

Children's Hospital of Pittsburgh

Annual Progress Report: 2011 Formula Grant

Reporting Period

July 1, 2013 – December 31, 2013

Formula Grant Overview

The Children's Hospital of Pittsburgh received \$228,401 in formula funds for the grant award period January 1, 2012 through December 31, 2013. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

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.

Duration of Project

1/1/2012 – 12/31/2013

Project Overview

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 propose two specific aims.

Aim 1. Enumerate Tregs from 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 and without GVHD.

Aim 2. Design new functional assays to characterize and quantitate the biological profile and suppressive capacity of purified Tregs 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, costimulatory and other critical suppressor molecules while Tcon cells will be tested in parallel for evidence of receiving suppressive signals.

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Expected Research Outcomes and Benefits

Graft-versus-host-disease (GVHD) is the most clinically significant immune mediated disease after allogeneic blood and marrow transplantation (BMT). If prevented or successfully treated, GVHD will be followed by tolerance characterized by the functional and 'peaceful' co-existence of host and graft tissues in the absence of any immunosuppressive (IS) drugs. Despite prophylactic drug combinations, GVHD still develops in 20-60% of BMT recipients and can cause mortality in excess of 20% of all allogeneic transplant recipients notwithstanding significant morbidity and severe reduction in quality of life. There is preliminary evidence that rapid reconstitution of regulatory T Cells (Tregs) confers protection from GVHD in humans, nevertheless, there is a great gap of knowledge regarding their functional and biological features after transplant, beyond apparent numerical associations. In fact, the role of Tregs is far less well defined in human immune disorders compared to laboratory animal models. The proposed studies would identify and discover biological characteristics of Tregs that are essential for human transplant tolerance and thus new biomarkers of GVHD may be discovered. There are

several centers that have developed ex vivo expansion platforms towards human clinical therapy with remarkably little functional knowledge of the manufactured products. Our studies could also aid to better define investigational products that are intended for human cell therapy trials. Importantly, a new in vitro laboratory model of testing Treg characteristics and function could become a valuable tool for researchers in the fields of rheumatology and gastroenterology to monitor disease activity and develop prognostic algorithms for autoimmune connective tissue disorders such as systemic lupus, Crohn's disease, rheumatoid arthritis, systemic sclerosis, diabetes, and others.

Summary of Research Completed

Aim 1A. Enumerate Treg cell distribution

In our first progress report covering the period January 1 – June 30 2012, we presented our newly designed 8-color FACS panel to detect Treg phenotypes and identify Treg subsets. In our 2nd progress report reflecting activity for the year ending June 30, 2013 we described how we further optimized our FACS gating strategies to increase the sensitivity in detecting the relationship between Treg sub-populations and their functions, or their activation. We have examined 7 healthy donors to define the normal range of Treg sub-populations including single positive Helios+, single positive Foxp3+, double positive Foxp3+/Helios+, and double negative Foxp3/Helios. We also looked at central memory phenotypes in those subsets.

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, during this reporting period we have been able to test two more BMT recipients with GVHD, for a total of three tested so far. 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. 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.

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. 1B) amongst all CD4+ T cells (p2 in Fig. 1A) 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+ dual

positive 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. 3D), 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 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.

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

(Manuscript in preparation).

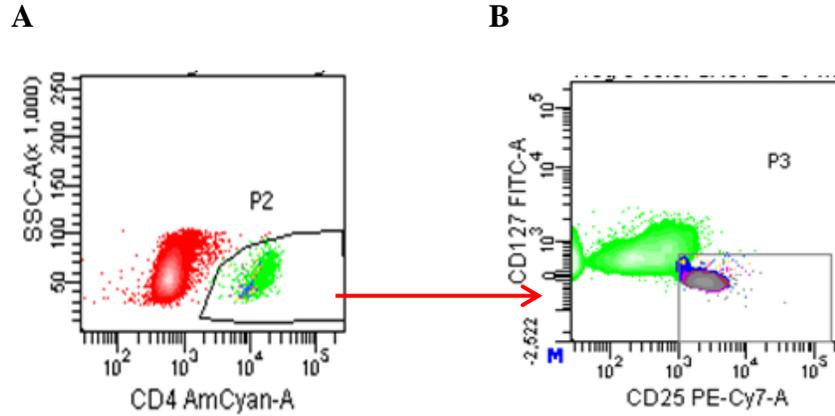


Figure 1. FACS gating scheme for Treg phenotypes. Lymphocytes were identified in dot plots by FSC vs SSC. Thereafter, CD4+ cells were gated (A). CD4+ T cells with CD25 positivity and CD127 low expression were gated next (B).

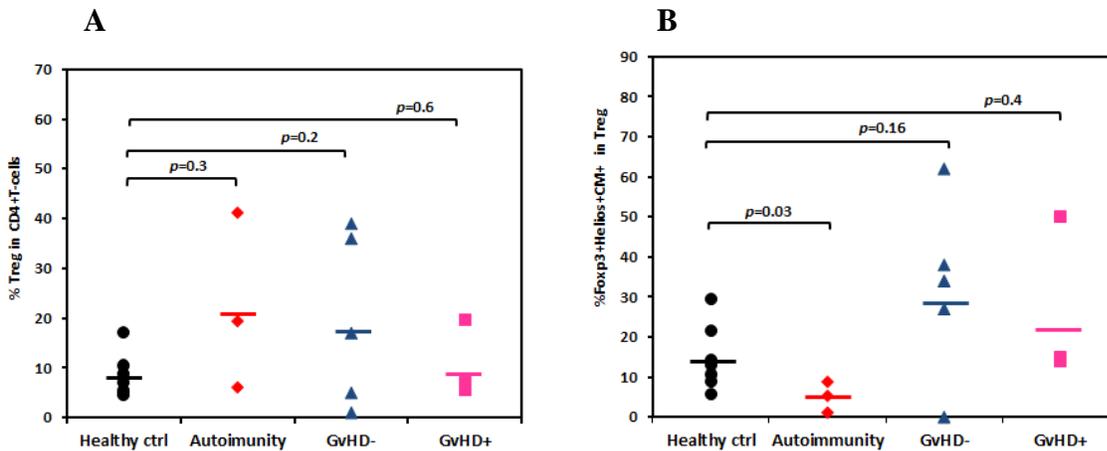


Figure 2. The comparison of the distribution for CD4+CD25+CD127 low population in CD4+ cells (A) or for Foxp3+Helios+ central memory Treg in CD4+CD25+CD127low cells (B) in healthy donors, in patients with active autoimmunity or transplant recipients with or without GVHD post-HSCT.

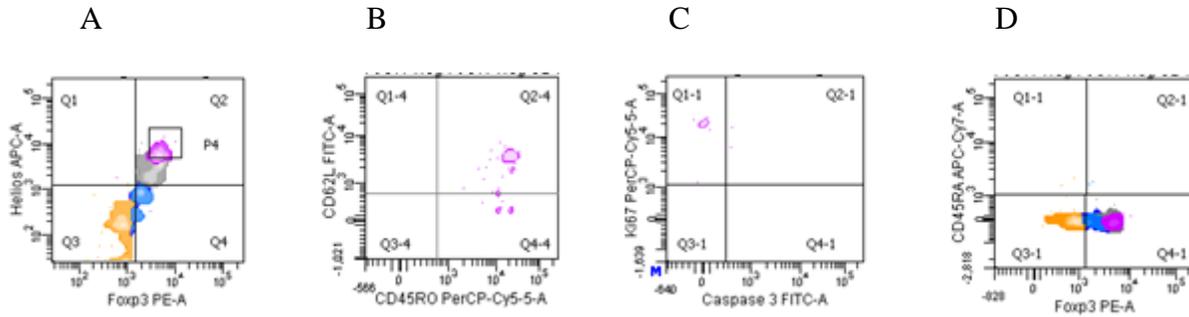


Figure 3. Characterization of Fcpx3+Helios+ Tregs with the highest expression of these transcription factors (A). Gating on the brightest dual positive Tregs to analyze expression of CD45RO+CD62L+ (B), and Ki67 (C); into “Sakaguchi region” II: CD45RA-Fcpx3^{high} active Treg (D).

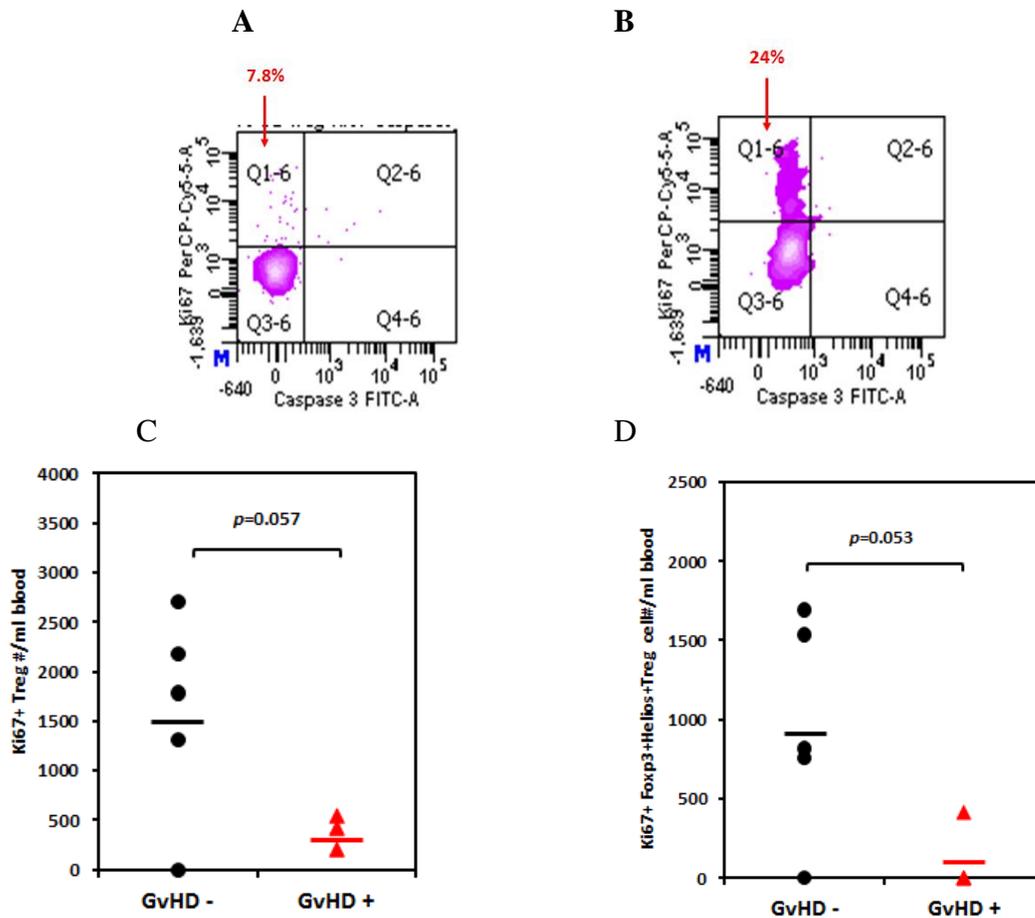


Figure 4. Comparison of intracellular (A) Ki67 expression in Treg cells of a patient with acute GvHD or (B) of a patient without GvHD. Absolute numbers of Ki67+ Treg/ml blood in (C) 5 patients without aGvHD vs 3 patients with aGvHD. (D) Contrasting values of absolute numbers of Ki67+ Fcpx3+Helios+Treg/ml within the central memory phenotype in the same patient groups as in (C).