioning', that may be responsible for homeostatic restoration. H_2O_2 deactivates phosphatases by removing a thiol group from the central core of cysteine-based phosphatases. Such inhibition promotes phosphorylation that influences cell signaling. Phosphorylation and de-phosphorylation positively or negatively regulate mechanisms within the pathways and are likely involved in crosstalk between NF-κB and JNK/AP pathways that affect the life or death destinies of cells. Pre-treatment with low non-toxic concentrations of H_2O_2 could generate a controlled adaptive immune response and protect individuals from the massive tissue damage from a terrorist nuclear attack. It is time for immunologists and radiobiologists to collaborate on such a worthwhile venture.

SIN1 SUPPRESSES *RAG1/2* AND *IL7R* GENE EXPRESSION THROUGH AKT2 IN B CELLS

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Mammalian target of rapamycin (mTOR) is an important mediator of phosphoinositol-3-kinase (PI3K) signaling. PI3K signaling regulates B cell development, homeostasis and immune responses however the function and molecular mechanism of mTOR mediated PI3K signaling in B cells has not been fully elucidated. Here we show that Sin1, an essential component of mTOR complex 2 (mTORC2), regulates B cell development. Sin1 deficiency results in increased IL-7 receptor (*il7r*) and RAG recombinase (*rag1 & rag2*) gene expression leading to enhanced pro-B cell survival and augmented V(D)J recombinase activity, respectively. Sin1 deficiency disrupts mTORC2 integrity and abolishes Akt hydrophobic motif (Ser473) phosphorylation in B cells. We identify Akt2 as the specific mediator of Sin1/mTORC2 dependent suppression of *il7r* and *rag* gene expression in developing B cells. Furthermore, we show that FoxO1 phosphorylation is impaired in both the Sin1 and Akt2 deficient B cells, and impaired FoxO1 phosphorylation correlates with increased expression of the FoxO1 target genes *il7r*, *rag1* and *rag2*. Finally, we provide evidence showing that the mTOR inhibitor rapamycin induces *rag* gene expression and promotes aberrant V(D)J recombination in developing B cells. Our study reveals that the Sin1/mTORC2-Akt2 signaling axis is a key regulator of FoxO1 transcriptional activity in B cells and shows that genetic or pharmacologic disruption of mTORC2 function results in the aberrant over-expression of key genes that regulate cell survival and immunoglobulin gene recombination in developing B cells.

MECHANISM OF TIM-3 SIGNAL TRANSDUCTION IN THE MODULATION OF TCR SIGNALING

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T-cell immunoglobulin and mucin 3 protein (Tim-3) is a type-I transmembrane protein expressed on murine T-helper type 1 (Th1) cells which regulates Th1 cell proliferation and development of tolerance. Tim-3 has six tyrosine residues in the cytosplasmic tail, two and three tyrosines residues are clustered in proximal and distal region of the cytoplasmic tail respectively. We demonstrated that deletion of the cytoplasmic tail region containing the distal tyrosine residues (Y250, Y251, Y253) slightly increased NFAT/AP1 and NF-κB-Luciferase reporter activity, while deletion of the region containing both distal and proximal (Y235, Y242) tyrosine residues abolished the reporter activity to basal level in Jurkat cells. Point mutation of both Y235 and Y242 decreased NFAT/AP1 and NF-κB reporter activity close to basal level while single point mutation of Y235 or Y242 did not result in significant changes in reporter activity. SH2 domain screen and co-immunoprecipitation identified P85 interaction with Tim-3. We conclude that phosphorylation of Y235 and Y242 residues of Tim-3 are responsible for potentiation of signal transduction initiated by the TCR, upstream of NFAT/AP1 and NF-κB pathways, in a redundant manner. Finally, Tim-3 signal transduction may be mediated in part by recruitment of P85 to the phosphotyrosine of Tim-3.

MAMMALIAN TARGET OF RAPAMYCIN COMPLEX 2 CONTROLS A PROTEIN KINASE C - NF-KB PATHWAY IN T HELPER TYPE 2 CELL DIFFERENTIATION

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Mammalian target of rapamycin (mTOR) is an essential serine/threonine kinase involved in cell growth, proliferation, and differentiation. Many cellular function of the rapamycin-sensitive mTOR complex 1 (mTORC1) have been defined, but relatively little is known about the biology of an alternative complex, mTORC2. Recently, we have found that mTORC2 selectively promotes helper T cell (Th) differentiation into a subset of CD4 effector types via regulation of protein kinases B and C. We further examined the relative role of mTORC2 in the axis of PKC/NF-κB pathway during Th2 differentiation. In the absence of rictor, an essential subunit of mTORC2, we detected impaired nuclear localization of NF-κB and decreased NF-κB-responsive promoter activity induced by antigen receptor and costimulation. The defect of NF-κB activity was reversed by transduction of a constitutively active PKCθ mutant. Importantly, the active PKCθ enhanced GATA-3 expression and restored normal Th2 differentiation of mTORC2-deficient T cells, whereas it was not sufficient to revert the defects of Th1 differentiation. In agreement with the evidence that PKCθ stabilizes the intercellular interaction of integrin LFA-1 to ICAM-1 shortly after antigen recognition, we also observed a decrease in T cell activation-dependent binding of ICAM-1. Our results provide evidence that a crucial mechanism by which mTORC2 regulates helper T cells is transduced via a PKC relay to signaling by NF-κB.

DNA END-JOINING PATHWAYS IN CLASS SWITCH RECOMBINATION

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Upon encountering pathogens, mature B cells undergo class switch recombination (CSR), a deletional recombination reaction that replaces the constant region of the expressed immunoglobulin molecule so that the B cell changes from expressing IgM to IgG, IgE or IgA. CSR requires the generation of DNA double-strand breaks (DSBs) at repetitive DNA elements, called switch (S) regions in the immunoglobulin locus, followed by ligation of DSBs between two different S regions. It is now clearly established that the activity of the DNA deaminase AID at S regions leads to the generation of DSBs. However, the mechanism by which the DSBs are ligated to complete CSR is not clearly understood. The non-homologous DNA end-joining (NHEJ) pathway was implicated to be required for CSR, but significant CSR still occurs in the absence of NHEJ components, suggesting the participation of additional processes, including a poorly characterized pathway, which is microhomology-mediated. To directly determine the requirement of this pathway in CSR, we have used the shRNA technique to knock-down a key player, CtIP, in the murine B-cell lymphoma line CH12. Additionally, we have knocked-down CtIP in combination with NHEJ components. The modified cells were then analyzed for CSR and for the nature of the S junctions. Our results provide interesting clues to the interplay of NHEJ and MMEJ pathways in CSR.

COLLABORATION BETWEEN MENIN AND MLL DURING HEMATOPOIESIS

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The *Mixed Lineage Leukemia (MLL)* gene is disrupted by chromosomal translocation in childhood and secondary leukemias. Its association with Menin, a tumor suppressor encoded by the multiple endocrine neoplasia (*MEN1*) gene, has been reported in cell line models. Importantly, this interaction is essential for MLL fusion oncogenes to transform hematopoietic cells; thus targeting this interaction has been proposed strategy for rational drug design. Our studies have demonstrated that *Mll* gene excision in a murine conditional knockout model results in defects in steady-state hematopoiesis. A similar experimental approach using a conditional Menin knockout mouse model revealed overlapping phenotypes particularly in maintenance of HSC function and homeostasis in the B cell lineage. If MLL is similarly dependent on Menin in stem cells and B cells, targeting the Menin-MLL interface may not prove an effective therapeutic strategy for selective leukemia targeting.

To determine the importance of *Menin* and *Mll* interaction to sustain hematopoiesis, we have undertaken a genetic analysis using compound conditional knockout mutants in the setting of an inducible Cre transgene (Mx1-Cre) or a developing lymphocyte Cre knock-in model (Rag1-Cre). We predicted that if MLL requires menin interaction for steady-state hematopoiesis, we may observe phenotypes in the double heterozygotes. In the Mx1-Cre system, normal HSC-enriched population and normal B cell progenitors were observed in animals lacking one copy of *Menin* and one copy of *Mll* ($Menin^{\Delta/+}$;$Mll^{\Delta/+}$). Stress hematopoiesis with 5-fluoro-uracil treatment exhibited similar recovery kinetics. Furthermore, we removed both alleles of *Menin* on a sensitized *Mll* heterozygous background ($Menin^{\Delta/\Delta}$;$Mll^{\Delta/+}$). Animals in which homozygous *Menin* deficiency was induced exhibited a reduction in B cells starting at the pro-B cell stage, as previously reported. However, *Menin* deficiency on the *Mll* heterozygous background did not result in reduction in HSCs or further reduction in B cells, an observation difficult to reconcile if Menin is a requisite component of the MLL complex. Ongoing experiments focus on *Menin-Mll* genetic interactions within developing lymphocyte populations using the Rag1-Cre knock-in to identify common or distinct Menin and MLL dependent pathways during lymphopoiesis.

In summary, our genetic evidence argues that Menin and MLL may act independently during steady-state hematopoiesis, suggesting that targeting this protein-protein interaction may be a promising strategy to treat leukemia.

TCR AND LAT ARE EXPRESSED ON SEPARATE PROTEIN ISLANDS ON T CELL MEMBRANES AND CONCATENATE DURING ACTIVATION

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The organization and dynamics of receptors and other molecules in the plasma membrane are not well understood. Here we analyzed the spatio-temporal dynamics of T cell antigen receptor (TCR) complexes and linker for activation of T cells (LAT), a key adaptor molecule in the TCR signaling pathway, in T cell membranes. Using high-speed photoactivated localization microscopy (hsPALM), dual-color fluorescence cross-correlation spectroscopy (dcFCCS) and transmission electron microscopy (TEM), we have found that both the TCR and LAT are preclustered into distinct, separate and spatially stable membrane domains (protein islands) on quiescent T cells. After antigen recognition, these protein islands transiently concatenate. The concatemers are identical to the previously described microclusters. Concatenation takes place without any substantial change in the size and number of the component domains. Surprisingly, no co-localization or mixing of TCR and LAT occurs on a molecular level. These data showed for the first time that the architecture of the plasma membrane is not only important for the exclusion or accumulation of T cell signaling molecules (for example, through rafts) but also controls the signaling process by the segregation and controlled association of particular membrane domains. This separation versus physical juxtapositioning of receptor domains and domains containing downstream signaling molecules in quiescent versus activated T cells may be a general feature of plasma membrane–associated signal transduction.

REGULATION OF TIM-1 SIGNALING AND LOCALIZATION IN T CELL ACTIVATION

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Transmembrane immunoglobulin and mucin 1 (Tim-1) belongs to a family of cell surface proteins with roles in immune regulation. Tim-1 cross-linking has been shown to enhance CD4+ T cell activation and differentiation. We and others have also demonstrated that Tim-1 cross-linking leads to interaction with important signaling molecules, including p85 PI3k, as well as induction of NFAT/AP-1 transcription, which is dependent on Tim-1 tyrosine 276 phosphorylation. However, the exact mechanisms by which Tim-1 alters T cell activation and differentiation remain mostly unknown.
We hypothesize that Tim-1 co-stimulation may impact T cell activation and differentiation by directing a unique signaling network after tyrosine phosphorylation and adopting a distinct pattern of localization upon activation. Work in this study focuses on identifying downstream targets of Tim-1 signaling as well as understanding Tim-1 localization during T cell activation.
Putative Tim-1 binding proteins were identified by motif scanning as well as pull-down with a phosphorylated 13 amino acid peptide based on the Tim-1 cytoplasmic tail. Confocal microscopy was used to visualize Tim-1 localization during immunological synapse and microcluster formation. We recently found that Tim-1 can recruit PLCγ1 in a phosphotyrosine-dependent manner. Furthermore, the downstream effects of Tim-1 on NFAT/AP-1 and markers of early activation may be mediated through PLCγ1. In addition, we have demonstrated that Tim-1 is diffusely expressed on the surface of resting T cells, and that it appears to be excluded from the immunological synapse after APC interaction. Understanding how Tim-1 signaling and localization are regulated will be important in establishing the underlying mechanisms by which Tim-1 specific ligands and antibodies modulate immune responses in autoimmunity and allograft tolerance.

DIRECT REGULATION OF HEMATOPOIETIC STEM CELL COMMITMENT BY Γ-SECRETASE COMPLEX ACTIVITY

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The γ-secretase complex is a protease complex responsible for the final intramembrane cleavage of various type I transmembrane proteins. It is composed of 4 proteins: APH1, PEN2, Presinilin and Nicastrin. The most renowned substrates are the amyloid precursor protein (APP) and Notch from which γ-secretase liberates amyloid β peptide and intracellular active form (ICN) respectively. The role of γ-secretase activity in the lymphopoiesis is well known through its effect on Notch signaling that specify T cell over B cell commitment. However the role of γ-secretase activity in hematopoietic stem cells (HSC) is unknown. To explore potential role of γ-secretase activity in HSC we generated a conditional knock-out mouse model of the Nicastrin gene. We show that lack of γ-secretase activity in HSC leads to a severe myeloproliferative disorder (MPD). We also show that this MPD arise through upregulation of a skewed myeloid-differentiation genetic program in HSC. Using a genetic model of conditional deletion of *Notch1* and *Notch2* genes in HSC, we show that the γ-secretase deficiency induced MPD is mediated by loss of Notch signaling and that restoring Notch signaling, using a conditional ICN1 knock-in mouse, is able to counteract Nicastrin deletion and inhibit myeloid commitment of HSC. We then further characterize the mechanism of Notch-induced myeloid inhibition by showing that this mechanism is mediated by the canonical Notch target gene, *Hes1*. We show that upon Notch activation, Hes1 is expressed in an early multipotent progenitor population and can directly bind the promoters and repress the expression of key genes involved in myeloid differentiation: *Cebpa* and *Pu.1*.
These findings indicate that Notch signaling negatively regulates myeloid commitment of HSC by inhibiting a critical genetic program involved in myeloid differentiation. Our study also unravels a new tumor suppressor role of γ-secretase activity/Notch in the hematopoietic system as loss of Notch signaling induces a severe myeloproliferative disorder.

THE FUNCTION OF THE RING DOMAIN OF AIRE IN ESTABLISHING IMMUNOLOGICAL TOLERANCE

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Immunological tolerance ensures that lymphocytes with specificity for self are prevented from initiating immune responses, and is maintained by "central" and "peripheral" mechanisms. In the case of T cells, central tolerance occurs in the thymus wherein differentiating thymocytes are exposed to a repertoire of self-antigens (Ags) that reflects those normally encountered in the periphery. This is accomplished by the expression and presentation of a large array of Ags on the surface of a subset of thymic medullary epithelial cells (MECs). The expression of these peripheral-tissue Ags (PTAs) is in large part under the control of the transcription factor Aire. Aire is a protein with several conserved domains typically found in transcription factors, and induces the transactivation of thousands of genes *in vitro*. However, the molecular mechanisms by which it induces PTA expression *in vivo* are not well understood. Our lab has recently demonstrated that Aire forms many functional interactions with proteins involved in DNA repair, chromatin remodeling, and RNA splicing/processing. Aire also has affinity for unmethylated lysine residues on histone H3 (e.g. H3K4) mediated by a PHD domain (PHD1). For this study, we chose to concentrate on the function of Aire's RING domain (previously called PHD2). RING domains often act as E3 ubiquitin ligases; however, the function of the RING domain of Aire has not been demonstrated *in vivo*.

Through the use of a previously described inducible-Aire double transgenic system, we are determining what effect loss of the RING domain has on the function of Aire in MECs in mice by expressing a RING-less (ΔRING) Aire transgene in otherwise Aire-deficient mice. We are comparing the repertoire of PTAs expressed in MECs as well as the incidence and severity of autoimmune disease in ΔRING Aire transgenic mice compared with WT Aire transgenic mice. As a complement to our *in vivo* studies using transgenic mice, we are determining by quantitative mass spectrometry whether the RING domain is required to form any of the previously described/novel protein-protein interactions. Preliminary experiments have shown that a ΔRING Aire mutant is stable and localizes similarly to WT Aire when expressed in a human thymic epithelial cell line (4D6).

FUNCTION OF MIR-146A IN CONTROLLING TREG CELL-MEDIATED REGULATION OF TH1 RESPONSES

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Foxp3+ regulatory T (Treg) cells represent a distinct cell lineage of T lymphocytes for maintaining immunological tolerance to “self” and restraining pathogenic immune responses to pathogens and commensal microorganisms. A class of short regulatory non-coding RNAs known as microRNAs (miRNAs) was recently shown to be pivotal in the development and function of Treg cells. Selective ablation of miRNAs in Treg cells resulted in the early onset autoimmune lethality indistinguishable to those were observed in mice devoid of Treg cells. Distinct miRNA expression profiles, of which many were directly targeted by Foxp3, in Treg cells further suggested specific miRNAs could control different facets of Treg biology. We found that elevated miR-146a expression in Treg cells is essential for effective control of a particular class of immune responses (TH1) dominated by IFNg Further studies suggested that miR-146 controls TH1 responses through targeting signal transducer and activator transcription 1 (STAT1), the key transcription factor involved in initiation of effector TH1 responses. Loss of miR-146a in Treg cells resulted in increased levels of both total and phosphorylated STAT1. As a consequence, mice harboring miR-146a-deficient Treg cells succumbed to IFNg dependent immunopathology. Our results provided strong evidence suggesting that specific aspects of Treg suppression function can be controlled by a single miRNA. Furthermore, our studies imply that miRNA-dependent post-transcriptional regulation enable Treg cells to control a particular type of effector immune responses.

IKAROS AND AIOLOS INHIBIT PRE-B CELLS PROLIFERATION BY DIRECTLY SUPPRESSING C-MYC EXPRESSION

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Pre-B cell expansion is driven by signals from the IL-7 receptor (IL-7R) and the pre-B cell receptor (pre-BCR) and is dependent on the cell cycle regulators cyclin D3 and c-Myc. We have shown previously that interferon regulatory factors 4 and 8 induce the expression of Ikaros and Aiolos to suppress pre-B cell proliferation. However, the molecular mechanism through which Ikaros and Aiolos exert their growth inhibitory effect is still not clear. Here, we provide evidence that Aiolos and Ikaros bind to c-Myc promoter in vivo and directly suppress c-Myc expression in pre-B cells. Downregulation of c-Myc is critical for the growth inhibitory effect of Aiolos, as sustained c-Myc expression antagonizes Aiolos-mediated growth inhibition. Aiolos also induces expression of p27 and downregulates cyclin D3 in pre-B cells, and growth inhibitory effect of Aiolos is compromised in the absence of p27. A time-course analysis further reveals that downregulation of c-Myc by Aiolos precedes p27 induction and cyclin D3 downregulation. Moreover, our results show that downregulation of c-Myc by Aiolos is necessary for the induction of p27 and downregulation of cyclin D3. Collectively, our studies identify a pre-B cell receptor signaling induced inhibitory network, orchestrated by Ikaros and Aiolos, which functions to terminate pre-B cell expansion.

PML AND KAKA NUCLEAR BODIES DETERMINE THE REPRESSIVE ENVIRONMENT AND LIMIT VIRAL GENE EXPRESSION IN HUMAN LYMPHOCYTES

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Various members of tripartite motif (TRIM) protein family display antiviral properties, targeting retroviruses in particular. The potential activity of TRIM19, better known as promyelocytic leukemia protein (PML) against several viruses has been well documented, yet it's role in HIV-1 infection remains elusive.
One of the most important cellular partners of HIV-1, P-TEFb kinase complex, composed of CDK9 and CyclinT1 plays a crucial role in regulation of HIV-1 transcription. We have previously demonstrated that both members of P-TEFb interact with PML protein and localize inside the PML Nuclear Bodies (PML NB). In particular, we found that the acetylated and enzymaticaly inactive form of CDK9 binds PML and can be separated with PML in the insoluble chromatin fraction. Our ChIP analysis revealed that acetylated form of CDK9 localizes to the integrated and transcriptionally silent viral genome, thus indicating that PML might have yet unidentified role in regulation of HIV-1 infection and latency.
In latently infected Jurkat clones, integrated viral genome was marked by H3K4Met3 at the promoter level and PML and KAP-1 proteins, both bound to the body of silent viral genome. Transcriptional activation of the provirus with TNF-α led to the displacement of PML and KAP-1 proteins. By three-dimensional immuno-fluorescent in situ hybridization (3D Immuno DNA FISH) we visualized the silent viral genome in close proximity to PML NB, at the distance of less that 0.6 Microns in 40-45% of the cells. Recently described KAKA foci, characterized by the presence of KAP-1 (or Trim28) and KRAB-Zinc Finger proteins, were also found to be spatially related to the silent viral genome, at a distance of 0.9 microns in 40% of the cells. Thus, silent viral genome resides in the close proximity to either PML NBs or to KAKA foci, that both might represent a repressive nuclear neighborhood. To verify this, we disrupted PML NBs by arsenic trioxide treatment or we stably knocked-down PML protein, and we observed a significant increase in the levels of viral transcription (from 8-15 times over a silent control). However, only a portion of cells with silenced PML showed an increased viral expression, whereas almost half of the cells still remained silenced. In these cells viral genome remained associated to the KAKA bodies. Double knock-down of both PML and KAP-1 proteins is expected to further increase the levels of viral gene expression.
Nuclear neighborhood seems to strongly influence viral gene expression, and we are currently monitoring the changes in spatial organization of the nucleus in CD4+ cells during the establishment of latent infection.

CONCERTED ACTION OF CELLULAR JNK AND PIN-1 RESTRICTS HIV-1 GENOME INTEGRATION TO ACTIVATED CD4+ T LYMPHOCYTES

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Long-standing evidence indicates that quiescent human peripheral blood T lymphocytes (PBLs) do not support efficient HIV infection. In resting PBLs, reverse transcription of viral RNA takes longer than in activated cells, partially because formation of the late products of reverse transcription is decreased by RNA binding by apolipoprotein B mRNA–editing enzyme, catalytic polypeptide-like 3G (APOBEC3G). In a subsequent step, integration of the viral complementary DNA that is eventually formed is markedly impaired. Here we show that cellular c-Jun N-terminal kinase (JNK), an enzyme that is lacking in resting CD4+ T cells, regulates permissiveness to HIV-1 infection, and we unravel a novel, sequential post-translational pathway of protein modification that regulates viral DNA integration. We found that, in activated T lymphocytes, viral integrase, which mediates HIV-1 cDNA integration into the host cell genome, is phosphorylated by JNK on a highly conserved serine residue in its core domain. Phosphorylated integrase, in turn, becomes a substrate for the cellular peptidyl prolyl-isomerase enzyme Pin1, which catalyzes a conformational modification of integrase. These concerted activities increase integrase stability and are required for efficient HIV-1 integration and infection. Lack of these modifications restricts viral infection in nonactivated, primary CD4+ T lymphocytes.

FUNCTIONAL GENOMICS STUDIES OF BOVINE MONOCYTE-DERIVED MACROPHAGES (MDM) STIMULATED *IN VITRO* WITH *MYCOBACTERIUM BOVIS*, *M. BOVIS*-BCG AND *M. AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP).

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To identify key host response genes to mycobacterial diseases in cattle, a functional genomics approach was undertaken to investigate bovine macrophage gene expression patterns in response to stimulation with *Mycobacterium bovis*, attenuated *M. bovis* bacillus Calmette-Guérin (BCG) and *M. avium* subsp. *paratuberculosis* (MAP) – the causative agent of Johne's disease. Purified monocyte-derived macrophages (MDM) from seven unrelated Holstein-Friesian females were used for separate *in vitro* challenge experiments using the three bacterial species (MOI – 2:1). Total cellular RNA was extracted from challenged MDM samples at intervals of 2 h, 6 h and 25 h post-infection and prepared for pan-genomic gene expression analyses using the high-density Affymetrix® GeneChip® Bovine Genome Array with features representing more than 23,000 gene transcripts. These gene expression data can be used for systems biology reconstruction of macrophage cellular pathways underlying host interactions with *M. bovis*, *M. bovis*-BCG and MAP. Important gene expression changes can be identified and validated using real time quantitative reverse transcription PCR (qRT-PCR). Analyses of these data will: (1) provide a panel of candidate resistance/susceptibility genes that can be used for subsequent population resistance/susceptibility studies in field-infected and control animals; (2) offer a greater understanding of the host responses against these mycobacteria; (3) lead to improvements in disease diagnostics; and (4) enhance vaccine development. Here we present preliminary analyses and results from these experiments.

A ROLE OF ARTEMIS/LIGASE IV COMPLEX IN EFFICIENT V(D)J RECOMBINATION AND POST-IRRADIATION CELL SURVIVAL

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Mutations in Artemis, the nuclease that opens hairpin coding ends during V(D)J recombination, result in radiosensitive severe combined immune deficiency (RS-SCID) syndrome. Radiosensitivity and genomic instability of Artemis deficient cells suggest its role in Non-Homologous End Joining (NHEJ), hence DNA double strand break (DSB) repair. We wanted to explore novel protein interactions of Artemis with the long term goal of understanding its multiple roles in V(D)J recombination and DNA DSB repair. We have discovered a direct interaction of Artemis with Ligase IV, a DNA ligase that plays essential roles in both NHEJ and V(D)J recombination. This interaction is observed with endogenous proteins in the physiological setting of developing B cell lines and is mediated by Artemis' Carboxy-terminal region and the DNA binding domain (DBD) of Ligase IV. It is a highly specific interaction since the DBD regions of other DNA ligases do not interact with Artemis, both *in vivo* and *in vitro*. Point mutants of Artemis that disrupt this interaction show impaired V(D)J recombination and increased sensitivity to ionizing radiations. Our data thus provides an important function for the uncharacterized C-terminal region of Artemis in both V(D)J recombination and post-irradiation cell survival through its interaction with Ligase IV. Previously identified hypomorphic disease mutants of Artemis that result in truncation of the Artemis C-terminal region showed undetectable Ligase IV interaction. Therefore, *in vivo* relevance of this interaction sheds light on the mechanism of immunodeficiency and development of lymphomas observed in these patients. Furthermore, by bringing processing and repair factors together, this interaction offers alternate models of NHEJ in which, in the presence of Artemis, a large protein complex might assemble, permitting highly efficient repair of DNA double strand breaks, both during V(D)J recombination and general DNA repair.

GENOME-WIDE ANALYSIS OF DIRECT AND FUNCTIONAL TARGETS OF EBF1: IMPLICATIONS FOR ROLES IN B CELL DIFFERENTIATION AND CHROMATIN STRUCTURE

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The transcription factor early B cell factor-1 (Ebf1) is expressed in B lineage cells, in addition to adipocytes and cells of the nervous system. Ebf1 is a key component of a transcription factor network that is necessary for the specification and commitment of B cell progenitors, including the restriction of alternative lineage choices. In an effort to identify both direct and functional targets of Ebf1, and to gain insight into the molecular basis of Ebf1 function in early-stage B cells, we utilized a genome-wide ChIP sequencing approach in combination with both gain- and loss-of-function transcriptome analyses. Among over 500 genes that are both occupied and regulated by Ebf1, we identified large sets involved in (pre)B cell receptor and Akt signaling. Moreover, we found that Ebf1 controls expression of multiple regulatory and structural components of cell adhesion and migration. Ebf1 binding was also detected at more than a third of previously described Pax5 targets, suggesting a functional synergy between Ebf1 and Pax5. In addition to Ebf1-activated and -repressed genes, we identified targets in which Ebf1 induces changes in chromatin structure that poise the genes for expression at subsequent stages of differentiation. Thus, multiple functions of Ebf1 contribute to the combinatorial gene control underlying B cell specification and commitment.

HISTONE MIMICRY BY THE NONSTRUCTURAL INFLUENZA PROTEIN

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The nonstructural protein 1 (NS1) of the human influenza virus is expressed early after viral infection and is used by the virus to counteract the anti-viral response mounted by the infected cell. We identified the presence of a histone H3K4-like sequence (histone mimic) within the carboxy-terminal domain of NS1. We showed that similar to histone H3, the histone mimic of the NS1 is methyated and acetylated in vivo in the nucleus of influenza virus-infected human cells. Using non-modified and modified cell permeable NS1 histone mimics, we identified the role of this sequence in negative regulation of type I IFN and IFN inducible gene expression. Consistently, the influenza virus that carries the mutated histone mimic is less virulent. We hypothesize that the presence of the histone mimic in NS1 allows the influenza virus to compete effectively with the host cell epigenome for transcriptional regulators of antiviral genes.

INTRA-IMMUNOGLOBULIN LOCUS TARGETING OF AID ACTIVITY

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Mature naïve B lymphocytes, upon encountering antigens in the peripheral lymphoid organs, can undergo two mechanisms of secondary immunoglobulin gene diversification: class switch recombination (CSR) and somatic hypermutation (SHM). Upon activation B cells begin to express the DNA editing enzyme activation-induced cytidine deaminase (AID), which is required for both SHM and CSR. AID initiates both of these mechanisms by deaminating cytosine residues to uracils, thus creating U:G mismatches. In SHM, transcription through the variable region, followed by AID activity and subsequent repair of the U:G mismatches by error-prone DNA repair proteins, leads to the introduction of point mutations throughout the variable region, and results in the generation of antibodies with higher affinity for antigen. In CSR, transcription through the highly repetitive switch (S) regions within the IgH constant region, followed by AID activity, results in DNA double-strand breaks and the exchange of the primary constant region, Cμ, for a downstream constant region and the excision of the intervening DNA sequence. Thus, CSR leads to the generation of antibodies with new effector functions.

CSR and SHM are independent processes, and it is unknown how AID is differentially targeted to distinct regions of the immunoglobulin locus to initiate either reaction. In order to address this enigma, we employed a murine B cell lymphoma line called CH12s, which have a propensity to switch from IgM to IgA upon stimulation with antiCD40, IL-4 and TGF-β. Since Cα is the final constant region in the murine IgH locus, we term IgA+ CH12s "terminally switched", meaning they can no longer undergo "classic" CSR. Using a purified population of IgA+ CH12s, we analyzed AID expression and targeting in stimulated "terminally switched" cells as compared to freshly stimulated CH12s. We also analyzed AID activity in stimulated "terminally switched" cells by looking at mutation rates at the VDJ, Cα and Sμ – Sα junction regions and comparing them to rates in unstimulated IgA+ cells. Despite seeing AID expression in stimulated "terminally switched" cells, we were only able to see AID recruitment and activity within the remaining switch region and never within the VDJ region of the locus. Using this system we conclude that either 1) the remaining switch region is acting as an AID sink, sequestering AID away from the VDJ or 2) a factor needed for AID targeting to the VDJ is not present in this system, either because of the stimulation used or the nature of the CH12 cell line.

IDENTIFICATION OF AID INTERACTING PROTEINS

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Activation induced cytidine deaminase (AID) initiates immunoglobulin class switch recombination (CSR) and somatic hypermutation (SHM) by deaminating transcribed double stranded DNA to produce U:G mismatches. Subsequent error prone repair serve to process AID induced lesions to produce SHM or CSR. Normally AID activity is targeted to the immunoglobulin locus however, AID induced lesions can produce off site DNA damage including double stranded breaks and chromosome translocations. Therefore, strict regulation of AID is important for maintaining genomic stability. Transcriptional regulation, nuclear export and phosphorylation have all been shown to modulate AID activity and it has been suggested that phosphorylation can facilitate interaction of AID with partner proteins. Furthermore, AID phosphorylation doesn't affect AID catalytic activity as measured in E coli, but augments AID function in vivo, providing evidence that post-translational modification facilitates AID interaction with partners. We have taken a proteomics approach to identify proteins that interact with AID. To ablate artifacts from overexpression and assay AID interactions in a normal physiological state where AID is properly phosphorylated, we created epitope tagged, Flag-AID, knockin mice. B cells from these mice develop normally and can undergo class switch recombination to IgG1 following stimulation by LPS and IL-4 in vitro. We identified candidate AID partners by detecting co-purifying molecules from B cells extracts. The Flag tag was used to purify AID and interacting proteins were identified via mass spectrometry. This approach identified several candidate proteins and AID interaction was confirmed via co-immunoprecipitation. The success of this approach and the current progress in assessing the role of these candidate partners in AID function will be discussed.

DOES HIV NEF DISTURB HUMAN T CELL DEVELOPMENT BY INTERFERING WITH CXCR4 AND IL-7 RECEPTOR SIGNALLING?

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HIV infection of the thymus results in a decreased output of naive T-cells. The HIV Nef protein functions as an adaptor protein to interfere with the trafficking of several surface molecules and intersects with intracellular signaling cascades. We previously showed that expression of the Nef protein alone is sufficient to disturb human thymopoiesis. Structure-function studies with mutant Nef alleles showed that Nef-sites, critical for disturbing intracellular signaling cascades are crucial for Nefs effect on T-cell development. Our recent studies with mutant HIV-1 Nef alleles that selectively disrupt the interaction with PAK2 (i.e. NA-7, F191R, SF2 F195A) showed that Nef-PAK2 is especially important for interfering with T cell development. In an attempt to unravel the exact mechanism by which this interaction disturbs human thymopoiesis, we evaluated these Nef alleles for their ability interfere with the signaling of CXCR4 and the IL-7 receptor, given their importance in regulating T-cell development.

Wild-type (WT) Nef alleles and Nef mutants were cloned into a retroviral vector expressing Nef and EGFP from a single bicistronic mRNA (NEF-IRES-eGFP). Primary $CD4^+$ T-cells were transduced with these Nef constructs and analysed for the expression of CD4, CXCR4, and CD127 (IL-7 receptor α-chain) by flow cytometry. The effect of Nef on thymopoiesis was studied by transducing $CD34^+$ hematopoietic progenitor cells and subsequent FTOC.

Primary $CD4^+$ T-cells and $CD34^+$ thymocytes, transduced with wild-type NA-7 and SF2 Nef showed a reduced surface expression of CD4, CXCR4 and CD127 (IL-7 receptor α-chain). As expected NA-7 and SF2 Nef severely impaired T-cell development in FTOC. Interestingly, F191R and F195I mutations, which completely abolish the interaction with PAK2, impair Nefs abilty to downregulate CXCR4 and CD127 and result in normal T-cell development. Treatment of FTOC with human recombinant IL-7 and SDF1α partially restored the effect of WT Nef on T-cell development. Our results show that Nefs ability to disturb human T-cell development is associated with the ability to downregulate CXCR4 and the IL-7 receptor, and support our hypothesis that Nef disturbs T-cell development by interfering with signalling of these receptors.

MICRORNA-BASED REGULATION OF T LYMPHOCYTES AND MAST CELL FUNCTIONS

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MicroRNAs (miRNAs) are an abundant class of conserved non-coding RNAs that control gene expression by targeting mRNAs for degradation or translational repression. Several human diseases have now been associated with dysregulated miRNA expression, however, it is unclear what influence miRNAs have on other diseases such as asthma. The balance between different inflammatory cytokines is an important determinant for asthma development and severity, and several cell types can contribute directly or indirectly to it. Among these are various subsets of CD4+ T lymphocytes (such as Th1, Th2 and Th17, each characterized by the ability to produce a specific panel of cytokines) as well as mast cells, which are central effector cells in asthmatic responses. It is now becoming clear that miRNAs may have fundamental roles in regulating immune cells differentiation and cytokine production, thereby also potentially influencing the cytokine and cellular milieu responsible for asthma. The molecular network that regulates mast cell differentiation and functions has not been investigated as widely as it has been in other hematopoietic cells. Such information could be used to determine potential therapeutic targets for the control of mast cell activation in disease. We identified a family of two miRNAs, miR-221-222, that is upregulated following acute activation of differentiated mast cells. We found that these miRNAs have pleiotropic effects, regulating mast cells proliferation, survival and effector functions, and we are now investigating a possible role also in cell differentiation. Since an alteration of the balance between Th1 and Th2 lymphocytes can lead to autoimmune diseases, allergy and asthma, we also analyzed the role of miRNAs in regulating the critical balance between these two cell types. We previously reported that very few miRNAs are differentially expressed between Th1 and Th2 cells in the mouse, indicating that they might be involved in fate determination of these cells. Indeed, our recent data now show that one particular miRNA has an important role in regulating T cell plasticity and Th1-Th2 balance in both human and mouse. Besides being of fundamental relevance to our understanding of cell differentiation and gene regulation, elucidation of the molecular mechanisms that control mast cells and T lymphocytes differentiation and activation have substantial potential to find new points of entry for clinical application in the treatment of asthma and allergy.

SPECIFICATION OF THE NF-KB TRANSCRIPTIONAL RESPONSE BY P65 PHOSPHORYLATION AND TNF-INDUCED NUCLEAR TRANSLOCATION OF IKKε

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Here we investigated the regulation of NF-κB activity by posttranslational modifications upon reconstitution of NF-κB p65-deficient cells with the wildtype protein or phosphorylation-defect mutants. Quantitative analysis of NF-κB target gene expression showed that p65 phosphorylations alone or in combination function to direct transcription in a highly target gene-specific fashion, a finding discussed here as the NF-κB barcode hypothesis. High resolution microscopy and surface rendering revealed serine 536 phosphorylated p65 predominantly in the cytosol, while serine 468 phosphorylated p65 mainly localized in nuclear speckles. TNF stimulation resulted in the translocation of the cytosolic p65 kinase IKKε to the nucleus and also to PML nuclear bodies. This inducible IKKε translocation was dependent on p65 and was prevented by the oncogenic PML-RARα fusion protein. Chromatin immunoprecipitation experiments revealed the inducible association of IKKε to the control regions of several NF-κB target genes. In the nucleus, the kinase contributes to the expression of a subset of NF-κB-regulated genes, thus revealing a novel role of IKKε for the control of nuclear NF-κB activity.

THE DEVELOPMENTAL RELATIONSHIP BETWEEN INTESTINAL LYMPHOID TISSUE INDUCER CELLS AND NATURAL KILLER CELLS.

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We and others recently identified a novel lymphocyte population in the lamina propria of the murine small intestine characterized by the expression of the transcription factor *retinoic orphan receptor γ t* (RORγt) and activating natural killer (NK) cell receptors (e.g., NKp46, NKG2D and NK1.1).
Similar to lymphoid tissue inducer (LTi) cells $NKR^+ROR\gamma t^+$ lymphocytes required RORγt for their development. Whether this innate lymphocyte subset is part of the NK cell or lymphoid tissue inducer (LTi) cell lineage or rather defines an independent third lineage of innate lymphocytes remains controversial and constitutes an important gap in the hematopoietic lineage maps.
We investigated the developmental relationship of LTi, $NKR^+ROR\gamma t^+$ and NK cells by using genetic fate mapping, in vitro differentiation and adoptive transfer assays. We demonstrate that NK cells and LTi cells are distinct and separate innate lymphocyte lineages. LTi cells were the direct progenitors of $NKR^+ROR\gamma t^+$ cells which constituted differentiated LTi cells. $NKR^+ROR\gamma t^+$ cells further differentiated into $NKR^+ROR\gamma t^-$ cells and RORγt expression determined their responsiveness to the IL-12 and γ_c family of cytokines. $ROR\gamma t^+$ cells produced high levels of IL-22 in response to IL-23 whereas $ROR\gamma t^-$ produced high levels of IFN-γ in response to IL-12. IFN-γ-producing LTi-derived NKR^+ lymphocytes but not NK cells were central mediators of experimental colitis.
These data provide direct evidence for the existence of two developmentally and functionally distinct lineages that constitute the pool of innate NK cell receptor-expressing lymphocytes in humans and mice and reveal previously unappreciated plasticity within the LTi cell lineage.

MICRORNA REGULATION OF MYELIN-AUTOREACTIVE CD4 T CELL GENERATION

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MicroRNAs are small, endogenous, single stranded RNA that represent a novel group of non-protein coding gene products. MicroRNAs play important gene-regulatory roles by influencing transcription, degradation or activation of mRNA produced by classical genes. It is estimated that microRNA dependent regulation of all classical gene expressions spans at least third of entire genome. MicroRNAs have also been demonstrated as a important regulatory principle in the immune system. However, a detailed role of miRNA in regulation of autoimmunity remains unclear. The aim of this study was to analyze microRNA involvement during the development of autoimmune demyelination. To this end we have performed a systematic miRNA profiling using global microarray analysis as well as individual miRNA assays. Sorted CD4 T cell from lymph nodes of C57Bl/6 mice immunized with MOG peptide 35-55 as well as T helper (Th) cell from MOG-TCR transgenic mice (2D2) were used for the analyses. We have found a very selective expression of miRNA in Th cell highlighting three miRNA that were consistently upregulated: mmu-miR155, mmu-miR21 and mmu-miR301a. Mmu-miR155, mmu-miR21 and mmu-miR301a have all also been selectively upregulated in brain infiltrating mononuclear cells isolated from animals with different stages of experimental autoimmune encephalomyelitis (EAE). The changes in the above miRNA correlated with both in vitro and in vivo Th17 differentiation. Blockage of microRNA function influenced the critical pathways of Th17 development. Search of potential targets of positively highlighted miRNA with both bioinformatics and microarray profiling approaches revealed a number of immune genes that could be regulated by miRNA in a process of myelin autoantigen recognition. Our results provide evidence for specific and selected role of several miRNA in the process of myelin antigen recognition and autoreactive Th cell activation suggesting a new venues for a possible therapeutical intervention for the autoimmune demyelination.

PROTEIN KINASE DS SET SIGNALLING THRESHOLDS FOR T LYMPHOCYTE HOMEOSTASIS

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Protein kinase Ds are a conserved family of serine/threonine kinases that includes PKD1, PKD2 and PKD3. PKDs are expressed ubiquitously but are particularly abundant in lymphocytes where they are selectively activated by triggering of antigen receptors. T lymphocytes express PKD2 and PKD3. Mice that lack PKD2 or express a catalytically inactive PKD2 can produce peripheral T cells but these cells have defects in antigen receptor induced proliferation and show an abnormal transcriptional program in response to engagement of their antigen receptor. In particular the cells are defective in the production of key cytokines including Interleukin 2 and Interferon gamma. We looked at the combined impact of PKD2/PKD3 gene loss In T cells by backcrossing PKD2-/- mice with PKD3fl/fl mice and mice expressing Cre recombinase specifically in the T cell lineage under the control of the p56lck promoter. In PKD2-/- PKD3fl/fl lck cre mice there is normal development of T cells in the thymus but a depletion of peripheral T cells in the blood, and peripheral lymphoid tissues such as the spleen and lymph nodes. Previous studies had noted that PKDs were activated by the pre T cell receptor in T cell progenitors and by the mature T cell receptor in peripheral T cells. The T cell phenotype of PKD2-/- PKD3fl/fl lck cre mice is consistent with the model that PKDs are dispensible for the preTCR function but important for the TCR signals that control mature T cell homeostasis.

IDENTIFYING LRR PROTEINS IN INNATE IMMUNE RESPONSE

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The intestinal mucosa is a dynamic environment of complex interactions between the host and the gut microbiota. Maintaining an effective barrier function against pathogens through pathogen-associated molecular patterns (PAMPs) ensures integrity of the gut mucosa. Certain protein domains such as leucine-rich repeats (LRRs) have been found to be key for sensing microbes as evidenced by Toll-like receptors (TLRs) which detect PAMPs through their LRR domain.

Although members of the TLR-family and their roles in pathogen recognition and innate immune signaling have been well-studied, the function of a large number of LRR proteins remains unknown. Where biological information or annotation is available through database interrogation and literature mining, broad functional diversity can be observed across LRR proteins. Because these varied functions are often determined by non-LRR domains in the protein, LRR proteins are typically grouped based on these non-LRR domains. This results in proteins with LRR-only domains being placed together in an isolated group unrelated to any specific function. Since most of these LRR-only proteins remain functionally uncharacterized, our aim is to devise a method for grouping LRR proteins such that LRR-only proteins would be distributed among those with non-LRR domains.

By employing an automated approach that uses pattern recognition and Hidden Markov models, we cataloged 375 human LRR proteins and classified them by LRR repeat-class composition. With few exceptions, human LRR proteins, in general, contain repeats of predominantly one class. We also observe a statistically significant co-occurrence of certain LRR repeat classes (when featured predominantly in the protein) with transmembrane domains or with non-LRR protein domains, eg. F-box, CARD or PYRIN domains. This provides a useful basis on which to classify LRR proteins of unknown function that co-cluster with better characterized members.

With this base classification for human LRR proteins, we were interested to identify from this, a subset that might have important roles in immunity and host defense pathways. We examined expression signatures for LRR genes across tissue types and identified about 80 LRR genes that are immune enriched, including NOD-like receptor and TLR genes. To identify bacterial-responsive LRR genes, we examined expression profiles of LRR genes in primary macrophages infected with a number of pathogenic bacteria including M. tuberculosis. LRR genes exhibiting both immune enrichment and induction by pathogens, eg MFHAS1 was found to be involved in key immune pathways including NF-kB-dependent signaling in epistasis and siRNA experiments.

SILAC DNA-PROTEIN INTERACTION SCREENING- A REVERSE CHIP APPROACH FOR IDENTIFICATION OF SEQUENCE SPECIFIC DNA BINDING FACTORS

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Mammalian genomes contain roughly 20000 protein-coding genes. However, in given cell types only about more than 5000 of these genes are expressed at detectable level. In order to understand this, much scientific effort has been focused on deciphering the gene regulatory network that consists of DNA sequence specific binding of transcription factors (TF), which are able to read the regulatory information. We have already reported a fast, scalable and sensitive MS based methodology (SILAC DNA-protein DNA interaction screening) that allow to identify protein-DNA interaction in an unbiased manner complementary to ChIP technology. Here we applied our reverse ChIP to discover the proteins, which bind to a novel evolutionary conserved mammalian core promoter-associated cis-element (termed M4) harboring the consensus sequence RRACTACANNTCCCRRNRNRC. A combination of biochemical and cellular in vitro studies recapitulates our MS data unequivocally demonstrating that the hematopoietic master regulator Ikaros and the pluripotency factor THAP11/Ronin are directly interacting with M4. We can further show that Ikaros is essentially required to recruit the NF-kappa B/rel family members p50, p52 to the M4 and that THAP11/Ronin is necessarily involved in bringing the epigenetic cell cycle regulator HCF1 (host cell factor 1) to M4 bearing genes. Since our reporter assays reveal that Ikaros binding is functional, we are continue to study biological role of M4 binders by knock-down experiments followed by global gene expression analysis.

REGULATORY T CELL LINEAGE STABILITY

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Regulatory T (T_{reg}) cells form a distinct cell lineage and act in a dominant manner to maintain immune homeostasis. Deficiency of T_{reg} cells is associated with the fatal lymphoproliferative disease, immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome in humans and the corresponding scurfy phenotype in mice. Despite this immunoregulatory role, T_{reg} cells have been demonstrated to co-express transcription factors normally expressed in effector cells, together with the lineage specifying transcription factor, Foxp3, and the T_{reg} cell T cell receptor repertoire is skewed towards autoreactivity. Together, this suggests that instability of the T_{reg} cell lineage could have pathogenic consequences. To assess the stability of T_{reg} cells *in vivo*, we utilized a mouse model in which Foxp3 expressing cells could be inducibly labeled with a stable YFP reporter and tracked over time. We demonstrate that, in the steady state, expression of Foxp3 and the associated T_{reg} cell phenotype is maintained for up to six months post labeling. Further, T_{reg} cells remained stable in vivo despite a variety of immunologic challenges, including irradiation-induced lymphopenia, infection with Listeria monocytogenes and treatment with CD40 agonistic antibody. Minimal loss of Foxp3 was observed *in vivo* following withdrawal of IL2. We report that, under homeostatic and inflammatory conditions, Foxp3 expression is stably maintained in T_{reg} cells, conferring faithful lineage identity.

POLYPYRIMIDINE TRACT BINDING PROTEIN 2 INTERACTS WITH AID AND IS REQUIRED FOR EFFICIENT CLASS SWITCH RECOMBINATION AND STABLE BINDING OF AID TO THE SWITCH REGIONS.

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Activation-induced deaminase (AID) is a B-cell specific cytidine deaminase that is required for class-switch recombination (CSR) and somatic hypermutation (SHM), resulting in the diversification of antibodies in germinal centers. Due to its potent mutagenic potential AID must be tightly regulated and specifically targeted to the Ig locus. Deregulated and mistargeted AID activity has been implicated in the ontogeny of a large number of mature B cell lymphomas in mice and humans. However, the mechanism via which this regulation and targeting is achieved is unknown. In order to gain insight into the regulation and targeting of AID, we have performed a screen for AID-interacting proteins using *in vivo* biotinylation tagging. This was accomplished by stably transfecting a B cell line (CH12) with an *Escherichia coli* biotin ligase (BirA) and AID containing a short sequence tag (biotag), which can be biotinylated by BirA *in vivo*. The biotinylated AID was then pulled down as a complex with its interactors in the context of CSR using streptavidin beads. Putative AID interacting proteins were subsequently identified by mass spectrometry. This screen identified the splicing factor polypyrimidine tract binding protein 2 (PTBP2) as an AID-interacting protein. We have verified this interaction in primary B cells using AID antibodies for co-immunoprecipitation. PTBP2 is an RNA-binding protein that plays a role in alternative splicing in neuronal cells but has not been studied in the immune system. We have shown that at least *in vitro*, PTBP2 can bind to the germline transcript at switch regions. We have also shown that knockdown of PTBP2 by shRNA in CH12s and primary B cells results in a significant defect in CSR. This decrease in CSR is not due to a defect in AID expression, cellular proliferation, transcription at the Ig locus or splicing of the germline transcript. However, we have shown by chromatin immunoprecipitation that shRNA knockdown of PTBP2 results in decreased AID binding to the switch regions after stimulation for CSR in CH12s and primary B cells. This defect in stable binding of AID to the switch regions could explain the defect in CSR observed in PTBP2 knockdown cells. This is the first study to show a direct role for splicing factors in the binding of AID to the switch regions.

TLR7 ENABLES CROSS-PRESENTATION BY LANGERHANS CELLS THROUGH A TYPE I IFN-DEPENDENT PATHWAY

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Activation of dendritic cells through TLR stimulation is a key component bridging the innate recognition of foreign entities to the induction of adaptive immune responses. Covalent conjugation of TLR7 agonists to protein antigens is an efficacious vaccine formulation that enhances the immunogenicity of the antigen and elicits adaptive immune responses. This study identifies divergent effects of two crucial cytokines induced by the TLR7 agonist-protein conjugate, IFNα and IL-12, in leading to efficient priming of CD8+ T cells. Here, we demonstrate that TLR7-driven CTL responses require not one, but both IFNα and IL-12. We found that signaling through the IFN-αβR was required for the timely recruitment and accumulation of activated dendritic cells in the draining lymph nodes. Surprisingly, we found that TLR7 induction led to cross-presentation of the conjugated protein by Langerhans cells. In contrast, IL-12 had no effect on the capacity of dendritic cells to respond to the conjugate. Instead, IL-12 appears to be crucial for the activation and expansion of CD8+ T cells. Collectively, the data suggest that TLR7-driven CD8+ T cell responses are dependent on IFNα for inducing migration and cross-priming in Langerhans cells, and IL-12 for eliciting the expansion of CD8+ T cells.

SILENCING NOTCH1 SPONTANEOUSLY ACTIVATES MACROPHAGES AND UPREGULATES NOTCH LIGANDS

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Notch signaling is a well conserved signaling pathway involved in cell fate decisions, proliferation, apoptotic cell death of various cell lineages, including myeloid lineage cells such as macrophages and dendritic cells. It also plays important roles in regulating effector functions of these cells in the periphery. In macrophages, activation of Notch signaling pathway regulates expression of pro-inflammatory genes, resulting in inflammatory responses upon TLR agonist-mediated activation. Inhibition of Notch signaling using pharmacological agents or genetic deletion of its DNA-binding partner, RBP-J, resulted in blunting of pro-inflammatory responses. Contributions of individual Notch receptors in this setting, however, were largely unknown. In this study, we specifically silenced expression of Notch1 in macrophage-like cell line, RAW264.7. Unexpectedly, knock down of Notch1 resulted in spontaneous activation phenotypes of macrophages. Increased expression of *iNOS*, *TNFα*, *IL-12p40* and *IL-10* were observed even in the absence of any TLR and/or IFNγ stimulation. Nitric oxide and TNFα productions correlated well with the mRNA expression. The microbiacidal activities of these Notch1 knock-down macrophages were also significantly higher, compared to the control cells, suggesting that Notch1 silencing leads to spontaneous activation of macrophages. The expression of one of target genes of the Notch signaling pathway, *deltex*, was significantly increased in the absence of any stimulation, indicating that Notch signaling pathway is activated when Notch1 is silenced. The expression of Notch ligands, i.e. *Dlk1*, *Dlk4* and *Jagged1*, were also upregulated in these cells. Taken together, we proposed that silencing Notch1 leads to activation of the Notch signaling pathway and macrophage activation, via upregulation of Notch ligands. The molecular mechanisms underlying the upregulation of the Notch ligands are under investigation.

PHLPP REGULATES THE DEVELOPMENT, FUNCTION AND MOLECULAR SIGNALLING PATHWAYS OF MOUSE AND HUMAN T REGULATORY CELLS

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T regulatory cells (Tregs) have a fundamental role in maintaining immune homeostasis. There is much interest in defining their unique properties in order to understand their mechanisms of action and identfy new therapeutic approaches to modulating their activity *in vitro* or *in vivo*. We previously found that Tregs have a reduced capacity to activate the PI3K/Akt pathway downstream of the TCR, and that low activity of Akt was necessary for their suppressive function. In order to define the molecular basis for their unique molecular phenotype, we investigated Treg expression of phosphatases that regulate the PI3K/Akt pathway: PTEN, SHIP and PHLPP. Although *ex vivo* Tregs and conventional T cells expressed similar amounts of the lipid phosphatases PTEN and SHIP, Tregs expressed significantly higher levels of PHLPP, a novel Ser/Thr protein phosphatase that negatively regulates Akt. Knock-down of PHLPP using siRNA completely restored TCR-mediated activation of Akt in Tregs. Moreover, knock down of PHLPP in naive T cells significantly impaired their capacity to develop into induced Tregs under the influence of TGF-β, indicating that PHLPP-mediated suppression of Akt is necessary for peripheral Treg development. To investigate the functional significance of PHLPP in Tregs, we analyzed T cells from PHLPP1-/- mice. Differentiation of peripheral Tregs was impaired in mice genetically deficient for PHLPP1, and the suppressive capacity of *ex vivo* Tregs was significantly reduced. These data define a novel mechanism used by T cells to control the Akt pathway and provide the first evidence for a molecular mechanism underlying the functionally essential reduction of Akt activity in Tregs.

HISTONE POSTTRANSLATIONAL MODIFICATIONS ON AUTOIMMUNE REGULATOR (AIRE) TARGET GENES

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The Autoimmune Regulator (AIRE) protein is expressed in thymic medullary epithelial cells, where it promotes the ectopic expression of tissue-restricted antigens. Expression of tissue-specific antigens is needed for efficient negative selection of developing thymocytes. Mutations in AIRE cause Autoimmune Polyendocrine Syndrome type 1 (APS1) , which is characterized by a breakdown of self-tolerance. The molecular mechanism by which AIRE increases the expression of a variety of different genes remains unknown. We have studied AIRE-regulated genes using whole genome expression analysis and chromatin immunoprecipitation. We show that AIRE preferentially activates genes that are tissue-specific and characterized by low levels of initial expression in stably transfected cell model but also in mouse thymic medullary epithelial cells. In addition, the AIRE-regulated genes lack active chromatin marks, such as histone H3 trimethylation (H3K4me3) and acetylation (AcH3), on their promoters. We also show that during activation by AIRE, the target genes acquire histone H3 modifications associated with transcription and RNA polymerase II. In conclusion, our data show that AIRE is able to promote ectopic gene expression from chromatin associated with histone modifications characteristic to inactive genes.

INTEGRATION OF DISTINCT INTRACELLULAR SIGNALING PATHWAYS AT DISTAL REGULATORY ELEMENTS DIRECTS T-BET EXPRESSION IN HUMAN CD4+ T CELLS

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The transcription factor T-bet (T-box expressed in T cells, TBX21) is a key regulator of Th1 cell development. Similar to the Th1-specific genes IL-12Rb2 and IFN-g, T-bet is not expressed in naïve CD4+ T cells, but is readily induced during Th1 cell differentiation. However, despite its crucial role in Th1 lineage commitment, the mechanisms controlling Th1-specific expression of this factor are incompletely understood.
We have investigated how T-bet expression is controlled at early time points following stimulation of naïve human CD4+ T cells. To identify potential cis-elements that control T-bet expression we mapped DNase I hypersensitive sites (HS) at this locus in naïve CD4+ T cells and in cells developing along a Th1 or Th2 pathway. Using conventional hypersensitive site assays we identified two strong HS in Th1 and Th2 cells. HS2 contains an NFAT binding site and was not detected in naïve CD4+ T cells, whereas HS3 was already present in naïve cells and represents the core promoter. Since we did not detect any Th1-specific HS within the 16.7kb surrounding the mRNA start site, we screened for potential Th1-specific HS using Quantitative Chromatin Profiling (QCP), a PCR-based approach, which is not limited by the presence of convenient sites for restriction enzymes. 12kb upstream of the transcription initiation site we identified an additional HS in Th1 cells (HS1), containing a STAT binding consensus sequence.
TCR/CD28 signaling induced rapid histone H4 acetylation at these elements and binding of NFAT to HS2 in vivo. H4 acetylation, NFAT binding and T-bet expression were strongly reduced when cells were stimulated in the presence of cyclosporine A (CsA), suggesting that TCR-induced NFAT activation and binding to the T-bet locus was required to initiate T-bet expression in naïve CD4+ T cells. IFN-g and IL-12 signaling alone were not sufficient to induce T-bet expression in naïve CD4+ T cells, but cytokine signaling enhanced T-bet expression in TCR/CD28-stimulated cells. We found that IL-12 signaling induced binding of STAT4 at HS1 and showed that siRNA-mediated knock-down of STAT4 expression reduced expression of T-bet and of IFN-g.
Collectively, our data suggest that T-bet locus remodeling and gene expression are initiated by TCR-induced NFAT recruitment and amplified by IL-12-mediated STAT4 binding to distinct distal regulatory elements during human Th1 cell differentiation.

KINOBEADS™ AS AN ENABLING TECHNOLOGY FOR THE DISCOVERY OF NOVEL JAK FAMILY INHIBITORS

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JAK family members are promising new drug targets for the treatment of inflammatory disorders. Here, we describe the use of Kinobeads™, our unique kinase-fingerprinting platform, for screening and selectivity profiling of JAK family inhibitors, using advanced clinical compounds CP690,550 and INCB018424 as examples. Using Kinobeads™ we can screen libraries of up to 100,000 compounds against native kinases directly in lysates from JAK expressing cell lines. For this purpose we have developed a primary assay based on Kinobeads™ that allows us to measure the potency of all four JAK family members (1, 2, 3, and Tyk2) by antibody detection on reversed phase microarrays. Subsequently, in lead optimization, we use Kinobeads™ as a primary assay system to drive optimization of the binding potency of candidate molecules towards the target, and selectivity over other, related kinases. Additionally, broad mass spectrometry based profiles against the protein and lipid kinome are performed on key molecules to confirm their selectivity in several different cell lines and tissues. Later in the drug discovery process, Kinobeads™ experiments can be used to determine the extent of target engagement in cells, organs and patient blood samples in efficacy and clinical studies.

NEUTROPHILS AND TH17 CROSS-TALKING IN AUTOIMMUNE DISEASES

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The identification of specialized subsets of effector CD4+ T cells has provided a paradigm to understand immunity and immunopathology. IL-17 producing T cells (Th17) have been first characterized in the mouse as a distinct lineage of CD4+ T cells that can differentiate from uncommitted naive T cell precursors under the aegis of the master transcription factors RORγt and the polarizing cytokines TGF-β, IL-6 and IL-23. IL-17 has been shown to mediate protection against extracellular pathogens by promoting neutrophil recruitment but also to cause immunopathology in different models of autoimmunity. Although it has been shown that neutrophils can infiltrate sites of bacterial infection and contribute to activation of T cells through induction of a pro-inflammatory environment, there is no evidence for a direct cross-talk between neutrophils and autoreactive Th17 cells. We will report on neutrophil dynamics in mice developing auitoimmune diseases and on how these cells contribute to shape T cell immune responses.

A NOVEL HIGHLY SPECIFIC SMALL MOLECULE INHIBITOR FOR BTK SUPPRESSES FC RECEPTOR FUNCTION IN MACROPHAGES AND PREVENTS INFLAMMATORY ARTHRITIS

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Activating Fc receptors and inflammatory cytokine production by macrophages have been implicated in the development and pathology of arthritis. However, the molecular components that mediate these events are not well characterized. Here we identify Bruton's tyrosine kinase (BTK) as an essential player not only in B-cell dependent but also in myeloid-cell dependent inflammatory arthritis using a novel highly specific small molecule inhibitor of BTK (BTK SMI). In rodent models of collagen-induced arthritis, treatment with BTK SMI inhibits disease by at least two mechanisms: preventing autoantibody production and suppressing inflammatory cytokine production. Specifically, BTK SMI treatment prevents signaling and inflammatory cytokine production in response to immune complex activation in macrophages. Pathways found in rodents translate to human as BTK SMI prevents Fc receptor induced cytokine production in human monocytes. These data indicate that BTK SMI may be beneficial for the treatment of rheumatoid arthritis and that BTK contributes substantially to myeloid cell-dependent disease pathogenesis.

INDUCTION OF HUMAN T-CELL DEVELOPMENT FROM CD34+ PROGENITORS BY EXPOSURE TO IMMOBILIZED NOTCH LIGAND DELTA-LIKE-4

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Notch signaling based culture system have become indispensable for studying T-cell development in vitro. Using the OP9/DL1 coculture system murine and human haematopoietic progenitors can be induced to engage towards T-cell differentiation in vitro. Transfer of murine T-cell precursors generated in vitro on OP9/DL1 into NOD/SCID/γc-/--mice (NSG) promoted reconstitution of the T-cell compartment. While most Notch based culture systems consist of stromal cells transduced with either DL1 or DL4, so far there is little evidence whether DL1 or DL4 alone can drive hematopoietic progenitors towards a T-cell fate in vitro. It was recently suggested that exposure of CD34+ HSC to Delta4 is sufficient to promote human T-cell differentiation in vitro. We applied this approach to further characterize early steps of Notch dependent induction of human T-cell differentiation.

We exposed human CD34+ progenitors to immobilized Delta4. Within 7 days a population of CD34+/CD7+ and CD34-/CD7++ T-cell precursors emerged in the presence of DL4. After 7 days the CD34+/CD7+ population subsequently declined while further amplification of the CD34-/CD7++ population was observed. Within the CD34-/CD7++ population, two distinct progenitor subsets, CD5+ and CD5-, emerged. The CD5+ subset partially acquired CD1a and thus adopted the phenotype corresponding to an intermediate between an early thymic progenitor (ETP) and a prethymocyte (pre-T), corresponding to the murine DN2/DN3 stage. The CD34-CD7++CD5- cells acquired a Natural Killer cell (NK) phenotype. Conversely to what observed in OP9/DL1-coculture, T-cell development did not progress beyond the pre-T stage. We neither observed more advanced stages of T-cell development nor TCR-rearrangements. 7-day exposure to immobilized Delta-lika-4 induced a 90-fold increase of T-precursor frequency in CD34+ progenitors (1/8800 vs. 1/90). All T-cell precursor activity was restricted to cells expressing CD34, CD7 or both (frequency: 1/9), whereas the CD34-CD7- population did not contain any detectable T-cell precursor. In vitro generated T-cell precursors were transferred into NSG to study their potential to enhance thymic reconstitution. Results of the in vivo-experiments are pending.

Our results provide further evidence that human T-cell development can be induced in vitro by a simple exposure to immobilized Notch-ligand DL4. In contrast to the OP9/DL1 system, T-cell development induced by immobilized DL4 is blocked at the preT-stage, suggesting that further signals distinct to Notch and not provided by the cultured human progenitors themselves are required for T-differentiation beyond the DN-stage.

OVEREXPRESSION OF SHORT CYLD CAUSING IMPAIRED T CELL DEVELOPMENT LEADS TO AUTOIMMUNITY

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CYLD is a tumor suppressor gene mutated in familial cylindromatosis, an autosomal-dominant condition that predisposes to multiple skin tumors. CYLD exhibits deubiquitinating activity and acts as a negative regulator of NF-κB signaling.
To analyse the function of CYLD *in vivo* we used the $CYLD^{ex7/8}$ mice, which are characterized by loss of the full-length transcript and overexpression of a short splice variant of *cyld* (sCYLD).
In $CYLD^{ex7/8}$ mice the overexpression of sCYLD resulted in splenomegaly and lymphadenopathy. Additionally, the B cell population in spleen and lymph nodes is increased at the expense of T cells.
Analysis of $CYLD^{ex7/8}$ T cells showed a significant reduction of CD4 single positive (SP) and CD8 SP T cells in the thymus and in the periphery. *In vitro* as well as *in vivo* we could show that $CD4^+$ T cells display a hyperactive phenotype, proliferating to a better extent than wild type (wt) cells and expressing high amounts of inflammatory cytokines like IL-6 and IL-17. Western blot analysis of steady state thymocytes and peripheral $CD4^+$ T cells were performed, showing that the alternative NF-κB pathway was highly upregulated visualized by the expression levels of RelB and p100/p52. In order to investigate the contribution of CYLD in positive and negative selection in the thymus *in vivo*, the HY-TCR transgene (HYtg) was crossed to $CYLD^{ex7/8}$ mice. The analysis of $CYLD^{ex7/8}$ HYtg males revealed an increase in $CD4^+CD8^+$ DP as well as in CD8 SP thymocytes, suggesting a less pronounced negative selection in CYLD mutant mice compared to HYtg control mice. Interestingly, the impaired negative selection in the thymus was accompanied by a strong colitis phenotype even at early age (4 weeks).
Since medullary TECs (mTECs) play an important role in the late stage of T cell development by negatively selecting autoreactive thymocytes, the levels of mTECs in the medullary compartment was investigated. Interestingly, low numbers of mTECs were observed, combined with decreased expression levels of the mTEC markers UEA-1, keratin-5, claudin-3 and claudin-4. The reduction of mTECs in the medullary compartment could explain the inflammatory phenotype of $CD4^+$ T cells in $CYLD^{ex7/8}$ mice. Taken together, these results show an important role of sCYLD in T cell development as well as in NF-κB signaling of T cells.

JAK1 AND JAK2 ARE CRITICAL FOR TSLP-MEDIATED STAT5 PHOSPHORYLATION.

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Thymic stromal lymphopoietin (TSLP) is a type I cytokine that plays essential roles in allergic/inflammatory skin and airway disorders, in helminth infections, and in regulating intestinal immunity. TSLP signals via IL-7Rα and a specific TSLPR subunit that is highly related to the common cytokine receptor γ chain, γ_c. Although TSLP has effects on a broad range of hematopoetic cells and can induce STAT5 phosphorylation, TSLP was reported to not signal via JAK kinases, and the mechanism by which TSLP regulates STAT5 phosphorylation has been unclear. We now demonstrate that TSLP activates JAK1 and JAK2 in mouse and human primary $CD4^+$ T cells, in contrast to the known activation of JAK1 and JAK3 by the related cytokine, IL-7. We show that just as JAK1 interacts with IL-7Rα, JAK2 is associated with TSLPR protein, and both JAK1 and JAK2 are indispensable for TSLP-mediated STAT5 phosphorylation. These findings clarify the basis for TSLP-mediated signaling and establish the first example wherein a cytokine uses JAK1 and JAK2 to mediate the activation of STAT5.

ON THE ROLE OF INFLAMMASOME IN TH17-MEDIATED IMMUNOPATHOLOGY

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IL-1β is a pleiotropic cytokine produced following cleavage of pro-IL-1β by interleukin-1 converting enzyme (caspase-1), which in turn is activated by the inflammasome. This cytokine has been shown to play a role in several inflammatory immune disorders in humans and in experimental animal models, including mouse experimental autoimmune encephalomyelitis (EAE). More recently, IL-1β was proposed to be involved in the differentiation of inflammatory Th17 cells. We observed that mice deficient for a component of the inflammasome (the apoptosis-associated speck-like protein containing a caspase recruitment domain, also known as ASC) did not develop EAE following immunization with MOG in CFA. However, T cells from ASC-KO mice could differentiate into Th17 cells in vitro and in vivo upon s.c immunization, indicating that in these conditions IL-1β is not required for priming of mouse Th17 cells. We hypothesized that IL-1β, or other inflammasome-generated cytokines, may be required in the effector phases of EAE. Consistent with this hypothesis we found that microglial cells and CNS-resident myeloid cells expressed caspase-1 and released active IL-1β upon stimulation with inflammatory cytokines or TLR agonists in vitro. To further investigate the role of IL-1β in Th17-mediated diseases we set up a different EAE model. Rag1 KO mice were adoptively transferred with 2D2 transgenic MOG-reactive T cells following induction of brain inflammation by head irradiation. After 4 weeks mice developed EAE, which was associated with a large infiltrate of inflammatory leukocytes including MHC class II+ microglia/inflammatory monocytes in the brain. We are now using this model in ASC-KO or IL-1 β KO mice.

ANALYSIS OF HUMAN ANTIBODY REPERTOIRES IN HEALTH AND DISEASE

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Antibodies are a major part of the immune system and consist of immunoglobulin heavy and light chains, which are encoded by rearranged genes assembled from subsets of V (variable), D (diversity, only heavy chains) and J (joining) gene segments. Antibody repertoire diversity, potentially of 10^8 or more unique molecules in a single individual, build the basis of antibody-based antigen recognition and protection. The diversity of antibody repertoires is further increased by somatic recombination and hypermutation to achieve high specificity and selectivity in the detection of foreign antigens. In some autoimmune disorders, as well as other diseases, a bias in V-gene usage has been reported and different patterns of V(D)J recombination are suggested. In an initial study we could verify that not only elevated levels of VH4-34 usage makes a difference between rheumatoid arthritis patients and healthy individuals, but a certain V(D)J recombination pattern is increasingly found.
To obtain a deeper insight into the nature of human antibody repertoires, we started developing a pyrosequencing method on a Roche Genome Sequencer FLX System yielding large sets of rearranged antibody sequences. We envisage to acquire V(D)J recombination patterns of heavy and light chains of all immunoglobulin classes in diseased and healthy individuals. To handle these datasets, engineering of a new database is ongoing, which will hold all collected sequence information as well as cover specific questions about antibody repertoires. We envisage that our study will conclude in new ways for sequencing and analysis of antibody repertoires in humans.

MICRORNAS REGULATE THE BALANCE OF TH1-TH2-TYPE CYTOKINES.

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CD4+ T helper (Th) lymphocytes play a central role in cell-mediated adaptive immunity. Upon encounter with the antigen, naïve CD4+ T cells can differentiate into various sub-types of effector cells including Th1 and Th2 cells, which are characterized by their cytokines profile (IL-4, IL-5 and IL-13 for Th2 cells, and IFN-γ by Th1 cells). MicroRNAs (miRNAs) are small, non-coding RNA molecules (22-24 nt in length) that typically function as negative regulators of the expression of protein-encoding genes. MiRNAs are transcribed in a PolII or PolIII-dependent manner and are processed in the nucleus from primary transcript (pri-miRNA) to a hairpin precursor (pre-miRNA). In the cytoplasm, mature miRNAs interact with target mRNA transcript, interfering with their translation into protein. Over the last few years, miRNAs has been shown to play key roles in regulating various immunological processes such as thymocytes maturation, differentiation of effector T cells (including Tregs), affinity maturation and selection of B cells in germinal centres. These findings suggest that miRNAs and the miRNA machinery may be crucial in the regulation of immunological response, development, function, and homeostasis. Since an alteration of the balance between cellular immunity (Th1) and humoral immunity (Th2) can lead to autoimmune and atopic diseases, we decided to analyze the role of miRNAs in regulating the critical balance between Th1 and Th2 lymphocytes. We previously reported that very few miRNAs were differentially expressed between Th1 and Th2 cells in the mouse, indicating that they might be involved in fate determination of these cells. Indeed, our recent data now show that one particular miRNA has an important role in regulating T cell plasticity and Th1-Th2 balance in both human and mouse. Specifically, in a Jurkat T cell line, miRNA overexpression leads to an increased sensitivity to activation inducing cell death (AICD), and altered IL-2 production. Most interestingly, in primary T cells from both human and mouse, the cytokine profile of miRNA-overexpressing cells was skewed towards a Th1-like phenotype, with increased IL-2 and IFN-γ production and reduced IL-4 production. Besides being of fundamental relevance to our understanding of cell differentiation and gene regulation, elucidation of the molecular mechanisms that control T lymphocyte differentiation and activation have substantial potential to find new points of entry for clinical applications.

ITK, A TEC FAMILY TYROSINE KINASE, CONTRIBUTES TO DIFFERENTIAL EXPRESSION OF IL17A AND IL17F

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Th17 cells are a subset of CD4+ T- helper cells important in autoimmunity. Although the cytokine environment required for Th17 differentiation and the transcription factors they activate have been widely studied; the signaling pathways that are responsible for Th17 differentiation are not fully understood. Recently we demonstrated that Itk, a Tec family tyrosine kinase involved in signaling after T-cell receptor engagement, helps modulate TCR signaling to ensure differential regulation of IL17A and IL17F. Th17 CD4+ T-cells lacking Itk showed decreased expression of IL17A resulting from a defective activation of NFATc1 due to impaired TCR-mediated activation of PLC-g1 and Ca2+ mobilization in the absence of Itk. Importantly, there was no change in expression of the master transcription factor RORgT or production of IL17F in CD4+ T cells from Itk -/- mice. Given that Itk couples TCR signaling to IL17A expression, we are examining differences in TCR-induced transcriptional complexes to understand how Itk-mediated pathways may regulate the differential expression of IL17A and IL17F.

NOTCH SIGNALING CRITICALLY REGULATES MULTIPLE EFFECTOR FUNCTIONS OF ALLOREACTIVE T CELLS DURING GRAFT-VERSUS-HOST DISEASE

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Notch signaling is essential during early stages of T cell development and can control peripheral T cell immunity with context-dependent effects. We have identified Notch as a new, critical regulator of donor-derived alloreactive T cells mediating graft-versus-host-disease (GVHD) after allogeneic bone marrow transplantation. To inhibit Notch-mediated transcriptional activation, we conditionally expressed the pan-Notch inhibitor DNMAML in mature $CD4^+$ and $CD8^+$ T cells ($ROSA^{DNMAML}$ x *Cd4-Cre*$^+$ mice). Notch-deprived alloreactive T cells were able to proliferate and to expand in major histocompatibility complex (MHC)-mismatched hosts, as well as in minor histocompatibility antigen-mismatched recipients. However, DNMAML T cells failed to differentiate into potent effector T cells, leading to reduced GVHD severity and markedly improved survival. Upon *ex vivo* restimulation with anti-CD3 and anti-CD28 antibodies, alloreactive DNMAML $CD4^+$ T cells produced markedly decreased amounts of multiple cytokines, including IFN-γ, IL-4, TNFα, IL-17 as well as IL-2. Similar effects were observed in MHC-mismatched recipients upon systemic administration of γ-secretase inhibitors or after conditional inactivation of *Rbpj*, encoding CSL/RBP-Jκ, a transcription factor mediating the canonical effects of Notch signaling. Alloreactive DNMAML $CD4^+$ and $CD8^+$ T cells had preserved expression of the master transcription factors, *Tbx21* (encoding T-bet) and *Eomesodermin*. When alloreactive DNMAML $CD4^+$ T cells were restimulated with PMA and ionomycin, bypassing proximal steps of signaling, *Ifng* mRNA was completely restored, and *Il2* mRNA was partially rescued. These observations differed from past observations of Notch signaling in mature T cells and were not consistent with classical $CD4^+$ helper or $CD8^+$ effector T cell differentiation defects. Our findings identify Notch as a novel, potent regulator of alloreactive T cell responses mediating GVHD and a promising therapeutic target after allogeneic bone marrow transplantation.

IDENTIFICATION OF TARGET GENES OF DC-STAMP/LUMAN PATHWAY IN DENDRITIC CELLS

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Dendritic cells (DC) are the highly specialized antigen-presenting cells that play a key role in regulating immune response. DC can efficiently initiate immune responses or induce tolerance. Regarding this dual function DC are studied in immunotherapy directed against cancer and autoimmune diseases. Characterization of DC-specific genes leading to better understanding of DC immunobiology will help to optimally apply DC in a clinical setting.
Dendritic Cell Specific TrAnsMembrane Protein (DC-STAMP) previously discovered in our lab, is a protein preferentially expressed by dendritic cells. Its expression is upregulated during differentiation and downregulated upon maturation of DC. DC-STAMP in immature DC localizes to the endoplasmic reticulum (ER). Interestingly, upon maturation it is transported out of ER towards a Golgi compartment. We identified a physically interacting partner of DC-STAMP in the endoplasmic reticulum (ER), called LUMAN (also known as CREB3 or LZIP). LUMAN was previously described as an ER-resident transcription factor with unknown function. It is activated in a process called regulated intramembrane proteolysis (RIP), which involves translocation to the Golgi and subsequent proteolytic cleavage. The proteolytically activated form of the protein then translocates to the nucleus. Our data suggest that DC-STAMP plays an important role in the modulation of LUMAN activity in DC.
To understand the importance of DC-STAMP/LUMAN pathway in DC immunobiology we set out to identify the target genes of LUMAN in DC. We introduced the active form of LUMAN into the mouse DC-like cell line D2SC1 and screened the altered gene expression in time using microarray. Two genes were selected as LUMAN specific targets and validated by qPCR in two independent experiments in D2SC1 cell line as well as in mouse bone marrow derived DC's (BMDC's). Currently, we are investigating the role of the DC-STAMP/LUMAN pathway, including the luman target genes, in DC.

IS CTLA-4 REALLY A NEGATIVE SIGNAL?

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CTLA-4 is an essential regulator of T cell immune responses whose mechanism of action is poorly understood. Whilst it has been proposed that ligation of CTLA-4 delivers an inhibitory signal to T cells, evidence for this process is hard to obtain. Indeed, T cells activated in the presence of natural ligands, all express CTLA-4, yet undergo robust cell division. Furthermore, studies of T cells lacking CTLA-4 fail to reveal obvious defects in T cell behaviour. In contrast, recent evidence increasingly supports the view that CTLA-4 can act cell-extrinsically to regulate the activity of T cells. This is consistent with a role for CTLA-4 as an effector of suppression on regulatory T cells. However, CTLA-4 expression (and presumably its functions) are not restricted to Treg cells. Using a model CHO cell system we have identified that a central molecular feature of CTLA-4 is its ability to capture and internalise the co-stimulatory ligand CD86 by a process of trans-endocytosis. Based on this observation we have found that all T cells expressing CTLA-4, including activated T cells and Treg and CTLA-4 -transfected Jurkat cells, have the ability to remove CD86 molecules resulting in impaired T cell costimulation by antigen presenting cells. Efficient removal of CD86 from target cells requires the C-terminal region of CTLA-4, which is responsible for protein trafficking and the removal process is stimulated by TCR engagement. Blocking lysosomal degradation reveals an accumulation of internalised ligand inside the T cell, suggesting that the fate of internalised ligands is lysosomal degradation.
Together, these results provide a new mechanism of immune regulation that uses CTLA-4 as a “ligand depleting pump” to remove costimulatory molecules from antigen presenting cells, thereby inhibiting T cell activation. Importantly, this model of CTLA-4 function accounts for many of the key features of the CD28-CTLA-4 system providing a cogent explanation for the sharing of ligands between CTLA-4 and CD28, the highly conserved nature of CTLA-4 endocytosis and the constitutive presence of CTLA-4 on regulatory T cells. Moreover, this model offers a simple intuitive mechanism of immune regulation that applies equally to all CTLA-4 expressing cells.

ROLE OF KRAB/KAP1-MEDIATED TRANSCRIPTIONAL REGULATION IN T AND B LYMPHOCYTE DIFFERENTIATION AND FUNCTION

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Recent studies have established the important role of chromatin modifiers in controlling the stage-specific networks of transcription factors regulating lymphoid cell differentiation. The vertebrate specific Krüppel-Associated Box-Zinc Finger Protein (KRAB-ZFP) family comprises more than 400 transcriptional repressors, which seem to be differentially expressed in a lineage-specific manner. Whereas the gene targets of these regulators remain mostly unknown, it has been demonstrated that they act through sequence-specific DNA binding and recruitment of their KAP1 (KRAB-Associated Protein 1) universal cofactor, which in turns recruits chromatin remodellers and induces heterochromatin formation.
In order to investigate the role of KRAB/KAP1-mediated regulation in the lymphoid lineage, we developed two conditional KAP1 knockout mouse lines by crossing KAP1flox^2 mice with strains expressing the Cre-recombinase under the control of either the CD4 or the CD19 promoters. Cytofluorimetric analyses of the resulting progeny indicate that CD4-Cre/Kap1flox^2 mice exhibit a significant accumulation of CD8+SP cells in the thymus and a reduction of the same population in the periphery. Also, in these mice, naturally occurring T regulatory and gd cell populations are expanded. CD19-Cre/ KAP1flox^2 exhibit a significant reduction of the marginal zone B cells and the B1 cell compartment. We are currently testing the function of the affected populations and performing transcriptome analyses to determine the molecular bases of the observed phenotypes. This work will shed interesting light on the role of epigenetic in the control of immune differentiation and function.

CLASS SWITCH RECOMBINATION DEFECTS IN MICE LACKING THE E3 UBIQUITIN LIGASE RNF8

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53BP1 is a well known mediator of the cellular response to DNA damage. Two alternative mechanisms have been proposed to explain 53BP1's interaction with DNA breaks (DSBs), one by binding to methylated histones and the other via an RNF8 E3 ligase-dependent ubiquitylation pathway. The formation of RNF8 and 53BP1 irradiation induced foci are both dependent on histone H2AX. To evaluate the contribution of the RNF8 dependent pathway to 53BP1 function we generated RNF8 knockout mice. We report that RNF8-deficiency results in defective class switch recombination (CSR) and accumulation of unresolved immunoglobulin heavy chain (IgH) associated DNA breaks. The CSR DSB repair defect is milder than that observed in the absence of 53BP1 but similar to that found in H2AX-/- mice. Moreover, similar to H2AX but different from 53BP1 deficiency, RNF8-/- males are sterile, and this is associated with defective ubiquitylation of the X-Y chromatin. Combined loss of H2AX and RNF8 does not cause further impairment in CSR, demonstrating that the two genes function epistatically. Importantly, although 53BP1 foci formation is RNF8-dependent, its binding to chromatin is preserved in the absence of RNF8. This suggests a two-step mechanism for 53BP1 association with chromatin, in which constitutive loading is dependent on interactions with methylated histones, whereas DNA damage inducible RNF8-dependent ubiquitylation allows its accumulation at damaged chromatin.

E47 REGULATES HEMATOPOIETIC STEM CELL PROLIFERATION THROUGH P21

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Bone marrow progenitor cells continuously replenish all blood lineages thus maintaining a functional immune system throughout adult life. The lineage negative, Sca^+, Kit^+ (LSK) subset of the bone marrow can be further resolved into long-term hematopoietic stem cells (HSC) and multipotent progenitors (MPP). The transition from HSCs to downstream MPPs is marked by loss of long-term self renewal as cells progress towards a more differentiated downstream progenitor. It is critical for HSCs to cycle at a steady yet low rate to continuously seed the marrow and at the same time keep their numbers constant. Insufficient stem cell renewal can lead to hematopoietic failure. In addition, disruption of mechanisms involved in controlling HSC proliferation can promote formation of cancer stem cells. Previous work from our lab has recently shown that HSCs from mice lacking E47 exhibit increased proliferation, loss of quiescence and decreased expression of the cell cycle inhibitor p21. These findings suggest that p21 is a possible downstream target of E47 in restraining the proliferative activity of bone marrow stem cells. However, formal evidence establishing a genetic interaction between E47 and p21 is lacking. We have generated $E47^{+/-}p21^{+/-}$ compound heterozygotes to test this hypothesis. While there are no detectable differences in the frequencies of LSKs, $flk2^-$ LSKs, $flk2^+$LSKs, common lymphoid progenitors and B cell progenitors from compound heterozygote mice compared to WT or single heterozygote controls, we found significant alterations in proliferative activity. In vivo BrDU incorporation assays reveal an increase from ~55% $BrdU^+$ LSKs in each WT and animals heterozygous for either gene, versus 65% $BrdU^+$ LSKs in the compound HETs. Sensitive resolution of individual HSC and MPP subsets within LSKs showed even more profound effects. Approximately 43% of $E47^{+/-}$ $p21^{+/-}$ $flk2^-$ LSKs are proliferating compared to 31-35% in WT or single heterozygotes. A significant increase from ~67% $BrDU^+$ $flk2^+$ LSKs from each WT and single HETS to 79% $BrDU^+$ $flk2^+$LSKs from compound heterozygotes was also observed ($p<0.05$, t-test). Consistent with the increased proliferation, total LSKs, $flk2^-$ LSKs, and $flk2^+$ LSK numbers from compound heterozygotes were more sensitive to 5-FU, a drug that is toxic to cycling cells. These results indicate that there is an increase in the proliferation of uncommitted progenitors in mice haploinsufficient in E47 and p21, providing evidence of a direct genetic link.

FOXO3 PROMOTES BCR-INDUCED APOPTOSIS IN IMMATURE B CELLS

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Either disruption of the tonic signal transmitted by an innocuous BCR or strong engagement of the BCR with antigen results in receptor editing and apoptosis in immature B cells. Inhibition of PI3K signaling promotes both of these outcomes. Expression of mRNA encoding the proapoptotic family of Foxo transcription factors is reduced by PI3K signaling in mature B cells, suggesting that changes in Foxo levels may also occur in and have functional consequences for immature B cells. anti-IgM stimulation was shown to upregulate Foxo family mRNA expression in primary immature B cells and WEHI 231 cells. Disruption of the tonic signal with PI3K inhibitors also increased Foxo mRNA. Since expression of Foxo3 was increased to a greater extent than that of Foxo1 or Foxo4 in primary cells, we examined its role in the response of immature B cells to BCR crosslinking. Overexpression of Foxo3 promoted BCR-induced cell death in WEHI 231 cells, while immature B cells from Foxo3-/- mice demonstrated reduced apoptosis in response to anti-IgM. In contrast, Igλ usage was normal, if not enhanced, in the absence of Foxo3 suggesting that receptor editing is unimpaired. This is consistent with previous studies demonstrating a role for Foxo1 in BCR-induced Rag expression. These results support a model in which Foxo3 and Foxo1 mediate apoptosis and receptor editing, respectively, in immature B cells expressing a non-functional or autoreactive BCR.

GFI-1B NEGATIVELY REGULATES RAG EXPRESSION IN V-ABL TRANSFORMED PRO-B CELLS

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Regulating RAG activity in B lineage cells is crucial to prevent deleterious events that can be caused by the presence of DNA double strand breaks. To identify negative regulators of Rag expression, we conducted an unbiased cDNA library screen in Abelson murine leukemia-virus transformed pro-B cells. We found that overexpression of the transcriptional repressor Gfi-1b downregulates RAG expression in pro-B cell lines and primary B lineage cells from bone marrow. Gfi-1b binds directly to a region of the RAG locus upstream of the B-cell specific Erag enhancer and its activity depends on its association with chromatin modifying cofactors. In addition, Gfi-1b's effect on RAG levels appears to be mediated in part by repression of Foxo1, a recently identified positive regulator of RAG expression. Gfi-1b-deficient cell lines exhibit increased RAG levels as well as an increase in the overall number of DNA double strand breaks per cell when compared to their wildtype counterparts, suggesting that Gfi-1B may be critical to maintain genome integrity. Moreover, microarray experiments revealed that Gfi-1b controls the expression of a suite of B lineage-specific genes, including the immunoglobulin kappa locus and the transcription factor SpiB. We identify Gfi-1B as a novel regulator of RAG expression that may also be involved in the execution of genetic programs that govern B cell development.

IN *NAÏVE* T CELLS THE *CTLA-4* GENE IS EMBEDDED IN A 20KB REGION ENRICHED IN H4AC WITH POCKETS OF H3K9ME2; POISED FOR TRANSCRIPTION, EARLY ACTIVATION INVOLVES A COMPETITION BETWEEN POSITIVE AND NEGATIVE REGULATORS FOR THE USAGE OF THE NFAT AND OCTAMER BINDING SITES

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Activation of CD4(+) Foxp3(-) T cells by Antigen Presenting Cells relies on the integration of both positive and negative signals. CTLA-4 is a negative regulator of T cell activation, expressed *de novo* after the integration of TCR/costimulation related signals. CTLA-4 is required for T cell homeostasis and tolerance and exerts its function by T-cell intrinsic and extrinsic mechanisms. Although multiple pathways have been shown to modulate T cell activation, in addition to TCR-mediated signals, the role of CD28 as positive and CTLA-4 as negative mediators remain critical.

Given the central role of CTLA-4 in regulation of T cell activation, surprisingly little is known about the regulation of *Ctla-4* transcription. We uncovered the presence of a positive regulatory cluster composed of NFAT- and Octamer-binding sites in the promoter element. In addition, we found that *Ctla-4* lies in an extended region enriched for H4Ac histones, with no much H3Ac, but with pockets of H3K9me2. Interestingly, this poised chromatin appears to have an added layer of negative regulation in that gene expression requires that the positive regulators NFAT and Octamer binding proteins out-compete negative regulators present in the naïve T cells that utilized the same binding sites.

In vitro, the *Ctla-4* promoter can be activated in an Octamer-dependent mechanism even in the absence of NFAT binding. Moreover, during the first hours of T cell activation, endogenous *Ctla-4* expression appears to occur in waves of transcription that seemed linked to differences in the NFAT family members present at the promoter. These observations are consistent with a key role for NFAT primarily in the “turn on” signal, and suggest a role for Octamer-binding proteins in the “maintenance of expression” (i.e. after NFAT activating signals have ceased). Finally, we uncovered a miR155 target site in the 3`UTR of the *Ctla-4* mRNA that *in vitro* decreases reporter protein levels, thus perhaps involved in “turning off” gene expression.

Altogether, these observations are consistent with a model where the transcription of the *Ctla-4* gene in T cells is regulated by NFAT-dependent and –independent mechanisms that out-compete negative regulators and miR155- rather than by extensive regulation of accessibility to the locus, which seems poised for transcription.

NFATC1 CONTROLS THE ACTIVITY AND SURVIVAL OF SPLENIC B LYMPHOCYTES UPON B CELL RECEPTOR STIMULATION

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Triggering of B cell receptor by anti-IgM antibodies (α-IgM Ab) induces a massive synthesis of NFATc1 in primary splenic B cells. This concerns mainly the short isoform NFATc1/αA which lacks a C-terminal peptide of approximately 245 aa residues and differs from other NFATc1 proteins by its N-terminal α-peptide. By the inactivation of the *Nfatc1* gene in B cells and re-expressing NFATc1/αA we show that NFATc1 levels are critical for the activity and survival of murine splenic B cells and chicken DT40 B lymphoma cells upon α-IgM Ab stimulation. While moderate NFATc1 levels support the proliferative capacity of splenic B cells, high concentrations induce their Activation Induced Cell Death (AICD) by stimulating the expression of pro-apoptotic *Fasl*/CD95 Ligand, *Pdcd1*/PD-1 and *Tnfsf14*/LIGHT genes. The diminished proliferative capacity of NFATc1-deficient B cells appears to be due to a decreased Ca^{++} mobilization and influx, and of calcineurin activity. NFATc1 ablation in bone marrow B cells did not markedly affect the differentiation of B cells in bone marrow and spleen, but suppressed the generation of peritoneal B1a cells and of germinal centre (GC) B cells upon immunization with sheep red blood cells. In addition, upon immunization with specific antigens NFATc1-deficient B cells produce less IgM antibodies and show a defect in switch to the production of other Ig classes. Ectopic expression of NFATc1/αA in NFATc1-deficient DT40 B induced Bcl-6 and Bach-2 but suppressed Blimp-1 expression, i.e. markers of GC reaction or plasma cell formation. These data find support in the constitutive nuclear expression of NFATc1/αA in GC B centrocytes expressing IgM. Incubations of NFATc1-deficient splenic B cells with T cells in mixed lymphocyte reactions or, upon loading of OTII T cells expressing an Ova-specific with transgenic TCR with an $Ova_{323-339}$ peptide showed a marked decrease in the capacity of B cells to stimulate the proliferation and IL-2 secretion of T cells. All these findings indicate that $NFATc1^{-/-}$ B cells acquire an anergy-like phenotype. Therefore, NFATc1 affects the strength and duration of immune responses, and its B cell-specific ablation impairs immune reactions *in vitro* and *in vivo*. This is shown for the mild clinical course of Experimental Autoimmune Encephalomyelitis (EAE) upon injection of EAE-inducing MOG_{35-55} peptide into mice bearing $NFATc1^{-/-}$ B cells. Taken together, our data indicate a crucial role for NFATc1 in the control of immune reactions. In addition, they suggest that NFATc1 and NFATc2, the two most prominent NFAT factors in peripheral lymphocytes, control the two features of the adaptive immune system, namely immunity (NFATc1/αA) or anergy (NFATc2).

DIFFERENTIAL REQUIREMENT FOR DELTA-LIKE-1 AND DELTA-LIKE-4 ENDOCYTOSIS IN T CELL DEVELOPMENT

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Specification of T cells requires signaling through the Notch receptor, which is mediated by Delta-ligand interactions. The endocytosis of Delta (Delta-like in mammals) in the signal-sending cell is thought to induce Notch activation in the signal-receiving cell. This work focuses on investigating the role of Delta-like 1 (Dll-1) and Dll-4 endocytosis in Notch activation and T cell development using the OP9-DL1 (and -DL4) stromal cells.

To address whether endocytosis of Delta-like is a requirement during T cell development, we have created different Delta-like mutants to specifically target Delta-like endocytosis to better understand its role in Notch activation. OP9 stromal cells retrovirally transduced to express the Dll-1 construct that lacks the intracellular domain (OP9-DL1-delta-ICD) or Dll-1 extracellular (ECD) domain fused to the transmembrane and cytoplasmic domain of CD25 (OP9-DL1-CD25), do not support T cell development nor do they inhibit B cell development. In contrast, OP9-DL4-delta-ICD and OP9-DL4-CD25 stromal cells retain the ability to support T cell development, but to a lesser degree compared to full-length Dll-4. In addition, quantitative PCR analysis revealed a differential induction of downstream Notch target genes. Constructs to promote endocytosis of Dll-1 or Dll-4 were created and over-expressed on OP9 stromal cells, by fusing the ECD of DL1 (or DL4) to the transmembrane and intracellular domain of the transferrin receptor (OP9-DL1-TFR1 or OP9-DL4-TFR1) and their ability to support T cell development was also analysed. Surprisingly, cell surface expression of both full length DL4 and DL4-delta-ICD are reduced compared to their respective DL1 constructs, suggesting that indeed Dll4 is able to support T cell development more efficiently than Dll1 and that it does not solely depend on endocytosis as a mechanism in which to initiate a Notch signal. To assess if the DSL domain of Dll4 has a greater affinity for Notch1 and is therefore better able to induce a Notch signal than Dll1, we swapped the DSL domains of Dll1 and Dll4 and over-expressed them in OP9 stromal cells and assessed their ability to support T cell development.

These studies not only demonstrate the importance of the endocytosis of Delta-like during hematopoiesis and its role in T cell specification, but also suggest the differential potential mechanism of Dll1 and Dll4 in this process.

CONFORMATIONAL CHANGES IN THE *TCRA/D* LOCUS DURING T CELL DEVELOPMENT

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The *Tcra/d* locus is composed of Dδ, Jδ and Cδ gene segments embedded between large arrays of Vα/δ and Jα gene segments. During T cell development, *Tcrd* gene segments recombine in DN thymocytes, whereas *Tcra* gene segments recombine in DP thymocytes. Notably, in DN thymocytes the Dδ-Jδ cluster has a single chance to recombine to widely separated Vδ gene segments, whereas in DP thymocytes, *Tcra* undergoes multiple rounds of Vα-to-Jα rearrangement and preferentially recombines proximal Vα gene segments first. The contraction of antigen receptor loci is thought to promote long-range interactions of V(D)J gene segments for synapsis and recombination. To investigate the changes in the conformation of the *Tcra/d* locus during T cell development, we used the 3-dimensional fluorescence in situ hybridization (3D-FISH) method with four DNA probes targeted to the distal V, central V, proximal V and 3'end of the *Tcra/d* locus. We found that the 3' portion of *Tcra/d* locus is highly contracted in both DN and DP thymocytes as compared to B cells. This result is consistent with the concept that locus contraction is necessary for Tcrd and Tcra rearrangement in DN and DP thymocytes, respectively. Remarkably, the 5' portion of the *Tcra/d* locus is highly contracted in DN thymocytes, but then decontracts in DP thymocytes. A potential explanation for this difference is that a highly contracted 5' conformation is essential for one-chance Tcrd rearrangement, involving proximal and distal V gene segments in DN thymocytes, whereas the relatively decontracted 5' conformation benefits *Tcra* rearrangement that is biased to more proximal V segments in DP thymocytes. In Murre's previous studies, the contracted conformation of the *Igh* locus in pro-B cells places all VH gene segments a similar distance from the DH-JH cluster, thereby providing all VH gene segments the opportunity for recombination. We suggest that the *Tcra/d* locus adopts a similar strategy in DN thymocytes, but a distinct strategy that is uniquely suited to promote multiple rounds of Tcra rearrangement in DP thymocytes.

ARVELEXIN ISOLATED FROM THE BRASSICA RAPA EXHIBITS ANTI-INFLAMMATORY PROPERTIES VIA THE DOWN-REGULATION OF NF-KB-DEPENDENT COX-2 AND INOS EXPRESSION IN LPS-INDUCED RAW264.7 CELLS

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Brassica rapa species (Brassicaceae) constitute one of the most important sources of food. We previously found that three indole acetonitrile compounds, namely, caulilexin, indole-3-acetonitrile, and arvelexin, comprise the active components of *B. rapa*. In the present study, arvelexin was found to have the most potent inhibitory effect on prostaglandin E_2 (PGE_2) and nitric oxide (NO) in LPS-stimulated RAW264.7 cells. Furthermore, arvelexin reduced levels of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and inflammatory cytokines (TNF-α, IL-6, and IL-1β) in LPS-stimulated cells. COX-2 and iNOS promoter activity assay revealed that arvelexin inhibited LPS-induced PGE_2 and NO production through the repression of their gene expression. In addition, arvelexin attenuated LPS-induced DNA binding and the transcriptional activities of nuclear factor-kappa B (NF-κB), which was associated with the prevention of the IκB kinase (IKK) phosphorylations and the inhibitor κBα (IκBα) degradation and consequently with decreased nuclear NF-κB protein levels. In rats, arvelexin (25 and 100 mg/kg, p.o.) inhibited carrageenan-induced paw edema, neutrophil migration, and PGE_2 production. These results demonstrate that arvelexin has an anti-inflammatory effect, which results from the inhibition of NF-κB activation in macrophages, thereby decreasing expression of COX-2, iNOS and pro-inflammatory cytokines. These findings showing inhibition by arvelexin on paw oedema as well as inflammatory gene induction will help to understand its pharmacology and mode of action.

PRDM1 IS A REGULATOR OF EFFECTOR CYTOKINE PRODUCTION IN HUMAN NATURAL KILLER CELLS

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PRDM1 (Positive Regulatory Domain zinc finger protein 1) is a DNA-binding transcription factor, originally identified as a suppressor of interferon-beta in virally-infected human osteosarcoma cells. It is most widely recognized as a key regulator of B-cell terminal differentiation into antibody-producing plasma cells. More recently, several reports have described functions in T-cell homeostasis and effector lineage differentiation. Here, we demonstrate that PRDM1 has a functional role in human natural killer (NK) cells. Using microarray profiling of primary NK cells stimulated with IL-2, IL-12 and IL-18, we observed strong induction of PRDM1 transcripts. Further characterization revealed high levels of expression predominantly in the $CD56^{dim}CD16^{+}$ subset in response to stimulation. Chromatin immunoprecipitation experiments reveal binding to novel regulatory sites in the IFNG and TNF loci in primary NK cells. Knockdown of PRDM1 leads to enhanced levels of IFNG and TNF mRNA and protein. Conversely, adenoviral-mediated over-expression of PRDM1 is sufficient to abrogate stimulation-induced IFNG and TNF transcription in cell lines. Furthermore, ChIP-on-chip analysis using promoter tiling arrays has allowed us to identify numerous novel PRDM1 target genes which may be relevant to NK function. The list of target genes identified thus far is quite divergent from genes previously shown to be regulated by PRDM1 in B and T cells, suggesting a unique functional role for PRDM1 in NK cells. Collectively, these data provide the first description and functional analysis of PRDM1 in NK cells, further extending the functional repertoire of this highly-conserved transcription factor.

HOMOLOGOUS PAIRING HELPS MAINTAIN GENOMIC STABILITY DURING CLASS SWITCH RECOMBINATION.

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Homologous pairing has been thought to have limited utility in higher eukaryotes. We recently discovered, however, that developing B cells undergo pairing during V(D)J recombination: homologous Ig alleles pair just prior to recombination, and monoallelic cleavage triggers ATM-dependent repositioning of the uncleaved allele to pericentromeric heterochromatin (PCH), halting further recombination until the first allele is repaired. Since genomic stability must also be maintained during class switch recombination (CSR) in B cells, we asked whether common processes regulate both recombination events. Not only do we find that Igh alleles pair during CSR, mediated by AID and the 3' enhancer, and that ATM repositions one allele to PCH, but our data suggest that AID and UNG provide a scaffold for the recruitment of repair proteins.

EPIGENETIC DYNAMIC AT DEVELOPMENTALLY REGULATED GENES DURING EARLY T-CELL DIFFERENTIATION

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It is now well established that different combinations of histone post-translational modifications influence the transcriptional activity of a given gene. However, how these epigenetic changes correlate with the regulation of gene expression during the process of cell differentiation has not been extensively studied. In order to address this issue, we have analysed locus-wide changes in gene expression and marks for “active” (H3K4me1/2/3, H3K36me3) and “repressive” (H3K9me2, H3K27me3) chromatin, at two critical stages of early T-cell differentiation, using primary cells from mouse model systems. We found that gene regulation was not associated with clear-cut switches between “active” and “repressive” marks. Instead, gene expression regulation between the two developmental stages resulted in a locus-specific combination of epigenetic marks at both proximal and distal regulatory regions. Importantly, both epigenetic priming and memory appears to be widespread properties of regulatory elements targeted by the pre-TCR signalling. Finally, we also describes the epigenetic dynamics at the complex TCR genes and highlight novel regulatory features related to the regulation of V(D)J recombination at these loci. In conclusion, our results show that gene regulation correlates with combinatorial changes of the epigenetic marks at both proximal and distal regulatory elements, with the strength of these modifications depending on the intrinsic features of each individual gene and associated regulatory elements.

PI3 KINASE SIGNALS BCR DEPENDENT MATURE B CELL SURVIVAL

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Previous work has shown that mature B cells depend upon survival signals delivered to the cells by their antigen receptor (BCR). To identify the molecular nature of this survival signal, we have developed a genetic approach in which ablation of the BCR is combined with the activation of specific, BCR dependent signaling cascades in mature B cells in vivo. Using this system, we provide evidence that the survival of BCR deficient mature B cells can be rescued by a single signaling pathway downstream of the BCR, namely PI3-Kinase (PI3K) signaling, with the FOXO1 transcription factor playing a central role.

AN UNBIASED SEARCH DETECTS REPRODUCIBLE AID-DEPENDENT DOUBLE-STRAND DNA BREAKS IN HUNDREDS OF GENES AND INTERGENIC REGIONS: THEIR POSSIBLE INVOLVEMENT IN B CELL LYMPHOMAGENESIS

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During B cell differentiation after immunization or infection, activation-induced cytidine deaminase (AID) initiates diversification of immunoglobulin (Ig) genes, initiating mutations within the antigen binding variable regions (somatic hypermutation), and recombination events resulting in a switch from one class of antibody to another (class switch recombination). Upon subsequent selection, mutations can improve the affinity of antibodies, and class switch recombination (CSR) leads to replacement of the μ constant region of the Ig heavy (H) chain (present in IgM) with another constant region, resulting in improved ability of the antibodies to remove the pathogens. AID initiates CSR by deaminating cytosines in special tandemly repeated switch (S) region sequences (ranging from 1 - 10 kb in length) located upstream of each of the IgH chain genes. The resulting dU bases are excised by uracil DNA glycosylase (UNG), which results in abasic sites that are converted to single-strand breaks (SSBs) by AP endonucleases. If the SSBs occur sufficiently near each other on opposite DNA strands, this can lead to the double-strand DNA breaks (DSBs) required for the deletional recombination occurring during CSR. S regions have numerous repeats of a sequence motif (WGCW), where W=A or T, which is a preferential target for AID. However, it is unknown if physiological levels of AID initiates DSBs at other sites in the genome, and if so, whether these DSBs can lead to genomic instability resulting in the translocations, deletions and amplifications found in B cell malignancies. AID has been shown to initiate mutations at non-Ig genes in Peyer's patch B cells, a location in which Ig variable region genes have undergone numerous rounds of SHM, but whether AID activity also leads to DSBs at sites other than IgH S regions is unknown. Using a non-biased genome-wide approach, we have identified reproducible AID-dependent DSBs in mouse splenic B cells induced to undergo CSR for two days in culture. Most interestingly, several of these DSB sites are syntenic with sites of translocations, deletions and amplifications in human B cell lymphomas.

MICRORNA FUNCTION IN CD4 T CELL PROLIFERATION AND CYTOKINE PRODUCTION

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MicroRNAs have been implicated as important regulators of many aspects of immune cell function. We and others have previously shown that deletion of the microRNA biogenesis enzymes Dicer and Drosha from T cells results in abnormal cytokine production and decreased proliferation of CD4 T cells *ex vivo*. We found that T cell specific deletion of the Drosha cofactor DGCR8 results in a similar phenotype. Reporter alleles and co-culture assays showed that these defects are cell intrinsic. Furthermore, retroviral expression of DGCR8 in these cells was able to restore miRNA expression and rescue the defects in proliferation and cytokine production. These data suggest a complex role for the miRNA pathway in the regulation of cytokine production and cell differentiation in actively dividing and differentiating CD4 T cells, likely due to the contributions of many miRNAs. Utilizing newly developed techniques to deliver functional miRNAs and miRNA antagonists to both wildtype and DGCR8-deficient primary CD4 T cells, we aim to identify the specific functions of individual miRNAs during T helper cell activation and cytokine production.

PYK2 IN IMMUNE CELLS EXISTS AS TWO BIOCHEMICALLY AND SPATIALLY DISTINCT POPULATIONS.

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Pyk2 is a non-receptor protein tyrosine kinase involved in cytoskeletal-associated functions of immune cells. Pyk2 can act as a scaffold protein and has been shown to complex with many proteins including paxillin and several Src-family kinases. The activity and function of Pyk2 is regulated through a variety of mechanisms, including phosphorylation and alternative splicing. Our laboratory has generated polyclonal antibodies against the N-terminus and the proline-rich regions in the C-terminus of Pyk2. Both of these antibodies are able to immunoprecipitate Pyk2, however in macrophages the N-terminal antibody pulls down a higher molecular weight form of the protein. We have found that this is not due to differential phosphorylation as treatment with alkaline phosphatase does not alter the relative mobility of the protein. In addition, Pyk2 precipitated with either antibody is tyrosine phosphorylated at similar levels. Through immunoprecipitation and immunoblotting, we show that Pyk2 pulled down with the C-terminal antibody constitutively associates with paxillin, whereas essentially no association is detected with Pyk2 recovered with the N-terminal antibody. Moreover, we demonstrate that the C-terminal antibody identifies a form of Pyk2 that is localized at the microtubule organizing center (MTOC) and along microtubules, where it colocalizes with paxillin. Conversely, the N-terminal antibody binds to Pyk2 that is predominantly membrane proximal and absent from the MTOC. Finally, Pyk2 associated with the Pyk2 recovered with the C-terminal antibody is complexed with a large number of proteins, consistent with its cytoskeletal location, whereas the Pyk2 associated with the N-terminal antibody does not seem to form large protein complexes. Biochemical and functional differences between the two populations of Pyk2 distinguished by these antibodies are currently being explored. All things considered, we propose that these antibodies identify two populations of Pyk2 that differ oin their association with other proteins, their localization and, possibly, their function. Further characterization of these two populations will provide significant insight in the diverse means by which Pyk2 is regulated, and how this regulation translates into differential protein binding, localization and function.

EZH2 CONTROLS PHYSIOLOGICAL FUNCTION OF DENDRITIC CELLS

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Polycomb group protein Ezh2, one of the key regulators of embryonic development in different organisms, was not essential for the in vitro and in vivo differentiation of various dendritic cell (DC) subsets.

The functional study however indicated the requirement of Ezh2 for the Fc-receptor mediated endocytosis and cell migration in DCs. The compromised endocytosis and migratory ability in Ezh2-deficient DCs may be attributed by the reduced turnover rate of some adhesion structures.

Furthermore, a genome wide proteomic analysis of the Ezh2-deficient DCs revealed that the protein biosynthesis, but not the transcripts of several adhesion and cytoskeleton related molecules were regulated by Ezh2. The molecular mechanisms by which, Ezh2 regulates these molecules, and hence the cellular functions will be addressed in this study.

IDENTIFICATION OF FACTORS THAT PROMOTE V(D)J RECOMBINATION

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Lymphocytes assemble the genes that encode antigen receptors, by rearranging component gene segments in a coordinated process termed V(D)J recombination. Recombination signal sequences (RSSs) flank these gene segments and are recognized and cut by the RAG (Recombination Activating Gene) proteins, which initiates V(D)J recombination. V(D)J recombination is highly ordered, and maintains lineage and stage specificity during the development of lymphocytes. Chromatin modifications are widely believed to be important for this tight regulation but the mechanistic details have not been determined. Reports indicate that the nucleosome structure prevents RSSs access to RAGs in vitro. Using staggered primers, we determined that a significant fraction of Jκ1 (used in most primary κ rearrangements) is present on a nucleosome in vivo, prompting us to further explore how this apparent inaccessibility is overcome in rearranging loci. Covalent modifications of histone tails in these nucleosomes have been shown to be associated with an increase or decrease in V(D)J recombination. To determine if any of the known enzymes that covalently modify histones are involved in the regulation of V(D)J recombination, we decreased their expression in pro B cells by shRNAs and assessed the effect on the frequency of κ rearrangement. We identified p300/CBP-associated factor (PCAF), a known histone acetyltransferase, as a promoter of κ rearrangement. Additionally, we have generated a pro-B cell line containing a chromatinized reporter that expresses a surface fluorescent marker when it undergoes RAG mediated recombination to perform an unbiased screen for factors that promote V(D)J recombination. When RAG expression was increased in these cells using a chemical inducer, these cell lines efficiently rearranged the reporter and expressed significant amounts of Green Fluorescent Protein (GFP). We are currently using these cells to screen a cDNA expression library for factors that promote V(D)J recombination. The reporter system is designed to recruit the expressed proteins to the rearranging locus, which increases the likelihood of identifying chromatin modifying factors.

A NOVEL IMMUNOGLOBULIN-LIKE RECEPTOR, ALLERGIN-1, INHIBITS IGE-MEDIATED ALLERGIC RESPONSES

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Immune responses are regulated by positive and negative signaling mediated by cell-surface receptors expressed on both lymphoid and myeloid cells. However, the molecular mechanism of these signaling, and patho-physiological roles in immune responses have been incompletely understood. Here, we identified and characterized a novel ITIM-bearing Immunoglobulin-like receptor, ALGN-1.
We identified human and mouse ALGN-1 by the signal sequence trap method. We generated monoclonal antibodies against ALGN-1, and found that it is highly expressed on peritoneal exudative and bone marrow-derived cultured mast cells. The cytoplasmic portion of ALGN-1 was tyrosine-phosphorylated upon pervanadate stimulation, and associated with tyrosine-phosphatases, including SHP-1, SHP-2 and SHIP. Moreover, co-ligation of ALGN-1 with FcεRI resulted in inhibition of FcεRI-mediated degranulation from bone marrow-derived mast cells. ALGN-1-deficient mice showed significantly enhanced IgE-mediated allergic responses, including passive systemic and cutaneous anaphylaxises.
These results indicate that ALGN-1 is a novel ITIM-bearing receptor expressed on mast cells, which inhibits FcεRI-mediated activating signal. Thus, ALGN-1 may be a novel therapeutic target for allergic disorders.

YEAST BASED IMMUNOTHERAPY INDUCES IL-6 PRODUCTION THEREBY REDUCING ANTIGEN SPECIFIC CYTOTOXIC T LYMPHOCYTES

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Saccharomyces cerevisiae are taken up by antigen presenting cells (APCs) while also binding to pattern recognition receptors (PRR) of the APC thus providing phagocytic and danger signals to the APC. The yeast are then processed and peptides are presented on the APC. Thus activation of APCs by yeast antigens provides both innate and adaptive immunity and under the appropriate conditions, yeast delivered with antigen can be cross-presented to induce a productive, antigen specific CD8 T cell response. Previous data with other TLR agonists and PRRs document the usefulness of adding an agonistic CD40 antibody to enhance the productive CD8 T cell response. We show the usefulness of this agonistic antibody with a yeast-based vaccine in providing an enhanced antigen specific CD8 T cell response. Additionally, the yeast-dependent antigen specific CD8 T cell responses produced are at a markedly higher frequency in IL6-/- mice than in the wild-type. Evidence indicates a link between immune responses to yeast and Th17 cell production. Th17 cells and the IL-17 they produce lead to the recruitment of neutrophils that eliminate fungal and certain bacterial infections. IL-6 and IL-23 are critical cytokines necessary for Th17 cell differentiation, maintenance and sustained IL-17 production. Thus, while Th17 cells may cause inflammation necessary to rid the host of fungal infection this appears to occur concomitant with Th1-mediated induction of CD8 T cells. Th17 cells share an avidity for TGF beta with regulatory T cells (Treg). IL-6 mediates the transition from naïve CD4 T cells into either Th17 or Treg cells by contributing to the differentiation of CD4s into Th17s and blocking the differentiation into Tregs. Indeed, Th17 are now considered to be dominant participants in autoimmunity in part through their negative effects on Treg development. We also provide evidence that boosting with yeast does not impair antigen specific CD8 T cell production in the IL-6-/- mouse. These data suggest that the experimental reduction of IL-6 may not necessarily promote excess Tregs that act to suppress the CD8 response. We conclude that counteracting the Th17 pathway by reducing availability of IL-6 can strengthen and provide a durable yeast-induced antigen specific CD8 T cell response. Both Th17 cells and CD8 cytotoxic T cells likely have direct effects on tumor growth and Th17 mediated IL-21 production may enhance memory CD8 responses that could also target infectious diseases. Therefore, modulation of yeast induced Th17, Th1, and CTL responses may provide opportunities in which whole yeast based immunotherapy can be tuned to preferentially target cancer or infectious disease.

TRANSCRIPTION FACTOR OCT1 STABILIZES AND AMPLIFIES BOTH REPRESSED AND INDUCIBLE STATES AT *IL2* IN NAÏVE AND RESTING HELPER T CELLS

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Although Oct1 has been intensely studied, and although it targets a number of genes encoding key immunological regulators and effectors, surprisingly little is known about how it regulates gene transcription. Here, we identify two components of the NuRD chromatin remodeling complex associated with Oct1 bound to *Il2* promoter DNA in vitro. Using ChIP we show that the NuRD subunit MTA-2 is bound to *Il2* in highly purified naïve splenic CD4 cells, and that association is lost upon T cell activation. In contrast, NF-AT associates only in activated cells and Oct1 is constitutively bound. NuRD is not associated with *Il2* in naïve T cells lacking Oct1, suggesting a regulated recruitment mechanism. Consistent with these findings, purified Oct1 deficient naïve T cells show inappropriate *Il2* promoter demethylation as assessed by bisulfite sequencing, and aberrant *Il2* activity as assessed by qRT-PCR. These findings indicate that Oct1 helps maintain a repressed state at *Il2* in naïve cells. Surprisingly, we also identify an opposing phenomenon: in resting but previously stimulated CD4 T cells, Oct1 is required to maintain a demethylated state and promote the stronger *Il2* expression associated with secondary stimulation. These findings indicate that Oct1 also helps maintain a poised state at *Il2* through an anti-repression mechanism. We propose that, rather than acting as a primary trigger of gene activation or repression, Oct1 acts as a bipotential "molecular transistor" that reinforces both repressed and inducible states at individual genes. We show that Oct1 is required for a strong CD4 (but not CD8) T cell response following challenge with LCMV and Listeria.

A BALB/C LOCUS ON CHROMOSOME 12 CONFERS RESISTANCE TO LUPUS IN FCΓR2-/- MICE.

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FcγR2B-deficient (R2-/-) mice develop autoantibodies and glomerulonephritis with a pathology closely resembling human lupus when on the C57Bl/6 (B6) background. The same mutation on the BALB/c background does not lead to spontaneous disease, suggesting differences in lupus susceptibility between the BALB/c and B6 strains. An F2 genetic analysis from a B6/BALB cross identified regions from the B6 chromosomes 12 and 17 with positive linkage for IgG autoantibodies. We have generated a congenic strain (B6.R2-/-sbb2) that contains the suppressor allele from the BALB/c centromeric region of chromosome 12 in an otherwise B6.R2-/- background. We have observed that the presence of sbb2, even as heterozygote BALB/B6, renders B6.R2-/- mice resistant to lupus disease by suppressing all antibody autoreactivity and spontaneous immune cell activation. Mixed bone marrow reconstitution experiments indicated that the sbb2 resistant allele is most likely T cell intrinsic. The sbb2 congenic interval contains several candidate genes that are currently being investigated. We generated transgenic lines containing BAC DNA insertions spanning the sbb2 interval. The new BAC transgenic strains were tested for lupus suppression in the induced model of lupus by pristane administration. Newly generated BAC transgenic strains were also bred to B6.R2-/- to test for suppression of lupus in spontaneous disease. The goal of our current experiments is to identify a new lupus susceptibility gene within the sbb2 interval that is able to suppress lupus disease by altering T cell function.

GENOME-WIDE MAPPING OF RAG1 AND RAG2 PROTEIN BINDING

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V(D)J recombination assembles the antigen-binding domains of immunoglobulin (Ig) and T cell receptor (TCR) genes from arrays of V, D, and J gene segments. The RAG1 and RAG2 proteins drive this process of somatic DNA rearrangement, inducing DNA breaks adjacent to recombination signal sequences (RSSs) in the antigen receptor loci. The full extent and patterns of RAG binding and activity, both within the antigen receptor loci, and at cryptic RSSs elsewhere in the genome, have not been comprehensively described. We have previously shown that the RAG proteins localize at discrete foci centered over the J segments in several antigen receptor loci. We are now in the process of extending our studies to a genome-wide survey of RAG1 and RAG2 binding using ChIP-seq. We have analyzed the RAG2 binding pattern in thymocytes of wild type mice and also in transgenic mice expressing a mutant version of RAG1 (D708A) that retains binding capacity but does not catalyze DNA breakage (thus preserving the germline configuration of sites bound by RAG proteins). We have found that RAG2 binds very broadly in the genome in conjunction with peaks of tri-methylated histone H3 lysine 4. We aim to correlate these maps of RAG binding to sites of oncogenic translocations that have long been associated with lymphoid malignancies arising from aberrant V(D)J **recombination.**

PLASMACYTOID DENDRITIC CELLS RESIDENT IN HUMAN THYMUS DRIVE NATURAL TREG CELL DEVELOPMENT

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Generation of natural regulatory T cells (nTregs) is crucial for the establishment of immunological self-tolerance and the prevention of autoimmunity. Still, the nature of nTreg precursors and the mechanisms governing their differentiation within the steady-state thymus are poorly understood, especially in humans. It has recently been shown that conventional dendritic cells (cDCs) resident in the human thymus are efficient inducers of nTreg generation in an allogeneic setting. However, the tolerogenic function of the major DC intrathymic subset, i.e., plasmacytoid DCs (pDCs) remains unknown, despite the fact that pDCs are key regulators of immunity and tolerance in the periphery. Here we report that, like cDCs, human thymic pDCs efficiently induced CD4+CD25+Foxp3+ nTreg cells from allogeneic CD4+CD25- single positive (SP) thymocytes, but in response to different signals (TSLP or CD40L plus IL-3, respectively). Thus activated pDCs and cDCs played non-redundant roles in the induction of functionally heterogeneous nTreg subsets, which displayed reciprocal IL-10/TGFβ expression profiles. Supporting their physiological relevance, activated pDCs and cDCs also induced nTreg cells from autologous thymocytes. Notably, pDC-primed nTreg cells selectively differentiated de novo from CD4+CD8+ double positive (DP) thymocytes undergoing positive selection (CD69hi TCRhi), which up-regulated CD40L upon TCR engagement, thereby promoting the activation of autologous pDCs. Visual proof of pDCs-nTreg contacts in the thymic medulla is provided in vivo, supporting the physiological relevance of pDCs in nTreg cell generation in the human thymus.

CD8+ T CELLS RESPONDING TO PULMONARY SELF-ANTIGEN DEMONSTRATE ALTERED PHENOTYPE AND IMPAIRED EFFECTOR FUNCTION

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Due to their cytotoxic potential CD8+ T lymphocytes are involved in the pathogenesis of many pulmonary disorders, e.g. chronic obstructive pulmonary disease (COPD), sarcoidosis and lymphoid interstitial pneumonia (LIP). However, immunological mechanisms underlying activation of lung-reactive CD8+ T lymphocytes and subsequently leading to induction or exacerbation of pulmonary disorders remain elusive to date. Using the transgenic SPC-HA mouse model which expresses self antigen (hemagglutinin, HA) exclusively in the lung epithelium, we aim to characterize phenotype and function of auto-reactive CD8+ T cells in the respiratory system. Surprisingly, naïve HA-(lung) specific CD8+ T cells ignored their antigen and did not expand clonally when adoptively transferred into SPC-HA mice. Histologically, no evidence of lung damage was observed. However, when pre-activated in vitro, self-reactive CD8+ T cells caused severe pulmonary inflammation. To investigate, which conditions may support in vivo activation of self-specific CD8+ T cells in the lung and promote their phenotypic switch towards cytotoxic effector cells, we combined adoptive transfers with proinflammatory stimuli. LPS stimulation, DC-licensing or provision of CD4+ T cell help all facilitated CD8+ T cell proliferation in the lung-draining lymph node but failed to trigger pulmonary inflammation. Phenotype and effector function of CD8+ T cells primed by self HA antigen (with help of CD4+ T cells) were compared to that of CD8+ T cells which recognize HA provided by influenza virus during respiratory infection. Though in both cases (autoimmune activation and infection) CD8+ T cells proliferated in the lung-draining lymph nodes, only those primed by the virus displayed classical effector phenotype by means of IFN-γ production and upregulation of activation markers (CD25, CD43). CD8+ T cells proliferating upon auto-antigen encounter displayed only marginal expression of activation markers and much lower IFN-γ secretion. Thus, we demonstrate that lung-reactive CD8 T cells can reside quiescently in close proximity to self-antigen, display high priming requirements and their in vivo expansion is uncoupled from acquisition of effector phenotype.

DIRECT CONTACTS WITH REGULATORY T CELLS INDUCE CAMP-DEPENDENT NUCLEAR LOCALIZATION OF ICER IN EFFECTOR CD4$^+$ T AND B CELLS

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High levels of cyclic adenosine monophosphate (cAMP) are typical for naturally occurring regulatory T (nTreg) cells and appear to contribute to their suppressive capacity. ICER (inducible cAMP early repressor) is a potent transcriptional repressor that is constitutively expressed in nuclei of nTreg cells. In effector CD4$^+$ T or B cells, ICER is strongly induced by increasing cAMP levels followed by nuclear translocation. We show here that upon re-stimulation of effector CD4$^+$ T cells by anti-CD3/CD28 antibodies, ICER is sequestered to the cytoplasm whereas in nTreg cells its nuclear location remains unchanged. In two mice models we demonstrate that the cellular localization of ICER in effector CD4$^+$ T cells is closely linked to Nuclear Factor of Activated T Cells (NFAT) – dependent IL-2 synthesis: while depletion of nTreg cells in DEREG ("depletion of regulatory T cell") mice resulted in cytosolic localization of ICER and a strong increase in IL-2 synthesis, direct contact between nTreg cells and effector CD4$^+$ T and B cells led to cAMP infusion followed by nuclear accumulation of ICER. These findings suggest that nTreg cells suppress T cell responses by the cAMP-dependent nuclear accumulation of ICER and subsequent suppression of IL-2 expression.

ALTERED CD40 SIGNALING AND CELLULAR DISTRIBUTION IN EBV-INFECTED B CELLS

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Lymphoblastic cell lines (LCLs) infected with Epstein Barr virus are constitutively activated through the CD40 signaling pathway through the expression of latent membrane-1 protein (LMP1). This protein has been shown to engage downstream effector pathways by binding to TRAF adaptor proteins 2,3 and 6 that are critical for CD40 signaling. It has previously been shown that signaling through CD40 in the presence of LMP1 can down regulate proliferation in EBV-generated LCLs. We wished to extend this work by asking how EBV infection in general and in particular LMP1 affects the signaling pathways and cellular location of CD40 in the infected B cell. Specifically, we found that signaling through CD40 results in a marked increase in NF-κB and MAPK-p38 but little to no increase in MAPK-JNK, ERK, PLCγ2, and Akt3. Relative to B cells conditionally expressing LMP1, we observe that LMP1 expression results in heightened phosphorylation of CD40. Additionally, past work revealed that both LMP1 and CD40 engages TRAFs in lipid rafts either constitutively (LMP1) or upon binding by CD40L. In analyzing the localization of CD40 in B cell lines infected with WT or recombinant EBV, we observed a distinct movement of CD40 to the lipid rafts in the absence of CD40L signals. We also observed differences in TRAF localization with and without CD40 signaling in the presence or absence of EBV or LMP1. Whether the expression of LMP1 is sufficient for this observed redistribution of CD40 into the lipid rafts is currently being investigated. These findings suggest that EBV infection alters both the phosphorylation status and the sub-cellular distribution of CD40 in the cell. Also, distinct CD40 pathways are differentiallly saturated by signals through LMP1 suggesting differences in the overall response of B cells to LMP1 and CD40L. Supported by AI37081.

NOTCH LIGANDS REVEAL A SIGNAL STRENGTH HIERARCHY DURING HEMATOPOIESIS

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Notch signaling is essential for inducing T-lineage commitment in hematopoietic progenitor cells. The mammalian genome encodes for 4 Notch receptors (Notch1-4) which can be activated through interaction with 2 serrate-like ligands (Jagged1 [Jag1] and Jagged2 [Jag2]) or 3 Delta-like ligands (DL-1, DL-3 and DL-4). It is clear that DL1 and DL4 can support early T lineage development but the role of the Jagged ligands remains unclear. Using the OP9 coculture system, we investigated the ability of the Notch ligands DL1, DL4, Jag1 and Jag2 to induce T cell commitment and maturation of human cord blood (CB) CD34+CD38-lin- progenitors. While all these ligands can bind Notch1, the most critical Notch receptor for inducing T-lineage commitment , our results show that the Notch ligand Jag2 is almost as efficient as DL1 and DL4 at initiating T cell development, in contrast to Jag1 which lacks this potential. However, Jag2/Notch signaling is less efficient in the generation of CD4+CD8+ double positive (DP) cells as well as in suppressing B cell differentiation. Furthermore, dose-dependent inhibition of Notch signaling with a gamma-secretase inhibitor demonstrates that B and myeloid differentiation is faster restored when Notch activation is induced by Jag2 compared to by Delta-like ligands. In contrast, Jag1/Notch signaling is not influenced by the Notch inhibitor. Strikingly, Fringe mediated glycosylation increases Jag2 induced Notch signaling in human HSC, similar as the DL ligands, but in contrast to expected for Serrate-like ligands. Thus, using hematopoiesis as a model system, our results reveal a signal strength hierarchy between the different Notch ligands and show important functional differences between Jag1 and Jag2.

THE NOVEL BLIMP-1 HOMOLOGUE HOBIT REGULATES THE MATURE POPULATION OF NKT CELLS IN THE THYMUS

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The transcription factor Blimp-1 is expressed in T and B cells and regulates their terminal differentiation. Recently, we identified a novel homologue of Blimp-1 in T cells (Hobit) in a screen for transcription factors that are involved in human T cell differentiation. Hobit was strongly up-regulated in effector CD8 T cells as well as NK cells, indicating that it mediates transcriptional control of cytotoxic function.

We found that the expression profile of Hobit is narrower in mice than in men, as expression of murine Hobit was confined to NKT cells. To further analyze the role of Hobit in mice, we have developed Hobit KO mice.

In contrast to Blimp-1-deficient mice, formation of effector and memory T cells was normal in Hobit KO mice. However, within the thymus but not in the periphery reduced numbers of NKT cells were present in Hobit KO mice compared to WT mice. Hobit-dependent NKT cells were defined as the $NK1.1^{high}CD44^{high}$ thymus-resident NKT population. Immature $NK1.1^{low}$ subsets of NKT cells were normally present within the thymus. This shows that Hobit transcriptionally regulates thymic retention of NKT cells or late stages of thymic NKT cell development.

EXAMINING THE ROLE OF CIS ELEMENTS HS5, 6 AND 7 IN IMMUNOGLOBULIN HEAVY CHAIN REGULATION THROUGH A KNOCKOUT MODEL

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The immune system generates a large repertoire of antibodies to protect mammals from the numerous constellations of attacking pathogens. This diversity is achieved by genomic alterations of the IgH gene cluster, which include VDJ joining, class switch recombination (CSR) and somatic hypermutation (SHM). Such DNA modifications require tight regulation to ensure proper functioning of the immune system and to prevent potentially oncogenic processes if such modifications extend beyond the IgH locus.
A key cis regulator of B cell specific expression of IgH genes is a large regulatory region that lies downstream of the IgH gene cluster (3'RR). The 3'RR contains multiple DNaseI hypersensitive sites (hs3a, hs1,2, hs3b and hs4) and is required for expression and class switching of the IgH genes. A new extension of the 3'RR, hs5, 6 and 7, has been identified and its role in IgH regulation is unknown. Hs5-7 display marks of open/active chromatin as early as the pro-B stage of B cell development and is as cultured cell insulator assay, hs5 and 7 exhibit insulator activity. We hypothesize that hs5-7 are vital regulators of the IgH locus by impacting on the DNA modifications specific to the IgH locus and acting as a boundary element to insulate the IgH locus from neighboring, non-IgH genes.
To test this hypothesis, I have generated a mouse knockout model. Preliminary data show that VDJ-joining, SHM and CSR occur in the hs5-7 deleted mice. FISH data suggest that the hs5-7 deletion may affect aspects of IgH locus contraction and nuclear localization, giving rise to a 'molecular phenotype'. Deletion of Hs5-7 does not increase expression of downstream non-IgH genes, therefore, the deletion does not diminish insulation of the IgH locus. We are currently evaluating the hypothesis that hs5-7 promotes looping and regulates aspects of chromatin architecture in the IgH locus.
Further elucidation of the role of hs5-7 will provide insight to mechanisms that regulate the IgH locus, and, in a more general sense, because hs5, 6 and 7 are located 40-200kb away from DNA targets in the IgH gene cluster that they may regulate, understanding their role will elucidate aspects of long range gene regulation mechanisms. (Supported by NIH AI13509 and Immunology and Immuno Oncology Training Grant T32CA009173.)

INFLUENCE OF DNA SEQUENCES ON AID TARGETING DURING SOMATIC HYPERMUTATION

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Activation induced deaminase (AID) activities are differentially targeted to initiate, respectively, DNA double-strand breaks (DSBs) in IgH switch (S) regions during class switch recombination (CSR) and point mutations in the nearby V(D)J exons during somatic hypermutation (SHM) in germinal center (GC) B cells. AID can also target S regions to induce DSBs during CSR in the in vitro stimulated B cells but does not induce mutations in the adjacent V(D)J exons. It is unknown how AID activity is differentially regulated to induce different types of mutations or specifically target one region but not another within IgH locus. Here, we replace the V(D)J exon sequence in the endogenous IgH locus with a portion of core Sμ (cSμ) sequence and find that the replaced cSμ sequence, strikingly, can induce mutations in the in vitro activated B cells. Furthermore, the replaced cSμ sequence harbors much more frequent internal deletions indicative of DSBs generation in the position of V(D)J exon locus in GC B cells. Thus, our results reveal that DNA sequence plays a major role in determining the specificity of AID targeting.

LOSS OF SNAI3 ALTERS THE TRANSCRIPTIONAL PROFILE OF CD8+ T CELLS WHILE OVER-EXPRESSION IN HEMATOPOIETIC CELL LINEAGES SUPPRESSES LYMPHOCYTE DEVELOPMENT AND ENHANCES MYELOID LINEAGES

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The role of the Snai3 protein in the derivation of mouse thymocytes and other cells of the hematopoietic system was investigated. Snai3 is highly expressed in skeletal and cardiac muscle, and in the thymus. Snai3 expression in the thymus is restricted to early stages of thymocyte maturation and to the CD8+ T cell lineage cells in the thymus and periphery. Maturing thymocytes lacking Snai3 (either via a conditional knockout or germline deficiency) demonstrated normal thymocyte development and CD8+ T cell release from the thymus indicating Snai3 was not critical for T cell lineage development. However, gene expression array analysis of Snai3 knockout CD8+ T cells showed significant changes in the transcriptome indicating that there are specific gene targets for this transcription factor in T cells. Forced expression of Snai3 by retroviral transduction of hematopoietic stem cells demonstrated a block in lymphoid cell development and enhanced expansion of myeloid lineage cells. Analysis of Snai3 expressing hematopoietic precursor cells showed normal numbers of stem, myeloid, and lymphoid precursor cells, but a block in the development of cells committed to T and B cell lineages.

BIFIDOBACTERIUM BIFIDUM ACTIVELY CHANGES THE GENE EXPRESSION PROFILE INDUCED BY *LACTOBACILLUS ACIDOPHILUS* IN MURINE DENDRITIC CELLS

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Dendritic cells (DC) play a pivotal regulatory role in activation of the innate as well as the adaptive immune system by responding to environmental microorganisms and conducting an appropriate immune response. We have previously shown that individual lactic acid bacteria exert differential effects on DC, as some lactobacilli induce a strong production of the pro-inflammatory cytokine IL-12, whereas bacteria of the bifidobacteria genus do not. In addition, bifidobacteria are able to inhibit the IL-12 production induced by lactobacilli. In the present study, we used genome wide microarrays to obtain a comprehensive view on how the two bacteria, *Lactobacillus acidophilus* NCFM and *Bifidobacterium bifidum* Z9, stimulate maturation and up-regulation of immune regulatory genes in murine bone marrow derived DC. *L. acidophilus* NCFM strongly induced a pro-inflammatory response including a high expression of the gene encoding IFN-β, multiple virus defence genes and genes related to both the adaptive and the innate immune response (cytokines IFN-β, IL-12, TNF-α, chemokines Cxcl9, Cxcl10, Cxcl11, Ccl12, etc.). Contrary, *B. bifidum* Z9 induced an anti-inflammatory response and mostly up-regulated genes related to the innate immune response (cytokines IL-10, IL-33, chemokines Cxcl1, Cxcl2, Cxcl5, etc.). When DC were stimulated with both *L. acidophilus* NCFM and *B. bifidum* Z9 simultaneously, *B. bifidum* Z9 strongly inhibited the expression of the genes initiating the adaptive immune response induced by *L. acidophilus* NCFM and significantly upregulated the expression of genes of the innate immune response. A number of genes were only induced by *B. bifidum* Z9 stimulation. In particular, the gene encoding Jun dimerization protein 2 (JDP2) was strongly upregulated. The use of MAPK kinase inhibitors (MAPK p38, JNK1/2, MEK1/2) revealed that blocking of the JNK1/2 pathway completely inhibited the expression of *Ifn-β*, and blocking of the p38 pathway resulted in a downregulation of *Ifn-β* by 60%. We therefore suggest that *B. bifidum* Z9 employs an active mechanism to inhibit the induction of genes in DC triggering the adaptive immune system and that JPD2 is involved in this regulatory mechanism.

EARLY B LINEAGE CELLS DISPLAY CLASS SWITCH PREFERENCE TO IGE

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A subset of primary immune deficiency diseases such as Omenn syndrome, are associated with elevated IgE levels despite a severe reduction in lymphocyte numbers, but the mechanism for this is not clear. We show that mice with the Omenn syndrome-like hypomorphic Rag1 mutation have severely reduced peripheral B cells, which are skewed toward an early developmental phenotype. In vitro stimulation of these Rag1 hypomorphic B cells with anti-CD40/IL4 results in a strong class-switch preference to IgE. In contrast, wild-type littermate control B cells prefer switching to IgG1 with the same stimuli. To test whether B cell immaturity plays a role in IgE switch preference, early B lineage cells were prepared by developing wild-type fetal liver cells on IL7-producing fibroblasts. These fetal liver-derived early B lineage cells proliferate, form blasts, and switch in response to anti-CD40/IL4 stimulation, similar to mature splenic B cells. However, the immature B cell population displays a strong preference for switching to IgE, similar to the hypomorphic Rag1 splenic B cells. Bone marrow B cells also demonstrate increased anti-CD40/IL4-induced IgE switching compared to more mature, splenic B cells. Additionally, mouse splenic B cells separated based on the immature B cell marker, AA4.1, show that B cells within the AA4.1-positive fraction prefer switching to IgE compared to cells in the more mature AA4.1-negative fraction. Moreover, splenic B cells from very young mice also prefer switching to IgE compared to splenic B cells from older mice. Quantitative real-time PCR reveals twice as much germline ε switch region transcript levels in anti-CD40/IL4-stimulated immature B cells compared to more mature B cells, despite an attenuated Stat6 phosphorylation in the former. We conclude that B lineage cell maturity influences isotype switch preference, with immature B cells favoring IgE. These data also suggest that peripheral B cell immaturity found in some types of primary immunodeficiency states due to lymphocyte developmental blockade may be a contributory mechanism leading to increased IgE levels in some of these disorders. Preliminary data suggest that developmentally distinct levels of Bcl6 may mediate this process. Ongoing studies are addressing the mechanism mediating this switch preference.

NAB2 REGULATES THE SECONDARY RESPONSE OF CD8+ T CELLS THROUGH CONTROL OF TRAIL EXPRESSION

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CD4+ “helper” T lymphocytes play a key role in the generation and maintenance of CD8+ T cell responses. CD4+ T cell help appears to operate through a “programming” mechanism during primary CD8+ T cell activation, resulting in specific patterns of gene expression. One key feature of this program occurs in “helpless” CD8+ T cells primed in the absence of CD4+ T cells, and involves the rapid induction of the pro-apoptotic molecule TRAIL upon restimulation that results in their subsequent death by activation-induced cell death (AICD). The “helped” CD8+ T cells, in contrast, do not induce TRAIL expression upon restimulation, and instead undergo a robust secondary response. We sought to understand the molecular events that define these disparate outcomes on the regulation of the TRAIL gene in CD8+ T cells by transcriptional profiling of helped versus helpless cells. The result of these studies reveals that TRAIL expression is inhibited during secondary antigenic stimulation in the helped CD8+ T cells through IL-2 dependent expression of the transcriptional co-repressor/co-activator NGFI-A binding protein 2 (Nab2). These findings show that Nab2 acts a crucial molecular switch in the provision of CD4+ T cell help for secondary responses in CD8+ T cells.

THE ROLE OF NOTCH-INDUCED DEGRADATION OF JAK3 IN LYMPHOPOIESIS

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Notch signaling promotes T and inhibits B cell differentiation. However, the underling mechanism remains unclear. We previously found that Notch induces the degradation of Janus Kinase 3 (Jak3) in B but not T cells in a MAP kinase-dependent manner. Given that Jak3 is essential for both B and T cell development, we hypothesize that Notch signaling inhibits B lymphopoiesis by inducing Jak3 degradation in B-linage cells. To test this hypothesis, we need Jak3 mutants that are insensitive to Notch-induced degradation but retain regulated kinase activity. Our laboratory also showed that Notch activates transcription of the gene encoding ankyrin-repeat SOCS box containing protein 2 (Asb2), capable of stimulating ubiquitination of several substrates including Jak3. Asb2 is known to associate with Cul5/ElonginB/C to form E3 ligase complexes. We have designed four Jak3 mutants by disrupting potential interaction with E3 ligases (V979R and R980W) or eliminating predicted MAP kinase recognition sites (S985A and S1025A). These mutants were found to be able to phosphorylate its substrate, STAT5, upon IL-7 stimulation. Next, all mutants except S1025A were shown to be resistant to Asb2-induced ubiquitination and degradation. While the mechanism underlying Asb2-mediated Jak3 degradation will be further investigated, suitable mutants will be expressed in hematopoietic cells in transgenic mice. We will examine B cell differentiation from transgenic progenitors in the presence of Notch signals. Lymphopoiesis in the bone marrow and peripheral lymphoid organs of these transgenic mice will be monitored to explore if Notch signaling plays a role in maintaining homeostasis of lymphoid differentiation and proliferation.

ADENO-ASSOCIATED VIRUS-MEDIATED CCL11 shRNA REDUCES LUNG INFLAMMATORY RESPONSES IN A MITE ALLERGEN-SENSITIZED MOUSE MODEL

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Asthma is considered as a chronic pulmonary inflammatory disease, characterized by the infiltration of eosinophils. Eotaxin-1 (CCL11) is secreted by lung epithelium cells and functions as the major chemokine for the recruitment of eosinophils. Hybrid adeno-associated virus (AAV) serotype 2/9, composed with AAV2 Rep and AAV9 Cap proteins, is one of the viral vectors that might achieve long-term gene delivery to lung epithelium cells. Thus, this study tested whether hybrid AAV2/9 virus carrying small hairpin RNA specific for CCL11, sh47 and sh137, could reduce eosinophilia in a mite allergen-induced asthmatic mouse model. Both sh47 and sh137 viruses significantly reduced CCL11 level in CCL11-transformed NIH 3T3 cells, compared with AAV2/9, shRNA and no virus-treated controls. We delivered 5X1011 genome copies (GC) of AAV2/9 sh47 or sh137 viruses by intratrachea injection (IT) into Dp2 (group 2 allergen of Dermatophagoides pteronyssinus) sensitized mice three days before the first challenge. The results showed that AAV2/9 sh47 and sh137 viruses significantly reduced airway hyperresponsiveness (AHR), CCL11 levels and eosinophilia in the lungs of Dp2-sensitized mice. Th2 cytokines, including IL-4, -5, and -10, were also significantly reduced in the bronchoalveolar lavage fluid (BALF) of AAV2/9 sh47 virus-treated mice. The levels of IL-4, -5, -10 and -13 were also reduced in Dp2-stimulated mediastinal lymphocytes of AAV2/9 sh47 and sh137 virus-treated mice. However, serum levels of Dp2-specific IgG1 and IgE as well as Th2 cytokine levels in Dp2-stimulated splenocyte culture supernatants were at the comparable levels to the untreated group. The results suggest that AAV2/9 sh47 and sh137 viruses relieved local instead of systemic inflammatory responses. Therefore, the hybrid AAV2/9 viral vector, which is preferable to target lung epithelia cells, might be applied as a novel therapeutic approach for asthma.

TRACKING GENE EXPRESSION PATTERNS AND EPIGENETIC HISTONE MODIFICATION DURING CD4-CD8 LINEAGE CHOICE IN DIFFERENTIATING THYMOCYTES

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CD4 and CD8 T cells differentiate from CD4+CD8+ double positive thymocytes (DP) that have received adequate intrathymic TCR signaling. DP thymocytes expressing MHC I-restricted TCRs mature into the CD8 lineage, and function as cytotoxic effector cells upon antigen encounter; whereas thymocytes carrying MHC II-restricted TCR mature into CD4 T cells, which generally have helper functions. It is now clear that CD4 and CD8 surface protein expression, which is commonly used to track the emergence of CD4 or CD8 lineages, is in fact a poor indicator of early lineage differentiation. To escape this limitation, the current study has tracked expression of CD4- or CD8-lineage specific genes and defined histone modifications (H3K4me3 and H3K27me3) to investigate CD4-CD8 lineage divergence in the thymus. Analysis of gene expression profiles and histone methylation changes revealed complex patterns with imperfect correlation between expression and histone methylation status. CD4-specific genes CD4, CD40L and Thpok were marked with H3K4me3 when actively transcribed, whereas they displayed heterogeneous enrichment in H3K27 trimethylation when they were repressed. Similar analysis were performed on CD8-lineage specific genes, including the use of a fluorescent Runx3 reporter transgene that assesses activity of both distal and proximal Runx3 promoters, and will be presented at the meeting. The significance of these observations for lineage choice mechanisms will be discussed.

GATA3 ACTIVELY REPRESSES STAT4-/T-BET-INDEPENDENT RUNX3-MEDIATED IFNΓ PRODUCTION

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GATA3 is crucial for the differentiation of naïve CD4 T cells into Th2 cells. Deletion of Gata3 allowed the appearance of the IFNγ-producing cells in the absence of IL-12 and IFNγ. Such IFNγ production is T-bet-independent both in vitro and in vivo. Eomes, but not T-bet expression is upregulated both in GATA3-deficient CD4 T cells differentiated under Th2 conditions and in Th2 cells with enforced Runx3 expression, contributing to IFNγ production. GATA3 overexpression blocks Runx3-mediated Eomes induction and IFNγ production, and GATA3 protein physically interacts with Runx3 protein through its N-terminus and zinc fingers. Furthermore, Runx3 directly binds to multiple regulatory elements of Ifng gene and blockage of Runx3 function both in Th1 and GATA3-deficient "Th2" cells results in diminished IFNγ production by these cells. Thus, the Runx3-mediated pathway, actively suppressed by GATA3, induces IFNγ production in a STAT4/T-bet-independent manner.

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REQUIREMENT FOR CYCLIN D3 IN GERMINAL CENTER FORMATION AND FUNCTION

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The D-type cyclins constitute an important component of the cell cycle engine that enables the cell proliferation program to respond to various physiological and pathological changes. Cell type- and developmental stage-specific use of the D-type cyclins has been described in certain situations but the cyclin D requirement during germinal center (GC) reaction has not been addressed. In this study, we first demonstrate that cyclin D3 is absent in resting follicular B cells but upregulated in GC centroblasts. In the spleen of Ccnd3-/- mice, a two-fold increase in marginal zone B cells is detected and is accompanied by a slight reduction in follicular B cells. Proliferation and Ig class switching of in vitro activated B cells are largely unperturbed by cyclin D3 deficiency. However, immunized Ccnd3-/- animals are profoundly impaired in GC development and in their T cell-dependent Ab response. In the few Ccnd3-/- GC B cells that can be identified, the cell cycle transition from G1 to S phase is notably delayed despite an apparent compensatory increase in cyclin D2 expression. Lastly, although cyclin D3 inactivation did not disrupt BCL6 expression in GC B cells, it completely blocked the GC promoting effect of BCL6 overexpression suggesting that cyclin D3 acts downstream of BCL6 to regulate GC formation. This is the first demonstration that cyclin D3 plays an important and unique role at the GC stage of B cell development.

IL-21 AND CD40L SYNERGISTICALLY PROMOTE PLASMA CELL DIFFERENTIATION THROUGH UPREGULATION OF BLIMP-1

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After undergoing Ig somatic hypermutation and antigen selection, germinal center (GC) B cells terminally differentiate into either memory or plasma cells (PCs). IRF4 and Blimp-1 are two transcription factors critically required for this process. It is also known that the NF-κB and STAT3 signaling pathways play major roles in directing B cells to exit the germinal center program and to commit to plasma cell fate. It is unclear, however, how the B cell transcription program interprets and integrates these two types of T cell derived signals. The goal of this study is to define the key target genes of STAT3 and to understand how STAT3 and NF-κB collaborate to promote plasma cell differentiation. Using normal tonsilar B cells and a cell line-based PC differentiation system, we demonstrate that continued STAT3 activation is required for differentiating B cells to move beyond the pre-plasmablast stage, and that IRF4, a transcription factor involved in the early stage of PC differentiation is a direct target gene of STAT3. Our analysis also reveals that CD40/NF-κB synergizes with IL-21/STAT3 by potentiating Jak/STAT3 signaling at the cytokine receptor level. Furthermore, STAT3 can directly transactivate Blimp-1 by binding to two separate regions in the human Blimp-1 locus and this effect is also enhanced by CD40 signaling due to its ability to relieve the suppressive effect of BCL6. Taken together, our study demonstrates that STAT3 activation plays a central and unique role during the early stage of post-GC plasma cell differentiation.

C101 HAS ANTI-INFLAMMATORY EFFECTS THROUGH THE DOWN-REGULATION OF NF-KB IN LPS-INDUCED RAW 264.7 MACROPHAGE CELLS.

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We investigated the anti-inflammatory effects of C101, which is the oroxylin A derivative on lipopolysaccharide (LPS)-induced RAW 264.7 cells. We synthesized 39 compounds containing similar structure of oroxylin A which is already well known having anti-inflammatory and anti-fungal effect. We conducted MTT, nitric oxide (NO) and prostaglandin E_2 (PGE_2) assay to select the most significant anti-inflammatory candidates. C101 is the only one compound which effects on the production of NO and PGE_2 is significantly reduced in a dose-dependent manner. We measure some cytokines such as TNF-α, IL-6 and IL-1β induced by LPS was also markedly reduced in a dose-dependent manner. In addition, C101 suppressed the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the protein and iNOS, COX-2, TNF-α, IL-6 and IL-1β at the mRNA levels. We studied NF-κB translocation because NF-κB plays important role as transcription factor. C101 substantially inhibited NF-κB p65 nuclear translocation and IκBα degradation in a dose-dependent manner. Based on these data, we suggested that C101 inhibited pro-inflammatory gene expression via down-regulating NF-κB signaling pathway.

YY1 REGULATES NUCLEAR LEVELS OF AID AND CLASS SWITCH RECOMBINATION

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Activation induced deaminase (AID) is an enzyme required for somatic hypermutation (SHM) and class switch recombination (CSR), processes that ensure antibody maturation and expression of different immunoglobulin isotypes through mutation of Ig variable and switch regions. It has long been hypothesized that AID needs specific cofactors to initiate SHM and CSR. Here we show that AID physically interacts with PU.1, PAX5 and YY1, transcription factors that bind Ig heavy and light chain enhancers. Several studies demonstrated that AID is predominantly localized in the cytoplasm even though in order to deaminate DNA it has to be translocated to the nucleus. We found that overexpression of YY1, but not PU.1 or PAX5, led to accumulation of nuclear AID in transfected 293T cells. YY1 conditional knock-out in activated splenic B cells interfered with CSR. Knock-out of YY1 did not affect B cell proliferation, transcription of the AID gene, or levels of various germline transcripts. Instead, we found that YY1 modulated levels of AID nuclear protein and thus compromised CSR. We show for the first time that YY1 plays a novel role in CSR by controlling AID nuclear protein levels.

DOWNSTREAM CLASS SWITCHING LEADS TO IGE ANTIBODY PRODUCTION BY B LYMPHOCYTES LACKING IGM SWITCH REGIONS

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Ig heavy chain (IgH) class-switch recombination (CSR) replaces the IgH Cμ constant region exons with one of several sets of downstream IgH constant region exons (e.g., Cγ, Cε, or Cα), which affects switching from IgM to another IgH class (e.g., IgG, IgE, or IgA). Activation induced cytidine deaminase (AID) initiates CSR by promoting DNA double-strand breaks (DSBs) within switch (S) regions flanking the donor Cμ (Sμ) and a downstream acceptor CH (e.g., Sγ, Sε, Sα) that are then joined to complete CSR. DSBs generated in Sμ frequently are joined within Sμ to form internal switch region deletions (ISD). AID-induced ISD and mutations have been considered rare in downstream S regions, suggesting that AID targeting to these S regions requires its prior recruitment to Sμ. We have now assayed for CSR and ISD in B cells lacking Sμ ($S\mu^{-/-}$ B cells). In $S\mu^{-/-}$ B cells activated for CSR to IgG1 and IgE, CSR to IgG1 was greatly reduced; but, surprisingly, CSR to IgE occurred at nearly normal levels. Moreover, normal B cells had substantial Sγ1 ISD and increased mutations in and near Sγ1, and levels of both were greatly increased in $S\mu^{-/-}$ B cells. Finally, $S\mu^{-/-}$ B cells underwent downstream CSR between Sγ1 and Sε on alleles that lacked Sμ CSR to these sequences. Our findings show that AID targets downstream S regions independently of CSR with Sμ and implicate an alternative pathway for IgE class switching that involves generation and joining of DSBs within two different downstream S regions before Sμ joining.

THE V(D)J RECOMBINATION MACHINERY IS ASSOCIATED WITH THE NUCLEAR MATRIX

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Somatic recombination of immunoglobulin or T cell antigen receptor genes occurs through recombination activating gene product (RAG)-mediated cleavage at recombination signal sequences (RSS) and subsequently, the broken DNA ends are repaired by non-homologous end joining (NHEJ) enzymes. Here, we show that RAG1, RAG2, and many NHEJ factors are associated with the nuclear matrix in mouse and human early B lineage cells and in mouse thymocytes. The core RAG1 and RAG2 proteins have their own nuclear matrix targeting regions. RAG-mediated double stranded DNA breaks at the Jκ4 RSS in murine B lineage cells or at the Dβ1 RSS in thymocytes can be readily detected in the nuclear matrix fraction, indicating that the recombination reaction is ongoing on the nuclear matrix. In the HEK 293 cell-based recombination system, artificial recombination substrates are recruited to the nuclear matrix for recombination. Moreover, nuclear matrix proteins purified from 293 cells expressing the core RAG proteins or human B lineage cells expressing endogenous RAG proteins support cleavage of RSS substrates in vitro. Based on these results, we propose that the V(D)J recombination machinery is associated with the nuclear matrix.

DISRUPTION OF IMMUNE SURVEILLANCE OF EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1-EXPRESSING B CELLS RESULTS IN PTLD-LIKE DISEASE IN MICE

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Epstein-Barr virus (EBV), a γ-Herpes virus infecting human B-lymphocytes and establishing a life-long latent infection in the host, has been associated with several diseases, including mononucleosis and B-cell lymphomas such as Hodgkin's lymphoma and Burkitt lymphoma. EBV is also associated with Post-Transplant Lymphoproliferative Disorder (PTLD) and AIDS-associated B-cell lymphoma in immunosuppressed patients. The biological function of the EBV latent membrane protein 1 (LMP1) is of special interest since LMP1 mimics constitutive CD40 signaling, transforms fibroblasts in vitro and increases the incidence of B cell lymphomas in mice. Using a system of conditional targeted gene activation, we induced LMP1 expression in B lineage cells in mice in vivo, and found that the LMP1-expressing B cells were eliminated by an immune surveillance mechanism, which was induced postnatally and involved T and NK cell activation. Interference with the immune surveillance of LMP1-expressing B cells resulted in a B-cell lymphoproliferative disorder resembling PTLD, with frequent development of B cell lymphomas. The LMP1-positive lymphoma cells expressed ligands for the NK cell receptor NKG2D on the surface and were thus targets for a novel NKG2D/Fc fusion protein, whose application to tumor-bearing animals resulted in dramatically reduced tumor growth in vivo. This work reveals a central role of LMP1 in the pathogenesis of PTLD and validates a promising therapeutic agent for this and similar diseases.

THE ROLE OF MICRORNA-146A IN MURINE B-CELLS

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Recently, our lab has successfully generated a knockout mouse with targeted germline deletion of microRNA-146a (miR-146a). The miR-146a knockout mouse developed a progressive immunoproliferative phenotype involving multiple hematopoietic lineages, massive splenomegaly, enlarged lymph nodes, and eventually splenic tumors. To our knowledge, this is the first case that knocking out a single miRNA has led to the development of frank hematopoietic tumors. Histological analysis shows that some spleens from the older knockout mice have the characteristics of follicular hyperplasia, follicular lymphoma, and large-cell lymphoma morphology. Although the cellular lineage and molecular properties of these tumors haven't been extensively characterized, some of them are likely to be of B-cell lineage. Furthermore, B-cells isolated from knockout mouse spleen show increased production of inflammatory cytokines, such as IL6, and enhanced proliferation, over wild-type control, when stimulated with various toll-like receptor ligands. This shows that miR-146a is an important negative regulator of the inflammatory pathways, and suggests that increased IL6 production is at least partially responsible for the chronic inflammatory and hyperproliferative phenotype observed in the miR-146a knockout mouse.

IDENTIFICATION OF PROTEINS ASSOCIATED WITH DNA BREAKS DURING CLASS-SWITCH RECOMBINATION

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Class-switch recombination (CSR) is an important event in antibody diversification. Prior to activation, the VDJ region in naïve B cells is proximal to the constant region μ (Cμ), with the other constant region genes located further downstream. These constant region genes are each preceded by a highly repetitive sequence known as the switch region. Upon activation, double strand breaks (DSBs) occur in these switch regions and repair between two different switch regions results in recombination and concomitant deletion of the intervening genomic sequences. This results in the association of the VDJ region to a new constant region gene (Cγ, Cε or Cα depending on the participating switch region), thereby coupling one antigen specificity to a broad spectrum of effector functions through these various isotypes.

Defects in this process have been shown to give rise to hyper-IgM syndrome and dysregulated CSR has also been implicated in the pathogenesis of B cell lymphomas. However, this clinically important process has not been well characterized. In particular, the molecular mechanisms for the targeted generation and repair of DSBs in switch regions are still not fully understood. Identification of proteins localized to DSBs in switch regions during CSR would further our understanding of this process and offer insights into treatment of relevant diseases.

For this, we have chosen to establish a reverse chromatin immunoprecipitation (ChIP) proteomic screen. This strategy involves labeling of DSBs in cells undergoing CSR with biotin, pull-down of biotinylated fragments of DNA, followed by identification of proteins associated with these labeled fragments by mass spectrometry. The strength of this screen lies in its unbiased approach and can thus potentially uncover novel proteins which play a role in CSR. At present, several proteins have been identified and their functions in CSR are currently being investigated.

THE ROLE THE ARYL HYDROCARBON RECEPTOR IN MODULATION OF THE TH17 AND TREG BALANCE

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The immune system must maintain a delicate balance. On the one hand, inflammation is required to fight harmful microorganisms, and on the other hand, too much inflammation can cause damage to healthy tissue. A complex mix of immune cells regulates this balance, including effector and regulatory T cells. Among effector cells, Th17 cells, characterized by the expression of the orphan nuclear receptor RORγt, secrete the signature cytokines IL-17, IL-17F, and IL-22. Th17 cells are required to clear certain bacterial or fungal infections at mucosal surfaces. Dysregulated Th17 responses result in autoimmune diseases in mice and in humans. Regulatory T cells (Tregs), marked by the expression of Foxp3, control T cell responses, including Th17 cells. The balance between Th17 and Treg cells is influenced by the local cytokine microenvironment and is achieved through the concerted action of transcription factors such as RORγt and Foxp3. The aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor best known for mediating the effects of environmental toxins, is one of the most highly upregulated transcription factors in Th17 cells. AhR can be activated by environmental toxins and by natural compounds generated by bacteria normally living in the intestines. Therefore, identification of a role for AhR in Th17 biology provides a fascinating link among environmental factors, gut microbiota, and human immunological diseases. The precise role for AhR in inflammation and chronic inflammatory disease remains to be determined.

We have found that AhR can modulate the RORγt and Foxp3 balance thereby promoting Th17 over Treg differentiation. AhR is synergistically induced by the Th17 polarizing cytokines IL-6 and TGF-β, but cannot induce IL-22 or IL-17 expression in the absence of both RORγt and RORα (another member of the ROR family of transcription factors). Induction of Th17 cytokines, especially IL-22, requires cooperative action of AhR and RORγt, presumably through protein interaction. AhR inhibits Foxp3 expression in vitro, consistent with the notion of a reciprocal relationship between Th17 cells and Tregs. Lack of AhR leads to impaired expression of the Th17 cytokine IL-22, and AhR-deficient mice are more susceptible to Citrobacter rodentium infection whose clearance is dependent on IL-22. We propose that AhR and RORγt have a differential impact on individual Th17 cytokine expression and play an important role in gut immunity. Since AhR is a ligand-dependent transcription factor, modulation of AhR activity may offer a novel means to fine-tune the Th17-Treg balance to prevent or treat human diseases.

T-BET-GFP REPORTER REVEALS A DEFAULT TH2 PROGRAM IN "TH1-WANNABE" CELLS

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T-bet is the master regulator for Th1 cells. It is also expressed in $CD8^+$ T cells, NK cells, NKT cells, dendritic cells and B cells. To identify and study T-bet-expressing cells *in vivo*, we generated BAC transgenic T-bet-GFP reporter mice in which GFP transcription is driven by DNA elements similar to those controlling endogenous T-bet. GFP expression, reporting the expression of T-bet, was detected in a subset of memory $CD4^+$ T cells and all memory $CD8^+$ T cells as well as NK and NKT cells in naïve mice. The $CD4^+GFP^+$ memory cells expressed T-bet mRNA while $CD4^+GFP^-$ memory cells did not. T-bet-GFP was preferentially induced in Th1 but not Th2 or Th17 cultures *in vitro*. *Toxoplasma gondii* infection, eliciting Th1 responses *in vivo*, dramatically induced GFP expression in both $CD4^+$ and $CD8^+$ T cells, which correlated with T-bet induction. The T-bet-reporter mice were then crossed onto a T-bet KO background. Both *in vitro* and *in vivo* experiments showed the existence of "Th1-wannabe" cells, which express GFP in the absence of endogenous T-bet. These "Th1-wannabe" cells produced little IFNγ but high levels of IL-4 correlated with GATA3 up-regulation, implying that when T-bet is absent, a default Th2 program can be activated even under a strong Th1-biased environment.

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TRACKING MOLECULAR INTERACTIONS WITHIN A T CELL "SYNAPSE"

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Cell-cell interactions mediated by specific surface molecules are a general feature of multicellular organisms, but research in this area is hampered by the inaccessibility of these interactions to classical biochemical or molecular analyses. One of the best-studied systems for cell-cell recognition involves specific T lymphocytes, whose antibody-like T cell receptor (TCR) molecule recognizes fragments of antigens (e.g., peptides) bound to major histocompatibility complex molecules on the surfaces of other cells. Here, we report that we are able to use bulk and single-molecule FRET to measure the affinity and kinetics of T cell receptor binding to peptide-MHC complexes within an "immunological synapse." We find that the association rate of this interaction is much faster (~ 100x) than in solution, apparently because of the orientational congruence of the receptor and ligand on their respective membrane bilayers. We also find that the dissociation rate is significantly accelerated (5-10x) compared to solution measurements, and that this is due to an actin polymerization activity that accompanies synapse formation.

We have also developed a photoactivatable form of the ligand to study the initiation of TCR binding and are working on extending this system to other T cell receptor systems and to include reporters for the "co-receptors" CD4 and CD8. In addition, we have used a high speed form of the PALM technique to demonstrate that TCRs congregate on specific "protein islands" prior to T cell activation and that these islands concatenate with islands containing the downstream adaptor molecule, LAT, to form signaling entities in the early stages of T cell recognition. These protein islands are a general feature of protein organization in the plasma membrane and are particularly well suited to "scanning" the surfaces of other cells for rare ligands, keeping unstable ligands engaged and in segregating all surface receptors from downstream signaling complexes until activation is initiated.

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THE N-TERMINUS OF VAV1 PLAYS A GEF-INDEPENDENT ROLE IN THE STABILIZATION OF SLP-76 MICROCLUSTERS.

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T cell activation requires the efficient recruitment of SLP-76 into persistent signaling microclusters (SLP-MC). Mutations eliminating the primary tyrosine phosphorylation sites within SLP-76 impair T cell activation and prevent the stabilization of SLP-MC. Biochemical studies have established that Vav1 binds directly to these sites via its SH2 domain. Here, we show that Vav1 enters SLP-MC, but is not appreciably enriched in distinct microclusters containing the T cell receptor (TCR). Using a Vav1-deficient T cell line (J.Vav1) and by performing knockdown/addback experiments, we demonstrate that Vav1 regulates the stability of SLP-MCs. The Vav1 SH2 domain plays a crucial role in recruitment of Vav1 into SLP-MC, and is required for the stabilization of SLP-MC by Vav1. The C-terminal fragment of Vav1, which contains an SH3-SH2-SH3 cassette, is efficiently recruited into SLP-MC. However, this C-terminal fragment does not stabilize SLP-MC. Thus, N-terminal domains within Vav1 must contribute to the stability of SLP-MC. The N-terminus of Vav1 contains a GTP exchange factor (GEF) domain specific for Rho-family GTPases, and contributes to the initiation of calcium elevations via its N-terminal calponin homology (CH) domain. These effector functions are separable and are contingent on the recruitment of Vav1 into SLP-MC. The deletion of the GEF domain does not impact the recruitment of Vav1 to SLP-MC, or the stabilization of SLP-MC by Vav1. In contrast, a point mutation inactivating the GEF domain prevents the stabilization of SLP-MC by Vav1. We propose that the N-terminus of Vav1 contributes to microcluster stability via a module that is sequestered through an intramolecular interaction with the GEF domain, and is revealed by GEF-dependent conformational changes upon the recruitment of Vav1 into SLP-MCs via its C-terminal SH3-SH2-SH3 cassette.

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REGULATION AND AUTOINHIBITION OF THE B CELL ANTIGEN RECEPTOR ON RESTING B CELLS

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At presence most studies on the B cell antigen receptor are conducted with activated B cells whereas little is known about the structure and function of the BCR on resting B cells. By employing the bifluorescent complementation (BiFC) assay in S2 Schneider and in normal resting B cells, we have found evidence that the undisturbed BCR has an auto-inhibited oligomeric structure. Furthermore, we have identified critical amino acids which are involved in auto-inhibition of the BCR and the stable expression of the receptor on resting B cells. This study is the first description of the structural requirements for the regulation of the BCR on resting cells. In a parallel study we have found that in resting B cells the tail of the Ig-α signalling subunit of the BCR is methylated on a highly conserved arginine by the protein arginine methyl transferase RPMT1. Interestingly, the arginine methylation is highest in resting B cells and declines after BCR activation. Therefore, this is the first protein modification at the BCR which is associated with the resting state of the receptor. By mutating this arginine to lysine we found that the BCR becomes more active in supporting the proliferation and preventing the differentiation of pre-B cells. Together, our data suggest that on resting B cells the B cell receptor is regulated by several layers that control its activation. Currently we are investigating how other proteins on the B cell surface are helping the BCR to maintain its resting state.

PERTURBING THE OPPOSING ACTIONS OF CD45 AND CSK REGULATES TCR SIGNALING IN THE BASAL STATE

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T cell antigen receptor (TCR) signaling initiates T cell responses by inducing downstream tyrosine phosphorylation dependent pathways. The Src family kinase Lck is the most upstream kinase in the TCR dependent pathway. Lck function is controlled, in part, by its phosphorylation at two tyrosines, Y394 in its activation loop and Y505 near its C-terminus. Phosphorylation of Y394, the result of trans-autophosphorylation, stabilizes the activated conformation of the catalytic domain. The Y505 site is regulated by the opposing actions by the cytosolic kinase Csk and the CD45 receptor-like protein tyrosine phosphatase. Using an allelic series of mice with expression levels of 0-125% of wt CD45 levels and a genetically controlled inhibitor of Csk catalytic function, we have studied the relative requirements of CD45 and Csk during basal and induced TCR signaling. Our studies reveal dynamic signaling in the basal state which is subject to regulation by the level of the opposing activities of CD45 and Csk and suggest that basal signaling is subject to negative feedback control. Induced signaling must overcome a threshold set by Csk and is subject to be negatively regulated by high levels of CD45.

THE IMMUNOLOGICAL SYNAPSE: A FOCAL POINT FOR EXOCYTOSIS AND ENDOCYTOSIS.

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There are many different cells in the immune system. In order to mount an effective immune response they need to communicate with each other. One way in which this is done is by the formation of 'immunological synapses' between cells. The role of the immunological synapse has been controversial, with studies suggesting roles in both enhancing and down-regulating signaling. Our own work has focused on the intracellular organization at the immune synapse. In cytotoxic T lymphocytes (CTL), we have found that the centrosome (which is the microtubule organizing center in T cells) polarizes to the cSMAC of the immune synapse, delivering the specialized lytic granules to the secretory domain where they release their contents and destroy their target. We have now begun to dissect the signals required for successful polarized secretion and find that centrosome and granule polarization are separately controlled. Our studies point to remarkable similarities between the structure formed at the immunological synapse and those formed at cilia and flagella. Our studies suggest that the immune synapse serves as a focal point for exocytosis and endocytosis.

MECHANISMS OF IL-17 RECEPTOR SIGNALING

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The IL-17 receptor superfamily is unique in structure and sequence from other known cytokine subfamilies, and as such has a distinct profile of signaling mechanisms. The prototypical receptor of this family is IL-17RA, which is a common subunit for IL-17 and IL-17F. IL-17RA encodes a domain with homology to Toll-IL-1 receptors (TIR) domains, termed "SEFIR." We have shown that the SEFIR domain is critical for activation of NF-κB, C/EBPδ and subsequent downstream signaling events. In addition, we have also identified an extension of this domain with homology to TIR BB-loops, which is also essential for signal transduction by IL-17. Moreover, we have defined a distal functional region within IL-17RA that mediates a distinct signaling pathways leading to control of the CCAAT/Enhancer Binding Protein (C/EBP) transcription factor, both at the level of alternative translation and inducible phosphorylation. The IL-17RC subunit is also needed for IL-17-mediated signaling and contains a predicted SEFIR domain, but less is known about its ability to mediate signaling. We have defined subregions within the IL-17RC cytoplasmic tail that are distinct from IL-17RA in terms of molecular function.

LYMPHOCYTE HOMEOSTASIS: ‘TIS DEATH THAT MAKES LIFE LIVE

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The B and T lymphocytes of the specific immune response use complex gene rearrangement machinery to generate a wide diversity of antigen receptors capable of recognizing any pathogen in the universe. Binding to receptors on both innate and specific immune system cells triggers intricate intracellular signaling pathways that lead to new gene transcription and effector cell activation. And yet, regulation is imposed on these responses so that paradise is not lost to the turning of the immune system onto self-tissues, the spectre of autoimmunity. Lymphocyte activation requires multiple signals and intercellular interactions. Mechanisms exist to establish tolerance to self by the selection and elimination of cells recognizing self-antigens. Immune system cell populations are reduced by programmed cell death once the pathogen threat is resolved. Once good health is reestablished, memory cells remain in the body to sharply reduce the impact of a second exposure to a pathogen. Over the last 15 years, our laboratory had been engaged in studying the molecular signaling pathways that confer cell survival or cell death in these lymphocytes. The special role of fas in germinal centre B cells survival, the function of the CARMA1/Bcl-10/Malt1 complex in the differentiation and survival of the various subsets of lymphocytes, the importance of nfil3 the development of NK cells and the intriguing task of the vagal nerves in maintaining lymphocyte homeostasis will be presented.

AID-INDUCED DECREASE IN TOPOISOMERASE 1 INDUCES DNA STRUCTURAL ALTERATION AND DNA CLEAVAGE FOR CLASS SWITCH RECOMBINATION

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To initiate class switch recombination (CSR) activation-induced cytidine deaminase (AID) induces staggered nick cleavage in the S region which lies 5' to each immunoglobulin constant region gene and rich in palindromic sequences. Topoisomerase 1 (Top1) controls the supercoiling of DNA by nicking, rotating, and religating one strand of DNA. Curiously, Top1 reduction or AID overexpression causes the genomic instability. Here we report that the inactivation of Top1 by its specific inhibitor camptothecin drastically blocked both the S region cleavage and CSR, indicating that Top1 is responsible for the S region cleavage in CSR. Surprisingly, AID expression suppressed Top1 mRNA translation and reduced its protein level. In addition, the decrease in the Top1 protein by RNA-mediated knockdown augmented the AID-dependent S region cleavage as well as CSR. Furthermore, Top1 reduction altered DNA structure of the Sμ region. Taken together, AID-induced Top1 reduction alters S region DNA structure probably to non-B form, on which Top1 can introduce nicks but cannot religate, resulting in S region cleavage.

MODELING EPSTEIN-BARR-VIRUS IMMUNE SURVEILLANCE AND B CELL TRANSFORMATION IN MICE

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Epstein-Barr-Virus (EBV) is endemic in the human population, where after clearance of an initial infection, it persists in a small population of B cells for life. Spreading of the virus is prevented by efficient immune surveillance through T and NK cells. In immunosuppressed individuals, such as post-transplantation or AIDS patients, immune surveillance may wane and severe EBV-caused pathologies develop, such as Post-Transplant Lymphoproliferative Syndrome (PTLD), often associated with EBV+ B cell lymphomas, or B cell lymphomas in AIDS patients. Burkitts lymphoma and, in about half of the cases, Hodgkin's Disease are also associated with EBV. In attempts to model such diseases in mice, we have expressed latent membrane protein 1 (LMP1), an EBV-encoded protein critical for B cell transformation and mimicking constitutively active CD40 (a major B cell co-stimulatory receptor), conditionally in the B cell lineage. In the mutant mice, the developing LMP1+ B cells were efficiently eliminated by T and, likely, NK cells. Ablation of T and NK cells led to rapid death of the animals due to accumulation of LMP1+ B cell blasts. B cell-specific LMP1 expression in T cell-deficient mice resulted in mild lymphoproliferation, rapidly followed by the development of aggressive, fatal LMP1+ B cell lymphomas. The lymphoma cells expressed activation-induced cytidine deaminase (AID) and stress antigens recognized by the activating NK cell receptor NKG2D. Accordingly, the in vivo growth of these tumors could be inhibited by a complement-binding NKG2D-Fc fusion protein. Taken together, we present evidence for a critical role of LMP1 in EBV immune surveillance and B cell transformation in vivo, and have generated a preclinical mouse model of LMP1-driven B lymphomagenesis under conditions of immunosuppression.

IGH MRNA EXPRESSION IS INFLUENCED BY TRANSCRIPTION ELONGATION

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Alternative processing of the Immunoglobulin heavy chain (Igh) mRNA fundamentally regulates antibody production. When B cells differentiate into plasma cells the Igh mRNA is both alternatively processed to the secretory-specific form and increased in abundance; this leads to the production of large amounts of mature Ig protein. We recently showed that this shift to high levels of secretory-specific Igh mRNA production is accompanied by an increase in serine-5 phosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II near the Igh promoter. There is also an unexpected increase in ser-2 phosphorylation near the promoter, an event that increases polymerase processivity. These changes in serine phosphorylation of the CTD are accompanied by an increased association of the polyadenylation factors with the RNA pol II that is dependent on ELL2, a transcription elongation factor. This allows the polymerase to elongate more effectively, producing more mRNA per initiation cycle, and to increase the local concentration of polyadenylation factors, thereby facilitating the use of the secretory poly(A) site, to the exclusion of splicing to Ig membrane-specific exons (Martincic et al Nature Immunology, 2009). Thus transcription elongation influences RNA processing.
We are now addressing two related questions: First, are other plasma cell genes affected by ELL2? We have evidence by Chromatin IP that ELL2 is associated with RNA pol II on essential plasma cell genes including IRF-4 and blimp-1. There are concomitant changes in promoter-proximal CTD phosphorylation and histone modifications on these genes that indicate more efficient transcription. For IRF4 there is a shift to use of a proximal poly(A) site that could influence IRF4 mRNA abundance. The second issue is: what is the molecular mechanism leading to the increase in ser-2 modification of the CTD? Hexim protein plus 7SK RNA sequester the factor that phosphorylates CTD at the ser-2 position (Cyclin T1 and cdk9, collectively known as pTEFb). Our results show higher levels of hexim in B cells which may be responsible for decreased pTEFb activation. In plasma cells with a paucity of hexim, splicing factors can be sequestered with the now available 7SK. This could lead to the decease in splicing we and others have seen for the Igh pre-mRNA in plasma cells. Thus changes in elongation can influence mRNA processing both directly at the polymerase by increasing polyadenylation and indirectly by squelching splicing. Together, these studies define a fundamental role for transcription elongation machinery in plasma cell differentiation.

MECHANISMS UNDERLYING NOTCH-INDUCED DEGRADATION OF DIVERSE SUBSTRATES

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Notch signaling pathways controls a variety of differentiation processes including several lineage decisions in lymphopoiesis, thus demanding versatile functions of Notch in these diverse situations. We have previously shown that signaling from Notch receptors accelerates ubiquitin-mediated degradation of several important regulators of lymphopoiesis, such as E2A and Janus kinases (Jak). Ubiquitination of E2A and Jak proteins has been known to be catalyzed by distinct E3 ubiquitin ligases, SCFSkp2 (Skp1-Cul1-Skp2) and ECS (elongin B/C-Cul5-SOCS), respectively. How Notch enhances the activity of both types of E3 ligases remained to be understood. We have now found that Notch signaling dramatically activates the transcription of the ankyrin repeat and SOCS box-containing protein 2 gene (Asb2). Over-expression of Asb2 stimulates the ubiquitination of both E2A and Jak proteins whereas a dominant-negative mutant of Asb2 blocks Notch-induced degradation of these proteins. Furthermore, we demonstrate that Asb2 bridges the formation of a dimeric E3 ligase complex through interaction not only with elongin B/C and Cul5 via its SOCS box but also with Skp2, which in turns brings Skp1 and Cul1, as well as its substrates. In addition, Asb2 is able to associate with Jak2 with a high affinity. Together, these results explain how Notch signaling has significant impact on the turnover of different substrates. Our study helps establish a paradigm that biological processes can be controlled by influencing the stability of crucial regulators through a dimeric E3 ligase complex, which is thought to have higher activities based on structural studies. By shifting monomeric E3 ligase complexes to dimeric ones through activation of Asb2 expression, Notch effectively exert its diverse effects on cell differentiation by controlling the turnover of a group of substrates previously known to be ubiquitination by Cul1 and Cul5-based E3 ligases. Among them, are E2A, SCL/Tal1, p27, IκBα and Jak2/3, which play important roles in various aspects of Immunobiology.

ROQUIN IS AN RNA-BINDING PROTEIN THAT FORMS COMPLEXES WITH P BODY COMPONENTS TO POST-TRANSCRIPTIONALLY REPRESS ICOS IN A MIRNA-INDEPENDENT MANNER

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Mice with a homozygous point mutation in the rc3h1 gene that encodes the Roquin protein develop severe autoimmune disease (Vinuesa et al., 2005, Nature 435, 452-8). It has been shown that Roquin represses ICOS by promoting mRNA decay and increased ICOS expression in the mutant mice contributes significantly to pathology (Yu et al., 2007, Nature, 450, 299-303).
We investigated the molecular mechanism by which Roquin represses ICOS. Knockdown approaches revealed that ICOS expression is placed under the control of Roquin in Th1 and Th2 cells. In a deletion mutagenesis we identified a carboxy-terminal sequence in Roquin with a high content of asparagine and glutamine residues that is functionally important and specifies localization of Roquin to P bodies, but is not required for enrichment of Roquin in stress granules. Similarly, we determined that Roquin-mediated ICOS repression does not require the essential stress granule protein TIA-1, instead functionally depends on the P body component Rck. We genetically tested a requirement for cellular RNAi by using Dicer-deficient mouse embryonic fibroblasts clones, which are incapable of microRNA biogenesis, and by using mouse embryonic stem cells with gene deletion of argonaute 1-4, which are unable to form microRNA-induced silencing complexes (miRISC). Our data show no requirement for microRNAs and miRISC formation in Roquin-mediated ICOS repression. Instead, we demonstrate direct physical interaction of Roquin protein with ICOS mRNA in vitro and binding of Roquin to ICOS mRNA in primary T cells. Our experiments uncover a novel mode of RNA-protein interaction, in which the amino-terminal ROQ domain cooperates with its adjacent zinc finger. Analyzing Roquin-interacting proteins we find specific and RNase-insensitive interactions of Roquin with Rck and Edc4 proteins in mouse T cells, which are critical factors in P body-associated mRNA-decay. We therefore propose that Roquin prevents autoimmunity through interaction with ICOS mRNA and by coordinating assembly of functional messenger ribonucleoprotein complexes that downregulate ICOS expression posttranscriptionally.

REGULATION OF IL-17 AND IL-10 IN HUMAN TH17 CELLS

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T helper cells that produce IL-17 (Th17 cells) have recently emerged as a new T cell lineage. They can induce recruitment of neutrophils and trigger production of pro-inflammatory cytokines and chemokines by a broad range of cellular targets. Although these effector functions confer upon Th17 cells the ability to protect against certain extracellular bacteria and fungi, a dys-regulated Th17 response can induce severe tissue destruction and autoimmunity. Therefore, mechanisms must be in place to shield the host from immune-mediated damage. The aim of this study was to investigate the stability and flexibility of the pro-inflammatory human Th17 subset and to identify factors that can modulate the cytokine profile of differentiated Th17 cells. We found that Th17 cells can produce IL-10, an immuno-suppressive cytokine that confers regulatory functions. IL-10 can be induced in circulating $CCR6^{+}CCR4^{+}$ Th17 cell subset as well as in Th17 clones under certain stimulatory conditions, which is accompanied by down-regulation of IL-17. We also found that the Th17 phenotype is subject to a complex modulation by other cytokines. IL-23, for example, which accounts for pathogenicity in the EAE mouse model by IL-10 suppression, promotes the regulatory Th17 phenotype in the human system by up-regulation and maintenance of IL-10/IL-17 co-expression. This reciprocal regulation of pro- and anti-inflammatory cytokines might constitute a self-regulatory mechanism that allows for self-limitation of the inflammatory Th17 cell response. The results of this study may have important implications for immune vaccination strategies and contribute to a better understanding of immuno-regulation in general.

PLZF+ CELLS REGULATE THE DEVELOPMENT OF INNATE-LIKE CD8 T CELLS THROUGH IL-4

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Several gene deficiency models, including ITK, CBP, KLF2, and Id3, result in the development of CD8+ T cells that resemble memory cells and exhibit immediate effector cytokine production. Such “innate-like” CD8+ T cells express the transcription factor eomesodermin and have been shown to play an important role early in infections. We previously showed that in KLF2 deficient mice, wild-type bystander CD8 T cells also adopt this phenotype, caused by high levels of IL-4 in vivo. Here we show that the source of increased IL-4 in KLF2 deficient mice is an expanded subset of T cells that express the transcription factor PLZF, including αβ NKT and γδ NKT cells. Indeed, in KLF2 deficient mice that also lacked PLZF, CD8+ T cells reverted to a normal phenotype. ITK, CBP, and Id3 deficient mice also display an expanded PLZF+ population that produces IL-4. Like KLF2 deficiency, we show that the innate-like CD8+ T cell phenotype in ITK deficient mice is not cell-intrinsic, but dependent on overproduction of IL-4. Interestingly, humans and different inbred strains of mice have an expanded population of PLZF+ cells and a larger subset of innate-like CD8+ T cells. Indeed, in BALB/c mice, the innate-like CD8+ T cell population is dependent on IL-4. Thus we propose that PLZF+ αβ and γδ NKT cells regulate the population size of innate-like CD8+ T cells that rapidly produce cytokines early during infection.

THE FUNCTION OF PLZF IN INNATE CELLS OF THE IMMUNE SYSTEM.

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Natural killer T cell (NKT cells) have an innate immunity-like rapidity of response and the capacity to modulate effector functions of other cells. We have shown that NKT cells specifically express the BTB-ZF transcriptional regulator PLZF. In the absence of PLZF, NKT cells developed, but lacked many innate T cell features. Factors that control the normal expression of PLZF in lymphocytes are unknown. We now have shown that PLZF expression is not restricted to NKT cells, but is also expressed by a subset of γδ T cells, functionally defining distinct subsets of this innate T cell population. Next, a second BTB-ZF gene, ThPOK, is shown to be important for the phenotype of the PLZF-expressing γδ Vγ1.1-Vδ6.3 T cells. Most importantly, it is shown that TCR signal strength and expression of Id3 control the frequency of PLZF-expressing γδ T cells. Therefore, our studies define factors that control the propensity of the immune system to produce potentially disease causing T cell subsets.

REGULATION OF CD4 EFFECTOR T CELL FUNCTION IN MOUSE MODELS OF PRIMARY IMMUNODEFICIENCIES

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Our laboratory's work focuses on the signaling pathways that help govern T cell differentiation with an emphasis on molecules and pathways implicated in human genetic immune disorders and how they affect responses to infectious diseases. To this end, we are studying mice deficient in SH2D1A/SAP, mutations of which are associated with X-linked lymphoproliferative disease as well as mice deficient in the Tec kinases Itk, Rlk, Tec and Btk. SH2D1A/SAP encodes a small SH2 domain containing adaptor molecule that binds to the intracellular tails of receptors related to SLAM, including SLAM, Ly108, CD84, Ly9, and 2B4. We have found that mice deficient in SAP recapitulate and moreover, have revealed new phenotypes of XLP, including T cell hyperproliferation post-infection and impaired antibody responses with defective germinal center formation. Both SAP-deficient mice and XLP patients also fail to develop NKT cells, an innate-type T cell lineage. We have recently shown that SAP and SLAM family members are required for proper T:B lymphocyte adhesion required for germinal center formation. Our recent work is focused on how SAP and SLAM family receptors regulate distinct types of lymphocyte adhesion and their effects on lymphocyte development, function and homeostasis. We are also examining the role of the Tec kinases in T cell development and regulation of effector T helper cell function and cytokine production, with a focus on the role of TCR signaling on differential regulation of Th17 cytokines. Together these studies provide insight into the differentiation of distinct T helper cell populations and how these cell populations contribute to the phenotypes of primary immune disorders.
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PROTEIN KINASE B/AKT : DOGMA VERSUS REALITY

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In T lymphocytes the activation of Protein Kinase B /Akt via the p110delta catalytic isoform of phosphatidyl inositol 3 kinase and phosphoinositide dependent kinase 1 occurs in response to triggering of antigen receptors,cytokines and chemokines. There is a dogma that Akt controls T cell glucose metabolism and is thus essential for T cell survival and proliferation. However, experimental analysis of this concpet reveals that the Akt dependence of T cell metabolism and proliferation applies to some but not all T cell subpopulations and is dependent on the exogenous stimulus. For example, in cytotoxic T cells the loss of Akt function has no impact on cell survival or proliferation but rather co-ordinates a transcriptional program that causes CTL to decrease the expression of cytolytic effector molecules while increasing expression of adhesion molecules and cytokine and chemokine receptors diagnostic of memory T cells. Strikingly, inhibition of Akt re-programmes CTL trafficking to secondary lymphoid organs. PI3K and PKB/Akt are thus at the core of the molecular mechanisms that regulates the effector/memory fate of peripheral T cells but are not essential for cell survival and proliferation.

MAPPING THE DECISION SPACE OF TH1-TH2 DIFFERENTIATION

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Upon activation, naïve CD4+ T-cells can differentiate into one out of few possible lineages, each invoking a specific immune response. This decision is influenced by the spectrum of cytokine signals the cells sense. However, the differentiating cells also actively alter these signals by producing relevant cytokines, thus influencing the differentiation process through extracellular feedbacks. IL-12 and IL-4 direct differentiation into the main fates of helper CD4+ T-cells, Th1 and Th2, respectively. This system is usually studied under polarized conditions, applying only one signal, typically together with antibodies against other cytokines thus interfering with intercellular communication. However, cells in vivo are expected to sense a more complex environment, in which they may be exposed to combinations of cytokines. How do cells respond under such mixed conditions is not well understood.

Here, we explore differentiation under mixed input conditions by exposing cells to varied combinations of the two cytokines, experimentally mapping the "decision space" of the differentiation process. We measured, at the single cell level, eight key parameters relating to the state of the cells, from which we can deduce properties of the molecular network describing the system. Our data indicate that cells exposed to combinations of both IL-12 and IL-4 give rise to a mixed response at the single cell level, with subpopulations of cells secreting either IFNγ, IL-4, or both upon restimulation. This is preceded by co-expression of the two lineage specific transcription factors T-bet and GATA3 at earlier time points. We build a mathematical model describing the system, which can reproduce our experimental data under specific assumptions regarding the underlying molecular network structure and kinetic constants. The model is extended to describe a multicellular system using a computational model in which each cell is simulated as a node on a dynamic network, whose links describe the propagation of intercellular cytokine signals.

Our experiments provide information also on levels of heterogeneity in the population in response to mixed conditions. We find that heterogeneity in cytokine expression upon restimulation is much larger under mixed conditions than under a single cytokine input. Interestingly, the level of heterogeneity (or noise) of the transcription factors in the mixed state is much lower than that observed at the output stage. These findings support a model by which decision under mixed conditions follows a fuzzy logic, where cells project their internal state onto the output phenotype stochastically, with probabilities which depend on the system's input.

FOXO CONTROL OF T LYMPHOCYTE HOMEOSTASIS AND TOLERANCE

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The forkhead-box O family of transcription factors (Foxos) are mammalian orthologs of Caenorhabditis elegans DAf16 and Drosophila melanogaster dFoxo with critical functions in control of ancient cellular processes such as nutrient sensing and stress responses. Three of the four Foxos (Foxo1, Foxo3, and Foxo4) are regulated via an evolutionarily conserved PI(3)K/Akt pathway, in which Akt activated by PI(3)K phosphorylates Foxos resulting in their nuclear exclusion and inactivation. Using a combination of genetic, genomic, and immunological approaches, we have started to dissect the functions of Foxos in T lymphocytes. T cell-specific deletion of Foxo1 gene results in compromised migration and survival of naïve T cells associated with defective expression of L-selection and IL-7Rα. Functional studies validate IL-7Rα as a Foxo1 target gene essential for Foxo1 maintenance of naïve T cells. In addition, T cell-specific ablation of both Foxo1 and Foxo3 proteins triggers a fatal multifocal inflammatory disorder due in part to the defects of Foxp3+ regulatory T (Treg) cells. Foxos function in a Treg-intrinsic manner to control the expression of a subset of Treg-associated genes critical for Treg function. These findings reveal Foxos as pivotal regulators of T lymphocyte homeostasis and tolerance under the steady state, and suggest that the Foxo pathway may have been rewired to regulate highly specialized activities in the immune system.

THE TRANSCRIPTION FACTOR BLIMP1 REGULATES DIFFERENTIATION AND FUNCTION OF EFFECTOR REGULATORY T CELLS

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Regulatory T (Treg) cells are required for peripheral tolerance. Recent evidence indicates that they can undergo specialized differentiation programs that in addition to Foxp3+ are controlled by other transcription factors, including T-bet and IRF-4.
We and others have previously shown that the transcriptional repressor Blimp1 is required for the differentiation of plasma cells and short-lived CD8+ effector T cells. Here we demonstrate that expression of Blimp1 defines a distinct population of Treg cells that localize predominantly to mucosal sites and constitute cells actively engaged in suppression. We show that Blimp1 is required for effector functions of Treg cells, for their correct tissue localization and for their homeostasis. Blimp1-deficient Treg cells fail to express the effector cytokine IL-10 and do not adequately regulate survival proteins and chemokine receptors, ultimately leading to accumulation and severe functional deficiencies of these cells. Our study illuminates some of the signals leading to Blimp1 expression in Treg cells and identifies molecular pathways required for effector Treg cell differentiation. It further demonstrates a remarkable conservation of the molecular requirements for the effector differentiation of different lymphocytic lineages.

MECHANISMS CONTROLLING TH2 CONVERSION OF FOXP3-EXPRESSING TREG CELLS *IN VIVO*

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Regulatory T cells (Treg) were originally discovered as immune suppressors critical for self-tolerance and immune homeostasis. Foxp3, an X-linked transcription factor highly and specifically expressed in Treg, is regarded as the master regulator and genetic marker for Treg cells. Treg cells were traditionally thought to be terminally differentiated and fully committed lineage specializing in immune suppression. However, accumulating evidence suggest that these cells have much more diverse functions than immune suppression. In fact, Treg cells are “plastic”, being able to convert into Th1, Th2, Th17 and Tfh cells to promote immune response. In particular, through yet-to-be-defined mechanisms, Treg cells become IL-4 producing Th2 cells *in vivo* upon the attenuation of Foxp3 expression. Here we will discuss our recent findings on the underlying mechanisms of Treg-to-Th2 conversion.

TLR SIGNALING IS REQUIRED FOR VIRULENCE OF AN INTRACELLULAR PATHOGEN

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Activation of innate immune receptors, such as the Toll-like receptors (TLRs), leads to induction of antimicrobial mechanisms that restrict pathogen growth. A lack of TLR function results in impaired host defense and increased susceptibility to pathogens. The host response applies strong selective pressure on microbial pathogens, and many virulence mechanisms work by subverting aspects of this innate immune response. Here we have examined the consequence of TLR deficiency on the replication and pathogenesis of the intracellular bacterial pathogen Salmonella enterica serovar typhimurium (S. typhimurium). Unexpectedly, we find that TLR function is required for S. typhimurium virulence. While mice lacking both TLR2 and TLR4 were highly susceptible to S. typhimurium infection, mice lacking additional TLRs were as resistant to infection as wildtype mice. Bacteria were unable to form a replicative compartment in macrophages lacking TLR function, and analysis of bacterial gene expression revealed a failure to upregulate virulence genes in these cells. Our results indicate that S. typhimurium requires cues from the innate immune system to regulate virulence gene expression and pathogenesis. This work provides a compelling example of host/pathogen coevolution, in which the pathogen has evolved virulence mechanisms whose induction depend on the very signals that selected their emergence.

TLR4 SIGNALING REGULATES MACROPHAGE BACTERICIDAL ACTIVITY THROUGH MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION

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Reactive oxygen species (ROS) are an essential component of the innate immune response against intracellular pathogens. Although ROS production by the phagosomal NADPH oxidase is well established, the contribution of mitochondrial-derived ROS (mROS) in the innate immune response and their interplay with phagosomal ROS remains unclear. We have found that engagement of Toll-like receptor 4 (TLR4) by bacterial lipopolysaccharide leads to increased production of mitochondrial and phagosomal ROS through a novel pathway. Disruption of this pathway in macrophages results in decreased levels of TLR4-induced ROS and reduced killing of intracellular bacteria. Furthermore, mitochondrially-targeted catalase expression results in significantly reduced ROS production and defective bacterial killing. Therefore TLR4 signaling activates a novel route to ROS production that is utilized by the innate immune system for robust bactericidal responses.

TOLL-LIKE RECEPTOR ACTIVATION OF XBP1 REGULATES INNATE IMMUNE RESPONSES IN MACROPHAGES

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Sensors of pathogens, such as Toll-like receptors (TLRs), detect microbes to activate transcriptional programs that orchestrate adaptive responses to specific insults. Here we report that TLR4 and TLR2 specifically activate the ER-stress sensor kinase IRE1α and its downstream target, the transcription factor XBP1. Previously described XBP1 ER stress target genes are not induced by TLR signaling. Instead, TLR-activated XBP1 is required for optimal and sustained production of proinflammatory cytokines in macrophages. Consistent with this finding, IRE1α activation by ER-stress synergizes with TLR activation for cytokine production. Moreover, XBP1 deficiency markedly increases bacterial burden in animals infected with the TLR2-activating human pathogen *Francisella tularensis*. Our findings uncover an unsuspected critical new function for the XBP1 transcription factor in mammalian host defenses.

DSIF CONTROLS THE DURATION OF NF-KB SIGNALING BY COORDINATING ELONGATION WITH MRNA PROCESSING OF NEGATIVE FEEDBACK GENES

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NF-κB is a transcriptional activator regulating inflammation, immune responses, cell survival and cell cycle. Deregulation of the NF-κB is linked to inflammatory diseases and tumorgenesis and therefore NF-κB has become an important target for drug development. We are studying the mechanisms underlying transcription activation by NF-κB and have reported that NF-κB controls the re-initiation and elongation stages of transcription. Most recently we found that NF-κB differentially affects regulation by the elongation factors P-TEFb and DSIF in a manner dependent on core promoter type and pre-initiation complex. The significance and the molecular basis of this mode of regulation among NF-κB target genes are as yet unknown. Here we report that DSIF selectively attenuates transcription elongation of the NF-κB negative feedback genes IκBα and A20. Surprisingly, inhibition of these genes by DSIF is actually essential for their productive protein synthesis. Investigating this apparent discrepancy between the effect of DSIF on mRNA and protein levels, we found that DSIF attenuation of elongating RNA Pol II is required for coordinating transcription elongation with mRNA processing. In the absence of DSIF large amounts of mature and immature mRNAs of IκBα and A20 are produced upon NF-kB induction but these mRNAs are not being exported into the cytoplasm. Consequenly much less IκBα and A20 proteins are synthesized and NF-κB activity is not properly terminated. Our findings revealed a previously unknown interplay between NF-κB, transcription elongation factors and mRNA processing that operates selectively on NF-κB negative feedback regulatory genes. Thus, these results identify new targets for selective manipulation of specific subsets of genes in the NF-κB pathway which could be significant in NF-κB related diseases.

THE ROLE OF LOCUS POSITIONING IN THE REGULATION OF CYTOKINE GENE EXPRESSION

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Th1 effector cells secrete potent proinflammatory cytokines involved in the induction of inflammation, apoptotic cell death and inhibition of tumorigenesis. Uncontrolled secretion of these leads to autoimmunity. Here we investigated the temporal and spatial regulation of cytokine gene expression in activated T-lymphocytes. Using three-dimensional DNA fluorescence in situ hybridization (3D-DNA FISH), we mapped the positions of the cytokine loci in interphase nuclei of T cells. We found that cytokine alleles were nonrandomly juxtaposed within the nuclear volume following T cell receptor (TCR) stimulation. We explored the functional significance of homologous associations by correlating this pairing event with the allelic transcription status. Our data suggests that alleles of a gene may cluster in the interchromosomal domain compartment, where both alleles are transcribed. We propose that locus repositioning and clustering may bring the homologous cytokines alleles into physical proximity to coordinate their activities and enhance the transcription output

CHARACTERIZATION OF A CRYPTIC CYCLOPHILIN A-DEPENDENT INNATE SIGNALING PATHWAY IN HIV-1 INFECTED DENDRITIC CELLS

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Dendritic cells (DC) serve a key function in host defense, linking innate detection of microbes to the activation of pathogen-specific adaptive immune responses. Whether there is recognition of HIV-1 or other retroviruses by host innate pattern-recognition receptors and subsequent coupling to antiviral T cell responses is not yet known. DC are largely resistant to infection with HIV-1, due to a block in reverse transcription, but this host restriction can be overcome by co-infection with SIVmac pseudovirions. Under such conditions, HIV-1 induced maturation of monocyte-derived DC and peripheral blood DC, resulting in expression of costimulatory molecules and in a strong type- I interferon response. Productively infected MDDC induced potent activation of HIV-specific T cells. This innate response was dependent on the interaction of newly-synthesized HIV-1 capsid (CA) with cellular cyclophilin A (CypA) and the subsequent activation of the transcription factor IRF3. Because the peptidyl-prolyl isomerase CypA also interacts with CA to promote HIV-1 infectivity, our results suggest that CA conformation has evolved under opposing selective pressures to balance effective infectivity against potential activation of host antiviral immunity. Thus, an innate response to HIV-1 exists, but is cryptic in the absence of dendritic cell infection. Manipulating this response may be essential to generate a HIV-1 vaccine.

MSKS ACT AS NEGATIVE REGULATORS OF INNATE IMMUNITY.

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The innate immune system acts as an initial line of defense against invading pathogens. Activation of innate immunity results in inflammation and pro-inflammatory cytokine production, processes essential to combat pathogens. Uncontrolled inflammation and excess pro-inflammatory cytokine production are however deleterious and can lead to tissue damage and septic shock. As a result, negative feedback mechanisms and anti-inflammatory pathways are necessary to keep inflammatory signaling in check.
Several pathways, including the ERK1/2 and p38 MAPK pathways are known to regulate pro-inflammatory cytokine production in innate immune cells. It is now apparent that these pathways also have roles in the negative feedback pathways controlling inflammation. MSK1 and MSK2 are nuclear kinases that act downstream of ERK1/2 and p38 in vivo, and their primary functions is to regulate the transcription of specific immediate early genes. We have found that these kinases regulate important negative control mechanisms in inflammation. MSK1/2 knockout mice are more sensitive to LPS induced endotoxic shock, and produce elevated levels of several pro-inflammatory cytokines including TNF, IL-6, IL-12. MSKs do not directly regulate the transcription pro-inflammatory cytokine genes, but instead exert their effects via activating negative feedback mechanisms. For instance, MSKs directly regulate the transcription of the anti-inflammatory cytokines IL-10 and IL-1ra. The use of compound IL-10 / MSK knockouts has shown that the increase in IL-6 and IL-12 seen in MSK knockouts is mainly due to the role of MSK in regulating IL-10 production. Not all of MSKs effects are however IL-10 dependent, as MSKs regulate LPS induced TNF production in an IL-10 independent manner.
MSKs can regulate transcription via both the phosphorylation of either histone H3 or transcription factors such as CREB and ATF1 and possibly NFκB. CREB is phosphorylated by MSKs on Ser133, which creates a binding site for the co-activator proteins p300 and CBP. Knockout of CREB in mice results in embryonic lethality, however unexpectedly we have found that mice with a knockin mutation of Ser133 to Ala are viable. Further study of these mice has shown that, similar to MSK knockouts, CREB Ser133Ala macrophages produce elevated levels of pro-inflammatory cytokines in response to TLR stimulation. Array screening has shown that most, but not all, of the MSK regulated genes in macrophages are also dependent on CREB phosphorylation.

IDENTIFICATION OF THE ORGANIZATIONAL PRINCIPLES OF ENHANCERS CONTROLLING INFLAMMATORY GENE EXPRESSION IN MACROPHAGES

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Enhancers and other distant-acting cis-regulatory sequences determine tissue specific gene expression programs. Enhancers are marked by high levels of histone H3 lysine 4 mono-methylation (H3K4me1) and by binding of the acetyltransferase p300, which has allowed genome-wide enhancer identification. However, the regulatory principles by which discrete subsets of enhancers become active in specific developmental and/or environmental contexts remain to be unraveled. Here we exploited inducible p300 binding to identify, and then mechanistically dissect, enhancers involved in a prototypical environmental response, endotoxin-stimulated inflammatory gene activation in macrophages. The fundamental organizational principle of these enhancers is the combination of binding sites for the lineage-restricted and constitutive Ets family member PU.1, with those for ubiquitous stress-inducible transcription factors such as NFkB, IRF and AP-1. PU.1 was required for the maintenance of H3K4me1 at macrophage-specific enhancers, independently from inflammatory stimuli. Reciprocally, ectopic expression of PU.1 caused re-activation of these enhancers in fibroblasts. Thus, the combinatorial assembly of tissue- and signal-specific transcription factors determines the activity of a distinct group of enhancers. We suggest that this may represent a general paradigm in tissue-restricted and stimulus-responsive gene regulation.

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Ambulance	**(9) 742-3300**	**(9) 692-4747**
Poison	**(9) 542-2323**	**(9) 542-2323**
Police	**(9) 911**	**(9) 549-8800**
Safety-Security	**Extension 8870**	

Emergency Room **Huntington Hospital** 270 Park Avenue, Huntington	**631-351-2300** **(1037)**
Dentists Dr. William Berg Dr. Robert Zeman	 **631-271-2310** **631-271-8090**
Doctor MediCenter 234 W. Jericho Tpke., Huntington Station	**631-423-5400** (**1034**)
Drugs - 24 hours, 7 days Rite-Aid 391 W. Main Street, Huntington	**631-549-9400** (**1039**)

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Dial the four numbers (********) from any **tan house phone** to place a free call.

GENERAL INFORMATION

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BOOKSTORE 367-8837 (hours posted on door)
Located in Grace Auditorium, lower level.

Photocopiers, Journals, Periodicals, Books, Newspapers
Photocopying – Main Library
Hours: 8:00 a.m. – 9:00 p.m. Mon-Fri
10:00 a.m. – 6:00 p.m. Saturday
Helpful tips - Obtain PIN from Meetings & Courses Office to enter Library after hours. See Library staff for photocopier code.

Computers, E-mail, Internet access
Grace Auditorium
Upper level: E-mail only
Lower level: Word processing and printing.
STMP server address: mail.optonline.net
To access your E-mail, you must know the name of your home server.

Dining, Bar
Blackford Hall
Breakfast 7:30–9:00, Lunch 11:30–1:30, Dinner 5:30–7:00
Bar 5:00 p.m. until late
Helpful tip - If there is a line at the upper dining area, try the lower dining room

Messages, Mail, Faxes

Message Board, Grace, lower level

Swimming, Tennis, Jogging, Hiking

June–Sept. Lifeguard on duty at the beach. 12:00 noon–6:00 p.m. Two tennis courts open daily.

Russell Fitness Center

Dolan Hall, west wing, lower level
PIN#: **Press 64330 (then enter #)**

Concierge

On duty daily at Meetings & Courses Office.
After hours – From tan house phones, dial x8870 for assistance

Pay Phones, House Phones

Grace, lower level; Cabin Complex; Blackford Hall; Dolan Hall, foyer

CSHL's Green Campus

Cold Spring Harbor Laboratory is pledged to operate in an environmentally responsible fashion wherever possible. In the past, we have removed underground oil tanks, remediated asbestos in historic buildings, and taken substantial measures to ensure the pristine quality of the waters of the harbor. Water used for irrigation comes from natural springs and wells on the property itself. Lawns, trees, and planting beds are managed organically whenever possible. And trees are planted to replace those felled for construction projects.

Two areas in which the Laboratory has focused recent efforts have been those of waste management and energy conservation. The Laboratory currently recycles most waste. Scrap metal, electronics, construction debris, batteries, fluorescent light bulbs, toner cartridges, and waste oil are all recycled. For general waste, the Laboratory uses a "single stream waste management" system, removing recyclable materials and sending the remaining combustible trash to a cogeneration plant where it is burned to provide electricity, an approach considered among the most energy efficient, while providing a high yield of recyclable materials.

Equal attention has been paid to energy conservation. Most lighting fixtures have been replaced with high efficiency fluorescent fixtures, and thousands of incandescent bulbs throughout campus have been replaced with compact fluorescents. The Laboratory has also embarked on a project that will replace all building management systems on campus, reducing heating and cooling costs by as much as twenty-five per cent.

Cold Spring Harbor Laboratory continues to explore new ways in which we can reduce our environmental footprint, including encouraging our visitors and employees to use reusable containers, conserve energy, and suggest areas in which the Laboratory's efforts can be improved. This book, for example, is printed on recycled paper.

1-800 Access Numbers

AT&T	**9-1-800-321-0288**
MCI	**9-1-800-674-7000**

Local Interest

Fish Hatchery	631-692-6768
Sagamore Hill	516-922-4447
Whaling Museum	631-367-3418
Heckscher Museum	631-351-3250
CSHL DNA Learning Center	x 5170

New York City

Helpful tip -

Take Syosset Taxi to Syosset Train Station ($8.00 per person, 15 minute ride), then catch Long Island Railroad to Penn Station (33rd Street & 7th Avenue). Train ride about one hour.

TRANSPORTATION

Limo, Taxi

Syosset Limousine	516-364-9681 (**1031**)
Super Shuttle	800-957-4533 (**1033**)
To head west of CSHL - Syosset train station	
Syosset Taxi	516-921-2141 (**1030**)
To head east of CSHL - Huntington Village	
Orange & White Taxi	631-271-3600 (**1032**)
Executive Limo	631-696-8000 (**1047**)

Trains

Long Island Rail Road	822-LIRR
Schedules available from the Meetings & Courses Office.	
Amtrak	800-872-7245
MetroNorth	800-638-7646
New Jersey Transit	201-762-5100

Ferries

Bridgeport / Port Jefferson	631-473-0286 **(1036)**
Orient Point/ New London	631-323-2525 **(1038)**

Car Rentals

Avis	631-271-9300
Enterprise	631-424-8300
Hertz	631-427-6106

Airlines

American	800-433-7300
America West	800-237-9292
British Airways	800-247-9297
Continental	800-525-0280
Delta	800-221-1212
Japan Airlines	800-525-3663
Jet Blue	800-538-2583
KLM	800-374-7747
Lufthansa	800-645-3880
Northwest	800-225-2525
United	800-241-6522
US Airways	800-428-4322