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Achievement of Transplantation Tolerance: Novel Approaches and Mechanistic Insights

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Achievement of Transplantation Tolerance: Novel Approaches and Mechanistic Insights

by

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ABSTRACT

Current immune suppressive strategies fail to induce donor-recipient immune tolerance after allogeneic hematopoietic cell transplantation. Accordingly, patients suffer morbidity and mortality from graft vs. host disease (GVHD) and prolonged immune suppressive therapy. Biologic insight into transplantation tolerance is needed, and translation of such insight to novel clinical strategies may improve clinical outcomes. We report original investigation at seminal phases of this process including initial prophylactic immune suppression, onset of acute graft vs. host disease, and ultimate immune suppression discontinuation: In a controlled randomized clinical trial, we demonstrate that sirolimus-based immune suppression reduces risk for acute GVHD, ameliorates the severity of subsequent chronic GVHD, and supports reconstitution of functional regulatory T cells. Study of tissue-infiltrating CD4+ T cell subsets in acute GVHD target organs supports a pathogenic role for Th17 cells. Finally, we demonstrate that peripheral blood transcriptional biomarkers provide mechanistic insight into human transplantation tolerance. These data signal progress, and suggest rational translational efforts to achieve transplantation tolerance.

CHAPTER ONE :

INTRODUCTION

Allogeneic hematopoietic cell transplantation and graft vs. host disease: The goal of allogeneic hematopoietic cell transplantation (HCT) is cure of malignancy and ultimate achievement of donor-recipient immune tolerance. Two major syndromes present the clinical manifestation of immune intolerance, namely acute and chronic graft vs. host disease (GVHD). Current standard immune suppressive (IS) approaches fail to prevent acute and chronic GVHD in most patients, prolonged administration of IS medications is required after HCT, and GVHD commonly develops or reocurs upon attempted IS discontinuation. Thus, donor-recipient immune tolerance is not effectively induced by current approaches, and consequently patients suffer morbidity, disability, and death. These shortcomings speak to the need for further insight into the biology of immunologic tolerance and translation of such discoveries to more effective immune-modulatory approaches in HCT. The following sections describe current understanding of acute and chronic GVHD, and immune tolerance after HCT.

Acute GVHD is a clinico-pathologic syndrome, which remains a major source of morbidity and mortality following HCT. Immunogenetic determinants of acute GVHD include disparity between HCT donor and recipient in major and minor histocompatibility antigens,[1-3] as well as polymorphism in non-HLA genes, including cytokines such as tumor necrosis factor (TNF), IL-10, and interferon gamma (IFN-γ),[4-6] KIR polymorphism,[7,8] and NOD2/CARD15 gene polymorphism.[9] Pathogenesis has been summarized in multi-phase model. These phases include tissue damage from conditioning therapy and activation of antigen presenting cells, activation of donor T cells resulting in differentiation and migration, and an effector phase in

which host tissue damage is mediated by inflammatory cytokines including TNF-α and IL-1, and effector cells, including cytotoxic T cells. These inflammatory mechanisms are tempered by suppressive factors, including regulatory T cells (Treg). Insight into the biology of the syndrome has afforded some advances, but considerable progress is needed.[10,11]

Clinically, the syndrome of manifests with erythematous skin rash, cholestatic liver disease, and upper or lower gastrointestinal involvement either together or in isolation. Clinical severity scoring takes into account severity stage of individual organs, which inform an overall grade.[12] Clinical predictors of the syndrome include donor relation (greater incidence following unrelated donor HCT) and HLA disparity (greater incidence and severity in mismatched HCT) between donor and recipient. Investigators have also demonstrated that biomarkers may predict acute GVHD and have prognostic ability independent of GVHD severity.[13] Importantly, severe acute GVHD is associated with refractoriness to glucocorticoid therapy and mortality.[14,15] In an analysis of 4174 recipients of matched sibling HCT, increasing acute GVHD grade (reference of no GVHD) was associated with risk for mortality: grade I, HR = 1.52 (1.19-1.96); grade II, HR = 2.48 (1.95-3.14); grade III, HR = 5.76 (4.44-7.48); grade IV, HR = 14.7 (10.9-19.9).[16] Failure to respond to therapy results in poor prognosis: In an analysis of 740 recipients of bone marrow allografts with grade II-IV acute GVHD, those with complete response to primary therapy had non-relapse mortality (NRM) comparable to those without acute GVHD, whereas NRM significantly worsened for those with only partial response, no response, or progressive manifestations on therapy.[15] Recently, investigators have reported that non-response at 28 days following initiation of steroid therapy for acute GVHD was associated with a relative risk (RR) for NRM of 2.32 (1.44 – 3.73), $p < 0.001$, and RR for OS of 2.79 (1.71 – 4.55), $p < 0.001$ in multivariate analysis.

Clinical investigation has led to some improvement in the prevention of acute GVHD. Early efforts established the superiority of combination (cyclosporine and methotrexate) therapy

over single agent methotrexate.[17] The current standard of care in acute GVHD prevention is the combination of tacrolimus and methotrexate. Two large randomized phase III trials demonstrated that tacrolimus and methotrexate (TAC/MTX) were superior to cyclosporine and methotrexate (CSA/MTX) in the prevention of acute GVHD. Grade II-IV acute GVHD was significantly lower with TAC/MTX compared to CSA/MTX in both sibling donor (32% vs 44%; p=0.01), and unrelated donor (56% vs 74%; p=0.0002) trials.[18,19] However, it is clear that further progress is needed: Protection from acute GVHD is incomplete, chronic GVHD remains a common problem, and these competing GVHD prevention strategies have not led to important differences in achievement of immune tolerance after HCT. As well, toxicity associated with methotrexate in particular has led investigators to examine the activity of alternative agents,[20] such as the combination of tacrolimus with either mycophenolate mofetil,[21-25] or sirolimus (SIR).[26-29]

As currently available prophylactic strategies insufficiently prevent GVHD, many will require additional immune suppressive therapy for control of the syndrome. The currently accepted standard primary therapy consists of high dose (≥ 1mg/kg/day of prednisone, or dose equivalent of alternative steroid agent) glucocorticoids. However, only 30-50% of will achieve complete resolution of acute GVHD with this standard therapy.[14,15,30] Acute GVHD which fails to respond to primary therapy is associated with an adverse prognosis. Most available therapeutic agents provide resolution in the minority of cases of refractory acute GVHD, and impose additional toxicity. These agents broadly include anti-lymphocyte antibodies, immunotoxin-based agents, agents targeting cytokines including tumor necrosis factor alpha, pharmacologic agents including mycophenolate mofetil, pentostatin, and sirolimus, and extracorporeal photopheresis (ECP).[31] Given the burden of acute GVHD that develops despite the current standard prophylaxis regimen, the limited complete remission achieved with glucocorticoids,

and the poor outcomes in those with refractory GVHD, there is a clear need for the development of a more effective GVHD prophylaxis regimen.

Evidence for the combination of sirolimus and tacrolimus for prevention of acute GVHD: Evidence suggests that the regimen of sirolimus (SIR)/tacrolimus (TAC) may be effective in the prevention of acute graft vs. host disease (GVHD). Investigators from Dana Farber Cancer Institute reported low cumulative incidence of grade II-IV acute GVHD (26%, and 20%, respectively) in two sequential phase II studies of combined SIR/MTX/TAC, and later SIR/TAC.[26-28] Investigators from City of Hope have also published on their experience with the acute GVHD prophylaxis regimen, drawing particular attention to the risk for thrombotic microangiopathy.[29] Investigators from the Fred Hutchinson Cancer Research Center reported contradictory results in two successive GVHD prophylaxis trials utilizing SIR (SIR/MTX/cyclosporine (CSA), and SIR/MTX/TAC); both were halted for lack of efficacy and toxicity: 77% developed grade II-IV aGVHD, and 42% stopped SIR early on account of toxicity, most prominently thrombotic microangiopathy (TMA) or myelosuppression.[32] Thus, data available at the time of our trial development indicated potential promise for this regimen, however randomized comparative data were lacking.

Activity of sirolimus as sole primary therapy of acute GVHD and as secondary therapy of glucocorticoid resistant acute GVHD: In patients with biopsy confirmed grade II-III acute GVHD after HCT, SIR induced complete remission (CR) in 50% of cases.[33,34] These data support the activity of this agent in control of acute GVHD, notably in the absence of glucocorticoid therapy. As well, among a series of 34 patients with glucocorticoid-refractory or intolerant acute GVHD, SIR induced complete remission in 44% of cases.[35] Taken together, these reports demonstrate the activity of this agent in GVHD control, and further strengthen the rationale for use of SIR in the primary prevention of GVHD.

Role of CD4 T helper (Th) subsets in GVHD pathogenesis: Naïve CD4+ T cells differentiate into distinct lineages (Th1, Th2, Th17, Treg) under the influence of antigen-presenting cells and specific cytokine signals (figure 1). These individual lineages have important roles in immunity and immune regulation, and a growing body of literature supports diverse functional roles in GVHD pathogenesis. Donor Th1 CD4+ T cells have been demonstrated to have a central role in acute GVHD.[11] In addition, specific Th1 cytokines, including IFN-gamma, have been implicated in the development and maintenance of acute GVHD.[11,36-38] Pre-clinical data has demonstrated the importance of IL-12 in particular.[39-41] In murine transplantation models, neutralization of IL-12 prevented the development of acute GVHD, polarized CD4+ cells toward a Th2 phenotype, and provided long-term protection from GVHD.

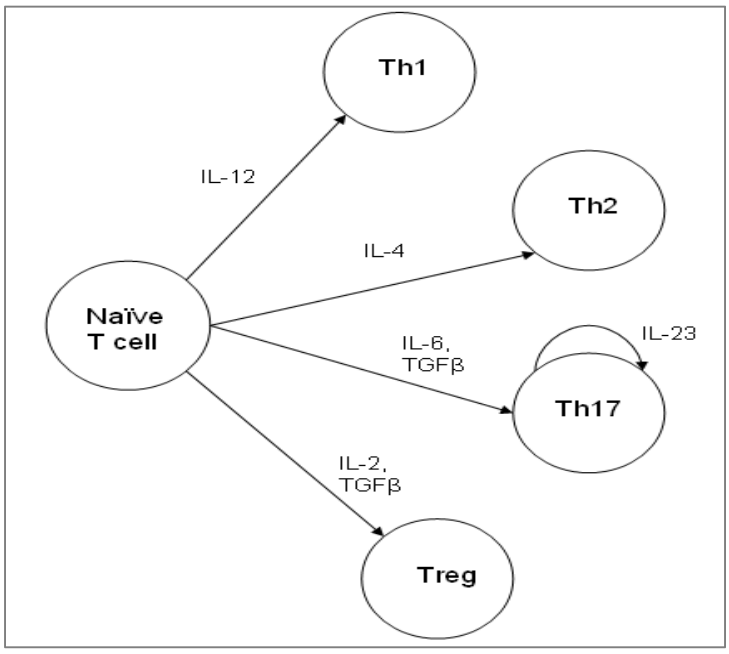


Figure 1: Schema representing CD4 T helper cell differentiation from naïve T cell to Th1, Th2, Th17, and regulatory T cell (Treg) lineages.

Th17 cells have been implicated in solid organ allograft rejection and autoimmunity,[42-46] and emerging evidence implicates Th17 cells in the pathogenesis of GVHD. In murine transplantation models, Th17 cells infiltrate target organs, and are sufficient for the generation of GVHD.[47-49] Data suggest unique contributions of these CD4+ T cell subsets to the target manifestations of GVHD,[50] and the relative balance of CD4+ subsets appears to be important. Loss of Treg and expansion of donor Th1 and Th17 CD4+ T cells was associated with the release of inflammatory cytokines, and autoimmune manifestations of chronic GVHD in one model.[51] In addition, investigators have identified secretion of IL-23 by antigen-presenting cells to be an essential component of GVHD induction,[52] indicating the relevance of this cytokine in particular as a therapeutic target. Simultaneous blockade of Th1 and Th17 (through targeted disruption of T-bet and RORγt transcription factors) prevented GVHD in a major MHC mismatched murine model, and did not impair graft-versus-leukemia (GVL) activity.[53] These data implicate both Th1 and Th17 CD4 lineages in the pathogenesis of acute GVHD, and thus identify these as potential targets of novel therapeutic approaches.

Regulatory T cells (Treg) are a naturally occurring subset of T cells that are distinguished by their constitutive expression of CD25, and expression of transcription factor FoxP3.[54-56] They are potent suppressors of immune responses, and therefore, have potential application in the prevention and treatment of GVHD. Treg suppress alloreactive T cells in vitro and prevent lethal acute GHVD in MHC-mismatched allogeneic transplantation models.[57-60] Given the low frequency (< 5%) among human T cells, several groups have performed ex-vivo expansion for therapeutic applications. Accordingly, translation to human clinical trials has become more feasible.[55-57,61-68] Ex-vivo expanded Treg demonstrate increased suppressive potency.[69,70] As well, antigen-specific Treg achieve selective suppression of allo-responses with no suppression of third-party responses.[71-78] In experimental systems, Treg appear to abrogate GVHD, while preserving graft vs. leukemia (GVL) responses.[79,80] This is a critical consideration

in the application to human studies, as the major goal of HCT is often control of malignancy. Pre-clinical data support that SIR supports the expansion and suppressive function of Treg; conversely, calcineurin inhibitors including CSA and TAC, have an adverse impact on regulatory T cell survival and function, given their interference with IL-2 production.[81-83]

Thus, current pre-clinical evidence implicates Th1 and Th17 in GVHD pathogenesis, and support the potent immune regulatory role of Treg. Human clinical evidence largely supports this paradigm: HCT recipient peripheral blood,[84-86] and donor graft,[87,88] Treg frequency has an inverse relationship with the occurrence and severity of GVHD, and resultant risk for mortality.[89] Donor allograft Th17 numbers have been associated with acute GVHD following HCT,[90] and Th17 have been associated with inflammatory bowel disease.[91] Studies in GVHD target organ tissues suggest a predominance of Th1 and not Th17 cells in cutaneous GVHD.[92] Th17 have been implicated in intestinal GVHD,[93] as well as inflammatory bowel disease,[94] and allied immune-mediated disorders.[46,95] FoxP3+ Treg in cutaneous GVHD,[96] as well as FoxP3+Treg/CD8+ T cell ratio in acute and chronic GVHD,[97] have an inverse relationship with GVHD and its severity. Thus, while discrepant findings have been reported,[98,99] this overall paradigm suggests targeted approaches to deplete Th1/Th17 and augment Treg may mitigate risk for GVHD after human HCT.

Sirolimus suppresses alloreactive T cells, inhibits differentiation of Th17 cells from naïve CD4 T cells, and promotes generation of Treg: Sirolimus (SIR), or rapamycin, is a naturally occurring inhibitor of mammalian target of rapamycin (mTOR). Treatment with SIR leads to inhibition of transcription and decreased kinase activity of cyclin-dependent enzymes. SIR also inhibits dendritic cell development and function. In T cells, SIR produces at least partial blockade of CD28 mediated co-stimulatory signaling. However, SIR is permissive for Treg: SIR permits expansion of Treg, preserves the potent CD27+ subset of Treg, does not impair Foxp3 expression, and allows for greater suppressor function as compared to Treg treated with CSA.[81-

[83,100-102] While effector T cells are sensitive to the inhibitory effect of SIR, Treg expand. Treg in murine and human systems do not activate the phosphatidyl inositol 3-kinase (PI3-K)/AKT pathway after activation through the T cell receptor. As SIR inhibits mTOR in this pathway, it may selectively inhibit effector T cells.[100,103,104] As well, SIR inhibits differentiation of naïve CD4 T cells to Th17, and promotes generation of Treg.[105] Thus, SIR may favorably modulate the immune system and mitigate GVHD risk and promote immune tolerance.

Chronic graft vs. host disease: Chronic graft-versus-host disease (GVHD) is a major long-term problem after HCT. The syndrome is associated with significant morbidity, mortality, impaired quality of life (QOL), greater symptom burden, and prolonged duration of immune suppressive therapy following HCT.[106-115] Pre-clinical and clinical observations suggest some insight into the pathogenesis of the syndrome, but much remains to be elucidated. Prevailing hypotheses suggest that chronic GVHD may be driven by alloreactive donor T cells and countered by regulatory T cells,[84,116-119] loss of tolerance,[120] altered B cell homeostasis, [121-124] and activation of pro-fibrotic pathways.[125,126] Chronic GVHD occurs in the majority of patients at risk, up to 60-80% of those who survive more than 100 days after transplantation.[108,109] The syndrome is characterized by diverse manifestations; the most commonly occurring manifestations arise in the skin, eyes, mouth, and liver. Major changes in the classification and severity grading of the syndrome have been suggested by the NIH Chronic GVHD Consensus Conference,[127] and severity has been validated as a determinant of survival.[128] Risk factors for development of chronic GHVD include increasing age of the donor or recipient, donor/recipient HLA disparity and donor relation, male recipients of allografts from alloimmunized female donors, prior occurrence of acute GVHD, and the use of peripheral blood mobilized stem cells vs. bone marrow.[116,129-131]

Importantly, the majority of approaches for initial GVHD prevention have failed to alter the incidence or severity of chronic GVHD.[18,19,28] One major exception – ex-vivo T cell depletion

from allografts – is complicated by risk for poor immune reconstitution and serious infections after HCT, as well as malignancy relapse. Others have attempted to decrease risk for chronic GVHD development through prolonged administration of calcineurin inhibitor after HCT, however these studies have failed to show any consistent benefit.[132-135] Thus, most will experience the syndrome, and therapy is often required to control symptoms and prevent progressive organ damage from chronic GVHD. Systemic steroid treatment is required to control established moderate-severe chronic GVHD, and 1mg/kg/day of prednisone remains the standard initial therapy. Trials examining novel combination therapies (prednisone in combination with either azothioprine, thalidomide, hydroxychloroquine or mycophenolate mofetil) have not shown benefit.[136-139] Complete resolution of chronic GVHD following initial therapy, however, is limited (by 6-9 months of prednisone therapy, complete response occurs in 30%, and complete + partial response occurs in only 60%).[136-139] Most patients will require additional lines of immune suppressive therapy to control chronic GVHD, and outcomes of secondary (and beyond) therapy for chronic GVHD are poor; a recent major analysis suggests that failure-free survival (i.e. freedom from death, malignancy relapse, and treatment change) for such patients is only 31% by 2 years, and 25% by 4 years after initiation of secondary therapy.[140] Thus, chronic GVHD is a major obstacle to the success of HCT, and novel strategies to prevent the syndrome are needed.

Development of immune tolerance following HCT – Biologic Mechanisms:

Experimental evidence demonstrates that multiple cellular and molecular mechanisms actively support the state of immune tolerance.[141-144] T cells have a central role, including induction of T cell anergy [145-147], central (thymic) and peripheral deletion,[148] mixed chimerism,[149-154] external influences including Treg,[54,59,155-159] the balance of cytopathic and regulatory T cells,[160] and co-stimulatory molecule signaling.[161-166] Several other important mediators of immune tolerance include dendritic cells,[167,168] B cells,[169] and components of the innate immune system,

importantly including NK cells.[170] Based on central findings from the investigation later presented, the following content focuses on dendritic cells, NK cells, and evidence surrounding their cooperation in immune tolerance.

Dendritic cells, professional antigen-presenting cells, sense environmental signals and orchestrate competing immune responses: Pro-inflammatory responses (up-regulation of HLA, co-stimulatory molecules, and inflammatory cytokines) drive antigen-specific responses of the adaptive immune system. Ligation of toll-like receptors (TLR) and downstream signaling (MyD88, TRIF, NFkB) result in up-regulation of co-stimulatory molecules and pro-inflammatory cytokines.[171] In contrast, a coordinated tolerogenic program (reduced co-stimulatory molecules and pro-inflammatory cytokines, and elaboration of tolerogenic signals including IL-10, IDO, TGF-β, among others) promotes T cell anergy, deletion, and induction of regulatory T cells (Treg).

NK cells are major components of the innate immune system that mediate killing of virally infected and malignant cells, and regulate other immune cells through elaboration of cytokines and chemokines. NK cells express multiple activating and inhibitory cell surface receptors, and the integration of these signals directs NK cell function.[172] Numerous killer immunoglobulin-like receptors (KIR) have been described, and KIR/KIR-ligand mismatch have been identified as key in NK licensing/education, and killing of allogeneic targets.[172] In the setting of HLA mismatched haploidentical HCT, this mechanism is central to alloreactive donor NK killing of recipient DC, facilitation of donor engraftment, and effective control of malignancy.[173] Additional NK cell receptors include natural cytotoxicity receptors (NCR) and lectin receptors (heterodimers of CD94:NKG2 family members). The CD94/NKG2 complex is expressed on NK and T cells. NKG2C and NKG2A interact with the non-classical MHC class Ib molecule, HLA-E, which presents peptides derived from sequences of other HLA class I molecules (while NKG2D interacts with ULBP, MICA, and MICB). Generally, NKG2C/D/E/F

have been classified as activating receptors, while NKG2A has been deemed inhibitory. However, diverse functional roles have been demonstrated for NKG2A: Qa-1 (the murine homologue of HLA-E) – together with Qdm – is expressed on activated CD4+ T cells. Qa-1 binding to TCR activates and expands antigen-specific CD8+ T cells. Conversely, Qa-1 binding to NKG2A/CD94 on CD8+ T cells, NK, and NKT leads to reduced activation of these cells. Qa-1 on activated CD4+ T cells has divergent interactions with NK and CD8+ Treg: Engagement of CD94/NKG2A on NK cells protects CD4+ T cells from lysis, while engagement of TCR on Qa-1 restricted CD8+ Treg leads to expansion of these CD8+ Treg and suppression of CD4+ T cell activation.[174] NKG2A in human γδ T cells inhibits NKG2C based effector function.[175] NKG2A signaling has been reported to have tolerogenic activity: Human NK-hepatocyte interaction via NKG2A led (through TGF-β) to DC-mediated induction of CD4+CD25+ Treg that suppressed T cell activation through PD-1/PDL-1 interaction.[176] As well, a NK subset identified in lymph nodes expressing CD94/NKG2A but not KIR controlled self-DC activation through killing of immature DC.[177]

NK can promote immune responses through promoting DC maturation and cytokine production through NK-DC interaction, promote Th1 polarization of CD4+ T cells via IFN-γ, promote cytotoxic T cell responses, promote B cell isotype switching through IFN-γ, and augment inflammatory responses executed by monocyte and macrophages.[178-180] NK cells have been implicated in both autoimmunity and solid organ transplant rejection.[180-185] In contrast, NK cells have a major role in immune regulation. Major proposed mechanisms have included production of IL-10, competition with CD8+ T cells for IL-15, killing of dendritic cells (in particular immature DC), and killing of activated T cells.[180,186-192] As well, NK cells are predominant in tolerant organs (e.g. liver, lung, intestine, and uterus),[178] have a central role in maternal-fetal tolerance (through regulation of Th17,[193] and expansion of Treg),[194] mitigate allograft rejection in experimental models,[180] appear to be central mediators of transplantation tolerance in human

hepatic allografts,[195] may exert a protective function in a number of autoimmune disorders,[196-199] and their deficiency is a risk factor for chronic GVHD development after HCT.[200]

Bi-directional interaction between DC and NK cells leads to activation and cytokine production, DC maturation, and NK proliferation and cytotoxicity.[179,201] DC promote NK activation, cytokine secretion and survival through multiple mechanisms: DC produce IL-15, which is essential for NK survival and differentiation;[202] DC also produce IL-12, which enhances NK cytotoxicity and IFN-γ production,[203] as well as IL-1 and IL-18, which potentiate the effect of IL-12 through induction of IL-12R on NK cells; DC also stimulate antigen-specific T cells which secrete IL-2 and activate NK;[204] DC and T cells also up-regulate ligands (e.g. MICA and MICB for NKG2D) for NK receptors. In turn, NK stimulate DC through cytokine (TNF, IFN-γ, GM-CSF) production, and NKp30-NKp30 ligand interaction.[179] In contrast, NK can also kill DC, and this is thought to be dependent upon NKp30 and NKp46 receptor ligation.[205-207] Immature DC are the primary target of NK cell killing: Both mature and immature DC express surface HLA class I molecules, but the surface density is increased in mature DC.[177] One hypothesis to explain the selection of NK stimulation of DC maturation vs. NK killing of DC is based on the ratio of NK to DC in experimental systems: With high NK:DC ratio, NK kill immature DC. In contrast, with low NK:DC ratio, DC maturation and cytokine (IL-12, TNFα) production is increased.[208]

Finally, emerging evidence supports the presence of NK cell subsets that may have divergent functional roles. One major sub-grouping is based on CD56 expression: While most human peripheral blood NK cells are $CD56^{dim}CD16+$, approximately 10% of human peripheral blood NK cells are $CD56^{bright}CD16-$. In contrast, $CD56^{bright}CD16-$ NK cells are enriched in tolerant organs, including human liver and uterus. These $CD56^{bright}CD16-$ NK cells may have an immunoregulatory role, and have been shown to control autoimmunity in part through APC-derived IL-27 driving NK cell IL-10 secretion.[209,210] $CD56^{bright}CD16-$ NK cells express high-affinity interleukin-2 (IL-2) receptor which enables them to proliferate and produce IFN-g in response to

low doses of IL-2, express CD94:NKG2A but low KIR (CD94:NKG2A+KIR- phenotype), and express lymph node homing molecules L-selectin, CXCR3 and CCR7. $CD56^{bright}$ NK cells with constitutive expression of high-affinity (IL-2Rαβγ) IL-2 receptor are present in human lymph nodes, stimulated by endogenous T cell derived IL-2, and secrete IFN-γ.[204] Human CD94/NKG2A+KIR- NK cells can kill autologous (primarily immature) dendritic cells, while NK cells that express KIR specific for self HLA class I do not kill autologous DC.[177] Multiple additional regulatory NK cell subsets have been identified, many of which produce established immunoregulatory cytokines including IL-10 and TGF-β.[178] Several have been identified as protective in auto-immune disorders including type I diabetes and multiple sclerosis. Thus, a growing body of experimental and human data demonstrates the toleragenic capacity of NK, and the importance of DC-NK cooperation.

Development of immune tolerance following HCT – Investigation into human transplantation tolerance biomarkers: Clinically, immune tolerance after transplantation has been defined by no ongoing immunologic injury due to incompatibility between donor and recipient following discontinuation of immune suppressive (IS) therapy.[211-213] While allograft rejection constitutes the major manifestation of immunologic injury in solid organ transplantation, acute and later chronic graft vs. host disease represent the major challenges after HCT.

In the setting of both solid organ transplantation and HCT, clinical judgment does not distinguish drug-suppressed immune response from development of immune tolerance. Thus, discontinuation of immune suppression (IS) is associated with serious risks, and individualized practice is not possible. There has been great interest in defining biologic markers of immune tolerance that may ultimately permit individualized management of IS. Investigators have reported changes in gene expression associated with the tolerant clinical phenotype in solid organ transplantation.[195,214-216] While there are potential challenges in the direct comparison of these studies, changes in gene expression in tolerant individuals recapitulate mechanisms of

immune tolerance supported by previous experimental evidence: One nearly consistent finding across these studies is that of reduced expression of genes important for immune activation and response, overall depicting a state of immune quiescence. Of particular importance in *Brouard, et al* are genes reflecting reduced immune response, apoptosis, and growth arrest that have been demonstrated to be under the control of TGF-β. Supporting another major mechanism of immune tolerance, *Brouard, et al* demonstrated decreased expression of genes related to co-stimulatory signaling.[214] Additionally, several of these reports support the importance of Treg: In the tolerant subjects, *Brouard* reported increased FOXP3 expression, *Martinez-Llordella* described increased Treg by immunophenotyping, and *Kawasaki* reported increased expression of STAT1, which has importance in Treg development.[214,216,217] Not specifically supported by the other studies, *Martinez-Llordella* demonstrated increased numbers and enrichment for genes expressed by γδT cells, enrichment for genes expressed by NK cells, and a polarization toward Vγδ1+ subtype predominance among the γδT cell population.[217] Thus, these efforts have begun to signal progress in the field.

Investigation into tolerance associated gene expression in solid organ transplantation has limited practical application, as the majority of solid organ transplant recipients require life-long IS. Conversely, these applications have great relevance in HCT, as most patients eventually discontinue IS therapy. However, IS discontinuation practice is empiric and often met with flares of graft vs. host disease and subsequent escalation in IS.[218] This makes clear an unmet need for an understanding of tolerance mechanisms, and an informed, rational approach to IS discontinuation after HCT.

CHAPTER TWO:

METHODS

Randomized phase II study to evaluate tacrolimus in combination with sirolimus or methotrexate after allogeneic hematopoietic cell transplantation:

Study design: We conducted a prospective, randomized comparison of sirolimus (SIR)/tacrolimus (TAC) vs. methotrexate (MTX)/TAC (NCT00803010). This trial was approved by the University of South Florida Institutional Review Board. Randomization was stratified for age ($\geq$ 50 vs. age < 50), and donor type (sibling vs. unrelated); these two factors were selected for stratification based on existing evidence supporting their impact on risk for GVHD. All patients received peripheral blood mobilized grafts. The primary objective of this trial was to evaluate the efficacy of SIR/TAC vs. MTX/TAC in prevention of grade II-IV acute GVHD. The study was powered to detect difference in the incidence of grade II-IV acute GVHD between SIR/TAC and MTX/TAC treated patients. Among MTX/TAC treated patients, we anticipated grade II-IV acute GVHD of 80%, based on that observed in MTX/TAC treated patients on a prior prospective clinical trial at our center.[219] Based on previously published single-arm phase II SIR/TAC trial results which demonstrated approximately 20% incidence of grade II-IV acute GVHD in comparison to previously reported incidence of 40-50% following MTX/TAC,[27,28] our *a priori* hypothesis was that we would observe a 50% reduction in this primary endpoint. With 56 evaluable patients without competing-risks, two-sided log-rank test would achieve 90% power at 0.1 significance level. We anticipated 20% of evaluable patients would develop competing-risk events within 100 days, and adjusted the total sample size to 70. We increased the sample size to 74 (37 in each arm) for possible 5% dropout.

Patients: Included patients were age 16 – 70 with cardiac ejection fraction ≥ 45%, FEV1, FVC, and DLCO ≥ 50% predicted values, AST and ALT < 3 times upper limit of normal, creatinine clearance ≥ 50 cc/min, and Karnofsky Performance Status ≥ 60%. Included disease were the following: Acute myelogenous leukemia of intermediate/high risk in first complete remission (CR1), or beyond CR1; myelodysplastic syndrome with IPSS score of ≥ 1.5; myeloproliferative disorders; chronic myelogenous leukemia; acute lymphoblastic leukemia; chronic lymphocytic leukemia; severe aplastic anemia; multiple myeloma; and Hodgkin or non-Hodgkin lymphoma. Patients with hepatitis B or C, human immunodeficiency virus (HIV), uncontrolled systemic infection, or HCT-comorbidity index ≥ 3 were excluded.[220]

Treatment protocol: Eligible donors were sibling or unrelated donors matched at HLA-A, B, C, and DRB1 by high-resolution typing. G-CSF mobilized peripheral blood products were targeted to a CD34+ cell dose/kg of 5-10 x 10^6. Use of anti-lymphocyte antibodies and cyclophosphamide-containing regimens was prohibited, but the conditioning regimen was otherwise not mandated. Institutional standards for bacterial, viral, and fungal infectious prophylaxis and monitoring were followed.

GVHD prophylaxis*:* TAC was administered from day -3 at 0.02mg/kg/day, then transitioned to oral formulation before hospital discharge. For patients receiving MTX, serum TAC target was 5-15 ng/mL. When given concurrently with SIR, target TAC was 3-7 ng/ml. According to protocol, patients without evidence of acute GVHD and not on therapy with systemic glucocorticoids were eligible to being TAC taper at day 50 following HCT. SIR was administered as a 9 mg oral loading dose on day -1, followed by maintenance to target 5-14 ng/ml. The protocol mandated that SIR should be continued through at least 1 year post-HCT. We aimed to determine if this prolonged course of SIR would impact risk for chronic GVHD development, severity, and ultimate discontinuation of all immune suppression. MTX was administered on day +1 at 15 mg/m^2, and then 10 mg/m^2 on days 3, 6, and 11. Beyond the

above specifications, the protocol did not mandate a particular taper schedule for TAC, SIR, systemic glucocorticoids, or other immune suppressive agents.

Study endpoints: Neutrophil and platelet engraftment were defined by standard methods. Mucositis was graded per CTC version 4.0. Diagnosis and severity grading of thrombotic microangiopathy (TMA) adhered to BMT Clinical Trials Network consensus.[221] Hepatic veno-occlusive disease (VOD) was diagnosed according to standard clinical criteria.[222] Acute GVHD was scored weekly from HCT to day 100; in keeping with established clinical practice, biopsy confirmation of acute GVHD was not required by the protocol.[12] However, biopsies were obtained according to usual practice when deemed necessary by treating clinicians. These GVHD biopsies were reviewed by Pathologists blind to study arm assignment. Chronic GVHD was scored per NIH consensus criteria.[127] Peripheral blood sorted (CD3 and CD33) and bone marrow donor chimerism were assessed at days 30, 90, 180, and 360 by PCR. Disease restaging occurred on days 30, 90, 180, and 360, 18 months, and 2 years following HCT. Patient reported quality of life (QOL) was assessed using the Functional Assesment of Cancer Therapy – Blood and Marrow Transplantation (FACT-BMT) questionnaire at baseline pre-HCT, and on days 30, 90, 180, 270, 360, 560, and 740 post-HCT.[223]

Treg repopulation post-HCT and suppressive function: Samples were drawn from peripheral blood of HCT recipients at the following time points: Baseline (prior to beginning conditioning regimen and HCT); day 0, 30, 90, 180, and 360 after HCT. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-hypaque gradient centrifugation. PBMC were stained with labeled antibodies (CD3PerCp, CD4FITC, CD25PE, CD127Alexa 647 and mouse IgG1 isotype controls from BD Biosciences). Samples were analyzed using FACS Calibur flow cytometer with CellQuest software (BD Immunocytometry Systems, San Jose, CA). T cells were identified by gating on CD3+ and CD4+ populations, and Treg were defined by CD4+, CD25(bright), and CD127(negative) phenotype. The reciprocal relationship between negative

surface CD127 and high intracellular FoxP3 expression was confirmed in a subset (n=15) of day 30 patient samples (r=0.94).

The suppressive potential of Treg was examined in a subset from both SIR/TAC and MTX/TAC groups from blood cells obtained between 90 and 180 days after HCT. CD4+CD25+CD127- Treg were isolated on a BD FACSAria II high-speed cell sorter (BD Biosciences, SanJose, CA). Treg were added in different ratios to 1 x 10^4 self CD4+CD25- T responder cells in the presence of 1:1 CD3/CD28 beads (Invitrogen Corporation, Carlsbad, CA) in 96-well round-bottom plates. Proliferation was analyzed by [^{3}H] thymidine incorporation using a gas scintillation counter (Matrix 96 beta counter, Canberra Packard, Meriden, CT). Cells were pulsed with 1μCi/well ^{3}H-thymidine for the last 18 hours in culture and harvested on day 5 to measure proliferation. Results are expressed in counts per minute (CPM) of triplicate measurements.

Statistical methods: The intent-to-treat population was used to conduct all analyses for all endpoints. Cumulative incidence of grade II-IV aGVHD was estimated and compared by the Gray test.[224] Survival was analyzed using the Kaplan-Meier method and compared using the log-rank test. Cumulative incidence of non-relapse mortality and relapse were estimated and compared. Pointwise 95% confidence intervals for survival curves and cumulative incidence curves were computed using log-log transformation. Analysis of association between GVHD outcomes and time-dependent measures (serial TAC and SIR levels, serial measures of Treg) utilized Cox regression model with time-varying covariates. Two-sided Wilcoxon rank-sum test was employed to test difference in percent Treg (% Treg/total CD4+ cells) on day 30, 90, 180 and 360 at significance of 0.05 (alpha of 0.025 at each time point using Bonferroni-Holm adjustment).

Tissue-infiltrating Th1, Th17, and Treg in GVHD target organs following human allogeneic hematopoietic cell transplantation:

Included patients: Patients were randomized to SIR/TAC or MTX/TAC on trial as described above.[225] Acute GVHD severity was scored per standard criteria weekly from HCT to day 100.[12] Those cases with GVHD who had diagnostic biopsy performed (including skin, gastrointestinal tract, or liver) were identified for this analysis. Pathologic GVHD grading was performed according to standard criteria with the Pathologist blind to study arm.

Processing and staining of GVHD tissue samples: Biopsies were preserved in neutral buffered formalin and processed in usual manner. Cylindrical punches were removed from paraffin-embedded tissue blocks to create a tissue microarray (TMA). Tissue microarray was utilized to improve experimental uniformity and ensure highly parallel analysis. Antibodies to RORγ (rabbit, 1:300 Abcam, Cambridge, MA), T-bet (mouse, 1:25, BD Biosciences, San Jose, CA), FoxP3 (mouse, 1:25, Abcam, Cambridge, MA) and CD4 (rabbit, 1:25, Cell Marque, Rocklin, CA) were utilized for immunohistochemistry (IHC) studies. Slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) per manufacturer's recommendations with proprietary reagents. Individual 4 μm sections were transferred to positively charged slides. The slides were deparaffinized on the automated system with EZ Prep solution (Ventana). Following heat-induced antigen retrieval methods in Cell Conditioning 1 (Ventana) for FOXP3 and T-bet and in RiboCC (Ventana) for CD4 and ROR-gamma, the samples were incubated with the selected antibodies using Dako antibody diluent (Carpenteria, CA). Then the Ventana UltraMap Anti-mouse secondary antibody was utilized for FOXP3 and Tbet, while the Ventana UltraMap Anti-rabbit secondary antibody was utilized for CD4 and ROR-gamma. Ventana ChromoMap kit detection system was used and the slides were then counterstained with hematoxylin. Finally the slides were dehydrated and coverslipped per normal laboratory protocol. Stained slides were scanned using Aperio™ (Vista,

CA, USA) ScanScope XT with a 200x/0.75NA objective lens at a rate of 3 minutes per slide via Basler tri-linear-array. Positivity for each marker was quantitatively scored using the TMA module of the TissueStudio v3.0 software platform from Definiens (Munich, Germany) for each TMA core (0.6mm diameter, or 1.13mm^2 area). Staining intensity thresholds were held constant throughout the study. In a subset of 10 randomly selected TMA cores, contiguous sections (4μm thickness) were stained with CD4 and RORγ for co-registration analysis; based on high degree of co-registration of CD4 and RORγ, we elected to only utilize RORγ to identify Th17 cells. T-bet was utilized to identify Th1, and FoxP3 identified Treg.

Statistical methods: Data are presented as absolute numbers for each CD4 subset (Th1, Th17, Treg), and ratio of each to total CD4+ cells. Analysis of variance (ANOVA) was utilized to compare tissue lymphocyte numbers according to clinical grade, pathologic grade, and GVHD prophylaxis group. Logistic regression analysis was used to study the association between tissue lymphocyte numbers and response to primary GVHD systemic glucocorticoid therapy; due to limiting number of events, only univariate relationships are described for this analysis.

Tolerance associated gene expression following allogeneic hematopoietic cell transplantation:

Identification of study patients and control subjects: From long-term survivors of allogeneic hematopoietic cell transplantation (HCT) in the Moffitt Cancer Center Blood and Marrow Transplantation Program, tolerant patients (TOL) were identified. The tolerant phenotype was defined by successful discontinuation of all IS agents (minimum time from complete discontinuation of IS to time of sample acquisition of 6 months), and sustained absence of any detectable clinical, radiographic, or laboratory manifestations of acute or chronic graft vs. host disease. The absence of manifestations of graft vs. host disease was confirmed by

at minimum two transplant physicians in each case to determine eligibility. Through systematic search of the program database including all allogeneic transplant recipients, matched non-tolerant comparator subjects (non-TOL) were identified who were not able to successfully liberate from immune suppression due to graft vs. host disease. An algorithm was developed wherein non-tolerant comparators were matched to the individual tolerant cases by date of HCT (+/- 6 months) and age at time of HCT (+/- 5 years). From all non-tolerant comparators for each case that met criteria, the best matched non-tolerant comparator was selected according to identity on the following factors in descending rank order: HLA matching between HCT donor and recipient (identical at HLA-A, -B, -C, and –DRB1 vs. mismatch), donor relation (sibling vs. unrelated donor), stem cell source (peripheral blood vs. bone marrow), GVHD prophylaxis agents, disease requiring transplantation, and conditioning regimen. Healthy volunteers were recruited to serve as control subjects. Minimum demographic information (age, gender) was collected, and volunteers completed a brief medical questionnaire to confirm they were not acutely ill for any reason, had no chronic medical conditions and were not taking any medications. These healthy control subjects were of interest, as they had not received HCT and were not treated with immune suppressive agents. All patients provided informed consent for participation in the study, which was approved by the University of South Florida Institutional Review Board.

Assessment of clinical data: For all participating tolerant and non-tolerant HCT recipients included in the study, standardized medical record abstraction was performed. Baseline demographic and transplantation variables included the following: age at time of HCT, condition requiring HCT, remission status at time of HCT, stem cell source, CD34+ cell dose/kg body weight, donor relation, donor age, gender matching of donor and recipient, HLA matching at HLA-A, -B, -C, and –DRB1 loci, cytomegalovirus serologic matching between donor and recipient, conditioning regimen, and GVHD prophylaxis agents utilized. Comprehensive

information was gathered on prior manifestations of acute and chronic GVHD including the following: Initiation and discontinuation dates of all immune suppressive agents (with indications for tapering and discontinuation of each agent) including both original prophylaxis agents, and those later employed for therapy of acute and chronic GVHD; onset, peak grade, biopsy confirmation, therapy delivered, resolution date, and recurrent manifestations for both acute and chronic GVHD;[12,127] outcome data including relapse date, date of death, and dates of last clinical follow up; and finally date of discontinuation of all systemic immune suppressive agents.

Sample processing, cell subsets and microarray analysis: Each subject consented to peripheral blood collection, which included two 10cc EDTA tubes. Freshly acquired samples were immediately processed. From one sample, peripheral blood mononuclear cells (PBMC) were isolated using the Ficoll-Hypaque method, and were immediately processed for characterization of cell phenotype by flow cytometry. PBMC were stained with labeled antibodies: T cell panel (CD3-Percp5.5, CD8αβ-FITC, CD8αα-PE, CD4-Alexa700, CD25-PE-Cy7, CD127-Alexa647); NK, B cell, and monocyte panel (CD3-Percp5.5, CD16-Alexa700, CD56-PE, CD19-PE-Cy7, CD14-FITC); Dendritic cell panel (HLA-DR-Percp-Cy5.5, Lin1-FITC, IL-3Ra (CD123)-PE, CD11c-APC). All antibodies were from BD Biosciences, except live/dead-yellow (Invitrogen). Red blood cells were lysed, samples washed, and samples were analyzed using the LSR II flow cytometer (BD Biosciences). We quantified immune cell subsets according to the following phenotypic markers: total CD4 T cells (CD4+); total CD8 T cells (CD8+); αβ CD8 T cells (CD8+, αβ TCR+); αα CD8+ cells (CD8+, αα TCR+); Treg (CD4+,CD25+,CD127(low)); NK cells (CD16+, CD56+); B cells (CD19+) ; monocytes (CD14+); type 1 Dendritic cell (HLA-DR+, CD11c+, Lin-); type 2 Dendritic cell (HLA-DR+, IL-3Rα+, CD4(low), CD11c-, Lin-). Due to multiple comparisons, we utilized a pre-defined level of significance ($p < 0.01$) for comparisons between groups.

PBMC were similarly isolated from the second sample, and total RNA was extracted to serve as the mRNA source for microarray analysis. RNA extraction was performed using the RNAeasy Mini Kit (Qiagen), and RNA was quantified using a NanoDrop 1000 spectrophotometer. The RNA quality was assessed using an Agilent 2100 Bioanalyzer. The poly(A) RNA was converted to cDNA, then amplified and labeled with biotin following the procedure initially described by Van Gelder et al.[226] Hybridization with the biotin labeled RNA, staining, and scanning of the chips followed the procedure outlined in the Affymetrix technical manual.[227] All analyses used the Affymetrix Human U133 plus 2.0 array, which contains approximately 48,000 probe sets designed from GenBank, dbEST, and RefSeq sequences clustered based on build 133 of the UniGene database and an additional 6500 transcripts identified from Unigene build 159. Scanned output files were visually inspected for hybridization artifacts and then analyzed by using robust multi-array average analysis (RMA). RMA is a well-established procedure that uses quantile normalization and a model-based signal calculation for determination of expression values in probe-based microarray gene expression.[228]

Statistical methods: Following the approach proposed in *Tibshirani, et al*, we used the SAM software to generate an estimate of power.[229] We utilized PBMC data run on the same platform (Affymetrix HG-U133Plus 2.0) from liver transplant patients in *Martinez-Llordella, et al* to generate estimates for sample size and power.[195] Using 10 TOL and 10 non-TOL liver transplant patients, and false discovery rate (FDR) of 10%, we estimated 99% power to detect an effect size of 1.5 for differentially expressed genes, assuming there are approximately 233 truly significant genes. Thus, we projected a minimum sample size of 20 total subjects.

The Significance Analysis of Microarrays (SAM) technique of *Tusher, et al* was employed to identify differentially expressed genes between phenotypic groups.[230] SAM was utilized for the two group (TOL vs. non-TOL) comparison with 10% FDR, and ≥ 1.5 fold difference in mean expression values. To account for confounding by immune suppression

(absent in TOL vs. present in non-TOL cases), we employed the following analyses: We first utilized SAM to identify differentially expressed genes between TOL and non-TOL groups. Second, we compared each group (i.e. TOL vs. control, and separately non-TOL vs. control) to the healthy control group using SAM. Shared genes (unidirectionally different in both TOL and non-TOL with reference to controls) were considered non-informative and filtered out, and thus unique gene lists that distinguished TOL and non-TOL from controls were developed. Finally, for each group of interest (TOL or non-TOL), we retained only those genes from the initial two-group comparison that also were identified as unique genes in each comparison to control. Thus, the final gene list for the TOL group were those that distinguished TOL from both non-TOL and control, and the final gene list for the non-TOL group contained those that distinguished non-TOL from both TOL and controls. Functional Ontology Enrichment (MetaCore by GeneGo) with 5% FDR filter was utilized to identify enriched canonical pathways and cellular process networks, and the biologic relevance of these genes was determined through examination of relevant literature. Finally, using these final TOL and non-TOL gene lists, a classifier was constructed using the leave-10%-out cross-validation method. The stability of this classifier was tested across configurations including a range of 20-80 total probe sets, and each iteration of the classifier included 10-fold cross-validation. Predictive accuracy was also assessed and visually presented in a receiver operating characteristic (ROC) plot. Gene set enrichment analysis (GSEA) was utilized to examine enrichment of the tolerance-associated gene set for cell lineage-specific gene expression (Hematology Expression Atlas of cell lineage-specific genes).

As a secondary analysis approach, a paired (matched TOL vs. non-TOL pairs) analysis utilizing Affymetrix MAS 5.0 comparison analysis for matched samples was performed, and differentially expressed genes were again mapped to pathways and process networks through functional ontology enrichment. We also investigated shared differentially expressed genes

between our data (TOL vs. non-TOL comparison) and previously published differential gene expression data following solid organ transplantation (TOL vs. non-tolerant comparator), and mapped shared genes to enriched pathways.[195,214-216]

CHAPTER THREE:

RESULTS

Randomized phase II study to evaluate tacrolimus in combination with sirolimus or methotrexate after allogeneic hematopoietic cell transplantation:

Patient characteristics and compliance with therapy: From September, 2008 through May, 2011, a total of 175 patients were assessed for eligibility. A total of 101 were excluded for the following: not meeting inclusion criteria (n=72), declined to participate (n=16), no insurance coverage for trial (n=8), and disease progression (n=5). Thus, 74 patients were randomized 1:1 to SIR/TAC vs. MTX/TAC. None were lost to follow-up, and all were included in the reported analyses. Baseline characteristics were well matched (Table 1). Among 37 patients treated with MTX/TAC, 34 completed all doses of MTX; three received 3 doses of MTX, followed in 2 cases by initiation of mycophenolate mofetil as substitute prophylaxis. The final dose of MTX was not given for grade 4 mucositis (n=2) and liver dysfunction (n=1). Overall compliance with SIR was excellent: At time of study analysis for original publication, a total of 2 patients had discontinued SIR (both for grade I TMA, at days 77 and 150 post-HCT, respectively). Among the 17 alive and beyond one year of follow up at the time of that analysis, 16 were receiving SIR as planned per protocol.

Table 1: Baseline characteristics of patients randomized to receive methotrexate or sirolimus in combination with tacrolimus for prevention of acute graft vs. host disease.

	Methotrexate	**Sirolimus**	
Recipient age (median, range)	48 (23-69)	49 (25-68)	p = 0.36
Gender Male Female	 23 14	 28 9	 p = 0.21
Diagnosis			P = 0.08*
ALL *CR1*	10 10	5 5	
AML *CR1* *CR2* *PIF* *REL 1* *no treatment*	8 5 2 1 0 0	15 8 3 2 1 1	
CLL *CR* *PR* *SD*	4 2 1 1	3 2 0 1	
CML *CP1*	0 0	2 2	
MDS *CR* *HI* *SD* *Not treated*	7 2 4 1 0	2 0 1 0 1	
MM *CR* *VGPR* *PR*	2 1 0 1	6 4 1 1	
MPD *SD*	2 2	0 0	
NHL *CR2* *CR3 or >* *PR1* *PR2* *PIF* *REL 1 (sensitive)*	4 0 1 1 0 1 1	4 2 0 0 1 0 0	

REL 3 or > (untreated)	0	1	

Table 1 continued.

CIBMTR risk category *High* *Intermediate* *Low* *Other*	 8 7 20 2	 7 7 23 0	p = 0.52
Donor *MRD* *MUD*	 18 19	 17 20	p = 0.82
Recipient:Donor CMV matching *NN* *NP* *PN* *PP*	 12 7 8 10	 10 1 16 10	p = 0.06
Donor gender *Female* *Male*	 21 16	 17 20	p = 0.35
Donor age (median, range)	37 (18-65)	37 (22-67)	p = 0.3
Conditioning regimen *FluBu* *Pento/Bu* *Flu/Mel*	 30 5 2	 26 4 7	p = 0.22

*Diagnosis: p = 0.08, Remission status: p = 0.69

ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; CLL = chronic lymphocytic leukemia; CML = chronic myelogenous leukemia; MDS = myelodysplastic syndrome; MM = multiple myeloma; MPD = myeloproliferative disease; NHL = non-Hodgkin lymphoma; CR = complete remission; PIF = primary induction failure; REL = relapse; PR = partial remission; SD = stable disease; CP = chronic phase; HI = hematologic improvement; VGPR = very good partial remission; MRD = matched sibling donor; MUD = matched unrelated donor; CMV = cytomegalovirus; N = negative, P = positive; Bu = busulfan; Flu = fludarabine; pent = pentostatin; Mel = melphalan

Engraftment and early toxicity: Time to neutrophil engraftment did not differ between SIR/TAC (median 16 days, range 11-22) and MTX/TAC (median 16, range 12-28), p = 0.57. Time to platelet engraftment was also similar for SIR/TAC (median 12, range 6-20) compared to MTX/TAC (median 16, range 10-33), p = 0.6. No significant differences were observed in donor

chimerism at any of the studied time points (day 30, 90, 360 post-HCT). Peak mucositis did not significantly differ for SIR/TAC vs. MTX/TAC (table 2). The cumulative incidence of hepatic VOD did not significantly differ (SIR/TAC 5% (95% CI 1-21%) vs. MTX/TAC 3% (95% CI 0.4-19%), p=0.56). VOD severity grading is presented in table 2. Notably, the observed incidence of VOD in this study is lower than that previously published.[231] The cumulative incidence of TMA did not significantly differ (SIR/TAC 25% (95% CI 14-44%) vs. MTX/TAC 20% (95% CI 10-38%), p=0.48). TMA occurred in 9 SIR/TAC patients and 7 MTX/TAC patients, p = 0.57. Maximal TMA grade for SIR/TAC vs. MTX/TAC is represented in table 2.

Table 2: Summary of mucositis, thrombotic microangiopathy (TMA), and hepatic veno-occlusive disease (VOD) according to randomized trial study arm.

Variable	Levels	MTX (%)	SIR (%)	P value
Mucositis CTC Grade	1	3 (8.1)	8 (21.6)	0.12
	2	9 (24.3)	13 (35.1)	
	3	21 (56.8)	15 (40.5)	
	4	4 (10.8)	1 (2.7)	
TMA	No	30 (81.1)	28 (75.7)	0.57
	Yes	7 (18.9)	9 (24.3)	
TMA grade	1	4 (10.8)	9 (24.3)	0.17
	2	2 (5.4)	0 (0.0)	
	4	1 (2.7)	0 (0.0)	
	N/A	30 (81.1)	28 (75.7)	
VOD	No	36 (97.3)	35 (94.6)	0.56
	Yes	1 (2.7)	2 (5.4)	
VOD grade	None	36 (97.3)	35 (94.6)	0.57
	Moderate	1 (2.7)	1 (2.7)	
	Severe	0 (0.0)	1 (2.7)	
	Total	37 (50.0)	37 (50.0)	

*TMA = thrombotic microangiopathy, VOD = hepatic veno-occlusive disease (sinusoidal obstructive syndrome), MTX = methotrexate/tacrolimus arm, SIR = sirolimus/tacrolimus arm

Acute graft vs. host disease: The cumulative incidence of grade II-IV acute GVHD at 100 days was 43% (95% CI 27-59%) in the SIR/TAC group, and 89% (95% CI 72-96%) in the MTX/TAC group, $p < 0.001$ (Figure 2). Adjusting for age > 50 vs. ≤ 50 and donor relation strata in a multivariable model, SIR/TAC was associated with reduced hazard for grade II-IV acute GVHD (HR 0.28, 95% CI 0.15-0.52, $p < 0.001$) compared to MTX/TAC. Significant reduction in grade II-IV acute GVHD was observed both for those with matched sibling donor (41% vs. 78%, $p = 0.02$) and matched unrelated donor (45% vs. 100%, $p = 0.001$). The cumulative incidence of grade III-IV acute GVHD did not significantly differ (14% vs. 11%), $p = 0.71$. While the observed incidence of grade II-IV acute GVHD in the MTX/TAC arm is higher than that reported in some published literature, it is consistent with that observed at our center in a previous randomized comparative trial.[219] The inter-institution variation in the observed acute GVHD incidence is in large part due to how aggressively diagnostic endoscopy is pursued to assess the etiology of gastrointestinal symptoms.[232]

Overall grade distribution significantly differed for SIR/TAC vs. MTX/TAC, based on reduction in overall grade II disease (Table 3). Among individual acute GHVD target organs, we only observed significant differences in GI stage (Table 3). When classified according to the site of GI involvement, SIR/TAC treated patients had reduction in both isolated upper GI (SIR n=3, MTX n=10) and combined upper/lower GI involvement (SIR n=5, MTX n=12), but not isolated lower GI involvement (SIR n=7, MTX n=7). Utilizing time-dependent Cox modeling, we could not detect significant relationship between immune suppressive drug (TAC, SIR) levels and grade II-IV or grade III-IV acute GVHD.

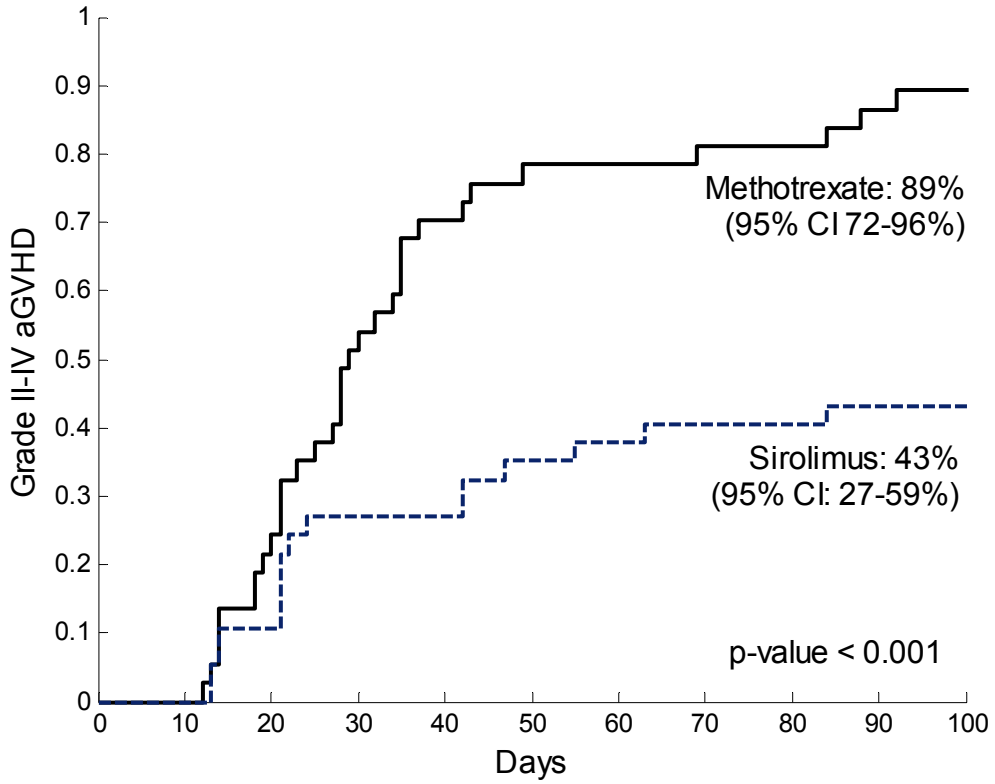


Figure 2: Cumulative incidence of grade II-IV acute GVHD over 100 days following HCT stratified according to initial GVHD prevention.

*GVHD = graft vs. host disease, HCT = allogeneic hematopoietic cell transplantation, methotrexate = methotrexate/tacrolimus study arm, sirolimus = sirolimus/tacrolimus study arm, days = number of days following date of HCT

Table 3: Acute and chronic GVHD characteristics of study population

(A) Individual acute GVHD organ staging and overall acute GVHD grade

	MTX	SIR	p value
Skin stage			
0	15 (41%)	16 (43%)	p = 0.48
1	17 (46%)	13 (35%)	
2	3 (8%)	7 (19%)	
3	2 (5%)	1 (3%)	
4	0 (0%)	0 (0%)	
GI stage			
0	8 (22%)	22 (59%)	p = 0.003
1	27 (73%)	10 (27%)	
2	1 (3%)	3 (8%)	
3	1 (3%)	1 (3%)	
4	0 (0%)	1 (3%)	
Liver stage			
0	30 (81%)	35 (95%)	p = 0.32
1	4 (11%)	1 (3%)	
2	2 (5%)	1 (3%)	
3	1 (3%)	0 (0%)	
4	0 (0%)	0 (0%)	
Overall Grade			
0	2 (5%)	11 (30%)	p < 0.001
I	2 (5%)	10 (27%)	
II	29 (78%)	11 (30%)	
III	4 (11%)	4 (11%)	
IV	0 (0%)	1 (3%)	

*MTX/TAC = methotrexate/tacrolimus arm, SIR/TAC = sirolimus/tacrolimus arm

(B) Chronic GVHD scoring according to NIH Consensus Criteria: Individual organ severity scores and global severity score

	MTX	SIR	p value
Skin			
0	20 (65%)	24 (73%)	p = 0.62
1	7 (23%)	5 (15%)	
2	3 (10%)	4 (12%)	
3	1 (3%)	0 (0%)	
Mouth			
0	18 (58%)	22 (67%)	p = 0.42
1	13 (42%)	10 (30%)	
2	0 (0%)	1 (3%)	
3	0 (0%)	0 (0%)	
Eyes			
0	21 (68%)	20 (61%)	p = 0.27
1	5 (16%)	11 (33%)	
2	4 (13%)	2 (6%)	
3	1 (3%)	0 (0%)	
GI			
0	24 (77%)	32 (97%)	p = 0.06
1	6 (19%)	1 (3%)	
2	0 (0%)	0 (0%)	
3	1 (3%)	0 (0%)	
Liver			
0	17 (55%)	29 (88%)	p = 0.03
1	5 (16%)	2 (6%)	
2	8 (26%)	2 (6%)	
3	1 (3%)	0 (0%)	
Lung			
0	27 (87%)	32 (97%)	p = 0.34
1	1 (3%)	0 (0%)	
2	1 (3%)	1 (3%)	
3	2 (7%)	0 (0%)	
Joints/fascia			
0	28 (90%)	31 (94%)	p = 0.81
1	1 (3%)	1 (3%)	
2	2 (7%)	1 (3%)	
3	0 (0%)	0 (0%)	
Genital			
0	0 (0%)	0 (0%)	
1	0 (0%)	0 (0%)	
2	0 (0%)	0 (0%)	
3	0 (0%)	0 (0%)	
Other			
0	30 (97%)	33 (100%)	p = 0.48
1	0 (0%)	0 (0%)	
2	0 (0%)	0 (0%)	
3	1 (3%)**	0 (0%)	
Overall global score			

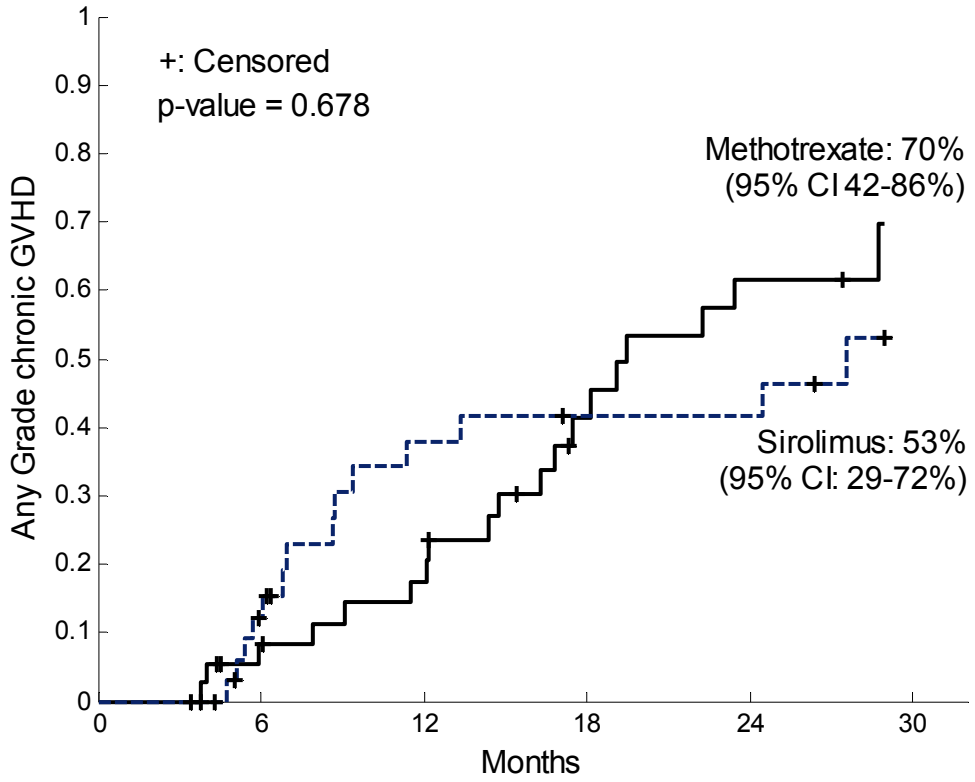
0 1 2 3	11 (36%) 1 (3%) 11 (36%) 8 (26%)	17 (52%) 10 (30%) 5 (15%) 1 (3%)	p = 0.001

*MTX = methotrexate/tacrolimus arm, SIR = sirolimus/tacrolimus arm
**pericardial effusion

Acute GVHD therapy: We captured comprehensive data on prednisone, beclomethasone and budesonide therapy for affected patients. The proportion of living patients on prednisone was not significantly different between groups compared weekly within 100 days and monthly following day 100. There was no significant difference in the proportion receiving systemic glucocorticoids at either 6 months (SIR/TAC 52%, MTX/TAC 59%) or 1 year (SIR/TAC 24%, MTX/TAC 25%) following HCT (p=NS). To spare systemic glucocorticoids, patients with acute upper GI GVHD were treated with beclomethasone and those with acute intestinal GVHD with budesonide, either alone or in combination with systemic glucocorticoids. Fewer patients in the SIR/TAC arm were treated with beclomethasone for manifestations of acute GVHD (p value for each weekly comparison for beclomethasone < 0.05 for weeks 5, 6, 9, 10 and < 0.01 for weeks 11-14); point-wise comparisons for budesonide were not significantly different. Ten patients in SIR/TAC and 6 in MTX/TAC discontinued TAC after intentional taper in the absence of primary disease relapse or TAC toxicity, including TMA. The cumulative incidence of intentional TAC discontinuation at 30 months post-HCT did not differ across groups (SIR/TAC 36%, MTX/TAC 30%, p = 0.16).

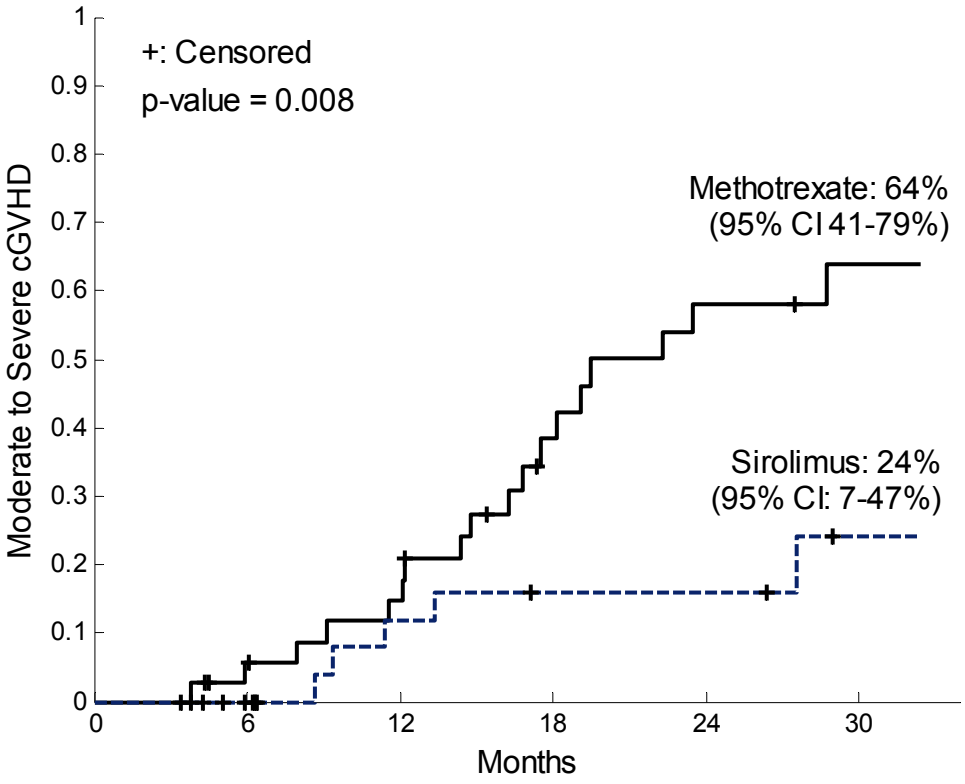
Chronic graft vs. host disease: The cumulative incidence of any grade chronic GVHD per NIH criteria was 53% (95% CI 29-72%) for SIR/TAC and 70% (95% CI 42-86%) for MTX/TAC, p = 0.68. Moderate to severe chronic GVHD was 24% (95% CI 7-47%) for SIR/TAC and 64% (95% CI 41-79%) for MTX/TAC, p = 0.008 (Figure 3). Cumulative incidence estimates

are provided at 30 months post-HCT. Adjusting for age/donor strata, moderate to severe chronic GVHD was significantly reduced among SIR/TAC patients (HR 0.27, 95% CI 0.1-0.72, p = 0.009).The predominant sites of organ involvement were skin, mouth, eye, and liver, recapitulating previously published estimates.[128] Maximum grade of chronic GVHD significantly differed for SIR/TAC vs. MTX/TAC (Table 3). Chronic GVHD therapy was not mandated by this protocol.



(A)

Figure 3: Cumulative incidence of any grade chronic GVHD and moderate to severe chronic GVHD according to NIH criteria



(B)

Figure 3: Cumulative incidence of any grade chronic GVHD and moderate to severe chronic GVHD according to NIH criteria

*GVHD = graft vs. host disease, NIH criteria = NIH Consensus Conference chronic GVHD diagnosis and severity scoring criteria,[127] methotrexate = methotrexate/tacrolimus study arm, sirolimus = sirolimus/tacrolimus study arm, months = number of months following date of HCT

Overall survival, non-relapse mortality, and disease relapse: Median follow-up for surviving patients at the time of study analysis was 20 months (range 4-32) for SIR/TAC, and 17 months (range 4-32) for MTX/TAC. Overall survival did not significantly differ between groups. Two year OS was 61% (95% CI 41-77%) for SIR/TAC and 69% (95% CI 48-83%) for MTX/TAC,

p = 0.66. We did not observe significant difference in primary disease relapse: The 2-year cumulative incidence of relapse was 18% for SIR/TAC and 31% for MTX/TAC, p = 0.09. Adjusting for age/donor strata, the hazard for relapse was not significantly different between the two arms (HR 0.41, 95% CI 0.15-1.14, p = 0.09). Relapse of malignancy was the primary cause of death for 2 patients in the SIR/TAC arm, and 7 patients in the MTX/TAC arm. The two year incidence of non-relapse mortality (NRM) was 28% for SIR/TAC and 8% for MTX/TAC, p = 0.025. Adjusting for age/donor strata, the hazard for NRM among SIR/TAC patients (reference MTX/TAC) was increased (HR 4.95, 95% CI 1.1-22.3, p = 0.04). Non-relapse causes of death occurred in 8 patients in the SIR/TAC arm (septicemia in 2, hepatic VOD, multi-organ failure, acute GVHD, chronic GVHD and hepatic failure, influenza and respiratory failure, and RSV pneumonia in one each), and 2 patients in the MTX/TAC arm (alveolar hemorrhage, and unknown).

Analysis of Patient-reported quality of life (QOL): The Functional Assessment of Cancer Therapy – Bone Marrow Transplant (FACT-BMT) was utilized to assess QOL at days 30, 90, 180, 270, and 360 after HCT.[223] The FACT-BMT is a 47-item measure with reliability and validity in HCT patients.[223,233] It yields a total score as well as subscales assessing physical well-being (PWB), functional well-being (FWB), social/family well-being (SWB), emotional well-being (EWB), and BMT-specific concerns (BMTS). A trial outcome index (TOI) is calculated by summing the PWB, FWB, and BMTS subscales. TOI was selected as the QOL outcome of interest due to its sensitivity to GVHD.[234,235] Higher scores indicate better QOL. As in previous research,[236,237] a difference of 5–9 points on the TOI was considered clinically meaningful. Because groups did not display equivalent QOL at baseline,[238] we examined the trajectory of QOL over the five post-HCT assessment points (i.e., days 30, 90, 180, 270, and 360), controlling for pre-HCT QOL. Thus, the analysis examines the effect of study arm on post-HCT change in QOL independent of baseline QOL. Three participants did not provide enough QOL

data to calculate trajectories, resulting in 71 participants who contributed data to the current analyses. BMT-TOI scores were normally distributed. Results indicated that TOI increased significantly over time in both study arms (p<.01). Nevertheless, study arm significantly predicted TOI at day 360 such that scores in the SIR/TAC group were a mean of 7.17 points lower than the MTX/TAC group (p=.03). There was also a significant effect of study arm over time indicating that the SIR/TAC arm showed smaller improvements in TOI than the MTX/TAC arm (p=.02). Multivariate analyses accounting for effects of acute and chronic GVHD and anemia demonstrated that the SIR/TAC group reported TOI scores 9.54 points lower at day 360 (p<.01) and demonstrated less improvement in TOI over time when controlling for potential clinical confounders (p<.01). These data indicate that prolonged administration of SIR after HCT is associated with inferior QOL through one year post-HCT, despite reduction in significant chronic GVHD.[239] This finding highlights a disparity between clinician and patient perception of benefit, and suggests the importance of inclusion of patient-reported outcomes in GVHD prevention trials.

Regulatory T cell reconstitution and suppressive function: Samples were obtained at the specified time points to characterize Treg in peripheral blood. There was significantly greater proportion of Treg/total CD4+ cells at day 30 and day 90 in SIR/TAC patients (Figure 4). There were increased absolute numbers of Treg and decreased absolute numbers of non-Treg CD4+ cells at these time points (figures 5 and 6). In a subset of patients from SIR/TAC (n=4) and MTX/TAC (n=5), functional assays were performed on samples obtained at day 90 (SIR n=2, MTX n=1), day 180 (SIR n=2, MTX n=3) and day 360 (MTX n=1). All patients were on systemic immune suppression at the time these samples were obtained: Of SIR/TAC patients, this included SIR (n=4), TAC (n=3), and prednisone (n=2), ranging from 0.17 – 1mg/kg/day. For MTX/TAC patients, this included TAC (n=5), SIR (n=1), and prednisone (n=2), ranging 0.1 – 0.83mg/kg/day. For escalating ratio of sorted Treg to T responder cells, we observed increasing

% suppression achieved. While these Treg were functional, we did not observe significant differences in suppressive function between the SIR/TAC and MTX/TAC treated patients (figure 7).

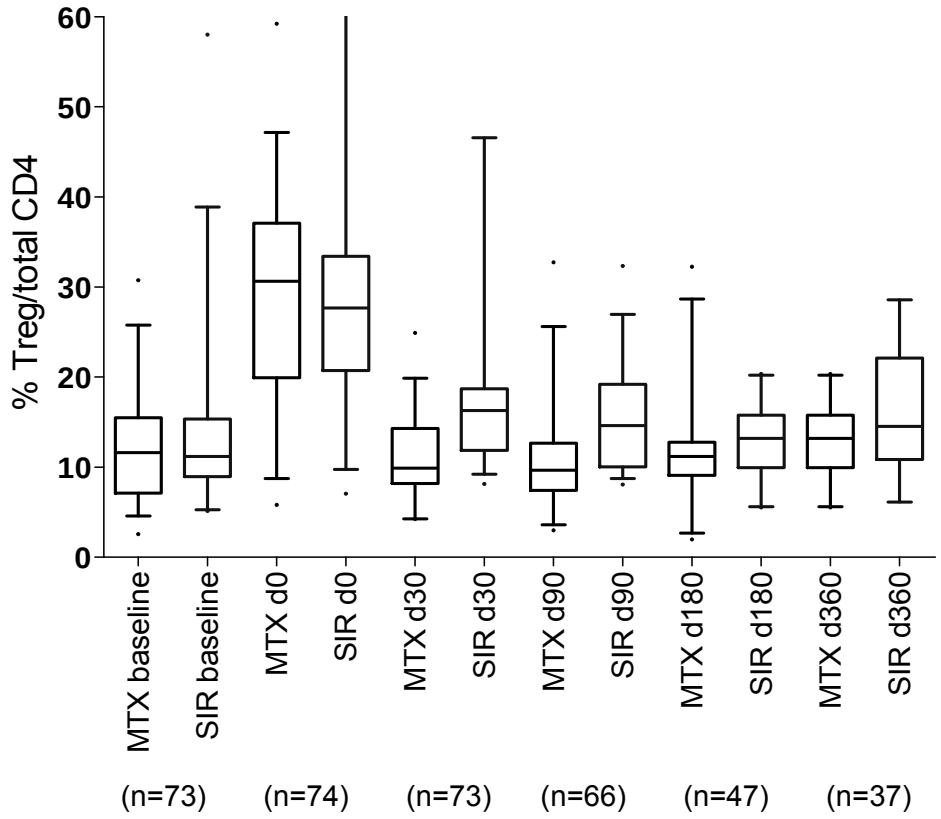


Figure 4: Reconstitution of Treg (Treg/total CD4+ cells) following transplantation according to GVHD prophylaxis regimen

* Day 30 ($p < 0.0001$), day 90 ($p = 0.0009$), day 180 ($p = 0.07$), otherwise, p = not significant. (*box and whisker plot: box margins = interquartile range, line = median value, whiskers = 95% confidence interval, dots = outliers*). Treg = regulatory T cells (defined by cell surface phenotype of CD4+CD25+CD127-), total CD4+ cells = total number of CD4 T cells (defined by cell surface phenotype of CD4+), HCT = allogeneic hematopoietic cell transplantation, methotrexate = methotrexate/tacrolimus study arm, sirolimus = sirolimus/tacrolimus study arm, days = number of days following date of HCT

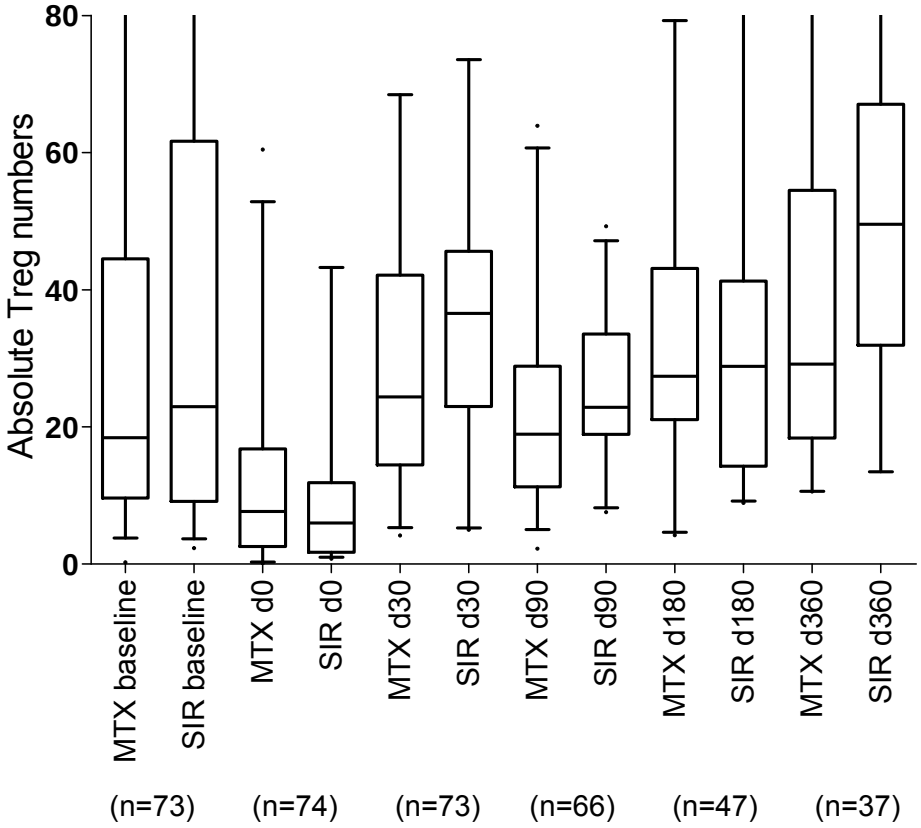


Figure 5: Reconstitution of Treg (absolute number of Treg) following transplantation according to GVHD prophylaxis regimen

***** P value = not significant for each comparison. (*box and whisker plot: box margins = interquartile range, line = median value, whiskers = 95% confidence interval, dots = outliers*).
MTX = methotrexate/tacrolimus study arm, SIR = sirolimus/tacrolimus study arm; Treg = absolute number of regulatory T cells/uL (Treg phenotype = CD4+CD25+CD127-), days = number of days following date of HCT.

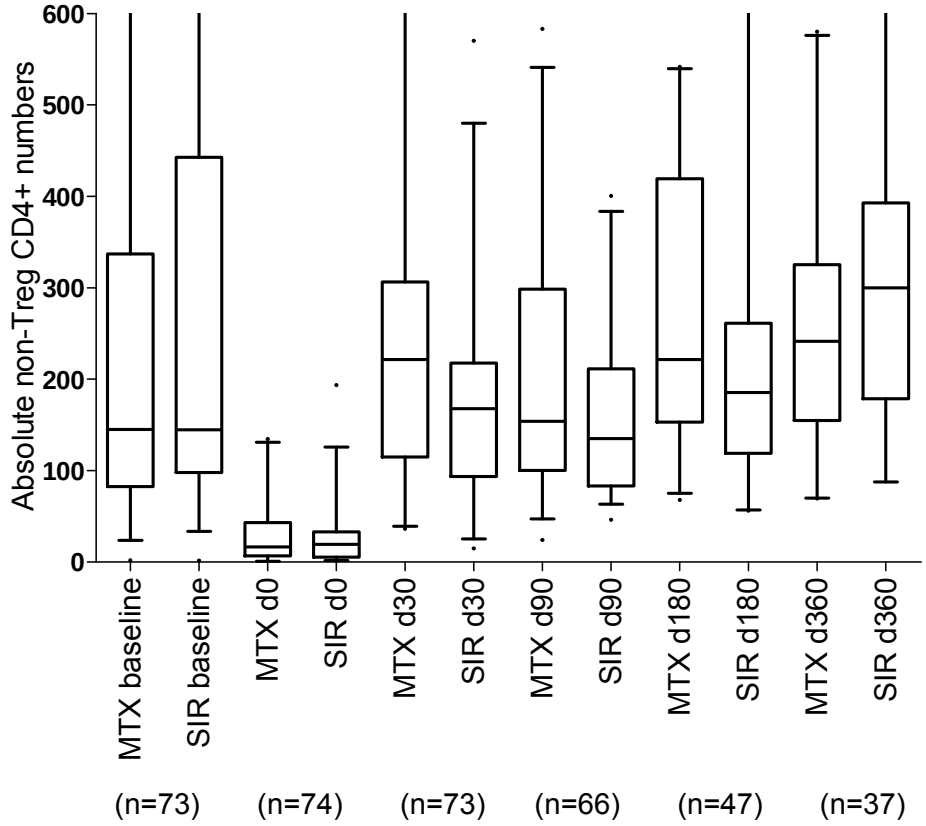
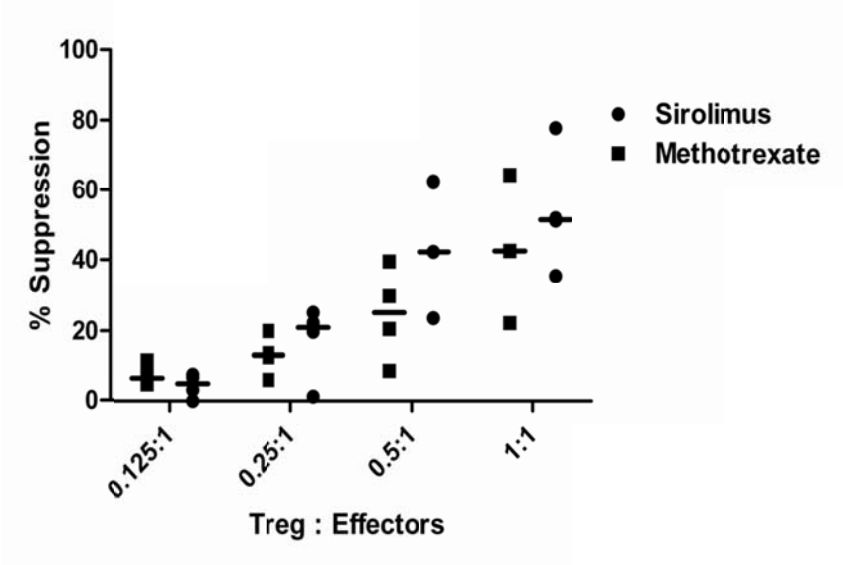


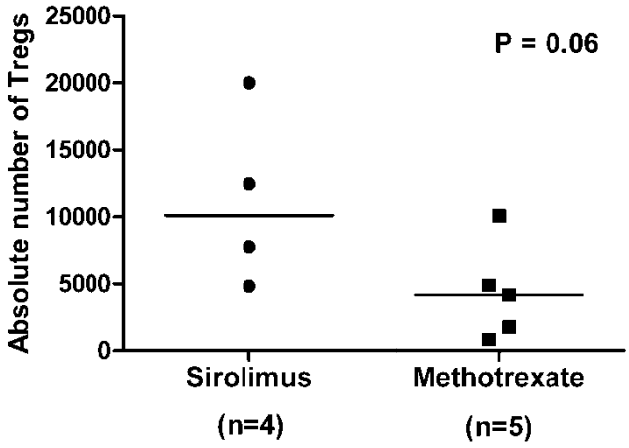
Figure 6: Reconstitution of non-Treg (absolute number of non-Treg CD4+) following transplantation according to GVHD prophylaxis regimen

***** P value = not significant for each comparison. (*box and whisker plot: box margins = interquartile range, line = median value, whiskers = 95% confidence interval, dots = outliers*).
MTX = methotrexate/tacrolimus study arm, SIR = sirolimus/tacrolimus study arm, non-Treg = absolute number of CD4+ cells minus absolute number of CD4+CD25+CD127+ Treg, days = number of days following date of HCT.

(A)



(B)



(C)

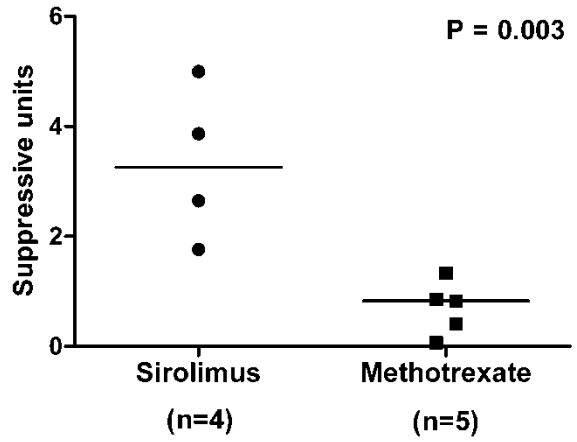


Figure 7: Suppressive function of Treg according to GVHD prophylaxis regimen

*(A) Sorted Treg were tested at different ratios to self CD4+CD25- T cell effectors in the presence of anti-CD3/CD28 beads. Results are shown as average CPM of triplicate measured by the incorporation of ^{3}H-thymidine in co-cultures at day 5 after subtracting the CPM of background wells without Treg (p = not significant for comparisons). (B) Absolute number of Treg per mL for SIR or MTX groups as determined by flow cytometry. (C) Suppressive units for SIR or MTX groups: IC25 of Treg was calculated for suppression of 1 x 10^{4} T effectors. One suppressive unit represents the measure of absolute number of Tregs per mL of blood divided by number Treg capable of suppressing 25% T effectors. SIR = sirolimus/tacrolimus study arm, MTX = methotrexate/tacrolimus study arm, Treg = sorted CD4+CD25+CD127- cells, T effectors = self CD4+CD25- T responder cells

Tissue-infiltrating Th1, Th17, and Treg in GVHD target organs following human allogeneic hematopoietic cell transplantation:

Included samples: A total of 48 patients (SIR: n=25, MTX: n=23) contributed 110 GVHD biopsies to the analysis. Acute GVHD organ biopsy sites, as well as clinical and pathologic grade are represented in table 4, and lymphocyte numbers per TMA core are presented in table 5. Time from GVHD biopsy to topical (p=0.17) or systemic glucocorticoid (p=0.55) therapy did not differ between SIR and MTX-treated patients. RORγ and CD4 co-

registration analysis demonstrated that the majority of RORγ^{+} cells were dual positive for CD4 (median 98%, range 89-99.6%).

Table 4: GVHD organ involvement, pathologic, and clinical grade of GVHD tissue biopsies according to GVHD prevention study arm.

		SIR (%)	MTX (%)	Total (%)	p value
Pathologic grade	1	23 (38.3)	15 (31.9)	38 (35.5)	NS
	2	26 (43.3)	21 (44.7)	47 (43.9)	
	3	11 (18.3)	9 (19.1)	20 (19.6)	
	4	0 (0)	2 (4.3)	2 (1.9)	
	Total	60 (56.1)	47 (43.9)	107 (100)	
Biopsy organ site	Gastric antrum	15 (23.8)	12 (25.5)	27 (24.5)	NS
	Duodenum	18 (28.6)	12 (25.5)	30 (27.3)	
	Rectum	19 (30.2)	15 (31.9)	34 (30.9)	
	Liver	1 (1.6)	2 (4.3)	3 (2.7)	
	Skin	10 (15.9)	6 (12.8)	16 (14.5)	
	Total	63 (57.3)	47 (42.7)	110 (100.0)	
Clinical grade	1	18 (28.6)	0 (0)	18 (16.4)	<.0001
	2	31 (49.2)	44 (93.6)	75 (68.2)	
	3	11 (17.5)	3 (6.4)	14 (12.7)	
	4	3 (4.8)	0 (0.0)	3 (2.7)	
	Total	63 (57.3)	47 (42.7)	110 (100.0)	

*SIR=rapamycin/tacrolimus GVHD prophylaxis group, MTX=methotrexate/tacrolimus GVHD prophylaxis group, NS=not significant

Table 5: Tissue-resident lymphocyte subsets according to GVHD prophylaxis group

	SIR Median (range)	**MTX** Median (range)	**p value**
Total CD4	315 (4-3229)	246 (9-2102)	NS
Th1	48 (7-344)	40 (4-504)	NS
Th17	4 (1-110)	9.5 (2-92)	0.01
Treg	5 (0-132)	6.5 (0-113)	NS

*SIR = sirolimus/tacrolimus GVHD prophylaxis group, MTX = methotrexate/tacrolimus GVHD prophylaxis group, NS=not significant

Association between tissue-resident CD4 subsets and GVHD severity and response to therapy: Th17 increased (median values - grade 1: 5, grade 2: 8, grade 3/4: 20.5) with pathologic grade (figure 8). Two-way ANOVA adjusted for GVHD organ site demonstrated that Th17 (p=0.033) and Th17/CD4 (p=0.021) were significantly associated with pathologic grade. No other subsets were associated with pathologic grade. We found no association of lymphocyte subsets with overall clinical GVHD grade. In subset analysis of GI stage, however, two-way ANOVA adjusted for site of GI organ involvement demonstrated that Th17/CD4 increased with greater GI organ stage (p=0.004). In comparison to MTX/TAC, SIR/TAC-treated patients had significantly lower Th17 cells (table 5, figure 9). Adjusted for clinical and pathologic

grade, SIR/TAC remained significantly associated with lower Th17 (p=0.04). Other lymphocyte subsets did not differ between SIR/TAC and MTX/TAC groups. Refractoriness to standard GVHD therapy (≥ 1mg/kg/day prednisone or equivalent) was defined as lack of complete or partial response by 28 days of therapy, as this is a validated predictor of subsequent non-relapse mortality.[30] Those with refractory acute GVHD had significantly increased (refractory median 27 vs. responsive median 5) number of Th17 present in affected tissues (figure 10). Logistic regression analysis demonstrated that tissue Th17 were significantly associated with refractoriness (OR 6.6, 95% CI 1.6-27, p=0.008), and clinical grade was also associated with refractoriness (grade 3-4 vs. 1: OR 4.4, 95% CI 0.7-25.7, p=0.019). Th17 was also significantly associated with refractoriness in a sub-group analysis limited to GI cases.

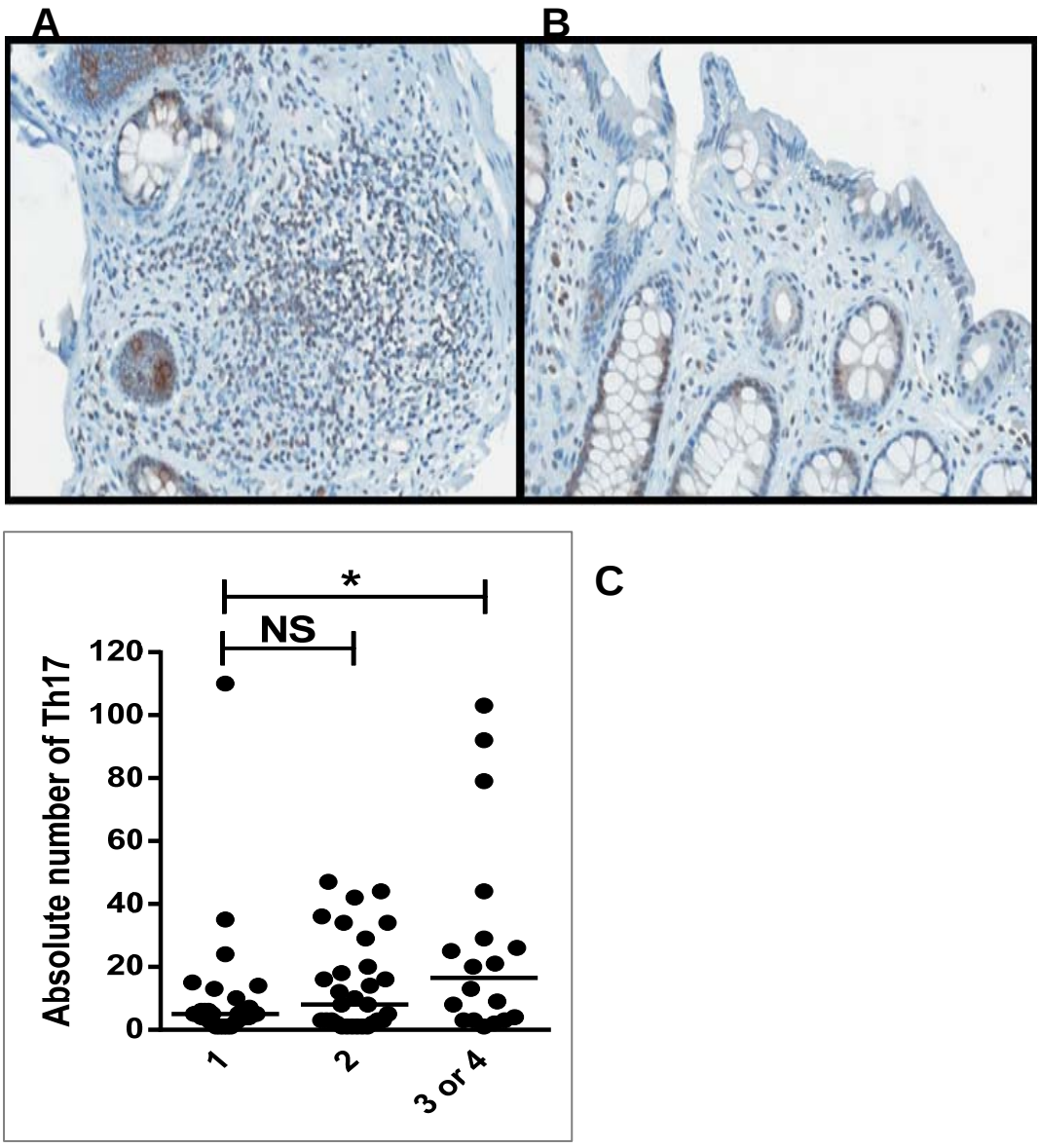
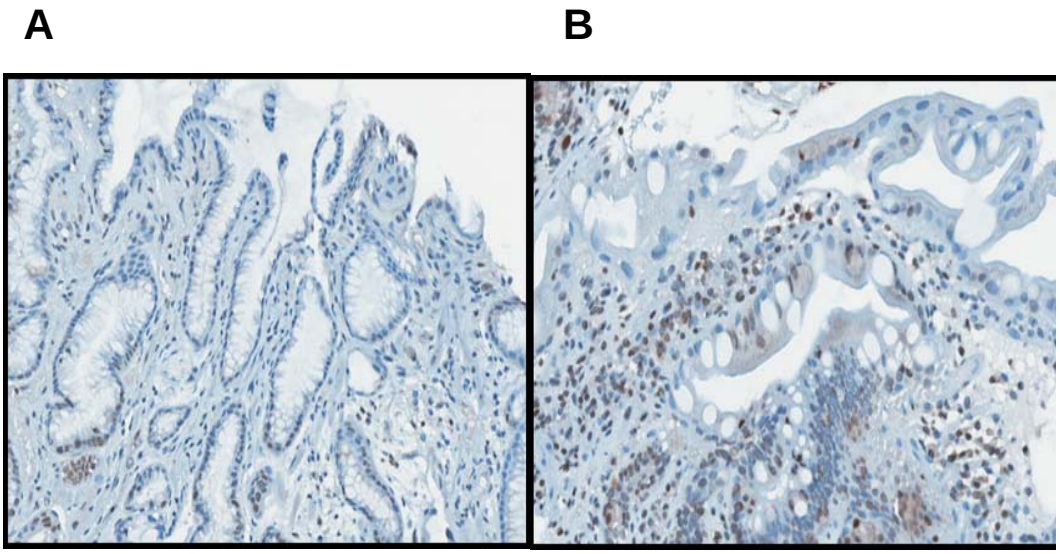


Figure 8: Tissue-resident Th17 cells according to GVHD pathologic grade

*(A) shows increased ROR gamma positive lymphocytes in a rectal biopsy from a patient with pathologic grade 3 GVHD. (B) shows fewer ROR gamma positive lymphocytes in a rectal biopsy from a patient with pathologic grade 1 GVHD. [ROR gamma, x400]. (C) Scatter plot shows absolute number of tissue-resident Th17 by pathologic GVHD grade . Line depicts median. NS=not significant, *P < 0.05.



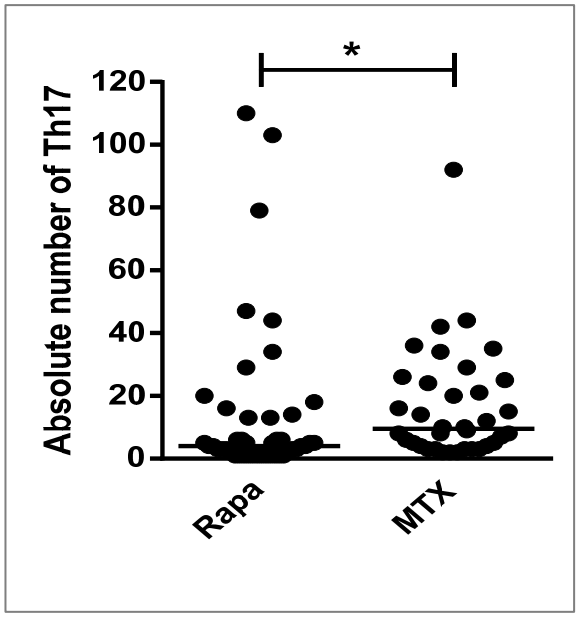


Figure 9: Target-organ Th17 cells according to GVHD prophylaxis regimen

*(A) shows ROR gamma positive lymphocytes in the duodenal lamina propria of a patient who received SIR/TAC. (B) shows increased ROR gamma positive lymphocytes in the duodenal lamina propria from a patient who received MTX/TAC. Both patients were diagnosed with pathologic grade 2 GVHD. [ROR gamma, x400]. (C) Scatter plot shows absolute number of tissue-resident Th17 by use of rapamycin (sirolimus) or methotrexate GVHD prophylaxis. Line depicts median. *P < 0.05.

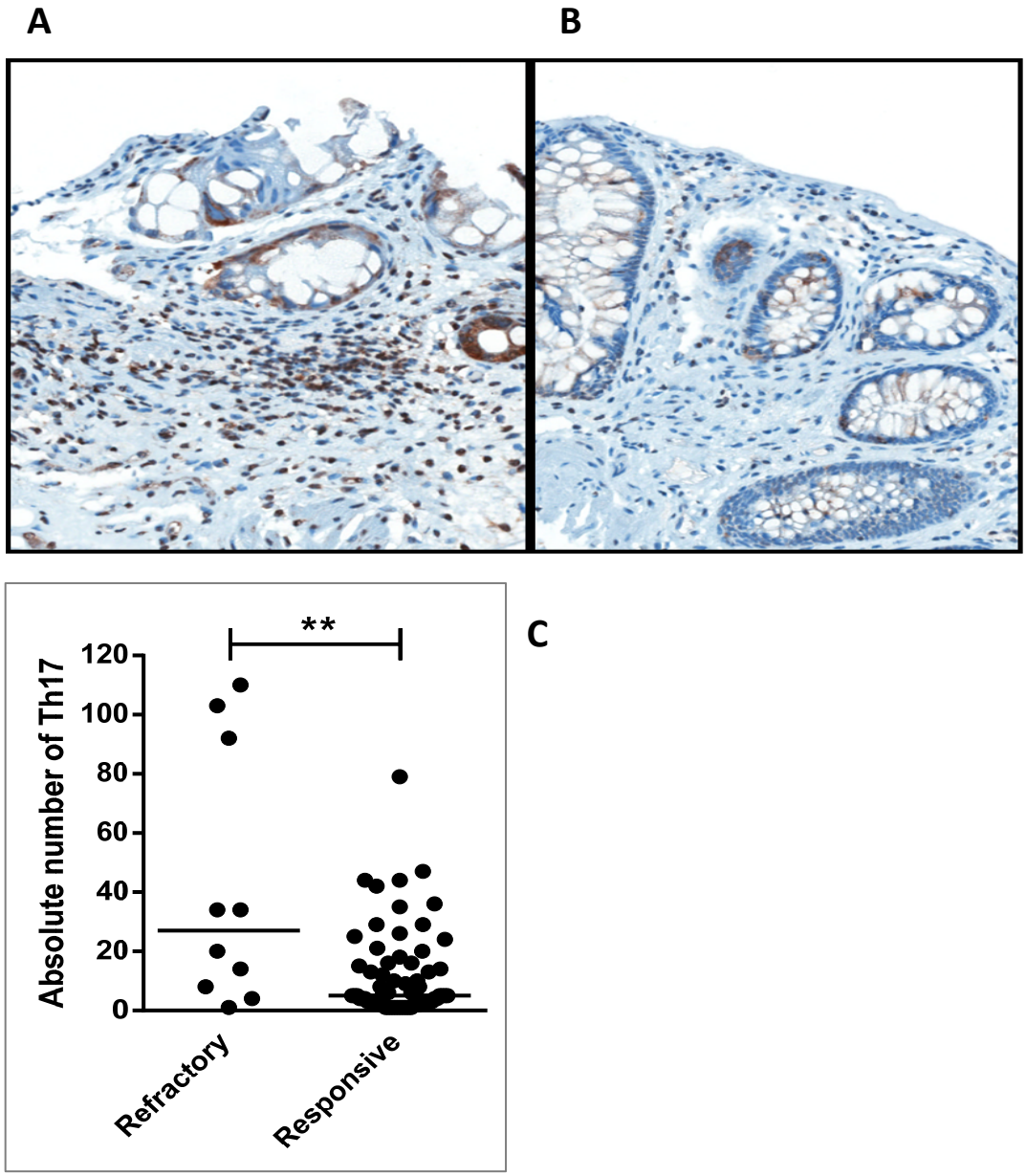


Figure 10: Tissue-resident Th17 cells according to GVHD therapy response

*(A) Increased ROR gamma positive lymphocytes in the lamina propria from rectal biopsy. Panel A patient was diagnosed with pathologic grade 3 GVHD and was refractory to steroid therapy. (B) Shows fewer ROR gamma positive lymphocytes in the lamina propria on rectal

biopsy. The panel B patient was diagnosed with pathologic grade 2 GVHD in the rectum and was responsive to steroid therapy. [ROR gamma, x400]. (C) Scatter plot shows absolute number of tissue-resident Th17 by response to corticosteroid therapy. Line depicts median. **P < 0.01.

Tolerance associated gene expression following allogeneic hematopoietic cell transplantation:

Patient characteristics: A total of 15 tolerant patients after HCT were identified and had sample collection. Two additional tolerant cases were identified, but were not able to participate in the study. A total of 17 non-tolerant comparators were selected based on age, time from HCT, and other clinical transplantation characteristics, and had samples collected. Demographic, transplantation, and GVHD characteristics of the included patients are detailed in table 6. Finally, a total of 10 healthy volunteer control subjects were recruited. These were without acute or chronic illness, and were not on any medications. Median age of controls was 32.5 (range 27-59) years, and included 7 females and 3 males. The TOL and non-TOL patients did not significantly differ according to demographic, disease, or transplantation characteristics (table 6). These were adult patients with hematologic malignancies and disorders predominantly treated with myeloablative chemotherapy-based conditioning. The majority received peripheral blood stem cells from either matched sibling or matched unrelated doors. Initial GVHD prophylaxis was a calcineurin inhibitor together with either methotrexate or mycophenolate mofetil, and acute GVHD severity and treatment did not differ between groups.

Table 6: Comparison of patient, transplantation, and GVHD variables across tolerant and non-tolerant groups in analysis of differential gene expression associated with immune tolerance.

Variable	Tolerant	Non-Tolerant	p value
Median age	50	49	0.79
Donor age	38	52	0.11
Condition			
AA	1	0	0.39
ALL	2	3	
AML	3	7	
CML	0	1	
FL	2	2	
HD	0	1	
IMF	0	1	
MCL	2	0	
MCL, MDS	1	0	
MDS	3	1	
MM	0	1	
MPD	1	0	
Stem cell source			
PBSC	15	16	0.34
BM	0	1	
Donor relation			
MMUD	1	0	0.51
MRD	10	11	
MUD	4	6	
Donor:recipient gender matching			
Female/female	3	6	
Female/male	2	5	0.36
Male/female	2	1	
Male/male	8	5	
HLA matching			
Matched	14	17	0.28
mismatched	1	0	
CMV serostatus recipient:donor			
Neg/neg	4	10	
Neg/pos	1	2	0.13
Pos/neg	5	1	
Pos/pos	5	4	
Conditioning			
Bu/Cy	1	2	0.35
Bu/Flu	8	14	
Bu/Flu/ATG	1	0	
Bu/Flu/R	1	0	
Cy/ATG	1	0	
Cy/BCNU/VP16	1	0	
Cy/TBI	1	1	
Flu/Cy/R	1	0	
Pento/Bu/R	1	0	
aGVHD prophylaxis agent 1			
CSA	1	2	0.51

CSA/TAC	1	0	
TAC	13	15	
aGVHD prophylaxis agent 2			
MMF	6	6	0.78
MTX	9	11	
Max grade aGVHD			
None	4	2	0.29
I	4	1	
II	5	11	
III	1	2	
IV	1	1	
aGVHD treatment agent 1			
none	7	7	
MMF	0	1	0.15
Pred < 1mg/kg	1	0	
Pred 1 mg/kg	4	9	
Pred 2 mg/kg	3	0	
aGVHD treatment agent 2			
none	10	13	
MMF	2	4	0.03
Rapa	3	0	
Max grade cGVHD			
None	9	0	0.0001
Mild	6	5	
Moderate	0	8	
Severe	0	4	
cGVHD treatment agent 1			
Prednisone	1	3	
ECP	0	1	0.13
MMF	0	3	
TAC	0	1	
Rapa	0	1	
none	14	8	
cGVHD treatment agent 2			
MMF	0	2	
MTX	0	1	0.001
Rapa	0	3	
TAC	0	2	
none	15	9	
cGVHD treatment agent 3			
CSA	0	2	
Prednisone	0	1	< 0.0001
Rapa	0	1	
none	15	13	

*Categorical data compared with Fisher's exact test or Chi-square, continuous data utilized wilcoxon rank sum test

* *AA – aplastic anemia; ALL – acute lymphoblastic leukemia; AML – acute myelogenous leukemia; CML – chronic myelogenous leukemia; FL – follicular lymphoma; HD – Hodgkin lymphoma; IMF – idiopathic myelofibrosis; MCL – mantle cell lymphoma; MDS – myelodysplastic syndrome; MM – multiple myeloma; MPD – myeloproliferative neoplasm; PBSC – peripheral blood stem cells; BM – bone marrow harvested*

stem cells; MMUD – mismatched unrelated donor; MRD – matched sibling donor; MUD – matched unrelated donor; HLA – human leukocyte antigen; CMV – cytomegalovirus; neg – negative; pos – positive; Bu – busulfan; Cy – cyclophosphamide; Flu – fludarabine; ATG – anti-thymocyte globulin; R – rituximab; BCNU – carmustine; VP16 – etoposide; TBI – total body irradiation; pento – pentostatin; CSA – cyclosporine; TAC – tacrolimus; MMF – mycophenolate mofetil; MTX – methotrexate; aGVHD – acute graft vs. host disease; pred – prednisone; rapa – rapamycin (sirolimus); ECP – extra-corporeal photopheresis; cGVHD – chronic graft vs. host disease

The TOL and non-TOL groups did significantly differ in their history of chronic GVHD, as the non-TOL patients had greater NIH Consensus global severity of chronic GVHD and greater extent of therapy delivered for chronic GVHD: Among the TOL patients, 9 had no history of chronic GVHD, and 6 had a prior maximum mild chronic GVHD. Of these, only one required the addition of any systemic IS for chronic GVHD therapy. Among the TOL patients with any history of chronic GVHD, this was completely resolved at a median of 25.3 months (range 17.6 – 39.7) prior to the study sample acquisition. In contrast, the maximum global severity of chronic GVHD among the non-TOL patients was 1 (n=5), 2 (n=8), or 3 (n=4). Chronic GVHD organ involvement included skin (n=11), eye (n=6), mouth (n=6), GI (n=5), liver (n=8), lung (n=2), and fascia/joints (n=1). Therapy delivered included prednisone and additional systemic immune suppressive therapies, and none had discontinued all IS by time of study sample acquisition. The median time from HCT to study sample acquisition (TOL 38.5 vs. non-TOL 39.5 months) did not differ between groups, p=0.97. The median time from complete IS discontinuation to study sample acquisition among TOL patients was 19.15 (range 7.1 – 68) months.

Immune cell subsets: Immune cell subsets were identified through evaluation of cell surface markers (table 7). There was a suggestion toward increased total CD8+ T cells, and specifically CD8 αβ T cells in the TOL group. However, based on our pre-specified significance level of 0.01 in the setting of multiple comparisons, we did not observe significant differences in any of the studied immune subsets between TOL and non-TOL groups. Accordingly, we did not incorporate cell subset composition into subsequent gene expression analyses.

Table 7: Comparison of immune cell subsets among tolerant and non-tolerant patients.

Cell subset	Phenotype	TOL	non-TOL	p value
Total CD3+	*CD3+*	62.4	56.3	0.28
Total CD4+	*CD4+*	25.9	31.9	0.52
CD4+ CD25-	*CD4+/CD25-*	22.7	30.6	0.48
Total CD8+	*CD8+*	32.1	18.2	0.052
CD8 αα (NK, DC, IEL)	*CD8 αα+*	1.85	1.4	0.27
CD8 αβ (alpha-beta CD8)	*CD8 αβ+*	18.6	6.8	0.03
Memory CD8	*CD8+/CD127+*	32.8	30	0.9
Effector CD8	*CD8+/CD127-*	67.2	69.9	0.9
Regulatory T cells (Treg)	*CD4+/CD25+/CD127-*	1.6	1.5	0.7
Treg/CD8 ratio	*Treg/CD8+CD25+*	1.1	0.9	0.82
Monocytes	*CD14+*	9.8	11.1	0.26
Total B cells	*CD19+*	12.9	8.1	0.1
Plasmacytoid DC	*IL-3RA+/HLA-DR+*	0.12	0.13	0.24
Monocytoid DC	*CD11c+/HLA-DR+*	0.23	0.57	0.27
NK cells	*CD16+/CD56+*	11.5	13.7	0.58
NKT	*CD3+/CD16+/CD56+*	0.05	0.025	0.16

**Values represent proportion of cells with indicated cell phenotype. NK – natural killer cell; DC – dendritic cell; IEL – intra-epithelial lymphocyte; Treg – regulatory T cell; NKT – NKT cells; TOL – tolerant patients; non-TOL – non-tolerant patients*

Two-group (TOL vs. non-TOL) analysis: In the initial two-group comparison, SAM identified 231 probe sets over- and 412 under-expressed in the TOL vs. non-TOL group. Enriched process networks included those related to NK cells (NK cell cytotoxicity), phagocytosis and antigen presentation (phagocytosis, phagosome in antigen presentation), B cell signaling (BCR pathway), and lymphocyte differentiation and signaling (T helper cell differentiation, TCR signaling, protein C signaling, anti-apoptosis mediated via MAPK and JAK/STAT, lymphocyte proliferation, JAK-STAT pathway, and Th17-derived cytokines) (figure 11). The secondary matched paired analysis identified 255 probe sets over- and 150 under-expressed in the TOL vs. non-TOL groups. Enriched process networks included TCR signaling ($p = 1.1E-05$), T helper cell differentiation ($p = 0.00086$), BCR pathway signaling ($p = 0.0029$), and NK cell cytotoxicity ($p = 0.009$). Differentially expressed genes in our analysis were compared with those identified in published comparisons of tolerant vs. non-tolerant comparators in liver and kidney transplantation, and these were mapped to enriched cellular process networks (figure 12).

cellular process networks	**p value**	**ratio** (involved) / (total)	
Inflammation_NK cell cytotoxicity	1.553E-09	22	164
Immune response_Antigen presentation	2.233E-07	21	197
Inflammation_Neutrophil activation	5.064E-06	20	219
Reproduction_Feeding and Neurohormone signaling	1.082E-05	19	211
Immune response_Phagocytosis	2.234E-05	19	222
Chemotaxis	4.077E-05	14	137
Cell adhesion_Amyloid proteins	4.853E-05	17	195
Cell adhesion_Platelet aggregation	1.924E-04	14	158
Immune response_T helper cell differentiation	2.050E-04	13	140
Inflammation_Interferon signaling	3.337E-04	11	110
Cell adhesion_Leucocyte chemotaxis	8.981E-04	15	205
Inflammation_Histamine signaling	1.264E-03	15	212
Proliferation_Lymphocyte proliferation	3.036E-03	14	209
Inflammation_IL-2 signaling	3.175E-03	9	104

Signal Transduction_Cholecystokinin signaling	3.610E-03	9	106
Autophagy_Autophagy	5.004E-03	6	55
Inflammation_IgE signaling	6.168E-03	10	136
Inflammation_Jak-STAT Pathway	8.599E-03	12	188
Proteolysis_Proteolysis in cell cycle and apoptosis	1.048E-02	9	125
Immune response_Phagosome in antigen presentation	1.122E-02	14	243
Immune response_TCR signaling	1.248E-02	11	174
Inflammation_Protein C signaling	1.318E-02	8	108
Apoptosis_Anti-Apoptosis mediated by external signals via MAPK and JAK/STAT	1.517E-02	11	179
Development_Neurogenesis_Axonal guidance	1.655E-02	13	230
Blood coagulation	1.937E-02	7	94
Cell adhesion_Cell junctions	1.960E-02	10	162
Inflammation_Amphoterin signaling	2.145E-02	8	118
Proliferation_Positive regulation cell proliferation	2.753E-02	12	221
Development_Regulation of angiogenesis	2.926E-02	12	223
Cardiac development_Wnt_beta-catenin, Notch, VEGF, IP3 and integrin signaling	3.068E-02	9	150
Signal transduction_WNT signaling	3.355E-02	10	177
Development_Blood vessel morphogenesis	3.390E-02	12	228
Immune response_IL-5 signalling	3.825E-02	4	44
Cell adhesion_Glycoconjugates	4.654E-02	9	162

Figure 11: Enriched cellular process networks for study of differential gene expression across tolerant and non-tolerant cases.

* Cellular process networks are ranked in descending order based on p value for magnitude of enrichment of experimental data to annotated networks using MetaCore by GeneGo software (limited to those with $p < 0.05$). Ratio of involved/total genes indicates the enrichment (number of genes involved per total number of genes annotated for each indicated process network) of differential genes for the indicated cellular process network.

cellular process networks	**p value** (solid organ) / (HCT)	**minimum (p value)**	**ratio** (involved) / (total)	
Inflammation_NK cell cytotoxicity	6.239e-3 / 2.916e-9	2.916E-09	30	164
Chemotaxis	8.023e-8 / 5.866e-5	8.023E-08	29	137
Inflammation_Neutrophil activation	5.617e-3 / 1.389e-7	1.389E-07	33	219
Cell adhesion_Amyloid proteins	2.722e-1 / 3.292e-7	3.292E-07	26	195
Immune response_Phagocytosis	1.065e-3 / 7.266e-7	7.266E-07	36	222
Immune response_Phagosome in antigen presentation	1.905e-6 / 9.972e-4	1.905E-06	37	243
Inflammation_IL-4 signaling	3.185e-6 / 2.647e-1	3.185E-06	18	115
Immune response_Antigen presentation	1.967e-3 / 6.449e-6	6.449E-06	31	197
Reproduction_Feeding and Neurohormone signaling	4.162e-2 / 1.739e-5	1.739E-05	27	211
Immune response_BCR pathway	3.083e-5 / 2.211e-2	3.083E-05	25	137
Cell adhesion_Leucocyte chemotaxis	1.140e-3 / 4.091e-5	4.091E-05	30	205
Cell adhesion_Platelet-endothelium-leucocyte interactions	5.144e-5 / 1.536e-1	5.144E-05	25	174
Development_Neurogenesis_Axonal guidance	3.878e-3 / 5.783e-5	5.783E-05	34	230
Development_EMT_Regulation of epithelial-to-mesenchymal transition	6.085e-5 / 7.855e-2	6.085E-05	31	226
Inflammation_Histamine signaling	4.106e-3 / 6.375e-5	6.375E-05	30	212
Cell adhesion_Platelet aggregation	2.115e-1 / 7.520e-5	7.520E-05	21	158
Inflammation_Amphoterin signaling	1.004e-2 / 2.085e-4	2.085E-04	20	118
Inflammation_TREM1 signaling	2.183e-4 / 7.069e-2	2.183E-04	21	145
Immune response_T helper cell differentiation	6.623e-2 / 2.839e-4	2.839E-04	19	140
Cell adhesion_Cell junctions	3.689e-1 / 3.518e-4	3.518E-04	20	162
Inflammation_Protein C signaling	1.950e-1 / 3.778e-4	3.778E-04	14	108
Inflammation_Interferon signaling	5.743e-4 / 1.677e-3	5.743E-04	19	110
Cell cycle_G2-M	1.203e-3 / 9.983e-1	1.203E-03	18	206
Signal Transduction_Cholecystokinin signaling	8.818e-2 / 1.264e-3	1.264E-03	14	106

Signal Transduction_TGF-beta, GDF and Activin signaling	1.291e-3 / 3.185e-1	1.291E-03	18	154
Cell adhesion_Integrin-mediated cell-matrix adhesion	1.822e-3 / 1.957e-1	1.822E-03	25	214
Cell adhesion_Glycoconjugates	2.092e-3 / 3.444e-3	2.092E-03	24	162
Proliferation_Positive regulation cell proliferation	1.753e-1 / 2.640e-3	2.640E-03	23	221
Apoptosis_Anti-Apoptosis mediated by external signals via MAPK and JAK/STAT	5.893e-2 / 2.808e-3	2.808E-03	23	179
Development_Regulation of angiogenesis	2.819e-3 / 1.683e-2	2.819E-03	27	223
Proteolysis_ECM remodeling	3.399e-3 / 2.528e-1	3.399E-03	12	85
Development_Blood vessel morphogenesis	3.546e-3 / 4.204e-2	3.546E-03	26	228
Inflammation_IL-2 signaling	1.732e-1 / 3.956e-3	3.956E-03	13	104
Proliferation_Lymphocyte proliferation	3.424e-1 / 4.093e-3	4.093E-03	20	209
Signal transduction_ERBB-family signaling	5.399e-3 / 1.889e-1	5.399E-03	11	75
Autophagy_Autophagy	1.379e-2 / 5.872e-3	5.872E-03	9	55
Immune response_TCR signaling	2.335e-2 / 6.104e-3	6.104E-03	22	174
Cytoskeleton_Regulation of cytoskeleton rearrangement	6.295e-3 / 1.003e-1	6.295E-03	22	183
Apoptosis_Apoptotic mitochondria	6.331e-3 / 9.061e-1	6.331E-03	9	77
Proliferation_Negative regulation of cell proliferation	6.599e-3 / 1.899e-1	6.599E-03	20	184
Cytoskeleton_Actin filaments	1.040e-1 / 6.673e-3	6.673E-03	20	176
Inflammation_Kallikrein-kinin system	6.915e-3 / 1.937e-1	6.915E-03	21	185
Inflammation_IgE signaling	1.184e-1 / 7.734e-3	7.734E-03	17	136
Immune response_Th17-derived cytokines	6.344e-2 / 9.123e-3	9.123E-03	15	98
Development_Cartilage development	9.340e-3 / 3.195e-1	9.340E-03	9	66
Inflammation_Jak-STAT Pathway	3.921e-2 / 1.103e-2	1.103E-02	21	188
Proteolysis_Proteolysis in cell cycle and apoptosis	4.732e-1 / 1.283e-2	1.283E-02	13	125
Signal transduction_Leptin signaling	1.411e-2 / 2.143e-1	1.411E-02	13	106
Signal transduction_WNT signaling	1.899e-1 / 1.759e-2	1.759E-02	19	177

Apoptosis_Anti-apoptosis mediated by external signals via NF-kB	1.860e-2 / 1.173e-1	1.860E-02	14	111

Figure 12: Enriched cellular process networks shared between experimental data and published tolerance-associated gene expression data in solid organ transplantation

*Cellular process networks are ranked in descending order based on p value for magnitude of enrichment (minimum p value for either solid organ or HCT data) to annotated networks using MetaCore by GeneGo software for each process network. Ratio of involved/total genes indicates the enrichment (number of genes involved per total number of genes annotated for each indicated process network) of differential genes for the indicated cellular process network. Solid organ = published solid organ transplant data,[195,214-216] and HCT = HCT experimental data.

Three group (TOL vs. non-TOL vs. control) analysis: SAM identified 655 probe sets differentially expressed between TOL and non-TOL groups. The TOL vs. control analysis identified 5,687 probe sets, of which 2,273 were unique after filtering out non-informative shared probe sets (those represented in both TOL vs. control and non-TOL vs. control lists and unidirectionally different from control). The non-TOL vs. control analysis identified 4,788 probe sets, of which 1,376 were unique. The final TOL list contained 281 probe sets, which were differentially expressed in the TOL group vs. both the non-TOL and control groups. The final non-TOL list contained 122 probe sets which were differentially expressed compared to both TOL and control groups.

Differentially expressed probe sets in the TOL and non-TOL groups were enriched for immune response genes focused in the innate immune response, NK cytotoxicity, lymphocyte signaling and regulation, apoptosis and cell cycle control. The direction and magnitude of differences with respect to each comparison group is represented in figure 13 and figure 14 for selected genes; from the total 281 TOL and 122 non-TOL probe sets, these genes were selected for presentation based on their association with top-scored cellular process networks, > 2-fold change vs. comparator groups, and relevance to established mechanisms of immune tolerance.

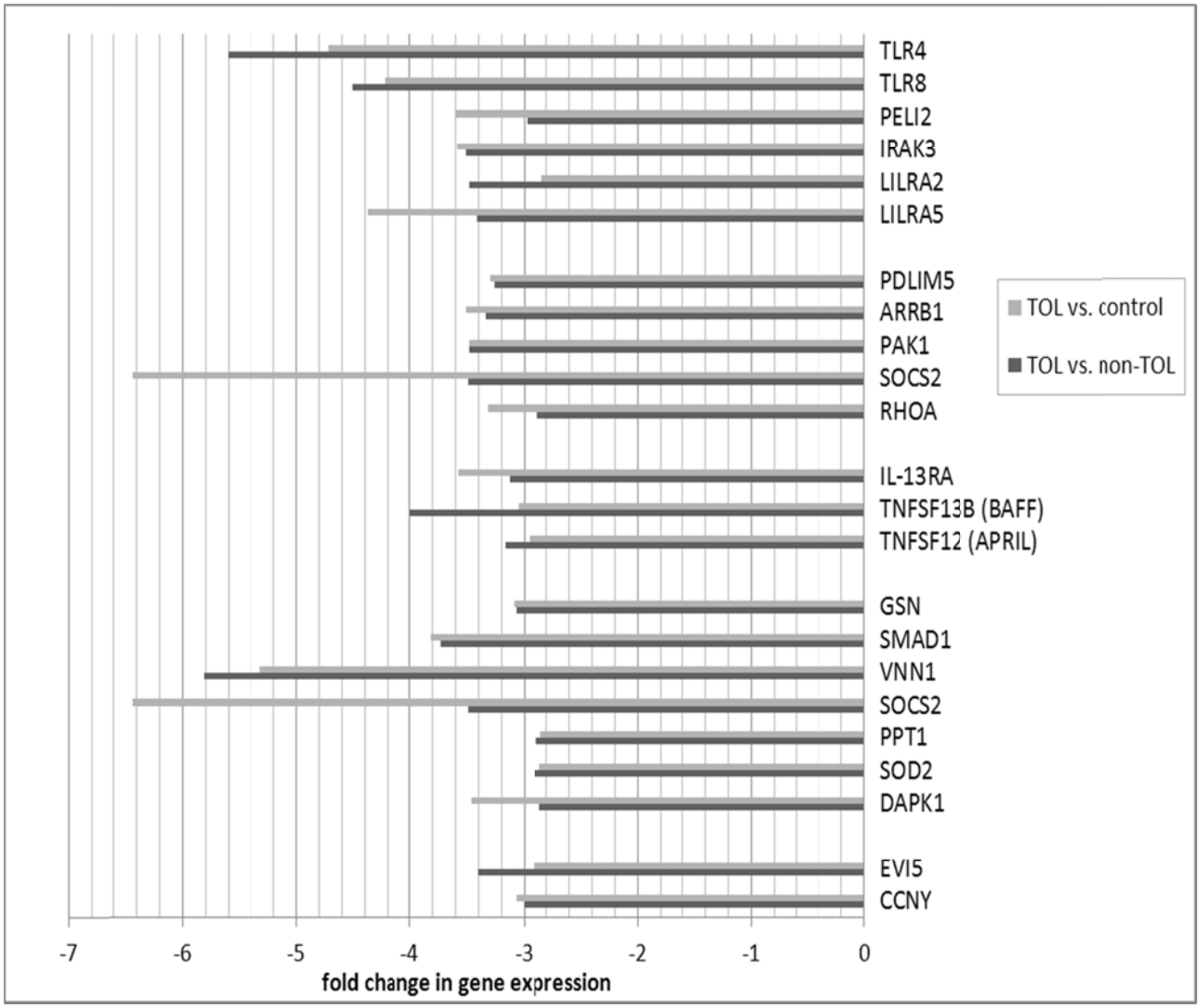


Figure 13: Direction and magnitude of change of selected genes in tolerant group vs. non-tolerant and control group.

(a) Genes with decreased expression in TOL group

*TOL = tolerant cases, non-TOL = non-Tolerant cases, control = healthy controls. TLR4 - toll-like receptor 4; TLR8 - toll-like receptor 8; PELI2 - pellino homolog 2; IRAK3 - interleukin-1 receptor-associated kinase 3; LILRA2 - leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 2; LILRA5 - leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5; PDLIM5 - PDZ and LIM domain 5; ARRB1 - arrestin, beta 1; PAK1 - p21 protein (Cdc42/Rac)-activated kinase 1; SOCS2 - suppressor of cytokine signaling 2; RHOA - ras homolog gene family, member A; IL-13RA - interleukin 13 receptor, alpha 1; TNFSF13B - tumor necrosis factor (ligand) superfamily, member 13b (BAFF); TNFSF12 - tumor necrosis factor (ligand) superfamily, member 12 (APRIL); GSN – gelsolin; SMAD1 - SMAD family member 1; VNN1 - vanin 1; PPT1 - palmitoyl-protein thioesterase 1; SOD2 - superoxide dismutase 2, mitochondrial; DAPK1 - death-associated protein kinase 1; EVI5 - ecotropic viral integration site 5; CCNY - cyclin Y.

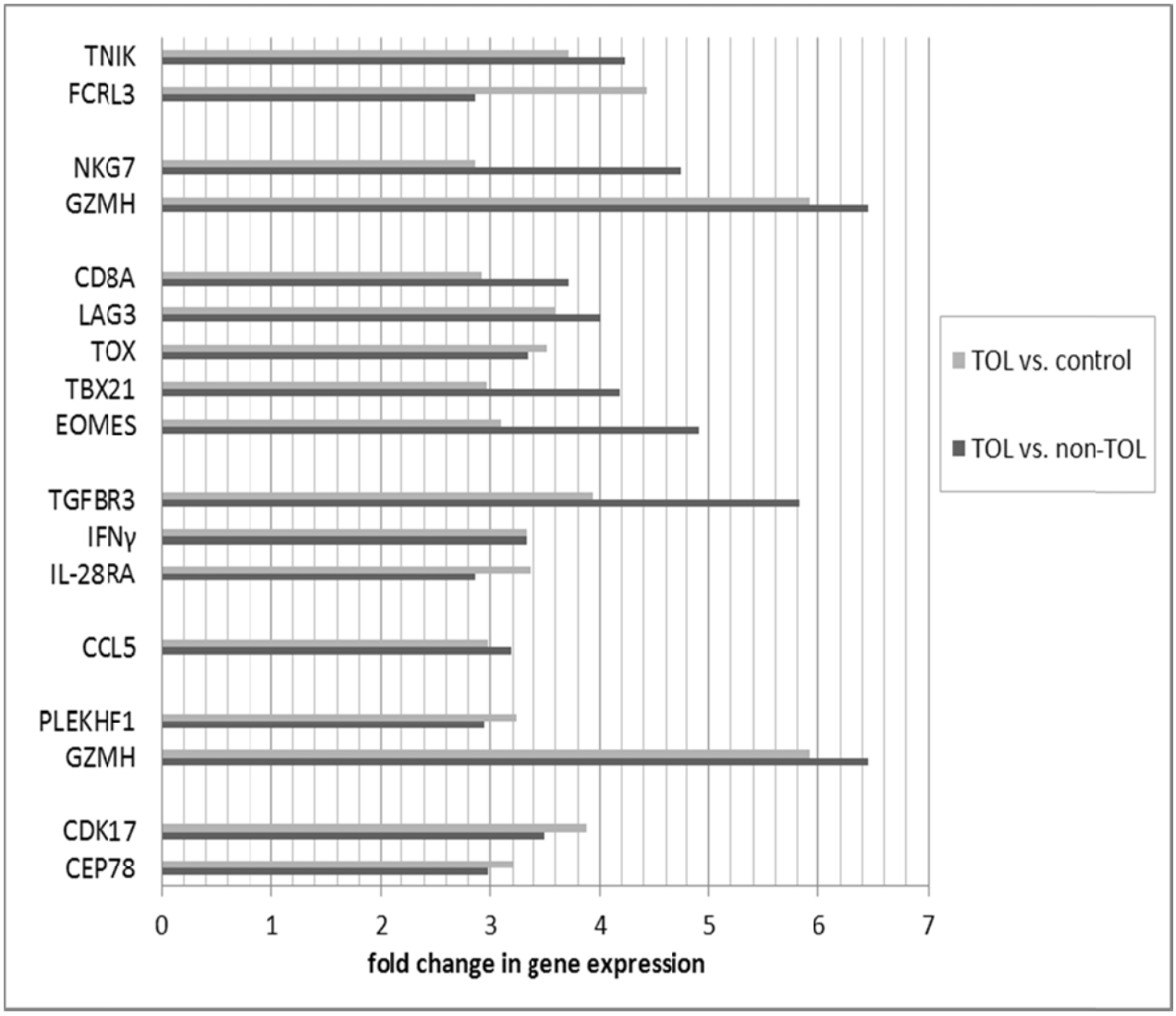


Figure 13: Direction and magnitude of change of selected genes in tolerant group vs. non-tolerant and control group.

(b) Genes with increased expression in TOL group

* TOL = tolerant cases, non-TOL = non-Tolerant cases, control = healthy controls. TNIK - TRAF2 and NCK interacting kinase; FCRL3 - Fc receptor-like 3; NKG7 - natural killer cell group 7 sequence; GZMH - granzyme H (cathepsin G-like 2, protein h-CCPX); CD8A - CD8a molecule; LAG3 - lymphocyte-activation gene 3; TOX - thymocyte selection-associated high mobility group box; TBX21 - T-box 21 (T-bet); EOMES – eomesodermin; TGFBR3 - transforming growth factor, beta receptor III; IFNγ - interferon, gamma; IL-28RA - interleukin 28 receptor, alpha (interferon, lambda receptor); CCL5 - chemokine (C-C motif) ligand 5; PLEKHF1 - pleckstrin homology domain containing, family F (with FYVE domain) member 1; GZMH - granzyme H (cathepsin G-like 2, protein h-CCPX); CDK17 - cyclin-dependent kinase 17; CEP78 - centrosomal protein 78kDa.

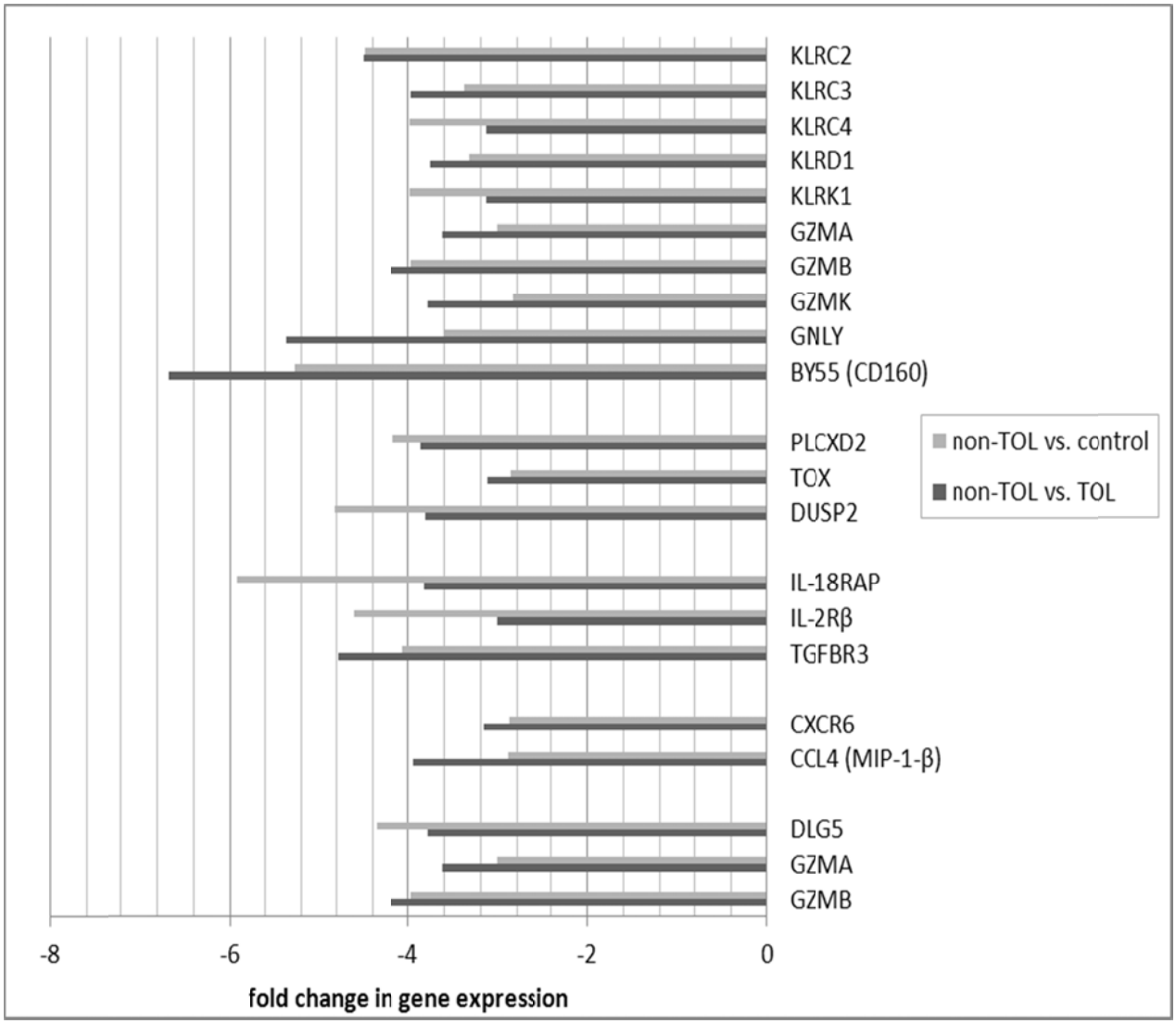


Figure 14: Direction and magnitude of change in non-tolerant group vs. tolerant and control groups

(a) Genes with decreased expression in non-TOL group

* TOL = tolerant cases, non-TOL = non-Tolerant cases, control = healthy controls. KLRC2 - killer cell lectin-like receptor subfamily C, member 2; KLRC3 - killer cell lectin-like receptor subfamily C, member 3; KLRC4 - killer cell lectin-like receptor subfamily C, member 4; KLRD1 - killer cell lectin-like receptor subfamily D, member 1; KLRK1 - killer cell lectin-like receptor subfamily K, member 1; GZMA - granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3); GZMB - granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1); GZMK - granzyme K (granzyme 3; tryptase II); GNLY – granulysin; BY55 (CD160) - CD160 molecule; PLCXD2 - phosphatidylinositol-specific phospholipase C, X domain containing 2; TOX - thymocyte selection-associated high mobility group box; DUSP2 - dual specificity phosphatase 2; IL-18RAP - interleukin 18 receptor accessory protein; IL-2Rβ - interleukin 2 receptor, beta; TGFBR3 - transforming growth factor, beta receptor III; CXCR6 - chemokine (C-X-C motif) receptor 6; CCL4 (MIP-1-β) - chemokine (C-C motif) ligand 4; DLG5 - discs, large homolog

5; GZMA - granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3); GZMB - granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1).

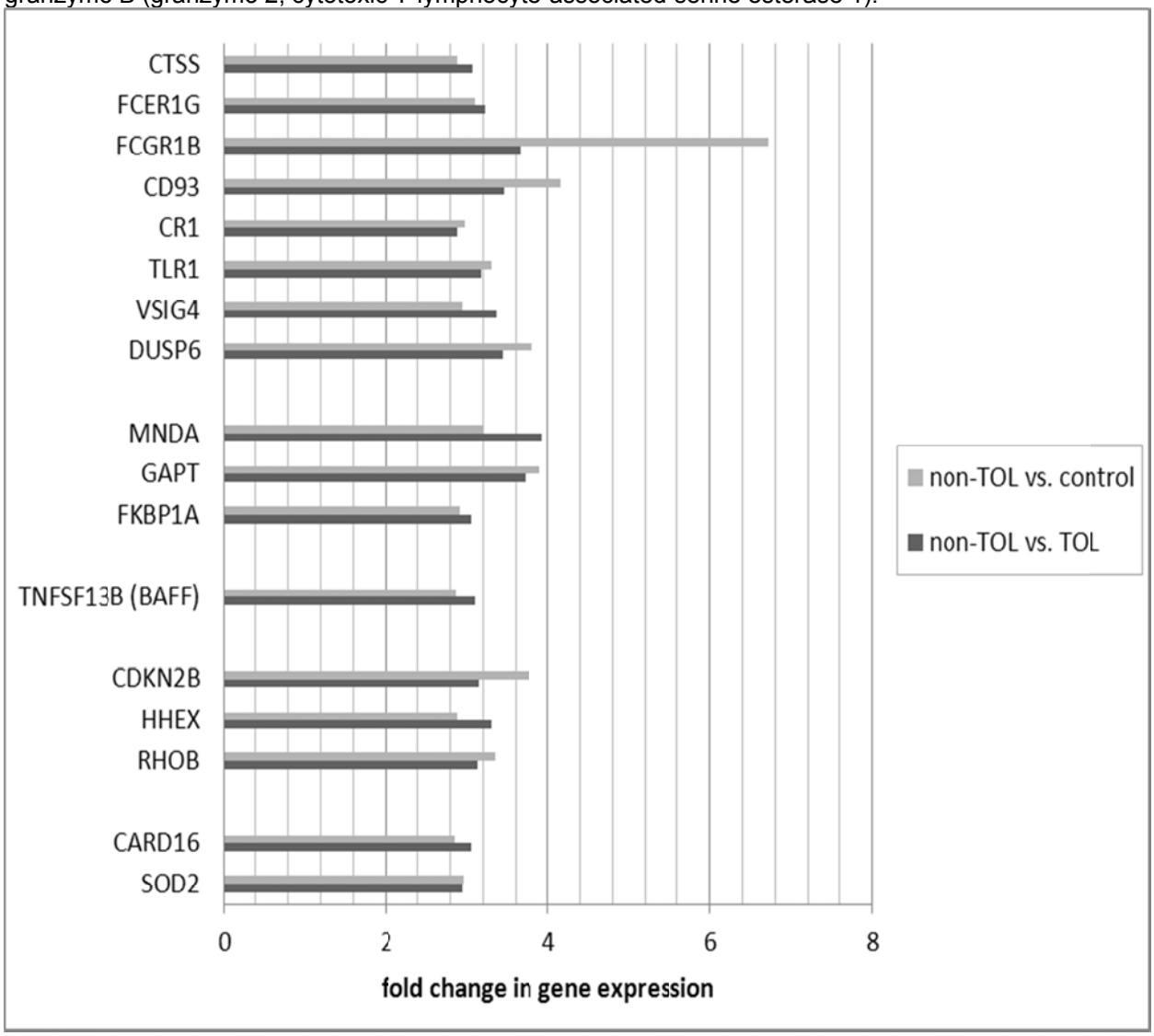


Figure 14: Direction and magnitude of change in non-tolerant group vs. tolerant and control groups

(b) Genes with increased expression in non-TOL group

* TOL = tolerant cases, non-TOL = non-Tolerant cases, control = healthy controls. CTSS - cathepsin S; FCER1G - Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide; FCGR1B - Fc fragment of IgG, high affinity Ib, receptor (CD64); CD93 - CD93 molecule; CR1 - complement component (3b/4b) receptor 1; TLR1 - toll-like receptor 1; VSIG4 - V-set and immunoglobulin domain containing 4; DUSP6 - dual specificity phosphatase 6; MNDA - myeloid cell nuclear differentiation antigen; GAPT - GRB2-binding adaptor protein, transmembrane; FKBP1A - FK506 binding protein 1A, 12kDa; TNFSF13B - tumor necrosis factor (ligand) superfamily, member 13b (BAFF); CDKN2B - cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4); HHEX - hematopoietically expressed homeobox; RHOB - ras homolog

gene family, member B; CARD16 - caspase recruitment domain family, member 16; SOD2 - superoxide dismutase 2, mitochondrial.

Classifier construction and cross-validation: The leave-k-out cross-validation method was utilized to train a classifier for the phenotypic groups (TOL vs. non-TOL) based on the observed differential gene expression. For each of 10 rounds of cross-validation, 10% of the total sample was left out for testing the classifier. An accurate classifier (90.6% accuracy, correctly classifying 14/15 TOL cases and 15/17 non-TOL cases) was developed only utilizing 20 probe sets, and classifier accuracy was stable (ranging from 87.5 to 90.6%) across the range of included (20-80 total) probe sets. The highest ranked (selected for classifier development 9-10 times out of 10 total rounds of cross-validation) probe sets and corresponding genes from the 20-probeset classifier are listed in table 8.

Table 8: Top probe sets and corresponding genes selected in classifier construction and leave-10%-out cross-validation.

Number of times selected	Probe set ID	Gene symbol	Gene name
10	235230_at	PLCXD2	phosphatidylinositol-specific phospholipase C, X domain containing 2
10	231776_at	EOMES	eomesodermin
10	226625_at	TGFBR3	transforming growth factor, beta receptor III
10	219566_at	PLEKHF1	pleckstrin homology domain containing, family F (with FYVE domain) member 1
10	214119_s_at	FKBP1A	FK506 binding protein 1A, 12kDa
10	206974_at	CXCR6	chemokine (C-X-C motif) receptor 6
10	206486_at	LAG3	lymphocyte-activation gene 3
10	204787_at	VSIG4	V-set and immunoglobulin domain containing 4

10	204731_at	TGFBR3	transforming growth factor, beta receptor III
10	204530_s_at	TOX	thymocyte selection-associated high mobility group box
10	1557985_s_at	CEP78	centrosomal protein 78kDa
9	218832_x_at	ARRB1	arrestin, beta 1

The accuracy of this gene expression based classifier is further demonstrated in the following ROC plot of sensitivity (true positive rate) vs. 1-specificity (false positive rate), indicating an AUC of 0.97 with 95% CI of 0.82-0.97 (figure 15).

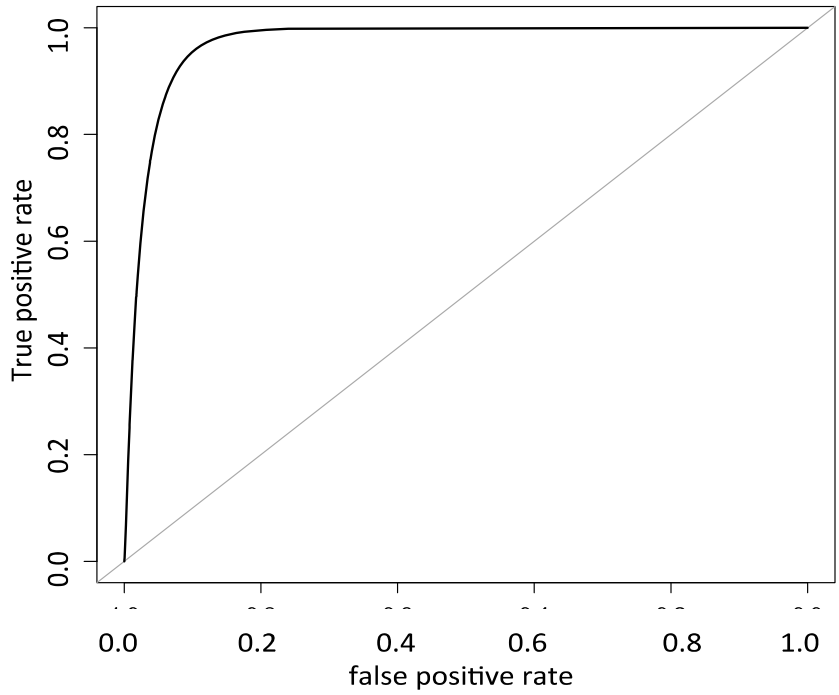


Figure 15: Receiver operating characteristic (ROC) curve demonstrating accuracy of developed gene classifier for tolerant phenotype.

Cell subtype enrichment analysis: The identified final unique gene sets for the TOL (n = 281) and non-TOL (n = 122) groups were studied for enrichment for lineage-specific gene sets previously identified through an analysis of sorted peripheral blood populations defined as follows: CD4+ Th lymphocytes, CD8+ Tc lymphocytes, CD14+ monocytes, CD19+ B lymphocytes, CD56+ NK cells, and CD66+ granulocytes. Appealing to this Hematology Expression Atlas of cell lineage-specific genes, we performed gene set enrichment analysis (GSEA Analysis, Broad Institute). The analysis demonstrated a high degree of enrichment for NK cell lineage-specific genes (Enrichment Score (ES) 0.84, p value < 0.0001, false discovery rate (FDR) < 0.0001) for tolerance-associated genes represented in our experimental data (figure 16). No significant enrichment was detected through this method for the other studied cell lineages (CD4+ and CD8+ T cells, monocytes, B cells, or granulocytes) for our tolerance genes.

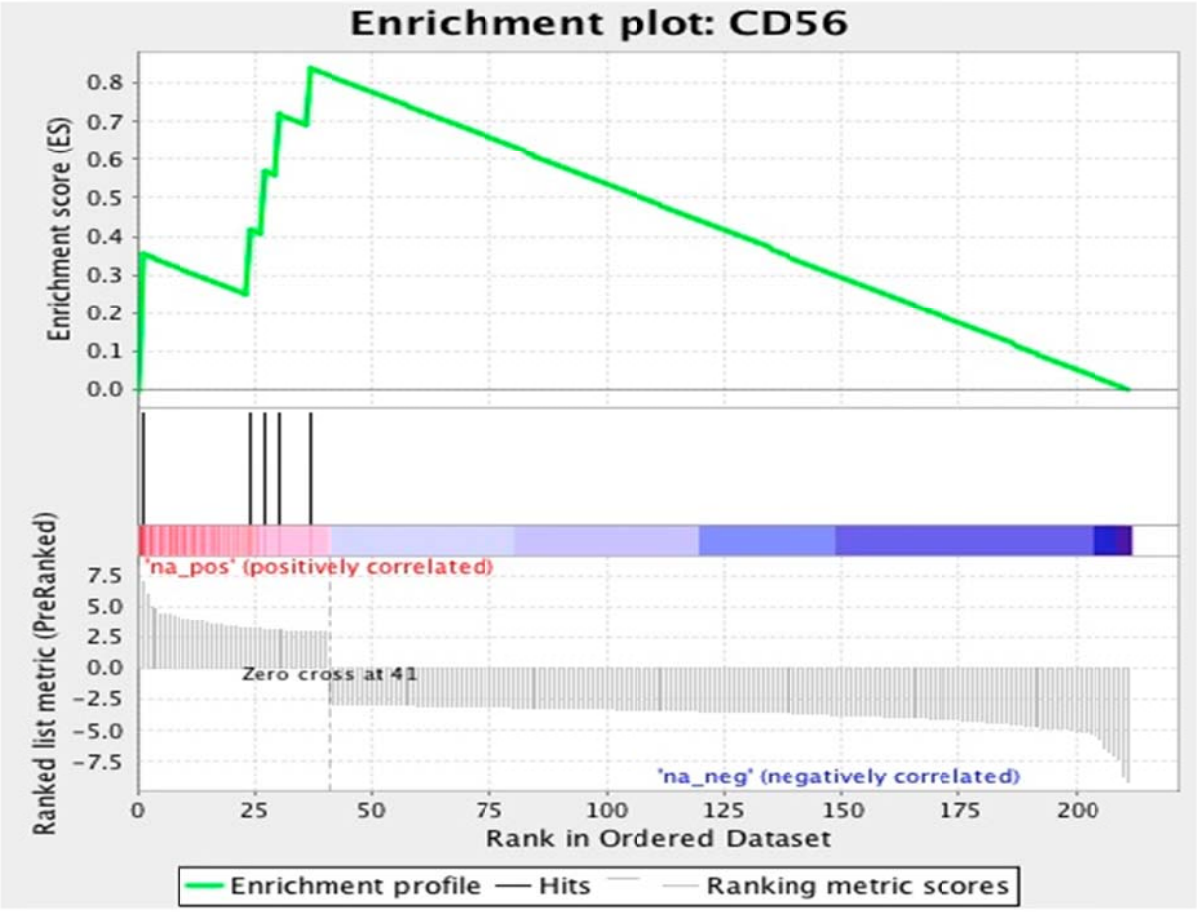


Figure 16: Gene set enrichment analysis for cell lineage-specific gene expression.

Enrichment Score (ES) 0.84, p value < 0.0001, false discovery (FDR) < 0.0001. P value = statistical significance of the enrichment score. FDR = estimated probability that the normalized enrichment score represents a false positive finding, accounting for multiple testing and gene set size.

Biologic relevance of differentially expressed genes: Differentially expressed genes in the TOL group were enriched for immune response pathways and recapitulated experimental mechanisms of immune tolerance: Expression of leukocyte immunoglobulin-like receptors (LILRA5, LILRA2) was decreased in the TOL group; these LILR are activating and associated with release of pro-inflammatory cytokines.[240] The Ig receptor superfamily member FCRL3 was

over-expressed in the TOL group; this molecule is involved in immune regulation, negatively regulates B cell receptor signaling,[241] and may distinguish a distinct subset of Treg.[242] Major components of the toll-like receptor signaling cascade (TLR4, TLR8, PELI2, IRAK3) were under-expressed as well; TLR/MyD88 signaling plays a key role in experimental models of transplantation tolerance,[243,244] and TLR4 inactivation protects against GVHD.[245] Several important cell signaling molecules were over-expressed: TOX is known to be involved in CD4 T cell lineage development, and important for Treg and CD1d-dependent NKT cells.[246] LAG3, a major negative regulator of CD4 and CD8 T cell activation and important for Treg homeostasis, function, and inhibition of DC activation, was over-expressed.[247-249] Conversely, SOCS2 (involved in DC maturation),[250] and beta arrestin 1 (involved in T cell activation, enhances transcription of IFN-γ and IL-17; increased in primary biliary cirrhosis patients)[251,252] were decreased. Among cytokines and their receptors, TOL patients had decreased expression of IL-13RA (IL-13 induces B cell proliferation and differentiation, and is expressed on Th17 cells), as well as BAFF and APRIL (major B cell activating TNF ligand family members implicated in human chronic GVHD).[122,124,253] Conversely, TGFBR3 (TGF-β co-receptor relevant to TGF-β receptor complex stability and signaling),[254] expression was increased. As well, IFN-γ was increased in TOL patients. This has been demonstrated to have both pro-inflammatory and immune regulatory actions,[255] importance in migration of Treg and conventional T cells to GVHD target organs,[256] and to mediate immune regulatory function in FoxP3+ Tregs in experimental GVHD.[257] In keeping with published data in solid organ transplantation tolerance, TOL patients had decreased expression of anti-apoptotic (DAPK1, SOD2, PPT1, SOCS2, VNN1, SMAD1, GSN), and increased expression of pro-apoptotic (GZMH, PLEKHF1) mediators, as well as involvement of cell cycle control genes.

Differentially expressed genes in the non-TOL group were strongly associated with NK cell cytotoxicity, antigen presentation, lymphocyte proliferation, and cell cycle and apoptosis

cellular process networks: Multiple NK cell/lectin receptors (By55/CD160, KLRK1, KLRD1, KLRC4, KLRC3, KLRC2) and cytolytic effectors (granulysin, and granzymes A, B, and K) were under-expressed in the non-TOL group with respect to both TOL and control subjects.[172] Tolerogenic activity of NK cells has been related to killing of activated T cells, production of IL-10, competition with CD8+ T effectors for IL-15, and killing of antigen-presenting DC.[258] While we did not detect decrease in absolute NK cell numbers, these gene expression findings are in keeping with a cohesive finding of NK deficiency in human chronic GVHD,[259,260] as well as the primacy of NK-associated gene expression changes (including specifically CD160 and NKG7) in distinguishing tolerant vs. non-tolerant liver transplant recipients in *Martinez-Llordella, et al.*[195] There was over-expression of TLR/MyD88 signaling (DUSP6, TLR1), complement receptors (VSIG4, CR1, CD93), and Fc receptors (FCGR1B, FCER1G), again highlighting the important role of the innate immune system. Among signaling mediators, GAPT (GRB2-binding adaptor protein associated with B cell activation),[261] and MNDA (myeloid cell nuclear differentiation antigen expressed in cells of the granulocyte-monocyte lineage and involved in response to interferon) were increased; interferon-inducible Ifi200-family genes (including MNDA) have been associated with autoimmune disorders, including systemic lupus erythematosis.[262,263] In contrast to TOL, the non-TOL patients had increased BAFF, and decreased TGFBR3. In keeping with findings after solid organ transplantation, non-TOL patients had increased expression of anti-apoptotic (SOD2, CARD16) and decreased expression of pro-apoptotic (GZMB, GZMA, DLG5) mediators, and involvement of molecules relevant to cell cycle control.

Unifying tolerance model: While diverse mechanisms have been established for immune tolerance development, the experimental data presented here highlight the central role of dendritic cell (DC) and natural killer (NK) cell interaction. Major supporting differential gene expression data for this hypothesis is presented in table 9. These data are consistent with

established bi-directional DC – NK interactions that shape DC and NK activity and subsequently, adaptive immune responses. A cohesive model supports the following: In the immune tolerant state, DC maturation and pro-inflammatory cytokine expression is decreased, thus dampening B and T cell adaptive responses. The major down-regulation of TLR (TLR4, TLR8, allied signaling molecules) is supportive of a tolerogenic program in DC, as well as other cell types that express TLR (e.g. B and T lymphocytes). Immature DC are susceptible to NK-mediated cytotoxicity, as demonstrated by NK degranulation (NKG7). In the non-tolerant state, TLR signaling and DC maturation are increased leading to productive B and T cell responses, and NK cytotoxicity is impaired with reduced activating NK lectin receptors and cytotoxicity effectors. Decreased NKG2A signaling in particular may result in diverse effects that support this paradigm (table 9).

Table 9: Dendritic cell and Natural Killer cell interaction: A unifying hypothesis for the observed immune tolerance-associated differential gene expression

	Immune Tolerant State		Non-tolerant (GVHD) State	
DC	↓TLR4, ↓TLR8 ↓LILR, ↓SOCS2 ↑LAG3	- ↓DC maturation, co-stimulatory molecule and pro-inflammatory cytokine expression[171] - Immature DC lysed by NK	↑TLR1, ↑DUSP6 ↑ VSIG4, ↑CR1, ↑CD93	- ↑ DC maturation, co-stimulatory molecule and pro-inflammatory cytokine □□□□□□□□□[171] - Mature DC not lysed by NK, stimulating adaptive immune response
NK	↑ NKG7	↑ Target cell-induced NK cell degranulation[264]	↓NKG2C/D/E/F, ↓CD160 ↓GZMA/B/K, ↓GNLY ↓NKG2 A (↓IL-2RB)	- ↓ Activating NK receptors - ↓ Cytolytic effectors - *
B cell	↑FCRL3 ↓LILR, ↓IL13RA, ↓TLR ↓BAFF, ↓APRIL	- ↓ BCR signaling, activation, and survival	↑GAPT ↑BAFF	- ↑B cell activation, survival
T cell	↑TGFBR3 ↑LAG3, ↓ARRB1, ↑TOX	- tolerogenic profile (↓activation, ↓Th1/Th17, ↑Treg)	↓TGFBR3	- Decreased TGF-β signaling/pro-inflammatory state

*Diverse potential mechanisms: (1) CD94/NKG2A+KIR- NK mediate immature DC killing (NKG2A+KIR-IL2-R+ CD56^{bright} NK subset may kill immature self-DC and express regulatory cytokines including IL-10) ; (2) murine Qa-1 (HLA-E in humans) binding of NKG2A regulates activity of CD8+ T cells, NK, and NKT; (3) NKG2A in human γδ T cells inhibits effector function; (4) NKG2A-DC mediated induction of CD4+CD25+ Treg.

CHAPTER 4:

CONCLUSION AND DISCUSSION

The desired end result of HCT is cure of the treated hematologic malignancy or disorder, effective prevention and therapy of GVHD, and development of immune tolerance. Major shortcomings exist in current practice: Existing pharmacologic immune suppressive GVHD prophylaxis regimens do not effectively prevent acute GVHD for many patients, severe acute GVHD is poorly responsive to therapy, and the majority will experience chronic GVHD. Finally, clinical judgment does not accurately identify the development of immune tolerance, practice surrounding discontinuation of immune suppression (IS) is empiric, and GVHD commonly develops or reoccurs in the setting of attempted IS discontinuation. These limitations undermine the potential of HCT as an otherwise curative therapy. The presented data directly address these areas of need, and suggest next steps in this line of investigation that promise to improve HCT outcomes.

We conducted a randomized trial comparing SIR/TAC vs. the commonly accepted standard MTX/TAC.[238] We demonstrated that SIR/TAC led to reduction in grade II-IV acute GVHD, however we did not observe significant reduction in grade III-IV acute GVHD, and benefit was restricted to reduction in GI acute GVHD (the most commonly represented organ site of acute GVHD involvement in this study). As well, over 40% of patients in the SIR/TAC arm experienced grade II-IV acute GVHD. Importantly, since completion of this study, a national BMT CTN phase III trial comparing SIR/TAC to MTX/TAC has shown only modest improvement in acute GVHD with SIR/TAC.[265] Important differences in the CTN trial (restriction to sibling

donor transplants, different conditioning regimen, and shorter duration of SIR exposure post-HCT) limit direct comparisons, however. These data speak to the need for additional advances in the field. We did observe, however, that prolonged administration of SIR was associated with significantly reduced incidence of NIH Consensus moderate to severe chronic GVHD. These findings are noteworthy, as previously published trials examining SIR/TAC (without this duration of SIR therapy post-HCT) have resulted in a greater burden of chronic GVHD.[28,29] Most importantly, SIR/TAC supported the reconstitution of functional Treg and suppressed non-Treg CD4+ T cells after HCT. These prospective data advance knowledge of Treg reconstitution following clinical HCT beyond previously reported correlative studies,[84,85] support the concept that sirolimus exerts suppression of non-Treg CD4+ cells,[82] and indicate that the combination of SIR/TAC may serve as a platform for Treg adoptive therapy. However, as Treg are dependent on IL-2 signaling, we recognize that the concurrent administration of TAC may counter beneficial effects of SIR on Treg. While a calcineurin inhibitor-free regimen would be most attractive, current evidence does not support the feasibility of this approach for GVHD prophylaxis after HCT.[266] Our team has developed a clinical trial testing the addition of low-dose subcutaneous IL-2 administration together with SIR/TAC (NCT01927120). This initial study may provide a foundation for subsequent investigation exploring whether elimination of TAC can be safely accomplished. Finally, as a direct extension of the presented work, we have developed technology to ex-vivo expand antigen-specific donor Treg,[267] and will test escalating dose of donor Treg (on platform of SIR/TAC) as adoptive therapy for GVHD prevention in a phase I trial (NCT01795573). This trial will provide a first-in-human test of the safety, clinical efficacy, and biologic activity of ex-vivo expanded antigen-specific donor Treg delivered for prevention of human GVHD. In total, these efforts promise to expand our scientific understanding, and may more effectively prevent GVHD and facilitate development of immune tolerance.

In the context of the parent randomized trial comparison of SIR/TAC vs. MTX/TAC, we have examined tissue-infiltrating CD4+ T cell subsets to discern mechanisms of failure. These data implicate Th17 cells in human GVHD target organs, support a reduction in Th17 under SIR treatment, and demonstrate that tissue-resident Th17 are associated with GVHD severity and refractoriness to standard primary GVHD therapy. Of note, we found no significant association of tissue-resident Th1 or Treg with GVHD prophylaxis type, pathologic or clinical severity grade of GVHD, or refractoriness to primary GVHD therapy. Our data are in keeping with evidence that supports a pathogenic role for Th17 in GVHD, and support the concept that interventions to reduce Th17 in vivo may lead to benefit in GVHD prevention and control. Through this and allied clinical and pre-clinical investigation (data not shown, manuscript under review), we have assembled a body of evidence implicating the STAT3/RORγ/Th17 axis in GVHD development and severity: In addition to the described human GVHD tissue work, we have demonstrated that STAT3 phosphorylation is significantly increased in CD4+ T-cells among human HCT recipients prior to the onset of grade II-IV GVHD. As well, we demonstrate that concurrent neutralization of TORC1 and STAT3 with rapamycin and S3I-201 (a STAT3 small molecule inhibitor) optimally suppresses RORγ expression, and that rapamycin-resistant T-cell proliferation can be inhibited by STAT3 blockade. Building from this concept, we have tested the activity of the IL-12/23p40 neutralizing antibody ustekinumab in the setting of advanced steroid-refractory acute GVHD,[268] and are currently conducting a placebo-controlled GVHD prevention trial that tests the addition of this agent to the SIR/TAC platform (NCT01713400). This trial promises to discern whether IL-12/23p40 neutralization will skew CD4+ T cell differentiation in vivo (diminish Th1 and Th17, augment Treg) and lead to beneficial reduction in GVHD.

Investigation into mechanisms of human immune tolerance after HCT is highly relevant to the body of work described above: There are currently no validated clinical or biologic determinants of immune tolerance after HCT, the required duration of IS therapy for any

individual patient is not known, clinical judgment can't distinguish drug-suppressed immune response from the development of donor-recipient immune tolerance, and clinical practice of IS discontinuation is empiric and fraught with a large burden of resultant GVHD (the major manifestation of donor-recipient immune intolerance). Any advances in prevention of GVHD are undermined by the development or recurrence of GVHD in the context of attempted IS withdrawal, however this phenomenon has been poorly studied to date. In a cross-sectional study, we have examined differential gene expression among tolerant, non-tolerant, and healthy control subjects to address this need. In this initial experiment, we have demonstrated that differential gene expression can provide mechanistic insight into immune tolerance, and that this data can be utilized to develop an accurate phenotypic classifier. Many of these candidates appear to have great biologic relevance based on previously published work in immune tolerance, and some are actionable targets of existing therapeutic agents. Despite inclusion of healthy controls and advanced computational work to stringently refine a list of informative candidate genes, we acknowledge that this single cross-sectional design does not completely recapitulate the clinical scenario of attempted IS withdrawal. A prospective trial (samples drawn at time of IS discontinuation and serial subsequent samples with observation for development of GVHD) is planned that will address this question further, and future work will investigate both advanced technology (RNA deep sequencing) and explore cell subset-specific gene expression changes.

CHAPTER FIVE:

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