Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Children's Hospital of Pittsburgh of the UPMC Health System

2. **Reporting Period (start and end date of grant award period):** 1/1/2012 – 12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** David H. Perlmutter, MD

4. **Grant Contact Person's Telephone Number:** 412-692-6081

5. **Grant SAP Number:** 4100057656

6. **Project Number and Title of Research Project:** Project #1 Regulatory T cells and Tolerance after Blood and Marrow Transplantation

7. **Start and End Date of Research Project:** 1/1/2012 – 12/31/2013

8. **Name of Principal Investigator for the Research Project:** Paul Szabolcs, MD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $227,201.68

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Chen, Xiaohua	Research Assistant Prof.	40%	$53,458.29
Szabolcs, Paul	Principal Investigator	10%	$40,037.29

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
Electronic multichannel pipettes 5-100ul	Eppendorf	$1333.80
Electronic multichannel pipettes 50-1200ul	Eppendorf	$1526.00
Electronic pipettes 50-1000ul	Eppendorf	$630.59

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No_____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes____X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Cadaveric Donor Lung and Bone Marrow Transplantation in Immunodeficiency Diseases	X NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	June 2013	$ 150,000 direct	Not Funded
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

We hope to extend our studies to patients and we would seek RO1 support from NIH to study Treg immune function in mixed chimerism and GVHD

12. Future of Research Project. What are the future plans for this research project?

1. We plan to perform experiments on newly accrued patients to extend and confirm our findings of Foxp3/Helios co-expressing Treg deficiency in GvHD by testing on more subjects.

2. Further characterize Treg subsets (cytokine responsiveness, functional pathways).

3. Refine our single cell Treg cloning strategy to develop therapeutic cell products in the future.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____ No__X____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other				
Unknown				
Total				

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X___

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____ No____X____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes__X___ No_____

If yes, please describe the collaborations:

We have entered into discussions how we may perform tolerance studies and Treg analysis on upcoming studies supported by the PIDTC consortium.
http://rarediseasesnetwork.epi.usf.edu/PIDTC/

16(B) Did the research project result in commercial development of any research products?

Yes_____ No___X____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Regulatory T Cells and Tolerance after Blood and Marrow Transplantation – Tolerance after blood and marrow transplantation (BMT) is achieved eventually in most patients after 1-2 years post-BMT as they become independent of drugs to avoid rejection or graft-versus-host-disease (GVHD). Regulatory T Cells (Tregs) are known to be important in sustaining tolerance, however, there is a great gap of knowledge after BMT in humans regarding their activity in disease state (GVHD) compared to health (tolerance). In this project we will isolate and analyze Tregs from patients experiencing GVHD and contrast these to Tregs isolated from patients free of GVHD. Once functional prerequisites for tolerance are discovered, novel targeted therapies can be devised for those patients who suffer from GVHD.

Tolerance after blood and marrow transplantation (BMT) is achieved eventually in most patients after 1-2 years post-BMT as they become independent of pharmacological agents to avoid rejection or graft-versus-host-disease (GVHD). The hallmark of tolerance is unresponsiveness between host and graft tissues in the absence of any immunosuppressive (IS) drugs. Regulatory T Cells (Treg) expressing CD25 and FOXP3 were identified over 10 years ago as critical players in sustaining tolerance. More Tregs in the transplant graft itself or in the blood of BMT recipients is associated with less GVHD. However, beyond these numerical associations there is a great gap of knowledge regarding their functional profile and features in GVHD compared to those patients without it. The proposed studies would discover new biological characteristics of Tregs that are essential for tolerance as they suppress the function of "conventional" T cells (Tcon). In this project we will develop new assays to analyze Treg cells from BMT patients and contrast the functional features of Tregs purified from patients with or without GVHD including those who never had GVHD. These studies may identify new biomarkers for the presence or absence of GVHD and should also identify specific features of Treg cells that are prerequisites for suppressing Tcon to induce tolerance. A new in vitro model could become a valuable tool to monitor other autoimmune diseases as well. We describe below the various aspects of our studies with the exciting new development on Treg cloning detailed in Aim I b below.

<u>Aim I. Enumerate regulatory T cells (Tregs) from blood and marrow transplant patients longitudinally and determine their T cell receptor (TCR) diversity by spectratyping after purification based on the expression of Foxp3 and Helios transcription factors. Contrast the profile of Tregs between BMT recipients with versus without GVHD.</u>

We have partially achieved our goals in Aim I. We continue to accrue new subjects to this study to permit well powered statistical conclusions. We are anticipating more adult patients in 2014 to enroll. Large volume of blood draw is permitted in adults and these samples should yield more T cells available for purification thus TCR repertoire analysis by spectratyping should become feasible. Nevertheless, we have developed the necessary techniques and have demonstrated proficiency as described below**.**

Aim 1A. Enumerate Treg cell distribution

In our first progress report covering the period Jan 1-June 30, 2012, we presented our newly designed 8-color FACS panel to detect Treg phenotypes and identify Treg subsets. Since then, we have further optimized our FACS gating strategies to increase the sensitivity in detecting the relationship between Treg sub-populations and their functions. The seven dotplots that are part of

Figure 1. depicts our gating scheme. A P1 region was drawn around cells conforming with known lymphocyte scatter properties, see **Fig 1a.** From those cells within the P1 region, CD4+ cells were identified (P2) as shown in **Fig. 1b**. Putative Tregs were identified amongst the CD4+ T cells by gating on a CD25+ and CD127 dim subset identified now by the P3 region, see **Fig. 1c**. The distribution of FoxP3 and Helios transcription factor expressing Tregs amongst all Putative Tregs ($CD4+CD25^{high}CD127^{low}$) P3 region was then analyzed by quadratic gating, see **Fig. 1d**. The distributions of naïve, central memory, and effector memory phenotype in each of the Treg subsets identifiable by their Foxp3/Helios expression can be further subdivided by additional quadratic gating, see example for FoxP3+/Helios+ double positive Tregs in **Fig.1e.** The percentile to their parents of each population was applied, and thereby the influence from the variation of parent numbers was excluded in this analysis.

To examine Treg activation which reflects the functional status of the cells, the past months we designed another 8-color FACS panel to include anti-Ki67 (proliferation marker)/activated-Caspase3 (apoptosis marker) Abs **(Fig.1f).** According to recent publications from Shimon Sakaguchi, we also added a combination of CD45RA vs Foxp3 to distinguish activated Tregs (region II) from resting Treg (region I) and Foxp3+ non-Tregs (region III), see **Fig. 1g.**

With these newly developed panels in place we went to characterize the distribution of Treg phenotypes in healthy donors. A total of seven healthy volunteer donors were tested so far. An average of 8.3±4.4% CD4+CD25+CD127low fraction of CD4+ population conforms with the "bulk" Treg phenotype. Notably, amongst these bulk Tregs identified four subpopulations based on different Foxp3 and Helios co-expression, see **Fig. 1d**. These include single Helios+/FoxP3- (25.5±15.7%), subsets within the Q1 quad of **Fig. 1d** , the double positive Foxp3+/Helios+ (45.9±13.4%) within the Q2 quad of **Fig. 1d**, the single Foxp3+ /Helios- (9±4%) Q3 quad of **Fig. 1d**, and finally the double negative Foxp3/Helios (19.5±8.1%) cells in Q3 quad of **Fig. 1d**. Circa 14.8±8.1% of Foxp3+/Helios+ Tregs carried central memory phenotypes (CD45RO+CD62L+) depicted within the Q2 quad of **Fig. 1e**.

More recently, we started to monitor these phenotypes from patient samples. Pre-transplant serotherapy by Alemtuzumab or ATG is routine in the allogeneic transplant population in our clinical practice; therefore we have a fairly low frequency of grade II or higher acute GvHD. Consequently, we have been able to test so far only three BMT recipients with GVHD at the end of this reporting period. As a comparison and control population, we have also examined seven cord blood recipients without GvHD. Another population of patients with severe autoimmunity was also enrolled on our IRB-approved protocol and was tested. So far three patients with autoimmunity were studied at the end of this reporting period. Undoubtedly, we are at a rather limited sample size at this point; nevertheless, we have started formal comparisons of the measured Treg subsets between these different subject categories. We plan to increase accrual by obtaining blood samples from UPCI/Hillman Cancer Center transplant patients besides continuing to accrue from patients transplanted at CHP of UPMC.

In comparison with healthy donors, so far we have found no significant alteration in average proportion of CD4+CD25+CD127low "bulk" Tregs (p3 in **Fig. 1c)** amongst all CD4+ T cells (p2 **in Fig. 1b)** in patients with autoimmune diseases and patients with or without GVHD, **Fig. 2A.** The same held true for both single Foxp3+ Treg subsets (data not shown), and single

Helios+ Tregs (data not shown). However, unexpectedly, compared to healthy controls, we found, a significant decrease in the Foxp3+/Helios+ (double positive) Treg fraction and their central memory compartment in patients with autoimmune diseases, while this population was comparable to healthy volunteers in cord blood transplant patients with or without GvHD (**Fig. 2B).** These results suggest that Foxp3 and Helios co-expressing "double positive" central memory Tregs, rather than single Foxp3+ or Helios+ Treg, may play an important role in the maintenance of immune tolerance.

When we restricted the analysis on the brightest subpopulation within the Foxp3+Helios+ central memory Tregs **(Fig.3A)** displaying the highest fluorescence intensity for these transcription factors (depicted P4 in Q2) and analyzed their distribution within the "Sakaguchi regions" (as defined by CD45RA/Foxp3 expression amongst CD4+/CD25 bright cells), we found no resting CD45RA+ Tregs (**Fig. 3 D**), however, the majority of them belong to "Sakaguchi region II"-aka "activated Tregs" which are CD45RA-/Foxp3^{high} **(**Q4-1 in **Fig. 3D**). These cells have undergone significant proliferation, identifiable by the strong intranuclear expression of Ki67 (see events in Q1-1 in **Fig. 3C**).

When we examined the Treg profile of those subjects with recent acute GvHD, we found little if any proliferation (low expression of intracellular (ic) Ki67 in Q1-6 **Fig. 4A**) amongst CD4+Treg and Foxp3+/Helios+ Treg carrying central memory phenotype, in comparison with that in a cord blood patient in good clinical condition, free of any GVHD, (Q1-6 **Fig. 4B)** . The analysis was performed at the same time-point, ~ 100 days post-transplantation. There was statistically significant reduction in proliferating "bulk" Tregs (**Fig. 4C**) in those with GVHD impacting just as well the Foxp3+Helios+ co-expressing Treg subset that displays (CD45RA-/CD45RO+/CD62L+) central memory phenotype **(Fig. 4D)**. It is plausible that reduced proliferative capacity and possible exhaustion of Foxp3+Helios+ Tregs is a critical event in the development of acute GvHD. We hypothesize that the subjects' immune environment, e.g. cytokine milieu that influence Treg function may also play an important role on Treg survival and proliferative capacity, which in turn may feed back into the pathogenesis of GVHD. Recent publications (Shamim et al Human Immunology, 2013;10:1111; Kim et al Transplantation 2012;94:1250) have suggested that single nucleotide polymorphism (SNP) in several cytokines are associated with occurrence of acute GvHD. Targeted modulation of the immune environment to support Treg function may become a new way to favorably modulate and treat acute GvHD. Methods that would favorably impact Treg kinetics and function may be as effective or more than infusion of bulk Tregs expanded ex vivo from allogeneic healthy donors.

Importantly, these new findings were detected directly from unmanipulated patient samples to reflect human disease state without experimental influence leading to accidental bias. If the findings were confirmed in a larger cohort, future Treg expansion strategies should focus on generating Tregs with stable and functionally and phenotypically defined subsets. This approach may result in superior therapeutic responses for GvHD and possibly in autoimmune disease as well where autologous Tregs would be expanded without fears of GVHD. In the immediate future we plan to confirm on larger patient populations these findings on functionally and phenotypically distinct Treg subsets, we could 1) devise more powerful biomarker assays to predict the presence or absence of overall tolerance versus exaggerated/pathological immune responses (i.e. GVHD) post-HSCT, and 2) design new therapeutical interventions.

In summary, these studies have yielded some exciting findings sufficient for generating new hypotheses. We plan to submit these for new funding agencies to extend and validate the above preliminary findings in a large patient cohort. Towards these goals, we have successfully reached out to colleagues at the Hillman Cancer Center of the University of Pittsburgh Cancer Institute (UPCI) to enroll their patients on these IRB approved studies.

Aim 1B. Treg single cell cloning

Because Foxp3 and Helios genes are transcription factors it is not feasible to purify viable primary human Treg sub-populations by gating on these intracellular markers. Therefore, we have developed a novel single cell cloning technique with the goal to characterize Treg sub-populations. After successful cell expansion, we may sacrifice a fraction of the clone for characterization while many more are available for functional studies. Conventional method of T-cell cloning requires feeder cells, in particular using PBMC to support single cell growth, may cause contamination with unwanted cells or more likely it may lead to additional cell contacts and soluble signals that could result in functional bias of Tregs expanding from single cells in an uncontrolled milieu. After months of pilot experiments, we have made progress towards a new protocol for single Treg cloning (**Fig. 5** and **Fig. 6),** with a remarkable ~ 20% cloning efficiency for single Treg clones, (manuscript in preparation). To verify single cell clonality, we established TCR qPCR and TCR immunoscope for fast screening single cell cultures, another technical advance in this field, (manuscript in preparation). **Figure 6** shows TCR qPCR profiles tested from the PBMC of a healthy donor (**Fig. 6A**) or from the single cell cloning **(Fig. 6B);** and TCR immunoscope profiles representing the PBMC of a healthy donor **(Fig. 6C),** or the clonal progeny of a single cell **(Fig. 6D).**

Aim IB TCR repertoire analysis

Our original hypothesis has been that the TCR repertoire of circulating Tregs from patients with GvHD will differ from that seen in patients not having experienced GVHD. The main obstacle to test this hypothesis has been the low cell numbers of Tregs we have isolated, reflecting both CD4+ lymphopenia post transplant and the overall small volume of blood samples obtained from young children (4-8 ml range). We have nevertheless titrated the assay to the lowest cell number sufficient for efficient RNA purification. By adding carrier during the purification procedure, we can purify RNA from 1000 cells (data not shown). However, we have not yet obtained reproducibly clear signals on TCR immunoscope with RNA purified from 1000 cell. We are pursuing experiments with pre-PCR amplification (Life Technologies) to enhance PCR signals.

<u>Aim II. Design and implement functional assays to characterize and to quantify the biological profile and suppressive capacity of purified Treg in vitro as they are mixed with conventional T-cells from the same BMT recipient. Following non-specific and transplant recipient specific activation of Tregs and Tcon in the same co-cultures to model the in vivo scenario, we will analyze Tregs by qPCR for cytokine and homing receptors, co-stimulator, and other critical suppressor molecules while Tcon will be tested in parallel for evidence of receiving suppressive signals.</u>

We have partially achieved our goals as described in Aim II, We have developed and validated a sensitive functional assay as described below. We are anticipating more adult patients to enroll between the Summer of 2014 and Spring of 2015 . These new datasets will permit correlative analysis of Treg function and clinical correlates, most importantly the presence or absence of GVHD. In adult subjects larger blood draw is permitted and these newly enrolled adult subject can provide more T cells for purification and for analysis. Nevertheless, we have developed the necessary techniques and have demonstrated proficiency as described below.

After initially setting up a "standard" CFSE proliferation assay developed by others years ago, we have further optimized the conditions to obtain differentiation peaks to give much distinct proliferation features. **Figure 7A** shows differentiation peaks generated at day 4 of a Tcon culture. We are currently titrating the dose of antigen presenting cells (APC), employing both live cells and artificial CD3/CD28 T cell stimulatory beads to determine the best conditions for *in vitro* Tcon activation to sufficiently quantify the suppressive features from Treg. We have had surprising proliferative responses from Tregs in the presence of unnecessarily potent stimulatory signals.

We are also in the midst of developing other functional assays that may directly reflect other Treg cell functions rather than inhibit of cell proliferation in response to non-antigen-specific signals.

i. We have started monitoring Treg suppression of cytokine secretion by Tcons. IFNγ is one of main cytokine secreted by functional Th1 type Tcons. Reduced IFNγ production by Tcons reflects an inhibition by Tregs in the co-culture. Importantly, Tregs usually secrete very low levels of IFNγ. Therefore, any alteration in IFNγ secretion in Tcon/Treg co-culture medium will indicate changes of Tcon function. We are performing experiments to establish and validate cytokine suppression assay(s). After 3-5 days co-culture of Tcon and Treg, the supernatant of the cultures are collected for cytokine analysis by Bioplex protein assay.
ii. While Bioplex assay is well suited to test batched Treg/Tcon culture supernatants, we will also establish real-time PCR which is very specific, quantitative, and sensitive. It can be applied to test individual samples.
iii. Multicolor FACS panel will be broadened in scope by examining the CFSE labelled Tcons for de novo expression of surface CD69, CD154 activation markers in parallel with intracellular IL-2 and IFNγ in response to APC. The putative Treg populations would inhibit these functional events and their percent suppression can be measured with titrated dose of Treg.

In summary, by using our newly established multi-color FACS panels and gating strategy, we found preliminary evidence for reduced proliferation of Foxp3+Helios+ central memory Tregs in the tested patients with acute GVHD (allogeneic responses). In the case of poorly controlled and clinically active autoimmune disease, we found a significant decrease in the Foxp3+Helios+ central memory Treg population. These findings suggest a positive correlation between the presence of Foxp3+Helios+ central memory Tregs above a threshold limit in achieving and sustaining immune tolerance. If these findings are confirmed in sufficiently large patient groups, these new findings could have significant clinical impact in the future. We may be able to

modulate the environment to promote survival and function of specific Treg subsets while in parallel we are developing methods to single cell clone and expand Tregs with favorable phenotype and suppressive function. Importantly, the preliminary dataset supported by CURE was obtained directly from non-manipulated patient samples to accurately reflect the actual state of human disease such as GVHD and/or autoimmunity.

Materials and Methods

Flow cytometric analysis of Treg phenotypes-for Aim I.
Peripheral blood mononuclear cells (PBMCs) were collected from the heparinized blood by standard Ficoll-Hypaque density gradient centrifugation. The immune phenotype of cells was tested by analysis on an 8 color FACS Canto II flow cytometer (BD Bioscience). The monoclonal antibodies used were anti-CD4-BV510, CD8-APC-H7, CD25-PE-Cy7, CD127-BV421, Foxp3-PE, Helios-APC, CD45RO-PerCP 5.5, CD62L-FITC, CD45RA-APC-H7, Ki67-PerCP-Cy5.5, Caspase 3-FITC from BD Bioscience. The lymphocytes were first gated in SSC/FCS, followed by gating on CD4 T cells, and the CD25+CD127low Treg was then selected. The central memory Treg was determined by quadratic gating on CD45RO vs CD62L on the Tregs. The proliferation vs apoptotic cells were distinguished by quadratic gating of Ki67 vs Caspase 3 on a giving population.

Treg isolation and culture -for Aim II.
The T cells were enriched from PBMNC by using EasySep® human T cell enrichment kit (Stem Cell Technologies). The CD4+/CD25+/CD127low/CD49d lowTregs were then isolated with EasySep® human CD4+CD25+CD127lowCD49d- enrichment kit (Stem Cell Technologies). Isolated Tregs were then cultured in XIVIVO 15 medium (Life Sciences) with 10% human serum.

TCRβ immunoscope -for Aim I.
The RNA was purified from cells with RNeasy Mini Kit (QIAGEN). Complementary DNA (cDNA) was synthesized by using Superscript II reverse transcripts and random hexamers rimer (Life Technologies) according to the manufacturer's instructions. PCR was performed by using Hotstar PCR Kit (QIAGEN). The PCR products were then run on an ABI Prism 3130 Genetic Analyzer (Life Technologies).

CFSE-labeled T cell proliferation assay - for Aim II.
T cells were labeled with CellTrace CFSE cell proliferation kit (Life Technologies) and cultured in XVIVO 15 with 5% human serum. On day 4, the cells were harvested and the division peaks of CFSE fluoresce were detected on FACSCanto II.

Treg suppression assay - for Aim II.
PBMNC were labeled with CFSE CellTrace kit (Life Technologies) and were cultured with Treg at ratio 1:1. On the day 4^{th}, both PBMNC and Treg were collected to determine inhibition of division peaks with FACSCanto II.

Figure Legends

Figure 1. **FACS gating scheme for Treg phenotypes.** Lymphocytes were identified in dot plots (a) by FSC vs SSC. Thereafter, CD4+ cells were gated (b). CD4+ T cells with CD25 positivity and CD127 low expression were gated next (c). The distribution of Foxp3+ and Helios+ cells in CD4+CD25^{high}CD127^{low} Tregs were analyzed by quadratic gating (d). The distribution of CD45RO+/CD62L+ (central memory) versus other phenotypes in each Foxp3/Helios population were analyzed by additional quadratic gating (e); intracellular Ki67 and Activated Caspase 3 expression (f) and CD45RA/Foxp3 (g) were also tested.

Figure 2. The comparison of the distribution for CD4+CD25+CD127low population in CD4+ cells (A) or for Foxp3+Helios+ central memory Treg in CD4+CD25+CD127low cells (B) in healthy donors, in patients with active autoimmunity or transplant recipients with or without GVHD post-HSCT.

Figure 3. **Characterization of Foxp3+Helios+ Tregs with the highest expression** of these transcription factors (A). Gating on the brightest dual positive Tregs to analyze expression of CD45RO+CD62L+ (B), and Ki67 (C); into "Sakaguchi region" II: CD45RA-Foxp3^{high} active Treg (D).

Figure 4. **Comparison of intracellular (A) Ki67 expression in Treg cells** of a patient with acute GVHD or (B) of a patient without GVHD. Absolute numbers of Ki67+ Treg/ml blood in (C) 5 patients without aGVHD vs 3 patients with a GVHD. (D) Contrasting values of absolute numbers of Ki67+ Foxp3+Helios+Treg/ml within the central memory phenotype in the same patient groups as in (C).

Figure 5. **Single Treg clone expansion** as reproduced by phase contrast inverted microscope at 20x after 7 days of culture (A) or at 12 days of culture (B).

Figure 6. Identification of single cell clonality by TCRβ qPCR in single cell cloning (B) in comparison with bulk unselected PBMNC in a healthy donor (A); or by using TCR immunoscope in single cell cloning (D), in comparison with PBMNC obtained from a healthy volunteer donor (C).

Figure 7. Treg suppression assay based on CFSE-labeled Tcon proliferation as detected by dilution of CFSE in dotplot (A) or division peaks in histogram represent successive generations of Tcon (B). Compared to Tcon alone (C and E) when Treg is added at 1:1 ratio, proliferation (D) and IFNε secretion (F) are both depressed in autologous Tcon cells.

Figure 1.

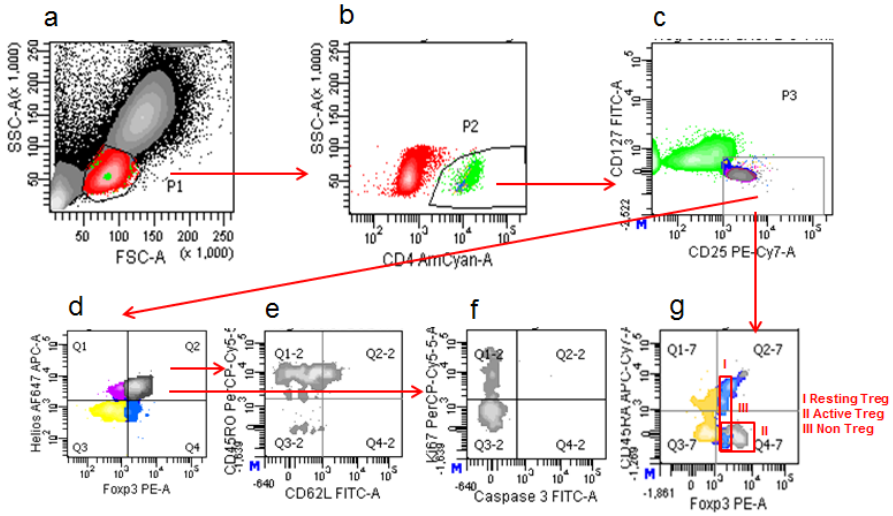


Figure 2.

A **B**

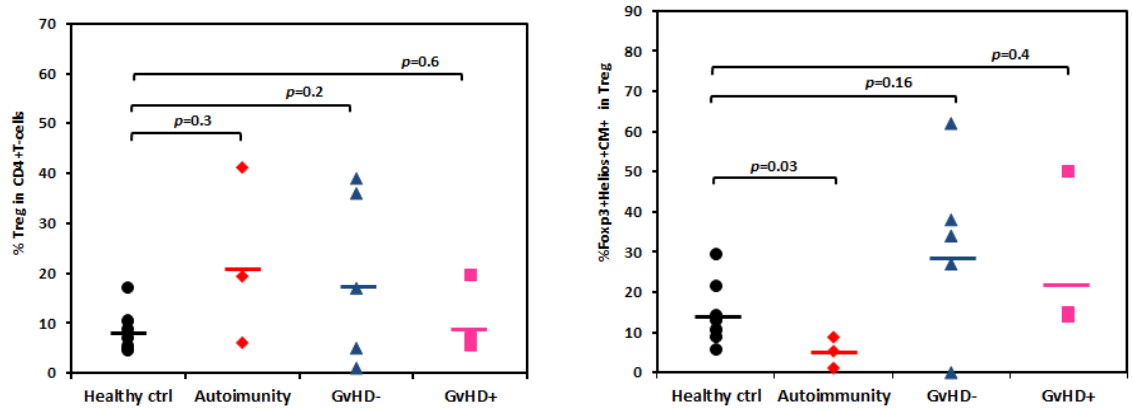


Figure 3.

A B C D

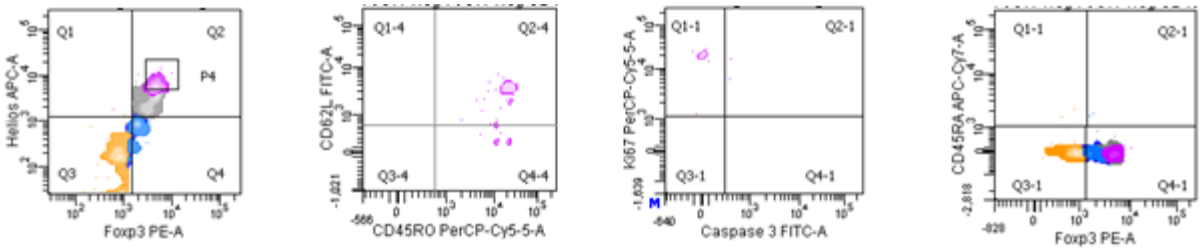


Figure 4.

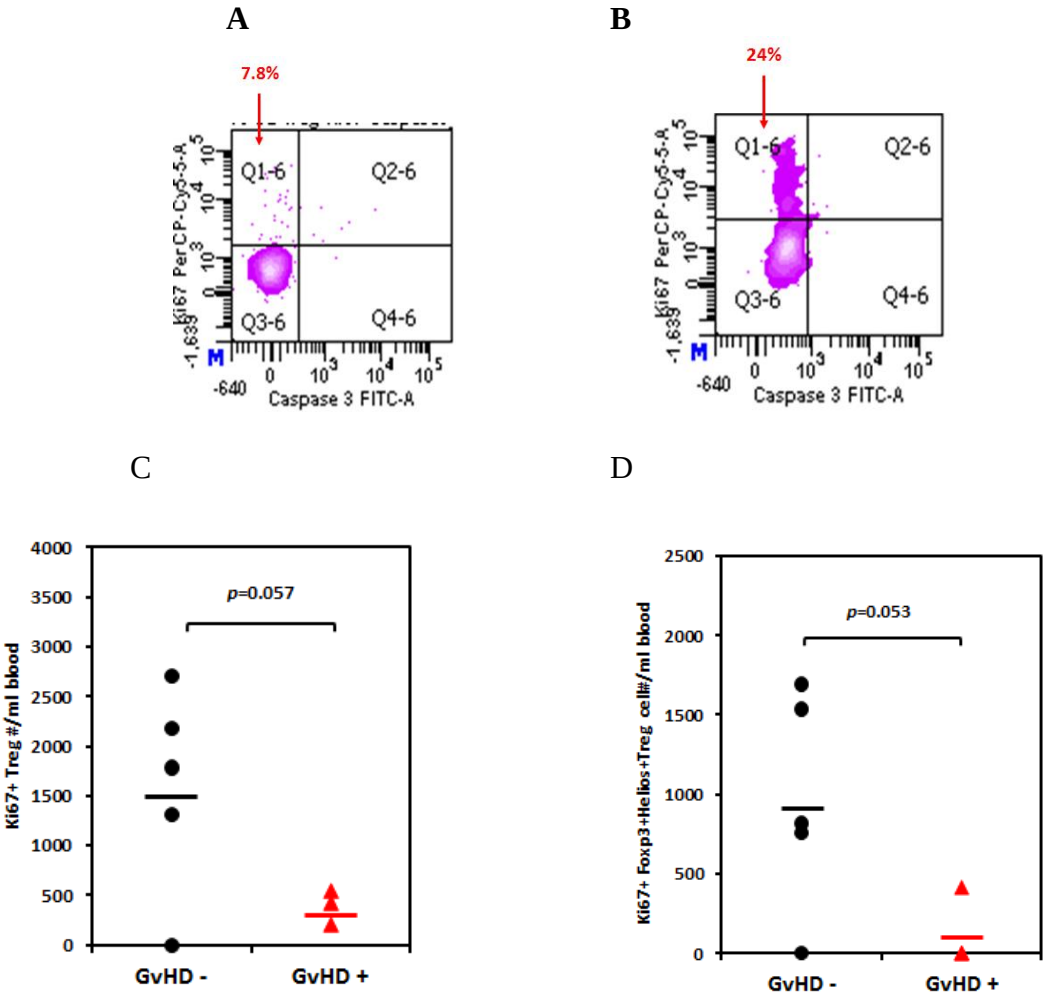


Figure 5.

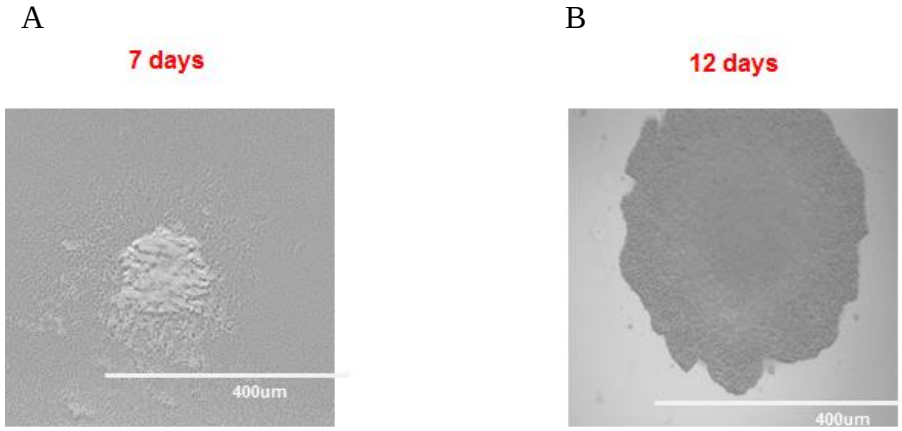


Figure 6.

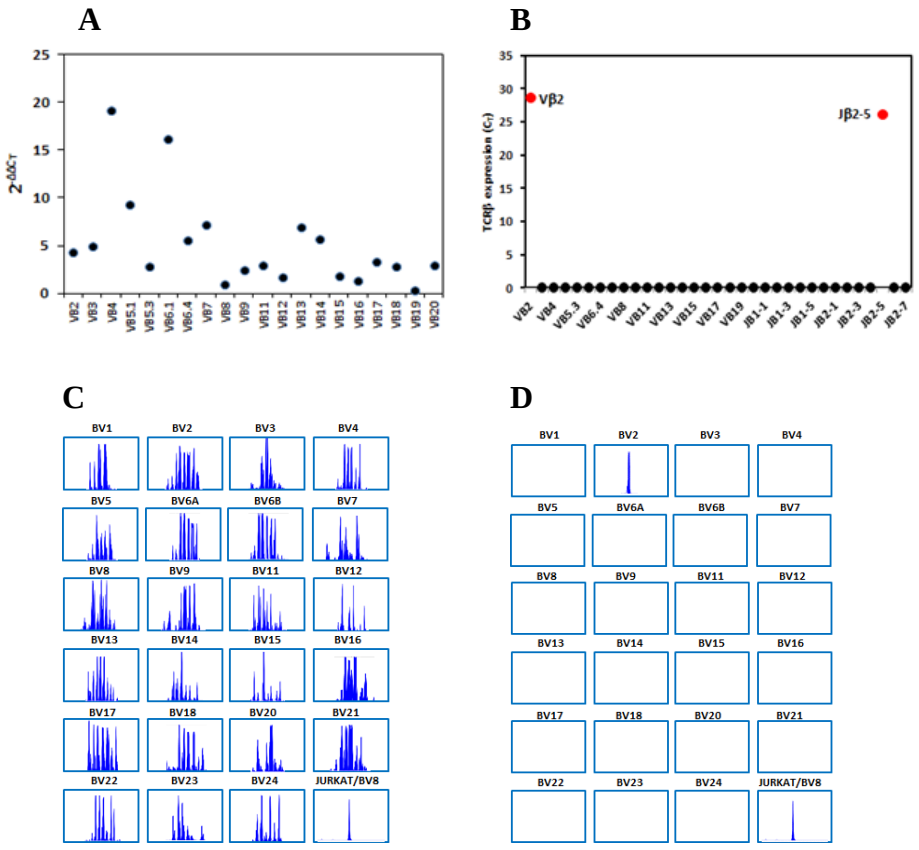
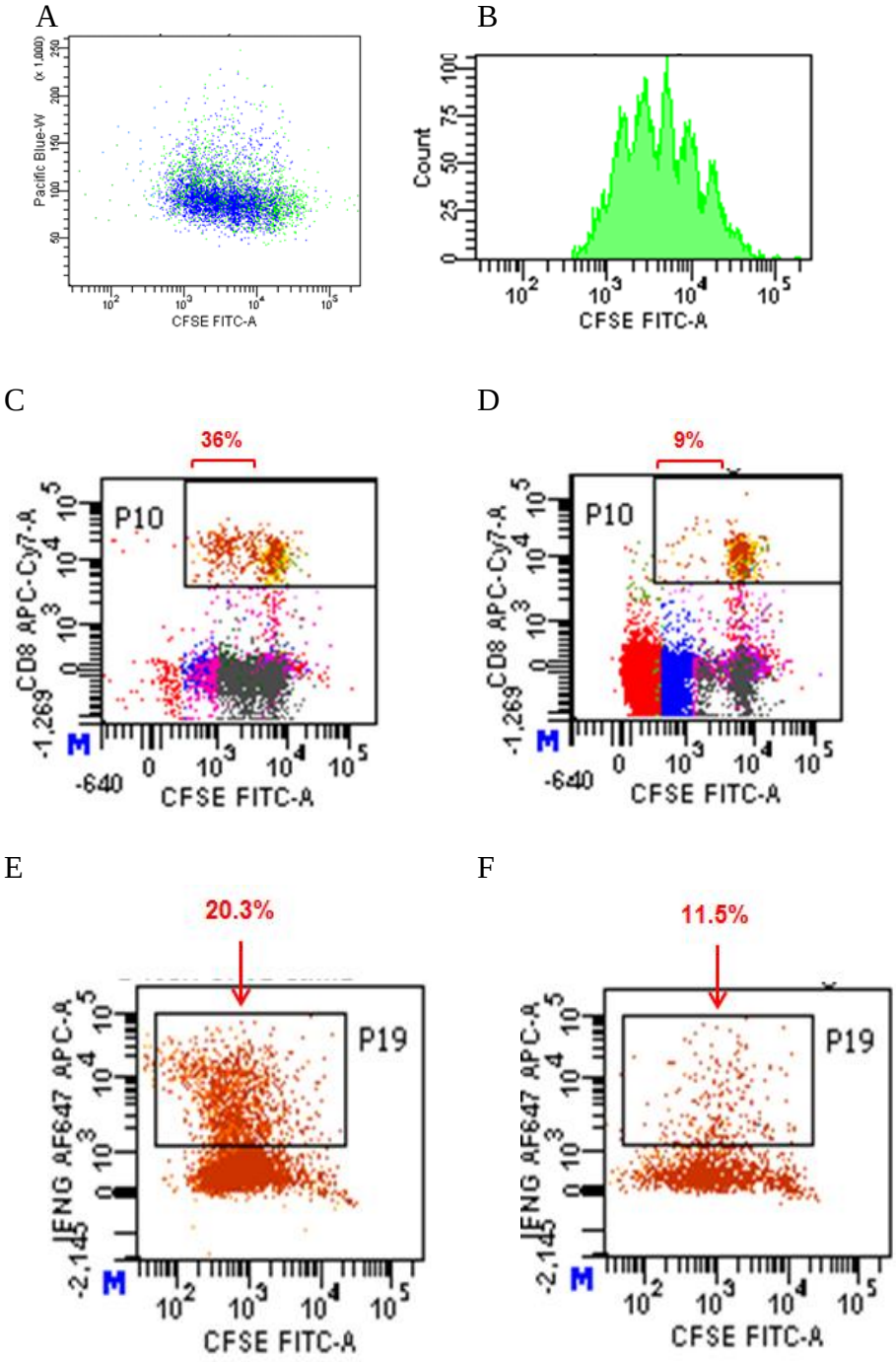

A
B
C
D
Vβ2
Jβ2-5
TCRβ expression (Cт)
BV1
BV2
BV3
BV4
BV5
BV6A
BV6B
BV7
BV8
BV9
BV11
BV12
BV13
BV14
BV15
BV16
BV17
BV18
BV20
BV21
BV22
BV23
BV24
JURKAT/BV8

Figure 7


A
Pacific Blue-W
CFSE FITC-A
B
Count
CFSE FITC-A
C
36%
P10
CD8 APC-Cy7-A
CFSE FITC-A
D
9%
P10
CD8 APC-Cy7-A
CFSE FITC-A
E
20.3%
P19
ENG AF647 APC-A
CFSE FITC-A
F
11.5%
P19
ENG AF647 APC-A
CFSE FITC-A

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
X____No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X_ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one

publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.				☐Submitted ☐Accepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X____ No_____

If yes, please describe your plans:

The following manuscripts will be published if they are confirmed with large data sets:

1. Deficiency of Foxp3/Helios co-expressing regulatory T-cells correlates with activity in autoimmunity. (an abstract was submitted to annual AAI meeting)
2. Inefficiency in proliferation of Foxp3/Helios co-expressing regulatory T-cells in those with GvHD post-blood and marrow transplantation.
3. Heterogeneity of regulatory T-cell single clones in healthy donors.
4. Characterization of regulatory T-cell clones in the patients with acute GvHD.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project.
Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis,

or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Our major findings so far are the lack of proliferative capacity for foxp3/helios co-expressing Treg in the patients with acute GVHD, and overall decrease of those Tregs in autoimmune diseases. If these very preliminary findings were to be confirmed, it may guide the treatment of GvHD by focusing on restoring the proliferative potential of Foxp3/Helios co-expressing Tregs as this subset may become a potential biomarker for onset of and activity autoimmunity.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes__X____ No_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

   Generation of regulatory T cell clones with superior function

b. Name of Inventor(s):

   Paul Szabolcs MD

   Xiaohua Chen PhD

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

   A new strategy in single regulatory T cell cloning from bulk Treg population or special sub-population, which also includes identifying single clones by TCRB immunoscope and TCRB real-time PCR.

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?

e. Yes_____ No X

If yes, indicate date patent was filed:

f. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?

g. Yes_____ No X
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

h. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No X

If yes, how many licenses were granted?_____

i. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No X

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes___X_____ No_____

If yes, please describe your plans:

Single cell cloning of regulatory T-cells

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Paul Szabolcs	Professor of Pediatrics and Immunology, University of Pittsburgh School of Medicine
eRA COMMONS USER NAME (credential, e.g., agency login) szabo001	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Pecs University School of Medicine, Pecs, Hungary	Transferred	1983	Medicine
Semmelweis University School of Medicine, Budapest, Hungary	M.D. *Summa cum laude*	1985	Medicine
Bellevue Hospital, New York	Residency	1987-90	Pediatrics
Cornell University Medical College, New York Hospital, Memorial Sloan Kettering Cancer Center, New York, NY	Fellowship	1990-93	Pediatric Hematology/Oncology
Sloan-Kettering Cancer Center, New York, NY	Postdoctoral Fellowship	1991-93	Molecular Biology
New York Hospital, Memorial Sloan-Kettering Cancer Center, New York, NY	Chief Fellow	1992-93	Pediatric Hematology/Oncology
Memorial Sloan-Kettering Cancer Center, New York, NY	Special Fellow	1993-94	Department of Pediatrics

1987 - 90 Intern, Resident, Department of Pediatrics, New York University-Bellevue Hospital Center, NY, NY
1989 - 90 Teaching Assistant, New York University Medical Center-Bellevue Hospital Center, NY,NY
1990 –93 Clinical Fellow, Pediatric Hematology-Oncology, Cornell University Medical College,(CUMC) New York Hospital/Memorial Sloan-Kettering Cancer Center (MSKCC), NY, NY
1992 –94 Chief Fellow, Pediatric Hematology-Oncology, MSKCC/CUMC
1994 –98 Instructor, Bone Marrow Transplant Service, Department of Pediatrics, MSKCC, NY
08/98-06/06 Assistant Professor Pediatrics, Stem Cell Transplant Program, Duke University Medical Center, Durham, NC
2001-2011 Assistant Professor of Immunology, Duke University Medical Center, Durham, NC
2006-2011 Associate Professor Pediatrics, Blood and Marrow Transplant Program, Duke University Medical Center, Durham, NC
2011-2014 Visiting Professor of Pediatrics, University of Pittsburgh School of Medicine
Chief, Division of Blood and Marrow Transplantation and Cellular Therapies,
Children's Hospital of Pittsburgh of UPMC
2014-present Professor of Pediatrics and Immunology, University of Pittsburgh School of Medicine

Other Experience and Professional Memberships

1986 – 87 Research Assistant, The Hospital of the University of Pennsylvania Cancer Center, Philadelphia, PA
8/91 - 2/93 Postdoctoral Fellow, Department of Molecular Biology Sloan-Kettering Institute, NY, NY
3/93 – 5/94 Guest Investigator, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, NY
1994 – 97 Research Associate, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, NY

1995+	American Society of Hematology (ASH)
1998+	American Society for Blood and Marrow Transplantation (ASBMT)
1998+	American Association of Immunologists (AAI)
1998+	Fellow, American Academy of Pediatrics
2004+	Center for International Blood and Marrow Transplant Research (CIBMTR): Immune Deficiencies/Inborn Errors Working Committee
2004+	Center for International Blood and Marrow Transplant Research (CIBMTR): Graft-vs-Host Disease Working Committee
2005+	International Society for Cellular Therapy (ISCT)
2006+	Society for Pediatric Research (SPR) Member
2009-14	Center for International Blood and Marrow Transplant Research (CIBMTR): Infection and Immune Reconstitution Working Committee – *Co Chair*
2010+	Clinical Immunology Society
2012+	University of Pittsburgh Cancer Institute
2013	American Pediatric Society

Honors and Awards:

1980	1st Prize for Critical Thesis, *Human globin genes,* Pecs University of Medical Sciences, Pecs, Hungary
1990	Elected *"Best Student of the Year"*, Pecs University of Medical Sciences, Pecs, Hungary
2000	Lisa Stafford Memorial Research Prize, Duke University Comprehensive Cancer Center, Durham, NC

C. Selected Peer-reviewed Publications

1. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, **Szabolcs P**, Bernstein SH, Magro CM, Williams AD, Hall RP, St Clair EW, Tedder TF. Characterization of a rare IL-10-competent B cell subset in humans that parallels mouse regulatory B10 cells. *Blood,* 2011, Jan 13;117:530-41 PMCID: 3031478
2. **Szabolcs P**. T-lymphocyte recovery and function after cord blood transplantation. *Immunol Res.* 2011 April, 49; 56-69. PMID: 21128006
3. Kamani, N. R., Kumar, S., Hassebroek, A., Eapen, M., Lerademacher, J., Casper, J., Cowan, M., Sanchez de Toledo, J., Ferster, A., **Szabolcs, P.,** Wingard, J. R., Horwitz, E., Filipovich, A. H. Malignancies after Hematopoietic Cell Transplantation for Primary Immune Deficiencies: A Report from the Center for International Blood and Marrow Transplant Research. *Biol. Blood and Marrow Transplantation,* 2011 epub May 20, PMID: 21658461.
4. Parikh S, **Szabolcs P**. Reduced-intensity conditioning (RIC) in children with nonmalignant disorders (NMD) undergoing unrelated donor umbilical cord transplantation (UCBT). *Biol Blood Marrow Transplant*. 2012 Jan; 18(1 Suppl):S53-5. PMID: 22226113
5. **Szabolcs P**, Burlingham WJ, Thomson AW. Tolerance after solid organ and hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2012 Jan; 18(1 Suppl):S193-200. PMID: 22226107
6. Bunin N, Small T, **Szabolcs P**, Baker KS, Pulsipher MA, Torgerson T. NCI, NHLBI/PBMTC first international conference on late effects after pediatric hematopoietic cell transplantation: persistent immune deficiency in pediatric transplant survivors. *Biol Blood Marrow Transplant*. 2012 Jan; 18(1):6-15 PMID: 22100979
7. Eapen M, Ahn KW, Orchard PJ, Cowan MJ, Davies SM, Fasth A, Hassebroek A, Ayas M, Bonfim C, O'Brien TA, Gross TG, Horwitz M, Horwitz E, Kapoor N, Kurtzberg J, Majhail N, Ringden O, **Szabolcs P,** Veys P, Baker KS. Long-term survival and late deaths after hematopoietic cell transplantation for primary immunodeficiency diseases and inborn errors of metabolism. *Biol Blood Marrow Transplant.* 2012 Sep; 18(9):1438-45. PMID: 22430083
8. Kanda, J, Chiou LW, **Szabolcs P**, Sempowski GD, Rizzieri DA, Long GD, Sullivan KM, Gasparetto C, Chute JP, Morris A, McPherson J, Hale J, Livingston JA, Broadwater, G, Niedzwiecki D, Chao NJ, Horwitz ME. Immune recovery in adult patients after myeloablative dual umbilical cord blood,

matched sibling, and matched unrelated donor hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* 2012 Nov 18. PMID: 22698485

9. Parikh SH, Mendizabal A, Benjamin CL, Komanduri KV, Antony J, Petrovic A, Hale G, Driscoll TA, Martin PL, Page KM, Flickinger K, Moffet J, Niedzwiecki, D, Kurtzberg, J, **Szabolcs P.** A Novel Reduced-Intensity Conditioning Regimen for Unrelated Umbilical Cord Blood Transplantation in Children with Nonmalignant Diseases. Biol Blood Marrow Transplant. 2013 Dec 1. pii: S1083-8791(13)00558-2. doi: 10.1016/j.bbmt.2013.11.021 [Epub ahead of print]. PMID: 24296492 [PubMed – as supplied by publisher]

ACTIVE

None