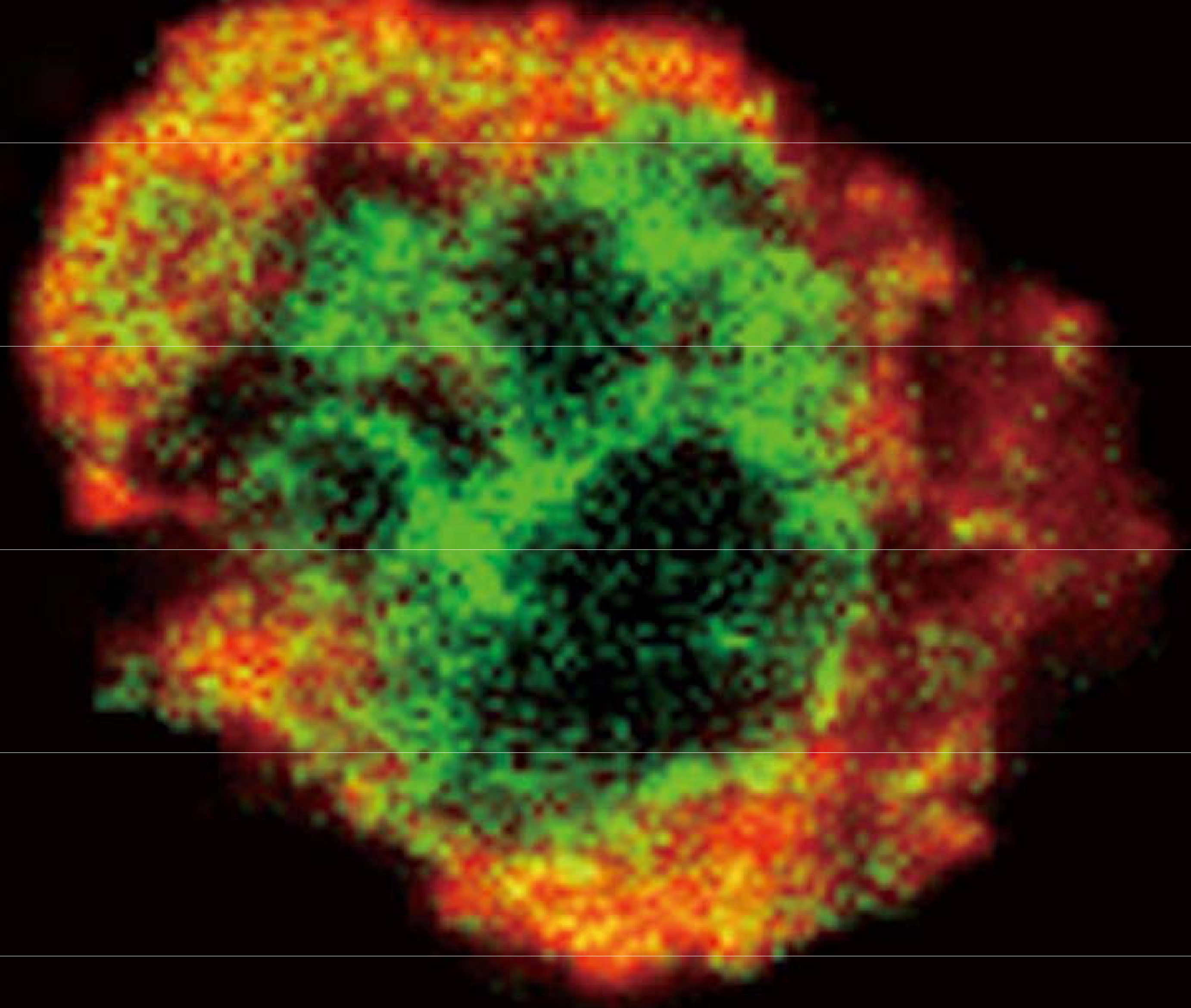
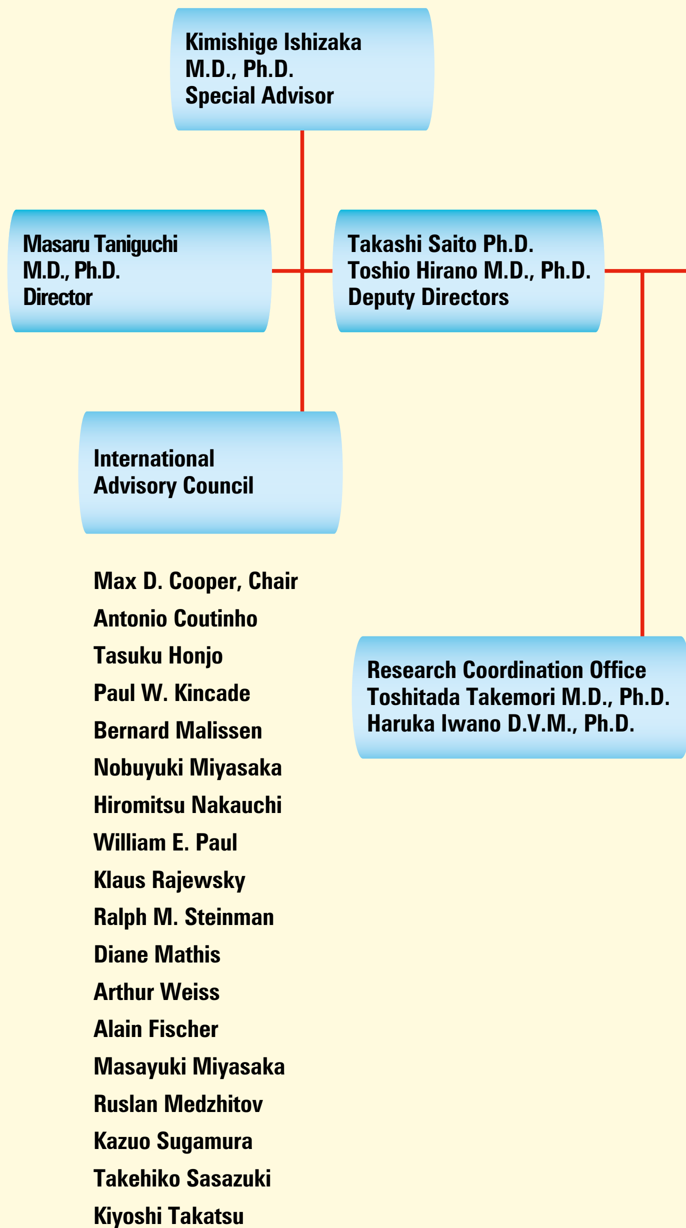


RIKEN RCAI Annual Report 2006

RIKEN Research Center for Allergy and Immunology



Research Center for Allergy and Immunology (RCAI) Organization

Core Research Program

- Lab. for Cell Signaling **Takashi Saito Ph.D.**
- Lab. for Lymphocyte Differentiation **Tomohiro Kurosaki M.D., Ph.D.**
- Lab. for Immunogenomics **Osamu Ohara, Ph.D.**
- Lab. for Developmental Genetics **Haruhiko Koseki M.D., Ph.D.**
- Lab. for Immune Regulation **Masaru Taniguchi M.D., Ph.D.**
- Lab. for Autoimmune Regulation **Osami Kanagawa M.D., Ph.D.**
- Lab. for Cytokine Signaling **Toshio Hirano M.D., Ph.D.**
- Lab. for Immunological Memory **Toshitada Takemori M.D., Ph.D.**

Strategic Research Fundamental Focus

- Lab. for Antigen Receptor Diversity **Jiyang O-Wang Ph.D.**
- Lab. for Signal Network **Masato Kubo Ph.D.**
- Lab. for Immunochaperones **Hei-ichiro Udono M.D., Ph.D.**
- Lab. for Mucosal Immunity **Sidonia Fagarasan M.D., Ph.D.**
- Lab. for Lymphocyte Development **Hiroshi Kawamoto M.D., Ph.D.**
- Lab. for Epithelial Immunobiology **Hiroshi Ohno M.D., Ph.D.**
- Lab. for Innate Cellular Immunity **Masato Tanaka M.D., Ph.D.**
- Lab. for Host Defense **Tsuneyasu Kaisho M.D., Ph.D.**
- Lab. for Dendritic Cell Immunobiology **Katsuaki Sato Ph.D.**
- Lab. for Infectious Immunity **Satoshi Ishido M.D., Ph.D.**
- Lab. for Transcriptional Regulation **Ichiro Taniuchi M.D., Ph.D.**
- Lab. for Immunogenetics **Hisahiro Yoshida M.D., Ph.D.**

Strategic Research Fundamental Focus

- Unit for Single Molecule Immunoimaging **Makio Tokunaga Ph.D.**
- Unit for Human Disease Model **Fumihiko Ishikawa M.D., Ph.D.**
- Unit for Allergy Transcriptome **Hirohisa Saito M.D., Ph.D.**

Strategic Research Clinical Focus

- Lab. for Vaccine Design **Yasuyuki Ishii Ph.D.**
- Unit for Cellular Immunotherapy **Shin-ichiro Fujii M.D., Ph.D.**
- Unit for Clinical Immunology **Hitoshi Kohsaka M.D., Ph.D.**

Adjunct Research Program

- Unit for Immune Surveillance **Takeshi Watanabe M.D., Ph.D.**
- Unit for Immune Homeostasis **Shohei Hori Ph.D.**

International Research Program

- Unit for Immuno-imaging **Michael Dustin Ph.D.**
- Unit for Lymphocyte Recognition **Facundo Damian Batista Ph.D.**
- Unit for Immunogenetics **Miguel Vidal Ph.D.**
- Unit for Immuno-pathlogy **Kenneth M. Murphy Ph.D.**
- Unit for B Cell Biology **Peter D. Burrows Ph.D.**
- Unit for Cytokine Gene Regulation **Mark Bix Ph.D.**
- Unit for Thymic Environment **Willem van Ewijk Ph.D.**
- Unit for Inflammatory Response **Michael Karin Ph.D.**
- Unit for Thymocyte Differentiation **Wilfried Ellemeier Ph.D.**
- Unit for Immune Tolerance **Yun-Cai Liu Ph.D.**

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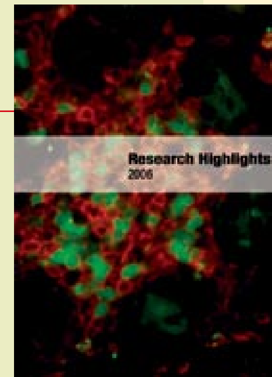

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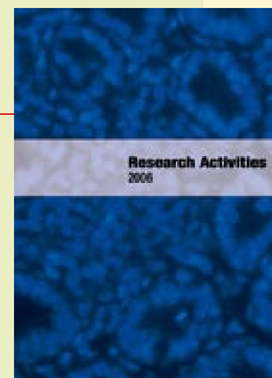

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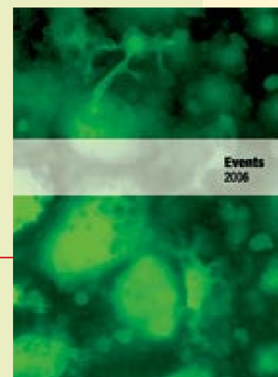

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Director's Report

This is the third Annual Report of the RIKEN Research Center for Allergy and Immunology (RCAI). Almost five years have now passed since I was first approached regarding the RCAI directorship by the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) and RIKEN, and it was almost three years ago that the research groups moved into the new building at the RIKEN Yokohama Institute. The second full meeting of the RCAI Advisory Council provided the initial assessment of our progress.

Second RCAI Advisory Council Meeting

The RCAI Advisory Council is a crucial component in managing the Center. Every laboratory at RCAI is reviewed on an annual basis by a team of 2-3 domain experts. These annual reviews are intersected by a full meeting of the RCAI Advisory Council every 2-3 years. In April 2006, the full RCAI Advisory Board met for the second time and asserted that the Center had "kept its promises". In the conclusion of their report, the RCAI Advisory Council said that "although RCAI moved into the new facilities at the RIKEN Yokohama Institute barely two years ago, the achievements at the Center are impressive and the researches undertaken at the Center are outstanding". In addition, the Advisory Council also broadly endorsed our future research strategy, most notably new efforts centering on humanized mouse models and our plan to build a regional database of human immunodeficiency patients in Asia. With its call to reinforce basic science, both in areas of strength and in those areas where RCAI has a clear research "mission", such as the fight against allergy, the recommendations of the Advisory Council are very much in line with the core research strategy of the Center.

Some concerns were voiced by the Advisory Council with respect to the involvement of RCAI in pre-clinical or clinical research. Although the strength of the Center is in basic science and not in applied medical research, an explicit and stated goal of the Center is to contribute proactively to the development of new therapeutic approaches. In line with the recommendations, we plan to do so by aggressively building linkages and networks with clinical scientists and industry. In this context, we have established two areas in addition to basic programs, a program from basic to medication (translational section) and the medical application program. The former includes projects using humanized mice, with the goal of developing new therapies for leukemia and primary immunodeficiency diseases, and the establishment of artificial lymphoid organs. The medical application program is targeted toward vaccine development in allergy and immunological therapy for cancer and is being done in close collaboration with university hospitals. We actively support such studies through our basic experimental research programs and by the development of methodologies and evaluation techniques that are so crucial in translational research.

In the summary of their report, the Advisory Council also stated that its members were "impressed by the rapid and appropriate RCAI response to the Advisory Council scientific evaluations and advice". Working with the Advisory Council has been extremely helpful for my work as director and we at RCAI are certainly extremely fortunate to be able to rely on such a knowledgeable and experienced group of scientists.

New Focus Areas : Advanced Imaging and Bioinformatics

In last year's report, I mentioned our single molecule imaging program as an example of a highly successful activity within the framework of RCAI's Central Facilities. The extremely positive

response to this program has convinced me of the importance of further strengthening advanced imaging activities at the Center and I am pleased that the RCAI Advisory Council has also noted in its report that imaging is an area where RCAI's basic research capabilities and Central Facilities platform provide for a superb environment for future world class research.

The value of a novel research technique also depends upon its "usability" and simply upon the number of scientists who have access to it. New enhancements of the basic technique, such as multi-color fluorescent imaging, together with novel fluorescent dyes developed at RIKEN and sophisticated new software tools developed by the Research Unit for Single Molecular Immunoimaging in cooperation with the Olympus Corporation, will make the technology available to a much larger number of non-expert users.

We will continue to invest into technology development and our goal over the next years is to build up an extensive imaging focus at the Center, providing access to the most advanced techniques in single-molecule imaging, single-cell imaging, and *in vivo* imaging. Based on these continuing investments and partnerships with scientists and instrumentation companies worldwide, I believe there is a real opportunity for RCAI to become the one of the world's leading centers for advanced imaging research in immunology.

I would also like to mention our increased investment into bioinformatics. Last year we established a new database tool, Reference Database for Immune Cells (RefDIC) [http://refdic.rcai.riken.jp/welcome.cgi]. RefDIC is a database with a host of "omic" data on various types of immune cells that has been prepared by the Laboratory for Immunogenomics in cooperation with various users within RCAI. While far from complete, RefDIC offers a broad variety of mRNA and protein profiles for a growing number of immune cell types and this collection will continuously expand over the next few years. Over time, and as more data and also different types of data are added, RefDIC will become a powerful informatics platform for immunologists worldwide.

Over the past year, we have also been strengthening our bioinformatics capabilities through various domestic and international partnerships. In this context, we mention here especially a partnership with the leading bioinformatics center in India through which RCAI scientists will gain access to advanced bioinformatics services. Various other projects at the Center also fall within the area of bioinformatics. I have already mentioned a project to develop an Asia-wide database of human immunodeficiency patients that eventually should be linked to both clinical and genetic information. Such a database will be of immense value to translational research in many areas of immunology. And again, these new investments are backed by the recommendations of RCAI's Advisory Council.

RCAI's New Research Coordination Office (RCO)

Another innovation at RCAI that has received high marks from the Advisory Council is the Center's Research Coordination Office (RCO). The primary role of the RCO is to provide professional support to scientists in all aspects of grant acquisition and management. In addition, the RCO is responsible for a broad variety of activities, including evaluation, dedicated support for postdoctoral fellows and young scientists, planning of events and education activities, and even outreach and public relations.

One important motivation to set up the RCO relates to research funding. RIKEN's budget is set to shrink rather than increase. This annual reduction in base funding allocations is not particular to RIKEN or RCAI. Rather, it is motivated by the fact that most research organizations, including RIKEN, also fall under legislation which requests a minimum annual decrease in base funding of at least 1% per year.

In order to increase research activities, we now need to compete for funding from both internal sources (such as RIKEN) and external grant programs. When we started RCAI, the total amount of external funding acquired by all research groups, which, during 2001-2003 were still primarily located at their respective universities, was well over JPY 800 million (US$ 7 million). After moving to the Center, external funding decreased somewhat to less than US$ 5 million per year. Now, with base funding of the institute declining for the second year in a row, there is little choice but to raise our share of competitive funding again. But, while the large, experienced research groups have considerable experience in funding acquisition, the same is not true for most of the smaller groups. The activities of the RCO are especially important in supporting young investigators as they compete for outside funding.

Hardly a year in its existence, the output of the RCO is already impressive. In the case of grant applications, over the past year the RCO has supported 19 applications for various types of grants under the highly competitive RIKEN President's Fund. In 2006, 4 applications from RCAI among a total of 226 applications from all of RIKEN have been funded for a total of over JPY 220 million (US$ 2 million). Since the average acceptance rate for all RIKEN applications was 12.0%, the RCAI acceptance rate (21.1%) was significantly higher. The RCO has also supported numerous applications to external grant programs and scientific prizes and the success rate has been well above average. In summary, the relatively small investment that has gone into setting up the RCO has already paid off handsomely. And, for me as the director of RCAI, the RCO is providing important support for a number of critical tasks.

A second rationale for setting-up RCO relates to the way research centers are administrated within RIKEN. At the Yokohama Institute, a generic administrative service provides highly component support to all four research centers located on the campus. RCAI has profited enormously from this core support. However, the focus within the RIKEN administration is on highly standardized tasks that are not different for any of the centers. Therefore it seemed necessary to create a small, versatile research management organization that caters more specifically to the needs of RCAI investigators and RCAI laboratories. RCO fulfills a number of crucial functions at the Center. To start with, RCO supports scientists with the acquisition of external funding. In Japan, this is an innovation. While a standard practice in the US and increasingly also in Europe, support staff for grant acquisition or grant management simply does not exist yet in Japan. I add here that RCO also serves as an interface with the RIKEN research promotion division. Secondly, RCO fulfills a variety of internal coordination tasks, from organizing regular seminars for internal postdoctoral fellows to the planning of our new RCAI International Summer Program in Immunology. Finally, RCO assists the director and RCAI management team in formulating a long-term research strategy for the Center. This is a most crucial task and, given the new funding environment for the Center, certainly not a trivial one.

While research organizations in Japan typically look at research management as yet another administrative function no different from accounting or facility management, at RCAI we have set the standards as high as the standards we set for hiring scientists. We have spent considerable time in selecting the best staff for the institute — PhD scientists with an experience in research and even a track record in immunological research. I am especially glad that Dr. Toshi Takemori (working on Immunological memory) has agreed to join the institute as both a research scientist and an

coordinator with broad duties. And, with Dr. Haruka Iwano, we have been able to recruit a young, aggressive research coordinator with a strong background in both research and scientific publishing.

Within RCAI, the role of the RCO will further expand over the next few years, and in Japan, RCO is simply unique. In fact, I have no doubt that this model of lean and efficient, yet highly professional support to bench scientists will set a new standard for research management in this country.

Young Scientists at RCAI

The Advisory Council was highly favorable in its review of our efforts to support young scientists.

My most important concern in building RCAI has always been with young scientists. Immunology is by now reasonably well established in Japan's universities. Yet, even today Japanese universities do not offer young scientists at the very beginning of their career enough breathing space to develop their own scientific identity, let alone to undertake challenging and risky research projects of their own. I strongly believe that the university system, while of course the main basis for public sector research in Japan, also remains an important limiting factor when I think about the future of biomedical research and immunology in Japan. Research organizations like RIKEN provide an alternative that, rather than competing with the universities, can complement the university research system.

What is most significant with RCAI is the focus on young investigators at the beginning of their career. Well over two-thirds of the research laboratories at the Center are lead by young investigators, many of whom are managing a research laboratory for the first time. With the strong focus on shared services and infrastructure and minimal hierarchy, the entire Center was designed to combine a maximum of support with a minimum of control for research teams lead by young investigators. This really distinguishes RCAI from universities where young research groups typically lack not only the freedom to pursue their own interests— but also the means to do so.

It has been our explicit policy to provide these young scientists with as much freedom as possible during their first few years at RCAI. For example, while all groups at the Center are evaluated annually, especially the younger groups are granted a period of five years during which they have all the freedom they need to concentrate on their research. The way we evaluate young scientists is not in any sense related to the number of publications.

Last year, I reported on a number of significant publications by many of the senior groups at the Center. This year, I am especially pleased to announce a number of important publications by several of the younger groups at the Center. The Center's publication record in 2006 provides clear evidence that some of the junior researchers have already clearly established themselves as leading scientists in their respective areas of specialization, e.g. Drs. T. Kaisho, S. Fagarasan, M. Tanaka, S. Ishido, K. Sato, T. Yokosuka, and F. Ishikawa published in Nature, J. Exp. Med., J. Clin. Inv., EMBO J., Blood, Nature Immunol., and Nature Rev. Immunol., respectively (see details below). Although RCAI's research highlights during the past two years were mostly by well-established groups, not only is the number of publications by junior groups increasing, several groups have now produced papers that, I believe, are not only of significant scientific value but are also highly innovative.

New Initiatives: Building a Global Presence

Throughout 2006, one of our main concerns had been to improve the research environment for young, talented scientists at the Center. By this I mean not simply those young scientists who occupy unit or team leader positions. Most of the actual research at RCAI is done by postdoctoral fellows who come to work at the Center for a number of years. By any definition, the output of the Center is highly dependent on the hundred scientists at the very beginning of their scientific careers. In addition, I am pleased to say that the number of graduate students working at the Center has now increased to well over 40. Yet, there is considerable room for improvement in the way we select and hire graduate students and postdoctoral fellows—and perhaps also in the way we care for them.

In order to better advertise the Center to students in their last years of graduate school, we have started a new International Summer School Program that was held for the first time last September. Despite very little lead-time for preparation, the RCAI International Summer Program 2006 was by any standard an important success for the Center. We selected 38 participants from 21 countries, and some of the participants were invited to stay for another week of laboratory training and practical research. This event would not have been possible without the outstanding efforts by a distinguished international faculty—which included several members of the RCAI Advisory Council—and I can only thank all those involved in this outstanding effort for their contributions. But, most of all, I must thank all these young and bright students for their participation, their enthusiasm, and the fresh perspective they brought to the RCAI.

Also, we have just launched a new initiative, the RIKEN RCAI Distinguished International Research Grant program, which aims at strategically recruiting foreign scientists, who will be helpful for development of RCAI activity. Our plan with this new program is also to link to the RCAI Collaborative Research Awards program. When launching the Collaborative Research Awards program in 2003, our goal was to recruit a small number of distinguished international scientists through existing collaboration networks rather than through an open competition. We chose this somewhat unusual approach for the simple reason that, at the time, the Center was brand new and little known outside of Japan. It is my conviction that, some three years later, RCAI has successfully established an international reputation for high quality immunology research. We are thus in a much better position to recruit some of the most brilliant young scientists today through a process of open competition. That said, I am fully aware that considerable efforts will be needed to make this new funding scheme a real success for the Center. After all, for a young, ambitious scientist the immediate merits of coming to Japan for a few years may seem fairly limited.

Still, I believe that RCAI offers unique opportunities for young scientists with an already well established research interest. RCAI is probably the best-equipped immunological research center in the world today. Home to more than 30 research groups in a broad variety of specialties, RCAI offers a highly productive environment for research in many areas of immunology. It is our goal with this new program to offer the best young investigators access to the Center and all its resources. And, in turn, it is my hope that the presence of these young scientists will make RCAI in an ever more open and accommodating place—and a true “Center” of world-class research!

Masaru Taniguchi
March, 2007

Research Highlights
2006

Missing link in antiviral immune response revealed

Deciphering of a key step in a biochemical pathway is a mighty leap forward in understanding how mammalian immune systems work

Tsuneyasu Kaisho

Katsuaki Hoshino

Immunologists at RIKEN have characterized a key component of the biochemical chain of events that leads to the release of interferon-α, an antiviral factor, following detection of viruses by specialized sensors expressed on the surface of dendritic cells, a kind of immune cell.

Humans and other animals are born with a sophisticated defence mechanism against viral and other microbial invasions. This so called 'innate immune response' consists of first detecting an invading particle via specific, preprogrammed sensors, also called 'receptors', followed by transmission of the signal through a unique intracellular signalling pathway, a 'signalling cascade', which leads to a 'first line of defence' immune response such as the release of interferon-α.

Tsuneyasu Kaisho from RIKEN's Research Center for Allergy and Immunology in Yokohama and colleagues from the University of Tokushima, the Research Institute for Microbial Diseases and ERATO in Osaka and Kazusa DNA Research Institute in Kisarazu, Japan, have worked out the key molecular step that mediates interferon induction by two members of the Toll-like receptor (TLR) family, TLR7 and TLR9. These receptors specialize on detecting singlestranded RNA and DNA viruses such as the influenza virus and herpes virus, respectively.

As reported recently in *Nature*, Kaisho and colleagues have now determined that a kinase, IκB kinase-α, an enzyme that could potentially activate IRF7 by chemically attaching a phosphate group to it, is the key player in this step. In a series of experiments with human kidney cells, the RIKEN authors first show that IRF7 is indeed modified by IκB kinase-α. They then proceed to demonstrate that an inactivated version of the kinase inhibits MyD88-induced interferon- α production. Finally, they prove that in mice lacking IκB kinase- α, interferon- α production is greatly reduced.

The mechanistic insights gained by Kaisho and colleagues could aid the design of drugs that activate or block the TLR7- and TLR9-induced interferon-α pathway. This would be useful in the treatment of viral infections where interferon-α serves as an antidote. In addition, the treatment of autoimmune diseases such as lupus, in which the interferon response is constantly activated, could benefit from drugs that reduce IκB kinase-α activity.

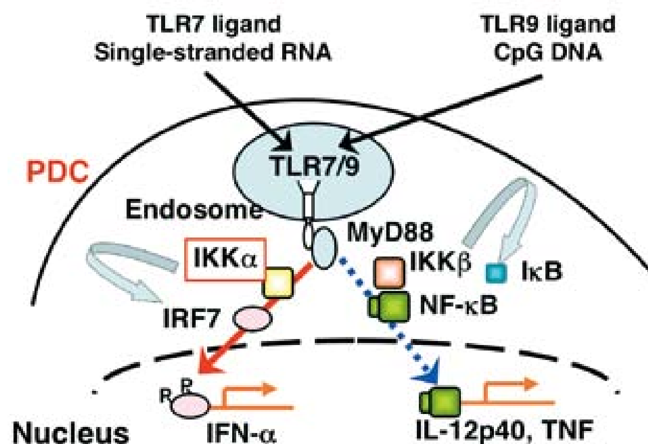

Figure TLR7/9 signaling mechanism in PDC.
TLR7/9 signaling depends on a cytoplasmic adapter, MyD88. Downstream of MyD88, the pathways split into the NF-κB and IRF-7 activation pathways. NF-κB is activated by IKKβ which can induce phosphorylation and degradation of IκB. This pathway is critical for the induction of inflammatory cytokines such as IL-12p40. IRF-7 is phosphorylated and activated by IKKα. This pathway is critical for the induction of type I IFN.

ORIGINAL RESEARCH PAPER

Hoshino, K. et al. IκB kinase-α is critical for interferon-α production induced by Toll-like receptors 7 and 9. *Nature* 440: 949 53 (2006).

FURTHER READING

T. Kaisho, S. Akira. TLR function and signaling. *J. Allergy Clin. Immunol.*117:979-987 (2006).

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Uncovering a secret messenger

Scientists reveal first evidence that zinc plays an active role in immune response and, possibly, cellular signaling

Toshio Hirano

It is well-established that zinc is vital to cellular function, although it has long been assumed that this is primarily due to its role as a cofactor for a variety of essential proteins, including metabolic enzymes and transcription factors. However, recent research by a team from the RIKEN Research Center for Allergy and Immunology, in Yokohama, and Osaka University suggests that zinc may be far more than just a passive biological bystander.

In 2004, the team, led by Toshio Hirano, found that proper cell migration during zebrafish development is mediated in part by increased expression of a zinc transporter protein in response to cytokine signaling. This surprising finding led the investigators to explore whether zinc regulation might also play a role in the maturation of dendritic cells (DCs). This is the process by which these cells present foreign antigens to T cells to trigger a cellular immune response. This process is also a major focus of Hirano's team.

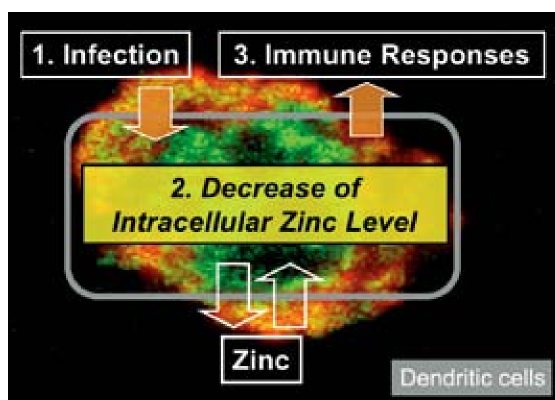

Figure A schematic of zinc's role in mediating immune response in dendritic cells (red = a fluorescent indicator of dendritic cell maturation; green = a fluorescent indicator of zinc).

Initial experiments showed that exposure of isolated mouse DCs to the bacterial endotoxin lipopolysaccharide (LPS), a stimulant of immune response, led to a decrease in intracellular zinc concentration; by the same token, artificial depletion of intracellular zinc triggered DC maturation (Figure). Artificial elevation of zinc levels, on the other hand, suppressed the ability of DCs to respond to LPS. Hirano and colleagues found that LPS affects the expression of a number of zinc import and export proteins, with the net effect of increased zinc transport out of the cell. The group observed similar effects in live animals, where injection of LPS or zinc-depleting agents led to increased DC maturation.

According to Hirano, this work offers important progress in understanding zinc's role in the immune system. "It is known that zinc deficiency causes immunodeficiency, but these observations are all phenotypic ones—no precise mechanisms are known," says Hirano. "This is the first evidence that zinc plays an active role in immune response."

However, the implications could go well beyond the immune system, and these data may offer evidence of a previously unrecognized mode of cellular signaling. "Our findings indicate that the level of intracellular free zinc changes in response to extracellular stimuli, such as cytokines and growth factors. This strongly suggests that zinc acts as a signaling molecule like calcium," he says. "If this process can be generalized to other cell types, this would be one of the most exciting findings in the field of signaling pathways."

Hidemitsu Kitamura

ORIGINAL RESEARCH PAPER

Kitamura, H., Morikawa, H., Kamon, H., Iguchi, M. Hojyo, S., Fukada, T., Yamashita, S., Kaisho, T., Akira, S., Murakami, M. & Hirano, T. Toll-like receptore-mediated regulation of zinc homeostasis influences dendritic cell function. *Nature Immunology* 7, 971–977 (2006).

FURTHER READING

Yamashita, S., Miyagi, C., Fukada, T., Kagara, N., Che, Y.S. & Hirano, T. Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. *Nature* 429, 298–302 (2004).

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Mobilizing front-line immune defenses

New data highlight ways in which immune cells are quickly pulled into the fight against microbial intruders

Sidonia Fagarasan

Researchers report fresh insight into the events required for rapid recruitment of immune cells, called B1 lymphocytes, to lymphoid organs. In quiescent conditions, B1 lymphocytes reside between the membranes lining the abdominal cavity—the peritoneum. Post-infection, these immune cells are among the first to arrive in lymphoid organs, where later phases of the immune response develop. Due to their exquisite sensitivity to the presence of immune 'danger signals', B1 lymphocytes constitute ideal first responders.

Now a research team led by Sidonia Fagarasan, a scientist at RIKEN's Research Center for Allergy and Immunology in Yokohama, has determined precisely how B1 cells sitting within the peritoneum quickly traffic towards immune responses developing at distant locales throughout the body.

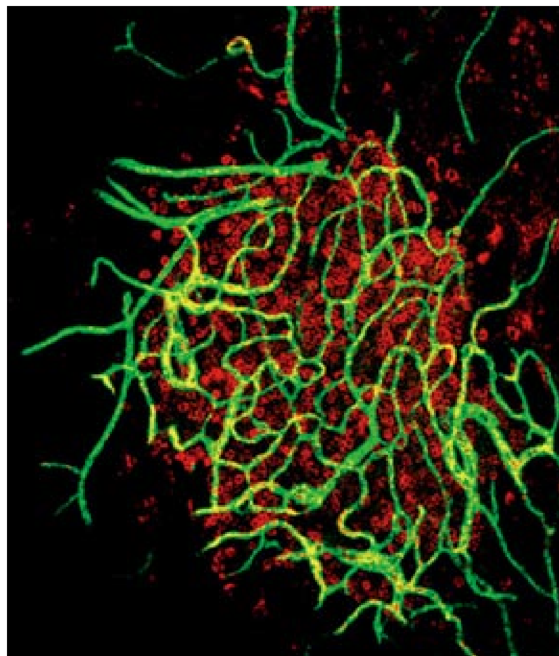

Figure An image of omentum, the exit route from the peritoneal cavity, with B1 cells (red) and blood vessels (green).

To simulate conditions of an infection, the team injected mice with a drug that injures the gut epithelium, thereby allowing microbes normally present within the small intestine to penetrate tissues close to the peritoneum. Dramatically, within six hours of drug injection, a substantial portion of B1 cells proceeded from the peritoneum to the spleen and gut-associated lymphoid organs (Figure).

The researchers hypothesized that proteins decorating the surface of bacteria might somehow provoke B1 cells to leave the peritoneum. Indeed, B1 cells departed the peritoneum after injection of lipid A, a component of many types of bacteria. In contrast, B1 cells lacking the sensor required for recognition of lipid A, known as Toll-like receptor 4, remained in the peritoneum even after lipid A injection.

Seon-ah Ha

The team also revealed the underlying mechanism. B1 lymphocytes express high amounts of proteins that allow cells to adhere or stick to tissues. However, exposure to lipid A triggered a drop in the expression of two such adhesion proteins known as integrins and CD9. Blockade of these adhesion proteins, even in the absence of bacterial components, allowed B1 cells to 'disengage' and leave the peritoneum.

However, B1 cell egress from the peritoneum was not the result of passive drift alone. After injection of bacterial components, cells lining the B1 cell departure route ramped up production of soluble proteins called chemokines, which actively attract B1 cells. Accordingly, B1 cells unable to receive chemokine signals remained within the peritoneum.

Unraveling the complexity of the series of events required to disengage B1 cells from the peritoneum presents multiple opportunities for therapeutic intervention. "These findings will open a new phase in immunology and hopefully in cancer research, because adhesion proteins on tumor cells may be regulated by similar mechanisms," says Fagarasan.

ORIGINAL RESEARCH PAPER

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Death to allergy-inducing cells

How attenuated tuberculosis infection stems allergy

Masaru Taniguchi

Michishige Harada

Worldwide—but particularly in industrialized countries—incidence of allergy is on the rise. Although the reasons for this are not clear, some scientists have speculated that excessive use of antibiotics and heightened cleanliness prevent beneficial infections early in life that would normally drive immune system development in a way that lessens the possibility of allergy in adulthood.

Michishige Harada in the team led by Masaru Taniguchi now demonstrates that vaccination with *Mycobacterium bovis* bacillus Calmette Guerin (BCG), the current vaccine against human tuberculosis, stimulates the immune system to dampen allergic responses. Working with mice as a well-characterized model of induced allergy, the team characterized the types of immune cells that respond to BCG vaccination. “BCG, ethically approved in Japan, was used to mimic bacterial infection in allergy patients or healthy volunteers,” says Taniguchi. The team found that NKT cells—a special subset of immune cells found especially in the liver—were responsible for the anti-allergy effect. Using genetically modified mice that lack all NKT cells, they demonstrated that NKT cells are required to suppress allergy after BCG vaccination. They also found that interleukin 21 (IL-21) expressed by the NKT cells was important, and that another cytokine, IL-12 made by dendritic cells that respond directly to BCG, was required to stimulate the NKT cells to produce IL-21. Exactly how BCG stimulates dendritic cells is not clear. “Because BCG is a live vaccine, many candidate BCG proteins can stimulate dendritic cells,” says Taniguchi. “We are currently collaborating with biochemists to purify BCG-derived products and to identify essential molecules.” The key to control of allergy after BCG vaccination is the IL-21 produced by NKT cells, which affects allergy-producing B cells in a way that causes them to die.

The team found that IL-21 induces activation of the death-promoting factor Bmf in Bε cells, which blocks the death-preventing factor Bcl-2 from keeping the cells alive (Figure). The importance of the study by Harada et al. lies in the characterization of mechanism by which BCG decreases allergy, which may have therapeutic implications. It also shows that infection (or vaccination) can reduce allergy. “It is easy to imagine that the IL-21 pathway in B cells provides a target for developing anti-allergy drugs,” notes Taniguchi. “If we can successfully identify a low molecular weight compound or a small molecule that stimulates the pathway, we can develop a drug for a treatment of allergy.”

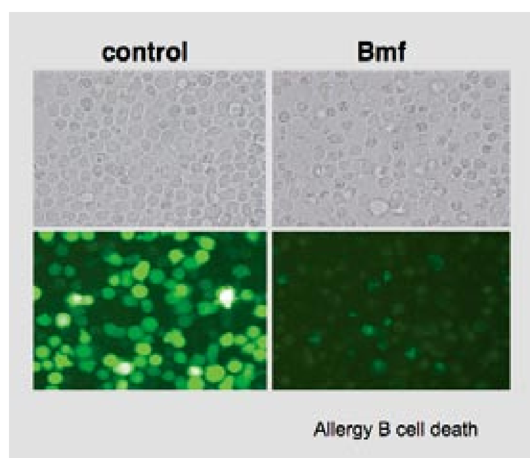

Figure A microscopic image showing that allergy-inducing B cells (green cells, left) undergo cell death when the death promoting factor Bmf is active (right).

ORIGINAL RESEARCH PAPER

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A DNA sequence not to be sneezed at

A sequence of DNA required for generating specialized lymphocytes regulates onset of allergy and asthma

Masato Kubo

Shinya Tanaka

In the June issue of *Immunity*, a team led by Masato Kubo, at the RIKEN Research Center for Allergy and Immunology in Yokohama, characterize a DNA sequence in lymphocytes responsible for controlling the development of a specific type of 'T helper lymphocyte' commonly associated with allergy and asthma.

Allergic diseases occur when the immune system is out of balance. Many of the mechanisms responsible for allergy and asthma are well-known and include over-production of immunity mediators such as interleukins, key signaling molecules in the immune system, and fast-acting histamine, which causes sneezing and constricted airways. T lymphocytes in particular are important producers of interleukins.

Inflammation leading to allergy and asthma can occur if too many specialized T lymphocytes called T helper type 2 (Th2) cells are generated. Th2 cells develop from naïve T cells, or T cells responding to newly encountered pathogens or allergens, after a program of differentiation that triggers the production of interleukins, such as IL-4, IL-5, IL-9 and IL-13. In large amounts these interleukins predispose people to allergy and asthma.

The critical finding by the team is that without the initial production of IL-4 by the memory $CD4^+$ T cells, the generation of Th2 cells does not occur. They also show that the specific DNA sequence required for IL-4 production in the memory $CD4^+$ T cells is regulated by the Notch pathway—a signaling pathway known to be important for development (Figure).

Determining that the Notch pathway regulates the DNA sequence in the memory $CD4^+$ T cells required for generating Th2 cells may provide a target for therapeutically regulating the development of Th2 cells and allergic responses. According to Kubo, "the memory $CD4^+$ T cells uniquely use the Notch pathway for IL-4 production," which suggests that it may be possible to specifically target the cells therapeutically and develop treatments for debilitating forms of Th2-mediated diseases like asthma, the incidence of which continues to increase.

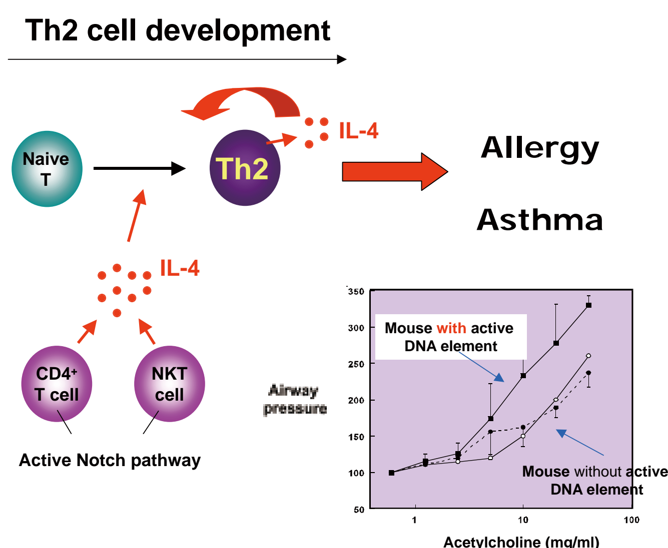

Figure a) Notch signal regulates primary IL-4 production from $CD4^+$ and NKT cells through the unique DNA sequence in those cells. The IL-4 produced primes the differentiation of Th2 cells from naive T cells. The Th2 cells then produce IL-4 and other factors that mediate allergy and asthma.
b) The graph shows data from an experiment in which mice that have the IL-4-producing memory $CD4^+$ cells have more airway obstruction than mice that don't have the cells.

ORIGINAL RESEARCH PAPER

Tanaka, S., Tsukada, J., Suzuki, W., Hayashi, K., Tanigaki, K., Tsuji, M., Inoue, H., Honjo, T., & Kubo, M. The interleukin-4 enhancer CNS-2 is regulated by Notch signals and controls initial expression in NKT cells and memory-type CD4 T cells. *Immunity* 24, 689-701 (2006).

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Regulating shock: immune cells that protect

Researchers find that an immune cell can protect against a deadly form of inflammation

Katsuaki Sato

When immune responses are effective against a viral or a bacterial infection beneficial amounts of inflammation occur, leading to elimination of the pathogen but also to some familiar side effects, including fever, chills and muscle aches. In rare situations, however, inflammatory responses can build to the danger point, called shock. Sepsis—a type of shock—occurs when inflammation is systemic and dangerous, potentially leading death. In the May 1st issue of *Blood*, Katsuaki Sato and colleagues at the RIKEN Research Center for Allergy and Immunology in Yokohama describe a type of immune cell that can protect against shock, which, in principle, could be used clinically to treat this deadly form of 'hyper' inflammation.

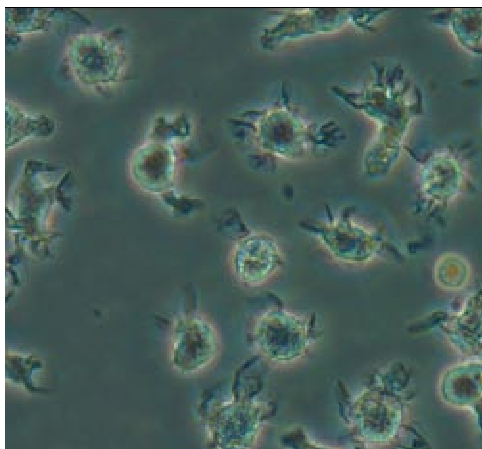


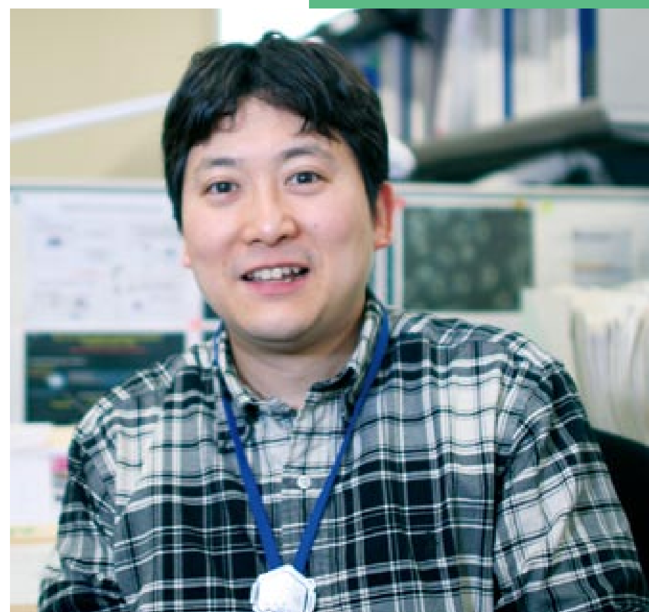
Figure Mrophology of dendritic cells

The immune cells in question are a subtype of dendritic cells, a family of cells that act as immune sentinels and initiate immune responses (Figure). Of the several subtypes of dendritic cells, Sato and colleagues work with ones that, instead of priming immune responses via expression of particular cell surface molecules, promote immune suppression in other immune cells. Using various mouse models of septic shock, the authors show that mice given the so called 'regulatory' dendritic cells before inducing shock prevents it.

How the regulatory dendritic cells mediate the suppression is through production of cytokines such as interleukin 10. Cytokines are intercellular mediators of immune responses. In certain circumstances, interleukin 10 reduces inflammation. Moreover, production of interleukin 10 is correlated with known intracellular signalling pathways in the regulatory dendritic cells that are associated with suppression. Thus, unlike their stimulatory cousins, regulatory dendritic cells are 'programmed' to provide suppression signals to other immune cells.

A frequent cause of septic shock is the accumulation of high amounts of bacterial components that over-stimulate the immune system. Infections that initially cause appendicitis, for example, can lead to an infection called peritonitis that is caused by bacterial invasion of the gut. If untreated, peritonitis can cause excessive stimulation of gut cells, sepsis and death. Sato and colleagues test the ability of regulatory dendritic cells to prevent shock in a mouse model of peritonitis that closely resembles the clinical syndrome in humans and find them to provide significant protection.

The therapeutic implications of the work by Sato and colleagues include clinical use of the regulatory dendritic cells in patients who suffer from acute shock. In principle, the dramatic effects seen in the mouse models mean that similar results could be seen in humans.



Shigeharu Fujita

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Understanding optimized immune therapy

A single agent efficiently activates multiple arms of the immune response

Shin-ichiro Fujii

In clinical trials worldwide, therapies capable of harnessing the power of the immune system show promise in treating infections and malignancies.

At the RIKEN Research Center for Allergy and Immunology in Yokohama, Shin-ichiro Fujii and colleagues have identified molecular mechanisms underlying the potent immunostimulatory capability of one such therapy. Their focus was on the glycolipid α-galactosylceramide (α-GalCer), which triggers strong protective immune responses.

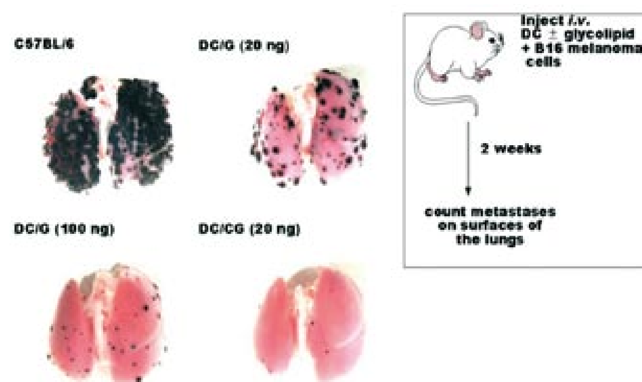

Figure α-C-GalCer inhibits tumor metastasis in mice. When co-injected with tumor cells, DCs pulsed with low doses of α-C-GalCer (CG/DC) effectively suppress the spread of tumor cells (blue spots) to the lungs. Higher doses of α-GalCer (G/DC) are required to achieve the same effect.

Within the body, 'foreign' substances such as microbes or abnormal tumor cells are captured by and displayed on the surface of dendritic cells (DCs). Passing immune cells called natural killer T (NKT) cells are equipped with sensors that allow them to recognize bits of foreign substances decorating the surfaces of DCs. NKT cells that detect foreign substances relay warning signals, in the form of cytokines and co-stimulatory molecules. These signals alert other immune cells to the presence of 'non-self'.

It has been established that when α-GalCer is presented by DCs, it activates NKT cells, and exhibits therapeutic activity in mouse models of cancer and infectious disease. However, a slightly modified version of α-GalCer, called α-C-GalCer, exhibits 100 and 1,000-fold greater potency in fighting tumor metastases and malaria infections, respectively.

Fujii and colleagues examined the consequences of intravenous injections of graded amounts of α-GalCer or α-C-GalCer into mice (Figure). As reported in the *Proceedings of the National Academy of Sciences*, they found that substantially lower doses of α-C-GalCer were needed to effect release of cytokines from DCs and NKT cells. Further, α-C-GalCer selectively amplified production of cytokines that are particularly adept at killing transformed and infected cells.

Although both glycolipids were equally capable of promoting the appearance of co-stimulatory molecules on the surface of DCs, less α-C-GalCer was needed to trigger expression of co-stimulatory molecules on NKT cells.

When co-injected with tumor cells, low amounts of α-C-GalCer were sufficient to elicit immune protection against subsequent challenges with tumor cells.

Less time was required to 'load' α-C-GalCer onto DCs, and DCs loaded with α-C-GalCer retained their ability to stimulate NKT cells for a prolonged period of time. "These observations suggest that more efficient and stable presentation underlie the enhanced potency of α-C-GalCer, although factors other than stability might also contribute," says Fujii.

The team's work highlights facets of the immune response that correlate with clinical efficacy, which may be important parameters to consider during future experiments aimed at designing optimal immune therapies.

Kanako Shimizu

ORIGINAL RESEARCH PAPER

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Artificial lymph node transplanted into mice

Takeshi Watanabe

An artificial lymph node has been transplanted into mice, where it successfully produced immune cells. The new form of bioengineered tissue marks a significant step towards transplanting an entire immune system into patients dying of AIDS, cancer or other diseases, say the researchers who carried out the transplant.

Takeshi Watanabe at the RIKEN Institute in Japan and colleagues used a "bioscaffold" made of collagen impregnated with stromal and dendritic cells extracted from the thymus of newborn mice. The entire package – a collagen sponge about 3 to 4 millimetres across – was then implanted into mice with healthy immune systems that had been vaccinated against a harmless antigen (something that triggers an immune response).

In a natural lymph node, stromal cells act as "organizer" cells, arranging the various components of the node and aiding its development. Watanabe found that the same was true of the artificial nodes. The implanted stromal cells attracted T and B immune cells (lymphocytes) that were already circulating in the healthy mouse, then organised them into compartments segregated from one another, just as they appear in natural nodes.

Chiori Shimizu *(left)* and Risa Chihara *(right)* (Technical staff)

Empty nodes

After the artificial node had filled with antigen-specific T and B cells, Watanabe transplanted it into a mouse with no functioning immune system. The lymphocytes quickly spread out from the artificial node into the animals' own lymph nodes, which lay empty due to the lack of immune activity.

When Watanabe injected the same harmless antigen into the immuno-deficient mouse, its transplanted immune system responded vigorously, producing massive numbers of lymphocytes to neutralise the foreign molecule. After a month, these cells' "memory" was still maintained, and they were able to fight off challenges from the antigen.

"It's one tiny step towards use in humans," says Watanabe. "The next step is to use human cells in humanised mice. Then, maybe in four or five years, we might be able to make the first prototypes of a human model."

Eventually, Watanabe hopes this technology will provide a revolutionary treatment for patients with AIDS or cancer.

By implanting artificial nodes plump with healthy T and B cells in AIDS patients, he believes he might be able to revitalise their damaged immune systems. For cancer, he hopes to adopt a similar approach in which the transplanted nodes will contain T cells trained to hunt down the antigens produced by tumour cells and kill them off.

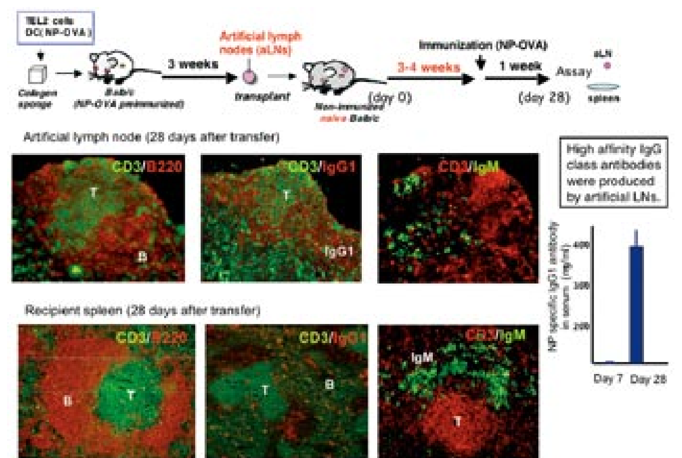

Figure Structure and immune function of the artificial lymph nodes are maintained for more than one month after transplantation into naïve or SCID mice.
Artificial lymph nodes containing antigen (NP-OVA)-primed immune cells were transferred into the renal subcapsular spaces of non-immunized naïve Balb/c mice. One month after transfer, mice were immunized with antigen. After 5 days, antigen-specific IgG class antibody formation was measured. In the artificial lymph nodes, we could detect numerous NP-specific antibody-forming cells of the IgG class but not IgM. On the contrary, only IgM antibody-forming cells were observed in recipient mouse spleen. In the serum, a high titer of IgG antigen-specific antibody was detected. These data indicate that the structure and immune function of the artificial lymph nodes are well maintained even long after transplantation.

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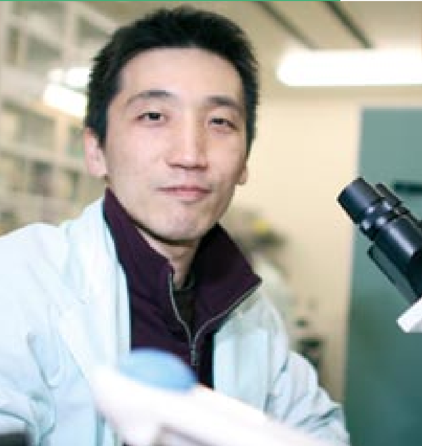
STIMulating calcium influx

Researchers demonstrate the critical role of a protein in mobilizing calcium ions

Tomohiro Kurosaki



Yoshihiro Baba

Calcium ions are used by various cell types, including immune cells, bone cells and neurons, to regulate their physiological response. Now, Tomohiro Kurosaki and colleagues from the RIKEN Research Center for Allergy and Immunology, Yokohama, and other academic institutions in Japan, provide new details of how the cell senses low intracellular calcium levels, potentially leading the way to future drug therapies.

Typically, calcium is sourced from stores in an intracellular compartment known as the endoplasmic reticulum (Figure). When these stores are depleted, the cell must transport more from the extracellular milieu. Important in this process is a protein in the endoplasmic reticulum called STIM1. Upon calcium depletion, STIM1 is transported to the region underneath the plasma membrane, where it communicates with calcium transport channels residing in the plasma membrane, thereby facilitating calcium influx into the cells.

Kurosaki and colleagues show for the first time that STIM1 normally resides in a special sub-compartment of the endoplasmic reticulum. Also, while confirming earlier studies demonstrating that calcium depletion stimulates STIM1 to translocate to regions near the cell's surface, the team shows that it is not inserted into the plasma membrane. They suggest that STIM1 must trigger calcium influx indirectly by activating calcium transport channels within the plasma membrane. How this actually occurs will be an important issue for future studies, says Kurosaki.

By removing specific portions of the protein's structure through genetic engineering, the team further shows that several different regions of STIM1 are required for calcium influx to occur. Interestingly, STIM1 appears to use these regions in different ways to facilitate its movement to the region underneath the plasma membrane. For example, removal of one region blocked the first step in the transport of STIM1, while removal of others either blocked subsequent transport steps or slowed its kinetics. But in all cases, none of the mutated protein made it to the region underneath the plasma membrane in appreciable amounts to properly trigger calcium influx.

Figure An artistic representation of endoplasmic reticulum.

Kurosaki points out future studies are needed to "clarify the mechanism of how low intracellular calcium levels stimulate STIM1 to leave the endoplasmic reticulum since the protein's movement is one of the critical points of its regulation". He also believes that some day drugs that target STIM1 may be therapeutic since many cell types, including immune cells and bone-degrading cells, depend on proper calcium oscillations for their activation and function. However, he notes that since STIM1 is expressed widely, these drugs would have to be well-targeted to avoid unwanted side-effects.

The team's work highlights facets of the immune response that correlate with clinical efficacy, which may be important parameters to consider during future experiments aimed at designing optimal immune therapies.

ORIGINAL RESEARCH PAPER

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Ubiquitination regulates MHC class II stability

Satoshi Ishido

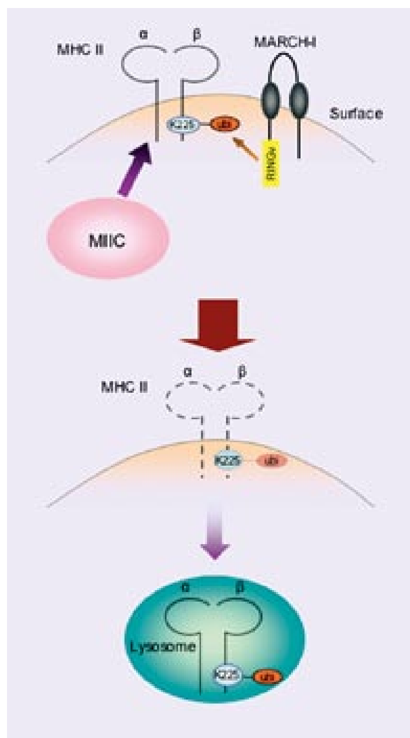

Figure Regulation of MHC II trafficking through ubiquitination of the beta chain. Newly synthesized MHC II molecules are transported and accumulated in MIIC (MHC II compartment). Once MHC II are transported to the cell surface, the lysine 225 of I-A beta chain (β) in MHC II is ubiquitinated by MARCH-I, and ubiquitinated MHC II are transported into lysosome for degradation.

Dr. Satoshi Ishido and his team discovered that an enzyme called "MARCH-I" plays an essential role in the regulation of MHC class II stability.

When pathogens invade the body, they are recognized and engulfed by immune cells named antigen-presenting cells (APCs). APCs degrade the pathogen and express fragments, usually peptides, of the pathogen on the cell surface. The Class II Major Histocompatibility Complex (MHC II) is the key molecule for this antigen presentation process. MHC II binds to the antigenic fragments within the APC and the MHC/peptide complex then moves to the cell surface. Antigens presented through this MHC II pathway are recognized by T cells, which induce immune reactions to eliminate the pathogens. Thus, regulation of MHC II is very important to protect the body from pathogens.

Ubiquitin ligase is an enzyme that attaches small proteins called ubiquitin to target proteins. This process is called ubiquitination and is a physiological system for protein turnover. Once a protein is tagged by poly-ubiquitins, it will be rapidly degraded by a variety of mechanisms.

The research team found that MARCH-I, a mammalian ubiquitin ligase, plays an important role in the regulation of MHC II. By analyzing MARCH-I knockout mice, in which MARCH-I is genetically eliminated, they found that MHC II is stabilized on the APC surface and the amount of MHC II is remarkably increased. Through further investigations, they found that MARCH-I ubiquitinates the β chain of MHC-II. This ubiquitination leads to sorting of MHC-II to the lysosomes, acidic organelles where protein degradation takes place. On the other hand, non-ubiquitinated MHC-II molecules are recycled to the cell surface.

These researchers had previously found that a virus called Kaposi's sarcoma-associated herpes virus (KSHV) uses a viral ubiquitin ligase, MIR1,2, to downregulate the host's MHC I expression. "MARCH-I might be a mammalian homologue of MIR1,2. Because many viruses capture and mimic host genes, it might be possible that KSHV uses the mammalian host's ubiquitin ligase to escape the immune system", says Dr. Ishido.

Their research received important hints from their previous studies of the immune modulation by the virus, but now it has been demonstrated that a similar physiological mechanism exists in mammals. In the future, it might be possible to utilize this mechanism to artificially regulate the immune system. For example, it is known that certain autoimmune disorders are strongly associated with the abnormal expression of MHC-II.

MHC-II regulation by MARCH-I ubiquitin ligase has provided important insight into a new level of regulation of the immune system.

(*From left*) Masami Aoki, Mari Hoshino, Mari Mito and Yohei Matsuki

ORIGINAL RESEARCH PAPER

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Award Winners

Toshio Hirano received the Medal with Purple Ribbon

RCAI Deputy Director, Dr. Toshio Hirano, received the Medal with Purple Ribbon on April 29, 2006. The ceremony was held on May 17 in the imperial palace. This medal is awarded for outstanding achievements in invention, improvement, or creation in the arts and sciences. Dr. Hirano was recognized for his long-term contributions and outstanding achievements in immunology.

Dr. Hirano began his work in immunology in the 1970s. He discovered B cell differentiation factor, which was later named Interleukin-6 (IL-6), in the culture supernatant of pleural effusion cells of patients of tuberculous pleuritis. The purification of IL-6 took eight years of effort, and in 1986 he succeeded in cloning IL-6 cDNA and determined its entire sequence. He continued research on IL-6 and his numerous accomplishments include the identification of the IL-6 receptor, IL-6 signaling pathways, and the relationship between IL-6 and autoimmune diseases and B cell malignancies such as rheumatoid arthritis and multiple myeloma.

Recently, Dr. Hirano's Cytokine Signaling Group discovered that zinc-dependent pathways play important roles in mast cell activation processes including degranulation and cytokine production. Their pioneering work on zinc and its transporters, in collaboration with Dr. Hiano's Laboratory of Developmental Immunology in Osaka University, is expected to develop into a new area of biology that may be involved in immune reactions, allergies, development, and cancer metastasis.

Shohei Hori awarded the MEXT Prize for Young Investigators

Dr. Shohei Hori, Leader of the Immune Homeostasis Research Unit won a Minister of Education, Sports, Culture, Science and Technology (MEXT) Prize for Young Investigators, 2006. This prize is awarded to young scientists (under 40 years old) in recognition of creative and original research and outstanding ability to develop research projects. Dr. Hori was awarded for his research on the "master" gene that controls the development of regulatory T cells.

Dr. Hori began his immunology career in 1998 at the Instituto Gulbenkian de Ciencia in Portugal under Dr. Antonio Coutinho and now continues his work at RCAI. His research currently focuses on the mechanism of immune regulation by regulatory T cells. In 2003, he discovered that the transcription factor Foxp3 functions as a master gene for immune tolerance. The *Foxp3* gene is mutated and no regulatory T cells exist in scurfy mice and in patients with IPEX syndrome, a human autoimmune disease. Hori et al. found that expression of the *Foxp3* gene converts naïve T cells into regulatory T cells. The discovery of *Foxp3* as a master gene for the development of regulatory T cells opened a new perspective in the analysis of regulatory T cells in immune tolerance.

Yosuke Murakami received the Excellent Poster Award at the 34th meeting of The Japan Society for Clinical Immunology

The 34th meeting of The Japan Society for Clinical Immunology (Oct. 2-3, 2006) presented its Excellent Poster Award to Yosuke Murakami, a visiting researcher in the RCAI Clinical Immunology Research Unit. The poster was entitled "Inhibition of CDK4/6 Induced Production of Inflammatory Cytokines by Macrophages and Suppression of Osteoclast Differentiation" (in Japanese).

Outstanding Contribution of the Year

RCAI has established a new annual award, the RIKEN RCAI Outstanding Contribution of the Year to recognize the most outstanding RCAI research. The determination of awardees depends on the Director Dr. Taniguchi, who selects nominees from all the members of the Center and names the awardees after consultation with the Group Directors. This year, three individuals, two unit leaders and one senior researcher, received the award. At a ceremony held on March 16, Drs. Fumihiko Ishikawa, Makio Tokunaga and Tadashi Yokosuka received honoraria from the Director, Dr. Taniguchi. The winners expressed their sincere gratitude to all the members of their laboratories and their collaborators.

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Dr. Fumihiko Ishikawa (photo 1), the leader of the Research Unit for Human Disease Model, was honored for his pioneering work in developing Humanized Mice and his success in reproducing human leukemia in this model (Shultz LD et al. *Nat Rev Immunol.* (2007), Ishikawa F. et al. *Blood.* (2005)). These are mice in which the human immune system is reconstructed; most of their immune cells including T, B, NK, NKT and dendritic cells are replaced by human cells. Human-derived immune cells are present in immune organs, and human antibodies are produced following immunization.

To create a humanized mouse, a three step process is used: 1. Prepare newborn immunodeficient mice as recipients, 2. Isolate human hematopoietic stem cells from human umbilical cord blood or human bone marrow, 3. Transplant the human stem cells into the recipient mice to replace the mouse immune cells with those of a human (Figure 1).

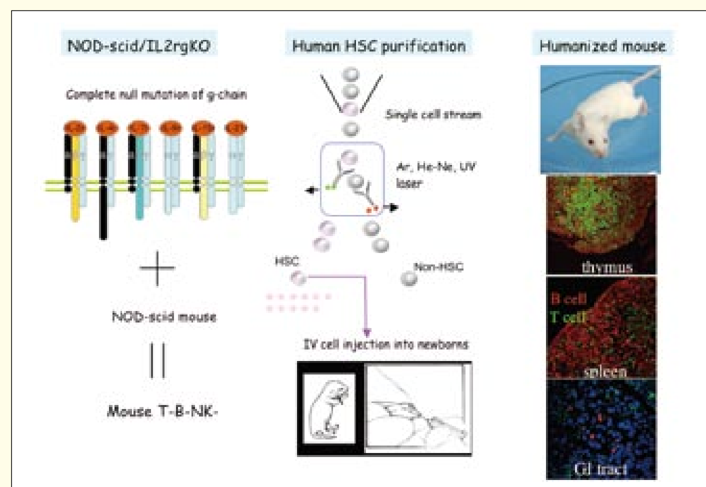


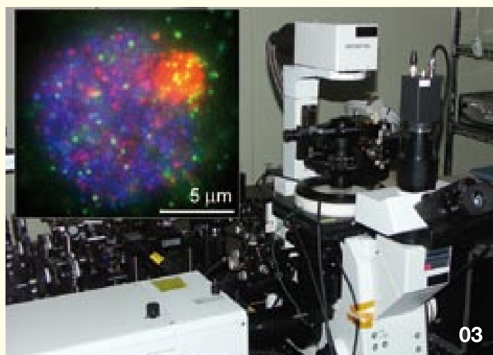
Figure 1　Development of human immunity in mice

"Two key points that made the creation of humanized mice possible were the availability of highly immunodeficient mice [non-obese diabetic/Severe combined immunodeficient/IL2 receptor γ chain deficient (NODscid/IL2rγ^{null}) mice], thanks to collaborative research with the Jackson Laboratory, and the use of newborn mice as recipients of human stem cells." says Dr. Ishikawa.

It is anticipated that immunologically humanized mice will play a major role in increasing our understanding of the human immune system and various diseases, leading to their use in clinical applications, such as developing new medical therapies. One example of their potential use is performing experiments that cannot be conducted on humans, such as testing the safety of new drugs under development. It will be possible to use the mice to conduct experiments that have previously proven difficult due to technical problems, safety issues, or ethical reasons.

More than that, it should be possible to reproduce the unique disease of a specific patient in the humanized mice by transplanting the patient's stem cells. An example is leukemia, which is regarded as cancer of the blood cells. It progresses through two processes, the

Outstanding Contribution of the Year



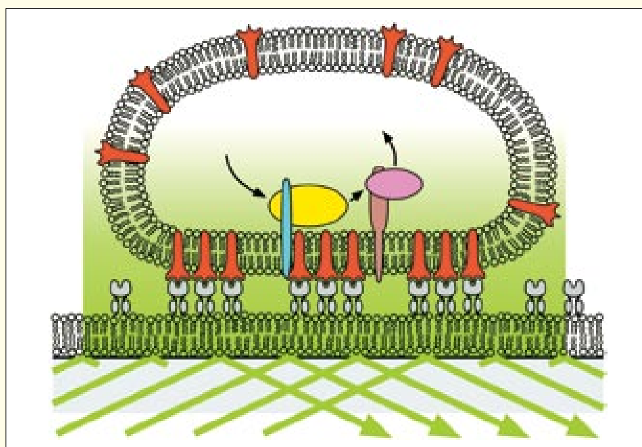


Figure 2 Single molecule imaging by total internal reflection illumination microscopy

rapid growth of leukemic cells and the slow self-reproduction of the leukemia stem cells. When Dr. Ishikawa transplanted these leukemia stem cells from a patient into immunodeficient mice, the recipient mice reproduced patient leukemia. In contrast, transplantation of non-stem leukemia cells did not result in reproduction of leukemia in mice. These results indicate that it is the slowly self-renewing stem cells that are the real cause of leukemia.

"Reproducing the immune status of a specific patient in a laboratory animal could lead to entirely new research methods and medical therapies. We are now using these mice to develop leukemia treatment models" says Dr. Ishikawa. "The efficacy and risk evaluation of anticancer drugs using these mice has important similarities to *in vivo* clinical tests on actual leukemia patients. This makes it possible to develop drugs best suited for the patient. This research using humanized mice has great potential to fulfill the need for tailormade medicine."

Dr. Makio Tokunaga (photo 2), the leader of the Research Unit for Single Molecule Immunoimaging, and Dr. Tadashi Yokosuka, a senior researcher in the Laboratory for Cell Signaling were honored for their collaborative studies to visualize the molecular dynamics within living lymphocytes and for the discovery on the immunological function of T cell microclusters (Yokosuka T. et al. *Nat. Immunol.* 2005).

Dr. Makio Tokunaga developed a single-molecule imaging microscope that made it possible to observe molecular dynamics and the interactions of individual molecules in living cells (photo 3). In single-molecule imaging, subject molecules labeled with fluorescent dyes are excited by a laser beam, and the resulting fluorescence is examined using an optical microscope. Three key advances led to the successful imaging of single discrete molecules: reducing background light, increasing camera sensitivity, and using bright fluorescent light. "We had had the greatest difficulty in reducing the background light", says Dr. Tokunaga. Usually, a laser beam is applied at right angles with respect to the glass plate carrying the specimen. However, because this allows the laser to reach deep into the specimen, fluorescent dyes at many points throughout the sample become excited to the extent that the brightness is diffused over the entire specimen. In this situation, the fluorescence from individual molecules cannot be distinguished from each other. "Hence, we attempted to apply a new method known as total-reflection illumination", says Dr. Tokunaga. When applied obliquely to the glass plate carrying the sample, the laser beam is totally internally reflected, producing near-field light (evanescent light), which emanates shallowly from the surface of the sample. Because the distance the near-field light can travel is only up to 200 nm from the specimen surface, the fluorescence from each molecule can be clearly visualized against the background, which remains dark (Figure 2).

Dr. Tokunaga set down as his next target the intracellular imaging of single-molecules. He used thin-layer oblique illumination to reduce the thickness of the exposure field. Thus

the laser strikes an extremely narrow area in the cell. The new type of microscopy termed “Highly inclined and laminated optical sheet” (HILO) microscopy visualizes single molecules not only on cell surfaces but also inside living cells. Further, it has been shown that single molecule imaging and quantification of molecular interactions are useful new tools to elucidate the molecular mechanism of cellular functions. “Single molecule imaging enables us to determine the intensity of the fluorescence from each molecule, we can obtain numerical quantitative values of the number of molecules that have gathered, the number of molecules required for the reaction, and the intensity of the interactions.”, says Dr. Tokunaga. “I am planning to watch how reactions on the cell surface are transmitted to the inside of the cell, and then into the nucleus, and what happens in the nucleus”

Dr. Tadashi Yokosuka (photo 4) discovered that molecular microclusters trigger and maintain immune responses. Immune responses start when antigen-presenting cells (APCs) take in and decompose foreign substances (antigens), such as viruses and pollens, and then provide molecular information about the antigens to T cells, so that the T cells can recognize antigens. Utilizing the microscope developed by Dr. Tokunaga, Dr. Yokosuka succeeded in observing dynamic movements of molecules at the interface between T cells and APCs. What they saw was different from the conventional model: it was previously believed that the immunological synapse (IS) was the place where the immune response starts, however, microclusters actually triggered the immune response immediately after contact with an APC, much earlier than the formation of the immunological synapse.

A microcluster is a small conglomerate of 50-200 molecules that gather on the surface of a T cell. A microcluster is comprised of receptors that recognize antigens and signaling molecules that transfer information. After being generated, microclusters migrate toward the center of the interface and form the central part of an immunological synapse (c-SMAC). Signaling events, such as protein phosphorylation and other molecular processes, are completed before the microcluster reaches the center. Once the immune synapse is formed, other microclusters continue to emerge along the periphery of the contact surface (p-SMAC) (Figure 3). Thus, microclusters are responsible not only for initiating but also for sustaining immune responses.

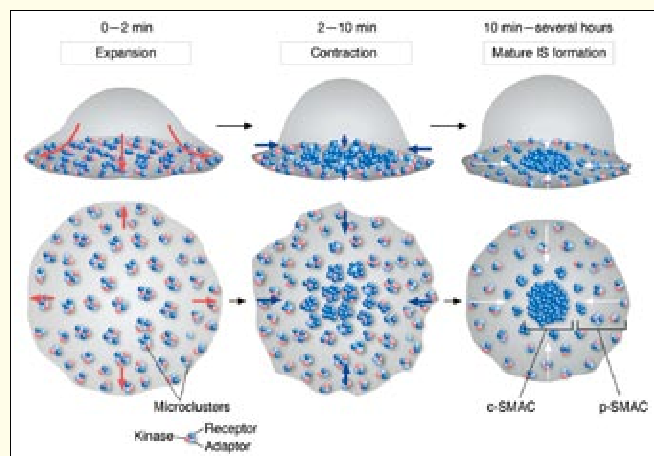


Figure 3 Spatiotemporal regulation of T cell activation through microclusters
(*Curr Opin Immunol.* 18, 305-313, 2006)

Microclusters are expected to become a target for the development of advanced drugs that can regulate T cell activation. Many people suffer from allergies and autoimmune diseases such as rheumatoid arthritis and nephritis, which are caused by an over-activated immune system. On the other hand, immune activators that can boost the immune system have been drawing attention in cancer treatment and health promotion for elderly people. Microclusters may be the target to artificially control the activation of T cells in either direction to suppress or promote immune responses.

Related publications

Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol.* 7, 118-130 (2007).

Ishikawa F, Yasukawa M, Lyons B, et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor γ chain^{null} mice. ***Blood.*** 106, 1565-1573 (2005).

Yokosuka T., Sakata-Sogawa K., Kobayashi W., Hiroshima M., Hashimoto-Tane A., Tokunaga M., Dustin M.L., Saito T. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap 70 and SLP-76. *Nat Immunol.* 6, 1253-1262 (2005)

Excellent Paper & Excellent Poster of the Year

01 K. Tanaka

02 S. Yoshida

03 K. Hase

04 Y. Miyake

05 H. Kitamura

06 A. Hijikata

07 H. Watarai

08 S. Yamasaki

09 N. Suzuki

10 T. Yokosuka

2006 Award for Excellent Paper of the Year

The RCAI Excellent Paper Award was established in 2004 to recognize exceptional publications of RCAI scientists. The award fund is supported by donations from Dr. Masaru Taniguchi and Dr. Toshio Hirano. This year, six papers were selected. Five of them were published by rather small size research teams lead by young team leaders. This reflects the fact that their research activities launched at the Center are bearing the fruits.

Drs. Katsuaki Hoshino and Tsuneyasu Kaisho received the award for their Nature paper “IκB kinase-α is critical for interferon-α production induced by Toll-like receptors 7 and 9”. They determined that a kinase IκB kinase-α, an enzyme that could potentially activate IRF7 by chemically attaching a phosphate group to it, is the key player in the step that mediates interferon induction by TLR7 and TLR9.

Drs. Hidemitsu Kitamura and Toshio Hirano were awarded for their paper published in Nature Immunology “Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function” in which they discovered that zinc plays an active role in the maturation of dendritic cells.

Drs. Shinya Tanaka and Masato Kubo were awarded for their work on “The interleukin-4 (IL-4) enhancer CNS-2 is regulated by Notch/RBP-J signals and controls initial IL-4 expression in NKT cells and memory-type CD4 T cells” (Immunity). In this paper, they determined that Notch pathway regulates the DNA sequence in the memory $CD4^{+}$ T cells, and the generation of Th2 cells does not occur without the initial induction of IL-4 by the memory $CD4^{+}$ T cells.

In the JEM paper “Regulation of B1 cell migration by signals through Toll-like receptors”, Drs. Seon-ah Ha and Sidonia Fagarasan determined precisely how B1 lymphocytes within the peritoneum quickly traffic towards immune responses developing at distant locales throughout the body.

Drs. Yohei Matsuki and Mari Ohmura-Hoshino were awarded for their paper “Novel regulation of MHC class II function in B cells” published in The EMBO journal, in which they discovered a ubiquitin ligase MARCH-I plays an important role in the regulation of MHC II in antigen presenting cells.

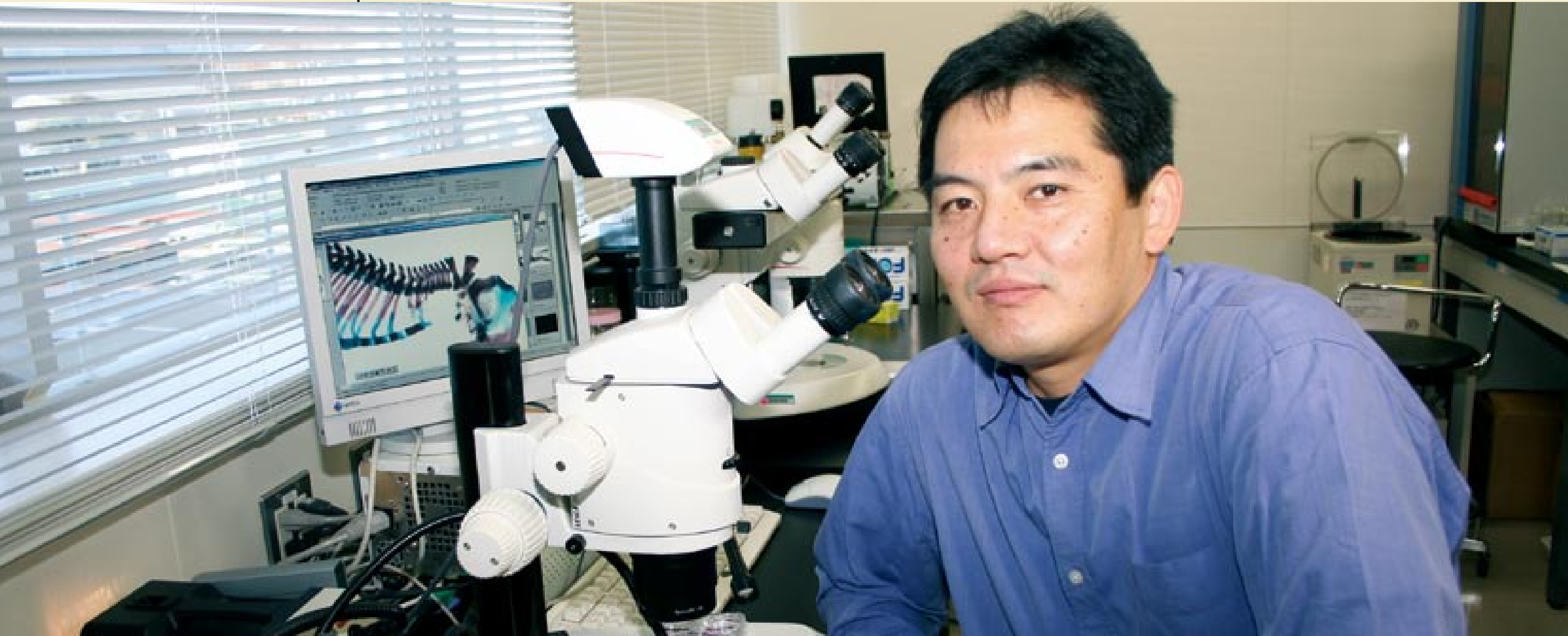
Drs. Shigeharu Fujita and Katsuaki Sato were awarded for the research on “Regulatory dendritic cells act as regulators of acute lethal systemic inflammatory response.” Published in Blood. In this paper, they showed that mice given the so called ‘regulatory’ dendritic cells prevents the septic shock. (For more information, see Research Highlights.)

2006 Award for Excellent Poster

During the retreat meeting held on 28-29, August, 95 posters were presented in the two poster sessions that lasted for three hours each. During the sessions, both new unpublished data and on-going projects were introduced to the attendants. As people participated in critical and thought-provoking discussions about the posters, the principle investigators of each laboratory reviewed them and voted for “excellent posters of the year” . Ten awardees were selected; Koji Tanaka (Research Unit for Immune Surveillance)(01), Shuro Yoshida (Research Unit for Human Disease Model) (02), Koji Hase (Lab. for Epithelial Immunobiology) (03), Yasunobu Miyake (Lab. for Innate Cellular Immunity) (04), Hiroshi Kitamura (Lab. for Immunogenomics) (05), Atsushi Hijikata (Lab. for Immunogenomics) (06), Hiroshi Watarai (Lab. for Immune Regulation) (07), Sho Yamasaki (Lab. for Immune Signaling) (08), Nobutaka Suzuki (Lab. for Immune Signaling) (09), and Tadashi Yokosuka (Lab. for Immune Signaling) (10). Reviewers commented that selecting only 10 poster presentations was quite difficult because the quality of the poster presentations has considerably improved since previous RCAI retreats. Winning the award was not only a happy surprise but also a great chance for each winner to introduce his/her project in the 10-minute English presentations which they gave the next day in front of 170 attendants.

Research Activities
2006

Laboratory for Developmental Genetics



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Tamie Morisawa
Atushi Kajiwara
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Masahiro Mutô

Administrative Staff :
Ryôko Moriizumi
Hiroko Iwamoto

The Developmental Genetics Research Group fulfills a double role within RCAI. A large portion of the manpower and financial resources of the group is devoted to the maintenance of a high-standard mouse facility. Through the Animal Core Facility, the group is also responsible for the generation of knock-out and transgenic animals for the various research laboratories at the center. At the same time, the laboratory is pursuing a research program to elucidate the molecular mechanisms underlying the epigenetic regulation mediated by Polycomb group (PcG) genes in development. PcG genes have been first isolated in Drosophila Melanogaster as a group of genes required in the maintenance of segmental identity and shown to be structurally and functionally conserved in mammals. Genetic analyses of mammalian PcG proteins revealed their roles not only in the anterior-posterior specifications but also in cellular proliferation, differentiation, and senescence. Particularly, the crucial role of PcG in lymphocyte and lymphoid organ development has repeatedly been reported. *PcG* gene products form at least two different multimeric protein complexes. First complex mediates trimethylation of histone H3 at K27 whereas second one ubiquitinylates histone H2A at K119 on the chromatin. How PcG complexes mediate transcriptional repression is, however, unknown. To elucidate the molecular basis of PcG-mediated transcriptional regulation, we have been focusing on the following issues, namely (1) the regulatory mechanisms of binding of *PcG* gene products to their targets, and (2) the regulation of the cellular differentiation by PcG and the interacting proteins.

Regulatory mechanisms of binding of *PcG* gene products to their targets

To address the molecular mechanisms underlying Polycomb group (PcG)-mediated repression of *Hox* gene expression, we have focused on the binding patterns of *PcG* gene products to the flanking regions of the *Hoxb8* gene in expressing and non-expressing tissues. In parallel, we followed the distribution of histone markers of transcriptionally active, H3 acetylated on lysine 9 (H3-K9), and transcriptionally inactive chromatin, trimethylated on lysine 27 (H3-K27). Chromatin immunoprecipitation revealed that the association of PcG proteins, and H3-K9 acetylation and H3-K27 trimethylation around *Hoxb8* were distinct in tissues expressing or not the gene. We show that developmental changes of these epigenetic markers temporally coincide with the misexpression of *Hox* genes in PcG mutants. Functional analyses using mutant alleles impairing the PcG class 2 component Rnf2, or the *Suz12* mutation decreasing H3-K27 trimethylation, revealed that interactions between class 1 and class 2 PcG complexes, mediated by trimethylated H3-K27, play a decisive role in the maintenance of *Hox* gene repression outside their expression domain. Within the expression domains, class 2 PcG

complexes appeared to maintain the transcriptionally active status via profound regulation of H3-K9 acetylation. Present studies indicate distinct roles for class 2 PcG complexes between transcriptionally repressed and active domains of *Hoxb8* gene. (Fujimura et al., *Development*)

Regulation of cellular differentiation by PcG and the interacting proteins

We identified the Homeodomain-interacting protein kinase (*Hipk*)-1, -2, and -3 as interacting proteins for Phc2, a component of Polycomb repressive complexes 1 (PRC1). Hipks are evolutionary conserved nuclear serine/threonine kinases, which were originally identified as interacting proteins with homeodomain-containing proteins. Hipks have been repeatedly identified as interacting with a vast range of functional proteins including, not only transcriptional regulators and chromatin modifiers, but also cytoplasmic signal transducers, transmembrane proteins and the E2 component of SUMO ligase. Gain-of-function experiments using cultured cells indicate regulatory roles of Hipks in cell growth upon receipt of morphogenetic and genotoxic signals. However, *Hipk1* and *Hipk2* single-deficient mice were grossly normal most likely due to a functional redundancy between Hipk1 and Hipk2. Therefore, we addressed the physiological roles of Hipk family proteins by using *Hipk1/Hipk2* double mutants. *Hipk1/Hipk2* double homozygotes are progressively lost between 9.5 and 12.5 days post coitus and frequently fail to close the anterior neuropore and exhibit exencephaly. This is probably due to a defective proliferation in the neural fold and underlying paraxial mesoderm, particularly in the ventral region, which may be attributed to a decreased responsiveness to Sonic hedgehog signals. The present study indicates the overlapping roles for Hipk1 and Hipk2 in mediating cell proliferation and apoptosis in response to morphogenetic and genotoxic signals during mouse development. Hipks could be involved in the qualification of stressed cells by various extracellular inputs, which would secure the further development and survival.

We also identified the product of the *Scmh1* gene, a mammalian homolog of *Drosophila* Sex comb on midleg, as a protein interacting with Phc2 . We have shown that Scmh1 was an indispensable component of the Polycomb repressive complex-1 (PRC1). During progression through the pachytene stage, Scmh1 was shown to be excluded from the XY body at late pachytene, together with other PRC1 components such as Phc1, Phc2, Rnf110, Bmi1 and Cbx2. We have identified the role of Scmh1 in mediating the survival of late pachytene spermatocytes. Apoptotic elimination of *Scmh1*$^{-/-}$ spermatocytes is accompanied by the preceding failure of several specific chromatin modifications of the XY body, whereas synapsis of homologous autosomes is not affected. It is therefore suggested that Scmh1 is involved in regulating the sequential changes in chromatin modifications at the XY chromatin domain of the pachytene spermatocytes. Restoration of defects in *Scmh1*$^{-/-}$ spermatocytes by *Phc2* mutation indicates that Scmh1 exerts its molecular functions via its interaction with PRC1. Therefore, for the first time we have been able to indicate a functional involvement of PRC1 during the meiotic prophase of male germ cells and a regulatory role of Scmh1 for PRC1. The regulation of PRC1 functions by Scmh1involves the sex chromosomes. (Isono et al., *Mol. Cell. Biol.*; Takada et al., *Development*)

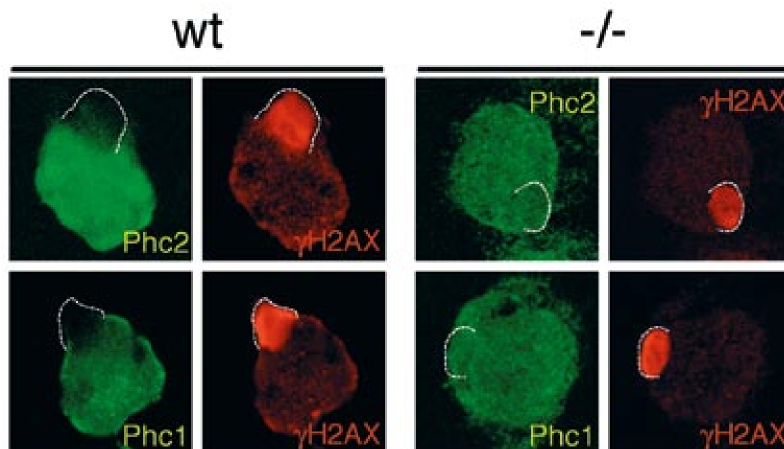

Figure Exclusion of Polycomb proteins (Phc1, Phc2) from the sex chromosomal domain (indicated by dotted lines) during male meiosis is affected in Scmh1-deficient mice (-/-).

Recent major publications

Takada Y., Isono K., Shinga J., Turner J.M.A., Kitamura H., Ohara O., Watanabe G., Singh P.B., Kamijo T., Jenuwein T., Burgoyne P.S., Koseki H., Mammalian Polycomb Scmh1 mediates exclusion of Polycomb complexes from the XY body in the pachytene spermatocytes. *Development*, 134:579-590 (2007).

Fujimura Y., Isono K., Vidal M., Endoh M., Kajita H., Mizutani-Koseki Y., Takihara Y., van Lohuizen M., Otte A., Jenuwein T., Deschamps J., Koseki H. Distinct roles of Polycomb group gene products between transcriptionally repressed and active domains of Hoxb8. *Development*, 133:2371-2381 (2006)

Isono, K, Nemoto, K, Li,Y., Takada, Y, Suzuki, R, Katsuki, M, Nakagawara, A, and Koseki, H, Overlapping roles for homeodomain-interacting protein kinases Hipk1 and Hipk2 in the mediation of cell growth in response to morphogenetic and genotoxic signals. *Molecular and Cellular Biology* 26:2758-2771 (2006)

Isono K, Fujimura Y, Shinga J, Yamaki M, O-Wang J, Takihara Y, Murahashi Y, Takada Y, Mizutani-Koseki Y, and Koseki H Mammalian polyhomeotic homologues Phc2 and Phc1 act in synergy to mediate Polycomb-repression of Hox genes. *Molecular and Cellular Biology.* 25:6694-6706 (2005)

Isono K., Mizutani-Koseki Y., Komori T., Schmidt-Zachmann M.S., Koseki H Mammalian Polycomb-mediated repression of Hox genes requires the essential spliceosomal protein sf3b1. *Gene Dev.*, 536-541 (2005)

Laboratory for Transcriptional Regulation

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Developmental programs in metazoan species present daunting challenges of spatial and temporal control of gene expression. Cell fate determination of progenitor cells differentiating distinct lineage cells involves several steps. Following lineage specification, cell identity is established during a process known as lineage commitment. In fully differentiated cells, specific cellular function is maintained in part by epigenetic maintenance of gene expression patterns. Researches in my laboratory try to understand (a) how progenitor cells sense external or intrinsic stimuli and turn on a genetic program for regulating lineage decision and (b) how an established gene expression pattern is maintained by epigenetic mechanisms. We address these questions by studying *CD4/CD8* gene regulation and lineage decision of $CD4^+CD8^+$ double-positive (DP) thymocytes as a model system. Our previous findings have shown that the Runx transcriptional factor complex plays an essential role in *CD4* gene silencing by binding to the intronic silencer during cytotoxic T cell development. To further extend these findings, we have been analyzing the roles of the Runx transcriptional factor complex by genetic approaches. Our preliminary results obtained for last three years revealed multiple roles of Runx complexes in development and regulation of the immune system

Essential role of Runx complexes for cytotoxic T cell development

The two distinct lineages of T lymphocytes expressing αβTCR, helper- and cytotoxic-lineage cells, differentiate from common progenitor $CD4^+CD8^+$ DP thymocytes. While cells expressing class II-restricted TCR differentiate into helper lineage and cease to express CD8, cells expressing class I-restricted TCR differentiate into cytotoxic linage and silence CD4. However, molecular pathways regulating cell fate determination of $CD4^+CD8^+$ DP thymocyte remain poorly understood. The lineage-specific *Cd4* silencing is controlled by the intronic silencer that requires binding of Runx transcriptional factor complexes for its activity. Interestingly, Runx3 is mainly expressed in cytotoxic lineage cells and is shown to bind mature specific enhancer at the *Cd8* locus to reactivate CD8 expression. These results suggest an important function of Runx complexes for lineage specification toward cytotoxic lineage. On the other hand, current genetic approaches have identified a central regulator for helper-lineage development, the Th-POK transcriptional factor. Therefore, it is important to clarify the roles of Runx complexes in CD4/CD8 lineage choice. For this purpose, we have generated mouse strains harboring single or combined mutations of Runx family genes, *Runx1, Runx3 or Cbfβ*. These approaches revealed that loss of Runx complexes function in $CD4^+CD8^+$ DP thymocytes induced re-directed differentiation of class I-restricted cells into $CD4^+CD8^-$ T cells that partially possess helper function due to dysregulated expression

of the *Th-POK* gene. Furthermore, we detected a direct binding of Runx complexes to the *Th-POK* locus at two regions, which contains stage- and lineage- specific enhancer activity detected in transgenic reporter assay These results provide a novel insight of transcriptional factor network during cell fate determination process of $CD4^{+}CD8^{+}$ DP thymocytes.

Role of Runx transcriptional factors in T helper cell differentiation

Upon encountering antigen, naïve $CD4^{+}$ T helper cells differentiate into effector cell subsets, T helper type 1 (Th1) cells expressing IFNγ and T helper type 2 (Th2) cells expressing IL-4. It is unclear how the exclusive expression pattern of each hallmark cytokine is established. We observed that T cell-specific inactivation of the *Cbf*β gene in mice led to a spontaneous elevation of serum IgE and airway infiltration by lymphoid and eosinophilic cells, two features which resemble what is seen in the case of human asthma mediated by enhanced Th2 responses. To understand the molecular basis for these symptoms, we examined the *in vitro* differentiation of Th1/Th2 cells and found de-repression of IL4 in Runx-deficient Th1 cells producing IFNγ. Furthermore, binding of Runx complexes to the *Il4* silencer was detected in naïve $CD4^{+}$ T cells and Th1 cells, but not in Th2 cells. Thus Runx complexes are involved in the repression of not only the *CD4* or *Th-POK* gene but also a cytokine gene by regulating negative *cis*-regulatory region through direct binding. These results demonstrate the critical role of Runx complexes in regulating immune responses, at least in part through the repression of the *Il4* gene during T helper cell differentiation.

Roles of Runx transcriptional factors in lymphoid organogenesis

Two isoform proteins, Cbfβ1 or Cbfβ2, are generated from the *Cbf*β gene by RNA alternative splicing, while transcriptions from two promoters, P1 and P2, at the *Runx1* locus generate the Runx1 isoform proteins, P1-Runx1 and P2 Runx1. By using gene targeting in ES cells, we have generated isoform-specific knockout mice. Since lacking either isoform of Cbfβ or Runx1 proteins did not affect early mouse development, we were able to examine the effect of the compromised function of the Runx complex in mice. Interestingly, mice lacking Cbfβ2 or P1-Runx1 proteins showed a severe defect in Peyer's patches formation and had fewer numbers of peripheral lymph nodes. In the fetus of these mice, differentiation of lymphoid tissue inducer (LTi) cells, which initiate lymphoid tissue formation with lymphoid organizer cells, is severely impaired. These findings revealed a new role of Runx complexes in the development of the immune system by regulating non-lymphoid lineage cell differentiation.

Our phenotypic analyses of *Runx* gene family mutant mice revealed that Runx complexes are involved in many processes during T lymphocyte development and in the regulation of immune responses. These solid genetic evidences encourage us to pursue our attempt to understand Runx-mediated regulatory pathway by identification of target genes and associated molecules of Runx complexes and post-translational modification of the Runx complexes. These approaches would shed a new light on the transcriptional factor network regulating lineage decision and would be of general interest for developmental biology and gene regulation.

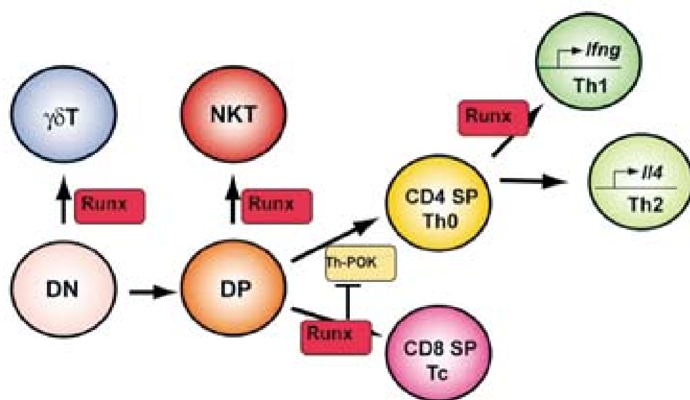

Figure Our genetic approaches revealed multiple functions of Runx complexes during the development of the immune system. For example, dysfunction of Runx complexes resulted in a developmental block of γδT cell, NKT cell, and CD8 single-positive (SP) cytotoxic cells. Runx complexes play also an essential role in *Il4* repression in Th1 cells.

Recent major publications

Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, Bendelac A, Littman D.R. Genetic evidence supporting selection of the Va14i NKT cell lineage from double positive thymocyte precursors. *Immunity* 22, 705-716 (2005)

Fukushima-Nakase Y, Naoe Y, Taniuchi I, Hosoi H, Sugimoto T, Okuda T. Shared and distinct roles mediated through C-terminal subdomains of acute myeloid leukemia/Runt-related transcription factor molecules in murine development. *Blood* 105, 4298-4307 (2005)

Wang X, Blagden C, Fan J, Nowak S, Taniuchi I, Littman D.R and Burden S.J. Runx1 prevents wasting, myofibrillar disorganization, and autophagy of skeletal muscle. *Genes & Dev.* 19: 1715-1722 (2005)

Grueter B, Petter M, Egawa T, Laule-Kilian K, Aldrian C.J, Wuerch A, Ludwig Y, Fukuyama H, Wardemann H, Waldschuetz R, Moroy T, Taniuchi I, Steimle V, Littman D.R and Ehlers M. Runx3 regulates integrin E/CD103 and CD4 expression during development of $CD4^{-}/CD8^{+}$ T Cells. *J. Immunol.* 175:1694-1705 (2005)

Kramer I, Sigrist M, de Nooij J.C, Taniuchi I, Jessell T.M and Arber S. A role for Runx transcriptional factor signaling in dorsal root ganglion sensory neuron diversification. *Neuron,* 49:379-393 (2006)

Laboratory for Lymphocyte Development

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Nagahiro Minato
Yoshimoto Katsura

In hematopoiesis, pluripotent hematopoietic stem cells (HSC) are sequentially restricted to give rise to a variety of lineage-committed progenitors. The major aim of the Laboratory for Lymphocyte Development is to elucidate the molecular mechanisms that regulate cell fate decisions in the process of lineage restriction from HSC to unipotent progenitors. We have previously established a clonal assay system that makes it possible to examine the developmental potential of individual progenitor cells toward T, B and myeloid cell lineages. This work has led to a fundamental redefinition of lymphoid progenitors and the ontogeny and phylogeny of T- and B-cell development.

Process of lineage commitment in hematopoiesis

The classic dichotomy model of hematopoiesis postulates that the first step of differentiation from the HSC generates the common myelo-erythroid and common lymphoid progenitors (CLP). Our previous studies in fetal mice, however, indicated that the first step of lineage restriction in HSC is the generation of myelo-lymphoid lineage progenitors and myelo-erythroid lineage progenitors (Figure 1). Most importantly, the myeloid potential is retained even after the segregation of myelo-lymphoid progenitor towards T and B cell lineages (Figure 2). Thus, each process of specification towards T, B and erythroid lineages appears to proceed according to the prototypical myeloid program. Recently we call this model "myeloid-based model". The concept of CLP has, however, persisted in models of adult hematopoiesis, since several groups have provided experimental results supporting the presence of CLP in the bone marrow. We have been analyzing the potential of progenitors in adult mice and have recently obtained evidence that the myeloid-based model is also applicable to adult hematopoiesis.

Prethymic stages of T cell development

We previously reported that T cell lineage-restricted progenitors are present in prethymic organs such as the AGM (aorta-gonad-mesonephros) region, fetal liver and fetal blood. We then examined the developmental potential of the ontogenically earliest thymic progenitors in day 11 murine fetus, which reside in the surrounding mesenchymal region and have not yet encountered thymic epithelial components. We analyzed the developmental potential of these cells, and the results provide direct evidence that the progenitors restricted to the T/NK/DC lineage selectively immigrate into the thymus. Furthermore, we have found that the prethymic T cell progenitors express PIR (paired-immunoglobulin like receptors) on their surface. This finding not only provides a tool for the isolation of prethymic T cell progenitors but also defines the prethymic stage of T cell development as a distinct stage from intrathymic stages. The isolated T cell progenitors will be a useful source for gene therapy and regenerative medicine.

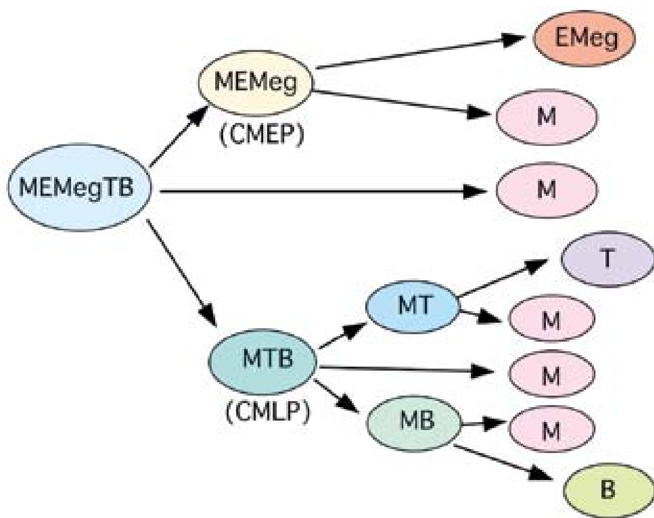

Figure-1 Myeloid-based model: this model proposes that the developmental programs towards T, B and erythro/megakaryocytic (EMeg) lineages proceed independently on the basis of the prototypical myeloid (M) program (Kawamoto H., *Trends in Immunol.*, 2006).

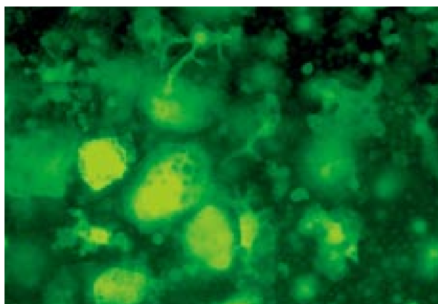

Figure-2 Macrophages and dendritic cells produced from a single intrathymic T cell progenitor. The finding that T cell progenitors in the thymus retain the potential to generate macrophages and dendritic cells after shutting off B cell potential supports our myeloid-based model.

Perspectives

Besides the two above-mentioned projects, we are currently interested in whether environmental factors instructively induce lineage commitment or selectively support autonomously committed progenitors in lymphopoiesis. To address this issue, it is important to establish an experimental system in which lineage commitment of progenitors can be monitored by real-time imaging. We have previously shown that the earliest T cell progenitors in the thymus retain the potential to generate NK cells and dendritic cells, and that the discontinuation of this tri-potentiality and clear committment to the T cell lineage happens before the initiation of TCRβ chain gene rearrangement. In order directly to visualize this differentiation step, we use GFP transgenic mice in which the expression of GFP is controlled by the proximal promoter of the T cell-specific tyrosine kinase lck.

Our research activities include the study of human lympho-hematopoiesis. Cord blood cells are used as a progenitor source, and the basic culture system is a modification of the co-culture with murine stromal cells. We are also interested in the development of thymic epithelial cells. Thymic epithelial cells support thymocyte development, and it is known that thymocytes in turn support the development of thymic epithelial cells. This mutual interaction is called thymic crosstalk. In collaboration with Prof. van Ewijk, we are studying the molecular mechanism of how thymocytes regulate the development of thymic epithelial cells, using the organ culture system. This project also includes studies on progenitors of thymic epithelial cells.

The information and technology that will be acquired through these studies can directly be applied to regeneration therapy. We are trying to induce mature T cells from progenitors on monolayers of stromal cells or three-dimensional "artificial thymus" *in vitro* or *in vivo* which could be a source for immune cell therapy in the future.

Recent major publications

Ikawa, T., Kawamoto, H., Goldrath A.W., Murre C. E protein and Notch signaling cooperate to promote T cell lineage specification and commitment. *J. Exp. Med*. 15:1329-1342, (2006)

Kawamoto, H. Close developmental relationship between lymphoid and myeloid lineages. *Trends in Immunology*. 27: 169-175, (2006)

Masuda K., Kubagawa H., Ikawa T., Chen C.C., Kakugawa K., Hattori M., Kageyama R., Cooper M.D., Minato N., Katsura Y., Kawamoto H. Prethymic T-cell development defined by the expression of paired immunoglobulin-like receptors. *EMBO J.* 24, 4052-4060 (2005)

Masuda K., Itoi M., Amagai T., Minato N., Katsura Y, Kawamoto H. Thymic anlage is colonized by progenitors restricted to T, NK and dendritic cell lineages. *J. Immunol.* 174: 2525-2532 (2005)

Ikawa T., Masuda K., Lu M., Minato N., Katsura Y., Kawamoto H. Identification of the earliest prethymic T cell progenitors in murine fetal blood. *Blood.* 103: 530-537 (2004).

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The long-term goal of the Cell Signaling group is to determine the molecular mechanisms of activation and homeostasis of T cells in order to be able to modulate T cell activation/function in immunological disorders. Therefore, the group is involved in a variety of projects related to the basic mechanisms of antigen recognition, activation, differentiation, functional regulation of T cells from the viewpoint of signal transduction. Particularly, the group recently concentrated on the dynamic regulation of molecular assembly of the TCR signalosome and immunological synapse, and related downstream signaling pathways upon antigen recognition using real-time imaging analysis. The signaling events that diversify the antigen recognition signals into NF-AT vs. NF-κB activation is investigated through the function of the Carma1- and Card9-containing signaling complex. The group also analyzes the regulation of cell migration as a consequence of T cell activation to establish peripheral antigen-specific repertoire for effector function. *In vivo* imaging analysis will combine the mechanism of cell signaling and *in vivo* cell movement and function.

Dynamic regulation of T cell activation

We have recently studied the dynamic movement of signaling molecules in the process of the formation of the immunological synapse and T cell activation upon antigen recognition at the single-cell level. Using a novel single-molecule imaging technique developed by Single Molecule Immuno-imaging Unit, we visualized single molecules in the dynamic process of T cell activation.

We have visualized CD3ζ, ZAP-70 and SLP-76 as GFP-fusion proteins, as representatives of the receptor, kinase and adaptor, respectively, in the TCR signaling complex. Consequently, we visualized the entire process of synapse formation and discovered microclusters containing TCR, kinases and adaptors as the site for initial and sustained activation of T cells. The discovery of microclusters leads to a revision of the mechanism of T cell activation such as the downstream signal molecules involved in microclusters, the function of lipid rafts, the regulation of co-stimulation signals, and the functional involvement of self-peptide recognition. We analyzed the temporal and spatial regulation of the co-stimulation receptor CD28. CD28 is recruited to TCR microclusters in the initial phase, but is accumulated in the outer region of cSMAC, indicating that CD28-mediated co-stimulation for sustained activation signals may be induced through spatially different areas in the immunological synapse from c-SMAC.

We also analyzed LFA-1-mediated co-stimulation, particularly in actin/cytoskeletal rearrangement, upon T cell activation. TCR stimulation induces a vigorous actin polymerization in the peripheral region of the spreading membrane. In contrast, LFA-1 crosslinking either by anti-LFA-1 or ICAM-1 induces a unique actin rearrangement, named “actin cloud”. The actin cloud was induced by LFA-1 crosslinking through the activation of ADAP and JNK, and it recruited LFA-1 and phosphoproteins in the absence of antigen stimulation,

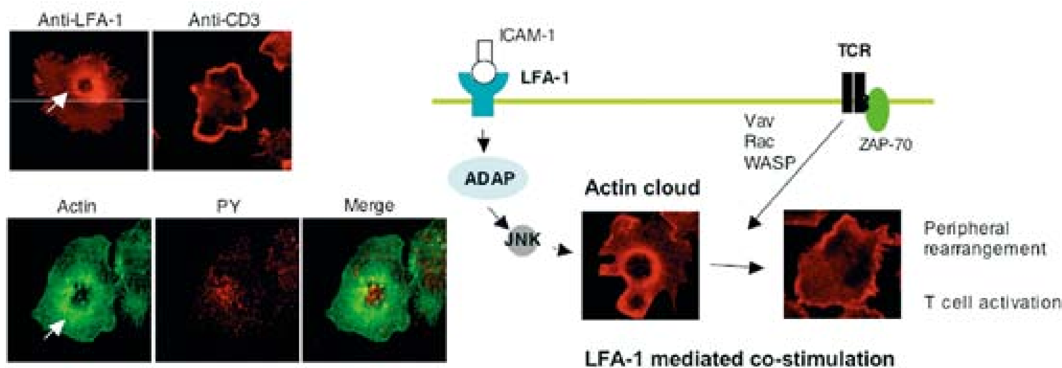

Figure (left)
LFA-1 stimulation of T cells by anti-LFA-1 or ICAM-1 induced actin cloud formation above the contact area around the center of T cells (white arrows), while CD3 stimulation induced peripheral actin rearrangement.
(right)
LFA-1-induced actin cloud recruits phosphorylated proteins, providing the possible platform for LFA-1-mediated co-stimulation

which resembles immature immunological synapses. Since the actin cloud formation augments further actin rearrangement at the periphery and increases the co-stimulation function of LFA-1, the actin cloud may be the basis of the LFA-1-mediated co-stimulation. Under physiological condition, the LFA-1-induced actin cloud may be initially induced by local and weak TCR stimulation or chemokine stimulation which in turn induces the formation of the immunological synapse (Figure)

Signal interface between innate and acquired immunity

The group has analyzed TCR- and FcR-mediated activation signals in T cells and mast cells, respectively, which share a common signaling feature, namely the ITAM+ adaptor molecules such as CD3s and FcRγ. ITAM-mediated signal transduction is a prototype of lymphoid-specific signaling mechanism. TCR stimulation induces signal transduction leading to several signaling pathways including activation of NFAT and NF-κB. Carma1-Bcl10-Malt1 (CBM) complex has been found to mediate NF-κB activation upon TCR stimulation since Carma1-deficient T cells fail to induce cell proliferation due to the lack of NF-κB activation. To understand the regulation of CBM-mediated NF-κB activation, Card9, a Bcl10 binding molecule, was analyzed by establishing Card9-deficient mice. T cells from Card9$^{-/-}$ mice exhibited normal activation and function. In contrast to lymphocytes, myeloid cells such as dendritic cells (DC) and macrophages showed defects in cytokine production due to impaired NF-κB activation upon crosslinking of the receptors associated with ITAM-containing adaptors, FcRγ and DAP-12. These receptors include FcγR, Dectin-1 and the activating receptor of paired receptors. Our analysis indicates that stimulation of ITAM-containing receptors induces two distinctly regulated CBM complexes; lymphoid CBM (Carma1-Bcl10) functions only in lymphocytes upon TCR and BCR stimulation, while myeloid CBM (Card9-Bcl10) mediates signal transduction to NF-κB activation only in DCs and macrophages (in press).

Regulatory mechanism of migration/ recruitment of T cells

An intriguing novel new direction of research is the investigation of the dynamic regulation of T cell migration from the lymph node to effector tissues. Homing and dynamic recruitment of lymphocytes into peripheral tissues is mainly regulated by adhesion molecules and chemokines.

We have recently cloned an Ig domain-containing adhesion molecule, CRTAM from a subtraction cDNA library. CRTAM is expressed exclusively in activated CD8+T cells and NK cells during the early stage of activation. Using CRTAM-Ig fusion protein, we could clone a ligand, Necl-2, which also belongs to the Ig superfamily. Necl-2 exhibits homotypic assembly, and heterotypic binding to CRTAM. The latter occurs between T cells and splenic DCs with higher affinity. We established CRTAM-KO mice to analyze the function of CRTAM *in vivo*. Although T cells from CRTAM-KO mice showed no alteration of activation, proliferation and cytolytic function *in vitro*, *in vivo* the effector function and the number of effector cells in the systems of anti-tumor function against implanted tumor, anti-viral activity in influenza infection in the lung, and the induction of diabetes in RIP-OVA mice, were impaired. *In vivo* proliferation of antigen-specific T cells in the lymph node was not changed, but the overall number of T cells within the lymph node was reduced. Together with our finding that CRTAM+ T cells bind better to lymph node than CRTAM- T cells, these observations suggest that CRTAM plays a role in retaining T cells within the lymph node. In the absence of CRTAM, T cells may leave the lymph node in a premature activation stage that prevents them from being recruited properly to the peripheral tissues upon inflammation or infection (submitted).

Recent major publications

Yokosuka T., Sakata-Sogawa K., Kobayashi W., Hiroshima M., Hashimoto-Tane A., Tokunaga M., Dustin M.L., Saito T. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap 70 and SLP-76. *Nat Immunol.* 6, 1253-1262 (2005)

Yamasaki S., Ishikawa E., Sakuma M., Ogata K., Sakata-Sogawa K., Hiroshima M., Wiest D.L., Tokunaga M., Saito T. Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. *Nat Immunol.* 7, 67-75 (2006)

Suzuki N., Suzuki S., Millar D.G., Unno M., Hara H., Calzascia T., Yamasaki S., Yokosuka T., Chen N-J., Elford A. R., Suzuki J-I., Takeuchi A., Mirtsos C., Bouchard D., Ohashi P. S., Yeh W-C., Saito T. A critical role for the innate immune signaling molecule IRAK-4 in T cell activation. *Science* 311, 1927-1932 (2006)

Varma R., Campi G., Yokosuka T., Saito T., Dustin M.L. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity* 25, 117-127 (2006)

Suzuki J-I., Yamasaki S., Wu J., Koretzky G.A., Saito T. The actin cloud induced by LFA-1—mediated outside-in signals lowers the threshold for T-cell activation. *Blood.* 109, 168-175 (2007)

Research Unit for Single Molecule Immunoimaging

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Based on a novel approach to single molecule imaging, our team has pioneered the application of single-molecule studies to immunology. The goal of single-molecule immunoimaging is to visualize immunological responses and signaling processes at the single-molecule level and within single cells. To this end, a new type of fluorescence microscopy, termed "Highly Inclined and Laminated Optical Sheet" (HILO) microscopy, was developed. A microscope system was installed at RCAI and various collaborations with different groups at the center have been initiated. Our group has focused on the development of novel types of fluorescence microscopy for use in single-cell/single- molecule studies. In addition, we are developing analysis software tools for imaging and quantitative analysis. Presently, more than eight external research collaborations including international collaborations are ongoing.

HILO microscopy

We have been involved in the development of total internal reflection fluorescence (TIRF) microscopy, a light-microscopic technique that uses evanescent light to illuminate single molecules. TIRF has become a widespread technique for single-molecule imaging at surfaces, but cannot be used for single-cell imaging due to the very limited depth of evanescent light. HILO is built upon the TIRF approach but enables the imaging of single molecules within living cells. Most importantly, HILO visualizes single molecules not only on cell surfaces but also inside living cells. Further, it has been shown that single-molecule imaging and quantification of molecular interactions are useful new tools to elucidate the molecular mechanism of cellular functions. HILO microscopy can be used for observations within living cells that are sensitive to the illuminated light, and for time-lapse observation over long periods since only weak illumination is required. Using HILO, the dynamic movements of large numbers of single molecules inside a cell can be traced. Thus, HILO is also useful for quantitative studies of the distribution, dynamic movement, or interaction of large numbers of molecules. Whereas a single-color HILO was developed initially, a new multi-color system has been installed to observe intermolecular interactions in greater detail.

Visualization of initiation of T cell activation

Although the immunological synapse has been thought to mediate antigen recognition and activation of T cells, the sites for initiating and sustaining T cell receptor (TCR) signals remain elusive. We have developed the "time 0 method" and used it to clearly visualize the initiation of T cell activation from the moment of the activation. Using molecular imaging, we have demonstrated that T cell activation is initiated and sustained in TCR-containing microclusters generated at the initial contact areas and at the periphery of the mature immunological synapse. Microclusters containing TCR, kinase

ZAP-70 and adaptor SLP-76 are continuously generated at the periphery. Whereas microclusters migrate toward the central region, SLP-76 disappears completely and ZAP-70 is mostly lost before the microclusters join the TCR-rich central region. These results suggest that TCR microclusters initiate and sustain TCR signals.

Application to nuclear pore complex

Nuclear transport is a highly dynamic process occurring in the nuclear pore complex (NPC). Using novel microscopy, we have clearly visualized single fluorescent molecules inside cells during nuclear import. Molecular interactions with the assembled NPC were quantified by single-molecule analysis. The retention times, the number of associated molecules, the dissociation constant, and the stoichiometry of import were all determined. Simulation based on a model with two types of multi-binding sites using these parameters well explained the molecular kinetics in cells. The combination of single-molecule quantification and modeling opens new approaches for the development of molecular systems biology.

Other ongoing projects

In addition to the above, a number of other internal and external collaborations are ongoing. For example, together with Takashi Saito's group, we are studying single-molecule imaging of lipid rafts. Visualization of Vav protein function is ongoing in cooperation with Dr. Wojciech Swat at the University of Washington. Further, in cooperation with Haruhiko Koseki, we are attempting to visualize chromatin modifier proteins and nuclear dynamics. Studies on STAT4 signaling molecule and visualization of zinc signaling are likewise ongoing.

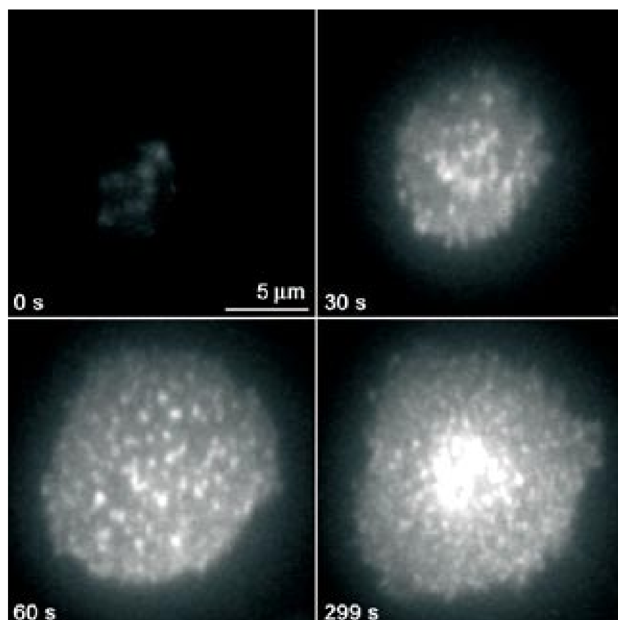

Figure-1 Single-cell molecular imaging: Microclustering of T cell receptor by signaling activation.

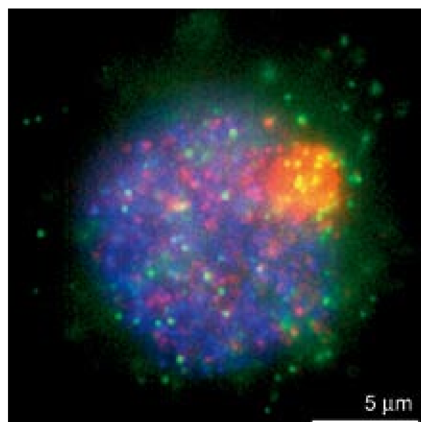

Figure-2 Image of immunofluorescence staining at a single-molecule level. Green: NFκB, red: phospho-c-jun, blue: acetylated H3.

Recent major publications

Miletic A. V., Sakata-Sogawa K., Hiroshima M., Hamann M. J., Gomez T. S., Ota N., Kloeppel T., Kanagawa O., Tokunaga M., Billadeau D. D., Swat W. Vav1 Acidic Region Tyrosine 174 Is Required for the Formation of T Cell Receptor-induced Microclusters and Is Essential in T Cell Development and Activation. *J Biol Chem.* 281, 38257-38265 (2006)

Yamasaki S., Ishikawa, E., Sakuma, M., Ogata, K., Sakata-Sogawa K., Hiroshima, M., Wiest, D. L., Tokunaga M., Saito, T. Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. *Nature Immunol.* 7, 67-75 (2006)

Yokosuka T., Sakata-Sogawa K., Kobayashi, W., Hiroshima, M., Hashimoto-Tane, A., Tokunaga M., Dustin, M. L., Saito, T. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nature Immunol.* 6, 1253-1262 (2005)

Shiina, N., Shinkura, K. Tokunaga, M. A novel RNA-binding protein in neuronal RNA granules, regulatory machinery for local translation. *J Neurosci.* 25, 4420-4434 (2005)

Sakata-Sogawa, K., Shimamoto, N., RNA polymerase can track a DNA groove during promoter search, *Proc. Natl. Acad. Sci. USA* 101, 14731-14735 (2004)

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B lymphocytes are the central mediators of humoral immunity. They differentiate through highly regulated pathways before becoming mature plasma cells that secrete antigen-specific antibody. B cells depend on cues from their extracellular microenvironment for development, homeostasis, activation, proliferation, survival, and effector function. These functions are regulated through cell surface molecules that generate transmembrane signals, regulate intercellular communication, and direct lymphocyte localization within tissues. These events are thought to primarily rely on signals generated by the B cell antigen receptor (BCR) composed of the membrane immunoglobulin noncovalently associated with disulfide-linked Igα/Ig heterodimers. In addition to the BCR, other cell-surface receptors such as CD40 contribute to B cell responses by functioning as co-receptors in their interactions with other types of cells, including T cells and stromal cells. Our laboratory aims at understanding the molecular basis of signaling complexes that contribute to cell fate decisions such as differentiation, proliferation, or apoptosis in B cells. Among various signaling pathways, we have particularly focused on how PLC-γ2/calcium signal is regulated and translated into biological outputs.

Regulation of calcium signaling by STIM1

Cytosolic calcium signals are key to the regulation of various physiological events. Two stages of calcium mobilization have been distinguished in lymphocytes. The first stage involves activation of PLC by the trimeric G protein- or the tyrosine kinase-coupled receptors. This enzyme hydrolyzes phosphatidylinositol bisphosphate to release the second messenger inositol-1,4,5-trisphosphate, which binds to its receptor in the endoplamic reticulum (ER) membrane, thereby causing a rapid and transient release of calcium from the ER stores. The second stage involves a sustained influx of extracellular calcium across the plasma membrane (PM) in a process termed store-operated calcium (SOC) entry. In this process, depletion of calcium within the ER lumen serves as the primary trigger to open SOC channels residing in the PM.

STIM1 has recently emerged as a key player in coupling the first and second stage of calcium mobilization. To elucidate the mechanism by which STIM1 activates SOC channels, we first established and analyzed STIM1-knockout B cells. Then, we constructed various STIM1 mutants and assessed their dynamic movement and functional consequences. What we found was that STIM1 moves in a tubulovesicular shape on the ER and its subcompartment in resting living cells, whereas, upon store depletion, it is rapidly redistributed into discrete puncta that are located underneath, but not inserted into the PM. Three functional domains of STIM1 (coiled-coil, Ser/Thr-rich, and sterile α-motif) were essential for activating SOC channels. Because deletion of these domains aborted depletion-mediated redistribution of STIM1 into puncta underneath the PM, our results

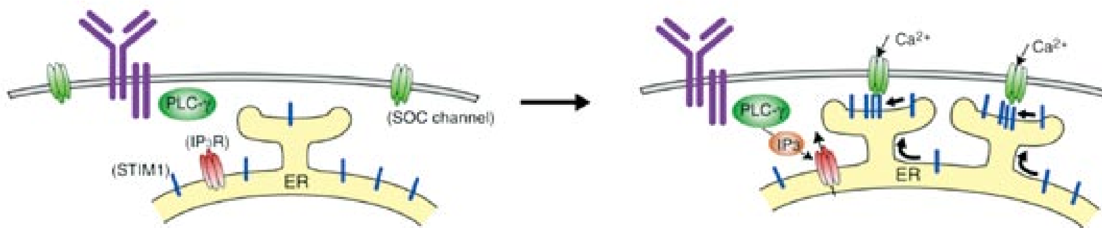

Figure Proposed mechanisms by which STIM1 activates SOC channels residing on the plasma membrane.
Once IP3 is generated, the IP3 receptor on the ER (red tetramers) can release calcium from the ER pools into cytoplasm. Then, calcium depletion in the ER makes STIM1 (blue monomer) redistribute just beneath the plasma membrane, thereby activating SOC channels (green tetramers) in the plasma membrane.

highlight the importance of the intracellular movement of STIM1 to activate SOC channels (Figure). (See Research Highlights.)

Function of ERK2 in B cell development and activation

Like other cell types, Ras is activated by BCR stimulation and the introduction of dominant-negative Ras inhibits BCR-mediated ERK activation, demonstrating that Ras functions as an upstream regulator for subsequent ERK activation. It was thought that Sos (one type of nucleotide exchange factor for Ras) participates in BCR-mediated Ras activation. However, data emerging from our and other laboratories indicate that RasGRP3 (another type of exchange factor for Ras) rather than Sos, plays a more dominant role for coupling BCR to Ras activation. The requirement of RasGRP3 in Ras activation well explains the previous findings that PLC-γ2 was necessary for BCR-mediated Ras activation. Since RasGRP3 possesses the C1 domain that binds to diacylglycerol (DAG), a product of the activity of PLC-γ2, the idea has been evoked that PLC-γ2 participates in the recruitment of RasGRP3 in a DAG-C1-dependent manner. Our previous analyses (Oh-hora et al; *J. Exp. Med.* 2003; Aiba et al; *PNAS* 2004) demonstrated that this hypothesis was indeed correct.

Based on the above evidence that PLC-γ2 regulates ERK activation in the BCR signaling context, we have tried to identify the functional roles of ERK2 in B cell development and activation. By using B cell-specific ERK2-targeted mice, we found that B cell development takes place normally even in the absence of ERK2, but that ERK2 is required for an efficient IgG production in T-dependent immune responses. In its absence, the proportion of antigen-specific IgG1-bearing cells and the subsequent number of IgG1 antibody-secreting cells were decreased, despite apparently unimpaired class switch recombination. Notably, this defect was countered by overexpression of the antiapoptotic factor Bcl2. Together, our results suggest that ERK2 plays a key role in the efficient generation of antigen-specific IgG-bearing cells by promoting their survival.

Conclusion

The above series of experiments demonstrate the importance of STIM1 in BCR-mediated calcium influx and of ERK2 in B cell biology. However, these new findings have raised interesting questions; 1) What is the biological role of STIM1 in B cell development and immune responses? ; 2) Why does ERK2 affect only IgG-switched, but not IgM B cells?; 3) What about the roles of ERK2 in B cell memory function? By establishing B cell-specific and stage-specific deletions of STIM1 and ERK2, we have been intensively pursuing these issues.

Recent major publications

Sanjo, H., Hikida, M, Aiba, Y., Mori, Y., Hatano, N., Ogata, M. and Kurosaki, T. Extracellular signal-regulated protein kinase 2 is required for efficient generation of B cells bearing antigen-specific Immunoglobulin G. *Mol. Cell. Biol.* 27, 1236-1246 (2007)

Baba, Y., Hayashi, K., Fujii, Y., Mizushima, A., Watarai, H., Wakamori, M., Numaga, T., Mori, Y., Iino, M., Hikida, M. and Kurosaki, T. Constitutive and inducible movement of STIM1 on ER and its sub-compartment contributes to coupling STIM1 to store-operated $Ca2^{+}$ entry. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16704-16709 (2006)

Yamada, T., Hikida, M. and Kurosaki, T. Regulation of cytokinesis by mgcRacGAP in B lymphocytes is independent of GAP activity. *Exp. Cell Res.* 312, 3517-3525 (2006)

Aiba, Y., Yamazaki, T., Okada, T., Gotoh, K, Sanjo, H., Ogata, M. and Kurosaki, T. BANK negatively regulates Akt activation and subsequent B cell responses. *Immunity* 24, 259-268 (2006)

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Memory B cells show a unique morphology and phenotype and acquire several intrinsic properties that differ from other stage of B cells, such as longevity, preferential localization in the antigen draining sites, and rapid response to antigen reexposure by differentiating into plasma cells. However, how and when memory B cells are established in the immune system and the mechanisms underlying their maintenance and prompt differentiation remain obscure.

Our aim is to know the origin and developmental pathways of memory B cells and the regulatory network responsible for the acquisition of memory B cell function.

Pathways of Memory B cell Development

Concerning memory B cell generation, it has been widely accepted that memory B cells originate from the germinal center (GC), wherein somatic hypermutation takes place. However, our recent observations suggest that memory B cells generate at the early immune response, probably before or at the beginning of GC formation (Talahashi et al. *Immunity* 2001; Toyama et al. *Immunity* 2002: Inamine et al. *Int. Immunol.* 2005).

To know the regulatory network towards memory B cell development, we have utilized Affymetrix GeneChip analysis and attempted a characterization of gene expression profiling that takes place in the transition from a naïve B cell to either GC B cells, memory B cells or plasma cells upon antigen stimulation. We detected a set of 100-200 transcripts enriched in antigen-stimulated B cell subsets to naïve B cells. Q-PCR confirmation reveals prominent groups of genes with similar changes in expression pattern within GC B cells, memory B cells and plasma cells upon antigen stimulation.

Memory B cell population expressed a group of transcripts selectively enriched in this population, with similar time-dependent changes in their expression patterns. The first group of transcripts was retained throughout the immune response from day 7 to day 40 postimmunization, supporting the idea that memory B cell commitment may occur at the early immune response, probably before day 7 after immunization. The second group of transcripts increased in memory phenotype B cell level as the immune response progressed, probably associated with functional development at the later immune response.

Based on the expression of these genes, we characterized the generation of early memory B cell precursors in the spleen of mice immunized with NP-CG and suggest that antigen-activated B cells progress to memory phenotype and GC phenotype B cells, at least, from day 5 to day 6 postimmunization with expression of several transcripts peculiar to each lineages, accompanied by an IgG class-switch recombination and efficient proliferation (Takemori and Kaji, *Keystone Symposium*, 2007). The results raise the question as to whether the memory compartment is actually founded by GC B cells. We are currently establishing a system to clarify this possiblity.

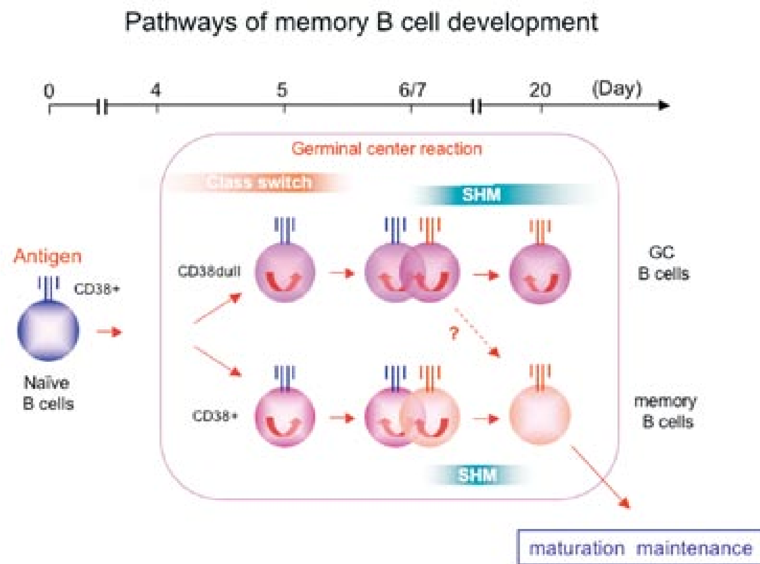

Figure Antigen-activated B cells progress to memory and GC phenotype B cells, at least, from day 5 to day 6 postimmunization with expression of several transcripts peculiar to each lineage, accompanied by class-switch recombination and efficient proliferation. Both the memory and GC compartments consisted of IgM+ and IgG+ B cell populations with similar gene expression profiling, raising the pssibilbity that both cell isotypes are involved in the primary memory and GC response.

Identify the molecules responsible for memory B cell functions

We have cloned several genes which are highly expressed in memory B cells, including E52, 3940, 3023 and 4010. Over expression of E52, 3940 and 3023 in B cell lymphoma cell lines prolonged cell survival in proapoptotic culture conditions, thereby, led us to speculate that these genes could be associated with the memory B cell survival.

We observed that 4010 encodes an adopter molecule and that its overexpression in splenic B cells results in augmentation of IgG1 response upon stimulation with anti-Igs and anti-CD40 mAbs *in vitro*. To examine the possibility whether 4010 could be involved in the signaling cascade responsible for terminal differentiation of memory B cells into antibody forming cells, we are currently establishing conditional knock out mice under the collaboration with Drs Kurosaki and Hikida (RCAI), and Dr. Rajewsky (Harvard Medical School, USA).

E52 was turned out to be a murine homologue of the survival of motor neuron (SMN) gene 1, which is known as a causative gene for Spinal mascular atrophy (SMA), characterized by a degeneration of a-motor neurons. SMA is clinically divided into severe and mild forms; the children in the type I disease are hypotonic and unable to sit unaided and their condition is accompanied with a rapid development of respiratory difficulty and serious respiratory infections, resulting in the child's demise, usually before the age of 18 months. It has been predicted that severe infection in SMA patients resulted from the secondary effect by defect in respiratory activity; however, we intended to know whether SMN deficiency might affect immune function.

Our extensive analysis showed that SMN associates with an effector molecule responsible for anti-oxidant activity and suggest that SMN plays a role in cell survival, probably through the contribution to the mitochondrial function (submitted). Furthermore, we observed that conditional deletion of a SMN function domain causes B cell deficiency in the T-cell dependent immune response (under the collaboration with Drs Takahashi (NIID), Melki (INERM), Rajewsky (Haravard Medical School, USA) and Casola (IFOM- The FIRC Institute of Molecular Oncology, Italy); manuscript in preparation).

The memory B cell population appeared to achieve functional development and higher affinities from day 7 to day 40 after immunization, accompanied by a unique time-dependent gene expression profiling. Such a phenotypical and transcriptional change in the memory B cell compartment may reflect a selection process among memory B cell populations and their localizations to different microenvironment at the early and late immune response.

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Laboratory for Antigen Receptor Diversity

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Germinal center (GC) B lymphocytes are a unique cell population that arises during a T cell-dependent antibody response. In GC B cells, the immunoglobulin (Ig) genes undergo two dynamic genetic alterations: first, somatic hypermutation (SHM), which is focused on the variable-region exons and can alter the antibody affinity for the antigen, and second, class switch recombination (CSR), which changes the constant region of the antibody to acquire different effector functions. Both SHM and CSR are initiated by a single enzyme, activation-induced cytidine deaminase (AID), which catalyzes the deamination of cytosine (C) to uracil (U); however, the precise mechanism of SHM still remains elusive. Our focus is on the study of genetic alterations in GC B cells and, in particular, on the role of various low-fidelity DNA polymerases. These polymerases are unique as they can replicate DNA past unrepaired lesions and have been implicated in both mutagenesis and mutation avoidance. We believe that the study of these polymerases will yield new insights into the molecular machinery of SHM and CSR. Further, we are investigating the roles of these polymerases in the maintenance of genome stability and in the development of lymphoid malignancies.

Role of the low-fidelity DNA polymerases in the somatic hypermutation of Ig genes.

As many as 10 low-fidelity DNA polymerases have recently been identified. At least four of these polymerases, Polθ, Polη, Polζ and Rev1, have been shown to participate in SHM of Ig genes. Polθ is a ~300-kDa family A polymerase with a unique structure, having a helicase domain in its N-terminal portion and a polymerase domain in its C-terminus. We have previously shown that inactivation of its polymerase domain resulted in a specific reduction of C/G mutations of Ig genes. To further understand the role of Polθ in Ig gene SHM, we have also generated mice that completely lack *Polq* expression (*Polq*-null). *Polq*-null mice showed a reduction of both C/G and A/T mutations. These results collectively suggest that Polθ is involved not only in C/G but also in A/T mutations of Ig genes.

Polη has been shown to play a dominant role in the generation of A/T mutations. To investigate how Polθ and Polη differentially participate in the generation of A/T mutations, we have analyzed the frequency and patterns of Ig gene mutations in mice deficient for both Polθ and Polη and compared them with the results from mice singly deficient for either enzyme. However, we did not observe a further decrease of the A/T mutations in Polθ Polη double-deficient mice. These results suggest that Polθ and Polη function in the same genetic pathway. Biochemical analyses using recombinant Polη and Polθ revealed that Polθ is capable of catalyzing the extension from mismatches opposite either template A or T much more efficiently than Polη. In addition, Polθ was able to extend Polη-generated mismatches opposite template A. The biochemical properties of Polη and Polθ, along with the genetic evidence, implicate that Polη and Polθ might function sequentially during SHM of Ig genes, with Polη first introducing a wrong nucleo-

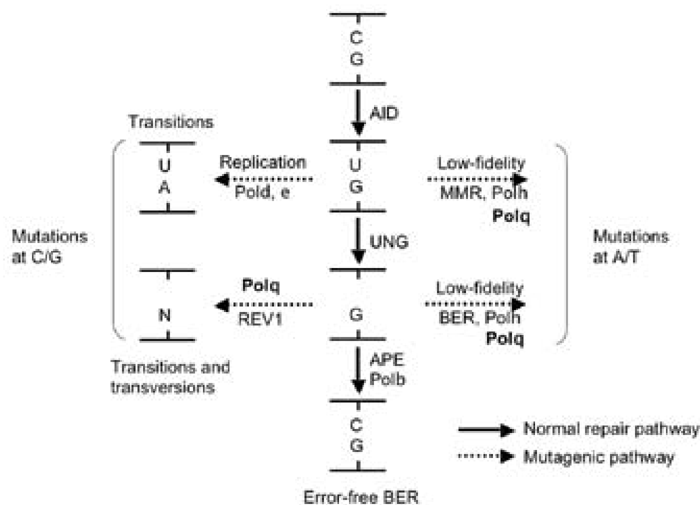

Figure Role of the low-fidelity DNA polymerases in the SHM of Ig genes. AID (activation-induced cytidine deaminase) deaminates cytosine (C) to uracil (U) and generates a U/G lesion. Under normal situations, this U/G lesion is correctly repaired by the base excision repair (BER) pathway. During Ig gene SHM, however, the intermediates of the BER pathway are either replicated to cause mutations at C/G or resolved by low-fidelity mismatch repair (MMR) or BER pathways, both of which result in the induction of A/T mutations. The proposed roles for Polθ (shown in bold) as well as several other polymerases are indicated. UNG, uracil DNA glycosylase.

tide opposite A or T, a fraction of which was then extended by Polθ. Further studies are required to reveal whether such a two-step polymerase action indeed occurs *in vivo*.

Why do GC B cells have increased mutations at A/T?

Using *lacZ*-transgenic mice in which the genome mutation frequency and patterns can be easily detected and with high sensitivity, we have examined the mutations in naïve and germinal center B cells. We found that GC B cells have a dramatic increase of mutation frequency as compared with naïve B cells, which may be related to the fact that GC B cells undergo rapid cell division and thereby are more likely to accumulate genomic mutations. More interestingly, we found a high frequency of mutations that occur at A/T in GC B cells. A number of experiments are now in progress to examine whether certain DNA polymerases might play a role in the induction of A/T mutations. Clarifying the molecular mechanism of the A/T mutations has significant implications in understanding how mutations are targeted to each nucleotide and how GC B cells acquire the ability to generate all the types of mutations necessary for the unfettered affinity maturation of antibodies.

Analysis of Clast6/Laptm5, a novel lysosomal protein involved in the regulation of T cell activation

Clast6/Laptm5 is a lysosomal protein that contains five membrane-spanning segments and a proline-rich domain at its C-terminus. Clast6 is specifically expressed in lymphoid tissues and its expression is rapidly and transiently down-regulated in both B and T cells by various activation signals. To explore its physiological role in the immune system, we have generated Clast6 knockout (Clast6-KO) mice by homologous recombination in embryonic stem cells. B and T cell development and maturation were normal in Clast6-KO mice although there was a moderate increase in the total cellularity in the bone marrow. Clast6-KO B cells showed no apparent abnormalities in terms of their proliferative responsiveness to various B cell activation signals. However, in comparison with wild-type (WT) T cells, Clast6 KO T cells exhibited elevated proliferation and cytokine production in response to CD3 stimulation. Both CD4 and CD8 T cells of Clast6 KO mice were hypersensitive to CD3-mediated proliferative signals. To explore the mechanism of the T cell hyperresponsiveness in the absence of Clast6, we examined the expression of molecules known to be involved in the signal transduction through the T cell antigen receptor (TCR). We found that the degradation of the TCRζ chain was impaired in Clast6-KO T cells, suggesting that Clast6 might directly or indirectly regulate TCRζ expression. Co-transfection of Clast6 and TCRζ into fibroblasts resulted in a dramatic reduction of the TCRζ protein level, compared with TCRζ-alone transfectants, without affecting its transcript level. Moreover, overexpression of Clast6 in a T cell hybridoma decreased the level of the endogenous TCRζ and abolished cytokine production induced by CD3 stimulation. These results collectively suggest that Clast6/laptm5 mediates the degradation of the TCRζ chain and thereby negatively regulates T cell activation.

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The mucosal epithelium that lines the inner surfaces of the body, especially within the intestine, is exposed to various macromolecules and microorganisms whose efficient uptake is crucial to maintaining normal immune regulation. Epithelial cells that cover the gut-associated lymphoid tissue (GALT), such as Peyer's patches (PP) and isolated lymphoid follicles, are both histologically and biochemically distinct from normal absorptive epithelial cells of the villi and are termed follicle-associated epithelium (FAE). FAE contains a specialized subset of epithelial cells, M cells, that play an important role in immune surveillance by delivering ingested macromolecules and microorganisms to the underlying lymphoid cells via transcytosis. Despite their significance, studies of M cells remain in their infancy, mainly because the low frequency of M cells and the lack of specific surface markers make it difficult to purify the M cells required for molecular/biochemical analyses. Accordingly, one of the primary aims of our laboratory is to understand the mechanisms that underlie the differentiation and function of FAE and M cells. Our research team is also investigating the recognition of commensal microbiota by intestinal epithelium and its influence on the mucosal and systemic immune system.

Identification of M cell-specific markers

Although M cells are believed to play an important role in mucosal immunity, the molecular mechanisms of antigen uptake and transport remain to be unraveled, and especially, the identification of endocytic receptors on the apical membrane of M cells. To solve the problem, we took advantage of transcriptome analysis using Affymetrix microarray in combination with real-time PCR and *in situ* hybridization. Consequently, we identified a gene encoding a GPI-anchored membrane protein, Glycoprotein-2 (GP-2), exclusively expressed in M cells among the intestinal epithelial cells. GP-2 has originally been reported to be abundantly expressed in pancreatic acinar cells and secreted in the pancreatic fruid, although its physiological role remains elusive. To examine its intracellular localization in M cells, both rat monoclonal and rabbit polyclonal antibodies directed against N-terminus of GP-2 were generated. Immunofluorescent staining with these antibodies proved the expression of GP-2 on the apical plasma membrane of M cells. The apical localization was also confirmed by confocal laser microscopic analysis on whole mount-stained specimens of murine PP. To examine whether it can act as an endocytic receptor, the anti-GP-2 monoclonal antibody was injected into the ligated intestinal loop containing PP. After incubation, the antibody bound to the surface-expressing GP-2 was endocytosed into the sub-apical region of M cells. The internalized molecule was not colocalized with Rab5a, an early endosomal marker protein, suggesting a role of this molecule in the non-conventional endocytic pathway of mucosal antigens in M cells. In conclusion, we found GP-2 as a novel M-cell marker

protein localized on the apical membrane. Our data suggest that GP-2 could work as a possible endocytic receptor of the luminal antigens and could serve as a potential target for M-cell-mediated oral vaccination and/or drug delivery.

Analysis of host-indigenous bacterial cross-talk via gene expression profiling of murine intestinal epithelium

In recent years, so many probiotics having beneficial effects on the hosts have been reported. However, we know little about these beneficial mechanisms including the host-bacterial or commensal-pathogenous bacterial cross-talk. In order to elucidate these host-commensal-pathogenous bacterial cross-talks, we investigated the role of bacterial protection against lethal infection of entero-hemorrhagic *Escherichia coli* O157:H7 in a mouse model. In our previously experiments, complete protection against O157 infectious death was found in *Bifidobacterium longum*-associated mice, although most of O157 mono-associated mice died within 7 days after infection. The protection afforded by *B. longum* is not due to the decrease in the number of O157, the production of Vero toxin, nor the pH in the intestine due to the presence of *B. longum* (unpublished data). Therefore, the protective role by *B. longum* against O157-infectious death is assumed to be an indirect one, through interaction with the host immune response and/or the host intestinal epithelium. As an initial approach, we examined the gene expression profiles of mouse small intestinal epithelial cells (IEC) and FAE of PP in each bacterium-associated mice with cDNA microarray. These analyses revealed that the gene expression profile in IEC and FAE of *B. longum*-associated mice and O157-associated mice was different, whereas that of *B. longum*- and O157- double associated mice (BE) and BL was similar. These results suggest that *B. longum* affects host IEC and FAE and prevent O157 infectious death . The difference was especially remarkable for those genes categorized as immune response, transcription factor activity, transcriptional repressor activity, and cytoskeleton by the Gene Ontology (http://www.geneontology.org/). We are now trying to deduce the biological pathways in each hierarchical clustering analysis-supervised gene cluster.

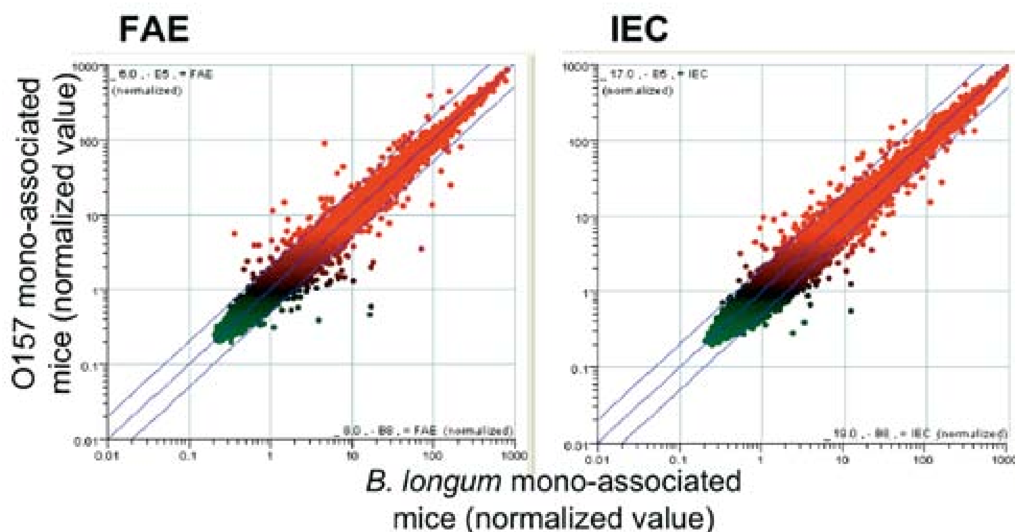

Figure Differential gene expression by intesitinal epithelial cells between *Escherichia coli* O157:H7 mono-associated mice and *Bifidobacterium longum* mono-associated mice. Normalized data obtained by microarray analysis are drawn in a scattered plot. Gene expression levels of follicle-associated epithelium (FAE) (left) and intestinal epithelium cells (IEC) isolated from O157 mono-associated mice and *B. longum* mono-associated mice were compared. The upper and lower blue lines indicate the threshold levels of 2-fold higher and lower expression, respectively. The data indicate that some genes are either up-regulated or down-regulated both in FAE and in IEC upon O157 association compared to *B. longum* association.

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Our intestine is colonized by an impressive community of commensals, that has profound effects on the immune functions. The relationship between gut microbiota and the immune system is one of reciprocity: commensals play an important role nutrient processing and education of the immune system and conversely, the immune system, particularly gut-associated lymphoid tissues (GALT) plays a key role in shaping the repertoire of gut microbiota. Our laboratory's aim is (a) to understand commensal host-bacterial relationships in the gut, with multiple feed-back and feed-forward controls involving both bacteria and immune cells and (b) to characterize cellular and molecular interactions responsible for tolerance or immunity in the intestinal mucosa.

Molecular mechanisms for migration of innate-like peritoneal B1 cells to the gut

Innate-like B1 cells residing mainly in the peritoneal cavity were found to generate a large amount of antibodies outside their niche (i.e. IgM in the spleen or IgA in the small intestine). These antibodies play a critical role in the early phase of infections, well before the adaptive immune responses are established. However, despite the importance of B1 cells, the molecular signals required for their mobilization remain unknown. We have determined precisely how B1 cells sitting within the peritoneum quickly traffic to the site of immune responses at distant locations throughout the body. We demonstrate that B1 cells express extremely high levels of integrins and that direct signals through toll-like receptors (TLRs) induce a massive egress of B1 cells from the peritoneal cavity that is associated with coordinated down-regulation of integrins and CD9. We show that the modulation of adhesion/motility-related proteins on B1 cells allows them to migrate with enhanced motility in response to chemokines. Thus, we reveal an unanticipated, new role for TLRs in regulation of trafficking of cells that connect innate with adaptive immune responses.

Homing of B1 cells to the peritoneal cavity and peritoneum-associated tissues depends mainly on the integrin $\alpha 4\beta 1$ and the B cell chemokine CXCL13. The main ligand for $\alpha 4\beta 1$ is VCAM-1, expressed by stromal cells in the omentum follicles and mesothelial cells of parietal peritoneum. Retention of B1 cells into these tissues is due to their high expression levels of integrins in association with tetraspanin CD9, which facilitate a stable attachment of B1 cells to the local matrix. Signals through TLRs induced by bacteria or bacterial components facilitate detachment of B1 cells, through down-regulation of integrin-CD9 complexes. CD9 modulation and activation of G-protein coupled receptors by yet undefined chemokines, enhance cell motility through the extracellular matrix and transmigration across the vascular endothelium. (See Research Highlights.)

Regulation of mucosal immune responses by lymphoid tissues-inducer cells (Lti) through their interactions with gut stromal cells

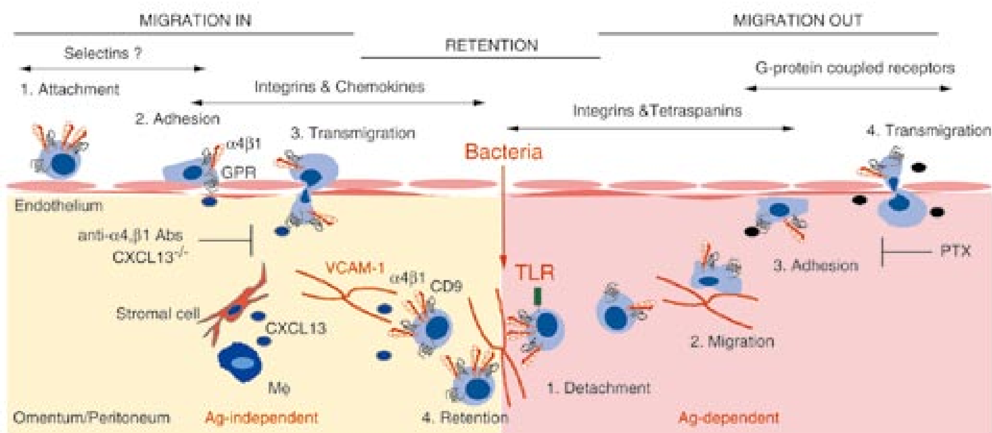

Figure-1 Proposed model for 'in' and 'out' migration of B1 cells

Lymphoid tissues-inducer cells were identified as hematopoietic-derived cells that are essential for lymph nodes and Peyer's patches formation during embryonic development. They express a transcription factor called RORγt, which is present besides LTi only in a subset of immature T cells and Th17 in the gut. Using RORγt-GFP knock-in mice (generated in Dan Littman's lab at NewYork University) we found that the function of LTi extend beyond embryonic life. We identified LTi in the adult intestine and found that these cells regulate mucosal immune responses, by inducing an “inflammatory" type of response in the gut. They facilitate both recruitment of lymphocytes in the gut lamina propria and IgA induction, through their interaction with gut stromal cells. LTi are thus “gut sensors” that control the gut plasticity required for maintenance of intestinal immune homeostasis.

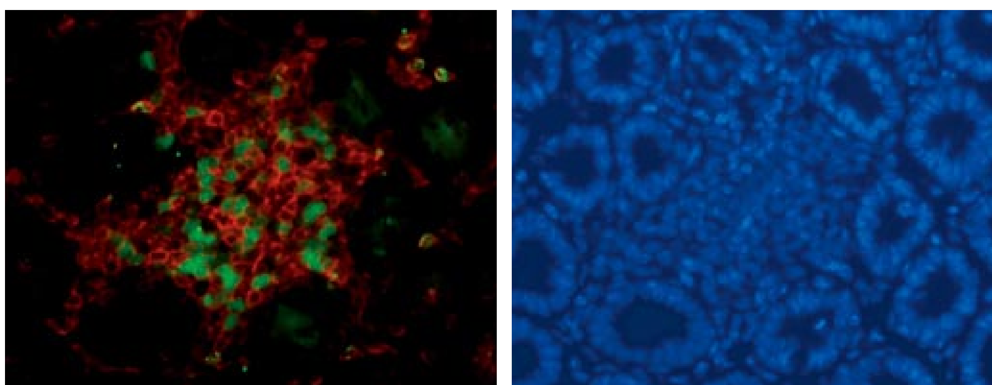

Figure-2 Section from adult small intestine, stained with GFP, CD45 (*red*) and DAPI (*blue*) and showing a cluster of RORγt$^+$ LTi cells and CD45$^+$ lymphocytes in a crypt patch.

Recent major publications

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Host defense in mammals relies on both innate and adaptive immunity. Innate immunity functions as a pathogen sensor and is involved in eradicating pathogens. Furthermore, innate immunity also contributes to the establishment of adaptive immunity. Dendritic cells (DCs) are antigen-presenting cells critically involved in a sequence of these immune responses. DCs sense various pathogen-derived molecular components and exert their immunostimulatory functions by producing inflammatory cytokines or upregulating expression of costimulatory molecules. Those components are considered as immune adjuvants based on these DC activating abilities. Immune adjuvants are recognized by various types of pattern recognition receptors including Toll-like receptors (TLRs). Identification of new types of immune adjuvants and characterization of the mechanism whereby they activate DCs should contribute to the development of novel immunoregulatory maneuvers. We are attempting to clarify how DCs are activated through pattern recognition receptors and to obtain critical information for manipulating the immune response effectively. Various immune adjuvants including TLR ligands are important tools for this purpose and so are gene-targeted mice.

How do TLRs have pleiotropic effects on DCs

TLRs can recognize a variety of immune adjuvants, which can be categorized into lipids, proteins, or nucleic acids. TLRs can transduce common immunostimulatory signals, but each TLR can also carry out its own function based on a specific molecular mechanism.

Notably, nucleic acid adjuvants are characterized by their ability to induce type I IFNs, especially IFN-α. Single-stranded RNA (ssRNA) and CpG DNA function as ligands for TLR7 and TLR9, respectively. Although TLR7 and TLR9 signaling can induce production of type I IFNs, the induction depends on a DC subset, called plasmacytoid dendritic cells (PDCs). PDCs express exclusively TLR7 and TLR9 among TLRs and are known also as type I IFN-producing cells during virus infection.

Type I IFN induction by TLR7/9 is obviously critical for antiviral immune responses. It is also noteworthy that TLR7/9 can recognize nucleic acids not only from virus but also from the host. In this regard, TLR7/9 signaling has a potential to provoke autoimmunity.

TLR7 and TLR9 show high homology in both extracellular and intracytoplasmic domains and trigger similar signal transduction pathways. The signaling depends on a cytoplasmic adapter, MyD88, because MyD88-deficient DCs fail to produce both inflammatory cytokines and type I IFNs in response to TLR7/9 agonists. Downstream of MyD88, the signaling pathway splits into two pathways for inflammatory cytokine and type I IFN induction. Type I IFN induction requires activation of a transcription factor, IRF-7 and IRF-7 activation requires its phosphorylation. However, how

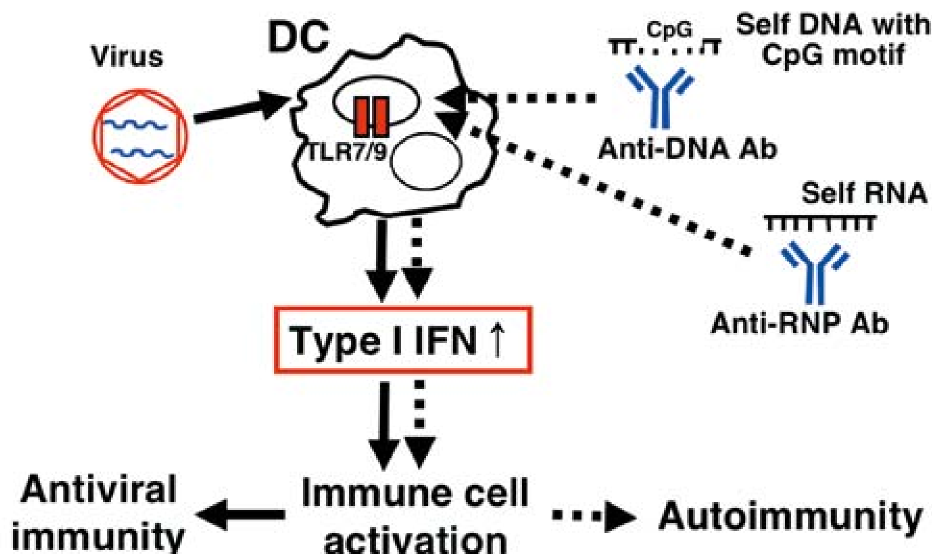

Figure Roles of Type I IFN induction by TLR7/9-stimulated DCs. Virus but also host nucleic acids can function as TLR7/9 agonists. TLR7/9-stimulated DCs produce type I IFN and this type I IFN induction contributes to the pathogenesis of autoimmune disorders as well as antiviral immunity.

IRF-7 is activated remains unclear. By analyzing IκB kinase-α (IKKα)-deficient mice, we have found that IKKα is a critical kinase selectively involved in the type I IFN induction pathway. IFN-α production was severely impaired in TLR7/9-stimulated IKKα-deficient PDCs, while inflammatory cytokine production such as IL-12p40 was only mildly impaired. IKKα could associate with and phosphorylate IRF-7. Importantly IRF-7 activation was impaired in IKKα-deficient PDCs. Thus, IKKα is critically involved in TLR7/9-induced type I IFN production through the interaction with IRF-7. IKKα should be a potential target not only for manipulating antiviral immunity but also for treating autoimmune diseases, in which IFN-α production is elevated. (See Research Highlights.)

How are innate immune responses regulated?

DC activation signal should be negatively regulated, because excessive and prolonged activation is likely to cause inflammatory diseases. TLR-mediated activation of NF-κB transcription factor is critically involved in the coordinate expression of immunoregulatory genes, including proinflammatory cytokines. The activation is negatively regulated not only by resynthesized IκB-mediated export of nuclear NF-κB to the cytoplasm but also by ubiquitination of NF-κB.

We have focused on a nuclear protein, termed SLIM, that can interact with STAT4, the transcription factor essential for IL-12-mediated T-helper 1 (Th1) cell differentiation (Tanaka et al. Immunity 22, 729-736, 2005). SLIM carries a PDZ domain at its N-terminus and a LIM domain at its C-terminus, and belongs to a large family of LIM proteins. SLIM can function as a ubiquitin E3 ligase toward STAT proteins through the LIM domain, which is similar to the RING finger domain, a well-known structure possessing ubiquitin E3 ligase activity. In $CD4^+$ T cells, SLIM can interact with STAT proteins in the nucleus, and promote their polyubiquitination and subsequent proteasomal degradation, thereby negatively regulating STAT-dependent signaling. Consistently, SLIM-deficient mice revealed increased STAT protein level and enhanced IFN-γ production by Th1 cells. Recent studies reported that nuclear protein level of the p65 subunit of NF-κB is also regulated by ubiquitin/proteasome-dependent degradation. Moreover, SLIM is expressed not only in T cells but also in innate immune cells, suggesting that SLIM may have some role in TLR-mediated signaling pathway. We have recently found that SLIM is also involved in innate immune responses by regulating the NF-κB activation. We are now testing whether and how SLIM functions as a ubiquitin E3 ligase for NF-κB, terminating TLR-mediated NF-κB activation.

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Dendritic cells (DCs), the most potent antigen (Ag)-presenting cells (APCs), are defined by their dendritic morphology and unique phenotype, and consist of heterogeneous subsets of myeloid- or lymphoid-lineages. DC differ in their maturation state in both lymphoid and peripheral tissues. Immature DCs (iDCs) sense the presence of invading pathogens via various pattern-recognition receptors (PRRs) and process the pathogens intracellularly in inflammatory tissues. They then develop into mature DCs (mDCs), which up-regulate major histocompatibility complex (MHC) and costimulatory molecules and produce various cytokines that activate innate immune cells in inflammatory microenvironments. Subsequently, mDCs home into secondary lymphoid tissues where they present the processed Ags to naïve T cells to generate type 1 helper T (T_H1) cells and T_H2 cells, depending on their lineage and activation signals. DCs thus play a crucial role in linking innate and adaptive immunity. On the other hand, accumulating evidence suggests that iDCs are involved in the induction of peripheral tolerance under steady-state conditions *in vivo*. In addition, the modification of iDCs with certain immunosuppressive molecules generates tolerogenic DCs, which not only show a reduced T-cell stimulatory capacity but also induce anergic T cells and regulatory T (T_R) cells. We have established human and murine modified DCs with more potent capacity to induce anergic T cells and T_R cells than the previously known tolerogenic DCs *in vivo* and *in vitro*, and we therefore designate them as regulatory DCs (DC_{reg}).

Our goal is: (1) to clarify the molecular mechanisms underlying the T-cell regulatory function of DC_{reg}, (2) to develop immunotherapy with DC_{reg} for immunopathogenic diseases, and (3) to characterize the specific DC subsets involved in immune regulation.

Regulatory dendritic cells act as regulators of acute lethal systemic inflammatory response

Bacterial infection triggers host inflammation through the activation of immune cells, normally leading to elimination of the bacteria. However, the regulatory mechanisms of the host inflammatory response remain unknown. Here we report that a subset of potent tolerogenic DCs, DC_{reg}, control the systemic inflammatory response. Unlike normal DCs, which produced proinflammatory cytokines in response to bacterial lipopolysaccharide (LPS), DC_{reg} produced less proinflammatory cytokines and instead preferentially produced IL-10. These events involved the expression of IκBNS and Bcl-3 as well as cyclic AMP (cAMP)-mediated activation of protein kinase A (PKA). In addition, DC_{reg} not only suppressed LPS-induced production of proinflammatory cytokines in macrophages, but also reduced the serum cytokine levels in mice. Furthermore, DC_{reg} prevented mice from dying of experimental endotoxemia and bacterial peritonitis. The inhibitory effect of DC_{reg} on inflammatory responses involved the production of IL-10. Moreover, naturally existing tolerogenic DC

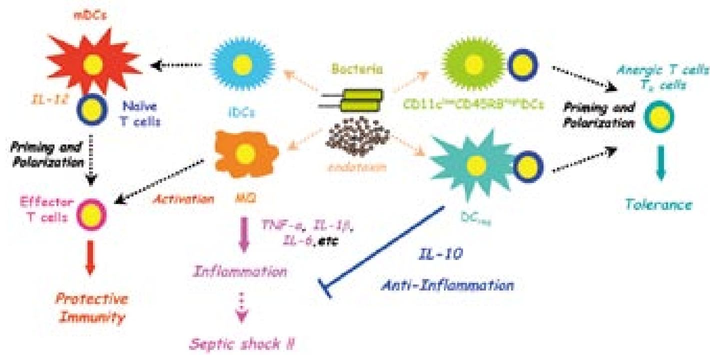

Figure-1 Regulation of immunity and inflammation by DC_{reg} and naturally existing tolerogenic DCs. Host innate immune responses to bacterial infections are primarily mediated by conventional iDCs and macrophages. These cells sense the presence of invading pathogens via various PRRs. Stimulation of the PRR signaling pathway initiates the secretion of proinflammatory mediators which promote host inflammatory responses, resulting in the elimination of microorganisms. DC_{reg} as well as naturally existing tolerogenic DCs act as potential regulators of the host inflammatory response and they may have preventive and therapeutic potential for the treatment of systemic as well as local inflammatory diseases.

subsets ($CD11c^{low}CD45RB^{high}$) producing IL-10 also suppressed LPS-induced host inflammatory responses. Thus, a subset of tolerogenic DCs act as potent regulators of the host inflammatory response, and they might have preventive and therapeutic potential for the treatment of systemic as well as local inflammatory diseases. (See Research Highlights.)

Impact of dendritic cells on T cell-polarization for the control of T type 2 helper cell-mediated response

DCs are crucial for the induction of immunity and tolerance. Despite our better understanding of DC-mediated control of T_H1-biased immunity, the role of DCs in the regulation of T_H2-mediated immunity is unclear. Here we report the impact of immunostimulatory mDCs and DC_{reg} on T_H2-driven allergic immunity involving IgE production. Treatment of Ag-primed mice with Ag-pulsed mDCs before and after immunization inhibited the production of Ag-specific and total IgE, whereas this treatment enhanced the production of Ag-specific and total IgG1 and IgG2a in primary and secondary responses. In addition, Ag-pulsed mDCs not only induced IFN-γ-producing T_H1 cells and IL-21-producing T follicular helper (T_{FH}) cells but also activated IL-4- and IL-10-producing T_H2 cells in Ag-primed mice. Analysis of *Ifng*$^{-/-}$ mice and *Il21r*$^{-/-}$ mice revealed that the IL-21-producing T_{FH} cells were involved in the inhibitory effect on IgE production whereas IFN-γ-producing T_H1 cells mediated the enhancement of IgG2a production. In contrast to the mDC effects, treatment with Ag-pulsed DC_{reg} before and after immunization markedly impaired the production of Ag-specific and total IgE, as well as IgG1 and IgG2a in primary and secondary responses.

Furthermore, Ag-pulsed DC_{reg} induced not only Ag-specific anergic $CD4^+$T cells but also Ag-specific $CD4^+CD25^+Foxp3^+T_R$ cells and IL-10-producing $CD4^+CD25^-$Tr1 cells. *In vivo* blockade experiments showed that the suppressive effect of Ag-pulsed DC_{reg} on IgE production was mainly mediated by Ag-specific $CD4^+CD25^+Foxp3^+T_R$ cells. Treatment of Ag-primed mice with Ag-pulsed mDCs before or after immunization not only promoted the airway inflammation but also enhanced airway hyperresponsiveness whereas treatment with Ag-pulsed DC_{reg} before and after immunization markedly suppressed the airway allergic pathogenesis. Collectively, our results suggest that DC_{reg} abolish T_H2-mediated IgE production and allergic inflammation based on Ag-specific dominant tolerance whereas mDCs exacerbate this pathogenesis despite the inhibition of IgE response through the activation of the diverse types of T_H responses.

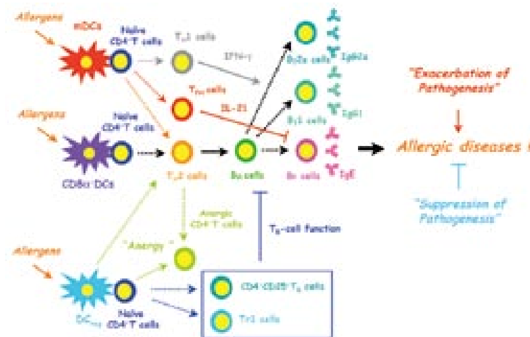

Figure-2 Regulation of allergic response by dendritic cells. Host connectional CD8α-DCs capture and process allergen, and they subsequently present them to naïve $CD4^+$T cells to instruct Ag-specific T_H2 cell differentiation. These T_H2 cells not only help the differentiation of naïve B cells to Bε cells in an Ag-specific manner but also induce local inflammation. These aberrant T_H2 immune responses might initiate and promote allergic diseases. Ag-pulsed mDCs not only generated Ag-specific IFN-γ-producing T_H1 cells and IL-21-producing T_{FH} cells but also caused further activation of Ag-specific IL-4- and IL-10-producing T_H2 cells in the T_H2-biased immune response. Activation of the diverse types of T_H responses exacerbates allergic diseases despite the inhibition of IgE response. In contrast, Ag-pulsed DC_{reg} suppresses T_H2-mediated IgE production and allergic inflammation mediated through the induction of Ag-specific anergic T_H2-polarized cells as well as $CD4^+CD25^-$Tr1 cells and $CD4^+CD25^+T_R$ cells. Thus, an immunotherapeutic strategy using Ag-pulsed DC_{reg} might provide a benefit for treatment of T_H2-driven allergic diseases.

Recent Major Publications

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Recently, we have discovered a novel E3 ubiquitin ligase family (MIR family) that consists of viral E3 ubiquitin ligases (E3) and their mammalian homologues. These novel E3s are membrane-bound molecules that share the secondary structure and catalytic domain for E3 activity. All family members have two transmembrane regions at the center and a RING-CH domain at the amino terminus. Forced expression of these novel E3s has been shown to reduce the surface expression of various membrane proteins through ubiquitination of target molecules. Initial examples of viral E3s were identified in Kaposi's sarcoma-associated herpesvirus (KSHV) and murine γ-herpesvirus 68 (MHV-68) and have been designated as Modulator of Immune Recognition (MIR) 1, 2 and mK3, respectively. MIR 1, 2 and mK3 are able to down-regulate MHC class I molecule expression, and mK3 is required to establish an effective latent viral infection *in vivo*. The first characterized mammalian homologue of MIR 1, 2 and mK3 is c-MIR. Forced expression of c-MIR down-regulates B7-2, a co-stimulatory molecule important for antigen presentation. Subsequently, several mammalian molecules related to c-MIR have been characterized and named as MARCH family. However, the precise physiological function of MARCH family members remains as yet unknown. Therefore, we plan to elucidate the physiological functions of MARCH family members by generating genetically modified mice.

At present, ubiquitination is thought to play an important role in the degradation of membrane proteins through the induction of endocytosis. In yeast, an E3, Rsp5p, has been reported to induce ubiquitination of the cytoplasmic tail of substrate proteins, a step that is necessary for the endocytosis and degradation of the substrate proteins. Similarly, we and other groups have recently identified a novel family of E3 enzymes termed MIR family, whose catalytic domain is a variant RING domain (RINGv domain). MIR family members have been shown to induce rapid endocytosis and degradation through the ubiquitination of the cytoplasmic tail of substrate proteins in mammals.

Most importantly, the overexpressed MIR family members could degrade immune-recognition-related molecules such as MHC class I (MHC I) and MHC II. However, the physiological substrates for these novel ubiquitin ligases remain completely unknown. Among the MIR family members, c-MIR and MARCH-I are of particular interest because they can efficiently degrade important proteins in the immune system, and the surface expression of MHC II has recently been shown to be regulated by ubiquitination in dendritic cells (DCs) which are potent antigen-presenting cells (APCs). Within the E3 catalytic domain and transmembrane regions, the amino acid identity between c-MIR and MARCH-I is > 80%, suggesting similar functions. Since MARCH-I expression was reported to be restricted to secondary lymphoid tissues such as spleen and lymph node, we have been especially interested in the elucidation of the function of MARCH-I.

In 2006, we demonstrated that in B cells, surface expression of MHC II is regulated through ubiquitination by MARCH-I, and ubiquitination does not contribute to the internalization of surface MHC II. In parallel with the stabilization of surface MHC II, we found that MARCH-I-deficient B cells expressed large amounts of antigen-loaded MHC II on their surface and showed a high ability to present exogenous antigens. Thus, our results suggest that the function of MHC II on the surface of B cells is regulated through ubiquitination by MARCH-I.

In 2006, we demonstrated two important novel findings in B cells: the presence of post-translational regulation of functional MHC II by ubiquitination, and the indispensable role of MARCH-I in MHC II ubiquitination. These important findings were based on the fact that the overexpressed MARCH-I down-regulated MHC II surface expression through ubiquitination of the lysine residue at position 225 in the I-A β chain, and that functional surface MHC II molecules were stabilized due to the complete loss of ubiquitination of the I-A β chain of MHC II in MARCH-I-deficient B cells. Given that MHC II molecules are indispensable for T-cell-mediated immunity, these novel findings serve as clues to understand the molecular basis of immune regulation. (See Research Highlights.)

In order to answer the question of the role of MHC II ubiquitination in B cells, the results from MARCH-I knockouts have to be carefully interpreted, because other molecules might be targeted by MARCH-I *in vivo*. For example, the interpretation of the results showing an increased ability of antigen presentation by MARCH-I-deficient B cells should be done with caution. Indeed, the overexpressed MARCH-I has been reported to down-regulate transferrin receptor, B7-2 and Fas. Therefore, to answer the question raised above, we are going to generate MHC II knock-in mice expressing in APCs a mutant I-A β whose lysine residue at position 225 was mutated to arginine..

MARCH-I is highly expressed in B cells. Why do B cells need MARCH-I? What happens when MHC II molecules are highly expressed in B cells? As long as the development of B cells was examined on the basis of the expression level of surface markers by FACS analysis, striking abnormalities could not be observed. In primary B cells, the molecular machinery for MHC II trafficking remains largely unknown. Our findings suggest that in naive B cells, surface MHC II molecules are replaced with newly synthesized molecules through the degradation of pre-existing molecules, as is the case in immature DCs. Is this “MHC II metabolism by MARCH-I” necessary for B cell homeostasis? We are going to try to answer these numerous questions.

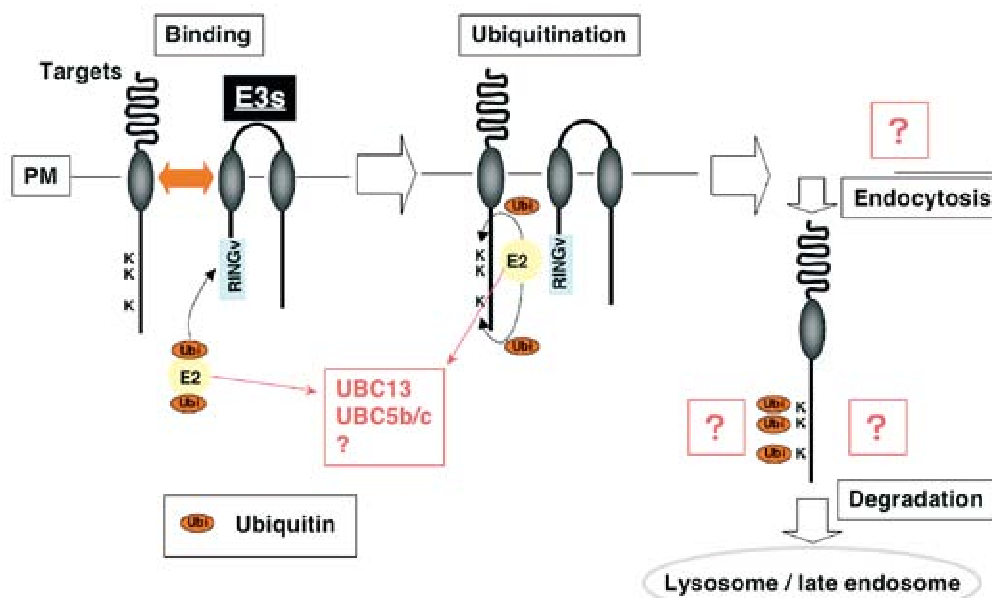

Figure Hypothetical model of downregulation by MIRs

Recent major publications

Matsuki, Y., M. Ohmura-Hoshino, E. Goto, M. Aoki, M. Mito-Yoshida, M. Uematsu, T. Hasegawa, H. Koseki, O. Ohara, M. Nakayama, K. Toyooka, K. Matsuoka, H. Hotta, A. Yamamoto, and S. Ishido. 2007. Novel regulation of MHC class II function in B cells. *Embo J 26:846.*

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Yonashiro, R., S. Ishido, S. Kyo, T. Fukuda, E. Goto, Y. Matsuki, M. Ohmura-Hoshino, K. Sada, H. Hotta, H. Yamamura, R. Inatome, and S. Yanagi. 2006. A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. *Embo J 25:3618.*

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Unfolded or denatured proteins are rapidly degraded in the cell, otherwise, they may cause cell death through apoptosis. The destruction of such proteins is mediated by the ubiquitin-proteasome pathway. Target proteins are first recognized by molecular chaperones like hsp90 and hsc70. These chaperones may simultaneously recruit the E3 ubiquitin ligase to polyubiquitylate the proteins, resulting in their delivery to the proteasome for degradation. In an immunological context, this chaperone-assisted quality control system for cellular proteins provides the main source of antigenic peptides presented by MHC class I molecules. Production of peptides depends on the structure of the 20S proteasome and its regulatory subunits such as PA28 and PA700 (19S cap). However, the precise regulatory mechanism by which the proteasome produces peptides is not well understood. Our laboratory focuses on the mechanisms, especially through PA28 and the molecular chaperone hsp90, that regulate the processing of cellular proteins and peptides.

HSPs in antigen processing

MHC class I ligands are mainly produced by the proteasome. Genetic ablation of PA28α and β results in complete loss of processing of certain types of peptides but not all. For example, *in vivo* processing of $TRP2_{181-188}$, a murine melanoma antigen, is completely abolished in $PA28\alpha^{-/-}\beta^{-/-}$ cells, while processing of the $OVA_{257-264}$ epitope is intact. Processing of the $OVA_{257-264}$ epitope is intact is because hsp90 is compensating for the loss of PA28 function in $PA28\alpha^{-/-}\beta^{-/-}$ cells. However, the molecular mechanism by which hsp90 participates in the processing has not been elucidated. We established a method by which the 26S type proteasome (RCR, RC and hybrid proteasome) as well as the PA28-assisted proteasome can be isolated from small amounts of any kind of cellular extracts. We found that Hsp90α pulled-down (or reconstituted) three distinct proteasomes from cell extracts *in vitro*; hsp90α-RC (singly capped 26S proteasome), hsp90α/Hop/hsc70/hsp40-20S, and hsp90α/CHIP-20S. (CHIP: carboxyl-terminus of hsc70 interacting protein, referred to as E3-ubiquitin ligase containing the U-box domain, is known to interact with hsp90 and hsc70 through its tetratricopeptide repeat (TPR) motif to ubiquitinylate proteins to be digested by the 26S proteasome.)The first two types of proteasomes produce epitopes *in vitro* that sensitize CTLs. We found that hsp90α-RC (thus, the 26S type) and hsp90α/Hop/hsc70/hsp40-20S could process the *Plasmodium yoelii* $CSP_{281-289}$ T cell epitope, however, the PA28-assisted proteasome could not. In order to further analyze the processing mechanism, we used several T cell epitopes spanning both N-and C-terminal flanking regions and established an ESI-based liquid chromatography/ mass spectrometry (LC/MS) mediated peptide digestion assay with the 26S and PA28-assisted proteasomes.

We found that *Plasmodium yoelii* $CSP_{281-289}$ could be processed by the 26S proteasome and was not properly processed by the PA28-20S

proteasome. On the other hand, the $TRP2_{181\text{-}188}$ epitope was processed by the PA28-20S and was not by the 26S proteasome, although the core 20S itself is the same in both types of proteasome. Our results indicate that regulatory particles of the proteasome like PA28 and 19S cap (PA700), not only deliver substrates into the 20S but also affect the digestion pattern, resulting in the production or destruction of particular T cell epitopes. We suggest that hsp90α stimulates the assembly of the 26S proteasome with the resulting augmentation of antigen processing. In fact, knock down of hsp90α but not hsp90β by siRNA transiently reduced the proteasome level (RC and RCR) and reduced expression levels of some MHC class I molecules. Moreover, knock down of hsp90α subsequently up-regulated the level of the PA28-associated proteasome (homo PA28-20S) Thus, in the absence of hsp90α, antigen processing mostly depends on PA28.

PA28 in antigen processing

IFN-γ stimulation increases the likelihood of football- (homo PA28-20S) and hybrid-shaped (PA28-20S-PA700) proteasomes. The former is a complex where PA28 is attached to both ends of the central 20S proteasome while in the latter, the 20S proteasome is flanked by PA28 on one side and PA700 (alias the 19S Cap) on the other. The hybrid-shaped proteasome functions as an ATP-dependent protease, similar to the 26S proteasome, which has PA700 on both ends. The fundamental role of PA28 is still largely unknown. One of the postulated mechanisms by which PA28 stimulates the proteasome is the so-called gate-opening, where the usually closed α-ring of the 20S subunit is immediately opened upon association with PA28 thereby enabling the translocation of substrates into the core catalytic 20S, followed by the production of antigenic -peptides. PA700 is also implicated as a chaperone complex that channels substrates into the core 20S. Based on that perspective, PA28 and PA700 may both open the α-ring of the 20S and load peptides into it. It is not known whether gate-opening is the only activity of these regulatory particles but, if so, the peptide repertoire produced by either PA28 or PA700 should be the same and depend on the 20S proteasome complex itself. The *Plasmodium yoelii* $CSP_{281\text{-}289}$ T cell epitope, however, was processed by the 26S but not by the PA28-20S proteasome, indicating distinct digestion patterns by these two proteasome complexes. To gain more insight into the mechanism of action of PA28, we performed an LC/MS based -peptide digestion assay by

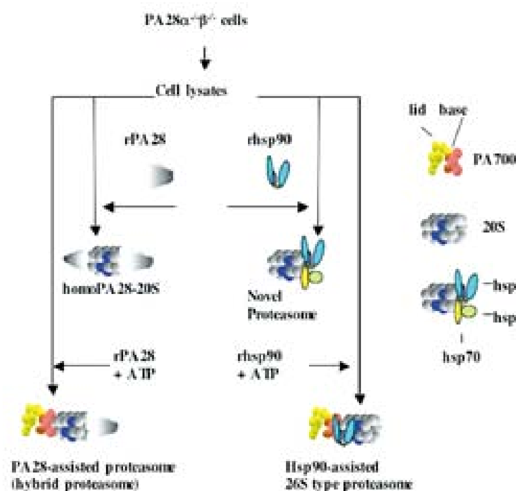

Figure Artificial reconstitution of each proteasome complex. PA28-deficient cells treated with or without IFN-γ were lysed with detergents. Recombinant PA28α or hsp90α pulled-down different kinds of proteasomes depending on the presence of ATP. By this method, PA28-assisted and hsp90-assisted protaeasomes were obtained. The unique functions of these proteasomes could be examined in terms of antigen processing.

the PA28-20S proteasome using many synthetic peptides harboring mouse MHC class I K^b and K^d ligands. We found that many of the K^b ligands were processed by PA28-20S but, in contrast, many of K^d ligands were not properly produced. We reasoned if K^d ligands are indeed improperly processed by the PA28-20S proteasome, then downregulation of PA28 by siRNA might augment the surface expression of K^d, which is dependent on peptide loading. Indeed, we found that knock-down of both PA28α and β by RNAi significantly augmented expression of K^d, indicating that PA28 negatively influences K^d expression. It is possible that the effect of PA28 on antigen processing depends on MHC polymorphism.

Antigen processing is regulated by the ubiquitin–proteasome system. The peptide repertoires produced by the proteasome are determined by the regulatory particles and structure of the 20S proteasome itself. Analyzing the structure of the proteasome and its digestion pattern of proteins harboring MHC class I peptide ligands will shed light on the processing mechanism. The proteasome structures are generally determined in cell lineages, tissues or organs, but can also be regulated by cytokines such as IFN-γ, suggesting that analysis of the relationship between cytokines and the structure of the proteasome should increase our understanding of the immune system.

Recent major publications

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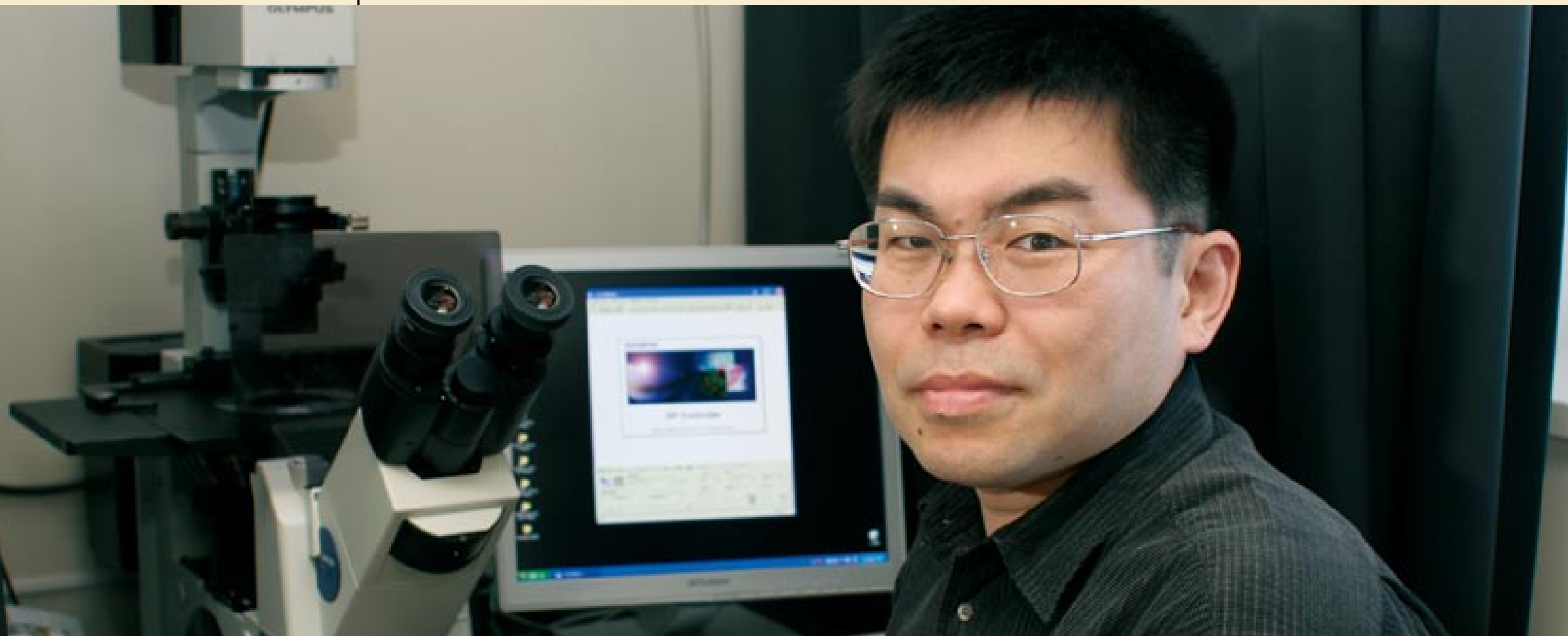
Laboratory for Innate Cellular Immunity



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Phagocytes such as macrophages and dendritic cells (DCs) exhibit a variety of activities including induction of inflammation, engulfment of microorganisms and dead cells, antigen presentation, and regulation of extracellular components. At the site of bacterial infection, phagocytes recognize invading microorganisms via a wide variety of surface receptors that bind cellular components common to many bacterial surfaces, thereby engulfing them for elimination. Phagocytes encountering bacteria also produce and secrete inflammatory cytokines and chemokines such as TNF-α, IL-6, and MIP-1α. These factors induce inflammation, and the inflammatory responses play a critical role in the effective clearance of invading bacteria and the induction of appropriate adaptive immunity. On the other hand, phagocytes also recognize cell debris and dead cells. Tissue injury and inflammation results in death of resident cells. In addition, infiltrating neutrophils are eliminated by apoptotic cell death during the late stages of inflammation. Phagocytes rapidly engulf these dead cells by means of specific phagocytic receptors for dying cells. The clearance of dead cells prevents their release of potentially toxic or immunogenic intracellular materials. Thus, the prompt elimination of dying cells is required for the resolution of inflammation and normal tissue repair. The Laboratory for Innate Cellular Immunity is investigating the molecular mechanisms for recognition and phagocytosis of dying cells by phagocytes, and the pathological relevance of impaired phagocytosis to inflammatory disorders including autoimmune diseases.

Protective role of alveolar macrophages in non-inflammatory lung injury

Macrophages have a wide variety of activities, and it is largely unknown how the diverse types of macrophages contribute to distinct pathological conditions in the different types of tissue injury *in vivo*. We established a novel animal model of acute respiratory distress syndrome caused by the dysfunction of alveolar epithelial type II (AE2) cells, and examined the role of alveolar macrophages in acute lung injury. Human diphtheria toxin receptor (DTR) was expressed under the control of the lysozyme M (LysM) gene promoter in these mice (LysM-DTR mice). When diphtheria toxin (DT) was administrated to the mice, they suffered from acute lung injury, and died within 4 days. Immunohistochemical examination revealed that AE2 cells as well as alveolar macrophages were deleted via apoptosis in the mice treated with DT. Consistent with the deletion of AE2 cells, the amount of surfactant proteins in bronchoalveolar lavage fluid (BALF) was greatly reduced in the DT-treated transgenic mice.

To examine how the ablation of alveolar macrophages contributed to lung pathology in the mice, we compared the severity of lung injury induced by DT administration between LysM-DTR mice reconstituted with wild-type bone marrow cells (W-L mice) and LysM-DTR mice reconstituted

with LysM-DTR bone marrow cells (L-L mice). Although both mice died after DT administration, the lung injury in W-L mice was less severe than that of L-L mice. These results indicate that injury of AE2 cells plays a major role in the respiratory failure in DT-treated LysM-DTR mice, and that the presence of alveolar macrophages diminishes this non-inflammatory lung injury caused by AE2 cell deletion.

Macrophages in the marginal zone regulate tolerance to cell-associated antigens

It is presumed that apoptotic cells injected intravenously are phagocytosed by antigen-presenting cells (APCs) in the spleen, and that the APCs then present foreign peptides derived from the apoptotic cells to T cells. Presentation in the absence of appropriate co-stimulatory signals could lead to antigen-specific T cell deletion or anergy. We found that intravenous injection of apoptotic cells expressing a fragment of myelin oligodendrocyte glycoprotein (MOG) induced MOG-specific T cell tolerance, and prevented the development of experimental autoimmune encephalomyelitis (EAE).

We observed that injected apoptotic cells first accumulated in the marginal zone (MZ) of the spleen. These results prompted us to determine the role of macrophages in the splenic MZ for induction of tolerance to apoptotic cell-associated antigens. For this purpose, we established transgenic mice (CD169-DTR mice) in which a human DTR cDNA was introduced into the CD169 gene, which is specifically expressed in marginal metallophilic macrophages. When DT was injected into the CD169-DTR transgenic mice, marginal metallophilic macrophages and marginal zone macrophages were transiently deleted, while red pulp macrophages, tingible body macrophages, T cells, B cells and DCs were unaffected. Injection of apoptotic cells expressing MOG no longer suppressed EAE in the CD169-DTR mice treated with DT, while DT treatment had no effect on the induction of tolerance to MOG by injection of these apoptotic cells into wild-type littermates. These results clearly indicate that macrophages in the MZ are indispensable for tolerance induction to cell-associated antigens. We next determined how injected apoptotic cells were cleared in the spleen of CD169-DTR mice using fluorescent-labeled apoptotic cells, and found that injected cell corpses accumulated in the MZ. However, there was a dramatic delay in corpse clearance in the MZ of the DT-treated mice compared with wild-type mice. These results indicate that macrophages in the MZ play a critical role in the clearance of intravenously-injected apoptotic corpses. We are now trying to elucidate the mechanisms for the failure of tolerance induction in DT-treated CD169-DTR mice.

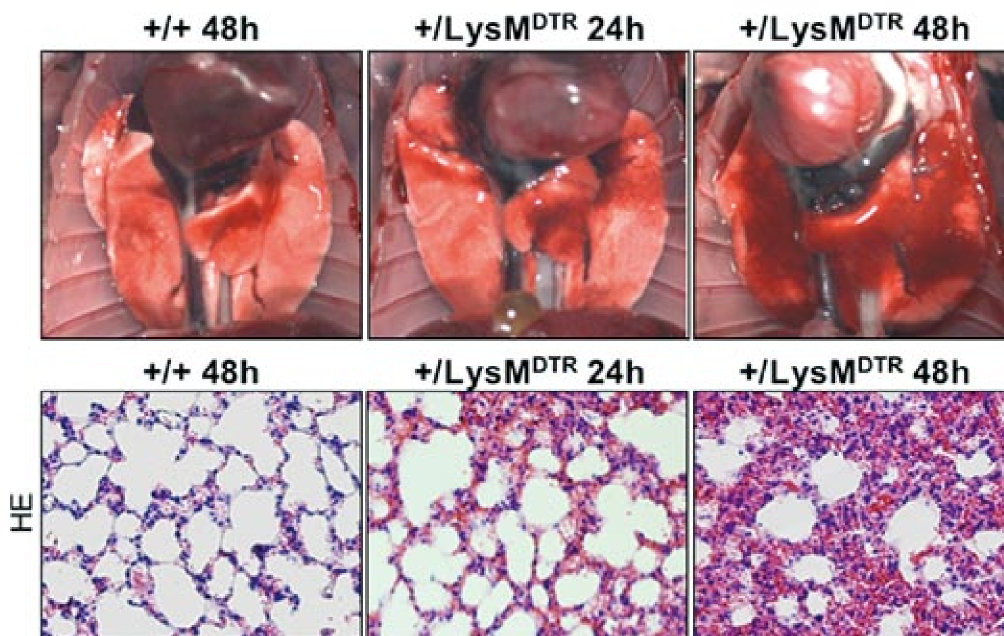

Figure Lung injury in LysM-DTR mice

Recent major publications

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TYPE 1 DIABETES mellitus is an autoimmune disease caused by the destruction of pancreatic beta cells by auto-reactive T cells. In both man and animal models of type I diabetes, class II major histocompatibility (MHC) antigens are the most important genetic factor for the susceptibility to the disease. Furthermore, various environmental factors such as infection and active immunization are known to influence disease development both positively and negatively. The goals of our research group are to understand disease process and to develop means to modulate this process. The disease process is shown in the Figure. Our projects are: (1) to identify the antigen(s) responsible for the initiation of the disease, (2) to elucidate the biochemical and molecular mechanisms by which certain MHC genotype confer susceptibility to type I diabetes, (3) to establish an effective method to regulate disease development *in vivo*, (4) to understand the final effector mechanisms by which pancreatic beta cells are destroyed and (5) to establish a series of methods to visualize each step from sub molecular, molecular, cellular to whole animal level in collaboration with different groups in RIKEN. All experiments are carried out in the NOD mouse model of type I diabetes, but we expect the results obtained in this mouse model to be relevant for the development of new strategies to treat human type I diabetes.

Screening of diabetogenic antigens

The search for diabetes antigens has haunted the world of immunology for many years. Although a number of antigens have been shown to play a role in the pathogenesis of type I diabetes in NOD mice, no antigen has been demonstrated to stimulate pathogenic T cells that cause diabetes *in vivo*. We use a saturation screening approach to identify pancreatic beta cell antigens that stimulate diabetogenic T cells. Using whole genome analysis, we have identified about 100 beta cell specific cDNAs. Proteins are produced from these cDNAs using a cell-free expression system and screened for their capacity to stimulate diabetogenic T cells *in vitro*. We also use retroviral-mediated expression of these cDNAs in an antigen- presenting cell. Antigen-presenting cells expressing beta cell antigens will also be tested with diabetogenic T cells. These approaches will cover the entire range of antigens expressed in pancreatic beta cells and are likely to succeed in identifying diabetogenic antigen(s) in the NOD mouse.

We are trying to extend this method for the identification of antigen(s) in human Type I diabetes.

Class II MHC and presentation of diabetogenic antigen

I-Ag7 in mouse and DQ8/2 in human are the most important genetic factors for the susceptibility to type I diabetes. These MHC molecules share non-Asp residue at position 57 of the beta chain. Also, these MHC molecules are known to bind

all peptide with a weak affinity. We hypothesize that i) poor peptide-MHC interaction generate unstable complex. ii) this unstable complex exhibits multiple transistional conformations and iii) T cells recognize this transitional conformation to form a stable trimolecular TCR/MHC/peptide complex for activation. To test this hypothesis, we are using diffracted X-ray tracking (DXT) to visualize movement of peptide in MHC groove.

Regulatory T cells in diabetes development

We have shown that regulatory T cells play an important role in the regulation of diabetes development in NOD mice. The following conditions favor the generation of regulatory T cells leading to the complete suppression of diabetes development I) MHC class II heterozygosity II) lack of interferon gamma signaling and III) exposure of T cells to soluble but not to cell bound form of antigen (manuscript in preparation). These results indicate that inappropriate activation of T cells preferentially induces regulatory functions. Currently, we plan to generate regulatory T cells from naïve beta cell antigen-specific T cells. These T cells will be tested for their ability to regulate autoimmune diseases as well as to inhibit rejection of transplanted pancreatic islets..

Regulation of T cell function by cytokines

We have shown that IL-2 and IL-15 which share all of the known signaling components of the receptor exhibit very different functions on T cells. IL-2 preferentially induces effector CD8 T cells and maintain regulatory CD4 T cells. IL-15 plays an important role in the maintainance of memory CD8 T cells but shows no effect on the function of CD4 T cells. We plan to examine the mechanisms by which these two cytokines differentially regulate the fate of antigen specific T cells *in vivo*. We have solved the structure of the IL-15/IL-15 receptor alpha chain (manuscript in preparation) and hope that this structural information contributes to the understanding of the different biological functions of these two cytokines.

In addition, we plan to investigate the kinetics difference between IL-2 and IL-15 for the activation of key signaling molecule STAT-5 using a single molecule microscope. At present, we have carried out preliminary experiments in the STAT4 system. Our results reveal that steady-state interaction between STAT4 and the surface receptor, which is so far unknown, plays a critical role for the ligand-induced signaling through STAT4 (manuscript in preparation). This result clearly demonstrates that real- time observation of single molecules in living cells will provide new insights into signaling processes and will become an essential method in biological research.

Monitoring cellular migration *in vivo*: Using transgenic mice expressing the photoconvertible fluorescence protein "KAEDE", we have established a method to monitor cellular movement *in vivo* with minimum external manipulations. Using this method, we have demonstrated that cells of the immune system re-circulate very rapidly *in vivo*. However, each cell type has its own kinetics which are determined by yet- unknown mechanisms. Upon initiation of an inflammatory response, each cell type again changes its migratory pattern to maximize cellular interaction and thereby mount an efficient immune response (manuscript in preparation). We are hoping to use this method to monitor the initiation as well as the effector phase of the autoimmune destruction of pancreatic beta cells.

Other ongoing projects in the laboratory

Production of mouse line from lymphocyte using nuclear transfer: We have generated mouse lines from various types of lymphocytes. These mice are totally normal and express receptor genes (TCR or BCR) from the original lymphocyte. We plan to use this technology to establish mouse lines from lymphocytes infiltrating into specific organs (e.g. pancreas-infiltrating cells in diabetes, joint-infiltrating cells in arthritis and nerve tissue-infiltrating cells in EAE). These mice will be a useful tool to characterize a pathogenic immune response in mouse and in human in the future.

Visualization of pancreatic beta cell mass ***in vivo*****:** Monitoring beta cell mass is the best method to predict the onset of type I diabetes. In collaboration with different centers in RIKEN, we are trying to develop a method to determine the actual bata cell mass with 3D visualization of pancreatic islets in thepancreas. This method will be combined with PET system to establish non-invasive methods to monitor pancreatic beta cell mass. This method could be used in clinical setting to monitor patients and people at risk.

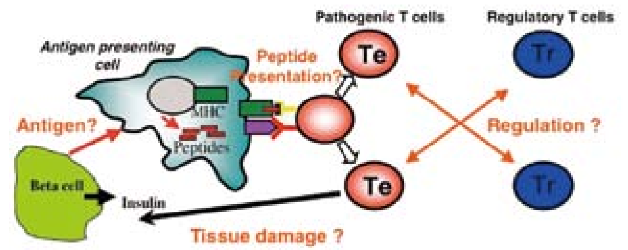

Figure: Pathogenesis and control of Type I diabetes

Recent major publications

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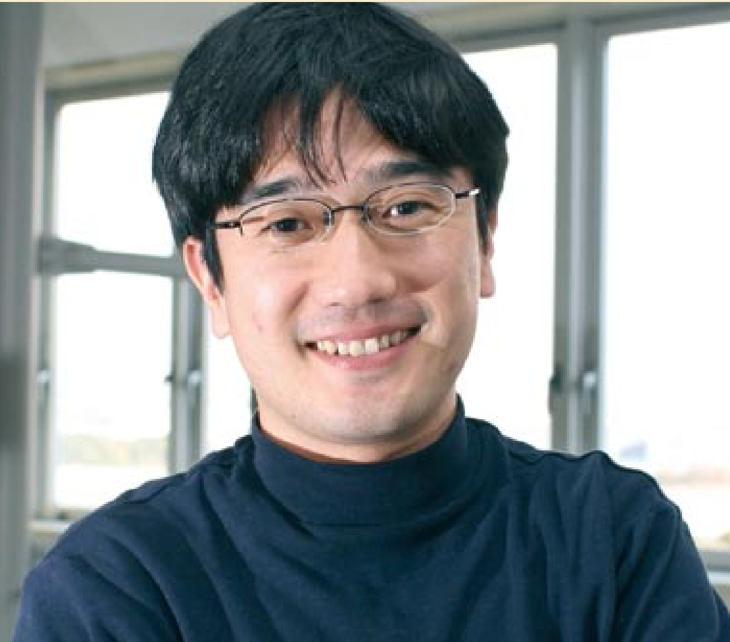
Research Unit for Immune Homeostasis



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This laboratory has been partially funded by the PRESTO program of the Japan Science and Technology Agency (JST).

A small subpopulation of T lymphocytes known as regulatory T cells (T_{reg}) play a central role in preventing pathological immune responses such as autoimmunity, inflammation and allergy, and thus ensure dominant tolerance to self- and innocuous environmental antigens. This has been best illustrated by our recent finding that the development and function of T_{reg} is controlled by the transcription factor Foxp3, a genetic deficiency of which leads to the development of a fatal autoimmune pathology in a natural mouse mutant strain called *scurfy* and human patients with the IPEX syndrome.

The identification of Foxp3 as a "master" regulator of T_{reg} development and function has been essential in addressing a number of questions concerning their role in tolerance and immune regulation, and their physiology including their origin, the molecular/cellular mechanisms controlling their development and function, and their antigen specificity. Resolving these issues is the goal of this laboratory.

Function of Foxp3$^+$ T_{reg} in the etiology of autoimmune pathology in *scurfy* mice

Since *scurfy* as well as Foxp3-deficient mice fail to generate T_{reg} cells, it is now believed that the defective T_{reg} generation is the primary cause of the immune dysregulation in Foxp3-mutant mice and humans. It has also been proposed, however, that Foxp3-deficient mice develop autoimmune pathology due to a defect in non-hematopoietic tissues, particularly the thymic epithelium which is essential for T cell development and repertoire selection. This notion has been supported by previous findings that reconstitution of lethally irradiated wild-type host mice with *scurfy* donor bone marrow cells fails to transmit the fatal immune pathology and that Foxp3 is reportedly expressed in thymic epithelium as well. To determine the relative contributions of these two proposed pathways to the autoimmune pathology in *scurfy* mice, we repeated this radiation chimera experiment and found that such chimeric mice still harbored normal numbers of host-derived Foxp3$^+$ T_{reg} cells. These host-derived Foxp3$^+$ T_{reg} cells are responsible for the disease protection as antibody-mediated or genetic ablation of host T_{reg} cells resulted in the development of a fatal immune pathology. Furthermore, our analyses of chimeras carrying the *scurfy* mutation only in non-hematopoietic cells have clearly demonstrated that Foxp3 deficiency in these cells does not contribute to the disease development in *scurfy* mice. Collectively, our analyses support the notion that the fatal autoimmune pathology observed in *scurfy* mice indeed results from defective T_{reg} development, and that Foxp3$^+$ T_{reg} cells plays an indispensable role in self-tolerance.

Further, our studies of the radiation chimeras reconstituted with *scurfy* bone marrow cells have also demonstrated that, in the absence of thymic generation, the peripheral Foxp3$^+$ T_{reg}

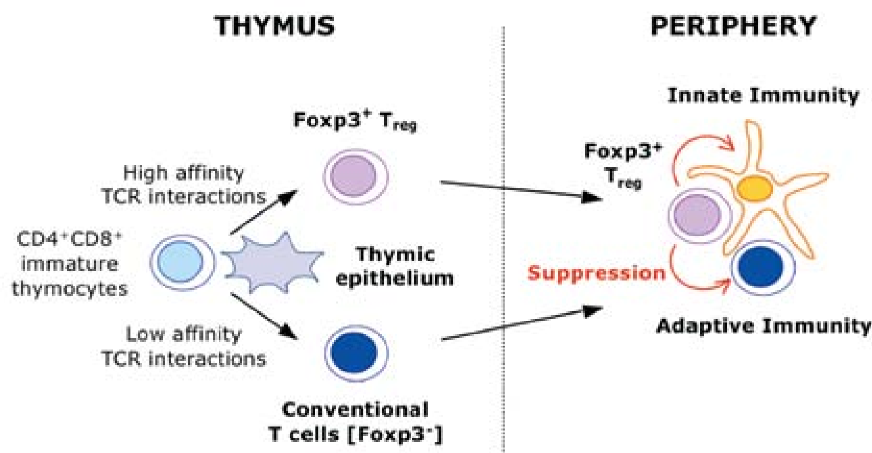

Figure Thymus-derived autoreactive Foxp3-expressing regulatory T cells (T_{reg}) establish and maintain dominant tolerance to self.

pool is fully restored and maintained by a small number of radioresistant endogenous T_{reg} cells or adoptively transferred exogenous T_{reg} cells through "homeostatic" expansion. This illustrates the robust homeostatic mechanisms that keep the size of peripheral Foxp3$^+$ T_{reg} cell pool constant and suggests that they occupy a specific and limited "niche" in the peripheral T cell repertoire. The molecular and cellular mechanisms controlling T_{reg} homeostasis are currently under investigation.

We are also generating a "knock-in" mouse model in which a human CD2/52 chimeric reporter is knocked into the Foxp3 locus. These mice will be a valuable tool not only to track Foxp3 expression *in vivo* but also to establish the functions of Foxp3$^+$ T_{reg} in tolerance and immune regulation in normal, non-lymphopenic animals because these mice will allow us to eliminate Foxp3$^+$ T_{reg} cells from the body by injecting depleting monoclonal antibodies against this surface antigen.

Impacts of IPEX mutations on T_{reg} development and function

Having established that Foxp3-deficient mice develop an immune pathology due to defective T_{reg} development, we then addressed whether and how Foxp3 mutations that had been identified in IPEX patients impact on T_{reg} development and function. To this end, we generated Foxp3 mutants carrying IPEX mutations and retrovirally transduced them into conventional T cells. Our analyses revealed that all the mutations which we examined were amorphic or hypomorphic in that T cells expressing these mutants failed to exert full suppressive activity *in vitro* and *in vivo*. Most of the mutations also affected the expression of T_{reg} phenotypic markers, suggesting defective T_{reg} development in patients carrying these mutations. Interestingly, T cells transduced with one of the mutations expressed normal levels of T_{reg} phenotypic markers despite their impaired suppressive activity, suggesting that the patients carrying this particular mutation develop IPEX syndrome due to impaired T_{reg} effector function rather than defective T_{reg} development. This observation prompted us to compare the gene expression profiles of T cells transduced with wild-type Foxp3 versus this mutant by DNA microarray analyses. Our results demonstrated that these two groups of T cells were strikingly similar in terms of gene expression, except for a small subset of Foxp3 target genes affected by this mutation. Since the expression of these genes is affected in a manner strongly associated with the loss of suppressive activity, we hypothesize that these genes may be important for T_{reg} effector function. We are currently undertaking genetic studies to test this hypothesis.

One of the central unresolved questions regarding T_{reg} cell biology is to understand the molecular mechanisms by which they suppress immune responses. Specifically, despite extensive worldwide studies by a number of laboratories, the identity of the molecule(s) that mediate(s) T_{reg} suppressor effector function still remains unknown. Our analyses on IPEX mutations appear to have provided a promising clue to approach this question; by comparing gene expression profiles of T cell transduced with wild-type and mutant Foxp3, we have identified a small number of Foxp3 target genes, which are affected by the mutation and are therefore strongly associated with T_{reg} suppressor effector functions. The identification of T_{reg} effector molecules will be particularly important in order to manipulate T_{reg} functions for the treatment of autoimmunity, inflammation, allergy, chronic infection and cancer.

Recent major publications

Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells by IL-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 201, 723-735 (2005)

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Our focus is the control of autoimmune diseases including rheumatoid arthritis (RA) and polymyositis (PM). The main pathological features of RA are inflammation and hypergrowth of synovial fibroblasts. The resulting hyperplastic tissue, called pannus, serves as a platform for tissue-degrading proteinase production and osteoclast activation, both of which contribute to destruction of the joint. Current approaches to regulate RA mainly focus on the inflammation, but such treatments often fail to control cartilage and bone destruction. Instead we have been trying to suppress RA by controlling the synovial cell cycle and via regulatory T cells.

PM is a systemic autoimmune disease that affects skeletal muscles and cytotoxic CD8 T cells play a major role in its pathology. Until we established a new animal model, C-protein-induced myositis (CIM) of mice, there was no animal model close to the human disease. This model is readily induced by a single immunization of a recombinant C protein fragment in several mouse strains, including C57BL6 mice which have been used to generate various gene mutant mice. In order to better understand PM, we have investigated the pathology of this model and have also explored new treatment approaches.

Control of RA

Intra-articular gene transfer of cyclin-dependent kinase inhibitors (CDKI) to suppress synovial cell cycling has shown efficacy in animal models of RA. CDKIs also modulate the immune function via a CDK-independent pathway. Accordingly, systemic administration of small molecules that inhibit CDK might ameliorate arthritis. In order to address this issue, a pan-CDK inhibitor, known to be tolerated clinically in treatment of cancers, and also a newly synthesized CDK4/6-selective inhibitor were tested for anti-arthritic effects. *In vitro*, both reagents inhibited the proliferation of human and mouse synovial fibroblasts without inducing apoptosis. *In vivo*, treatment of collagen-induced arthritis (CIA) mice with the pan-CDK inhibitor suppressed synovial hyperplasia and joint destruction while serum concentrations of anti- type II collagen (CII) antibodies and proliferative responses to CII were maintained. Treatment was effective even when therapeutically administered. Treated mice developed arthritis upon termination of the treatment. Thus, immune responses to CII were unimpaired. The same treatment ameliorated arthritis induced by K/BxN serum transfer to lymphocyte-deficient mice. Similarly, the CDK4/6-selective inhibitor suppressed CIA. Suppressing lymphocyte function was not how both small molecule (sm) CDK inhibitors exerted their effect in animal models of RA. We believe that smCDK inhibitors hold promise as a new class of anti-rheumatic drugs that inhibit a distinct phase of the rheumatoid pathogenesis.

The above-mentioned compounds are now under consideration of clinical trials. In parallel, we have investigated effects of these compounds on macrophage activation. With these compounds,

macrophages fail to produce inflammatory cytokines and differentiate into osteoclasts, suggesting that CDK4/6 inhibitors are potent anti-rheumatic drugs.

Secondly, we obtained considerable amelioration in a murine model of RA with Foxp3-transduced $CD4^+$ T cells. In recent years, several types of CD8 T_{reg}s have been described. We tried generating or expanding these CD8 T_{reg}s *in vivo* and used them for *in vitro* suppression of CD4 T cell proliferation. However, *in vitro* all CD8 T_{reg}s tested failed to suppress CD4 T cells as strongly as $CD4^+Foxp3^+$ T_{reg}s. This may suggest that $CD4^+Foxp3^+$ cells are the most powerful T_{reg}s to treat autoimmune diseases such as arthritis.

Control of PM

Using the new model, C-protein induced myositis (CIM), we found that CD8 T cells are primarily important for muscle damage. The myositis could be adoptively transferred to naïve mice by CD8 T cells from affected mice. Mice lacking the perforin gene, which is the effector molecule of cytotoxic CD8 T cells were resistant to muscle damage in CIM. Mice lacking TNF-alpha gene were susceptible to the disease, showing that this cytokine is dispensable for disease development. In contrast, mice lacking interleukins -1 or -6 were resistant. These observations suggest that selected cytokines can be target molecules when trying to control PM. We also found that intravenous injection of immunoglobulins (IVIG) was an effective treatment in this model as it is in human PM. Up to now, studies of IVIG have been carried out using human immunoglobulins in murine disease models. However, human immunoglobulins can bind to murine lymphocytes and induce proliferation *in vitro*. We believe that the results of such studies should be carefully interpreted. We will use pooled murine immunoglobulins to avoid any artifacts.

Overall, this model has proved to be useful for studying pathology of PM and developing new therapeutic approaches.

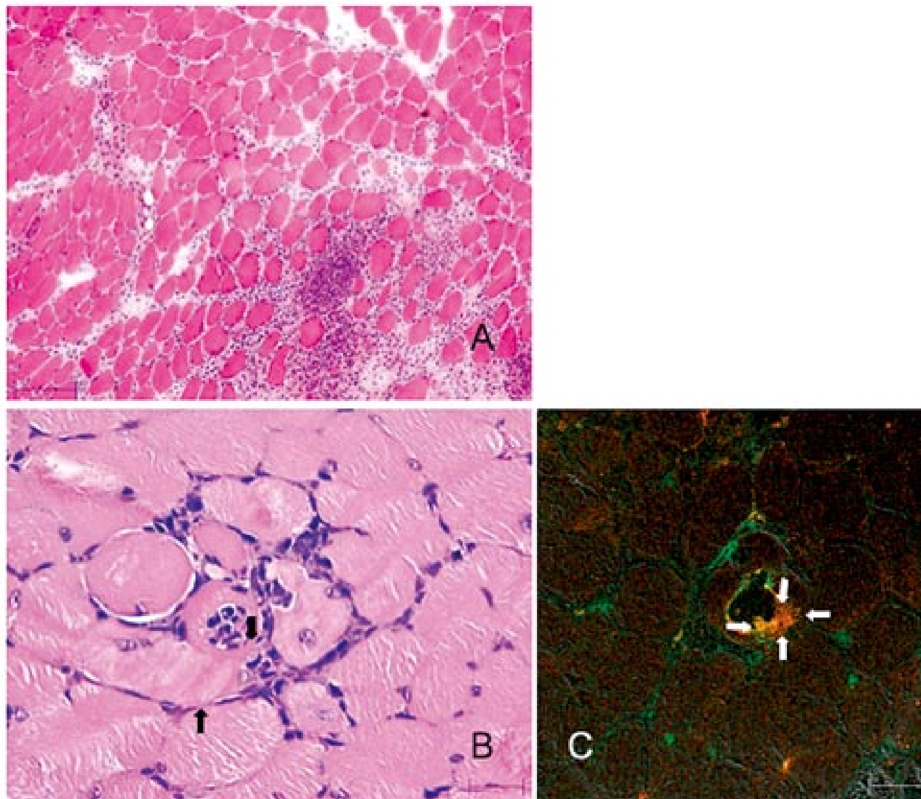

Figure Histology of CIM A and B: Haematoxylin and eosin staining of the affected muscles. C: Immunofluorescence staining of CD4 (green) and CD8 (red) cells. Arrows show T cells invading into the muscles

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CD1d-restricted natural killer T (NKT) cells, which express both a single invariant Vα14 antigen receptor (and are therefore also known as Vα14^{+} NKT or Vα14i NKT cells) and NK receptors, such as NK1.1, occupy a unique intermediary position between innate and acquired immunity. Further, because of their ability to quickly release large amount of cytokines, such as interferon (IFN)-γ and IL-4, and their apparent self-reactivity, NKT cells have been hypothesized to play important roles in the initiation and regulation of many types of immune responses. The multi-function of the NKT cells causes various autoimmune diseases such as rheumatoid arthritis, SLE, type I diabetes, systemic sclerosis, and allergic diseases such as asthma. In the case of organ transplantation, the control of NKT cells is required for the maintenance of allograft tolerance. Our group studies the regulation of immune responses, both beneficial and harmful, mediated by NKT cells, which would contribute to the development of new strategies for human immune therapy.

IL-21–induced Bε cell apoptosis mediated by natural killer T cells suppresses IgE responses

Epidemiological studies have suggested that the recent increase in the incidence and severity of immunoglobulin (Ig)E-mediated allergic disorders is inversely correlated with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) vaccination; however, the underlying mechanisms remain uncertain. We demonstrate that natural killer T (NKT) cells in mice and humans play a crucial role in the BCG-induced suppression of IgE responses. BCG-activated murine Vα14 NKT cells, but not conventional CD4^{+} T cells, selectively express high levels of interleukin (IL)-21, which preferentially induces apoptosis in Bε cells. Signaling from the IL-21 receptor increases the formation of a complex between Bcl-2 and

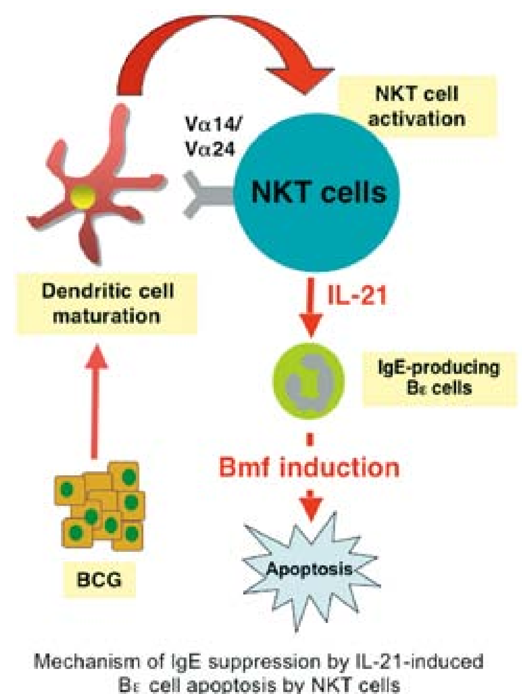

Figure 1 IL-21–induced Bε cell apoptosis mediated by NKT cells suppresses IgE responses

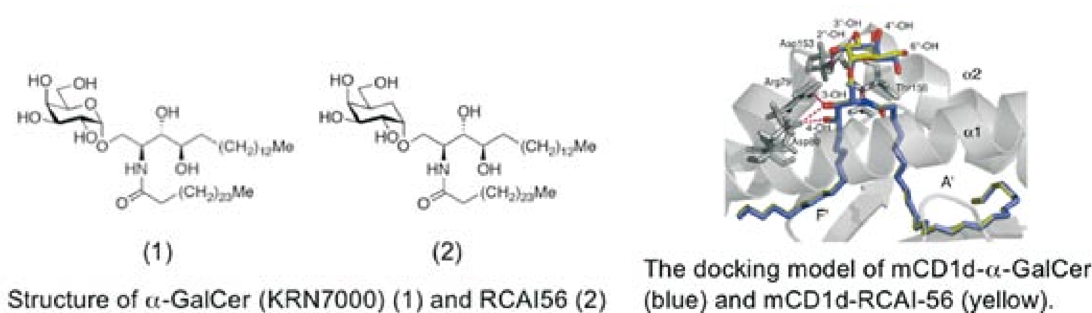

Figure 2 RCAI-56, a carbocyclic analogue of α-GalCer: its synthesis and potent activity for NKT cells to preferentially produce IFN-γ

the proapoptotic molecule Bcl-2–modifying factor (Bmf), resulting in Bε cell apoptosis. Similarly, BCG vaccination induces IL-21 expression by human peripheral blood mononuclear cells (PBMCs) in a partially NKT cell–dependent fashion. BCG-activated PBMCs significantly reduce IgE production by human B cells. These findings provide new insight into the therapeutic effect of BCG in allergic diseases. (See Research Highlights.)

Hyporesponsiveness to NKT-cell ligand α-GalCer in cancer-bearing state mediated by CD11b⁺ Gr-1⁺ cells producing nitric oxide.

CD1d-restricted NKT cells are a potential therapeutic target for cancer, for which several clinical trials have already been reported. NKT cells are specifically activated by a synthetic glycolipid, alpha-galactosylceramide (α-GalCer). However, it is known that, in human cancer patients, NKT cells express a degree of hyporesponsiveness to α-GalCer. In this study, we have examined the mechanism by which hyporesponsiveness to α-GalCer can be induced. In cancer-bearing mice, α-GalCer-induced NKT cell expansion, cytokine production, cytotoxicity, and antimetastatic effect *in vivo* were all significantly impaired. In fact, α-GalCer could eliminate metastatic disease in naive animals but failed to protect cancer-bearing mice. $CD11b^+$ $Gr\text{-}1^+$ cells were particularly increased in cancer-bearing mice and were necessary and sufficient for the suppression of the α-GalCer response in a nitric oxide-mediated fashion. Administration of a retinoic acid to cancer-bearing mice reduced the population of $CD11b^+$ $Gr\text{-}1^+$ cells and effectively restored α-GalCer-induced protection. These results show a novel feature of NKT cell function in cancer. Furthermore, our data suggest a new strategy to enhance NKT cell-mediated anticancer immune responses by suppressing $CD11b^+$ $Gr\text{-}1^+$ cell functions.

RCAI-56, a carbocyclic analogue of α-GalCer: its synthesis and potent activity for NKT cells to preferentially produce IFN-γ

In 1995 researchers at Kirin Brewery Co. developed an anticancer drug candidate α-GalCer (KRN7000) through the modification of the structures of agelasphins, which had been isolated in 1993 as anticancer sphingolipids from the extract of an Okinawan marine sponge, *Agelas mauritianus*. These sphingolipids exhibited antitumor activity *in vivo* in mice and human. It has been shown that α-GalCer is a ligand to make a complex with CD1d protein, a glycolipid presentation protein on the surface of the antigen presenting cells of the immune system. NKT cells are activated by recognition of the CD1d/α-GalCer complex with their invariant Vα14 antigen receptors, and release both helper T (Th) 1 and Th2 types of cytokines in large quantities at the same time. Th1 type cytokines such as IFN-γ mediate protective immune functions like tumor rejection, whereas Th2 type cytokines such as interleukin (IL) -4 or IL-10 mediate regulatory immune functions to ameliorate autoimmune diseases. Th1 and Th2 type cytokines can antagonize each other's biological actions. Because of this antagonism, use of α-GalCer for clinical therapy was unsuccessful. To circumvent this problem, we are trying to develop new analogues of α-GalCer, which induce NKT cells to produce only Th1 or Th2 type cytokines. RCAI-56 is a remarkably potent inducer of Th1 biased cytokine production *in vivo*. Further studies are in progress to clarify the structural requirements for a glycosphingolipid ligand in controlling the ratio of Th1/Th2 responses.

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*Dr. Fujii has served as a member of the ethics committee of RIKEN Yokohama Institute for FY 2005 and 2006.

We have studied dendritic cell (DC) biology and tumor immunology by focusing on the link between innate and adaptive immunity, especially through the interaction of DCs with NKT cells. The goal of our laboratory is to employ strategies leading to clinical studies that will ultimately lead to development of new therapies for cancer.,

First, we have analyzed a new type of NKT ligand, α-C-GalCer, to study the processes of induction of adaptive immunity by the "adjuvant effects" of NKT cells. α-GalCer's glycosidic linkage involves an O atom, whereas α-C-GalCer,'s glycosidic linkage involves a CH_2 group. We found that this new ligand can be stably loaded onto DCs and is more potent than α-GalCer, the prototypical ligand, in activating NKT cells. The mechanism of antitumor T cell immunity mediated by innate NKT cell activation was further analyzed and we found that the full maturation of DCs plays an important role in conjunction with CD40 signaling. Second, we have analyzed different types of antigen presenting cells (APCs) for loading α-GalCer to induce stronger innate immunity against tumors. When we previously studied the capture of α-GalCer in α–GalCer-injected mice, we found that only $CD11c^+$DCs could successfully capture the glycolipid for stimulation of NKT cells to produce IFN-γ. Recently, we have used *in vitro* loading to study the capacity of different $CD11c^-$ leukocytes to present α-GalCer and found that as long as different types of CD1d expressing leukocytes were able to capture the ligand *in vitro*, they could stimulate NKT cells, suggesting that the costimulatory properties of DCs are not essential in inducing innate immunity. Finally, when we used α-GalCer-loaded tumor cells instead of DC/Gal, we found significant augmentation of NK cell activity via "adjunct effects".

Study of the relation between augmentation of NKT cells and induction of adaptive immunity using a new ligand, α-C-GalCer.

We found that a new analogue, α-C-GalCer, is a more potent inducer than α-GalCer of innate and adaptive immune responses in mice. α-C-GalCer triggers IL-12 and IFN-γ production more efficiently than α-GalCer, whereas it elicits only minimal release of IL-4 and TNF-α into the serum. Also α-C-GalCer mobilizes NKT and NK cells to resist B16 melanoma cells better than α-GalCer. In addition, we found that DCs loaded with α-C-GalCer induce stronger NKT cell responses *in vivo* and with more prolonged kinetics than those observed with α-GalCer- loaded DCs. This effect may be due to the fact that α-C-GalCer binds more stably to DCs than α-GalCer. When these glycolipids are targeted to DCs in spleen together with antigens in dying cells, such as irradiated tumor cells, α-C-CalCer is active as an adjuvant for T cell immunity at lower doses, 20 ng/mouse, and it is also able to upregulate the required CD40L costimulatory molecule on NKT cells. Therefore α-C-GalCer rep-

resents a glycolipid that binds more stably to DCs and acts as a more effective link between innate and adaptive immunity *in vivo*. (See Research Highlights.)

Tumor cells loaded with α-GalCer induce innate NKT- and NK cell-dependent resistance to tumor implantation in mice

DCs loaded with α-GalCer are known to be active APCs for the stimulation of innate NKT and NK cell responses *in vivo*. In this study, we evaluated the capacity of non-DCs to present α-GalCer *in vitro* and *in vivo*, particularly α-GalCer-loaded tumor cells (tumor/Gal). Even though the tumor cells did not express the CD40, CD80 and CD86 costimulatory molecules, intravenous injection of tumor/Gal resulted in IFN-γ secretion by NKT and NK cells. These innate responses to tumor/Gal, including the induction of IL-12p70, were comparable to or better than to DC/Gal. B16 melanoma cells that were stably transduced to express higher levels of CD1d showed an increased capacity relative to wild-type B16 cells to present α-GalCer *in vivo*. Three different tumor cell lines, when loaded with α-GalCer, failed to establish tumors upon i.v. injection, and the mice survived for at least 6 months. The resistance against tumor cells was independent of CD4 and CD8 T cells but dependent upon NKT and NK cells. Mice were protected from the development of metastases if the administration of live B16 tumor cells was followed 3 h or 3 days later by the injection of $CD1d^{hi}$-B16/Gal with or without irradiation. Taken together, these results indicate that tumor cells loaded with α-GalCer are efficient APCs for innate NKT and NK cell responses, and that these innate immune responses are able to prevent the establishment of metastases *in vivo*.

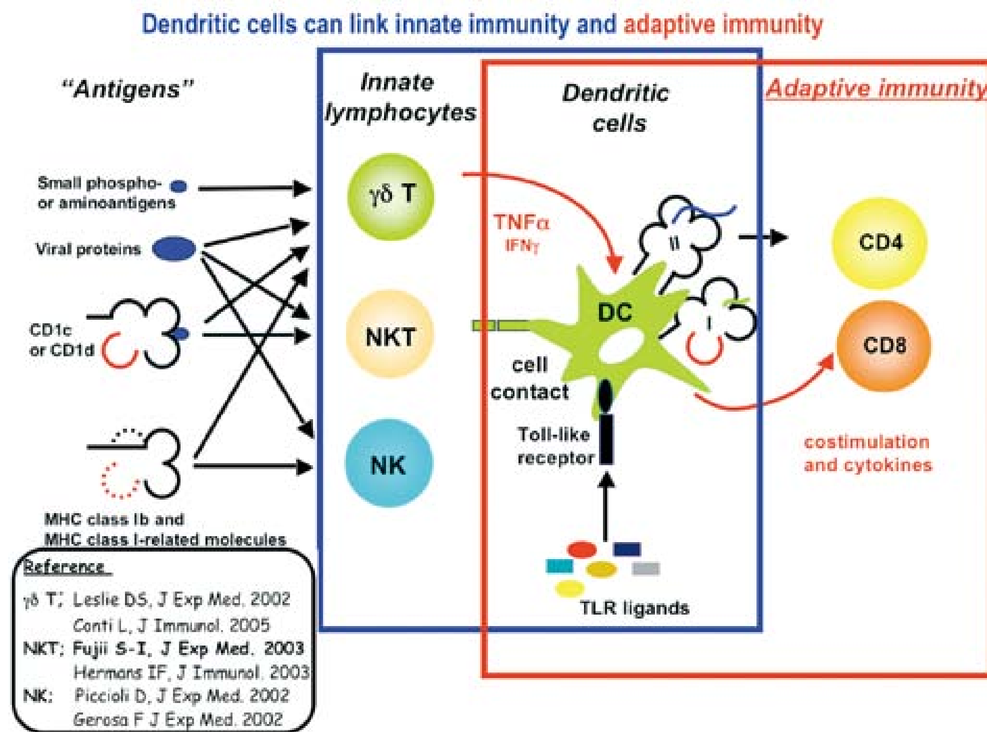

Figure DC maturation indudced in vivo by the innate immune system.
DCs can mature in situ through the activation of innate lymphocytes (γδ T cells, NK cells, and NKT cells) as well as via Toll-like receptor signaling.

Recent major publications

Shimizu K, Goto A, Fukui M, Taniguchi M, Fujii S. Tumor cells loaded with α-alactosylceramide induced innate NKT and NK cell-dependent resistance to tumor implantation in mice. *J. Immunol.* 178, 2853-61 (2007).

Shimizu K, Hidaka M, Kadowaki N, Makita N, Konishi N, Fujimoto K, Uchiyama T, Kawano F, Taniguchi M, Fujii S. Evaluation of the function of human invariant NKT cells from cancer patients using α-alactosylceramide-loaded murine dendritic cells. *J. Immunol.*177, 3484-92 (2006).

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Fujii S, Liu K, Smith C, Bonito AJ, and Steinman, RM. The linkage of innate to adaptive immunity via maturing dendritic cells *in vivo* requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J. Exp. Med.* 199, 1607-1618 (2004).

Laboratory for Cytokine Signaling

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Despite their increasing prevalence of autoimmune diseases and allergies in developed countries, the molecular mechanisms behind these diseases remain poorly understood. The eventual goal of the Cytokine Signaling Research Group is to contribute to the elucidation of the molecular and immunological mechanisms of autoimmune diseases and allergy from the viewpoint of signal transduction within the immune system. More than two decades ago we were involved in the cloning of "soluble factors" that were later to become Interleukin 6 (IL-6), and we continue to explore the molecular processes related to IL-6 and the IL-6 receptor, focusing especially on STAT3 (Signal Transducer and Activator of Transcription 3), various members of the Gab family of proteins and, most recently, the largely unknown universe of signaling through zinc transporter proteins, to reveal the role of zinc in immune functions.

Molecular mechanism of mast cell degranulation

In order to investigate the immunological and molecular mechanisms underlying the role of mast cells in allergy, inflammation, and autoimmune diseases, we dissected the degranulation process of mast cells. FcεRI stimulation first triggers microtubule polymerization and granule translocation to the plasma membrane in a calcium-independent manner. Second, the granules fuse with the plasma membrane in a well-characterized calcium-dependent manner. Furthermore, the Fyn/Gab2/RhoA—but not the Lyn/SLP-76 signaling pathway plays a critical role in the calcium-independent microtubule-dependent pathway (Nishida and Yamasaki et al., *J. Cell. Biol.* 2005). Present research concerns mainly the clarification of the molecular mechanism of calcium-independent microtubule-dependent granule translocation. Very recently, it was found that Zn is required for Fc ε RI-induced granule translocation to the plasma membrane, suggesting Zn chelators as potential new antiallergic agents. Zn is also required for cytokine production in mast cells. In addition, zinc deficiency in mast cell prevents the plasma membrane translocation of protein kinase C (PKC) as well as downstream events such as the phosphorylation of IκB and nuclear translocation of NF-κB and the production of the cytokines such as IL-6 and TNF α (Kabu et al., *J. Immunol.* 2006). Based on these findings, we are now searching for Zn-associated molecules that regulate granule translocation and cytokine production in mast cells.

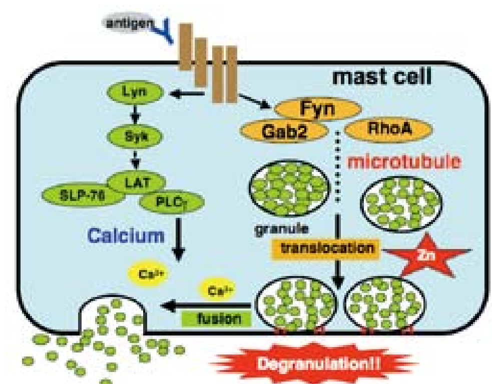

Figure-1 FcεRI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane: Zinc-dependent pathway

Further, we are trying to identify the tubulin-associated molecules involved in granule translocation and are establishing knock-in mice expressing a mutant Gab2 molecule in order to further dissect FcεRI-mediated signaling and to better clarify the *in vivo* function of mast cells in allergy, inflammation and autoimmune diseases.

The role of IL-6/gp130 and zinc signaling in dendritic cell functions.

The regulation of MHC class II (MHCII) molecules in dendritic cells (DCs) is a critical factor in the host defense system and activation of the adaptive immune system. Antigen presentation through MHCII in DCs is critical for CD4$^+$ T cell-mediated immune responses. We investigate the effect of IL-6/gp130 signaling on DC functions related to antigen presentation. We provided evidence that IL-6-STAT3 signaling decreased the intracellular MHCII αβ dimer, Ii, and H2-DM levels by enhancing the cathepsin S activity in DCs, even before the activation. These mechanisms explain, at least in part, how STAT3-mediated signaling suppresses the LPS-mediated surface expression of MHCII in DCs. The results indicate that a cathepsin S-mediated decrease in the MHCII αβ dimer level attenuated the subsequent CD4$^+$ T cell response (Kitamura et al., *Immunity*, 2005).

We previously identified the zinc transporter ZIP6 (also known as LIV1) as a target gene of the IL-6 cytokine signaling molecule STAT3 and further demonstrated that ZIP6 plays an important role in the massive cell migration that takes place during the gastrulation process in the early development of the zebra fish embryo (Yamashita et al,, Nature, 2004). Zinc is a trace element that is essential for the function of many enzymes and transcription factors. Zinc deficiency results in defects in innate and acquired immune responses. However, little is known about how zinc controls the function of immune cells. Very recently, we found that stimulation with the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) altered the expression of zinc transporters in DCs thereby decreasing intracellular free zinc (Kitamura et al., *Nature Immunol.* 2006). Intracellular zinc depletion with a chelator upregulated surface MHCII level and CD4$^+$T cell response, whereas zinc supplementation or overexpression of the gene encoding ZIP6, whose expression was reduced by LPS, inhibited LPS-induced upregulation of MHCII, costimulatory molecules, and CD4$^+$T cell response. These results strongly suggest that intracellular zinc homeostasis is closely related to DC-mediated immune responses. These findings establish a novel mechanism which links TLR and zinc signaling, something which may be crucial for immune regulation.(See Research Highlights.)

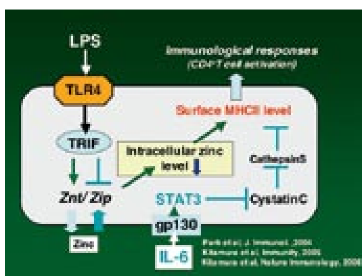

Figure-2 STAT3 and TLR-mediated zinc signaling controls DC maturation.

Autoimmune arthritis associated with mutated interleukin (IL)-6 receptor gp130 is driven by STAT3/IL-7–dependent homeostatic proliferation of CD4$^+$ T cells

IL-6 has been implicated in rheumatoid arthritis (RA) and other T cell-driven autoimmune diseases. Indeed, a previous study by our group showed that an activating mutation in the gp130 subunit of the IL-6 receptor caused a lymphocyte-driven arthritis in mice. But the mechanism was unclear. We showed that disease development in these mice depends on CD4$^+$ T cells, but not on cytolytic CD8$^+$ T cells or antibody-producing B cells. The CD4$^+$ cells did not appear to cause disease due to their affinity for joint-specific antigens. Rather, the cells simply proliferated excessively in the mutant mice. This hyper-proliferation was not intrinsic to T cell, as wild-type CD4$^+$ T cells also multiplied excessively and caused disease when transferred into irradiated mutant mice. Rather, the gp130 mutation caused non-hematopoietic cells to produce excess IL-7, a growth factor that triggers T cell proliferation. This is the first evidence that IL-6 family cytokines can trigger IL-7 production. These data suggest that IL-6, which is elevated in the serum and joints of patients with RA, might exacerbate disease by inducing IL-7 and driving T cell activation. These results show that an interaction between the immune system and other systems causes tissue-specific autoimmune diseases.

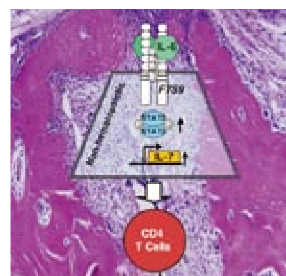

Figure-3 Interaction between the immune system and other systems induces autoimmune arthritis.Gp130 Y759 mutation (F759) in non-lymphoid tissues induces IL-7 production through STAT3 activation, giving rise into an enhanced CD4$^+$T cell homeostatic proliferation. This in turn induces autoimmune arthritis in a manner dependent on CD4$^+$T cells.

Recent major publications

Kitamura, H., H. Morikawa., H. Kamon., M. Iguchi., S. Hojyo., T. Fukada., S. Yamashita., T. Kaisho., S. Akira., M. Murakami., T. Hirano. Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. *Nature Immunol.* 7, 971-977 (2006)

Kabu, K., S. Yamasaki., D. Kamimura., Y. Ito., A. Hasegawa., E. Sato., H. Kitamura., K. Nishida., T. Hirano. Zinc is required for FcεRI-mediated mast cell activation. *J. Immunol.* 177, 1296-1305 (2006)

Sawa, S., D. Kamimura., G.-H. Jin., H. Morikawa., H. Kamon., M. Nishihara., K. Ishihara., M. Murakami., T. Hirano. Autoimmune arthritis associated with mutated IL-6 receptor gp130 is driven by STAT3/IL-7-dependent homeostatic proliferation of CD4$^+$ T. *J. Exp. Med.* 203, 1459-1470 (2006)

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T cells play a central role in the effector and regulatory functions in immunological surveillance, and aberrations of these functions can lead to various immunological disorders. T helper 1 (Th1) cells secrete IL-2, IFN-γ and TNF-α in cellular immune responses against intracellular pathogens and viruses. In contrast, Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 in humoral immune responses mainly against extracellular pathogens and account for allergic immune responses. Recently, Th17 cells have been characterized by their production of IL-17 and association with many aspects of autoimmune tissue inflammation. Cytokines are critical factors in the transmission of information from the receptor to the nucleus as well as in the communication between cells. Thus, the cytokines secreted from effector helper T cells play a critical role in controlling cell fate during immune surveillance. The main goal of our laboratory is to understand the molecular basis of the regulation of effector cytokine production and the cytokine-controlled signal transduction cascades that dictate the fate of T cells. We are interested in how expression of Th2 cytokine genes is controlled in various cell lineages including lymphoid and myeloid lineages during allergic responses. We are also interested in the role of negative regulator of cytokine signaling (SOCS) in the cytokine signal network .

Role of Notch signal in Th2 cytokine regulation

Notch signals have been shown to affect the cellular differentiation of tissue throughout the life of multicellular organisms. RBP-Jκ is a downstream common transcriptional mediator in Notch signaling. The T cell-specific deficiency of RBP-Jκ resulted in selective loss of Th2 development. The distal 3' enhancer in the *Il4* locus that corresponds to one of the conserved regions called conserved non-coding sequence (CNS)-2, contained multiple putative binding sites for RBP-Jκ. Using a transgenic GFP reporter system, we showed that binding of RBP-Jκ to the CNS-2 enhancer regulated the initial IL-4 production from NKT cells and a subset of $CD44^{hi}$ memory phenotype (MP) CD4 T cells. The *Rbp-j* deficient T cells exhibited a marked reduction of both CNS-2 enhancer activity and initial IL-4 production by NKT and MP CD4 T cells. Depletion of the CD4 T cells using CNS-2 enhancer markedly decreased Th2 differentiation from naïve CD4 T cells. These results underline the functional importance of Notch signal and MP CD4 T cells in facilitating Th2 differentiation of naïve CD4 T cells during the initial stage of antigen-induced allergic responses (Tanaka et al. *Immunity* 2006). (See Research Highlights.)

Regulation of *cis*-acting elements of the *Il4* locus among different cell lineages producing IL-4 and IL-13

IL-4 and IL-13 are canonical Th2 cytokines responsible for immunity to extracellular pathogens and helminth parasites and contribute to the pathology of allergy, asthma, and other atopic diseases. Several cell types have been reported to secrete IL-4 and IL-13, including CD4 T cells, Th2 cells, NKT cells, mast cells, basophils, and eosino-

phils. The initial production of IL-4 appears to be key to determining the subsequent course of immune responses. In order to understand how Th2 cytokine genes are controlled in myeloid lineages, we established a series of a reporter transgenic mice with deletions of cis-acting elements. We recently demonstrated that the conserved non-coding sequence-2 (CNS-2), located downstream of the *Il4* locus is a constitutively active enhancer in NKT cells as well as in a subset of CD44^{hi} memory phenotype CD4^{+} T cells. In the study of myeloid lineages, we demonstrated that CNS-2 is a common regulatory element for MP CD4 cells, NKT, mast cells and basophils, but is dispensable for IL-4 production in basophils. Our studies also indicated that the HS4 element instead is a specific enhancer for IL-4 transcription in basophils. Therefore, the expression of Th2 cytokine genes seems to be controlled by a different regulatory machinery in distinct cell lineages.

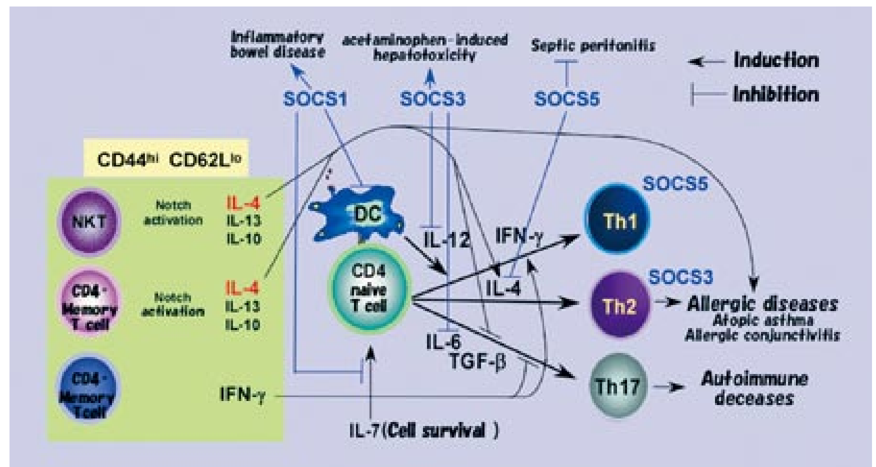

Figure Role of Notch signaling and SOCS proteins in helper T cell differentiation and disease development

Role of suppressor of cytokine signaling (SOCS) in immune regulation

The cytokine environment at the site of initial antigen stimulation determines the direction of helper T cell differentiation into either Th1 or Th2 cells. The SOCS3 and SOCS5 proteins are implicated in this process to control the balance between Th1 and Th2 cells through the inhibition of IL-12 and IL-4 signaling pathways, respectively. A Th2 environment accelerates the number of SOCS3-expressing CD4 T cells in allergic inflammatory sites, subsequently enhancing the pathogenesis of allergic diseases (Kubo et al. *Cur.Opin. Allergy Clin. Immunol.*2006). We demonstrated a role of SOCS3 and SOCS5 in allergic conjunctivitis, atopic asthma, septic peritonitis, and acetaminophen–induced hepatotoxicity. These findings suggest that the modification of SOCS3 and SOCS5 function and expression could become a target for therapeutic intervention in the case of allergies as well as of several immune disorders.

SOCS1 is a powerful negative regulator capable of inhibiting a wide range of cytokines through binding to all members of the Jak family of tyrosine kinases via its SH2 domain. In order to understand the role of SOCS1 in the development and maintenance of naive and memory CD4T cells, we generated transgenic (Tg) mice constitutively expressing SOCS1. The Tg mice developed experimental colitis and inflammatory bowel disease. Most peripheral CD4 T cells in SOCS1 Tg mice have a memory phenotype even in a DO11.10 TCR transgenic or *cd28*-deficient background. These results demonstrate that IL-7-mediated STAT5 activation and some other survival signal through a SOCS1-sensitive cytokine are essential for the maintenance of the naive CD4 cell pool in peripheral organs.

Although considerable research emphasis has been placed on understanding the molecular basis of *Il4* gene regulation in CD4 T cells, several other cell types have been reported to secrete IL-4 and IL-13, including NKT cells, mast cells, basophils, and eosinophils.. *Il4* gene expression has an impact on the regulation of other Th2 cytokines, but the mechanisms underlying this regulation remain unclear as do the effects of Th2 cytokine genes in distinct lineages. To resolve the problem, we are generating deletion mutant mice of each cis-acting element in the *Il4* and *Il13* loci. Our reporter transgenic mice and the cis-acting element deletion mice should allow us to understand the role of each cis-acting element in the regulation of the *Il4* gene and other Th2 cytokine genes. Moreover, our GFP reporter transgenic system should be a useful tool to visualize the behavior of Th2 cells, memory T cells, NKT cells, mast cells, and basophils in an allergic inflammatory site.

We have demonstrated that the SOCS family proteins are implicated in many aspects of acquired immunity through their control of the balance between Th1 and Th2 cells. Recently, a novel Th cell subset has been described that produces IL-17 (Th17 cells). In addition, FOXP3^{+} TGF-β^{+} regulatory T cells (Th3 cells or inducible Tregs) also developed after stimulation with APCs. Generation of all these subsets is promoted by cytokines and the cells are mutually inter-regulated by specific cytokines. Th17 differentiation can be promoted by IL-6 and TGF–β, and IL-23, while early differentiation of Th17 cells is suppressed by IFNγ and IL-4, and STAT3 has also been implicated in Th17 differentiation. These observations suggest that SOCS3 may play a critical role in Th17 differentiation. Therefore, we are investigating the role of SOCS3 in EAE and inflammatory bowel disease.

Recent major publications

Tanaka, S., Tsukada, J., Suzuki, W., Hayashi, K., Tanigaki, K., Tsuji, M., Honjo, T., and Kubo, M., ; The interleukin-4 (IL-4) enhancer CNS-2 is regulated by Notch/ RBP-J signals and controls initial IL-4 expression in NKT cells and memory-type CD4 T cells. *Immunity* 24, 689–701, 2006

Terada, M., Tsutsui, H., Imai, Y., Yasuda, K., Mizutani, H., Yamanishi, K., Kubo, M., Matsui, K., Sano, H., and Nakanishi, K.; Contribution of interleukin-18 to atopic dermatitis-like skin inflammation induced by *Staphylococcus aureus* product in mice *Proc. Natl. Acad. Sci. USA* 103: 8816-8821, 2006

Yamada, S., Tsukada, J., Yoshimura, A, and Kubo, M.; Computer Simulation of the Role of SOCS Family Protein in T Helper Cell Differentiation. *Int. Immunol*. 18, 335-345, 2006

Kubo, M, Ozaki, A., Tanaka, S., Okamoto, M., Fukushima, A. ; Role of suppressor of cytokine signaling in ocular allergy. *Current Opinion in Allergy & Clinical Immunology* 6, 361-366, 2006

Kubo, M., Inoue, H.; Suppressor of Cytokine Signaling 3 (SOCS3) in the Th2 cells evokes Th2 cytokines, IgE and eosinophilia, *Current Allergy and Asthma Reports* 9, 32-39, 2006

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The aim of the Laboratory for Vaccine Design is to clarify the immunological and molecular mechanisms underlying the prevention, or suppression, of allergic responses and to establish innovative technologies for the manufacturing of allergy vaccines against Japanese cedar pollinosis. Our focus is therefore on the modulation of IgE production, a potentially powerful, yet not well-explored approach to mitigate allergic diseases.

It is generally accepted that polarization of the immune response towards Th2 type is the most important prerequisite for the development of the IgE antibody formation and atopic disorders. Based on this principle, several attempts have been made to reverse the deviation of the T cell response to Th1. Another obvious approach for suppression of the IgE antibody formation is to induce immune tolerance and to develop antigen-specific regulatory T (T_{reg}) cells. Although various types of T_{reg} cells were suggested to be effective for the suppression of IgE antibody response, the mechanism of induction of T_{reg} cells *in vivo* has remained unclear. In the experimental model of mice treated with CD1d-ligand, i.e., α-galactosylceramide (αGalCer), however, the NKT cells expressing the invariant Vα14-Jα281 chain (Vα14 NKT) are required for the development of systemic immune tolerance. The accumulated findings suggested to us the possibility that the efficient delivery of αGalCer to proper antigen-presenting cells (APCs) might induce the regulatory function of Vα14 NKT cells and turn them into immunoregulatory cells *in vivo*. Since formulation of αGalCer in liposome enhances the uptake by various cells, we considered the possibility that the liposome might be captured and processed by some APCs that facilitate the differentiation of T_{reg}, rather than Th cells at the time of (or prior to) antigen presentation to naïve T cells. Therefore, we prepared liposomes which contained αGalCer in lipid monolayer with or without antigens.

Mechanisms of IgE suppression mediated by liposome allergy vaccine

Our previous findings had indicated that primary antibody responses and T cell priming were completely suppressed by pretreatment with α-GalCer-liposome. Although the suppressive mechanism might be related to the expansion of $CD11c^{low}CD45RB^{high}$ cells that developed in the presence of IL-10, the detailed mechanism underlying the suppression has remained unclear. Since the expansion of the $CD11c^{low}CD45RB^{high}$ cells was observed in the spleen of mice treated with αGalCer-liposome, but not aqueous αGalCer, the difference between the effects of αGalCer liposome and those of aqueous αGalCer on the immune response was probably ascribed to the difference in the APCs involved. It is well known that presentation of αGalCer by DCs to NKT cells induces the production of large amounts of IFN-γ and IL-4, and that the production of IFN-γ by the NKT cells in this system requires the formation of IL-12 by

the DCs. However, the present experiments also showed that αGalCer-liposome might be captured not only by splenic DCs but also by splenic low-density (LD)-B cells, and that the presentation of αGalCer by the B cells to NKT cells resulted in the formation of IL-10. Since marginal zone B cells, which are included in the LD-B cell fraction, express high levels of CD1d and CD21, we assumed that αGalCer associated with CD1d molecules on the B cells would be presented to NKT cells. Indeed, co-culture of sub-cellular fractions in LD-B cells from the αGalCer-liposome-treated mice with NKT cells resulted in the formation of IL-10 from mainly $CD21^{high}$ $CD23^{low}$ cell and $CD21^{low}$ $CD23^{low}$ cell fractions, but not from CD21middle $CD23^{high}$ cell fraction which include follicular B cells. These results suggest that αGalCer-liposome can be delivered to marginal zone B cells and iniate the development of the immunoregulatory cells that are involved in the suppression of the acquired immune responses.

Capacity of IgE suppression mediated by liposome allergy vaccine *in vivo*

Previous results have indicated that αGalCer-liposome containing OVA might induce regulatory $CD4^+$ T cells suppressing the IgE antibody response in an antigen-specific fashion. To evaluate the capacity of liposomes on the Japanese cedar pollen-specific IgE antibody response, we prepared a αGalCer-liposome containing Cry j1 which is one of major allergens of Japanese cedar pollen. Our results clearly showed that the secondary IgE antibody response in mice immunized with Cry j1 in alum was significantly suppressed by injection of the liposome, suggesting that αGalCer-liposome could perform antigen-specific IgE suppression by virtue of antigens in the liposome (Figure).

For practical use of the liposome to Japanese cedar pollinosis, we prepared recombinant Cry j1-Cry j2 fusion protein the tertiary structure of which might be destroyed, thereby preventing binding by IgE antibodies. *E. coli*-expressed recombinant Cry j1-Cry j2 fusion protein was encapsulated into αGalCer-liposome. The liposome was injected into mice treated with immunized with Cry j1 in alum and boosted with Cry j1 alone. Cry j1-specific ongoing titer and the IgE antibody response in the treated mice were remarkably suppressed compared to those in control mice. The results suggest that the tertiary structure of antigenic polypeptides in the liposome is not essential for the suppressive activity of the liposome.

Preliminary experiments using healthy Beagle dogs showed that αGalCer-liposome containing recombinant Cry j1-Cry j2 fusion protein could induce the proliferation of invariant NKT cells in their spleens and livers, and the development of $Foxp3^+CD4^+CD25^+$ T cells in the peripheral blood, indicating that canine immunoreguialtory cells were also responsive to the liposome. We are now preparing a dog model to evaluate the changes of allergic clinical signs in collaboration with Prof. Thierry Olivry's group of North Carolina State University. In this model, Maltese-Beagle atopic dogs have being sensitized with a mixture of both Cry j1 and Cry j2 antigens, and will be administered αGalCer-liposome containing recombinant Cry j1-Cry j2 fusion protein after the establishment of sensitization.

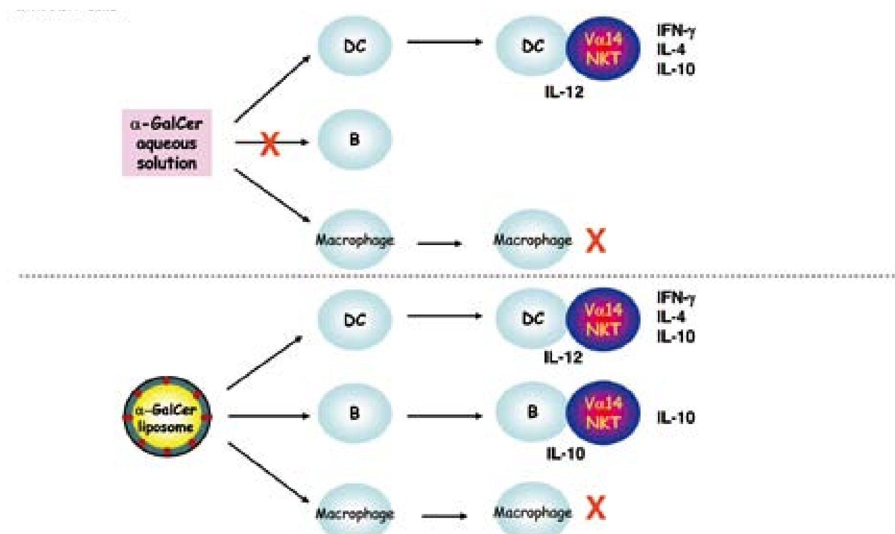

Figure Splenic $CD11c^-B220^+$ cells derived from αGalCer-liposome-treated mice were involved in IL-10 production after co-culture with NKT cells

Recent major publications

Michishige Harada, Kumiko Magara-Koyanagi, Hiroshi Watarai, Yuko Nagata, Yasuyuki Ishii, Satoshi Kojo, Shigetoshi Horiguchi, Yoshitaka Okamoto, Toshinori Nakayama, Nobutaka Suzuki, Wen-Chen Yeh, Shizuo Akira, Hiroshi Kitamura, Osamu Ohara, Ken-ichiro Seino, and Masaru Taniguchi, IL-21–induced B ε cell apoptosis mediated by natural killer T cells suppresses IgE responses (2006) *J. Exp. Med*. 203: 2929-2937

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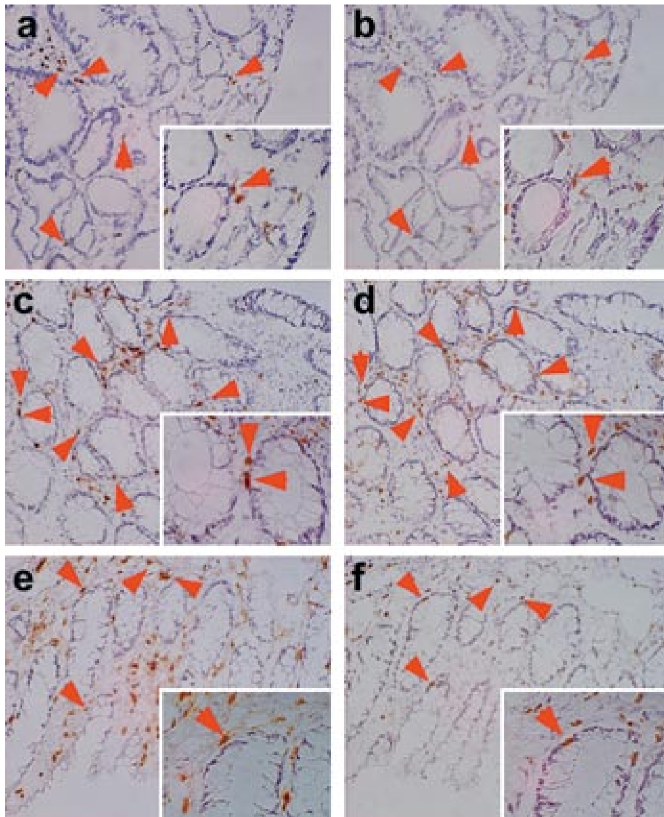
Mast cells (MCs) have a pivotal role in immediate-type and inflammatory allergic reactions via their high-affinity receptors for IgE (FcεRI). In addition, recent animal studies indicate that MCs protect the host against lethal enterobacterial infections via TLR4 or CD48-mediated activation. We have reported that human MCs express functional TLR4 and the high-affinity IgG receptor FcγRI in response to IFN-γ . Further, combined stimulation with LPS and IgG1 cross-linking synergistically increases TNF-α production (unpublished data). We thus hypothesized that human MCs change their phenotype in the Th1-cytokine microenvironment to protect the host against bacteria. Although we have previously reported that skin MCs express FcγRI in psoriatic tissue, it is unclear whether human MCs express TLR4 *in vivo* and what are the roles of FcγRI and TLR4 under pathophysiological conditions. Luminal pathogens are known to act through TLRs in the pathogenesis of inflammatory bowel diseases, and MCs have been shown to be the major source of TNF-α, with approximately 60% of mucosal TNF-α being derived from intestinal mucosal MCs. Crohn's disease (CD) is a chronic inflammatory bowel disease that is thought to be caused by genetic and environmental factors and that affects host-microbe interactions and the production of inflammatory mediators. Variants of the nucleotide-binding oligomerization domain 2 (NOD2), an intracellular sensor of bacteria-derived muramyl dipeptide (MDP), increase the susceptibility to CD up to 40 times. NOD2 mediates the intracellular recognition of MDP, a building block for bacterial cell walls, and activates NF-κB, which results in the production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6. The aim of our study is to investigate the role of MCs in inflammatory bowel diseases, especially CD.

Hyperexpression of FcγRI and TLR4 in the intestinal mast cells of Crohn's disease patients

We previously reported that human MCs express high-affinity IgG receptor (FcγRI) and TLR4 in response to IFN-γ *in vitro*. However, it is unclear whether human MCs express these receptors *in vivo* and whether these receptors are functional under pathophysiological conditions. The number of MCs is reported to increase in Crohn's disease (CD) and ulcerative colitis (UC), but these two diseases are associated with different cytokine profiles (a Th1 pattern in CD and a Th2 pattern in UC). The aim of this study was to examine the expression levels of FcγRI and TLR4 in MCs from CD patients, UC patients, and control patients, and to examine co-expression of TNF-α and FcγRI, and TNF-α and TLR4 in the intestinal MCs. Samples of colonic mucosa (CD: n = 6; UC: n = 6; control: n = 6) were immunohistochemically analyzed, and the density of lamina propria cells expressing FcγRI, TLR4, TNF-α, mast-cell-specific protease tryptase, or a combinations of these proteins were measured in adjacent sections of mucosa. TNF-α production by human MCs and monocytes was measured



Figure

Expression of mast cell tryptase and NOD2 in the intestinal mucosa. Tryptase (a, c, e) and NOD2 (b, d, f) in adjacent 3-µm sections were detected with specific antibodies. Red arrows indicate co-localization of NOD2 in MCs (MCs are defined as tryptase+ cells). Representative sections are shown (a, b: control samples; c, d: Crohn's disease; e, f: ulcerative colitis) (original magnification: x 400). The insets in the lower right corner are high-power views of the cells indicated by the arrows (original magnification: x 650).

by ELISA. The density of MCs expressing FcγRI, TLR4, or both proteins was significantly higher in the CD patients than in the UC patients or control patients ($p < 0.05$). The density of TNF-α^+ MCs expressing FcγRI or TLR4 was significantly higher in the CD patients than in the control patients ($p < 0.01$ or 0.05). LPS and IgG1-cross-linking synergistically induced a high level of TNF-α production in IFN-γ-treated human MCs. FcγRI and TLR4 were expressed in MCs *in vivo*. Hyperexpression of FcγRI and TLR4 on MCs was related to the high frequency of TNF-α expression in CD, suggesting the activation of MCs via these receptors *in vivo*.

Preferential expression of inflammatory cell-recruiting molecules via NOD2 in human mast cells

Variants of the nucleotide-binding oligomerization domain 2 (NOD2), an intracellular sensor of bacteria-derived MDP, increase susceptibility to CD. NOD2 is predominantly expressed by macrophages and dendritic cells. MCs are localized in the intestinal mucosa and have an important role in immunity. The present study shows that intestinal MCs in CD patients express a significantly high level of NOD2 and, in the presence of interferon-γ, human progenitor-derived and lung tissue-derived MCs express NOD2 *in vitro*. To identify specific NOD2-mediated functions in the MCs, GeneChip® analysis was used to compare gene expression profiles between the MDP-, lipopolysaccharides-, and IgE/anti-IgE-specific stimulation profiles, and to compare the MDP-stimulated gene expression profile of peripheral blood mononuclear cells to that of MCs. CXCL10 and urokinase-type plasminogen activator (uPA) upregulation was specific to MCs activated by MDP. Protein expression of these genes was confirmed using an ELISA. MDP-induced upregulation of the adhesion molecules ICAM-1, VCAM-1, and uPA was specific to the MCs. The number of CXCL10$^+$NOD2$^+$ MCs was significantly increased in patients with CD compared to the control patients. Our results suggest that NOD2$^+$ MCs have specific pathogenic roles in innate immunity that involve the recruitment of inflammatory cells through the expression of CXCL10, ICAM-1, and uPA.

Recent major publications

Saito H, Kato A, Matsumoto K, Okayama Y. Culture of human mast cells from peripheral blood progenitors. *Nat. Prot.* 1, 2178-2183 (2006)

Okumura S, Sagara H, Fukuda T, Saito H, Okayama Y. Fc epsilon RI-mediated amphiregulin production by human mast cells increases mucin gene expression in epithelial cells. *J. Allergy Clin. Immunol.* 115, 272-279 (2005)

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Research Unit for Immune Surveillance

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This laboratory is partially funded through the Immune Surveillance Project, a special grant in priority areas funded by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for a total of four years starting in 2003. This project which is coordinated by our laboratory funds 66 research groups all over Japan.

The goal of the Immune Surveillance Project is to mobilize the knowledge on the immune system accumulated recently for the study of immune surveillance. The projects are focusing on the following three major subjects: (1) the spatial and temporal regulation of the immune responses and immunosurveillance, (2) the clarification of molecular signatures of immune memory and approaches to regulate a long-term immune memory, (3) the immune surveillance against cancer cells, pathogens or any danger, and the development of novel methods to restore, reinforce, and/or regulate immune surveillance. We have been working on the construction of artificial secondary lymphoid tissues and the establishment of a novel strategy to restore and reinforce the ability of the immune system to overcome severe infections and to conquer cancer. Eventually, our project based on the generation of artificial lymph nodes has been part of the above-mentioned three topics.

Artificial Lymph Nodes and their immunological function

We previously demonstrated that the artificially generated lymph node-like tissues (artificial lymph nodes), could be constructed by the transplantation of stromal cell-embedded biocompatible scaffolds into the renal subcapsular space in mice. They possess a well-organized tissue structure similar to that of the secondary lymphoid organs. In artificial lymph nodes, the T cell domain and B cell domain are clearly distinguishable, and a follicle is formed. In addition, the existence of germinal centers is confirmed and they harbor both numerous B cells actively dividing and plasma cells. The artificially constructed lymph node-like tissue was transplanted to naïve normal as well as to severe combined immunodeficiency (SCID) mice. The artificial lymph nodes support extremely potent antigen-specific secondary antibody responses in SCID as well as naïve mice. Immune cells in the transplanted artificial lymph nodes also migrate to the SCID spleen and bone marrow (BM) where they drastically expand to generate large numbers of antigen-specific antibody-forming cells. The cells migrate to the empty spaces in immune tissues of SCID mouse and there they undergo clonal expansion upon antigen stimulation. It is thought that the migration of cells from artificial lymph nodes to the spleen and BM in SCID is mediated through signaling from pertusiss toxin-sensitive G-protein coupled receptors including chemokine receptors as well as sphingosine-1-phosphate receptors. Both the structure and the immune function of

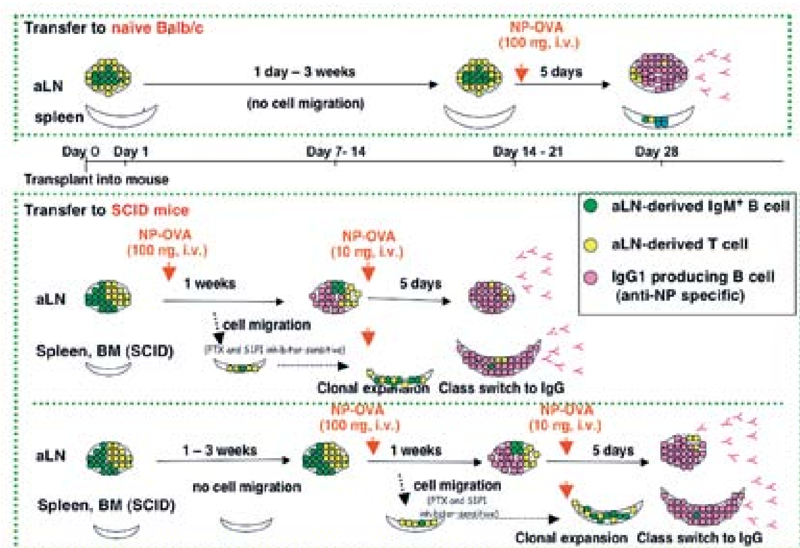


Figure Immune cells in the transplanted artificial lymph nodes migrated to the SCID but not naïve mouse spleen and bone marrow where they explosively expand to generate large numbers of antigen- specific antibody-forming cells. The migration and expansion of the immune cells are triggered by antigen stimulation.

the artificial lymph nodes are stable for over one month after transplantation into naïve and SCID mice. Antigen-specific antibody-forming cells persist after antigen challenge, indicating that artificial lymph nodes can support development of memory B cells and long-lived plasma cells. Surprisingly, memory type CD4$^+$ T cells are highly enriched in the artificial lymph nodes and in the spleens of SCID mice carrying the artificial lymph nodes.

We have also succeeded in constructing artificial lymph nodes possessing antigen-specific anti-cancer activity. In the artificial lymph nodes as well as in the spleens in SCID mice carrying the artificial lymph nodes, a large numbers of IFNγ-producing CD4 and CD8 T cells appear in the tumor in an antigen-specific manner when they are transplanted into tumor-bearing mice. The artificial lymph nodes strongly suppress tumor growth. Tumor-suppressive activity of the artificial lymph nodes was significantly stronger compared to the simple transfer of single-cell suspensions of spleen cells containing tumor antigen-specific immune cells.

This novel simplified system of lymphoid tissue construction will facilitate analyses of spatial and temporal cell-cell interactions required for the development of secondary lymphoid organs and an efficient induction of adaptive secondary immune responses. It may have applications in the treatment of immune deficiency, severe infections and cancer in the near future. (See Research Highlights.)

Regulatory roles of histamine receptor signaling in immune responses

We have been working on the role of histamine H1 and H2 receptors (H1R and H2R) -mediated signals in the regulation of immune responses. GSC in RIKEN Yokohama Institute synthesized *in vitro* and purified the histamine H2 receptor protein. We made monoclonal antibodies against the protein and could obtain a monoclonal antibody which can specifically recognize the natural form of the receptor. It strongly blocked binding of the ligand to the receptor. This monoclonal antibody may facilitate the purification of the natural H2 receptor protein (which is a G protein-coupled receptor), and the functional analysis of the H2 receptor-mediated signaling.

We have examined the role of histamine receptors in allergic inflammation *in vivo* using a mouse model of asthma. Allergen-stimulated splenic CD4$^+$ T cells from H1R-deficient mice exhibited enhanced Th2 cytokine production as we have previously reported. However, allergen-challenged H1R-deficient mice exhibited diminished lung Th2 cytokine mRNA level, airway inflammation, goblet cell metaplasia, and airway hyper-responsiveness (AHR). We found that H1 receptor-deficient CD4$^+$ Th2 cells failed to migrate to the local region of lung and to confer airway inflammation or AHR. Our work clearly established a role for histamine and H1R in promoting the migration of Th2 cells into sites of allergen exposure.

Recent Major Publications

Okamoto N., Nishimoto S., Chihara R., Shimizu C., Watanabe T. Artificial lymph nodes induce potent secondary immune responses in naïve and immunodeficient mice. *J. Clin. Invest.* 117, 997-1007 (2007)

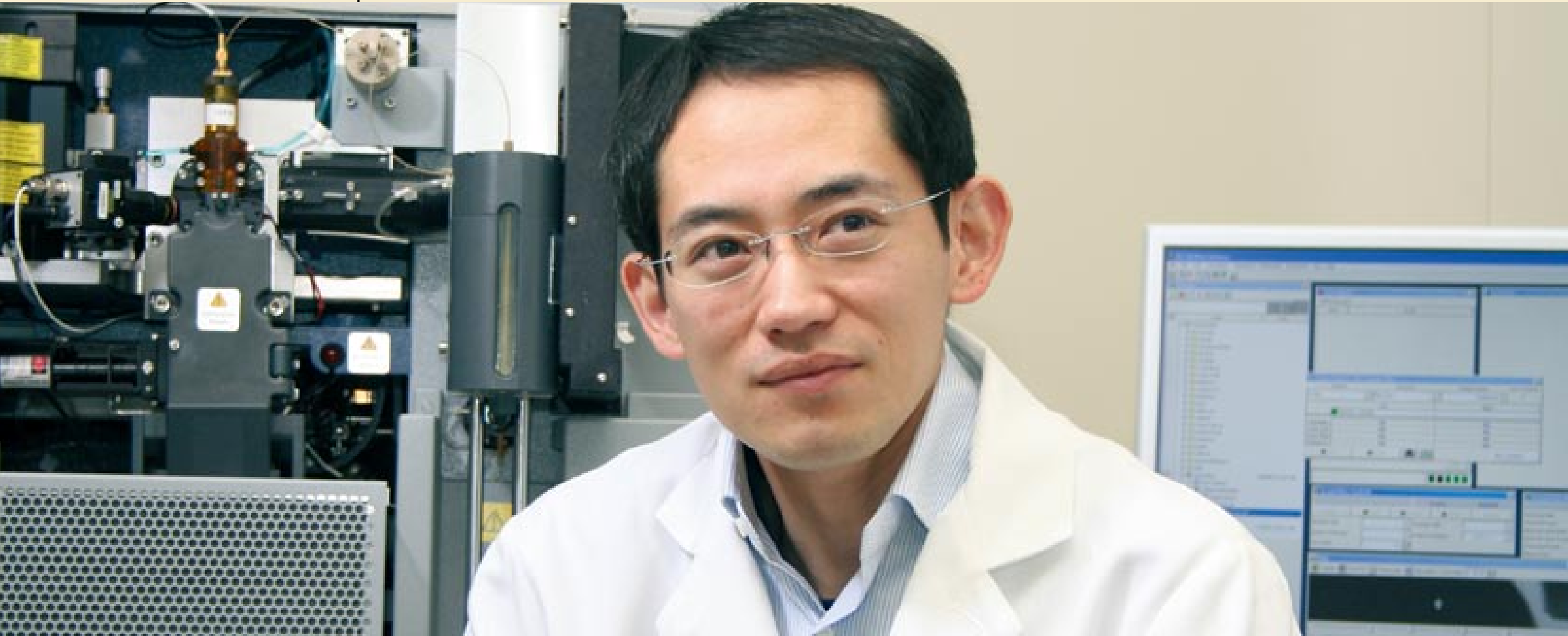
Bryce P.J., Mathias C., Harrison K., Watanabe T., Geha R.S., Oettgen H. The H1 histamine receptor regulates allergic lung responses. *J. Clin. Invest.* 116,1624-1632 (2006)

Huang Z-L, Mochizuki T., Qu W-M, Hong Z-Y, Watanabe T., Urade Y.,Hayaishi O,. Alterations of sleep-wake behavioral characteristics and lack of arousal response to H3 receptor antagonist in histamine H1 receptor knockout mice. *Proc. Natl. Acad. Sci. USA* 103, 4687-4692 (2006)

Ishikawa F., Yasukawa M., Lyons B., Yoshida S., Miyamoto T., Yoshimoto G., Watanabe T., Akashi K., Shultz L.D., Harada M. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor γ chainnull mice. *Blood* 106,1565-1573 (2005)

Suematsu S. , Watanabe T. Generation of synthetic lymphoid tissue-like organoid in mice. *Nature Biotechnology* 22, 1539-1545 (2004).

Research Unit for Human Disease Model



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Humanized mice are unique *in vivo* models for studying human normal hematopoietic and immune systems as well as recapitulating patient-specific disease states in mice. Transplantation of purified human hematopoietic stem cells into a novel immune-deficient mouse strain, NOD.Cg-*Prkdc*^{scid}*Il2rg*$^{tm1Wjl/J}$ (NOD-SCID/IL2rγ^{null}), carrying a complete ^{null} mutation of the common γ chain has enabled us to generate mice with a fully developed human immune system. The newborn NOD-SCID/IL2rγ^{null} mouse system is also a sensitive and efficient system to evaluate the differentiative capacity of human lineage-committed progenitors and to determine the lineage origins of each immune subset. In the past year, we have confirmed the development of two subsets of dendritic cells (DCs), conventional DCs and plasmacytoid DCs, by transplanting purified hematopoietic stem cells, myeloid progenitors and lymphoid progenitors. The DC subsets thus generated *in vivo* retain phenotypic characteristics and transcriptional profiles of the original human samples even in the mouse environment. This system is also expected to reproduce hematological and immunological disorders, facilitating the development of novel therapeutic modalities and the optimized therapy for individual patients. To date we have developed the disease models for acute leukemia, myelofibrosis and graft-versus-host disease. In addition to the development of personalized therapy model, we aim to identify disease-specific stem cells and examine their biology. To discover specific molecular targets for each disease, gene-expression profiles are being determined for disease-specific stem cells.

By using such high-fidelity models recreating normal human hematopoietic and immune systems and by using primary human samples *in vivo* for various diseases, we hope to determine the biology of normal and diseased human stem cells and the mechanisms of the development of the normal and diseased human immunohematopoietic systems leading to the discovery of novel therapies in the future.

Development of normal human dendritic cells in NOD/SCID/IL2rγ^{null} mice

DCs play a crucial role in maintaining the immune system. They are particularly efficient in presenting antigenic peptides in the context of MHC to T cells. Two distinct DC subsets, conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs), have been identified in murine and human hematopoietic tissues. The developmental pathways for human DCs still remain enigmatic, in part due to the lack of suitable animal models. For instance, NOD/SCID mice support human B cell differentiation from transplanted human hematopoietic stem cells (HSCs) but not T cells or DCs. In contrast, using the novel newborn NOD/SCID/IL2rγ^{null} HSC transplantation model, it became possible to analyze the developmental origins of human DCs *in vivo*. When purified human cord blood CD34^{+}CD38^{-} stem cells were transplanted into newborn NOD/SCID/IL2rγ^{null}

mice, both cDCs and pDCs were detected in the recipient bone marrow, spleen and peripheral blood. The bone marrow of the NOD-scid/IL2rγ^{null} recipients contained hCD34^{+}hCD38^{-} human HSCs as well as committed progenitors such as Lin^{-}hCD34^{+}hCD38^{+}hIL^{-}3Rα^{lo}hCD45RA^{-} CMPs and Lin^{-}hCD34^{+}hCD38^{-}hCD7^{+} CLPs, recapitulating the stepwise development of the human immuno-hematopoietic system. HSC-derived cDCs and pDCs could be isolated by flow cytometry based on their phenotype. Purified pDCs but not cDCs had basal levels of IFN-α2 and -β1 mRNA, and treatment with unmethylated CpG oligonucleotides induced a significant up-regulation of IFN mRNAs only in pDCs, indicating that a functional maturation of human DC subsets takes place in the recipients. Differentiation occurred via known intermediates such as common lymphoid and common myeloid progenitors, recapitulating normal human hematopoiesis. These findings suggest that the NOD/SCID/IL2rγ^{null} newborn transplant model is an excellent model to study the human DC development and function *in vivo*. Currently, we are conducting experiments to further define the origins of human DC development in the mouse model.

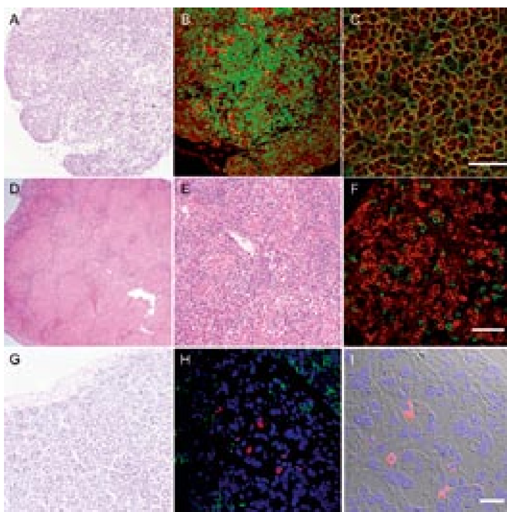

Figure Histology of lymphoid organs in engrafted NOD/SCID/IL2rγ^{null} recipients.
(A) The thymus shows an increased cellularity after reconstitution. (B) The thymus contains cells positively identified by anti-hCD3 (green) and anti-hCD19 (red) antibodies. (C) The thymus contains cells positively identified by anti-hCD4 (green) and anti-hCD8 (red) antibodies. The majority of thymocytes are double positive for hCD4 and hCD8. (D and E) Lymphoid follicle-like structures are present in the spleen of a recipient. (F) The lymphoid follicles mainly contain hCD19^{+} B cells (red) that were surrounded by scattered hCD3^{+} T cells (green). (G) Histology of the intestine in an engrafted NOD/SCID/IL2rγ^{null} recipient. (H) In the intestine, DAPI^{+} nucleated cells (blue) include both scattered hCD3^{+} T cells (green) and human IgA^{+} cells (red). (I) The DIC image of the same section shows that IgA^{+} B cells were mainly found in the interstitial region of the intestinal mucosal layer. White bars inside panels represent 80mm (C), 100mm (F), and 20mm (I).

Development of an *in vivo* model of idiopathic myelofibrosis

Idiopathic myelofibrosis (IMF) is a myeloproliferative disorder characterized by *de novo* development of massive collagen fibrosis, osteosclerosis and angiogenesis in the bone marrow. Clinically, this manifests in progressive anemia, massive splenomegaly, a myelophthisic peripheral blood smear, constitutional symptoms, cachexia and extramedullary hematopoiesis. Infection, bleeding, portal hypertension and leukemic transformation are common causes of mortality, and leukemic transformation occurs in 8-23% of the patients in the first decade of the disease. While allogeneic hematopoietic stem cell transplantation (HSCT) represents the only treatment option with proven curative potential, the majority of affected patients are not suitable candidates for standard-dose conditioning followed by HSCT because of a relatively high transplant-related mortality. If a transplantation is not an option, the currently available therapy does not affect the poor prognosis associated with the natural history of the disease. An *in vivo* model of human IMF in CB17^{-}scid mice or NOD/SCID mice has not yet been achieved. However, using the newborn NOD/SCID/IL2rγ^{null} mouse transplantation model, it became possible successfully to recapitulate human IMF disease *in vivo*. The injection of HSCs from primary IMF patient peripheral blood resulted in the development of bone marrow fibrotic changes characteristic of IMF. Transplanted IMF cells showed infiltration into the recipient liver and spleen with evidence for the development of human hematopoiesis in both organs, similar to extramedullary hematopoiesis leading to hepatosplenomegaly in IMF patients.

The NOD/SCID/IL2rγ^{null} mouse model is an excellent *in vivo* model to study normal and abnormal human hematopoietic and immune systems. It is superior to existing models as it supports high levels of human cell engraftment and full differentiation of immune components. Using this model, it is possible to recreate *in vivo* normal human hematopoietic and immune development by reconstituting normal human bone marrow or umbilical cord blood-derived hematopoietic stem and progenitor cells. It is also possible to recapitulate *in vivo* the pathogenesis of various diseases affecting the hematopoietic and immune systems by transplanting primary patient hematopoietic stem and progenitor cells. We are also developing the next-generation of immunosuppressed mice with an even greater capacity for human hematopoietic and immune reconstitution.

Recent major publications

Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol.* 7, 118-130 (2007).

Shimoda S, Ishikawa F, Kamihira T, et al. Autoreactive T-cell responses in primary biliary cirrhosis are proinflammatory whereas those of controls are regulatory. *Gastroenterology.* 131, 606-618 (2006).

Ishikawa F, Shimazu H, Shultz LD, et al. Purified human hematopoietic stem cells contribute to the generation of cardiomyocytes through cell fusion. *FASEB J.* 20, 950-952 (2006).

Ishikawa F, Yasukawa M, Lyons B, et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor γ chain^{null} mice. *Blood.* 106, 1565-1573 (2005).

Yoshida S, Ishikawa F, Kawano N, et al. Human cord blood-derived cells generate insulin-producing cells *in vivo*. *Stem Cells.* 23, 1409-1416 (2005).

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The main activity of our team is focused on screening a large ethylnitrosourea (ENU) mutant panel and is done in cooperation with the RIKEN Genomic Sciences Center. An important goal of this project for RCAI is the development of novel mouse models for various immunological disorders, notably, allergic and autoimmune disorders. Since the ENU mutagenesis introduces approximately 3,000 point mutations on a genome, we can expect 100 coding region mutations in one pedigree.

In parallel, we are screening those mutations under environmental bias, using a variety of approaches, including the immunization of mutants with various allergic antigens and adjuvants to identify the allergic disease modifier genes.

ENU mutant panel study

In order to identify the genetic basis for immune disorders, we are screening a pool of mutant mice generated by random chemical mutagenesis through ENU administration. ENU induces random single-base pair changes in genomic DNA at approximately 3,000 sites throughout the whole genome and approximately 100 sites in the coding regions of proteins per a first-generation (G1) mutant mouse.

It is expected that screening approximately 300 G1 pedigrees will be sufficient to establish a mutant mouse library with mutations in all gene products. Following the administration of ENU to C57BL/6 mice, the dominant mutant analysis was started in August 2003 and the recessive mutant analysis in December 2003 in the GSC research building as a pilot study. In this process, we screened 102 dominant inheritances from 16 pedigrees and around 400 recessive inheritances from 58 mouse pedigrees. After moving into the RCAI research building, we screened, within the first 3 years, 7,000 recessive inheritances from 70 mouse pedigrees. For basic phenotypic screening, we examined the levels of immunoglobulins, cytokines, and auto-antibodies, as well as peripheral blood cells surface marker expression. Using tissue sections from animals at 16 weeks of age, we clarified the mutant phenotype by pathological, cytological and molecular biological examinations of affected tissues, lymphoid organs and blood

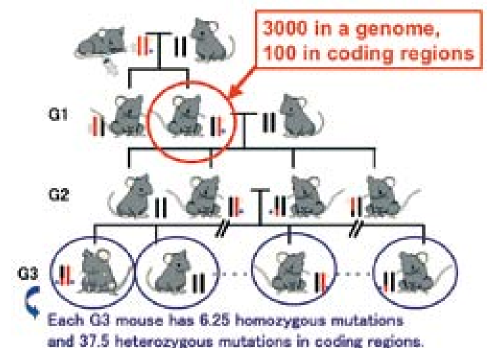

Figure-1 Random mutagenesis was developed by ENU injection into founder C57BL/6J male mouse, and its sperm was mated to wild-type eggs of C57BL/6J female mice, The third generation of the offspring was estimated to have more than 6 genes homozygoue mutations.

cells. Collaborating teams examined the dissected samples by their own methods. All screening data are updated on the internal database system in the RCAI web server, and we define the mutant phenotype at meetings with collaborating teams held every month. All the attendants discuss the selection of candidate mutant lines.

A phenotype identified as a mutant candidate line is reexamined in a later development stage, and if the same phenotype is present in both sexes, we start mating them for phenotype inheritance tests. If only single-gender mutant animals are obtained, the phenotype is disregarded but is noted as an interesting one. The responsible mutation loci are mapped by backcrossing mutant individuals with the C3H/HeJ strain for gene detection by single-nucleotide polymorphism analysis. After approximate mapping, candidate gene sequences are checked against public databases by means of the PosMed research system established by RIKEN GSC.

Allergic disease model mutant mouse model

By ENU recessive mutant screening, we identified and established a mutant reminiscent of a human allergic disease. The serum IgG1 or IgE level of these mice gradually increased during growth. The ear skin became thicker and red, and the mice started to scratch the ear skin or face continuously. Pathologically, the epidermal layer was hypertrophic and many lymphocytes were found in the dermis of the lesion. Mast cell numbers increased in the same region, and many of them became enlarged and emitted metachromatic particles. All these symptoms or findings seemed compatible with the criteria for human atopic dermatitis (AD). Therefore, we have started to study this mutant line intensively. The disease grade or onset timing of atopic dermatitis in the mutant mice varied within a litter . Since ENU induces multiple mutations in a pedigree, it is plausible that a combination of a few mutations affects the clinical symptoms. In order to identify every responsible gene for those phenotypes, we are collaborating with the Phenome Informatic Team and Mouse Mutation Resource Exploration Team in GSC, RIKEN. These teams can quickly detect the responsible region roughly to 5cM from 30 mutant DNA samples. Subsequently, we can focus on the candidate genes by means of database analysis using our own software program. We have mapped the responsible region into a few cM and are sequencing the candidate genes.

Perspectives

By ENU recessive mutant screening, we have identified and established more than 140 mutant lines of immune or blood disease models. In line with our expectation, more than 50 lines showed phenotypes related to allergic diseases. For example, more than ten lines exhibited elevated serum IgE levels, and some of them showed aberrant responses against the induction of artificial allergic disease. We still require more than a year to confirm the phenotype inheritance of all these mutants. We are now backcrossing some of them to the C3H/HeJ strain in order to map the responsible loci. As of December 2006, we have mapped four independent mutant-responsible loci to distinct regions, and three of them have been clarified to be point mutations of known genes. After mapping to 10cM in a chromosome, we have chosen a candidate gene using the PosMed system and are preparing to sequence it. We plan to map ten more loci responsible for allergic disease model mutations.

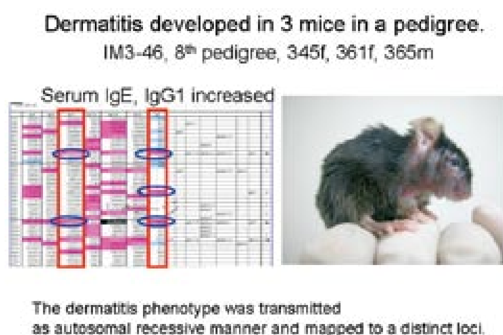

Figure-2 An atopic dermatitis-like mutant mouse was established from ENU screen. The study of precise mechanism of the responsible mutation in this disease onset is now underway.

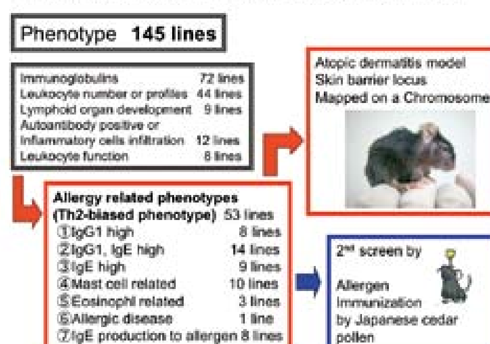

Figure-3 Summary of three years of ENU mutant screening. In total, approximately 7,000 mutant mice from 70 genomes have been screened and 140 phenotypes have been identified as mutant lines. The mouse shown presents a mutant phenotype reminiscent of human atopic dermatitis.

Recent major publications

Hamanaka S, Nabekura T, Otsu M, Yoshida H, Nagata M, Usui J, Takahashi S, Nagasawa T, Nakauchi H, Onodera M. Stable transgene expression in mice generated from retrovirally transduced embryonic stem cells. *Mol Ther.* 2007 Mar; 15(3):560-5.

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Administrative Staff :
Kazuyo Nomura

An important mission of our research group is to function as a "Gateway" to genomics for immunologists. In practice, our research activities thus consist of three categories: (1) central support activities; (2) strategic and collaborative research activities; and (3) exploratory research activities aimed at new technology development. Although the research goals for (1) and (2) vary considerably depending on the respective projects or collaboration, our mission is always to provide the best available expertise in transcriptomics, proteomics, and bioinformatics to researchers in this center. Although we do not require any authorship in research activity (1), our efforts under research activity (2) have lead to many publications this year (a part of which is shown in next page "Recent Publications"). Because we have always been involved in more than 10 collaborative projects in parallel inter- and intra-institutionally, we expect these to keep yielding results that can be presented as visible outcomes of the research activities under (2). On the other hand, although research activities under (1) and (2) are given the highest priority at present, research activities under (3) are equally important for achieving distinction in genomic researches in immunology in the future. Several different lines of research, in the fields of proteomics, transcriptomics, microfluidics, and bioinformatics, are going on under this research category. (A) The launch of Immunogenomics database and (B) the comprehensive analysis of translational control of gene expression are highlighted below.

Construction of an open-access database which integrates cross-reference information of transcriptome and proteome of immune cells

Although huge amounts of mammalian genomic data have been accumulated to date, immunologists still encounter obstacles that prevent them from using the data. One of the hurdles for obtaining biological insights from the genomic data is the inability of cross-referencing of transcriptomic and proteomic data on a single informational platform. This is because the correlation of protein profiles to mRNA profiles is usually not very high whereas protein profiles are expected to be directly relevant to biological phenomena. Thus, since mRNA and protein profiles cannot both be examined in parallel, it is very difficult to mine biological insights from the data. To address this, we constructed an open-access database that allowed us to cross-reference transcriptomic and proteomic data of immune cells. The database, named RefDIC (Reference genomics Database of Immune Cells; http://refdic.rcai.riken.jp/), currently contains (1) quantitative mRNA profiles of human and mouse immune cells/tissues obtained by using Affymetrix GeneChip, (2) quantitative protein profiles of mouse immune cells obtained by two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry and image analysis, and (3) various visualization tools for cross-referencing the mRNA and protein profiles of immune cells. RefDIC is, to our knowledge, the

first open-access database for immunogenomics and serves as an important information-sharing platform for a focused-genomic approach in immunology. Cross-reference of mRNA and protein profiles allows researchers convincingly to interpret modulation of gene expression from a biological viewpoint at a glance.

RefDIC is now open to the public and accessed by a large number of immunologists world-wide. To make RefDIC more attractive, we newly developed some informational tools and opened them to the public. However, this is only the first stage of our attempts towards constructing a meaningful bioinformatics platform in immunology. RefDIC will keep growing further and will integrate Immunogenomics database through linking to other bioinformatics databases and incorporation of new data yielded by our original experiments.

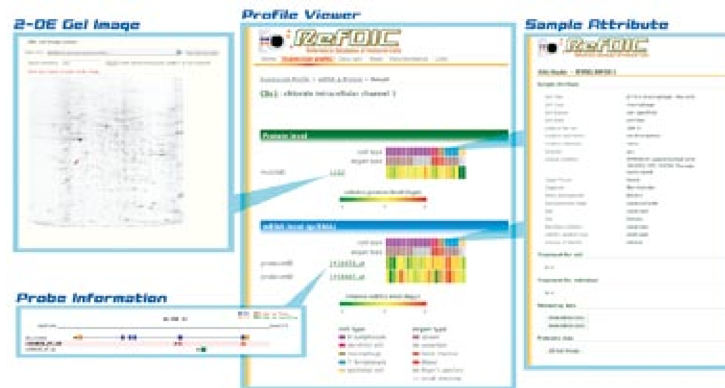

Figure-1 Visualization of mRNA/protein profiles in various types of immune cell. RefDIC provides a web-based query interface and tools for visualization of expression profiles of a particular gene at both mRNA and protein levels across various types of immune cell. The database also offers internal or external links to relevant information such as sample attribute, probe annotation of the microarray data, and protein spots on the 2-D gel.

Study of post-transcriptional events of macrophage-like cells stimulated by bacterial lipopolysaccharide

Although post-transcriptional regulation is believed to be pivotal in immune cells, it has not been fully elucidated yet. We are now attempting to uncover these events using comprehensive approaches for mRNA profiling. In particular, elucidation of translational control of gene expression can be expected to fill the gap between transcriptome and proteome because discrimination of translationally active transcripts from inactive ones is critical for interpretation of mRNA profiles from a biological viewpoint. So far we have focused our efforts on the analysis of bacterial lipopolysaccharide (LPS)-induced translational regulation in macrophages. To achieve this, we first collected quantitative profiles of polysomal (translation active) and sub-polysomal (translation-inactive) transcripts from murine macrophage-like J774.1 cells and compared the profiles before and after LPS stimulation. We found that the mRNA pools thus fractionated had distinguishable mRNA profiles from each other and behaved differentially in response to LPS stimulation; the effect of LPS on the translational state of mRNAs was specific to a limited number of transcripts and a total of 92 and 209 transcripts were up- and down-regulated after the stimulation. Many of genes encoding these transcripts were confirmed to be affected at their nascent protein levels and thereby eventually modulated at the accumulated protein levels. In our observations, these changes seemed in turn to affect indispensable cellular functions such as respiration and viability. Because the mechanism of the observed translational control of gene expression induced by LPS in macrophages is unclear at present, we are actively trying to identify molecular components participating in the LPS-induced translational regulation .

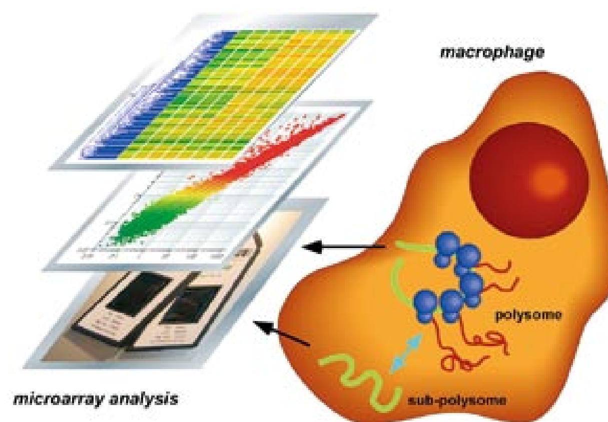

Figure-2 Genome-wide analysis of translational states of transcripts in macrophages. Translationally active and inactive transcripts can be isolated in polysome and subpolysome fractions, respectively. Combination of this fractionation with microarray enables us to assess translational activity of each transcript in a genome-wide manner.

Recent major publications

Nishikawa, Y., Hikida, M., Magari, M., Kanayama, N., Mori, M., Kitamura, H., Kurosaki, T., Ohmori, H. Establishment of lymphotoxin receptor signaling-dependent cell lines with follicular dendritic cell phenotypes from mouse lymph nodes. *J. Immunol.* 177, 5204-5214 (2006)

Nakayama M, Iida M, Koseki H, Ohara O. A gene-targeting approach for functional characterization of KIAA genes encoding extremely large proteins. *FASEB J.* 20, 1718-1720 (2006)

Kimura Y, Yokoyama R, Ishizu Y, Nishigaki T, Murahashi Y, Hijikata A, Kitamura H, Ohara O. Construction of quantitative proteome reference maps of mouse spleen and lymph node based on two-dimensional gel electrophoresis. *Proteomics* 6, 3833-3844 (2006)

Sasuga Y, Tani T, Hayashi M, Yamakawa H, Ohara O, Harada Y. Development of a microscopic platform for real-time monitoring of biomolecular interactions. *Genome Res.* 16, 132-139 (2006)

Hoshino K, Sugiyama T, Matsumoto M, Tanaka T, Saito M, Hemmi H, Ohara O, Akira S, Kaisho T. IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. *Nature* 440, 949-953 (2006)

Central Facilities

Central Facilities include the resource and competency pools created by RCAI to provide all researchers in the Center with access to the most advanced equipments and technologies. In April 2006, the RCAI Advisory Council Board reported that the services provided by the Central Facilities had been both timely and of the highest quality.
The Central Facilities are divided to five sections; FACS Lab, Confocal Lab and Monoclonal antibody Lab managed by Dr. Takashi Saito, Genomics Lab managed by Dr. Osamu Ohara, and the Animal Facility managed by Dr. Haruhiko Koseki.

FACS Lab

Technical Staff: Hanae Fujimoto and Yukiko Hachiman

Photo: FACS Vantage

The FACS Lab provides a variety of support for cell sorting and cell analysis, procedures essential for all immunological experiments. FACS Lab contains 4 Vantages, 1 Aria, 7 Caliburs and 1 LSR. FACS Lab is conducted under collaboration with Becton-Dickinson. Co. Ltd. Currently, 20 out of 28 research groups utilize the FACS Lab. The laboratory's activities are divided into three main parts; technical support and training for FACS users, cell sorting operation service, and management of the FACS machines.

1. Technical support and training

In FY2006, the facility offered 22 technical training courses (7 for cell analysis and 15 for cell sorting). Courses were held in seven levels, LSR, Calibur basic1, Calibur basic2, Calibur advanced, Vantage basic (two days), Vantage advanced, Aria basic (two days), and Aria advanced.

2. Cell sorting operation service

The FACS Lab provides a cell sorting operation service, in which the researchers can ask an experienced operator to conduct the cell sorting experiment. In FY2006, 22 research groups used this system.

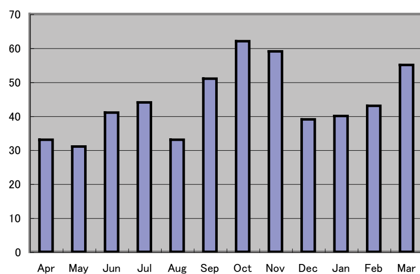

Figure: Numbers of users:
cell sorting operation service per month

3. Management of FACS machines

FACS machines are available for registered users 24 hours a day and reservations are accepted one month in advance through internal website. All the necessary information including instructions, reservations and users fee can be accessed via the intranet. In addition to the in house FACS Lab staff, engineers from Becton Dickinson visit once a week to provide maintenance and technical support.

Confocal Lab

Collaborative Researcher: Akiko Furuno (Leica)

Photo: Intravital microscopy

The Confocal Lab provides imaging equipments and support. Confocal Lab is conducted under collaboration with Leica Microsystems. There are 5 confocal microscopes; 1. Inverted system with visual and multiphoton (MP) laser which is suitable for time-lapse imaging of living cells and organs, 2. Inverted system with 405nm laser which is suitable for a time-lapse imaging of living cells in a controlled environment (CO_2, temperature, and humidity), 3. Inverted system with visual and UV laser which can be used for calcium detection, 4. Upright system with visual and UV laser which is suitable for standard fixed specimen, and 5. Intravital upright system with visual laser and MP laser which can be used for whole animal body. During FY2006, the total running time of the microscopes was over 1,300 hours and 460 researchers used this facility.

In addition to the management of the equipment, Dr. Furuno supports researchers in establishing suitable conditions to visualize their specimens. In particular, intravital imaging is one of the Center's interests. As the first step, they are setting up a standard conditions to observe immune cells in the peripheral lymph nodes or transferred lymph node in kidney capsule of mice.

Monoclonal Antibody Lab

Collaborative Researcher: Shinji Seki (MBL)

Technical Staff: Tomomi Aoyama and Hajime Inamochi

The Monoclonal Antibody (mAb) Lab produces mAbs requested by RCAI research groups. Since the Center's establishment in 2004, the lab has produced more than 70 mAbs to various antigens, which were synthetic peptides,

recombinant proteins or cells. The mAb Lab receives approximately 25 requests a year for this type of service.

The lab uses a variety of screening methods, including FACS and ELISA. Recently a high-speed 96 well-plate screening system that can be used in conjuction with the FACS Calibur HTS was introduced.

In addition to their contribution to many publications using mAb produced by the mAb Lab, two of their mAb are under patent application and one has already been commercialized.

Animal Facility

Senior Technical Scientist: Takanori Hasegawa
Technical Scientists: Shinobu Mochizuki, Yasuaki Murahashi (IT)
Technical Staff: Masashi Matsuda, Tomoyuki Ishikura, Isamu Hisanaga, Momoko Okoshi, Natsumi Saito, Atushi Kajiwara
Administrative Staff: Hiroko Iwamoto

The Animal Facility provides the following services for users.

1. Supports for the ENU mutant mice project

The Animal Facility supports the ENU mutant mice project (Collaborative project between RCAI and GSC) by conducting *in vitro* fertilization, cryopreservation and breeding. During FY2006, 10 3rd generation lines (334 mice) were generated. 14,628 3rd generation fertilized eggs and 8,246 2nd generation fertilized eggs were cryopreserved.

2. Generation of SPF mice and cryopreservation of fertilized eggs

In order to maintain its SPF level, the Animal Facility performs *in vitro* fertilization to sanitize all mice obtained from outside institutes. During FY2006, 1,041 mice of 97 lines were obtained by this procedure. Cryopreservation of fertilized eggs reached 46,893 of 278 lines.

3. Generation of transgenic mice

The Animal Facility generates transgenic mice requested by laboratories of the RIKEN Yokohama Institute. During FY2006, 77 transgenic mouse lines were generated using 37 vectors provided by eleven laboratories.

4. Generation of chimeric mice

1) Injection method

666 chimeric mice were generated using 24 vectors from 8 laboratories.

2) Aggregation method

789 male chimeras were obtained using 55 vectors from 10 laboratories.

5. Cryopreservation of sperms

258 tubes of 84 lines were cryopreserved in FY 2006.

Genomics Lab

The Genomics Lab provides the following support to the laboratories of RCAI.

1. DNA sequencing

Hiroshi Kitamura (senior research scientist) and Yuki Kobayashi (technical staff)

The Genomics Lab provides a DNA sequencing service using 36cm or 50cm capillary sequencers (ABI). During FY2006, a total of 22,530 samples from 24 research groups were sequenced.

2. DNA Microarray analysis

Hiroshi Kitamura (senior research scientist), Sachiko Matsuyama (technical stafft), Masako Mori (technical staff),
Tomoko Yuasa (technical staff)

Microarray analysis is conducted using the GeneChip system (Affymetrix) and the Genespring Workstation data mining software (Silicon Genetics). During FY2006, microarray analyses were conducted on 469 samples; 352 from mice, 96 from humans, 4 from chickens, and 15 from bacteria.

3. Proteome Analysis

Yayoi Kimura (research scientist), Ryo Yokoyama (technical staff), Yasuyo Utsugi (technical staff)

Mass spectrometry analysis (241 samples), 2-D gel electrophoresis (40 samples) and *in vitro* translation (11 samples) were carried out in FY2006.

4. Clone distribution

Hiroshi Kitamura (senior research scientist) and Yuki Kobayashi (technical staff)

The Genomics lab provides cDNA and *E. coli* clones in FANTOM (Functional Annotation of the Mouse) projects, lead by the RIKEN Genomic Sciences Center. In FY2006, 159 clones were provided to 11 research groups in RCAI.

Photo: Microinjection room

Photo: DNA sequencer

RIKEN Special Postdoctoral Researcher (SPDR) and Junior Research Associate (JRA)

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RIKEN Special Postdoctoral Researcher (SPDR) Program

RIKEN's program for Special Postdoctoral Researcher was instituted to provide young and creative scientists the opportunity to be involved in autonomous and independent research in line with RIKEN objectives and research fields. The positions are competitive, but if selected, researchers receive salaries and research budgets (1 million yen) from RIKEN, and they are able to conduct their research at one of its laboratories.

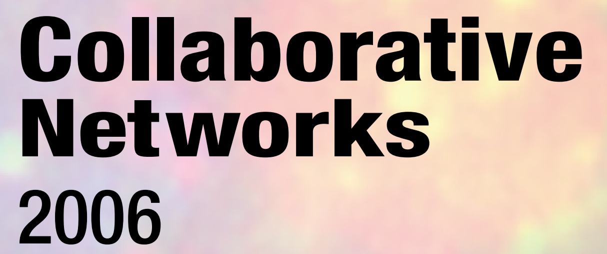
This year **Dr. Tomoyuki Suzuki** (Lab. for Cytokine Signaling) (01) and **Dr. Yu-ichi Fujimura** (Lab. for Developmental Genetics) (02) conducted their research at RCAI through the SPDR program.

RIKEN Junior Research Associate (JRA) Program

The Junior Research Associate program was launched in 1996 to encourage young scientists with fresh ideas and youthful enthusiasm to collaborate with, and learn from, veteran scientists with years of experience. This program provides part-time positions at RIKEN for young researchers enrolled in university PhD programs. The JRA program serves the dual purpose of fostering the development of these young scientists while also energizing RIKEN with their innovative thinking.

This year, eight JRA students studied in RCAI.

Xiangzhi Li
(Lab. for Developmental Genetics) (03)
Naoki Imajo
(Unit for Allergy Transcriptome) (04)
Shinya Tanaka
(Lab. for Signal Network) (05)
Masashi Tachibana
(Lab. for Transcriptional Regulation) (06)
Noriko Komatsu
(Unit for Immune Homeostasis) (07)
Naoko Okiyama
(Unit for Clinical Immunology) (08)
Takahiro Sugiyama
(Lab. for Host Defense) (09)
Masayo Harada
(Lab. for Developmental Genetics) (10)


Collaborative
Networks
2006

Human Immunodeficiency Network

Primary immunodeficiency (PID) is caused mutations in genes required for normal immunological development and/or immune responses and is often characterized by recurrent and severe infections. In the most severe cases there may be infection by several pathogens, and this can be life-threatening if they are resistant to antimicrobial therapy. Many causative PID genes has been identified, however, the genetic basis remains unknown in approximately 25% of patients at present. Depending on the type of PID, intravenous immunoglobulin substitution, careful anti-microbial therapies, and hematopoietic stem cell transplantation including bone marrow transplantation can provide a good prognosis, but in some patients the diseases lead to autoimmune disorders and cancer. The most effective therapy could be genetic correction of the responsible mutation (gene therapy), and this has been successfully applied in some types of PID However, additional basic research for the development of safe and efficient gene delivery vehicles is still needed.

In this context, RIKEN RCAI and the Clinical Study Group for Primary Immunodeficiency (table), together with Kazusa DNA Research Institute, established a platform to develop and combine genetic diagnosis and basic and clinical research, including the reconstitution of the human diseases in humanized mice and the search for causative genes for the primary immunodeficiencies of unknown etiology. Within this platform, systematic information concerning immunological, genetic and clinical features of these patients, including prognosis, will be accumulated. This database will be useful in providing a total view of the pathogenesis of the diseases and the establishment of critical diagnosis by multiple parameters. This information can be accessed by clinicians and researchers in the Clinical Study Group for Primary Immunodeficiency and utilized for clinical purposes. In the future, as the necessary software is developed, it should be possible to simulate disease progress and prognosis in silico before the onset of treatment. Thus this system could open up a completely new area in clinical research and patient care. The role of RCAI RIKEN here will be database construction, immunological diagnosis, preparation of genetic material, and basic science research for the development this new technology. The collaboration between clinical and basic researchers should lead to synergies that will help our quest for a cure for primary immunodeficiencies and contribute to our understanding the human immune system.

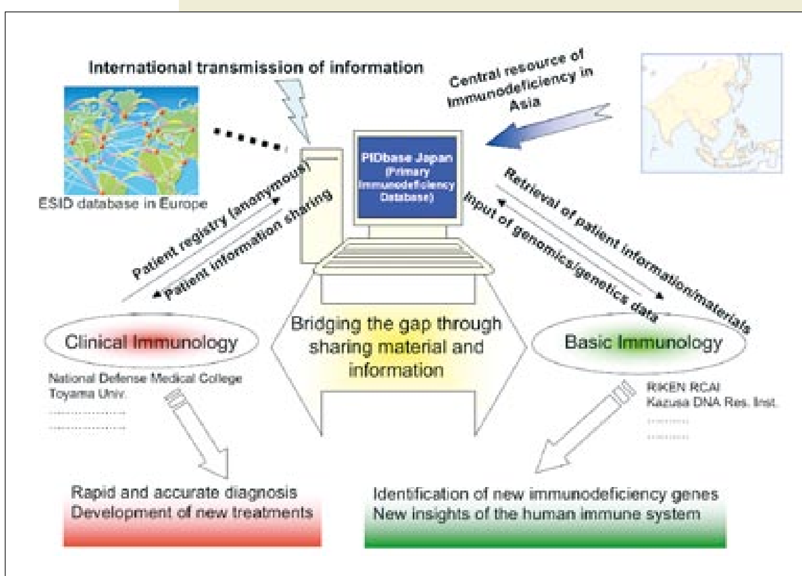


Figure: Construction of a database of primary immunodeficiencies at RIKEN RCAI

Table 1: Collaborating universities and institutes

1	Kazusa DNA research institute
2	Toyama Univ.
3	National Defense Medical College
4	Hokkaido Univ.
5	Tohoku Univ.
6	Tokyo Medical and Dental Univ.
7	Kanazawa Univ.
8	Gifu Univ.
9	Hiroshima Univ.
10	Kyusyu Univ.
11	Miyazaki Univ.
12	Nagoya Univ.
13	Kyoto Univ.

Two RIKEN Venture Companies Established

In 2006, RIKEN approved two biotech venture companies, REGiMMUNE and Animal Allergy Clinical Laboratories which are based on the knowledge and technologies developed at RCAI.

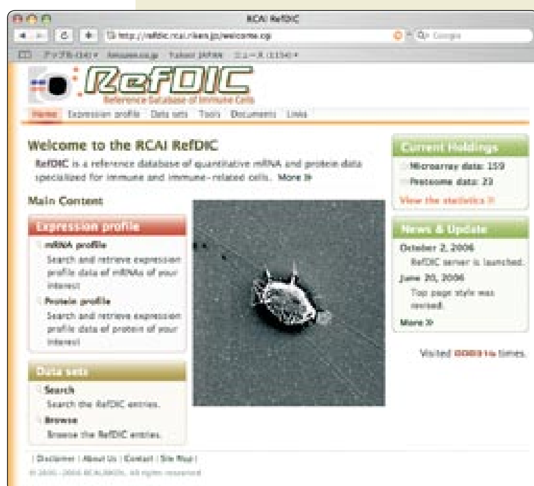
REGiMMUNE Corporation applies recombinant fusion antigen vaccine and liposome vaccine technologies invented by RCAI's Lab. for Vaccine Design to develop and commercialize immune-regulatory therapeutics. The recombinant fusion protein comprising of Cry j1 and Cry j2 mature polypeptides, which are major antigens of Japanese cedar pollinosis, was firstly established. This Cry j1/2 fusion protein has two unique characteristics. That is that the fusion protein is not recognized by IgE antibodies produced by pollinosis patients probably owing to the tertiary structure of the fusion protein, distinct from that of Cry j1 and Cry j2 protein themselves. Thus, it seems probable that this new type of vaccine is expected to be less anaphylaxis. In addition, the protein contains all epitope peptide sequences responsible for activation of allergen-specific T lymphocytes. "We are going to provide the recombinant Cry j1/2 fusion protein for sublingual immunotherapy (SLIT), which should be much safer and more efficient than pollen-derived crude extract." explains Dr. Yasuyuki Ishii, the company's Founder and Non-Executive Director, who is also the leader of the Laboratory for Vaccine Design at RCAI. The liposome vaccine technology, named "reVax", contains CD1d ligand, α -galactosyl ceramide (α -GalCer), in the lipid bilayer. This vaccine efficiently delivers α -GalCer to antigen-presenting cells (APCs). The APCs subsequently activate NKT cells by presenting α -GalCer through CD1d on their surface. It appears that activated NKT cells develop regulatory T cells, which play an essential role in the systemic immune tolerance. "We think the liposome vaccine will suppress the excessive immune reactions in allergy, autoimmune and graft rejection after organ transplantations. In this context, the efficacies for autoimmune diseases or transplantations are being confirmed in animal models at laboratory of REGiMMUNE Inc. CA, U.S. In Japan, we are focusing on research to develop the liposome vaccine specific for Japanese cedar pollinosis. Manufacture of GMP materials and preclinical studies are now underway." says Dr. Ishii.

So far, the investors in the company include NIF SMBC Ventures Co., Ltd., Japan Asia Investment Co., Ltd., JAFCO Co., Ltd., Orix Capital Co., Ltd., Yasuda Enterprise Development Co., Ltd., Nikko Antfactory, K.K., New Business Investment Co., Ltd., Tokyo Small and Medium Business Investment & Consultation Co., Ltd., Fund Creation Co., Ltd., Mitsubishi UFJ Capital Co., Ltd., and RIKEN Venture Capital Co., Ltd.

The second venture company, Animal Allergy Clinical Laboratories, was established by Dr. Kenichi Masuda, a research scientist in the Laboratory for Vaccine Design. The company will provide laboratory testing services to help veterinarians diagnose allergy and immune diseases in animals. Dr. Masuda has developed a novel method to measure dog IgE in a quantitative manner. Applying this method, the company plans to develop quantitative dog IgE ELISA system for various antigens. Furthermore, since Dr. Masuda has used several activation markers for dog lymphocytes in his studies, which will be also applied to monitor and diagnose allergic diseases in dogs for veterinarians. Those monitoring assays of dog immune system will be also useful to perform pre-clinical study using dogs for pharmaceutical companies and to find novel seeds for development of drugs in terms of a different approach from studies using mice. In addition to the testing services, the company is interested in developing new therapies for pets. There are many dogs suffering from allergy, cancer or autoimmune diseases. Amazing inventions at RCAI such as the liposome vaccine, regulatory dendritic cells, artificial lymph nodes may be useful for the development of new therapies for animals before application to humans.

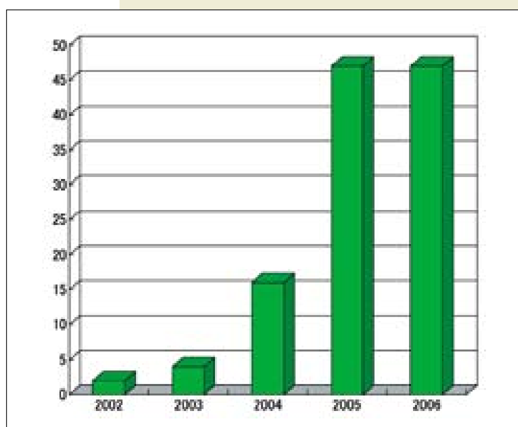
RefDIC is Launched

The RCAI RefDIC Consortium, directed by Dr. Osamu Ohara, has created an open-access database of immune cell mRNA and protein profiles. The Reference genomic Database for Immune Cells, abbreviated as RefDIC, is designed so that users can easily retrieve various features of mRNA/Protein profiles from a wide variety of immune cells through Internet. RefDIC currently includes data from 159 Affymetrix GeneChip microarrays and 23 proteomic analyses in 16 types of immune-related cells, and the numbers will continue to grow. "Users are able to perform queries on molecules, immune cells, or organs and retrieve the expression profile," explains Mr. Atsushi Hijikata of the Immunogenomics Research Group. "For example, users are able to determine which gene is expressed in which types of immune cells. More importantly, they can compare the levels of the mRNA and a corresponding protein in one view by displaying them in parallel."



RefDIC is a collaborative research project involving 15 RCAI laboratories. The aim is to construct an informational platform that enables immunologists to fully exploit "Omics" data. To generate the reference database, the Immunogenomics Research Group performed mRNA profiling experiments and 2D-gel-based proteomic analyses of various kinds of immune cells that were prepared by the collaborative laboratories in the Center. The cell types included representative cell lines and primary cells, either untreated or treated with various immunologically relevant stimuli. All the transcriptomic and proteomic data obtained were compiled in the database by the Immunogenomics Research Group for easy retrieval on demand. "We believe that accumulation of this type of essential basic data will help researchers to frame their hypotheses," says Dr. Osamu Ohara, the leader of Immunogenomics Research Group. "We hope that RefDIC will function as a data sharing platform for the immunology research community."

Support for Patent Application



Graph: Number of patent applications

RCAI regards the development of new technology in immunological studies as one of its missions. In order to support such innovations, RCAI has developed the following unique support system for patent application.

A Japanese patent law firm visits RCAI twice a year to conduct hearings on the candidate inventions for patent applications. This law firm prepares the necessary documents on the inventions that are suitable for application as patents. Scientists submit the reports to the Center for Intellectual Property Strategies of RIKEN.

The number of patent applications increased dramatically after this system was introduced. The number of applicatiions was kept high in FY 2006, suggesting that many research discoveries have potential for becoming patented, whereas most of them would have been neglected had there not been the support by the experts.

International Research Collaboration Award

The RCAI International Research Collaboration Award is a unique program supporting researchers outside of Japan in setting up semi-independent research units within the laboratory of their collaboration partner at the Center. The program provides 15 million JPY/year to each collaborative research projects for up to three years.

The program started in 2004, and 10 projects were carried out in 2006. Among them, three projects have already resulted in successful discoveries. Drs. Michael Dustin and Takashi Saito published their work on T cell receptor microclusters in *Immunity* (2006) and *Nat. Immunol* (2005). Drs. Willem van Ewijk and Hiroshi Kawamoto's work on thymic progenitors cells resulted in a paper in *Development* (2006). Drs. Haruhiko Koseki and Miguel Vidal published their work on mechanisms of Hox genes repression by PcG proteins in *Development* (2006).

In 2006, Drs. Michael Karin, Sunhwa Kim and Masato Tanaka launched their collaborative project, and two new collaborative programs by Drs. Cooper and Ohno and Drs. Brendolan and Watanabe have been selected to start in April, 2007.

This collaboration program has not only achieved scientific discoveries, but also promoted the international exchange of people and international visibility. After three years of collaboration, Dr. Ewijk and Dr. Vidal will join RCAI in 2007 as adjunct Unit Leaders, and Dr. Peter Burrows will become Science Advisor to the Director of RCAI.

Table 2: Awardees of RCAI International Collaboration Award Program

Year	Awardee	Title of Research	Host Lab.
2004-2006	**Michael Dustin** New York University School of Medicine	Analysis of dynamism and function of Iimmunological synapse using planar membrane and knock-in T cells	**Takashi Saito** Lab for Cell Signaling
2004-2006	**Willem van Ewijk** Leiden University Medical Center	Regulatory role of lymphoid pro-genitors during development of thymic microenvironments	**Hiroshi Kawamoto** Lab for Lymphocyte Development
2004-2006	**Miguel Vidal** Centro de Investigaciones Biologicas, CSIC	Genomic and functional analysis of the role of the Polycomb Ring1 genes in B-cell development	**Haruhiko Koseki** Lab for Developmental Genetics
2004-2005	**Steven F. Ziegler** Benaroya Research Institute at Virginia Mason Medical Center	Role of NKT cells in TSLP-Mediated Aallergic Iinflammation	**Masaru Taniguchi** Lab for Immune Regulation
2004-2006	**Peter D. Burrows** University of Alabama at Birmingham	Expression and function of FcRY-a novel Fc receptor-related gene expressed in B cells	**Jiyang O-Wang** Lab for Antigen Receptor Diversity
2005-	**Wilfried Ellemeier** Institute of Immunology, Medical University Vienna **Hilde Cheroute** La Jolla Institute for Allergy and Immunology	Study of T cell differentiation mediated by regulated expression of CD8 genes	**Ichiro Taniuchi** Lab for Transcriptional Regulation
2005-	**Mark Bix** University of Washington, Seattle, Washington	Understanding genetic regulation of interleukin 4 production by a CD4(+) T cell-intrinsic mechanism.	**Masato Kubo** Lab for Signal Network
2005-2006	**Yun-Cai Liu** La Jolla Institute for Allergy and Immunology	Gene-array analysis and proteomics of Th2 tolerance	**Yasuyuki Ishii** Lab for Vaccine Design
2005-	**Kenneth M. Murphy** Howard Hughes Medical Institute Washington University School of Medicine	Visualization of STAT protein in the cytokine mediated signaling at a single molecular level.	**Osami Kanagawa** Lab for Autoimmune Regulation
2005-	**Facundo Damian Batista** Cancer Research UK London	Role of signaling molecules in B cell synapse formation and its maintenance	**Tomohiro Kurosaki** Lab for Lymphocyte Differentiation
2006-	**Sunhwa Kim and Michael Karin** Department of Pharmacology, Univ. of California, San Diego	Identification of Novel Necrotic Molecules from Necrotic Hepatocytes and Examination of Its Effect on the Inflammatory Response	**Masato Tanaka** Lab for Innate Cellular Immunity
2007-	**Max. D. Cooper** University of Alabama at Birmingham	Identification and characterization of a mammalian homolog(s) for chCATH-B	**Hiroshi Ohno** Lab for Epithelial immunobiology
2007-	**Andrea Brendolan** Cornell University Medical Center, Department of Cell and Developmental Biology	A study on the spleen and lymph nodes mesenchymal cells that participate in the assembly of artificial secondary lymphoid organs	**Takeshi Watanabe** Research Unit for Immune Surveilance

Short Term Lectureship Program

The Center's Short-Term Lectureship Program invites internationally recognized investigators to give lectures and stay for one week to discuss ongoing research with young investigators. In 2006, the program invited seven distinguished researchers from four countries to have seminars and discussions.

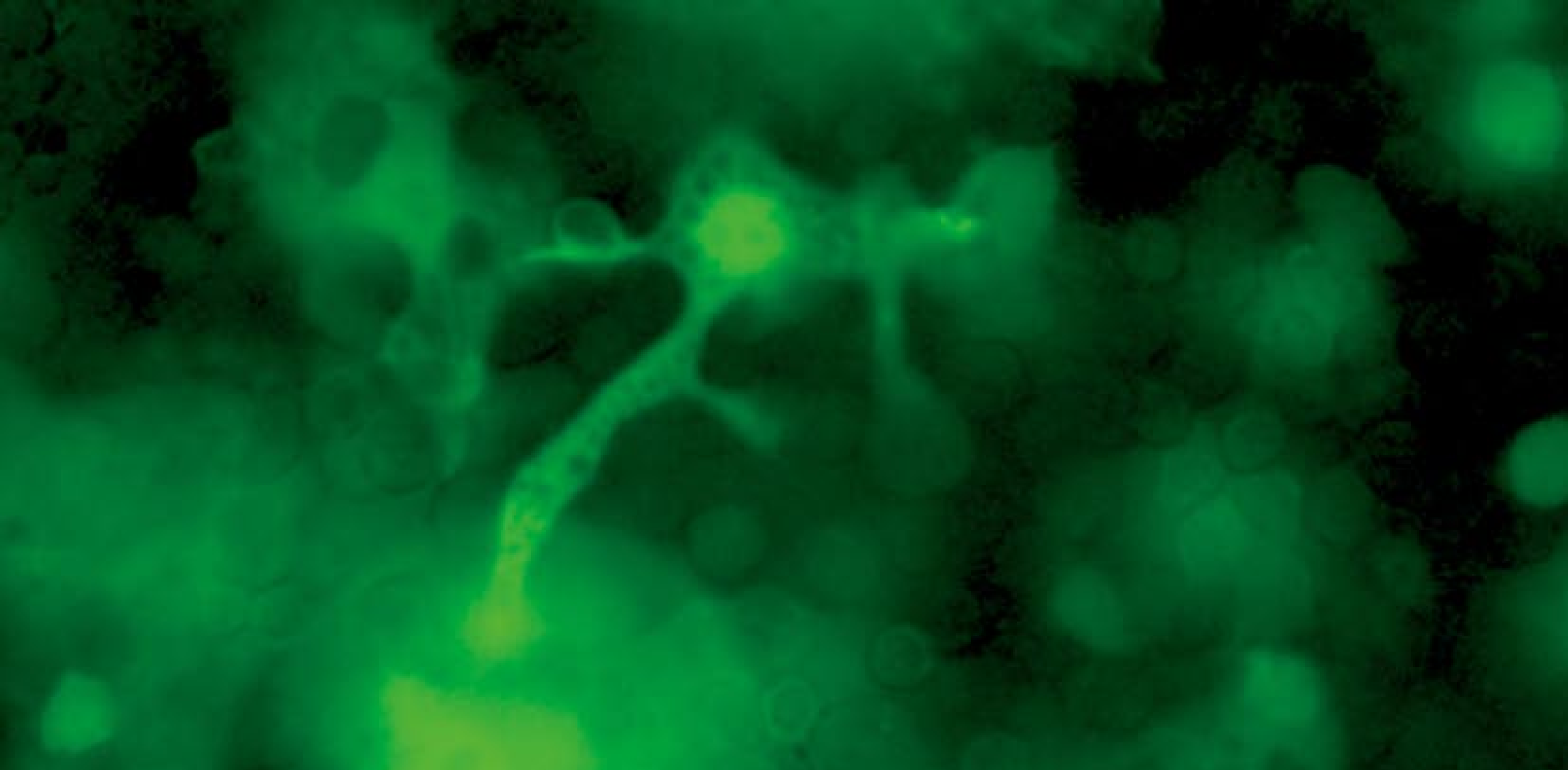
Table 3: Short-term Lectureship Program 2006

Month	Lecturer	
July	Ronald N. Germain	Deputy Chief of Lab Immunol & Section Chief, NIH/NIAID/Lab Immunology, USA
Oct.	Zi Hai Li	Associate Professor, University of Connecticut, Center for Immunotherapy, USA
Oct.	Andreas Radbruch	Director in General & Group leader, Deutsches Rheuma-Forschungszentrum Berlin/Cell Biology, Germany
Oct.	Markus G. Manz	Group leader, Institute for Research in Biomedicine (IRB)/ Haematopoietic Development, Switzerland
Nov.	Stefano Casola	Group leader, IFOM-The FIRC Institute for Molecular Oncology, Italy
Nov.	Hansjorg Schild	Professor, Institute for Immunology University of Mainz, Germany
Nov.	Chen Dong	Associate Professor, U. Texas, MD Anderson Cancer Center/ Immunology, USA

Collaboration with Foreign Institutes

In addition to the above programs, RCAI has signed a collaboration agreement with the Max Planck Institute of Immunology and the Max Planck Institute for Infection Biology in Germany, and the La Jolla Institute of Allergy and Immunology in San Diego, US. Under these agreements, the Center will carry out joint seminars and exchange young scientists in collaborative programs.

The first RIKEN-Max Planck joint workshop is planned for April, 2007 in Berlin. This will be the inaugural workshop for RCAI, Max Planck Institute of Immunology and Max Planck Institute for Infection Biology. In May 2007, a RIKEN- Singapore A*STAR (Agency for Science, Technology and Research) joint symposium is being planned. A*STAR groups such as the Singapore Immunology Network (SIgN), the Institute of Molecular and Cell Biology (IMCB), the Institute of Medical Biology (IMB), and the Genome Institute of Singapore (GIS) will join groups from RIKEN RCAI and the Center for Developmental Biology (CDB) in participating in the program.



Events
2006

RCAI Advisory Council Meeting 2006

The Second Core Meeting of the RCAI Advisory Council took place April 17 -18, 2006 at the RCAI research facility on the RIKEN Yokohama Institute. The RCAI review and evaluation policy calls for "core" meetings of the Center's scientific advisory board, the RIKEN RCAI Advisory Council, every 2-3 years. The agenda of Core Advisory Council meetings is to review the findings of previous Annual Scientific Review Meetings that evaluate research activities every year, and to take a broader look at the Center's research activities, research strategy, future plans, and operating policies. The meeting was attended by Drs. Max D. Cooper (U Alabama at Birmingham, USA, Chairman), Antonio Coutinho (Instituto Gulbenkian de Ciencia, Portugal), Tasuku Honjo (Kyoto U, Japan), Paul W. Kincade (OMRF, USA), Bernard Malissen (Centre d' Immunologie Marseille-Luminy, France), Nobuyuki Miyasaka (Tokyo Medical and Dental U, Japan), William E. Paul (NIH, USA), Klaus Rajewsky (Harvard Medical School, USA), and Ralph M. Steinman (Rockefeller U, USA).

Following greetings and introductions by Drs. Taniguchi (RCAI Director), Ogawa (RIKEN Yokohama Institute Director), and Cooper (AC chairman) (photo 1), there was a selection of "highlight" presentations of the most recent research at RCAI (photo 2-7). This initial session was followed by an in-depth discussion of the findings of annual individual scientific reviews held throughout the year from June 2005 to April 2006. The findings of these review meetings were evaluated together with the response from each group, team, and unit leader. In a final session, Dr. Taniguchi summarized the present status of the institute and presented the institute's plans for the next five years. Following extensive internal deliberations, Dr. Taniguchi answered questions from the Advisory Council in a final round of discussion. A summary of the findings of the meeting was presented by Dr. Cooper, the Advisory Council's chairman, to Mr. Omori, Deputy Director of the RIKEN Yokohama Institute.

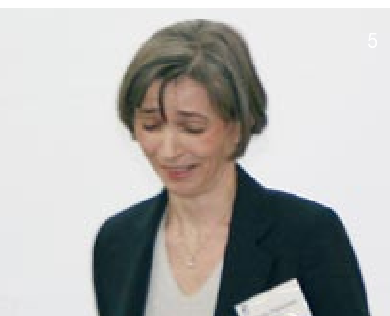
In general, the Advisory Council was extremely pleased with the recent development of the RIKEN Research Center for Allergy and Immunology. They noted that, considering the fact that RCAI moved into the new facilities at the RIKEN Yokohama Institute barely two years ago, the achievements at the center are impressive and research undertaken at the center is outstanding. RCAI's central facilities provide rapid services of high quality that empower smaller research groups by accelerating their internationally competitive research projects. The RCAI International Collaboration Award Program has enhanced international awareness of the Center.

We are grateful to the members of Advisory Council for their intensive discussions, recommendations and encouragement, and RCAI will continue to develop with their endorsement.

RCAI AC2006 REPORT EXECUTIVE SUMMARY

The Advisory Council is highly impressed with the rapid development and high quality of the research accomplishments of RCAI since its occupancy of the new RIKEN Yokohama Institute facilities. The senior investigator-led groups have taken full advantage of the excellent facilities that this new center offers. Most importantly, the younger investigator-led groups are also thriving in this favorable environment. RCAI's central facilities provide rapid services of high quality to RCAI scientists. Strategic investments in leading-edge technologies and approaches, such as single-molecule imaging and high through-put genomics, are paying off handsomely and will continue to do so. Access to these outstanding core facilities empower smaller research groups by speeding up their internationally competitive research projects. The strengths of the basic research enterprise places RCAI in a strong position for future investment in translational research and to serve as a source infrastructure for collaboration with clinical research centers. The RCAI International Collaboration Award Program has enhanced international awareness of the Center and, notably, collaborative results funded by this innovative scheme are being published in high profile international journals. We are supportive in general of the Center's future plans, an excellent example of which is the dedicated development of a mouse model with a functional human immune system. The difficulties of this endeavor notwithstanding, we believe the investment in this model promises to significantly enhance RCAI contributions to immunological research related to human diseases. In addition to an appreciation of the judicious administrative style of the RCAI Director, Dr. Masaru Taniguchi, we are impressed by the rapid and appropriate RCAI response to Advisory Council scientific evaluations and advice. Finally, we congratulate the Japanese government and RIKEN for its vision and support of this new scientific center of excellence.

AC Members
Max D. Cooper, Chair
Alain Fischer
Antonio Coutinho
Arthur Weiss
Bernard Malissen
Diane Mathis
Hiromitsu Nakauchi
Kazuo Sugamura
Kiyoshi Takatsu
Klaus Rajewsky
Masayuki Miyasaka
Nobuyuki Miyasaka
Paul W. Kincade
Ralph M. Steinman
Ruslan Medzhitov
Takehiko Sasazuki
Tasuku Honjo
William E. Paul



First International Summer Program

This new program aims to foster international interactions among young scientists and encourage future immunologists around the world. The program included lectures by eminent immunologists, poster presentations by the participants, discussions and opportunities for internship experience. Thirty-eight students from 21 countries were selected to join the five-day intensive lecture course, and 10 students participated in an additional internship course at the Center laboratories.

On the first day of the program, students were divided into four groups depending on the theme of their current projects. Every morning before lecture, each participant of one group gave a poster presentation in which the Center's principle investigators also took part. Dr. Bilic of Croatia, commented that the presentations were especially interesting because most of them included data from on-going projects that are close to publication. After the poster discussions, participants attended two-hour lectures by various prominent researchers. The seven PIs of the Center and the nine additional researchers who were invited to give talks covered a wide variety of immunological topics. Many graduate students studying at the Center also attended the lectures. Mr. van Hamburg of the Netherlands remarked that not only did the lectures cover all the major fields of immunology, but they also gave students an opportunity to listen to and talk directly with the major researchers of those fields. The attendants had opportunities to talk not only with the guest lecturers but also with the PIs of the Center. Dr. Kashyap of India explained that one of the greatest things about the program was that he was able to speak with Dr. Osamu Ohara, the head of the Immunogenomics Research Group.

On the last day of the program, the Excellent Poster Awards were announced. Five people, Dr. Haxhinasto of Albania, Dr. Cemerski of Yugoslavia, Dr. Pathak of India, Ms. Sather of the US and Dr. Bilic of Croatia received the award.

During the internship course, each member joined to a laboratory in the Center where they learned new immunological techniques. The members commented that the skills they had learned during the course would be helpful in their future research, and said that the program was a unique experience, both professionally and personally.

In an effort to support participation from as many countries as possible, all costs, including accommodation and travel expenses, were covered by RCAI. Dr. Taniguchi, Director of RCAI, made the decision to start the program: "It is very important to invite talented young scientists to Japan and provide opportunities to

discuss science with people from all over the world. Through the experience, they absorb a lot, and the program will foster future leaders in many countries." To realize this goal, the organizing committee, led by Dr. Kurosaki, worked extremely hard in selecting participants, inviting lecturers, deciding the program, and providing instructions to the participants, among many other tasks. The contributions of the administrative staff, Ms. Tanabe, Ms. Adachi, Ms. Sai and other RCAI assistant staff, were also crucial to the success of the program. "Although it was the first summer program of the Center and everything was a new experience, time for preparation was limited," said Ms. Tanabe of the rush to put everything in place before students arrived.

Owing to their tireless efforts, the first summer program finished a great success. According to the questionnaire, 100% of the attendants answered that they would recommend this program to their colleagues. "It was my first time to attend a summer program, but the experience totally widened my view." Ms. Schlenner of Germany said. In turn, the attendants' cooperative and grateful attitude also moved RCAI staff members. "The students' sincere attitudes toward people were impressive," said Ms. Tanabe. Thus, in expected and sometimes unexpected ways, RCAI's first summer program brought together scientists from around the world and opened new horizons in the minds of many young students.

Lecturer	**Title**
Prof.Paul W. Kincade, Oklahoma Medical Research Foundation	Initial Generation and Lifelong Replenishment of the Immune System
Prof. Ralph M. Steinman, Rockefeller University	Dendritic cells in immunobiology and medicine
Prof. Willem van Ewijk, Leiden University	The Thymus, classrooms, students and teachers
Prof. Arthur Weiss, University of California-San Francisco	T Cell Antigen Receptor Signal Transduction
Prof. Kenneth M. Murphy, Washington University	Development of T cell effector and regulatory subsets in the adaptive immune response
Dr. Yun-Cai Liu, University of California, San Diego	Protein Ubiquitination in the Immune Regulation
Dr. Tomohiro Kurosaki, RIKEN, RCAI	Regulation of B cell fates by BCR signaling components
Dr. Takashi Saito, RIKEN, RCAI	Initiation of Immune Responses
Prof. Ruslan Medzhitov, Yale University	Innate Immunity
Dr. Haruhiko Koseki, RIKEN, RCAI	Molecular mechanisms underlying epigenetic regulations mediated by mammalian Polycomb group complexes
Prof.Masayuki Miyasaka, Osaka University	How do lymphocytes and dendritic cells migrate to certain tissues? –Dogmas and Enigmas
Dr. Masaru Taniguchi, RIKEN, RCAI	Immune regulation mediated by NKT cells
Dr. Osami Kanagawa, RIKEN, RCAI	Autoimmune Regulation
Dr. Shohei Hori, RIKEN, RCAI	Regulatory T Cell
Dr. Takeshi Watanabe, RIKEN, RCAI	Generation of Artificial Lymph Nodes With Immunological Function
Prof. Shigeo Koyasu, Keio University	Role of dendritic cells in infectious immunity

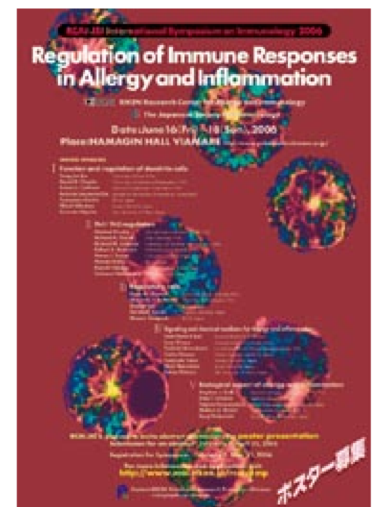
Participants (country)			
Stanley A. I. Adoro (Nigeria)	Manoj Kumar Kashyap (India)	Mahmoud A. Pouladi (Canada)	Yufeng Shi (China)
Pornpimon Angkasekwinai (Thailand)	Jieun Kim (South Korea)	Sandro Prato (Italy)	Olivia A. Simma (Austria)
Ivan Bilic (Croatia)	Juyang Kim (South Korea)	Alison Clare Pridans (Australia)	Jan Pieter van Hamburg (Netherlands)
Saso D. Cemerski (Yugoslavia)	Ira Kim (South Korea)	Linda Reiling (Germany)	Joris Vanderlocht (Belgium)
Weiqian Chen (P.R.China)	Amita Suresh Limaye (India)	Hai-Bin Ruan (China)	Demetrios Vassilakos (Greek)
Silvie Cloosen (Belgium)	Hua Liu (P.R.China)	Shinya Sakaguchi (Japan)	Yoshika Yamakawa (Japan)
Kylie T. Greig (Australia)	Adele M. Mount (Australia)	Blythe Duke Sather (US)	Noemí K. Yokobori (Argentina)
Sokol A. Haxhinasto (Albania)	Konstantin Neumann (Germany)	Susan Mariola Schlenner (Germany)	Ren-In You (Taiwan)
Caroline Houde (Canada)	Vanessa A. G. de Oliveira (Portugese)	Wooseok Seo (Canada)	Jun Zhong (P.R. China)
Jin Hyun Kang (Korea)	Sushil Kr Pathak (India)		

2006 RCAI-JSI International Symposium on Immunology

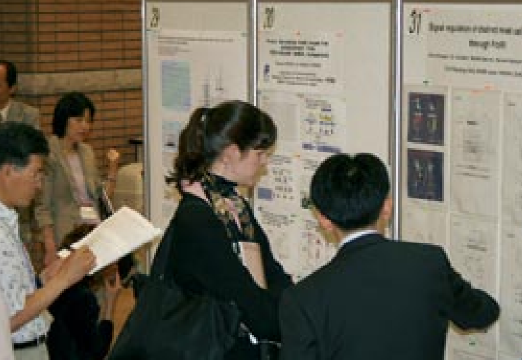
Regulation of Immune Responses in Allergy and Inflammation

An international symposium on immunology hosted by RIKEN Research Center for Allergy and Immunology (RCAI) in conjunction with the Japanese Society for Immunology (JSI) was held June 16-18 in Yokohama. This international symposium was started last year with the goal of providing young Japanese scientists and students with the opportunity to communicate with leading researchers and to learn about the most advanced topics in immunology. This year's conference, organized by Dr. Masaru Taniguchi, the director of RCAI, and Dr. Toshio Hirano, the JSI president and deputy director of RCAI, was a great success with more than 500 people in attendance. Thirty invited specialists, including 17 from the US and Europe, gave outstanding presentations. In addition, a poster session was introduced this year so that young scientists and students could have a chance to make their own presentations and discuss their data with world renowned researchers.

The organizing committee selected the theme of "Regulation of Immune Responses in Allergy and Inflammation" for its second symposium, which reflects the recent direction of immunologic research. Having dissected the individual constituents of the immune system, contemporary research aims to understand how the overall immune system is regulated. In keeping with this theme, important immunoregulatory components such as dendritic cells, regulatory cells, Th1/Th2 balance, signaling molecules and chemical mediators in allergy and inflammation, biological aspect of allergy and inflammation were the focus of the symposium. Speakers, including the prominent biochemists, Dr. Shuh Narumiya and Dr. Takao Shimizu, gave thought-provoking talks that included recent unpublished results, and many insightful questions were raised from the floor. Fifty posters were presented, and the participants had informal discussions and a very useful exchange of information during the poster session.



Nearly all of the seats in the Yokohama Hamagin Hall were occupied throughout the sessions, and the participants were able to bring back the most advanced beneficial information to their home laboratories. Research on regulation of immune responses has become a growing interdisciplinary field, and we expect more advances will be forthcoming at the third symposium next year.



Session I: Function and regulation of dendritic cells	**Chair: Y-J. Liu & T. Kaisho**
Robert L. Coffman, Dynavax Technologies, USA	Therapy of allergic diseases with Toll-like receptor 9 agonists
Tsuneyasu Kaisho, RCAI, Japan	Critical roles of IκB kinase-α in TLR7/9-induced type I IFN production
Kensuke Miyake, The University of Tokyo, Japan	Roles for accessory molecules in lipopolysaccharide recognition by toll-like receptor 4
David D. Chaplin, The University of Alabama at Birmingham, USA	Innate mechanisms that signal for CD4$^+$ T cell recruitment to the lungs and airways
Antonio Lanzavecchia, Institute for Research in Biomedicine, Switzerland	Signal integration in dendritic cells
Hitoshi Kikutani, Osaka University, Japan	The transmembrane semaphorin Sema6D and its receptor Plexin-A1 in dendritic cell functions
Yong-Jun Liu, University of Texas, USA	The function of TSLP-activated mDCs in positive selection of Treg and pDC-specific receptor ILT7 in regulating innate IFN-responses
Session II: Regulatory cells	**Chair: E.M. Shevach & S. Hori**
Ethan M. Shevach, National Institutes of Health, USA	Mechanisms of action of naturally occurring T regulatory cells
Shohei Hori, RCAI, Japan	How Foxp3 controls dominant tolerance: lessons from IPEX mutations
Haruhiko Suzuki, Nagoya University, Japan	The role of CD8Tregs in maintaining immune homeostasis and their application to immune-based disorders
Alexander Y. Rudensky, University of Washington, USA	Role of transcription factor Foxp3 in dominant tolerance
Masaru Taniguchi, RCAI, Japan	NKT cell-triggered IL-21 production induced selective Bε cell apoptosis and IgE-specific suppression
Session III: Th1/Th2 regulation	**Chair: R.M. Locksley & K. Takatsu**
Richard M. Locksley, University of California, San Francisco, USA	Initiating allergic immunity in vivo
Richard A. Flavell, Yale University, USA	Dynamic modulation of interchromosomal interactions during T cell differentiation
Toshinori Nakayama, Chiba University, Japan	Crucial role of MLL for the maintenance of memory Th2 cell identity
Michael J. Grusby, Harvard School of Public Health, USA	Regulation of STAT signaling by ubiquitination and dephosphorylation
Robert A. Kastelein, Schering-Plough Biopharma, USA	IL-23 and IL-27, cytokines with divergent functions
Masato Kubo, RCAI, Japan	Role of Notch signal and il4 3' enhancer in Th2 differentiation
Steven Reiner, University of Pennsylvania, USA	Making daughter T cells different during immunity and inflammation
Kiyoshi Takatsu, The University of Tokyo, Japan	Role of T-cell receptor signal and T-bet in the induction of Th1 differentiation and cross-priming of antigen
Session IV: Signaling and chemical mediator for allergy and inflammation	**Chair: J. Rivera & T. Kawakami**
Juan Rivera, National Institutes of Health, USA	Integration of signaling in mast cells and the allergic response
Toshiaki Kawakami, La Jolla Institute for Allergy and Immunology, USA	IgE-centric immunity
Toshio Hirano, RCAI and Osaka University, Japan	FcεRI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane: Zinc-dependent pathway
Shuh Narumiya, Kyoto University, Japan	Regulation of immune responses and allergy by prostanoids
Takao Shimizu, The University of Tokyo, Japan	Lipid mediators, fundamental regulators of immune responses inflammatory reactions
Session V: Biological aspect of allergy and inflammation	**Chair: S.J. Galli & H. Karasuyama**
Jean-Pierre Kinet, Harvard Medical School, USA	Calcium influx pathways in cells of the immune system: Identification of a CRAC channel component
Stephen J. Galli, Stanford University, USA	Mast cells: Versatile effector and potential immunoregulatory cells in health and disease
Dale T. Umetsu, Stanford University, USA	The Role of NKT Cells in the development of asthma
Hajime Karasuyama, Tokyo Medical and Dental University, Japan	Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation, independently of T cells and mast cells
Melissa A. Brown, Northwestern University, USA	Genetically-determined heterogeneity in mast cell IL-4 expression: variable influence on the character of T helper cell responses?
Kenji Nakanishi, Hyogo College of Medicine, Japan	The role of IL-18 in intrinsic allergic diseases
Toshiyuki Takai, Tohoku University, Japan	Role of paired immunoglobulin-like receptor (PIR)-B in allergic responses

First JSI-RCAI Workshop

Transcriptional Factors and T Lymphocytes Development

The Japanese Society for Immunology (JSI) and RIKEN RCAI will co-support a new program, JSI-RCAI workshop, with the aim of providing opportunities to young researchers for presenting their own works, exchanging information and establishing active collaborations.

In collaboration with RCAI, JSI organizes several meetings and courses, including the JSI-RCAI International Symposium and JSI's annual meeting. However, unfortunately, there is a little opportunity for researchers in their thirties and forties, who actually perform experiments and contributes to the scientific achievements, to organize meetings and to present their works. Thus, this new program should give the young researchers a valuable opportunity to make their own presentations and stimulate communication.

The first JSI-RCAI workshop was held on March 7, 2007 at RCAI, with 125 people attending. The 2007 JSI-RCAI workshop organized by Dr. Ichiro Taniuchi, RIKEN RCAI, was titled "Transcriptional Factors and T Lymphocytes Development", and was consisted of two sessions: "Transcriptional regulation of thymocyte differentiation" and "Transcriptional regulation of effecter T cell differentiation". T lymphocytes are composed of several cell subsets that possess distinct functions. Over the last decade there has been remarkable progresses in the identification of transcriptional factors that play an important role in differentiation of specific T lymphocytes subsets. Characterization of these factors provides a new insight into how different lineage cells are generated from progenitor cells. However, since it is becoming evident that the developmental program is controlled by a network of transcriptional factors, a major purpose of the workshop was to get perspective for further understanding of the transcriptional factor network during T lymphocyte development from the works focusing on individual factors. At the workshop, current unpublished works on the function of each transcriptional factor were presented.

Session I Transcriptional regulation of thymocyte differentiation	
Ellen Rothenberg, Caltech, USA	"Notch-dependent channeling of transcription factor activities in early T-cell development"
Tomokatu Ikawa, RCAI	"Roles of E2A in lymphoid lineage commitment"
Wilfried Ellmeier, Med. Univ. Vienna, Austria	"Epigenetic and transcriptional control of CD8 gene expression during thymocyte development"
Ichiro Taniuchi, RCAI	"Role of Runx complexes in CD4/CD8 lineage choice"
Jonathan Kaye Scrips, USA	"Development of all CD4 T Cell lineages requires nuclear factor TOX"
Session II Transcriptional regulation of effecter T cell differentiation	
Koji Yasutomo, Tokushima Univ.	"Notch-mediated regulations of T cell cytotoxicity"
Akemi Sakamoto, Chiba Univ.	"Role for Bcl6 in memory T cell differentiation"
Masakatu Yamashita, Chiba Univ.	"MLL-dependent maintenance of memory Th2 cell identity"
Shohei Hori, RCAI	"How Foxp3 controls dominant tolerance: lessons learned from naturally occurring foxp3 gene mutations"
Liang Zhou, NYU, USA	"The role of RORgt in Th17 dell development"
Masahiro Ono, Kyoto Univ.	"A mechanism of IL-2 repression by Foxp3 and a Foxp3-interacting protein."

Guidance Session for Adjunct Graduate School Program

May 26, 2006

Currently, 13 RCAI investigators hold adjunct professorship appointments at four universities (Chiba, Osaka, Tokyo Medical and Dental, and Yokohama City). However, because RIKEN is a research institute and not an educational institution, it is not well known that students are able to conduct their research at RCAI during their tenure in graduate school.

Thus, in order to introduce the program to the students who might be interested in studying at RCAI, the first orientation session was held on May 26, 2006. Twelve students from all over Japan attended the session held at RCAI research facilities in Tsurumi, Yokohama. At the start of the session, Deputy Director Dr. Takashi Saito explained the adjunct professorship program, and then current research highlights were presented by Dr. Kaisho (Lab. for Host Defense), Dr. Ishii (Lab. for Vaccine Design), Dr. Ishikawa (Research Unit for Human Disease Models) and Dr. Yoshida (Lab. for Immunogenetics). After these talks, the attendees moved to the poster session, where each laboratory gave a short introduction to their research, and the students had the opportunity to talk directly with researchers.

It seems that the participants were favorably impressed with both the orientation session and with RCAI. The students' comments indicated that the session had been one of their few chances to talk directly with investigators, and that they were impressed by the cutting edge research taking place at the center. There were requests from the participants to continue the session every year and to make information about the session more available to the public.

Table: RCAI investigators who hold adjunct professorships

University	Investigators
Faculty of Medicine, Graduate School of Medicine, **Osaka University**	**Osami Kanagawa** (visiting professor) **Tsuneyasu Kaisho** (visiting professor) **Toshiyuki Fukada** (visiting associate professor)
School of Medicine, **Chiba University**	**Takashi Saito** (visiting professor) **Haruhiko Koseki** (visiting professor) **Hiroshi Ohno** (visiting professor) **Shin-ichiro Fujii** (visiting associate professor) **Yasuyuki Ishii** (visiting associate professor) **Fumihiko Ishikawa** (visiting associate professor)
School of Biomedical Science, **Tokyo Medical and Dental University**	**Takashi Saito** (visiting professor) **Tomohiro Kurosaki** (visiting professor) **Sidonia Fagarasan** (visiting associate professor)
International Gaduate School of Arts and Sciences, **Yokohama City University**	**Hiroshi Ohno** (visiting professor)

RIKEN Yokohama Institute Open Campus

June 24, 2006

The RIKEN Yokohama Institute Open Campus took place on June 24, 2006. The good weather brought an estimated 1,640 visitors, including 300 children.

RCAI's Deputy Director, Dr. Takashi Saito gave a special lecture on "The Commandos of Immune System" in which the basics of immunology was plainly explained by analogy to the movie "Fantastic Voyage". The lecture room was nearly full, and after the lecture, audience members were lined up to ask questions. Although many of the questions were not directly related to his research field, and some were naive, Dr. Saito answered them with sincerity and clarity.

At RCAI, each laboratory presented a poster and explained their research to the visitors. The visitors' eager attitude to learn more about science moved the researchers to stand by their posters the entire day in rotation to answer questions. A highlight of the exhibition was the opportunity for hands-on learning. The Laboratory for Immunogenetics prepared stained samples of fetal mice at various developmental stages. The visitors, from elementary school students to retirees, were captivated by the chance to observe the specimens through stereomicroscopes. The Laboratory for Lymphocyte Development taught the concept and the technique of cell sorting to high school students and above. After the lecture by Dr. Kawamoto, the attendees were divided into groups and were able to directly experience cell-sorting experiments in a laboratory (photo).

This annual event to open the Center to the public enhances the awareness and understanding of the Center's immunological research to people of all ages and in different lines of work.

RCAI Retreat

August 28-29, 2006

The aim of the annual RCAI retreat is to improve the activities of the Center by gathering all the members in one place. This year, 173 people, including researchers, technical staff and assistant staff were invited to attend the August 28-29 retreat at the Nihon Aerobics Center in Chiba Prefecture.

The highlight of the retreat was the presentation of posters by researchers of the Center. Ninety-five posters were presented in the two poster sessions that lasted for three hours each. During the sessions, both new unpublished data and on-going projects were introduced to the attendants. As people participated in critical and thought-provoking discussions about the posters, the principle investigators of each laboratory reviewed them and voted for excellent posters of the year (See Research Highlights).During the banquet held

on the first night of the retreat, ten awardees were announced. Winning the award was not only a happy surprise but also a great chance for each winner to introduce his/her project in the 10-minute English presentations which they gave the next day.

During the retreat, assistant staff members had a meeting in which they discussed topics such as a new computer-based regulating system for experimental reagents, the Center events to be held in 2006-2007 and the supporting work that will be required for those events.

Thanks to the organizers, Dr. Ishido and Dr. Udono, and their assistants, Ms. Nakamura and Ms. Imaizumi, 2006 RCAI retreat was a great success. As hoped, participating in active discussions stimulated the interactions between many different people in the Center. The next RCAI retreat is scheduled for September 2007.

Super Science High School

Jan 13, 2007

“Super Science High Schools” are the schools designated by the Ministry of Education, Culture, Sports, Science and Technology to focus specifically on scientific education. There are currently 99 Super Science High Schools in Japan. Because more young Japanese are moving away from the sciences, the ministry aims to strengthen and improve scientific education especially at these schools. RCAI held a special workshop for students from five of these Super Science High Schools in Kanagawa prefecture; Hakuyoh, Seisho, Yokosuka, Zama and Kanagawa Sogo Sangyo High Schools. After lectures on “Aids Virus and Immunity” and “Immunological Organs” by Drs. Tomohiro Kurosaki and Hisahiro Yoshida, the students were divided into groups for hands-on practice of the dissection and histological staining of immunological organs. “I am impressed by the dissection practice,” a student said, “it was scary at first, but what I saw was different from I imagined from the text book”. According to the questionnaire, 12 out of 23 students participated this program because they hope to study biological science in the future. “Students gain valuable experience from this program”, said one of the high school teachers. “Students who had attended this program the year before last have graduated high school and now they are studying medicine, dentistry, veterinary medicine or agriculture at universities.”

Research Coordination Office (RCO) Established

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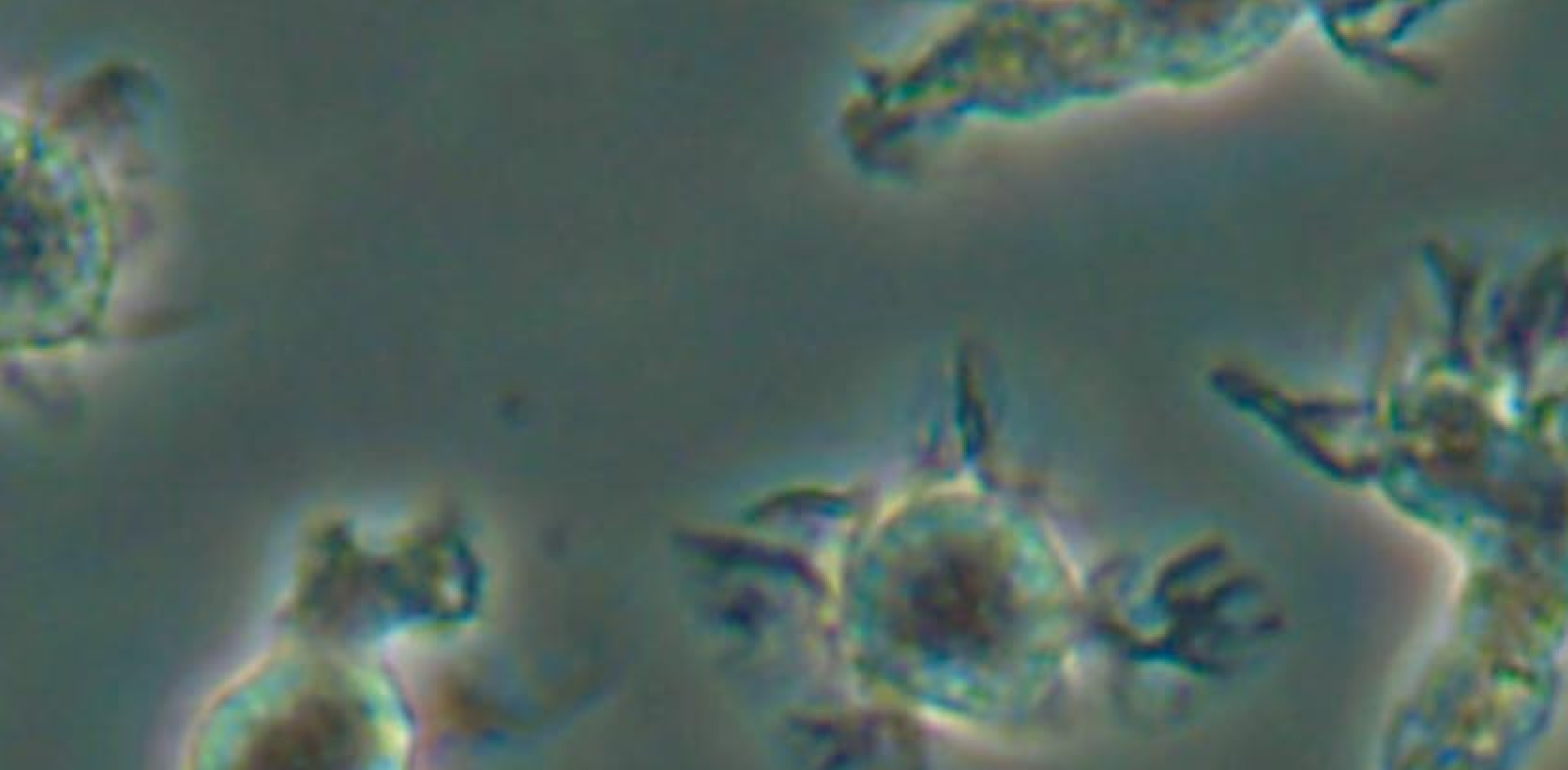
RCAI has established a new Research Coordination Office (RCO) in 2006. The primary role of RCO is to provide support for various activities including grant acquisition, future plans for the Center, collaborative projects with other universities and institutes, projects evaluation, planning events, education activities, public relations and support for postdocs and young scientists. The four members of RCO, Dr. Toshitada Takemori (01), Ms. Hiroko Tanabe (02), Ms. Yuuko Sai (03) and Dr. Haruka Iwano (04) share these tasks. Dr. Takemori joined RCAI in April 2006. He has two roles at the Center, as a research coordinator and as a director of the Lab for Immunological Memory. With his strong background in immunological research, he supports top priority matters such as future plans, grant acquisition, collaborative projects and support for young scientists. Ms. Hiroko Tanabe also has two roles: she is responsible for important events such as the international summer program and symposium and also provides direct secretarial support for the Director. Ms. Sai is responsible for management of the Center's clerical works and accounting, and Dr. Iwano is responsible for evaluation of projects, press releases and public relations.

During 2006, these members cooperated with each other to conduct numerous tasks at RCO and coordinate the various RCAI events and activities described in this Annual Report.

Furthermore, in less than a year after its creation, the RCO has had an impressive output in grant applications. RCO supported 19 applications for the highly competitive RIKEN President's Fund in 2006. Among the 226 applications from all the RIKEN centers, 4 from RCAI were successfully funded (over JPY 220 Million (US$ 2 Million) in total). Since the average funding rate for these applications was 12.0%, the RCAI funding rate (21.1%) was significantly higher.

The number of people working at RCAI has exceeded 350. As RCAI continues to grow, RCO's mission to coordinate various internal activities and outreach will become increasingly important in unifying the Center and developing its cooperative relations with external institutions.



Data and Statistics
2006

Publications FY2006

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Publication Table (Apr. 2006-Mar. 2007)

Journal Title	IF* (2005)	FY2006
Nature Reviews Immunology	30.5	1
Nature	29.3	2
Nature Immunology	27.0	2
Immunity	15.2	2
Journal of Clinical Investigation	15.1	2
Journal of Experimental Medicine	14.0	5
PNAS	10.2	3
Trends in Immunology	10.2	3
Blood	10.1	4
EMBO Journal	10.1	6
Current Opinion in Immunology	9.1	1
Cancer Research	7.6	1
Development	7.6	4
Molecular and Cellular Biology	7.1	3
Oncogene	6.9	1
Journal of Immunology	6.4	20
Proteomics	6.1	1
Journal of Biological Chemistry	5.9	4
International Immunology	3.3	2
Other Journals		59
Total		**126**

*IF: Thompson ISI Impact Factor

Invited Presentations

Meeting	Title	Date	Presenter
Max-Plank Institute for Infection Biology, Berlin	Signals through Toll-like receptors control innate memory B cells by modulation of integrins and CD9 expression.	Apr-06	**Fagarasan, S.**
2006 CSHL Meeting, "Gene expression & Signaling in the Immune System". CSHL, NY, USA	Roles of Runx proteins during T cell development.	Apr-06	**Taniuchi, I.**
16th Congress of Interasma Japan / North Asia. Tokyo, Japan	Allergic inflammation and childhood asthma.	Apr-06	**Saito, H.**
109 the Annual Meeting of the Japanese Society of Pediatrics. Kanazawa, Japan	Effects of genetic and environmental factors on allergic sensitization.	Apr-06	**Saito, H.**
Dr. Fritz Melchers' Memorial Symposium. Max-Planck Institute for Infection Biology, Berlin	Generation of artificial lymph nodes and their immunological function.	May-06	**Watanabe, T.**
26th Symposium of the Collegium Internationale Allergologicum. Malta, Italy	NKT cell triggered IL-21 production induced selective B cell ap-otosis and suppression of IgE responses in mice and humans	May-06	**Harada, M., Seino, K., and Taniguchi, M.**
26th Symposium of Collegium Internatio-nale Allergologicum. St. Julians, Malta	Gene expression profiling of human mast cell lines.	May-06	**Saito, H, Oboki, K, Okayama, Y.**
18 the Spring Meeting of the Japanese Society of Allergology	Mast cells in allergic diseases.	May-06	**Saito, H.**
20th IUBMB International Congress of Biochemistry and Molecular Biology/11th FAOBMB Congress, Kyoto, Japan	Physiological roles of clathrin adaptor AP complexes: lessons from mutant animals.	Jun-06	**Ohno, H., Nakatsu, F.**
CBR Seminar at Harvard Medical School, Boston, U.S.A.	Visualization and single molecule analysis of molecular functions in linving cells.	Jun-06	**Tokunaga, M. and Sakata-Sogawa, K.**
15th International Symposium on Molecular Cell Biology of Macrophages. 17th Annual Meeting of The Japanese Dendritic Cell Society. Tokyo, Japan	NKT cell immunotherapy by α-GalCer-loaded dendritic cells (DCs).	Jun-06	**Fujii, S.**
46th Annual Meeting of The Japanese Society of Lymphoreticular Tissue Research. Nagoya, Japan	Immunotherapy by NKT cells.	Jun-06	**Fujii, S.**
RCAI-JSI International Symposium on Immunology, Yokohama, Japan	How Foxp3 controls dominant tolerance: lessons from IPEX mutations.	Jun-06	**Hori, S.**
Lectureship in Graduate School, Tokyo Medical and Dental University, Tokyo, Japan	Immunobioligu of dendritic cells.	Jun-06	**Sato, K.**
JSI-RCAI joint International symposium	Role of Notch Signal and il4 3' enhancer in Th2 differentiation	Jun-06	**Kubo, M.**
RCAI-JSI International Symposium on Immunology 2006: Regulation of Immune Responses in Allergy and Inflammation. Yokohama, Japan	NKT cell-triggered IL-21 production induced selective B cell apoptosis and IgE-specific suppression.	Jun-06	**Taniguchi, M.**
20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress, Kyoto (Japan)	Dynamics of Polycomb group proteins in living cells	Jun-06	**Koseki, H.**
RCAI-JSI International Symposium on Immunology 2006	Critical roles of IκB kinase-α in TLR7/9-induced type I IFN production.	Jun-06	**Kaisho, T.**
Annual congress of the European Academy of Allergology and Clinical Immunology 2006. Vienna, Austria	Gene expression in eosinophils.	Jun-06	**Saito, H.**
Satellite Meeting of 20th IUBMB Joint Meeting of AOHUPO Symposium and 56th JES Symposium Tokyo, Japan	Filling the gap between transcriptome and proteome: An approach from transcriptomic side.	Jun-06	**Ohara, O.**
RCAI-JSI International Symposium on Immunology 2006, Yokohama, Japan	Fc epsilon RI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane: Zinc-development pathway.	Jun-06	**Hirano, T.**
The 71st annual meeting of Japanese Society of Interferon and cytokine, Nishinomiya, Japan	Construction of artificial lymph nodes (aLNs) and their immunological function.	Jul-06	**Watanabe, T.**
15th Japanese Society for Apoptosis, Kyoto, Japan	Physiological role of apoptotic cell clearance.	Jul-06	**Tanaka, M.**
Biophysics Summer School for Young Scientists, Kobe, Japan	Single molecule imaging and quantitative analysis, from molecule to system.	Jul-06	**Tokunaga, M.**
The Seminar on Sexual Transmitted Disease, control of AIDS and ATL). Kumamoto, Japan	Immunotherapy for HIV-1 and ATL	Jul-06	**Fujii, S.**
The 55th Fujihara International Seminar, Tomakomai, Japan	Function of CRTAM-TSLC1/Necl2 interaction in in vivo immunity	Jul-06	**Takeuchi, A. and Saito, T.**
MEXT Nanobio Summer School "Visualization of living activities", Fujiyoshida, Japan	Single molecule imaging and quantification, from molecule to system.	Aug-06	**Tokunaga, M.**
NEDO workshop "Development for Biomolecular Network Analysis", Tokyo, Japan	Single molecule imaging and quantification by HIRO microscopy, from molecule to system.	Aug-06	**Tokunaga, M.**
JSI Summer School 2006, Kazusa, Chiba, Japan	Process of lineage commitment in hematopoiesis.	Aug-06	**Kawamoto, H.**
The 18th Takato Symposium, Takato, Japan	Dynamic regulation of antigen-recognition and activation of T lymphocytes	Aug-06	**Saito, T.**
2006 Runx Meeting. Gettysburg, PA, USA	Roles of Runx proteins in CD4/CD8 lineage choice.	Aug-06	**Taniuchi, I.**
Toray Science Foundation 56th Science Symposium	Challenges to defeat cancer: immune cell therapies	Sep-06	**Taniguchi, M.**
Development and function of secondary and tertiary lymphoid tissues. Institute Pasteur, Paris	The role of intestinal immunity for establishment of gut microbiota.	Sep-06	**Fagarasan, S.**

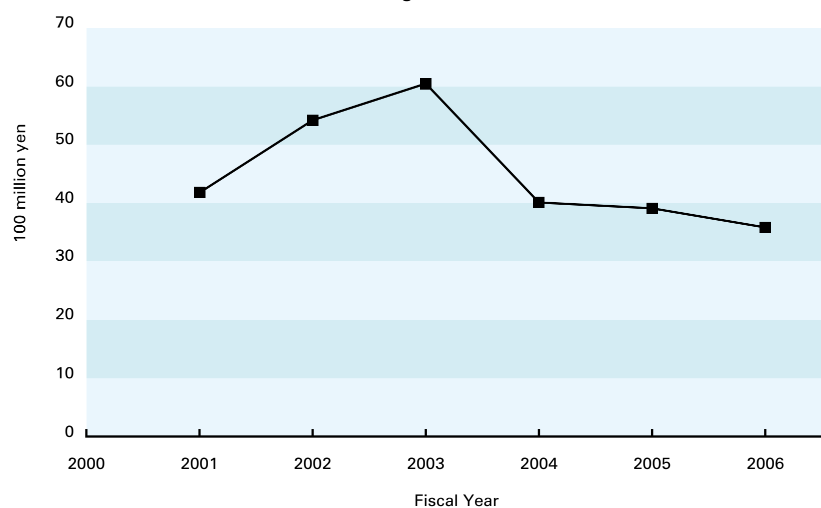
NEDO workshop “Development for Biomolecular Network Analysis”, Tokyo, Japan	Molecular imaging of microclusters responsible for initiating T cell receptor signaling.	Sep-06	**Sakata-Sogawa, K.**
Hiroshima Unv., Hiroshima, Japan	Lineage commitment in hematopoiesis/lymphopoiesis.	Sep-06	**Kawamoto, H.**
Nihon Unv., Fujisawa, Japan	Cell fate decisions during hematopoiesis/thymopoiesis.	Sep-06	**Kawamoto, H.**
“3rd International Conference on Gene Regulation in Lymphocyte Development”. Corfu, Greece	Roles of Runx Transcriptional factor complex in thymus. 2006 Aegean Conferences,	Sep-06	**Taniuchi, I.**
The 3rd Stage Surface Barrier Immunology Study Group 3rd Meeting, Miyazaki, Japan	Differentiation and function of intestinal M cells.	Oct-06	**Hase, K.**
4th Japan Consortium for Glycobiology and Glycotechnology, Shinagawa, Japan	Generation and analysis of siglec-1+ macrophage depletion mice.	Oct-06	**Miyake, Y.**
Rediscovering B cells: Protective and pathogenic roles in infectious and autoimmune diseases. Trudeau Institute, Saranac Lake, NY	B cell subsets and their function.	Oct-06	**Fagarasan, S.**
Japanese Association for Food Immunology (JAFI)	Dynamic interactions between bacteria and B cells in gut-associated lymphoid tissues (GALT).	Oct-06	**Fagarasan, S.**
Research meeting at National Institute of Physiology, Okazaki, Japan	Molecular imaging and analysis of nuclear structures and local translation in synapse.	Oct-06	**Tokunaga, M., Shiina, N., Hiroshima, M., Sakata-Sogawa, K.**
68th Annual Meeting of The Japanese Society of Hematology & 48th Annual Meeting of The Japanese Society of Clinical Hematology. Fukuoka, Japan	A role of NKT cells in immune responses and its application to cancer immunotherapy.	Oct-06	**Fujii, S.**
The 22nd International Kumamoto Medical Bioscience Symposium, Kumamoto, Japan	Memory B cell development and maintenance.	Oct-06	**Takemori, T. and Kaji, T.**
1st International Workshop on “Humanized Mice”, Tokyo, Japan	Development of human immunohematopoietic system in the NOD/SCID/IL2rg null mice.	Oct-06	**Ishikawa, F.**
4th Annual NKT Cell & CD1 Workshop. Tuscuny, Italy	IL-21-induced B cell specific apoptosis mediated by Vα14 NKT cells.	Oct-06	**Taniguchi, M., Harada, M., Nagata, Y., and Watarai, H.**
The 8th International Congress of Neuroimmunology. Nagoya, Japan	Regulatory function of NKT cells in the protection of autoimmune disease development.	Oct-06	**Taniguchi, M., Nakagawa, R., Kojo, S., and Watarai, H.**
The 2nd Annual Meeting of Japanese Association for Food Immunology, Tokyo, Japan	TLR-mediated activation and regulation of T cells	Oct-06	**Imanishi, T and Saito, T.**
Institute for Protein Research, Osaka University, Osaka (Japan)	Molecurar mechanisms for cellular memory mediated by Polycomb complexes	Oct-06	**Koseki, H.**
11th International Dental Congress on Modern Pain Control, IFDAS2006. Yokohama, Japan	Prediction and Prevention of Drug Allergy based on Basic Evidence.	Oct-06	**Saito, H.**
56th Annual Meeting of Japanese Society of Allergology, Tokyo, Japan	Vaccine research for Japanese cedar pollinosis by using the immunoregulatory system	Nov-06	**Ishii, Y.**
Gut Forum Tokyo	Dynamic interactions between bacteria and immune cells in gut.	Nov-06	**Fagarasan, S.**
High-quality camera essential for single molecule imaging. Hamamatsu Photonics Luncheon Semiar in EABS&BSJ2006	High-quality camera essential for single molecule imaging.	Nov-06	**Tokunaga, M.**
Fifth East Asian Biophysics Symposium & Forty-Fourth Annual Meeting of the Biophysical Society of Japan, Ginowan, Japan	Molecular imaging and analysis of microclusters responsible for initiating T cell receptor signaling.	Nov-06	**Sakata-Sogawa, K., Yokosuka, T., Hiroshima, M., Saito, T. and Tokunaga, M.**
Joint International Symposium in KFDA and KRIBB. Seoul, Korea	NKT cell immunotherapy by α-GalCer-loaded Dendritic Cells (DCs).	Nov-06	**Fujii, S.**
26th Translational Research Meeting of Osaka University Hospital. Osaka, Japan	Interaction between NKT cells and DCs for cancer immunotherapy.	Nov-06	**Fujii, S.**
21st Annual Meeting of The Eastern Network of Cancer Immunological Therapy. Tokyo, Japan	Development of dendritic cell therapy	Nov-06	**Fujii, S.**
University of Mainz, Germany	Survival of motor neuron gene (SMN1) promotes the biogenesis of mitochondria respiratory chain by binding to the complex I 39-kDa subunit and the apoptosis-inducing factor (AIF).	Nov-06	**Takemori, T. and Kaji, T.**
Kanazawa Cancer Stem Cell Center Foundation Symposium, Kanazawa, Japan	Identification of Leukemic Stem Cells.	Nov-06	**Ishikawa, F.**
RKI-Seminar(Robert Koch Institute) , Berlin, Germany	TCR microcluster as the site for antigen recognition and activation of T cells	Nov-06	**Saito, T.**
Tokyo Institute of Technology Bio Symposium, Yokohama, Japan	Spatio-temporal regulation of initial and sustained T cell activation Spatial and temporal regulation of starting and maintaining immune responses	Nov-06	**Saito, T.**
The 56th Annual Meeting of Japanese Society of Allergology	Molecular mechanism on type I interferon production from plasmacytoid dendritic cells.	Nov-06	**Kaisho, T.**
KAAACI-WAO Joint Congress 2006 & the 9th WPAS. Seoul, Korea	Mast cell-specific genes.	Nov-06	**Saito, H.**

KAAACI-WAO Joint Congress 2006 & the 9th WPAS, Seoul, Korea	Expression of TLR4, NOD2 and FcγRI in human mast cells: role of human mast cells in host defense and autoimmune diseases.	Nov-06	**Okayama, Y**
56 the Annual Meeting of the Japanese Society of Allergology. Nov. 2-4, 2006	Strategy for treating allergic diseases in post-genomic era.	Nov-06	**Saito, H.**
Fifth East Asian Biophysics Symposium & Forty-Fourth Annual Meeting of the Biophysical Society of Japan (EABS&BSJ2006) Okinawa, Japan	A perspective of immunogenomics from a biophysical viewpoint.	Nov-06	**Ohara, O.**
NIH-JSPS Symposium, Frontiers in 21st Century Biomedical Science: Highlights from Japan and the United States, Bethesda, U.S.A.	Zinc signaling regulates epithelial-mesenchymal transition and activation of immune cells: Zinc is a novel second messenger?	Nov-06	**Hirano, T.**
The 37th International Symposium of the Princess Takamatsu Cancer Research Fund "Cancer Micrioenviromnents", Tokyo, Japan	Zinc signaling regulates epithelial-mesenchymal transition.	Nov-06	**Hirano, T.**
12th Hidgut Club JAPAN Symposium, Tokyo, Japan	Analysis of transcytosis of mucosal antigens by intestinal M cells.	Dec-06	**Hase, K.**
Biofinance Guild "What is cellome?", Tokyo, Japan	Frontier in in vivo cellular imaging: single molecule imaging.	Dec-06	**Tokunaga, M.**
The Seminar of Saitama Medical University. Saitama, Japan	Interaction between NKT cells and DCs for cancer immunotherapy.	Dec-06	**Fujii, S.**
5th International Congress on Autoimmunity. Sorrento, Italy	Manipulation of NKT cell function by stimulation with α-GalCer for their disease control.	Dec-06	**Taniguchi, M.**
The 36th Annual Meeting of the Japanese Society for Immunology, Osaka, Japan	TCR microclusters initiate an sustain T cell activation	Dec-06	**Yokosuka, T.**
The 36th Annual Meeting of the Japanese Society for Immunology, Osaka, Japan	Critical roles of IKKα in TLR7/9-stimulated DC.	Dec-06	**Kaisho, T.**
2nd Congress of Asian Society for Pediatric Research, Yokohama, Japan	Molecular Mechanisms involved in the development of asthma and allergies.	Dec-06	**Saito, H.**
The 36th Annual Meeting of the Japanese Society for Immunology, International Symposium, Osaka, Japan	Zinc and dendritic cell maturation: Does zinc act as a signaling molecule?	Dec-06	**Hirano, T.**
The 36th Annual Meeting of the Japanese Society for Immunology, Late Prof. Yuichi Yamamura memorial symposium, Osaka, Japan	Interaction of immune system with non-immune system induces autoimmune diseases.	Dec-06	**Hirano, T.**
Lectureship in Graduate School of Medical and Dental Science, Kagoshima University, Kagoshima, Japan	Dendritic cells and regulation of immune response.	Jan-07	**Sato, K.**
2nd Uehara Memorial Biomedical Foundation Symposium. Tokyo, Japan	Immune System: Challenges to defeat cancer	Feb-07	**Taniguchi, M.**
Keystone Symposium. Vancouver, Canada	How Foxp3 controls dominant tolerance: lessons learnt from naturally occurring foxp3 gene mutations.	Feb-07	**Hori, S.**
Keystone Symposium, Banff, Canada	Pathways of Memory B cell Development. Biology of B Cells in Health and Diseas	Feb-07	**Takemori, T. and Kaji, T.**
2nd Uehara Memorial Biomedical Foundation Symposium. Tokyo, Japan	Revolutionary technology in immunology and the emerging medical therapy.	Feb-07	**Ishikawa, F.**
The Second International Symposium on Immune Surveillance	Role of BCAP and BANK in B cell development and activation.	Feb-07	**Kurosaki, T.**
The Second International Symposium on Immune Surveillance, Tokyo, Japan	Spatial regulation of initial and sustained T cell activation	Feb-07	**Saito, T.**
The 4th Osteoimmunology Forum Tokyo, Japan	Real-time imaging of the start and maintenance of immune responses	Feb-07	**Saito, T.**
The 10th International Membrane, The 10th Membrane Research Forum, Kyoto, Japan	Spatiotemporal regulation of T cell activation by TCR-CD28-microclusters	Feb-07	**Yokosuka, T.**
Deutsche Gesellschaft fur Immunologie 3rd Spring School on Immunology, Ettal, Germany	Imaging for recognition and activation	Feb-07	**Saito T.**
Keystone Symposia, Intracellular and Intercellular Signaling in Dendritic Cell Function, Colorado, USA	Critical involvement of IκB kinase-α in TLR7/9-induced type I IFN production.	Feb-07	**Kaisho, T.**
The 5th EAACI-GALEN Davos Meeting, Davos, Switzerland	Regulation of Innate Immunity.	Feb-07	**Kaisho, T.**
Hokkaido Univ. Research & Business Park "Next Generation Post-genome Seminar", Sapporo, Japan	Single molecule imaging in cells, from molecule to system.	Feb-07	**Tokunaga, M., Sakata-Sogawa, K.**
25th Annual Meeting of The Japan Society of Immunology & Allergology in Otolaryngology. Yamanashi, Japan	Interaction between DC and NKT cell response.	Mar-07	**Fujii, S.**
USA-Japan Cooperative Cancer Research Program, Hanalei, Hawaii	Animal models of hematological malignancies.	Mar-07	**Ishikawa, F.**
The 6th Annual Meeting of the Japan Society for Regenerative Medicine, Yokohama, Japan	Studying human hematopoietic stem cells and leukemia stem cells.	Mar-07	**Ishikawa, F.**
JSI-RCAI Workshop 2007, Yokohama, Japan	Roles of E2A in lymphoid lineage commitment.	Mar-07	**Kawamoto, H.**
JSI-RCAI workshop 2007. Yokohama, Japan	Role of Runx complexes in CD4/CD8 lineage choice.	Mar-07	**Taniuchi, I.**

Date	Title	Lecturer	Affiliation
12-Apr-06	Function of p63 in epithelial stem cells: focus on thymic epithelial cells	**Dr. Makoto Senoh**	Department of Cell Biology, Harvard Medical School, USA
13-Apr-06	Protein ubiquitination in immune regulation	**Dr. Yun-Cai Liu**	Associate member, Division of Cell Biology, La Jolla Institute for Allergy and Immunology, USA
19-Apr-06	Evolution of Adaptive Immunity	**Dr. Max D.Cooper**	Howard Hughes Medical Institute The University of Alabama at Birmingham, USA
24-Apr-06	Prospective reconstitution of hepatocellular carcinoma from isolated liver stem cells	**Dr. Hideki Taniguchi**	Yokohama City University School of Medicine, JAPAN
24-May-06	FOXP3 ensembles in T cell regulation	**Dr. Mark I.Greene**	Medeical Scinece University of Pennsylvania, USA
19-Jun-06	Externalized endosomal vesicles with many functions?	**Dr. Graca Raposo**	Institute Curie, Paris, France
20-Jun-06	Role of DNA double strand break response in IgH class switching and suppression of translocations	**Dr. Fredrick W.Alt**	Harvard Medical School Investigator, HHMI, USA
27-Jun-06	Mucosal T cell differentiation: the good, the bad and the ugly	**Dr. Hilde Cheroutre**	Associate Member, Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology
4-Jul-06	Membrane Traffic: life crucial intracel-lular trafficking network crucial for life	**Dr. Tamotsu Yoshimori**	Research Institute for Microbial Diseases, Osaka University, JAPAN
14-Jul-06	TLR-dependent and -independent mechanisms of anti-viral responses indendritic cells	**Dr. Marco Colonna**	Professor of Pathology & Immunology,Washington University school of Medicine,St.Louis
27-Jul-06	Dynamic in vivo 2-phonton imaging of immune cells in lymphoid and peripheral tissues	**Dr. Ronald N. Germain**	Chief of Immunology National Institute of Allergy and Infectious Diseases National Institutes of Health
28-Jul-06	New tools for spatially-resolved modeling and simu-lation: Focusing on the biology and not the Math	**Dr. Ronald N. Germain**	Chief of Immunology National Institute of Allergy and Infectious Diseases National Institutes of Health
21-Aug-06	Regulatory mechanism of TCRb allelic exclusion	**Dr. Yasutoshi Agata**	Graduate School of Medicine, Kyoto University, JAPAN
22-Aug-06	High Resolution Live-Cell Imaging using DeltaVision RT Imaging System	**Dr.George L. Kumar**	Sr. Scientist, Applied Precision LLC
13-Oct-06	HIV infection in human CD34+ cell transplanted Rag2-/-gamma-/-mice	**Dr. Markus G.Manz**	Institute for Research in Biomedicine(IRB), Switzerland
24-Oct-06	Toll-like receptor, heat shock protein gp96 and the myth in between	**Dr. Zihai Li**	Center for Immunotherapy of Cancer and Infectious Diseases, Department of Immunology, University of Connecticut Health Center (UCHC), USA
24-Oct-06	The analysis of NF-kappaB pathway in B cell development,maintenance and transformation	**Dr. Yoshiteru Sasaki**	The CBR institute for Biomedical Research Harvard Medical School, USA
30-Oct-06	Long-lived plasma cells in im-munity and immunopathology	**Dr. Andreas Radbruch**	Deutsches Rheumaforschungszentrum, and Charite, Humboldt University, Berlin, Germany
15-Nov-06	Amplification of TLR-mediated signals for the improved induction of adaptive immune responses	**Dr. Hansjoerg Schild**	Institute for Immunology, University of Mainz, Germany

16-Nov-06	Antigen processing beyond the level of CTL epitope generation	**Dr. Hansjoerg Schild**	Institute for Immunology, University of Mainz, Germany
21-Nov-06	Regulation of helper T cell immune responses.	**Dr. Chen Dong**	Department of Immunology MD Anderson Cancer Center, USA
29-Nov-06	The B-cell antigen receptor function in peripheral B cell differentiation and transformation: lessons from mouse models	**Dr. Stefano Casola**	Group leader IFOM-IEO campus Milan, Italy
14-Dec-06	New insights into TCR signaling	**Dr. Lawrence Samelson**	Chief, Laboratory of Cellular and Molecular Biology National Cancer Institute National Institutes of Health
14-Dec-06	Interplay of innate and adaptive immune receptors in B cell activation	**Dr. Susan Pierce**	Chief, Laboratory of Immunogenetics National Institute of Allergy and Infectious Diseases National Institutes of Health
14-Dec-06	New insights into TCR signaling	**Dr. Susan Pierce**	Chief, Laboratory of Immunogenetics National Institute of Allergy and Infectious Diseases National Institutes of Health
21-Dec-06	Visualization and manipulation of the tis-sue-specific alternative splicing of mRNAs	**Dr. Masatoshi Hagiwara**	Professor, Department of Functional Genomics, Medical Research Institute, Tokyo Medical and Dental Universit, Japan
11-Jan-07	Biochemical and Biological Consequences of Peptide Selection by Diabetogenic Class II MHC Molecules	**Dr.Anish Suri**	Assistant Professor of Pathology & Immunology,Washington University school of Medicine,St.Louis
18-Jan-07	C-type lectins in metastasis, infection and hypersensitivity	**Dr. Tatsuro Irimura**	Professor, Univ. Tokyo
18-Jan-07	Muc21: a novel mucin expressed the thymic medulla in an Aire-independent manner	**Dr. Katrin Ishi-Schrade**	Unv. Tokyo
18-Jan-07	MUC1 DNA vaccine to prevent colon cancer metastasis: Novel organ-specific effecter mechanisms	**Dr. Kaori Denda-Nagai**	Assistant Professor, Univ. Tokyo
18-Jan-07	On the road to a DC vaccine: generation of MUC1 Ag specific T cells in vitro	**Dr. Wilfred Germeraad**	Assistant Professor, Univ. Hosp. Maastricht
18-Jan-07	For clinical immunotherapy patients and doctors need the knowledge of real scientists	**Dr. Gerard Bos**	Hematologist and Head pre-clinical research, Dept Hemato-Oncology, Univ. Hosp. Maastricht
26-Jan-07	Lymphoid organ development in the human fetus	**Dr. Tom Cupedo**	Erasmus University Medical Center Department of Hematology
5-Feb-07	NetPath: A resource for analysis of signal transduction pathways	**Dr.S.Sujatha Mohan**	Institute of Bioinformatics, Bangalore, India
16-Feb-07	Structure and Function of the Immunological Synapse	**Dr. Andrey Shaw**	rofessor of Pathology & Immunology, Washington University school of Medicine, St. Louis
16-Feb-07	Function and action mechanisms of co-receptors on B lymphocytes	**Dr. Thomas F. Tedder**	Chairman of Dept. of Immunology, Duke University
27-Feb-07	Real-time visualization of biomolecular interac-tions in solution by atomic force microscopy	**Dr. Hiroaki Sugasawa**	Research Institute of Biomolecule Metrology, Co., Ltd.
7-Mar-07	Genetic and epigenetic control of antigen receptor gene assembly	**Dr. Eugene M. Oltz**	Professor of Dept. of Microbiology & Immunology, Vanderbilt University Medical School

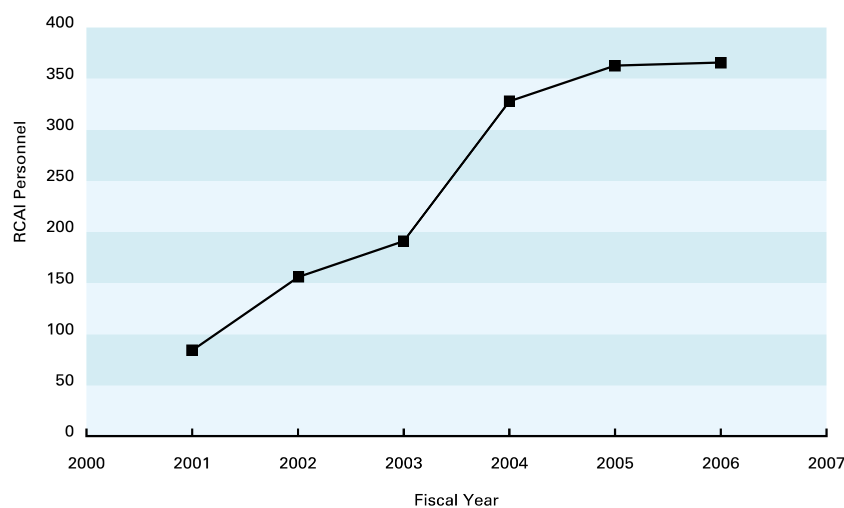
Budgets and Personnel



RCAI Budget 2001-2006 (JPY 100 Million)

Note:Budgets for 2001-2003 include construction expenses for RCAI facility

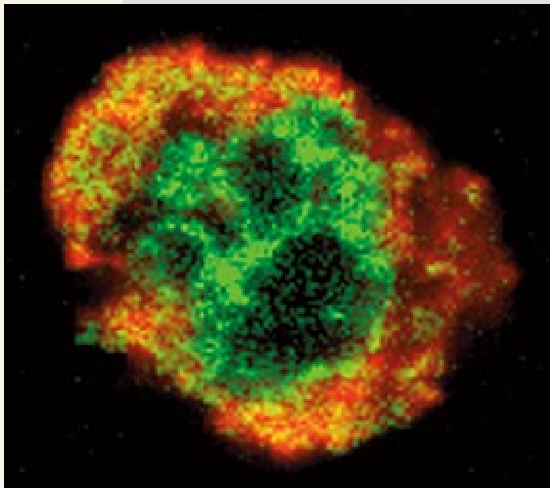
RCAI Budget (JPY 100 Million)	
2001	41.74
2002	54.23
2003	60.48
2004	40.10
2005	39.02
2006	35.90



RCAI Personnel	
2001	84
2002	156
2003	191
2004	328
2005	363
2006	366

RCAI Staff Composition (as of March 31, 2006)

Category	Number
Director	1
Special Advisor	1
Principal Investigators	27
Coordinator	1
Senior Scientist	9
Scientists	62
Senior Technical Scientist	1
Technical Scientist	4
Technical Staff I	2
Technical Staff II	98
Graduate Students	45
Research Associate	4
Visiting Scientists	34
Assistants	23
Part-time Staff	30
Temporary Employment	24
Total	**366**



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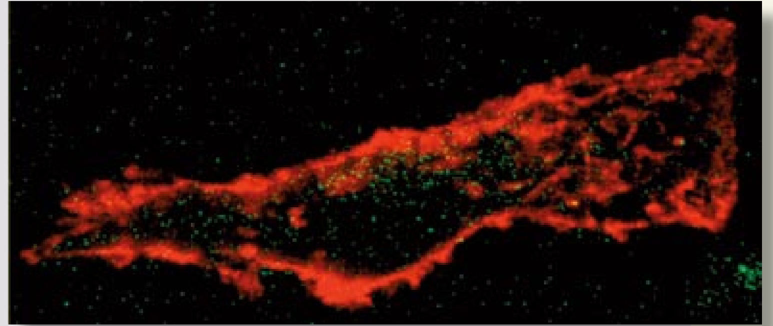
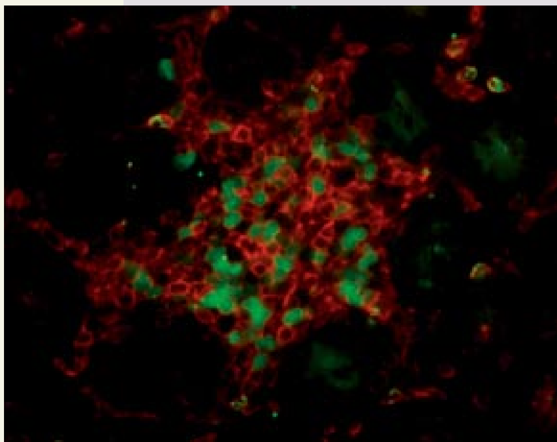


2

Front and back covers:
Changes in intracellular zinc levels in dendritic cells before (1) and after (2) LPS stimulation.

It is well-established that zinc is vital to cellular function and zinc deficiency causes immunodeficiency, although still no precise mechanisms are known. Recent research by the Lab for Cytokine Signaling suggests that zinc may be far more than just a passive biological bystander. Their data may offer evidence of a previously unrecognized mode of cellular signaling, indicating that the level of intracellular free zinc changes in response to extracellular stimuli such as cytokines and growth factors.

Dendritic cells (DCs) mature following exposure to the bacterial endotoxin lipopolysaccharide (LPS) and express major histocompatibility complex II (MHCII) (red) on the cell surface. The Lab for Cytokine Signaling found that the level of intracellular free zinc (green) is decimated in maturating DCs and that zinc-depleting agents lead to increased DC maturation (See Research Highlights).
Image courtesy of Lab for Cytokine Signaling



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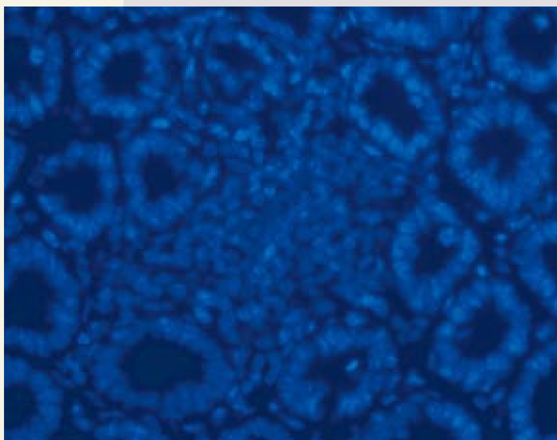
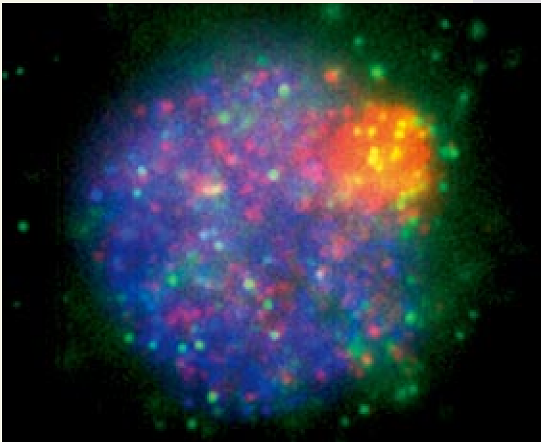


4

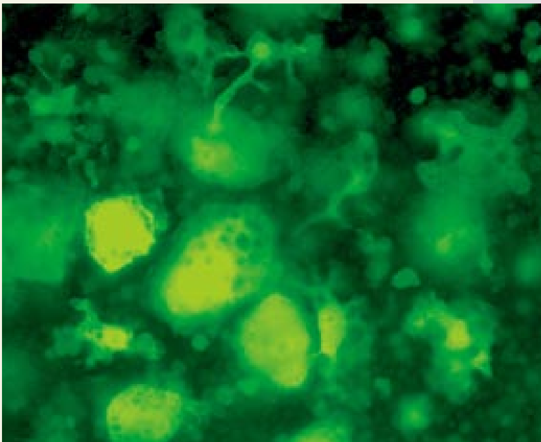
Front pages of Research Highlights (3) and Research Activities (4) sections:
Section from adult small intestine. RORγt-GFP (green) and $CD45^{+}$ lymphocytes (red) were identified in a crypt patch stained with DAPI (blue).

Lab for Mucosal Immunity found that Lymphoid Tissue-inducer cells (LTi) function as “gut sensors” and regulate gut plasticity for maintenance of intestinal immune homeostasis. Lti had been identified as hematopoietic-derived cells essential for lymph node and Peyer’s patch formation during embryonic development. LTi express a transcription factor called RORγt, which is also found in only a subset of immature T cells and Th17 cells in the gut. Using RORγt-GFP knock-in mice (generated in Dr. Dan Littman’s laboratory at New York University), they identified LTi in the adult intestine and found that these cells facilitate both recruitment of lymphocytes in the gut lamina propria and IgA induction through their interaction with gut stromal cells.
Image courtesy of Lab for Mucosal Immunity


5

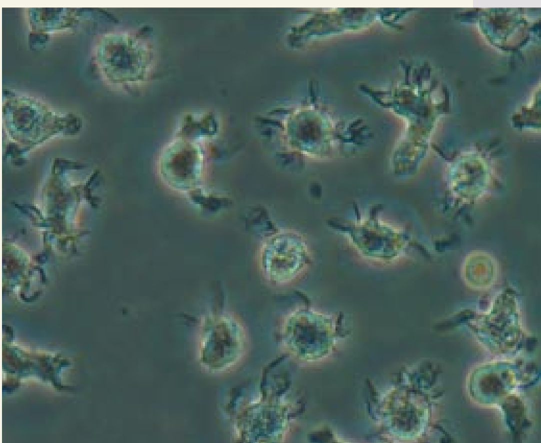
Front page of Collaborative Networks section (5):
Multi-color immunofluorescence staining at a single-molecule level. NF-κB (green), phospho-c-jun (red), acetylated histone H3 (blue).

The Research Unit for Single Molecule Immunoimaging developed a novel optical imaging technology, Highly Inclined and Laminated Optical Sheet (HILO) microscopy. HILO broadens the application of the Total Internal Reflection (TIRF) technique beyond flat surfaces, allowing the imaging of single biomolecules within three-dimensional structures such as cells, while conserving the high sensitivity of the TIRF approach. HILO can be used for single-molecule analysis of fluorescently tagged molecules inside or on the surface of single cells as well as for studies on the actual three-dimensional distribution of individual molecules within a cell. This technique is extremely sensitive and can also be used for quantitative and kinetic studies. HILO was initially developed for single color fluorescence imaging, but a new multiple color version has now been established (See Research Highlights).
Image courtesy of Research Unit for Single Molecule Immunoimaging


6

Front page of Events section (6):
Macrophages and dendritic cells produced from a single intrathymic T cell progenitor of a GFP (green) transgenic mouse.

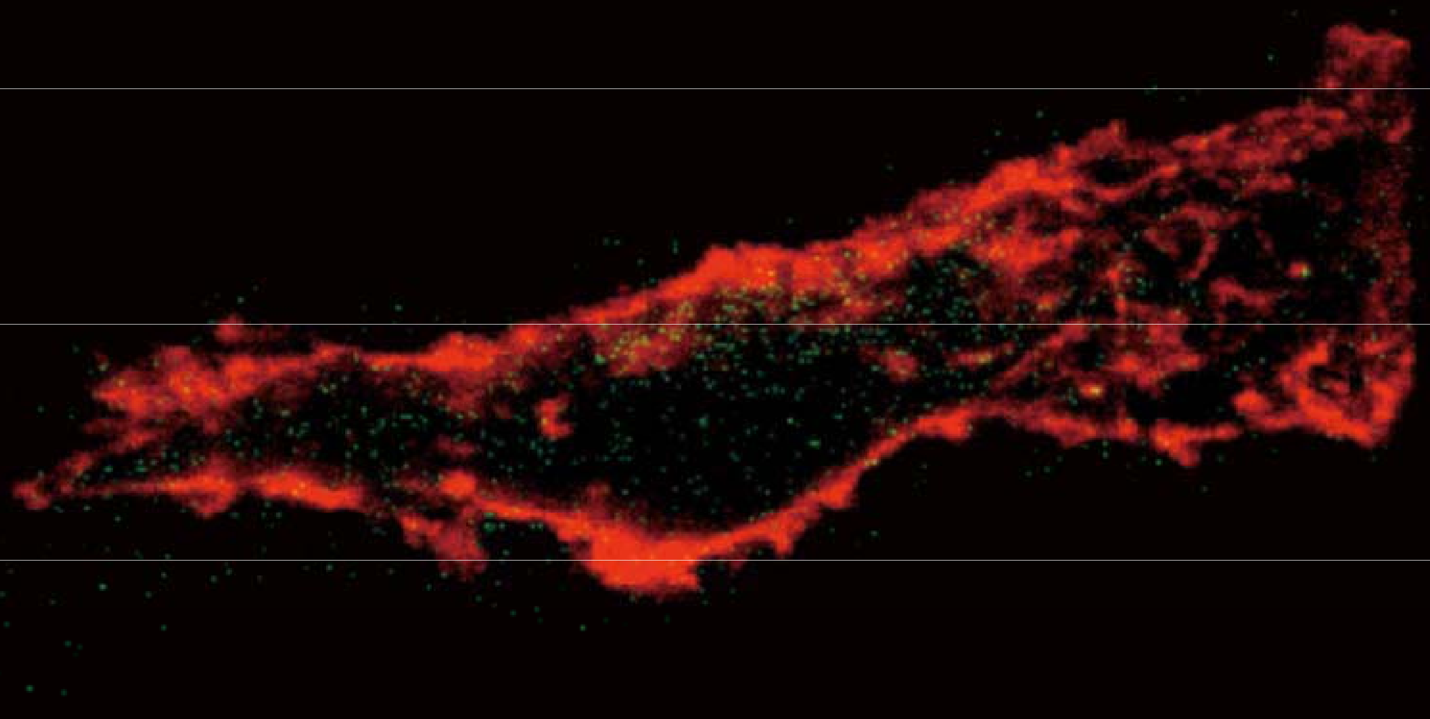
The classic dichotomy model of hematopoiesis postulates that the first step of differentiation beyond the multipotent hematopoietic stem cell generates the common myelo-erythroid progenitors and common lymphoid progenitors. However, the Lab for Lymphocyte Development found that T cell progenitors in the thymus retain the potential to generate macrophages and dendritic cells after shutting off B cell potential. This finding suggests that myeloid potential persists in the T- and B-cell branches even after these lineages have diverged, indicating that the simple dichotomy model is invalid, at least for fetal hematopoiesis. Thus, the Lab for Lymphocyte Development has suggested an alternative "myeloid-based model", in which each process of specification towards T, B and erythroid lineage appears to proceed according to the prototypical myeloid program. (See Research Activities)
Image courtesy of Lab for Lymphocyte Development


7

Front page of Data and Statistics section (7):
Morphology of dendritic cells.

Dendritic cells (DCs) are immune cells defined by their dendritic morphology and phenotype. DCs consist of heterogeneous subsets with different lineage derivation and maturation stage, and they are widely distributed in both lymphoid and nonlymphoid tissues. *In vivo*, immature DCs (iDCs) can recognize 'non-self', such as pathogens or tumor cells, and they subsequently become mature DCs (mDCs) under inflammatory conditions. mDCs are not only the most powerful antigen-presenting cells (APCs) for inducing acquired immunity but they also activate innate immunity though the production of various cytokines to adequately eliminate them. On the other hand, iDCs act as tolerogenic APCs to induce tolerance to 'self', and are thereby involved in the maintenance of immunological homeostasis. Therefore, DCs are thought to be intrinsic controllers of the immune system, depending on their lineage heterogeneity and functional plasticity.

At the Lab for Dendritic Cell Immunobiology, they modified DCs to establish tolerogenic APCs with potent immunoregulatory properties even under inflammatory conditions, and they designated them "regulatory DCs". Their goal is to identify the molecular mechanisms underlying the function of regulatory DCs and to develop immunotherapy with regulatory DCs for immunopathogenic diseases (See Research Highlights).
Image courtesy of Lab for Dendritic Cell Immunobiology



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