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Investigating host versus donor T cell chimerism in cutaneous graft versus host disease

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INVESTIGATING HOST VERSUS DONOR T CELL CHIMERISM IN CUTANEOUS GRAFT VERSUS HOST DISEASE

by

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ABSTRACT

BACKGROUND: Graft versus host disease (GVHD) is a significant cause of morbidity and mortality following stem cell transplantation. Donor T cells are thought to be the main mediators of this disease, although we have recently identified that host T cells are present and active during acute GVHD suggesting contributions from both donor and host T cells. Whether both donor and host T cells can survive GVHD and coexist harmoniously after disease resolves is unknown.

OBJECTIVE: The goals of this thesis are two-fold: (i) to study T cell chimerism in post-GVHD skin and (ii) to understand what effect, if any, treatment has on T cell chimerism in skin.

METHODS: Acute GVHD and post-GVHD skin samples were obtained from male patients that had been transplanted with female donor cells. Chimerism was assessed using fluorescence in situ hybridization for the X and Y chromosomes concurrently with immunofluorescence staining for CD3, a T cell marker. Regulatory T cells were stained by immunofluorescence for CD3, CD4 and Foxp3. Medical record data was collected for all patients.

RESULTS: We found that the percent of host T cells decreased significantly after resolution of acute skin GVHD compared to during active acute skin GVHD in

skin samples obtained from five male patients that had been transplanted with female donor cells. The T cell composition in these patients in post-GVHD skin was primarily donor. We identified chimerism shifted toward donor T cells in patients treated with systemic steroids and this correlated with an increased number of donor T cells infiltrating into skin rather than a decrease in the number of host T cells in skin. With regard to frequency of Tregs, there was no significant difference between the group that had been treated with systemic steroids prior to biopsy and the group that had not.

CONCLUSIONS: We discovered that donor chimerism predominates in post-GVHD skin and in active skin GVHD of patients who received systemic steroids, suggesting a role of donor cells in acute GVHD resolution. We were not able to identify a higher frequency of regulatory T cells in the treatment group. It is possible that the Treg recruited to skin by steroid treatment is Foxp3 negative, and therefore missed by our staining approach. The use of another marker is required for future studies.

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LIST OF ABBREVIATIONS

APC ..... Antigen Presenting Cell

FFPE ..... Formalin-fixed paraffin-embedded

FISH ..... Fluorescence in situ hybridization

GC ..... Glucocorticosteroid

GI ..... Gastrointestinal

GVHD ..... Graft versus host disease

HLA ..... Human Leukocyte Antigen

HSCT ..... Hematopoietic stem cell transplant

IF ..... Immunofluorescence

IL ..... Interleukin

MHC ..... Major Histocompatibility Complex

NIH ..... National Institute of Health

NK ..... Natural killer

NO ..... Nitrous oxide

Th ..... Helper T Cell

TNF ..... Tumor Necrosis Factor

Treg ..... Regulatory T Cell

INTRODUCTION

Graft versus host disease (GVHD) is a major complication and cause of morbidity and mortality among patients who receive allogeneic hematopoietic stem cell transplantation (HSCT). It is a severe reaction thought to occur when donor T cells mount an immune response against the immunocompromised host, causing a reaction in various organs such as the skin, liver, lungs, and gastrointestinal (GI) tract (Ferrara 2009). The incidence and severity of GVHD is directly associated with the level of human leukocyte antigen (HLA) mismatch between the donor and recipient (Atkinson 1990; Loiseau 2007). However even of the HLA-matched HSCTs, about forty percent of recipients develop systemic acute GVHD, requiring treatment with high dose steroids (Ferrara 2009). Thus both HLA and non-HLA factors play a role in the development of GVHD. While it has been widely believed that donor T cells mediate GVHD, Divito et al recently identified the presence of a substantial population of activated host T cells in skin and gut during acute GVHD, suggesting a possible contribution of host T cells to disease (Divito 2020, Accepted for Publication). This interesting dichotomy between host and donor T cells in the pathogenesis and resolution of GVHD requires further investigation.

Clinical features of acute and chronic GVHD

Historically, acute GVHD was classified as GVHD that occurs within the first one hundred days following transplant. However, recently the National

Institute of Health (NIH) proposed a new GVHD classification. Their definition includes cases of acute GVHD occurring within one hundred days of transplantation or persistent, recurrent or late acute GVHD that occurs after one hundred days. There is also an overlap syndrome (includes features of both acute and chronic GVHD) (Filipovich 2005; Griffith 2008; Jagasia 2015). Acute GVHD primarily affects the skin but also affects the GI tract, liver, lungs and can extend to the eyes, mouth and genitals. A characteristic feature of acute GVHD in the skin is a pruritic maculopapular rash. Pathologically, apoptosis at the base of the epidermis is characteristic. Other findings are dyskeratosis, exocytosis of lymphocytes, satellitosis (lymphocytes adjacent to dyskeratotic epidermal keratinocytes), and perivascular lymphocytic infiltration of the dermis (Ferrara 2009; Jamil 2015). GI tract involvement of acute GVHD typically presents as diarrhea but can also include vomiting, anorexia, abdominal pain, or a combination when severe. Radiologically, luminal dilation with thickening of small bowel wall and air or fluid levels suggestive of ileus are found. Histological findings are patchy ulcerations, apoptotic bodies in the base of crypts, crypt abscesses, and loss and flattening of surface epithelium (Ferrara 2009). Liver disease caused by GVHD, like skin and gut disease caused by GVHD, is difficult to distinguish from other causes related to the bone marrow transplantation such as veno-occlusive disease, toxic drug effects, viral infection, sepsis, or iron overload (Ferrara 2009). Though biopsies are rarely taken due to thrombocytopenia in hepatic GVHD patients, histological findings would be

endothelialitis, lymphocytic infiltration of the portal areas, pericholangitis, and bile duct destruction (Choi 2005; Snover 1984). Severity of acute GVHD is staged according to the clinical status of the patient and extent of individual organ involvement, ranging from stage 1 (mild) to stage 4 (very severe), which then are combined to yield an overall grade (Glucksberg 1974).

Chronic GVHD is more autoimmune in nature- its presentation involves various organs such as skin, GI tract, liver, lungs, mouth, eyes, nails, female genitalia, muscles, fascia, joints, kidneys, marrow, and heart. Based on the NIH classification, the diagnosis of chronic GVHD requires at least one diagnostic clinical sign of chronic GVHD present such as poikiloderma or the presence of one distinctive manifestation such as keratoconjunctivitis sicca that is confirmed by a biopsy or relevant test (Filipovich 2005). Clinical signs of chronic GVHD are first seen in the buccal mucosa. The greatest risk factors for chronic GVHD are old age of recipient and history of acute GVHD. Thus, prevention strategies for acute GVHD are also relevant for chronic GVHD. While the focus of this thesis is on acute GVHD, it is important to note that there is another type of graft versus host disease which should be differentiated due to its separate pathogenesis and presentation.

GVHD treatment

Steroids are the gold standard for treatment of acute GVHD. Topical steroids are usually sufficient to treat mild stages of GVHD, but in severe cases

systemic steroids are often administered in high doses. Extracorporeal photopheresis and blocking jak/stat signaling are other treatment options for acute GVHD. Administering immunosuppressants chronically is dangerous and can result in death from infection or malignancy recurrence (Ferrara 2009).

Pathophysiology of GVHD

Acute GVHD is a severe, inflammatory process thought to be mediated by mature donor lymphocytes. Studies have also shown the importance of the microbiome and intestinal epithelium in the pathogenesis of acute GVHD (Qayed 2016; Teshima 2016).

The pathophysiology of acute GVHD is classically defined by three phases: an afferent phase, an efferent phase, and an effector phase (Ball 2008). In the afferent phase, activation of antigen presenting cells (APCs) occurs before transplant, from the underlying disease and HSCT conditioning regimen. Damaged host tissues respond by producing proinflammatory cytokines like TNF-α and IL-1 from macrophages. Activated macrophages produce chemokines that activate neutrophils (Zhang 2016). These proinflammatory cytokines increase expression of MHC and adhesion molecules on APCs, enhancing the APCs' antigen-presenting capacity.

Donor T cells that recognize the APCs and get activated (Blazar 2012; Paczesny 2010) differentiate into Th1 cells and release proinflammatory cytokines that mediate GVHD (Carlson 2009). Th2 cells are also involved in

GVHD pathogenesis. Severity of GVHD is affected by the proportion of naïve cells maturing along Treg, Th1, Th2, or Th17 phenotypes (Henden 2015). An increase in secretion of proinflammatory cytokines by Th1 and Th17 cells has been associated with an unbalanced Treg loss (Chen 2013). Treg percentages and Foxp3 expression is significantly lower in severe acute GVHD patients than in patients without GVHD. Treg can also reduce the severity of and prevent GVHD in murine/rodent models. The role of Treg in GVHD resolution will be discussed further.

The second phase of GVHD is the core of the graft versus host reaction and is characterized by the activation of effector cells. Donor T cell activation further increases the expression of MHC and adhesion molecules, chemokines, and the expansion of host-specific cytotoxic donor CD8 T cells and CD4 T and B cells (Zhang 2016).

The effector phase involves a cascade of cellular inflammatory mediators (NK and cytotoxic T cells) and soluble inflammatory agents (TNF-a, interferon gamma, IL-1, and NO) that work together to further promote inflammation and target tissue destruction (Morris 2013).

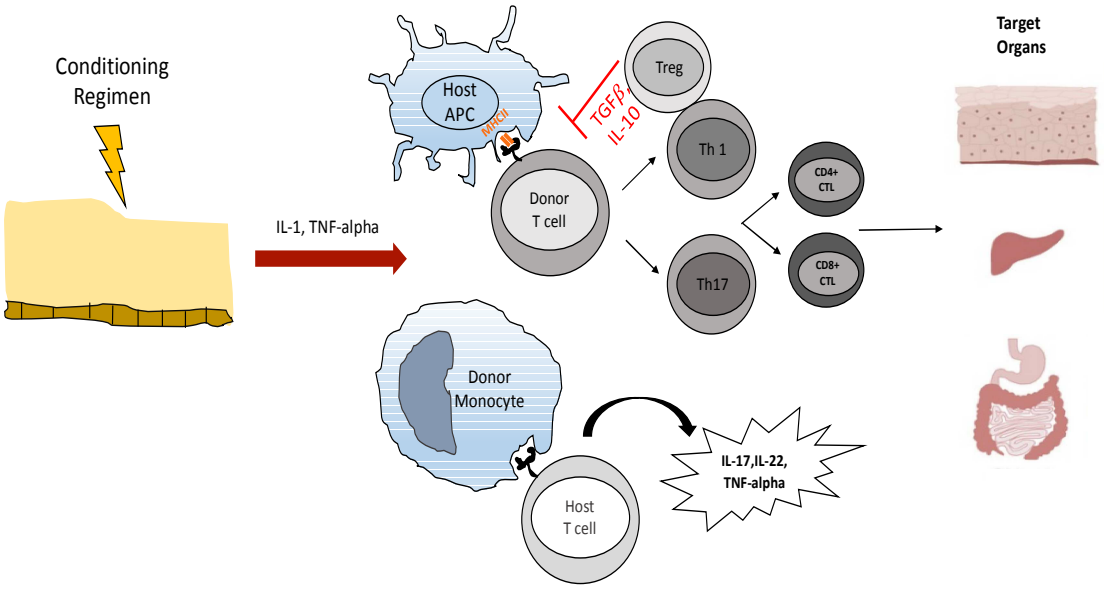


Figure 1. Steps in GVHD Pathogenesis. The conditioning regimen leads to the damage of host tissues, leading to the release of pro-inflammatory cytokines. These cytokines activate host antigen presenting cells, which activate mature donor T cells. While the above shown mechanism is what is currently the widespread understanding in the literature, the mechanism shown below is that suggested by the discovery of Divito et al- showing that donor APCs and their interaction with host T cells were also found to play a role in the pathogenesis of disease. T cells proliferate and differentiate into Th1 and Th17 cells, which activate CD4 and CD8 cytotoxic T lymphocyte and natural killer cells that mediate tissue damage. Effector T cells along with pro-inflammatory cytokines migrate to and damage the target organs.

T cell chimerism

In immunology, chimerism is defined as “the presence of two or more genetically distinct cell populations (from donor and recipient) in the same organism, as occurs following HSCT (Schroeder 2011). While the role of donor T

cells has been clearly identified and defined in GVHD pathophysiology, the role of host T cells is less well understood. Divito et al found that T cell chimerism in the skin and gut, does not reflect T cell chimerism in the blood (Divito 2020, Accepted for Publication). Their findings showed that host T cells survive HSCT conditioning regimens and are present and active in the skin during acute GVHD, suggesting their potential role in the pathophysiology of disease. Around fifty percent of the T cells in skin during active acute GVHD were of host origin.

The first experiment of this thesis will be a follow-up study of this paper aiming to reveal what happens to T cell chimerism in the skin after resolution of GVHD. Looking at the chimerism of T cells in the skin during active GVHD compared to post-GVHD can give us insight on the process involved in resolving disease.

Regulatory T Cells and their role in preventing GVHD

Understanding the mechanisms of the cell types and subsets involved in GVHD may provide insight to develop better therapeutic strategies (Beres 2013). I will focus on regulatory T cells, based on data presented below. The primary, originally defined function of regulatory T cells (Treg) was to prevent autoimmune diseases by establishing and maintaining self-tolerance. However, various other functions of Treg are now recognized such that they regulate the effector phase of the immune response (Corthay 2009).

The subsets of CD4+ regulatory T cells are natural and induced Treg cells. Natural Tregs develop in the thymus and upregulate Foxp3 during negative selection when they recognize self-antigen rather than undergoing clonal deletion (Beres 2013). They are responsible for maintaining immune homeostasis and tolerance to self antigen by inhibiting self reactive T cells in the periphery (Beres 2013). Induced Tregs are generated when conventional T cells are activated in the presence of the cytokines TGF-β and IL-2, resulting in the upregulation of Foxp3. They can also be induced independent of TGF-β. While their role is less well understood, induced Tregs are thought to be important for regulating peripheral T cell activation during infection and mediating the contraction phase of the immune response. They can be generated in vitro by activating naïve T cells with either anti CD3 or anti CD28 antibodies in the presence of TGF-β and IL-2. More work needs to be done to define the precise roles and extent of these two Treg subsets in regulating immune responses, however a study in murine models has suggested that the two subsets act in a complementary fashion to reduce inflammation (Beres 2013).

While CD25 is the classical marker for regulatory T cells, Foxp3 was thought to be the phenotypic marker whose expression is both necessary and sufficient for their suppressive function. Foxp3 was thought to be essential for immunosuppressive Treg differentiation, and the amount of Foxp3 protein in Tregs to be critical for their suppressive function (Devaud 2014). Although Foxp3 is more difficult to visualize because it is an intracellular marker and requires

fixation and permeabilization, this is the preferable marker for Treg (Le Texier 2017), and so we used it in our studies for this thesis. Furthermore, CD25 is problematic as a marker to define Treg because it is also expressed by activated T effector cells (Xin Chen 2011). Newer studies have identified a unique Foxp3 negative Treg population. This challenges the use of Foxp3 as a definitive marker for Treg, as not all of them express it. This will be discussed further in the following sections of this thesis.

Various studies have established a correlation between the amount of regulatory T cells and the risk/severity of acute GVHD. They have also suggested the potential for their use in therapeutics. Regulatory T cells that are CD4+(CD25+)Foxp3+ play a vital role in the regulatory control in graft versus host reactions mediated by both alloreactive and autoreactive lymphocytes and help to establish donor anti-host as well as self tolerance (Hess 2006). Rezvani's study tells us that the Treg content of the donor graft is important in determining GVHD severity (Rezvani 2006). Rezvani et al quantitated the coexpression of Foxp3 and CD4 T cells in 32 donor stem cell transplants infused into HLA matched siblings and examined the incidence of GVHD in their recipients. High CD4+Foxp3+ T cell count in the donor was associated with a reduced risk of GVHD. His study also looked at regulatory T cells during immune reconstitution and found that a low CD4+Foxp3+ T cell count early after stem cell transplant (day 30) was associated with an increased risk of GVHD. The donor CD4+Foxp3+ T cell count was significantly higher for patients who did not

develop GVHD compared with patients who developed GVHD (though there was no significant difference between two groups of patients who had different severity grading of GVHD). A proportion of the CD4+Foxp3+ Treg cells were found to be CD25low, further emphasizing the shortcomings of CD25 as a marker of Treg activity. This study and its elucidation of the role of the number of donor Tregs in predicting GVHD has serious implications for potential treatments, as their manipulation can play a role in preventing or reducing the severity of GVHD (Rezvani 2006).

Regulatory T cells have been shown to suppress acute graft versus host disease after allogeneic bone marrow transplantation (Hoffman 2002). Hoffman showed that CD4+CD25+ T cells isolated from donor spleen or bone marrow are potent inhibitors of the acute GVHD response in mice (Hoffmann 2002). Similarly, many studies have identified a decreased frequency of Treg cells in the peripheral blood of patients with high clinical grades of acute GVHD compared to patients with low grade or no GVHD (Beres 2013).

One paper by Chen et al identified the absence of CD4+Foxp3+ regulatory T cells in chronic GVHD patients (Xiao Chen 2007). This study demonstrated that autoimmunity, which develops as a consequence of GVHD, is attributable to donor-derived CD4+ T cells with Th1 and Th17 cytokine phenotypes, which emerge as a result of the disproportionate loss of CD4+CD25+Foxp3+ cells, unleashing Th1 and Th17 cells to expand, thus releasing proinflammatory cytokines and causing pathological damage. While the topic of this thesis is not

chronic GVHD, this paper confirms the role of regulatory T cells in protecting (mice) from developing autoimmunity, and identified that acute GVHD results in significant reduction in the ratio between CD4+Foxp3+ to CD4+Foxp3- T cells in the spleen and GVHD target tissues. The implications of this, for the purpose of this thesis, are that during acute GVHD there is not an adequate amount of Foxp3+ Treg cells (thus not enough suppression), but when treated we hypothesize that the amount of Foxp3+ regulatory T cells increase.

Impact of glucocorticosteroids on regulatory T cells

Glucocorticosteroids are widespread, effective drugs for transplantation and autoinflammatory diseases, however, the exact mechanisms of their immunosuppressive properties are not well understood, specifically their impact on Treg cells. Some studies have investigated the association between glucocorticosteroid treatment and enhancement of Foxp3+ regulatory T cells, which suppress the immune response.

Chen found that a glucocorticosteroid, dexamethasone, increased the proportion of Treg cells to T effector cells by suppressing IL-2 activation. Though IL-2 is important for the production of Treg cells, it is also important for the generation of T cell mediated immunity (Xin Chen 2006). This could be important therapeutically if it could be discovered how to use IL-2 to expand Treg cells while restraining the activation of T effector cells. Unexpectedly, they found that when dexamethasone was administered alone, the percentage of CD4+Foxp3+

T cells actually decreased, while IL-2 treatment alone caused an increase in Treg percentages. When used in combination, they found that dexamethasone further amplified IL-2's capacity to expand CD4+Foxp3+ T cells. They tested the effect of this combination on Treg cells in an experimental autoimmuno encephalomyelitis (EAE) murine model and found that they failed to suppress the proliferation of CD4+CD25- T cells, and pretreatment with dexamethasone and IL-2 increased the proportion of CD4+Foxp3+ cells and restored function of splenic CD4+CD25+ T cells and inhibited development of EAE (Xin Chen 2006). The implications of this study are that the combination of glucocorticoids and IL-2 could provide a novel approach for GVHD treatment or the treatment of autoimmune diseases. For the purposes of our project, this paper actually was not able to show that glucocorticoid treatment alone increased the amount of Treg- however the implications of this study may be clinically relevant.

Glucocorticoids were found to increase Foxp3 expression of regulatory T cells in patients affected by asthma. In a study by Karagiannidis et al, CD4+ T cells from healthy donors, and glucocorticoid treated asthma patients were isolated and their expression of Foxp3, IL-10, and TGF-β was determined (Karagiannidis 2004). They found that Foxp3 mRNA expression was significantly increased in asthmatic patients receiving inhaled glucocorticoid treatment, systemic glucocorticoid treatment, or both.

Glucocorticoids were also shown to restore the impaired suppressive function of regulatory T cells in patients with relapsing multiple sclerosis. In a

study by Xu et al, 14 patients relapsing from multiple sclerosis were treated with high dose IV injection of glucocorticoid, the effects of GC treatment on Treg function were evaluated ex vivo and in vitro compared to 20 healthy controls. Treg function was significantly enhanced after 5 days of GC treatment, and there was a trend towards increasing CD4+CD25+Foxp3+ T cells and IL-10 secretion with GC treatment when compared to healthy controls (Xu 2009).

A similar prospective study done on seventeen patients with active systemic lupus erythematosus found that patients taking oral prednisone had an increased proportion of circulating regulatory T cells than those who were not. This study found that high intravenous dose of methylprednisolone induces a rapid, transient increase in circulating Tregs, which may be an important part of methylprednisolone's preventive effect on subsequent flares in systemic lupus erythematosus (Mathian 2015).

While these papers show the positive effect of steroids on Tregs in the presence of autoimmune diseases, there is limited data on the effect of glucocorticoids on Tregs in the context of solid organ transplantation. Because steroid treatment causes various side effects, there have been many clinical efforts to minimize glucocorticoid therapy in transplant patients and it has actually been demonstrated that low dose IV methylprednisolone therapy has the same effect on renal graft rejection outcomes as high dose IV treatment. Seissler's study examines the effect of low dose methylprenisolone on the composition of the Treg population in renal transplant patients. This study found that

methylprenisolone has the ability to enhance the suppressive activity of the Treg pool by increasing the percentage of highly differentiated and highly suppressive HLA-DRhighCD45RA- Tregs (Seissler 2012).

One paper tried to investigate the possible mechanism by which glucocorticoids increase Treg cell frequency. They investigated glucocorticoid-induced leucine zipper (GILZ), which is a protein induced by glucocorticoids, which promotes Treg cell production. They found that in mice, overexpression of GILZ increased the number of regulatory T cells, while deficiency of GILZ impaired the generation of peripheral regulatory T cells associated with inflammation. This study discovered that GILZ is required in order for glucocorticoids to induce TGF-β and Foxp3, and it also enhances TGF-β signaling by binding to and promoting Smad2 phosphorylation and activating Foxp3 expression. The results of this study established a link between the GILZ mechanism and the TGF-β dependent Treg cell production by glucocortiocids (Bereshchenko 2014).

Finally in regards to the effects of glucocorticosteroids on skin, Stary et al. performed a study whereby nickel allergic patients were exposed to nickel for 48 hours followed by treatment with 40mg of prednisone for 10 days . This was done twice, with 30 days separating the two epidermal patch tests, and skin was assessed each time. After the second epidermal patch test, the prednisone group had reduced skin inflammation and had higher frequency of Foxp3 (Stary 2011). It was established that this response was Foxp3 specific because there was no

increased frequency of CD45, a generic leukocyte marker, in the treatment group post treatment with prednisone.

While these studies shine light on potential mechanisms by which glucocorticosteroids suppress an immune response, it remains important to elucidate their association with Tregs, especially in the context of graft versus host disease resolution and in the context of host versus donor T cell chimerism.

SPECIFIC AIMS

The purpose of this thesis is to understand T cell chimerism in the skin during and after resolution of acute GVHD. The first aim was to compare T cell chimerism in the skin during and after resolution of acute GVHD. The second aim was to investigate the effect of systemic steroids on host versus donor T cell chimerism and Treg cells.

METHODS

Patients

Subjects were selected from a database of adult male patients transplanted with female donor cells who underwent skin biopsy for acute GVHD at Brigham and Women's Hospital/Dana Farber Cancer Institute. Medical records were reviewed to confirm that each patient was diagnosed clinically with acute skin GVHD, and each patient's skin biopsy was read by an experienced dermatopathologist as consistent with GVHD. Samples from any patients with underlying T cell malignancy, history of prior transplant, or with limited available tissue sample were excluded.

Patients underwent skin biopsies for clinical purposes. As such, biopsies were obtained at variable time points after disease onset, so some biopsies were collected after initiation of systemic immunosuppression. In addition, 5 patients underwent additional skin biopsy for unrelated clinical reason (no active skin GVHD) after their acute skin GVHD had resolved.

Patient medical record data were collected including age, underlying diagnosis, timing between transplant and treatment, time (if any) between treatment and biopsy, prior therapies, conditioning regimen, GVHD prophylaxis, and GVHD treatment. Pathology reports were also read from each biopsy. This study was approved Institutional Review Board of the Partners Human Research Committee.

Fluorescence in situ hybridization-Immunofluorescence (FISH-IF) on FFPE tissue

FFPE skin sections 5-6 micrometers thick were baked, deparaffinized, rehydrated, and treated with sodium citrate 10mM pH 6 antigen retrieval buffer for 30 minutes at 96 degrees Celsius, then treated with pepsin. FISH probes for X and Y chromosomes (Abbott Molecular) were hybridized overnight at 37 degrees Celsius, requiring denaturation at 94 degrees Celsius prior to hybridization. Skin sections were washed after hybridization and then blocked for non-specific protein binding. The skin sections were then stained for mouse anti-human CD3 (Leica), then with anti-mouse IgG AF647 (ThermoFisher Scientific). All sections were counterstained with DAPI. A Mantra Quantitative Pathology Workstation was used to image the tissue and InFORM software (PerkinElmer) was used for analysis.

Quantification of Percentage T Cell Chimerism

Images were taken from multiple high-power fields (defined as an image taken at 400x) of each patient sample using the Mantra imaging system. Using the Inform analysis software, total T cells were manually identified and counted based on the CD3 T cell marker. Of the CD3 positive cells, the number of female or male T cells was manually counted based on the presence of a Y chromosome stain or two X chromosome stains. If the stain was not clear enough to be determined, it was not counted as identifiable. Of the identifiable

cells, the percentage of male and female cells was calculated for each image and then averaged for all the images taken for each patient sample. The percentage of host T cells from the biopsies during acute GVHD was compared to the percentage of host T cells from the biopsies of post-GVHD skin.

Immunofluorescence

FFPE skin samples were baked, deparaffinized, rehydrated, then exposed to Tris EDTA pH 9 antigen retrieval buffer at 96 degrees Celsius for 50 minutes, and were blocked for non-specific protein binding with TBS + 5% Normal Donkey Serum. An antibody panel was created and optimized to stain for regulatory T cells against CD3, CD4, and Foxp3 markers. We used rabbit IgG CD4 (Epitomics Ep204, 1:100 dilution), Rat IgG1 CD3 (BioRad CD3-12, 1:200 dilution), and mouse IgG1k Foxp3 (EBioscience 206D, 1:50 dilution) as our primary antibodies, and then applied donkey anti-rabbit IgG AF 488 (Invitrogen R37118, 1:500 dilution), donkey anti-rat IgG AF 594 (Invitrogen A-21209, 1:500 dilution), and donkey anti-mouse IgG AF 647 (Invitrogen A-31571, 1:200 dilution). The optimal dilutions were experimentally determined. Images were taken using the Mantra Imaging System of multiple high-power fields (400x), and frequency and percent of Tregs was calculated by manual analysis/counting using Inform software.

Statistical Analysis

GraphPad Prism was used to graph data and perform statistical tests. For the Post-GVHD study, a two-tailed paired parametric t-test was done (alpha=0.05). For the Treg study, a two tailed unpaired parametric t-test with Welch's correction was performed (alpha=0.05).

RESULTS

Assessment of T Cell Chimerism in Post-GVHD Skin

To compare T cell chimerism in skin during acute GVHD to post-GVHD skin, we identified five patients who had skin biopsies taken and available for analysis at both points. The clinical characteristics of these patients are detailed in Table 1. Most patients had underlying diagnoses different from one another, other than two with acute myeloid leukemia. Prior treatments were variable across all five patients. Conditioning regimens, GVHD prophylaxis, and GVHD treatment were similar but not equal in all patients, and timing between biopsies was dependent on the patients' clinical presentation so they were all different from each other.

Patient age at transplant	Underlying diagnosis	Prior treatment	HSCT Conditioning Regimen	GVHD prophylaxis	GVHD skin severity	GVHD treatment	Time between transplant and acute GVHD biopsy	Time between acute GVHD and post GVHD biopsy	Reason for post GVHD biopsy
27	Aplastic anemia	ATG + cyclophosphamide	Cytotoxan, radiation, ATG	Tacrolimus, sirolimus, MTX	Stage 1	Steroids + cellcept	20 days	2 years	Rash
23	CML	CVAPL/ASP; Gleevec; 6-MP; cyclophosphamide+ cytarabine+ intrathecal MTX/hydrocortisone vs asparaginase+ vincristine	Cytotoxan, radiation	Tacrolimus, MTX	Stage 3	Steroids+ cellcept	53 days	4 years	Rash
33	AML	AraC+ doxocubicin, Clofarabine+AraC	Cytotoxan, radiation	Tacrolimus, sirolimus	Stage 3	Etanercept and prednisone	21 days	7 months	Rash
59	NHL	Rituxan, CVP-R; rituxan;fludarabine+ mitoxantrone;zevalin ; RICE; cyclophosphamide+ AMD-3100; R-CHOP; autologous trx	Fludarabine, busulfan	MMF, sirolimus	Stage 3	High dose steroids + tac	13 days	1 year	Single lesion
54	AML	Idarubicin+AraC, MEC+Lenalidomide	Fludarabine, Melphalan	Tacrolimus, MTX	Stage 3	High dose systemic steroids	62 days	8 months	Rash

Table 1. Patient characteristic table for comparing active GVHD chimerism to post-GVHD (Cohort 1)

[1]

[1] ATG= anti-thymocyte globulin; MTX=methotrexate; CML=chronic myeloid leukemia; CVAPL/ASP=cyclophosphamide/cytarabine, vincristine, prednisone, L-asparaginase, 6-MP=mercaptopurine; AML= acute myeloid leukemia; AraC= cytarabine; NHL= non-hodgkins lymphoma; CVP-R= rituximab, cyclophosphamide, vincristine sulfate, and prednisone; RICE= rituximab, ifosfamine, carboplatin, etoposide; AMD-3100= plerixafor; R-CHOP=rituximab, cyclophosphamide, hydroxydaunomycin, oncovin, prednisone; MMF=mycophenolate mofetil; AML=acute myeloid leukemia; MEC=mitoxantrone, etoposide, cytarabine

The following figures illustrate representative images of staining, from which the raw data were collected: the total number of CD3+ T cells, CD3+ female T cells, and CD3+ male T cells was manually counted from various high-power fields of view for each patient.

The red stain is for the X chromosome. When two X chromosomes within the same nucleus are clearly seen as prominent red dots spaced apart, as shown in figure 2, the T cell can properly be counted as female. Good examples of Y staining can also be seen in the following figures. When one Y chromosome is seen, the cell is counted as male regardless of the presence of the X chromosome.

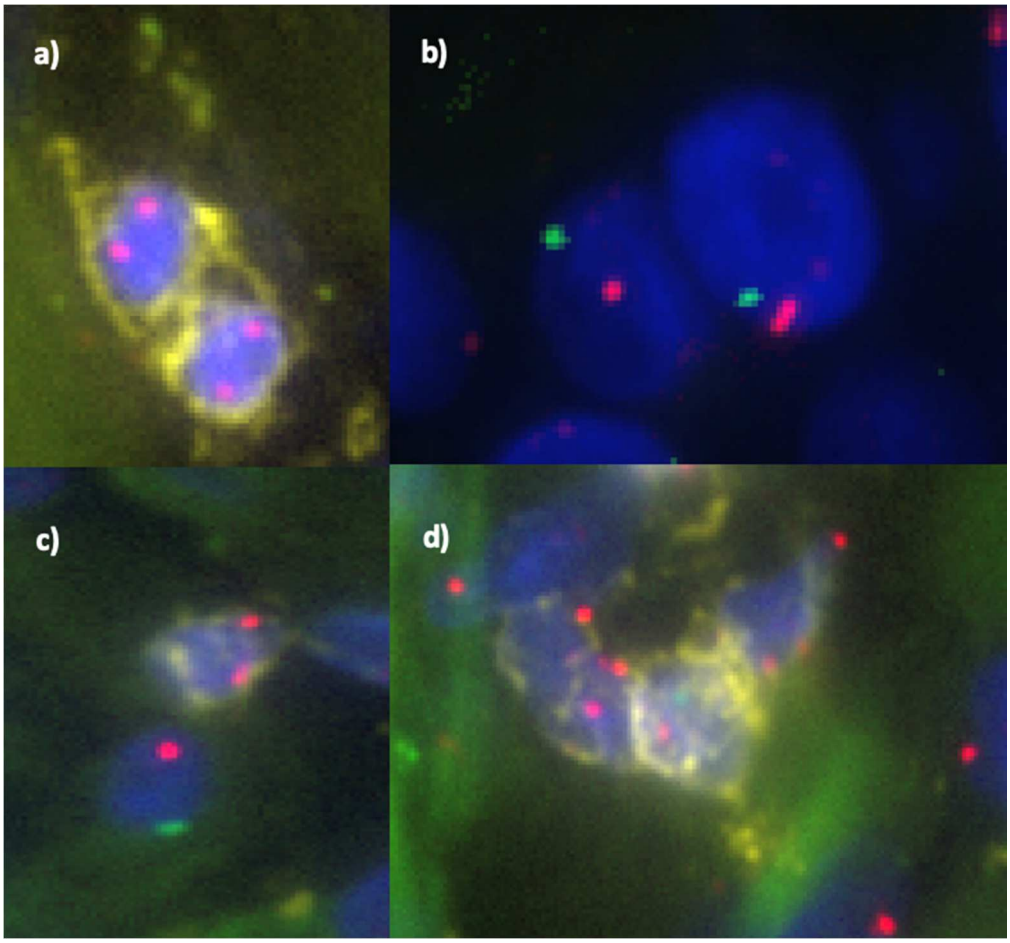
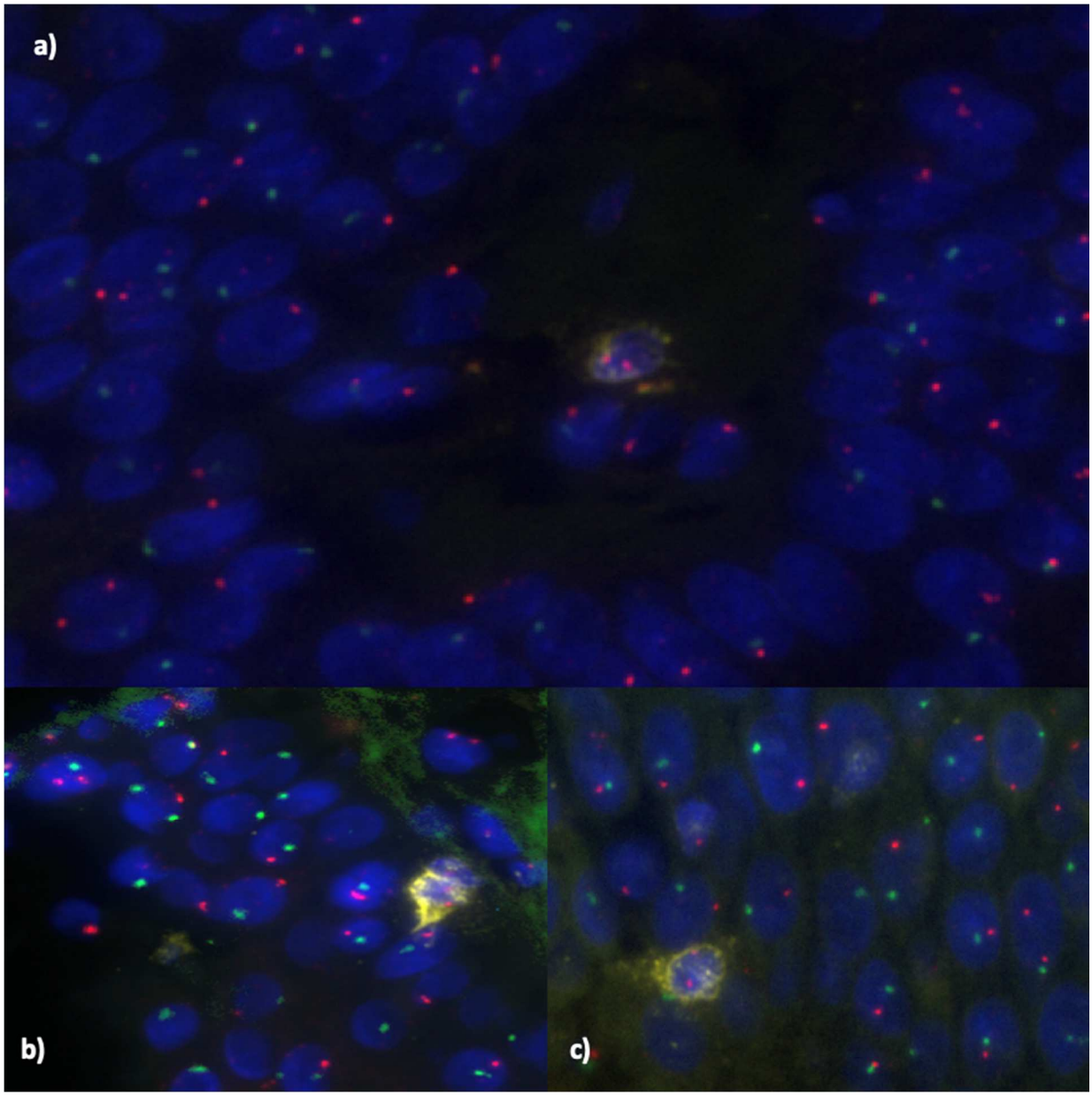


Figure 2. Example FISH-IF images for X and Y chromosomes with CD3 immunofluorescence stain in post-GVHD skin. Yellow=CD3, Green=Y chromosome, Red=X chromosome, Blue=DAPI nuclear stain. Imaged at 400x. A) Donor (female, 2 X chromosomes) T cells (CD3+) in a male host. B) Two host (male, XY) non T cells (CD3-). c) Close-up image of a donor (female, XX) T cell (CD3+) adjacent to a host (male, XY) non T cell (CD3-). D) Host and donor T cell. While most of the host cells in this study were not T cells, as donor chimerism predominated, this image shows an example of the very rarely seen male T cell.

Upon analysis of the fluorescence in situ hybridization-immunofluorescence of the post-GVHD samples in comparison to the same patients' biopsies taken during active acute GVHD, we found that there was a significant shift among the T cells in skin from host (male) to donor (female)

origin for all patients studied. Nearly all (ranging from 95-100%) of the CD3+ T cells in the skin that were imaged in post-GVHD skin were of donor origin, meaning that they stained for two X chromosomes (female).

The percentage of host versus donor T cells was calculated by dividing the number of female/male CD3+ cells by the total number of " identifiable" (either definitely male or female) CD3 cells. This calculation did not take into account any CD3+ cells that were not definitively identifiable as male or female. The percentage of host T cells during acute GVHD was then compared to the percentage of host T cells in post-GVHD skin, shown in figure 3d.


a)
b)
c)

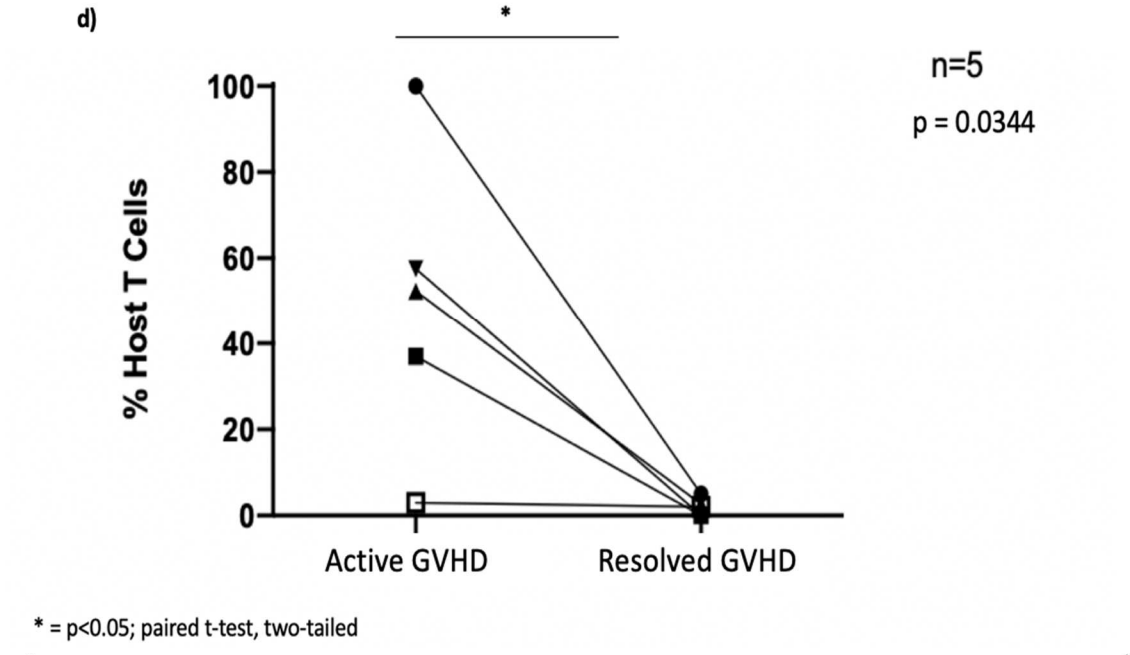


Figure 3. Sparse T cell infiltrate in post-GVHD skin is primarily donor. a-c: Yellow=CD3, Green=Y chromosome, Red=X chromosome, Blue=DAPI nuclear stain. Images taken at 400x. These three example images show very few T cells within patient skin showing that the only T cell present is donor-derived. d: Quantification of all five patient samples' T cell chimerism in skin biopsies during active GVHD versus post-GVHD.

Given the dramatic shift from mixed chimerism to nearly 100% donor T cells in skin after disease resolution, we wondered whether this was the natural consequence of mixed chimerism and active GVHD or a result of treatment. To address this, we studied patients with severe acute GVHD (stages 3 and 4), nine of whom received systemic steroid treatment prior to biopsy, and six who had not. Of these, we were able to stain five patient samples from each group. (Table 2). They had various underlying diagnoses, such as AML, CML, CLL, and lymphoma. There was a good distribution between myeloablative and non-

myeloablative HSCT, and most of the patients received similar GVHD prophylaxis.

Table 2. Patient characteristic table in treatment versus no treatment cohort

Clinical Parameters	GVHD Treatment Cohort	GVHD Non-Treatment Cohort
No. of Patients	5	5
Age, median (range)	41 (23-60)	54 (33-59)
Underlying Diagnosis	AML(n=2), CML, CLL, MDS	Follicular Lymphoma(n=3) MDS (post Hodgkins tx) AML
Conditioning		
Myeloablative, n (%) Non-Myeloablative, n (%)	3 (60%) 2 (40%)	2 (40%) 3 (60%)
MHC allele match/ mismatch		
Matched, n (%) Mismatched, n (%)	2 (40%) 3 (60%)	4 (80%) 1 (20%)
Relationship Status		
Related, n (%) Unrelated, n (%)	1 (20%) 4 (80%)	4 (80%) 1 (20%)
GVHD Prophylaxis	4x Tacrolimus + MTX 1x 4x Tacrolimus + Sirolimus MTX	2x Sirolimus + MMF 1x Tacrolimus + MTX 1x Tacrolimus + MTX+Velcade 1x Tacrolimus + Sirolimus

T cell chimerism in skin in treatment and non-treatment groups

Quantification using FISH-IF revealed that the percentage of host T cells was lower in skin of patients who had received systemic steroids prior to biopsy raising the question of whether steroids caused host T cells to die off (Figure 4a).

However quantification of total host T cell numbers per high-powered field showed that the number was not significantly reduced in the treatment group (Figure 4b). Rather, the total number of T cells per high-powered field was actually increased, as was the total number of donor T cells in the treatment group (Figure 4c and d).

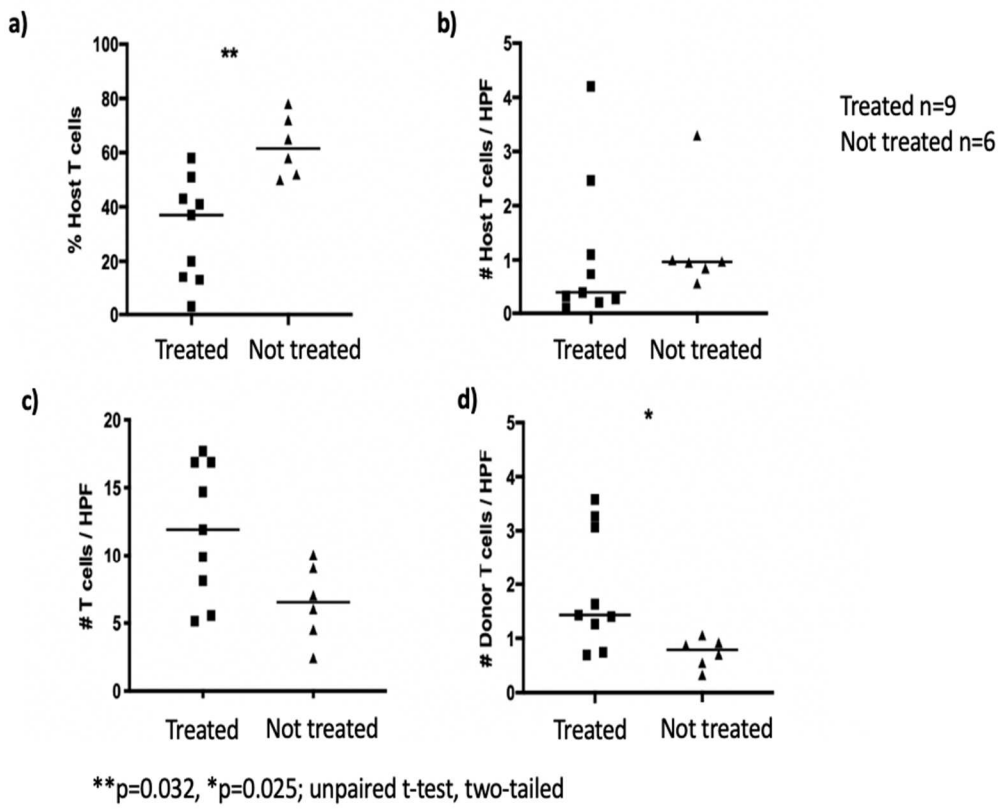


Figure 4. Effect of systemic steroid treatment on host versus donor T cell chimerism. a) The percentage of host T cells is higher for the non-treatment group. b) There is no significant difference in number of host T cells between the treatment and non-treatment group. c) There is no significant difference in the

total number of T cells per high power field between the treatment and non-treatment group. d) There is a significant increase in the number of donor T cells in the treatment group. This is notable especially because there is no corresponding increase in the number of host T cells.

This data suggested that donor T cells were recruited into the skin by systemic steroids and were actually mitigating disease. Donor T cells could theoretically resolve GVHD if they were Treg that suppressed the immune response or possibly if they were effector cells that destroyed host T cells, and the absence of host T cells reduced the stimulus for disease. To address this, available patient samples from treatment and non-treatment groups were stained for CD3, CD4, and Foxp3, and the frequency and percentage of Treg was quantified (Figure 5). There was no difference between either the percentage of Treg per HPF or the frequency of Treg per high-powered field suggesting that donor Treg are not the mechanism by which steroids resolve disease.

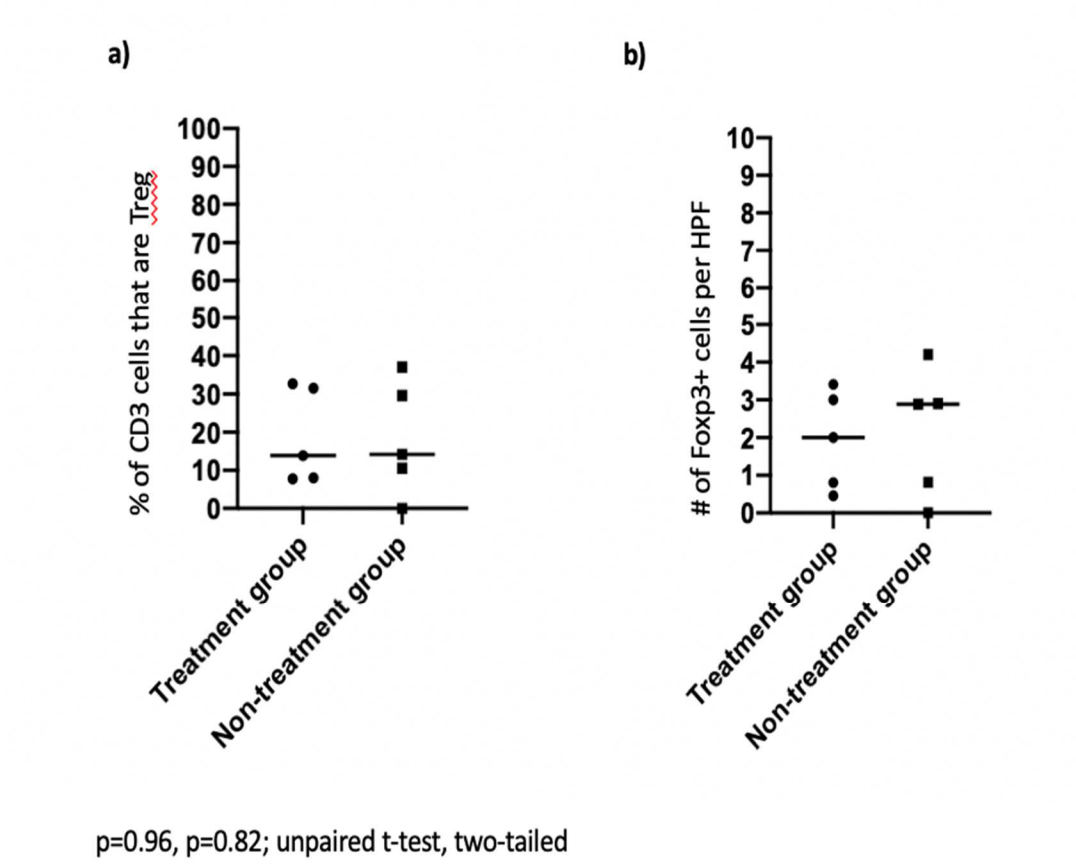


Figure 5. There is no difference in percentage and frequency of Treg between the treatment and non-treatment group. A) Graph showing percentage of CD3+ T cells that are CD4+Foxp3+ (Treg). B) Frequency of Foxp3+ Tregs per high power field (400x) for treatment and non-treatment group.

DISCUSSION

Most studies on chimerism after hematopoietic stem cell transplantation are done in blood. Studies on chimerism in peripheral tissues such as skin are much less common though extremely important especially in the context of graft versus host disease. This could be because it is more difficult to obtain biopsies of the peripheral tissues, especially from the same patients at various time points throughout their course of disease. We were able to do this with our retrospective patient samples and successfully study T cell chimerism in acute skin GVHD during and after resolution of GVHD. One limitation of this study, however, is that the biopsies taken post-GVHD were taken for other clinical purposes, i.e. active skin issues, rather than truly healthy skin. Despite the reason for the post-GVHD skin biopsy, all five patients clearly had a signficant shift toward donor T cells.

In blood, mixed chimerism, compared to complete donor chimerism, has been significantly associated with decreased risk of GVHD (Mattsson 2001). Frassoni et al also found that GVHD risk was significantly higher in patients with complete chimerism compared to mixed chimeras. These papers show that in blood, mixed chimerism is important for the development of tolerance. However, as has been identified by Divito et al, chimerism in the peripheral tissues does not reflect that in the blood, and our data suggest that mixed chimerism may promote GVHD.

The shift we observed in T cell chimerism in resolved GVHD skin led us to ask whether this was a natural result of GVHD or potentially a result of treatment

interventions. Analysis of treated versus non-treated groups support that in fact treatment results in a shift to donor T cell chimerism in skin, by recruiting in donor T cells, rather than by a direct loss of host T cells.

We performed our next study in part to identify what types of donor T cells are involved in the resolution of severe acute GVHD, hypothesizing that donor Tregs play a role and are recruited by the systemic steroid treatment that these patients received. Our results from this study, however, did not show a difference between the frequency or percentage of Tregs between treatment and non-treatment groups. It is possible that with a higher n value, a significant difference might be observed. Additionally, staining of additional skin sections would increase total number of T cells available for analysis and potentially reveal a difference between groups. Another limitation at the current phase of this study is that we were unable to retrieve all the relevant treatment information from the online medical records of these patients. This impedes our ability to make conclusions about the timing of the treatment in relation to the biopsy- for example, if treated immediately before biopsy, perhaps the treatment did not have time to take effect. Additionally, it is extremely important to take into consideration the dosage of the steroids given and the number of treatments received before biopsy. If possible, we will try to obtain this information so that conclusions can properly be made about the findings from this cohort of patients.

It is also possible that Foxp3 negative regulatory T cells are being recruited by the treatment, which we would not have identified using our staining

panel. It is now known that a Foxp3 negative population of regulatory T cells possess immunosuppressive properties. IL-10 was found to drive the generation of a subset of CD4+ regulatory T cells that suppress antigen-specific immune responses and downregulate a pathological immune response in vivo (Groux 1997). Zohar et al found that the induction of Foxp3 negative Treg cells suppresses autoimmune encephalomyelitis through binding of the CXCL11-chemokine. This immunotolerized state is characterized by IL-10 and IL-4 high cells. One thing notable about this study is that CXCL11 upregulated IL-10 only in Foxp3- T cells. It is therefore possible that systemic steroids could be promoting this unique Treg subset (Foxp3- IL-10 producing Tregs). Future work therefore is to stain tissue sections for CD3, CD4 and IL-10 to address this.

Finally, it is possible that the donor T cells recruited into skin as a result of systemic steroids are not regulatory at all, but in fact effector T cells that are directly targeting and eliminating host T cells. This would suggest the possibility that it is the presence of mixed chimerism that stimulates GVHD. We are currently working to interrogate these different possibilities.

CONCLUSION

The concept that host T cells survive and participate in acute GVHD is a novel one (Divito 2020, recently accepted for publication), and ours is the first study to interrogate better the presence and role of host versus donor T cells in GVHD resolution. Through retrospective FISH-IF studies, a dramatic shift toward donor T cell chimerism was observed in the skin after resolution of acute GVHD. Likewise, a significant increase in the number of donor T cells in a group of severe acute GVHD patients who received systemic steroid treatment at the time of biopsy compared to those who did not was seen, suggesting that treatment hastens disease resolution through recruitment of donor T cells. We are currently following up our results with further investigation into the effects of systemic steroids on the types of donor T cells recruited to skin (regulatory versus effector) and their interaction with host T cells. This study therefore has major implications for not only our understanding of the pathobiology of GVHD but for treatment approaches in the clinic.

LIST OF JOURNAL ABBREVIATIONS

J Immunol=Journal of Immunology

Nat Med= Nature Medicine

Transpl Immunol=Transplant Immunology

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CURRICULUM VITAE