Multifunctional Regulatory T Cells from Human Umbilical Cord Blood and the Role of Tumor Necrosis Factor in Immune Homeostasis

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MULTIFUNCTIONAL REGULATORY T CELLS FROM HUMAN UMBILICAL CORD BLOOD AND THE ROLE OF TUMOR NECROSIS FACTOR IN IMMUNE HOMEOSTASIS

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ABSTRACT

Regulatory T cells (Tregs) are required to suppress inflammation and prevent autoimmunity. During fetal development Tregs are crucial to maintain tolerance between mother and child. After birth, neonates require tolerance to avoid harmful immune responses to foreign antigens in food and allow colonization with commensal microbes. We demonstrate a propensity for T cells in human umbilical cord blood to differentiate into Tregs in response to antigen receptor stimulation ex vivo. Cord blood-derived Tregs potently suppress T cell proliferation, but also produce pro-inflammatory cytokines known to activate innate immune responses. These results suggest that antigen exposure during early life results in development of T cells with both regulatory and effector functions. Surprisingly, we observe expression of tumor necrosis factor (TNF) by cord blood and adult Tregs. We show a role for autocrine TNF signaling in survival of Tregs, suggesting an important function for TNF in immune tolerance and homeostasis.
CHAPTER ONE
INTRODUCTION

Immune Tolerance

The concept of immune tolerance was first demonstrated in 1945 by Ray Owen, who observed that dizygotic twin cattle shared red blood cells in the placenta which persisted into adult life. The absence of immune response to the foreign red blood cells in the blood of cattle suggested that immune tolerance could be generated in response to foreign cells acquired before birth. In 1953 Billingham, Brent, and Medawar demonstrated that mice inoculated in utero with cells from a different strain of mouse were tolerant to skin grafts from the same strain in adult life. Importantly, this study found that induction of tolerance for foreign tissues was dependent on the timing of exposure. Mice that were injected with foreign cells after birth demonstrated a lack of tolerance to skin grafts, suggesting that the developing immune system was somehow biased toward the acquisition of tolerance. These studies laid the groundwork for the concept of acquired immunological tolerance, for which Burnet and Medawar won the 1960 Nobel Prize in Physiology or Medicine. In 1961, Miller discovered the role of the thymus in generation of immune tolerance, which led Burnet to hypothesize that immune tolerance was maintained via the elimination of self-reactive lymphocytes.

In the late 1980s, Marrack and colleagues demonstrated that tolerance to self-antigens was the result of removal of autoreactive T cells during thymic selection, a process now called clonal deletion. Removal of autoreactive T cells in the thymus is now known to be mediated by
the transcription factor AIRE, or autoimmune regulator, which is expressed in the thymus\textsuperscript{6}.

AIRE drives medullary thymic epithelial cells (mTECs) to express self-antigens from peripheral tissues, resulting in apoptosis of T cells that bind to these proteins and thus preventing T cell responses to self-antigens in the periphery\textsuperscript{7}.

A second mechanism for immune tolerance was described by Nossal, who discovered the presence of mature, autoreactive B lymphocytes in circulation which failed to respond to antigen stimulation\textsuperscript{8}. Clonal anergy, as this new mechanism came to be called, was similarly described in T cells by Jenkins and Schwartz, who observed that the absence of costimulatory signals during antigen stimulation resulted in unresponsiveness upon subsequent stimulation\textsuperscript{9}. These studies demonstrate anergy as another means by which tolerance can be maintained during thymocyte development in the thymus.

Clonal deletion and anergy maintain tolerance by preventing peripheral immune responses by autoreactive lymphocytes. However, until 1970 an active mechanism for peripheral tolerance had not been identified\textsuperscript{10}. Gershon and Kondo discovered that T cells were able to suppress immune responses in addition to mediating them, identifying a distinct population of T cells initially referred to as suppressor T cells\textsuperscript{11}. However, due to difficulty in definitive identification of this new cell population and a lack of evidence of their role in disease, studies of suppressor T cells waned during the 1980s\textsuperscript{10}. Despite this, however, studies of autoimmune disease revealed a population of T cells that were responsible for maintenance of self-tolerance\textsuperscript{12}.

**Regulatory T Cells**

Beginning in 1969, a series of experiments revealed a population of T cells that inhibited the development and progression of autoimmune disease in mice and rats\textsuperscript{10}. Nishizuka and
Sakakura demonstrated that thymectomized neonatal mice developed autoimmune destruction of ovaries\(^{13}\). Further, other groups showed that thymectomy of adult rats in conjunction with radiation resulted in autoimmune thyroiditis and type 1 diabetes\(^{14,15}\). Importantly, syngeneic transfer of T cells, in particular CD4+ T cells, from normal animals abrogated disease\(^{15}\). These studies provided evidence for the hypothesis that the thymus produces CD4+ T cells with the ability to suppress T-cell mediated autoimmune disease, prompting investigations aimed at determining a way to identify these suppressive T cells\(^{10}\). In 1995, Sakaguchi et al. discovered that the suppressive population of CD4+ T cells expressed the IL-2 receptor CD25\(^{16}\). Depletion of CD4+CD25+ T cells resulted in the development of organ-specific autoimmune disease and reconstitution of this group of cells prevented the onset of symptoms, suggesting that expression of CD25 could be used to identify suppressive T cells\(^{16-18}\). Furthermore, several studies demonstrated the functional importance of CD25 expression as a receptor for IL-2. IL-2-deficient mice developed autoimmunity and exhibited a paucity of CD4+CD25+ cells while maintaining a normal composition and number of T cells\(^{19}\). IL-2 was also identified as a growth factor for CD4+CD25+ suppressive T cells, as antibody-mediated neutralization of endogenous IL-2 reduced CD4+CD25+ T cell numbers and resulted in onset of autoimmune disease\(^{20}\).

**Foxp3.**

Identification of the transcription factor forkhead box P3, or Foxp3, in 2001 provided a crucial phenotypic and functional marker for regulatory T cells (Tregs), as they came to be called\(^{21}\). The discovery came from investigations into the cause of the fatal lymphoproliferative disorder of scurfy mice, and identified a single gene mutation in the X-chromosome responsible for a loss of Foxp3 expression\(^{22}\). Subsequently, a mutation in Foxp3 was also identified as the
cause of the human disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), a fatal disease characterized by excessive inflammation and multi-system autoimmunity\textsuperscript{23}. The symptoms observed in scurfy mice and IPEX patients closely resembled those of mice deficient in CD4+CD25+ T cells, giving rise to the hypothesis that mutations in Foxp3 depleted Tregs and resulted in excessive lymphoproliferation and inflammation\textsuperscript{24}. By 2003, several studies supported the role of Foxp3 in generation and suppressive function of Tregs. Foxp3 expression was confirmed in CD4+CD25+ T cells with suppressive function, and retroviral transduction of Foxp3 induced suppressive function and Treg phenotype in naïve T cells\textsuperscript{25}. Furthermore, experiments in mice demonstrated the requirement of Foxp3 in Treg development, solidifying Foxp3 as the regulatory T cell lineage-specifying transcription factor\textsuperscript{26, 27}. While the precise function of Foxp3 in suppressive activity of Tregs remains poorly understood, whole genome analyses in mice indicate Foxp3 acts as both a transcriptional activator and repressor, acting in concert with other transcription factors to influence expression of hundreds of genes\textsuperscript{28}. Specifically, Foxp3 is known to form complexes with NFAT, repressing expression of IL-2 and upregulating Treg surface markers CD25 and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4)\textsuperscript{29, 30}. Interaction of Foxp3 with AML1 (acute myeloid leukemia 1 or Runx1) has also been tied to suppression of IL-2 and interferon-gamma production while activating suppressive activity of Tregs and upregulating glucocorticoid-induced TNF-receptor-family-related protein (GITR) \textsuperscript{31}. Importantly, while Foxp3 expression in mice is limited to Tregs, human T cells transiently express low levels of Foxp3 during T cell receptor (TCR) stimulation in contrast to stable expression in Tregs\textsuperscript{32}. 
Mechanisms of Suppression.

Despite extensive studies on the function of Tregs *in vivo* and *in vitro*, the mechanisms of by which Tregs suppress effector T cell proliferation and inflammation remain poorly understood. Furthermore, several studies suggest Tregs not only directly suppress T cell responses, but also alter the activity of antigen-presenting cells (APCs) as an indirect means of modulating immune responses. To date, many mechanisms of Treg-mediated suppression have been proposed, falling broadly into two classes: contact-dependent and contact-independent. Importantly, while Treg suppression requires antigen stimulation via TCR activation, activated Tregs can suppress in an antigen-nonspecific manner referred to as bystander suppression. Therefore, Tregs can modulate immune responses by T cells independent of TCR specificity.

Initial investigations into Treg suppression revealed that *in vivo* CTLA-4 blockade resulted in development of autoimmune disease in mice and abrogated *in vitro* suppression of T cell proliferation. Subsequent studies revealed that CTLA-4 on Tregs inhibited T cell activation by competitively binding B7 molecules on APCs, preventing costimulatory signal transduction via CD28 on T cells. Thus, Tregs can prevent activation and proliferation of conventional T cells in a contact-dependent manner. Direct cytotoxicity by Tregs has also been observed, demonstrating that under some conditions Tregs are capable of killing a variety of target cells to control immune responses by expression of granzyme A and perforin. Production of cyclic adenosine monophosphate (cAMP) by Tregs is implicated in contact-dependent suppression, particularly *in vitro*. A potent inhibitor of proliferation and IL-2 production, cAMP has been shown to transport across cell membranes through gap junctions formed between Tregs and conventional T cells. Moreover, the suppressive activity of Tregs is abrogated in the presence
of a cAMP antagonist as well as by the use of a gap junction inhibitor. While in vitro studies suggest contact-dependent mechanisms are the dominant form of Treg suppression, several disease models implicate the production of immunosuppressive cytokines are important for Treg function in vivo. Studies of inflammation in rodents demonstrate the requirement of interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) production by Tregs in control of immune pathology. Tregs produce both soluble and membrane-bound TGF-β, and notably blockade of TGF-β in vitro mildly reduces suppression of both human and mouse T cell proliferation. Control of intestinal inflammation also requires IL-10 and TGF-β production by Tregs. Taken together, these studies demonstrate the complex nature of Treg suppression.

Tregs employ a variety of mechanisms to maintain tolerance and downregulate inflammation, and the exact manner of suppression is likely dependent on many factors including the type of immune response, tissue site, and cell types involved. Furthermore, recent evidence reveals that Tregs are not a homogeneous population, but rather a complex group of distinct subsets with specific functions.

**Regulatory T cell subsets.**

The majority of Tregs are produced in the thymus (nTregs/tTregs), however Tregs can also be generated in peripheral tissues (pTregs) and induced in vitro from naïve T cells (iTregs). During development in the thymus, T cells reactive against self-antigens expressed on mTECs are either deleted by apoptosis, become anergic, or become Foxp3+ Tregs, which leave the thymus and enter peripheral tissues to maintain self-tolerance. Additionally, mature, naïve T cells in the periphery can become Foxp3+ Tregs when stimulated with high affinity cognate antigen in the presence of TGF-β and IL-2. Similarly, naïve T cells can be
induced to express Foxp3 and acquire suppressive function in cell culture by TCR stimulation in the presence of TGF-β and IL-2. Recent studies in mice and humans elucidated a heterogeneous population of CD8+CD25+Foxp3+ T cells with functional suppressive activity. While the role of CD8+ Tregs in immune homeostasis remains unclear, patients with asthma and inflammatory bowel disease display a paucity of CD8+ Tregs. Therefore, CD8+ Tregs may play distinct roles in maintenance of immune homeostasis, perhaps preferentially controlling specific types of immune responses. CD4+ T cells with suppressive activity in the absence of Foxp3 expression have been identified in mice, including IL-10-producing Tr1 cells and TGF-β-secreting Th3 cells. However, the suppressive activity of Foxp3- Treg subsets is limited to immunosuppressive cytokine secretion, and in the case of Th3 cells, driving peripheral Foxp3+ Treg induction through the expression of TGF-β. Due to the wide variety of immune responses required to defend against pathogens, the diversity of Treg function and phenotype is not surprising. Indeed, recent reports indicate that Foxp3+ Tregs exist in distinct populations with precise anti-inflammatory roles.

Naïve T cells differentiate into effector subsets based on TCR stimulation in the presence of a specific cytokine environment and can be distinguished based on the cytokines and transcription factors they express. Interleukin 12 (IL-12) drives Th1 differentiation, resulting in interferon-gamma (IFN-γ) production and expression of the transcription factor T-bet. Similarly, Th2 cells require interleukin 4 (IL-4) and express IL-4 and GATA-3, while Th17 cells are induced by TGF-β and interleukin 6 (IL-6) and express interleukin 17 (IL-17) and RAR-related orphan receptor gamma (RORγt). Several studies in mice and humans describe upregulation or activation of Th-associated transcription factors in Foxp3+ Tregs during
regulation of Th1, Th2, or Th17 immune responses, suggesting that Tregs differentiate into specialized subsets to modulate these responses\textsuperscript{57, 68-70}. Furthermore, pro-inflammatory cytokine production is associated with Th-like Treg subsets, despite the maintenance of suppressive activity and stable Foxp3 expression\textsuperscript{57, 71-73}. To describe these subsets of Foxp3+ Tregs in humans and mice, the terms Th1-like, Th2-like, and Th17-like have been posited to refer to Tregs with Th-associated phenotype or function\textsuperscript{57}. An ongoing difficulty in the study of Treg biology, particularly \textit{in vivo}, is the lack of known surface markers to distinguish between subsets. However, recent studies of human Tregs demonstrate that the surface phenotype of Treg subsets corresponds to their effector counterparts, providing a means by which to identify and isolate specific populations\textsuperscript{57, 73}.

**Chemokine Receptors and T cell Trafficking**

In addition to the expression of cytokines and transcription factors, effector T cell subsets express different chemokine receptors which, along with adhesion molecules, mediate the ability to migrate to peripheral tissues and sites of inflammation\textsuperscript{74}. Mature, naïve T cells exit the thymus and primarily migrate to the blood and secondary lymphoid tissues, where they encounter antigen presented by APCs and become activated\textsuperscript{75}. Subsequently, effector T cells are generated which mount a short-lived immune response in peripheral tissues while long-lived memory T cells migrate to tissues to participate in immune surveillance\textsuperscript{74}. Differential expression of chemokine receptors on T cell subsets mediate migration to inflamed tissues as well as trafficking to lymph nodes\textsuperscript{76}. In this way, chemokine receptor expression directs specific types of T cells to appropriate tissues and controls the type of immune response mounted against a particular pathogen. For this reason, expression of chemokine receptors can be used not only
to distinguish types of T cells, but also which tissues they localize to. Th1 cells, for example, preferentially express CXC chemokine receptor 3 (CXCR3) in contrast to Th2 cells which express CC chemokine receptor 3 (CCR3) and CCR4\textsuperscript{77, 78}. Th17 cells, on the other hand, express CCR4 and CCR6 which mediate migration to sites of Th17 inflammation and, in particular, entry into the central nervous system (CNS) in animal models of immune-mediated neuroinflammation\textsuperscript{79, 80}. CCR10, in combination with CCR4 and CCR6 is associated with skin-homing T cells, illustrating the importance of complex chemokine receptor signaling in determining the migratory ability of T cells\textsuperscript{81}. Finally, CCR7 confers the ability to migrate to secondary lymphoid tissue and its expression is associated with naïve and central memory T cells\textsuperscript{74}. Like conventional T cells, Tregs express chemokine receptors which control tissue localization and migration in response to inflammation\textsuperscript{82}. Maintenance of immune homeostasis in peripheral tissues is dependent upon colocalization of Tregs with effector T cells, meaning that Tregs respond to chemokines in the same manner as conventional T cells\textsuperscript{83, 84}. Furthermore, \textit{ex vivo} analysis of chemokine receptor expression by human Foxp3\textsuperscript{+} Tregs reveals the existence of distinct subsets which closely resemble effector T cell subsets despite \textit{in vitro} suppressive activity\textsuperscript{57}. Together, these studies suggest Tregs are functionally specialized to regulate immune responses by localizing with effector T cells \textit{in vivo}.

**Fetal Tolerance and Neonatal Immunity**

For humans and other placental mammals, pregnancy presents a significant immunological challenge for both the maternal immune system and the developing fetus. The maternal immune system must tolerate foreign antigens the fetus inherits from the paternal genome while the developing fetus must remain tolerant to maternal antigens that cross the
placenta. While development of T cells in mice is delayed, in the human fetus T cells can be detected in peripheral lymphoid tissue by 10 weeks of gestation, necessitating tolerance early in pregnancy. Tregs are critical to maintaining tolerance at the fetomaternal interface, as antigens are exchanged between mother and fetus throughout pregnancy. In the mother, T cells do not differentiate to pathogenic effector cells, but rather preferentially differentiate to Tregs and expand in response to fetal antigens. Notably, maternal Tregs specific for fetal antigen persist after delivery and rapidly expand upon subsequent pregnancy. The importance of Tregs during pregnancy is illustrated by cases of deficient Treg expansion, which result in spontaneous abortion and preeclampsia. The fetus also depends upon Tregs to maintain tolerance in response to maternal antigens and cells that cross the fetomaternal interface. Although T cells are present in fetal lymphoid tissues early during development, the fetal adaptive immune system was long considered immature, and therefore unresponsive to antigen. However, as early as 1945, seminal experiments demonstrated that antigens encountered in utero induced long-lasting tolerance, suggesting an active immunosuppressive response. Furthermore, the fetal immune system is highly responsive to stimulation under some conditions, and mounts adaptive immune responses in response to certain pathogens. Recent studies demonstrate that a high frequency of Tregs in fetal lymphoid tissues are responsible for dominant, tolerant responses observed during antigen exposure in utero.

By the time of birth, frequencies of peripheral blood Tregs are comparable to adult, however naïve T cells in neonates and infants display an enhanced propensity to differentiate to Tregs rather than conventional T cells. In agreement, studies of human umbilical cord blood demonstrate elevated Treg generation in response to antigen stimulation, suggesting an intrinsic
bias toward tolerance that persists into early life\textsuperscript{101}. Birth presents an immense challenge to the newborn, requiring maintenance of tolerance to avoid damaging responses to environmental antigens and commensals while also necessitating protective immune responses against pathogens\textsuperscript{102}. For this reason, neonatal immunity is dependent upon plasticity to adapt to the complex demands of early life\textsuperscript{102}. In addition, newborns mount immune responses differently from adults, including the well-documented bias toward Th2 responses and absence of Th1 responses\textsuperscript{103, 104}. Despite a downregulation of antibacterial Th1 responses, newborns display enhanced production of interleukin 8 (IL-8) by CD4+ and CD8+ T cells, a cytokine known to potently activate and recruit antimicrobial neutrophils\textsuperscript{105}. In contrast, IL-8 production by T cells in adults is rare, perhaps representing a unique adaptation of the neonatal immune system to early life\textsuperscript{105}. Infectious disease remains a significant threat to newborns and neonatal infection results in high morbidity and mortality even in the developed world\textsuperscript{106}. Despite this, neonatal immunity remains poorly understood. Understanding the balance between tolerance and inflammation is critical to preventing newborn mortality and improving outcomes of vaccination in early life\textsuperscript{102}.

**Tumor Necrosis Factor Biology**

Tumor necrosis factor (TNF) was first discovered in 1975 and named for its ability to induce necrosis of tumors \textit{in vitro}\textsuperscript{107}. Not long after, studies in mice demonstrated efficacy against both human and murine tumors \textit{in vivo}\textsuperscript{108}. Expression of TNF was identified in monocytes, macrophages, and activated T cells\textsuperscript{109, 110}. Recombinant TNF was quickly moved into clinical trials, where minimal anti-tumor effects were far outweighed by massive side effects and toxicity\textsuperscript{111}. Subsequently, studies in mice led to the observation that immunization against
TNF protected mice from lethal effects of endotoxin, demonstrating for the first time that TNF mediated inflammation\textsuperscript{112}. Over the past 30 years, a wide range of activities have been attributed to TNF, revealing diverse functions and solidifying TNF as perhaps the most pleiotropic cytokine known today\textsuperscript{111}.

**TNF signaling.**

TNF is produced as a 26 kDa type II transmembrane protein by macrophages, monocytes, microglia, and activated NK and T cells\textsuperscript{113}. The membrane form of TNF (mTNF) arranges in homotrimers and is released to the soluble form (sTNF) via proteolytic cleavage by the metalloprotease TNF alpha converting enzyme (TACE)\textsuperscript{114, 115}. Soluble TNF circulates in blood and elicits potent responses at distant sites while mTNF signals locally through cell-cell contact\textsuperscript{111}. Both forms of TNF bind two transmembrane receptors with distinct functions; TNF receptor 1 (TNFR1 or CD120a) contains a death domain and induces cell cycle arrest and apoptosis while TNF receptor 2 (TNFR2 or CD120b) promotes cell growth and survival\textsuperscript{116}. Importantly, the membrane form of TNF signals more potently than sTNF via TNFR2\textsuperscript{117}. Expression of TNFR1 is ubiquitous, expressed at low levels in most cell types and tissues, while TNFR2 expression is limited to lymphoid and myeloid cells\textsuperscript{118}. mTNF can also signal back to the TNF-expressing cell when ligated with TNFR, resulting in phosphorylation of intracellular domains and activating nuclear factor-kB (NF-κB) to modulate cytokine responses in a process called reverse signaling\textsuperscript{119, 120}.

**TNF in human disease.**

The role of TNF in inflammation is well established, and inhibition of TNF in treatment of inflammatory diseases is common and efficacious\textsuperscript{121}. Monoclonal antibodies or soluble
receptors against TNF are particularly effective in patients with rheumatoid arthritis (RA) and inflammatory bowel disease\textsuperscript{122, 123}. However, some patients treated with TNF inhibitors experience the onset or exacerbation of inflammatory diseases including psoriasis, uveitis, and Crohn’s disease\textsuperscript{124}. In addition, studies of inflammatory bowel disease in mice demonstrate that colonic inflammation is paradoxically enhanced in TNF knockout animals\textsuperscript{125}. Furthermore, treatment of multiple sclerosis (MS) patients with anti-TNF therapy resulted in exacerbation of disease symptoms\textsuperscript{126}. These unexpected complications suggest that TNF mediates anti-inflammatory effects as well as promoting inflammation.

TNF is reported to inhibit the function of Foxp3, thus reducing suppressive function of Tregs isolated from RA patients\textsuperscript{127, 128}. However, another study reports that suppressive activity of Tregs is not affected by TNF\textsuperscript{129}. In addition, TNF promotes proliferation of Tregs and enhances Treg differentiation by suppressing Th17 generation\textsuperscript{130, 131}. These contradictory results suggest that TNF signaling in Tregs is complex and probably dependent upon experimental approach. Tregs express TNFR2 and require TNFR2 signaling for stability of Foxp3 expression and suppressive activity\textsuperscript{132-135}. Furthermore, mTNF preferentially signals via TNFR2, indicating that contract-dependent TNF signaling may be crucial to immunosuppressive responses\textsuperscript{117}. The impact of TNF on Treg function and stability remains controversial. Therefore, the role of mTNF on Treg biology must be elucidated in order to understand paradoxical responses to anti-TNF therapeutics in human disease.
CHAPTER TWO
MATERIALS AND METHODS

Mononuclear Cell Isolation and Cell Purification

Umbilical cord blood samples were collected from healthy volunteer donors into citrate phosphate dextrose solution. Adult samples were collected from healthy volunteer donors into heparin solution. Mononuclear cells were isolated by density gradient centrifugation using Lymphocyte Separation Medium (Corning). Red blood cells were lysed using ACK lysis buffer (Gibco). Adult Tregs and CD4+CD25- T cells were isolated from mononuclear cells using EasySep Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit (Stem Cell Technologies). For mouse experiments, T cells were isolated from spleens of C57BL/6 mice and enriched for CD4+ cells using mouse CD4+ T cell enrichment kit (Stem Cell). Naïve T cells for suppression assay were obtained by FACS sorting for CD3+CD45RA+ and APCs by sorting for CD3- cells.

Treg Induction

Umbilical cord blood mononuclear cells were stimulated with α-CD3 (200 ng/ml unless otherwise stated) in the presence of IL-2 (10 ng/ml) in RPMI 1640 (Hyclone) supplemented with 10% fetal calf serum. Cells were cultured for 14 days with change of media every 2-3 days, maintaining IL-2 concentration throughout culture duration. Tregs were analyzed on day 14
after stimulation. For Th1 polarization cultures, IL-12 (20 ng/ml) was maintained in culture medium. For Th17 polarization cultures, IL-1β (20 ng/ml) and IL-6 (20 ng/ml) were maintained in culture medium. Recombinant human cytokines were obtained from Peprotech.

**Antibodies**

Antibodies used for flow cytometry were anti-CD4, CD8, CD25, Foxp3, CCR4, CCR6, CCR7, CCR10, CXCR3, TNFR2, TIM-3, CD27, CD26, OX40, CD31, IL-2, GM-CSF, IFN-γ, TNF-α, and IL-8 from Biolegend. Anti-TNFR1 was from Bio-Rad. Functional antibodies for cell culture were anti-CD3 (OKT3), TNF-α (Mab11), IL-2, and CD28 from Biolegend. Antibodies against mouse CD4, CD25, Foxp3, and TNF were obtained from Biolegend.

**Flow Cytometry**

Single cell suspensions were prepared in phosphate buffered saline with 0.5% fetal calf serum and 0.01% sodium azide. Staining was performed according to standard protocols. Prior to surface staining, cells were blocked with Human TruStain FcX blocking solution (Biolegend). Foxp3 staining was performed using Foxp3 Fix/Perm buffer set (Biolegend). For cytokine staining, cells were restimulated for 4 hours using phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (1 μM; Sigma Aldrich) in the presence of monensin (1 μM; Biolegend) in RPMI 1640 (Hyclone) supplemented with 10% fetal calf serum. For surface TNF-α analysis, stimulation was performed without monensin. Data were collected on FACS Canto II and LSR Fortessa (BD Biosciences) and analyzed by Flowjo software (Tree Star).

**Suppression Assay**

Adult Treg cells were enriched by magnetic sorting as described above. Naïve T cells and APCs were FACS sorted as previously described. Cord blood assays used total cord blood
from Treg induction cultures. Prior to stimulation, Tregs were labeled with Cell Trace Violet (Thermo Fisher) and naïve T cells labeled with CFSE (Thermo Fisher). 100,000 Tregs, 100,000 naïve T cells, and 50,000 APCs were added to a 96-well tissue culture-treated plate and stimulated with 5 µg/ml anti-CD3 for 5 days. Data collected on FACS Canto II.

**Survival Assay**

Adult Tregs were isolated from peripheral blood and isolated by magnetic sorting as previously described. Cells were labeled with CFSE and 100,000 cells added to each well of 96-well non-tissue culture-treated plate coated with anti-CD3. Costimulation was provided by 5 µg/ml anti-CD28. IL-2 (10 ng/ml) was provided to control wells only and all other conditions were treated with low endotoxin azide-free (LEAF) grade anti-IL-2 antibody (2 µg/ml). LEAF grade anti-TNF or isotype control LEAF mIgG1 (25 µg/ml) were added as indicated. After 5 days, cells were counted in triplicate in 0.1% trypan blue solution in PBS. Average cell number was calculated from triplicate values and divided by cell number of untreated control to generate plotted data. Apoptosis was assessed by staining with annexin V (BD Biosciences) and measured by flow cytometry.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism software. For TNF and IFN-γ expression were analyzed by student’s t-test (p<0.05). Treg survival assay was analyzed by paired t-test (p<0.05).
CHAPTER THREE
PROPENSITY FOR TREG GENERATION BY CORD BLOOD NAÏVE T CELLS

Introduction

Umbilical cord blood and adult blood display a similar frequency of Tregs, however naïve T cells from cord blood display an enhanced propensity for Treg differentiation when stimulated ex vivo in the presence of IL-2 (Lee et al., manuscript in preparation). Tregs can be generated from naïve T cells in peripheral tissues or in cell culture through TCR stimulation in the presence of TGF-β and IL-246. Previous work demonstrates that CD14+CD36hi monocytes in cord blood provide TGF-β and retinoic acid to naïve T cells, resulting in the majority of CD4 and CD8 T cells differentiating to Foxp3+ Tregs when stimulated in the presence of exogenous IL-2 (Lee et al., manuscript in preparation).

Recognition of cognate antigen in the context of MHC is critical for T cell activation and differentiation. Furthermore, many factors determine the fate of T cells during activation including affinity of TCR for antigen in the context of MHC, the extent of TCR ligation, costimulatory signals provided by antigen presenting cells (APCs), and the local cytokine environment58,136. In order to understand the in vivo role of cord blood Tregs, it is first important to gain an understanding for the factors that influence differentiation of naïve T cells from cord blood. For these reasons, we tested the impact of TCR stimulation strength and T lineage-polarizing cytokines on the differentiation of naïve T cells from cord blood.

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The Role of TCR Signaling in Treg Generation

Outside the thymus, TCR ligation is required for differentiation of Tregs\textsuperscript{46}. Numerous studies indicate affinity of antigen is an important determinant for T cell differentiation, and low dose, high affinity antigen is recognized as a potent driver of Treg generation \textit{in vivo}\textsuperscript{137, 138}. Furthermore, the extent of TCR ligation is known to determine differentiation of naïve T cells into specific lineages of effector T cells\textsuperscript{136}. However, the precise role of TCR ligation in Treg differentiation remains poorly understood.

To avoid the requirement for specific antigen, naïve T cells in cord blood were stimulated with monoclonal antibodies against CD3, the signaling domain of TCR. In this manner, APCs to provide costimulatory signals and cytokines as would occur \textit{in vivo}. However, the extent of TCR ligation as modeled by binding of anti-CD3 presented by APCs may impact the fate of T cells. Specifically, we aimed to address whether the extent of TCR ligation in \textit{ex vivo} stimulated T cells in cord blood affects their differentiation into Tregs. To determine this, several doses of anti-CD3 were tested and differentiation of cord blood T cells measured by flow cytometry. Human T cells transiently express low levels of Foxp3 upon antigen stimulation, so cells were expanded with change of media for 14 days to ensure that cells staining positive for Foxp3 were stable Tregs\textsuperscript{32}. We found that decreasing concentrations of anti-CD3 antibody reduced total Foxp3 expression in both CD4 and CD8 subsets, indicating that less TCR stimulation decreases T cell differentiation to Foxp3+ cells (Fig. 1).

To ensure that changes in Foxp3 expression were not due to lack of TCR stimulation at low concentrations of anti-CD3, total cell numbers were calculated at the end of 14 day culture period. Below 200 ng/ml anti-CD3, a two-fold reduction in cell number was found (Fig. 2).
Together, these data indicate that higher doses of TCR stimulation promote Foxp3 expression. Notably, even at low doses of stimulation the majority of CD4 and CD8 T cells express Foxp3.

Figure 1. Decreasing anti-CD3 reduces Foxp3 expression. Cord blood T cells stimulated with decreasing concentrations of anti-CD3 and stained for Foxp3 after 14 days. Data plotted are means of Foxp3+ frequency from 11 donors. Error bars indicate standard error of the mean.
Plasticity of Cord Blood Naïve T Cells in Response to Polarizing Cytokines

Differentiation of naïve T cells into effector lineages is dependent upon local cytokine milieu during activation. In the presence of IL-2, naïve T cells from cord blood upregulate Foxp3 and differentiate to functionally suppressive Tregs. Previous work demonstrates that the addition of exogenous IL-4 potently blocks Treg differentiation by cord blood T cells (Lee et al., manuscript in preparation). However, the plasticity of naïve T cells from cord blood in response to other lineage-polarizing cytokines remains unresolved. For this reason, we investigated the impact of Th1 and Th17 polarizing conditions on cord blood Treg differentiation.
Cord blood T cells readily upregulate Foxp3 and become Tregs in response to *ex vivo* stimulation, a propensity that is maintained in the infant throughout the first year of life\textsuperscript{139}. Like Treg differentiation, generation of inflammatory Th17 cells is dependent on TGF-β\textsuperscript{140}. However, while IL-2 promotes Foxp3 expression, Th17 differentiation is driven by the presence of inflammatory cytokines. Specifically, IL-6 and IL-1β are known to drive RORγt and IL-17 expression, which are key identifiers of inflammatory Th17 cells\textsuperscript{141,142}. Cord blood T cells fail to proliferate in the absence of IL-2 (data not shown), so the capacity for Th17 differentiation was tested by adding recombinant IL-6 and IL-1β to naïve T cells in the presence of exogenous IL-2. If Th17 differentiation occurred, we expected to see a decrease in Foxp3+CD25+ cells. After 14 days, Treg phenotype was assessed by flow cytometry after staining for Foxp3 and CD25. Addition of Th17-polarizing cytokines had no effect on the expression of these markers by CD4+ or CD8+ cells, indicating that Treg differentiation was not affected in either subset (Fig. 3). Furthermore, Th17 differentiation was assessed by intracellular staining for RORγt, revealing no difference between Treg-induced (IL-2 alone) and Th17-induced (IL-2+IL-1β+IL-6) conditions (data not shown). To test for IL-17 expression, Treg and Th17-induced cells were restimulated with PMA/ionomycin and culture supernatants assessed for soluble IL-17 by cytometric bead array. IL-17 was not detected in either condition, confirming the absence of Th17 differentiation by cord blood T cells (data not shown).
Figure 3. Treg differentiation in response to Th17 and Th1 polarizing cytokines. Cord blood mononuclear cells stimulated with anti-CD3 in the presence of indicated cytokines for 14 days. Data shown are representative of 3 donors. Data generated by size gating on live cells, then gating on CD4+ and CD8+ groups.

To test the plasticity of cord blood naïve T cells in response to Th1 polarizing conditions in vitro, recombinant human IL-12 and IL-2 were added to cord blood mononuclear cells at the time of stimulation and maintained throughout culture duration. Differentiation of Tregs was assessed by the expression of Foxp3 and CD25 using flow cytometry. After 10 days in culture, cessation of growth became evident in cells treated with IL-12, and by day 14 after stimulation cells exhibited widespread death. Flow cytometric analysis revealed markedly reduced expression of Foxp3 and CD25 in both CD4+ and CD8+ populations, indicating a reduction in
Treg differentiation in IL-12-treated samples (Fig. 3). Alternatively, reduction in Foxp3 expression in live-gated cell groups may be due to loss of expression by apoptotic cells.

**Discussion**

Th1 helper cells are critical for immune responses against intracellular bacteria. IL-12 and IL-2 drive Th1 differentiation, upregulating T-bet expression and the signature cytokine IFN-\(\gamma\), which activates macrophages to kill intracellular bacteria\(^{58,60}\). Appropriate immune responses to intracellular bacteria require differentiation of naïve T cells in response to polarizing cytokines, thus inducing expansion of Th1 effector cells in response to infection. Studies in mice and human cord blood naïve T cells demonstrate reduced Th1 differentiation compared with adult naïve T cells and a bias towards production of IL-4, IL-5, and IL-13 rather than IFN-\(\gamma\)\(^{143-145}\). A bias toward Th2 immunity combined with a deficiency in cell-mediated immunity make newborns susceptible to viral and bacterial infections and limit efficacy of vaccination against intracellular pathogens\(^{146}\). Furthermore, differentiation of Tregs rather than effector T cells may contribute to impaired immune responses to pathogens and vaccines. For these reasons, understanding the extent to which cord blood naïve T cells differentiate to Tregs in the presence of T helper-polarizing cytokines is crucial to improving outcomes for neonates in response to infectious disease. We demonstrate that Th1-polarizing conditions reduce Foxp3 expression and Treg development *in vitro*. Furthermore, Th1-polarizing cytokines resulted in cell death and reduced expansion in response to TCR stimulation. Th1 cytokines may promote cell death or attenuation of proliferation in newborns, contributing to the paucity of Th1 immunity in early life. This possibility, however has not been directly tested. Future work will
determine whether reduction in Foxp3 during Th1-polarization is associated with upregulation in Th1 cytokines and T-bet expression.

In contrast, Th17-polarization had no effect on Treg generation. IL-2 is shown to block Th17 differentiation, so the possibility remains that IL-2 addition inhibited Th17 generation\textsuperscript{147}. Without IL-2, however, cord blood T cells fail to proliferate. In agreement with our results, Th17 cells are not readily detected in the peripheral blood of infants and only begin to differentiate after the first few months of life\textsuperscript{139}. The propensity for Treg generation observed in cord blood and neonates may block Th17 differentiation, resulting in a tolerant, rather than pro-inflammatory, immune status.

The effect of antigen dose was modeled by stimulation with variable concentrations of anti-CD3, revealing a relationship between antigen dose and Treg differentiation. We observe a correlation between concentration of anti-CD3 and frequency of cells expressing Foxp3, suggesting that the extent of TCR ligations impacts cell fate during stimulation. Interestingly, even the lowest doses of anti-CD3 resulted in Foxp3 expression in a large fraction of cells, demonstrating the propensity for Treg generation by cord blood T cells. The role of antigen affinity on Treg generation, however, was not tested. Anti-CD3 stimulates TCR signaling, and therefore concentration of anti-CD3 affects the amount of TCR complexes activated, but does not directly provide any information about antigen affinity in Treg generation.

Together, these results demonstrate a propensity for Treg generation in cord blood that is at least partially affected by extent of TCR ligation. Furthermore, we demonstrate a failure to reduce Treg differentiation in response to inflammatory cytokines reported to induce Th17 cells. Therefore, in agreement with other studies, Treg differentiation may predominate neonatal
responses to antigen while Th17 inflammatory responses are absent. Th1-polarizing conditions, however, reduced Foxp3 expression and resulted in reduced growth and subsequent cell death. While the mechanism by which IL-12 attenuates growth and induces cell death was not tested, this phenomenon may represent a means by which the neonatal immune system avoids Th1 responses.
CHAPTER FOUR

PHENOTYPE OF CORD BLOOD TREGS IN COMPARISON TO ADULT PERIPHERAL BLOOD TREGS

Introduction

Cord blood naïve T cells readily differentiate to regulatory T cells after \textit{ex vivo} stimulation and exhibit \textit{in vitro} suppressive activity comparable to adult peripheral blood Tregs. However, recent studies demonstrate the existence of several distinct subsets of Foxp3+ Tregs with distinct functions in controlling immune responses\textsuperscript{57}. Tregs suppress T cell proliferation primarily by direct contact, and therefore tissue localization is critical to Treg function \textit{in vivo} \textsuperscript{43}. Therefore, analysis of chemokine receptor expression by cord blood Tregs is pivotal to predicting compartmentalization to peripheral tissues during immune responses in neonates. Furthermore, subsets of Tregs in adult peripheral blood have been identified and distinguished by differential expression of chemokine receptors\textsuperscript{57}. These subsets display surface phenotypes similar to effector T cell subsets and even share expression of characteristic pro-inflammatory cytokines when stimulated \textit{ex vivo} \textsuperscript{57}. Cell surface molecules are important not only to the phenotypic classification of cell types and subsets, but also to a multitude of important cellular functions. Specific molecular interactions between immune cells during cell-cell contact is crucial for many aspects of immune cell function, and T cells express various costimulatory and immune checkpoint molecules to regulate activation, proliferation, and survival\textsuperscript{148}. While adult peripheral blood Tregs have been investigated extensively, little is known about Tregs in fetal or
neonatal immunity. To understand the function of cord blood Tregs in neonatal immunity, the surface phenotype and cytokine profile must first be determined.

**Cord Blood Tregs Express Chemokine Receptors and are Distinct from Adult tTregs**

To characterize the phenotype of umbilical cord blood-derived Tregs, we compared chemokine receptor expression to Tregs isolated from adult peripheral blood (tTregs). Adult tTregs expressed high amounts of CCR4 and CCR6 and the majority were CCR4⁺CCR6⁺CCR7⁺⁻CXCR3⁺⁻ (Fig. 4). In contrast, cord blood CD4⁺ Tregs expressed lower amounts of CCR4 and CCR6, but exhibited elevated CCR7 expression in comparison to adult. The phenotype CCR4⁺⁻CCR6⁺⁻CCR7⁺⁻CXCR3⁺⁻ was shared between CD4⁺ and CD8⁺ UCB Tregs, although CD8⁺ Tregs expressed less CCR4. Furthermore, while only a subset of adult Tregs expressed CXCR3, the majority of cord blood CD4⁺ and CD8⁺ Tregs expressed CXCR3. Finally, low CCR10 expression was only observed in CD8⁺ cord blood Tregs.
Cord Blood Tregs Express Distinct Surface Antigens

To investigate functional differences between cord blood-derived Tregs and adult tTregs, we compared expression of surface antigens associated with T cell function. In particular, we tested the expression of surface molecules involved in T cell activation or contact-dependent interactions with other immune cells. We found that cord blood and adult Tregs shared high expression of CD27, a costimulatory surface receptor known to be critical for T cell expansion and formation of immunological memory\textsuperscript{149,150} (Fig. 5). To further analyze surface antigen expression, CD4\textsuperscript{+} and CD8\textsuperscript{+} populations were further gated on Foxp3\textsuperscript{+}CD27\textsuperscript{+} groups. Strikingly, we found high expression of CD26 on both CD4\textsuperscript{+} and CD8\textsuperscript{+} Tregs from cord blood, but in
contrast adult Tregs were all CD26−. We also tested the expression of surface receptors involved in T cell activation and immune regulation. We found that a fraction of CD8+ Tregs from cord blood express TIM-3 (~60%) in addition to a smaller fraction of CD4+ Tregs (~15%). In contrast, adult Tregs showed no expression of TIM-3. Additionally, expression of the TNF receptor superfamily molecule OX40 was detected on a group of CD4+ cord blood Tregs (~40%), but not on CD8+ cord blood Tregs or adult tTregs. Finally, nearly all CD8+ Tregs from cord blood expressed the platelet adhesion molecule CD31, in addition to a subset (~30%) of cord blood CD4+ Tregs. CD31 was not detected on adult Tregs. Together, these results demonstrate a surface antigen phenotype in cord blood Tregs that contrasts with adult CD4+ Tregs, suggesting cord blood Tregs possess distinct functions not observed in adults. Furthermore, phenotypes differed between CD4+ and CD8+ Tregs in cord blood, providing further evidence for functional specialization of these subsets.
Figure 5. Surface antigen expression by cord blood and adult Tregs. Cord blood-derived Tregs and adult peripheral blood Tregs stained for surface antigen expression. Samples gated on total CD4+ or CD8+ populations prior to gating on Foxp3+CD27+ for further analysis.
Cord Blood Tregs Express Distinct Cytokine Profile

Recent reports demonstrate inflammatory cytokine production by subsets of human Tregs\(^5\). To assess possible functions for cord blood-derived Tregs in vivo, we tested the ability of these cells to produce cytokine. Naïve T cells were induced to generate Tregs as previously described, then restimulated with PMA and ionomycin in the presence of monensin to induce activation and cytokine expression while blocking golgi transport to prevent secretion of cytokines. Subsequently, cytokines were detected by intracellular staining along with Foxp3 co-staining and analyzed by flow cytometry. We found that cord blood Tregs express a distinctive profile of cytokines (Fig. 6A). Foxp3\(^+\) CD4\(^+\) cells produced TNF (>80%), IL-2 (~30%), GM-CSF (~50%), and IL-8 (~30%). Similarly, Foxp3\(^+\)CD8\(^+\) Tregs from cord blood expressed TNF (>80%), GM-CSF (~70%), and IL-8 (~20%). CD8\(^+\) Tregs did not express IL-2, but expressed IFN-\(\gamma\) (25%). Expression of IL-10 and IL-17 were not detected (data not shown). The majority of CD8\(^+\) Tregs from cord blood expressed both IL-8 and GM-CSF, while a fraction (~20%) of cord blood CD4\(^+\) Tregs expressed both cytokines (Fig. 6B). In contrast, conventional adult T cells (CD4\(^+\)CD25\(^-\)Foxp3\(^-\)) display subsets of IL-8 or GM-CSF producing cells (~10%), however the production of these cytokines were mutually exclusive. Furthermore, Foxp3\(^+\) Tregs from adult blood did not express the cytokine profile of cord blood Tregs. Unexpectedly, however, TNF expression was observed in the majority of adult Foxp3\(^+\) Tregs (>70%). These data demonstrate distinct cytokine profile differences for cord blood Tregs in comparison to adult Tregs. Furthermore, cytokine expression by cord blood Tregs supports functional differences for cord blood Tregs, suggesting Tregs in cord blood are able to mediate both anti-inflammatory and pro-inflammatory immune responses.
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GM-CSF
Figure 6. Distinct cytokine profile of cord blood Tregs. Cord blood and adult Tregs stimulated with PMA and ionomycin for 4 hours in the presence of monensin. (A) Cytokine profile of cord blood and adult Tregs (B) Co-expression of IL-8 and GM-CSF by cord blood Tregs in contrast to adult Tregs (CD4+CD25+Foxp3+) and conventional T cells (CD4+CD25-Foxp3-). Representative data from 6 donors for cord blood Tregs, and 3 donors for adult samples. (C) Frequency of cells staining positive for cytokines. Cells gated on CD4 or CD8 as indicated, followed by gating on Foxp3+ and Foxp3- populations prior to cytokine analysis. For adult samples, Foxp3+ were also gated on CD25+ and Foxp3- are gated on CD25-. Bars represent means of each sample.

Discussion

Chemokine receptor expression.

Cord blood Tregs express a distinct phenotype of chemokine receptor expression in comparison to adult Tregs, suggesting differential roles in tissue localization and trafficking ability. Expression of CXCR3 is limited predominantly to Th1 cells and mediates migration to sites of inflammation in response to CXCR3 ligands CXCL9, CXCL10, and CXCL11\textsuperscript{151}. Recently, a subset of Th1-like CXCR3\textsuperscript{+}Foxp3\textsuperscript{+} Tregs have been identified in adult peripheral blood with the ability to express IFN-\(\gamma\) and suppress Th1-mediated immune responses\textsuperscript{57}. In support of this, we observe a small population of CXCR3\textsuperscript{+} Tregs in adult peripheral blood. In contrast, however, the majority of CD4\textsuperscript{+} and CD8\textsuperscript{+} Tregs in cord blood express CXCR3, suggesting a role for these cells in suppressing Th1 immune responses and localizing to sites of Th1-mediated inflammation. During fetal development and early life, Th1 responses are mostly absent with the exception of responses to specific pathogens\textsuperscript{146}. In utero, Th1 responses are toxic to the placenta and may result in termination of pregnancy\textsuperscript{152}. Therefore, expression of CXCR3 by Tregs during fetal development may be important to control Th1 responses by trafficking to sites of Th1 inflammation and suppressing T cell proliferation and cytokine production.
Elevated CCR7 expression distinguishes cord blood Tregs from adult. CCR7 is required for T cell trafficking from peripheral tissues to lymphoid tissue in response to CCL19, the ligand for CCR7\textsuperscript{153}. CCL19 is expressed in T-cell zones in lymphoid tissue, suggesting a role for CCR7\textsuperscript{+} Tregs in modulation of T cell activation in germinal centers\textsuperscript{154}.

While adult peripheral blood Tregs are known to express high levels of CCR4 and CCR6, expression of these receptors was reduced in cord blood Tregs\textsuperscript{155}. CCR4\textsuperscript{+} Tregs are associated with residence in epithelial tissue, and in mice the loss of CCR4 in Tregs results in lymphocyte infiltration and inflammation in skin and lungs\textsuperscript{84}. Expression of CCR6 mediates migration of both Th17 and Treg cells to areas of inflammation, suggesting a role of CCR6\textsuperscript{+} Tregs in suppressing Th17-mediated inflammation\textsuperscript{156}. Decreased expression of CCR4 and CCR6 on cord blood Tregs in comparison to adult suggests a diminished ability to migrate to epithelial tissue and sites of Th17-mediated inflammation, however this hypothesis has not been tested.

Together, these data demonstrate a distinct phenotype of cord blood Tregs in comparison to adult and suggest that cord blood-derived Tregs would migrate differently than tTregs during immune responses and inflammation.

**Surface antigen phenotype.**

CD26, also known as dipeptidyl peptidase 4 (DPPIV), is a surface antigen associated with T cell activation and possesses enzymatic activity\textsuperscript{157}. CD26 can cleave a variety of hormones, growth factors, and chemokines which share proline or alanine residues at the N-terminus, thus modulating many physiologic processes including immune regulation and responses to infection\textsuperscript{157}. In particular, adenosine deaminase (ADA) is a known substrate for CD26\textsuperscript{158}. ADA is involved in purine metabolism and immune homeostasis, and genetic
deficiencies in ADA result in severe combined immune deficiency (SCID) and eventually death\textsuperscript{159}. As our data corroborate, CD26 is not expressed on adult Tregs, but in contrast we demonstrate high expression on cord blood Tregs, suggesting an important role for this enzyme in fetal or neonatal immune regulation or development\textsuperscript{160}. Future work will determine whether CD26 expression by cord blood Tregs modulates fetal or neonatal immune responses.

Expression of TIM-3 is associated with Th1 cells in humans and regulates Th1 cytokine production as well as maintenance of tolerance \textit{in vivo} \textsuperscript{161}. Specifically, interaction of TIM-3 with TIM-3 ligand is shown to inhibit Th1 responses in mice, thus maintaining peripheral tolerance\textsuperscript{162}. In humans, a paucity of TIM-3 expression is observed in multiple sclerosis (MS) patients compared with healthy controls and blockade of TIM-3 increases IFN-\(\gamma\) secretion by CD4\(^+\) T cells\textsuperscript{163}. Furthermore, expression of TIM-3 on cytotoxic CD8\(^+\) T cells is associated with anti-atherogenic cytokine production in human patients with atherosclerosis and blockade of TIM-3 in this context aggravates inflammation\textsuperscript{164}. Studies of tumor-bearing mice implicate tumor-resident TIM-3\(^+\) Tregs in tumor progression, identifying TIM-3\(^+\) Tregs as highly suppressive of effector T cell responses\textsuperscript{165}. Together, these studies demonstrate the importance of TIM-3 in the maintenance of tolerance in both human and murine disease. Expression of TIM-3 on cord blood Tregs, particularly CD8\(^+\) Tregs, suggests an important immunoregulatory role for this receptor. Future work will determine the function of TIM-3 in immune homeostasis in early life.

TNF receptor superfamily members are highly conserved and control a wide variety of immune responses\textsuperscript{166}. One of these members, OX40, is important to regulation of CD4 and CD8 T cells responses as well as the differentiation and activity of regulatory T cells\textsuperscript{167}. OX40 is
expressed on activated T cells, where interaction with OX40 ligand (OX40L) on APCs induces NF-κB activation, enhancing T cell survival and proliferation\textsuperscript{168}. Ligation of OX40 with OX40L has been shown to reduce Foxp3 upregulation to block Treg differentiation and inhibit production of IL-10\textsuperscript{169,170}. An interesting, but untested, possibility is that expression of OX40 on a subset of cord blood CD4\textsuperscript{+} Tregs may be a mechanism to limit Treg differentiation in neonates.

CD31, or platelet endothelial cell adhesion molecule-1, is a transmembrane receptor expressed on a variety of immune cells, endothelial cells, and platelets\textsuperscript{171}. While the precise role of CD31 in Treg function has not been elucidated, some studies link CD31 expression to reduction in T cell activation and proliferation and others demonstrate a loss of Treg-mediated suppression following loss of CD31 \textit{in vivo}\textsuperscript{172,173}. In addition, CD31 signaling is suggested to limit clonal expansion by negatively regulating proliferation and inhibiting apoptosis\textsuperscript{171}. Expression of CD31 by cord blood Tregs, but not adult tTregs suggests that cord blood Tregs may be responsive to signaling which adult Tregs are not. However, the function of CD31 in neonatal immune homeostasis and Treg function remains unresolved.

\textbf{Cytokine profile.}

Cord blood Tregs exhibit potent suppressive activity \textit{in vitro} but also produce inflammatory cytokines known to activate innate immune responses. Granulocyte macrophage colony-stimulating factor (GM-CSF) activates monocytes/macrophages and induces differentiation to modulate immune responses in peripheral tissues\textsuperscript{174}. Importantly, GM-CSF induces monocytes to differentiate into inflammatory M1 macrophages rather than anti-inflammatory M2 macrophages\textsuperscript{175}. GM-CSF is produced by a variety of immune cells including B and T cells, macrophages, neutrophils, and eosinophils\textsuperscript{176}. Production of GM-CSF by T cells
is associated with pathology in several human diseases and their mouse models. In MS and the mouse model experimental autoimmune encephalomyelitis (EAE) CD4+ T cells drive inflammation and tissue destruction, and specifically Th17 cells have been tied to pathology of neuroinflammation\textsuperscript{176}. However, studies in mice demonstrate IL-17 is not required for development of EAE, but rather GM-CSF inhibition ameliorates progression of disease\textsuperscript{177}. In addition, production of GM-CSF by pathogenic T cells is associated with Th1 inflammation in rheumatoid arthritis and Th2-mediated allergic lung disease\textsuperscript{176}. Therefore, GM-CSF not only regulates immune homeostasis, but also exacerbates inflammation. GM-CSF production by T cells in cord blood may be important to differentiation of myeloid cells in responses to infection, although this possibility has not been tested. Expression of GM-CSF by Tregs suggests a multifunctional role for these cells, possibly negatively regulating adaptive immune responses while simultaneously driving innate immune responses and inflammation. Future work will test the ability of cord blood Tregs to induce M1 macrophage differentiation from monocytes. Furthermore, previous work demonstrates the requirement for monocytes in the differentiation of Tregs in cord blood (Lee et al., manuscript in preparation). Therefore, GM-CSF may induce differentiation of monocytes and reduce Treg differentiation in favor of effector T cell generation in a negative feedback loop to control Treg homeostasis in neonates.

IL-8 also mediates innate immunity, resulting in recruitment and activation of neutrophils during immune responses\textsuperscript{178}. Neutrophils serve as the first line of defense against many pathogens, making IL-8 expression an essential aspect of initiation of immune responses during infection\textsuperscript{179}. In newborns, T cells readily produce IL-8 upon antigen encounter and infection, however production of IL-8 by T cells is rare in adults\textsuperscript{105}. Infants do not readily mount Th1
immune responses, so IL-8 production may be a compensatory mechanism to recruit neutrophils during bacterial infections. IL-8 production by Tregs has been identified in humans, and is hypothesized to modulate the recruitment of immune cells to inflamed tissues\textsuperscript{180}. Together, these data suggest IL-8 production by Tregs may be important for effective defense against bacterial infections in early life, recruiting neutrophils to peripheral tissues and activating innate immunity.

Interferon gamma (IFN\textsuperscript{-}γ) is produced by activated T helper and CD8\textsuperscript{+} cytotoxic T cells in response to viral and bacterial infections\textsuperscript{181}. Along with TNF, IFN\textsuperscript{-}γ is considered a prototypic Th1 cytokine and contributes to pathogen recognition while initiating antiviral responses by inhibiting cell proliferation and inducing apoptosis\textsuperscript{181}. Regulatory T cells are generally considered unable to produce IFN\textsuperscript{-}γ, however recently a subset of Th1-like Tregs have been described by expression of IFN\textsuperscript{-}γ during \textit{ex vivo} stimulation\textsuperscript{57}. We observe IFN\textsuperscript{-}γ expression by CD8\textsuperscript{+}Foxp3\textsuperscript{+} Tregs from cord blood, suggesting these cells mediate immune homeostasis in response to intracellular pathogens. Viral infections are particularly dangerous during fetal development and in early life, with recent cases of Zika virus infections demonstrating a paucity of antiviral immune responses in the fetus\textsuperscript{182}. An interesting, but untested possibility is that CD8\textsuperscript{+} Tregs suppress adaptive immune responses to prevent damaging inflammation while modulating antiviral immunity by secreting IFN\textsuperscript{-}γ during infection.

Both cord blood and adult Tregs robustly express TNF in response to stimulation. The pro-inflammatory effects of TNF are well-described, however recent studies provide clear evidence of anti-inflammatory roles for this pleiotropic cytokine. In mice, TNF is important to development of inflammatory bowel disease. Paradoxically, however, TNF gene knockout
results in more severe intestinal inflammation rather than reducing pathology. Similarly, in humans inhibition of TNF in RA and Crohn’s disease patients results in onset of autoimmune inflammation in some cases\textsuperscript{124}. These results suggest an important role for TNF in immune homeostasis that remains poorly understood. Expression of TNF by Tregs from both cord blood and adult peripheral blood raises the possibility of an important function for TNF in Treg homeostasis.

Together, these results suggest a multifunctional role for Tregs in fetal and neonatal immunity. Expression of cytokines known to recruit and activate innate immune cells to sites of inflammation, along with distinct chemokine receptor expression may allow Tregs to migrate to peripheral tissues to control immune responses to pathogens. Tregs may be important to control adaptive immune responses during development while simultaneously protecting the newborn from infection by recruiting and activating macrophages and neutrophils.
CHAPTER FIVE

FUNCTION OF TUMOR NECROSIS FACTOR SIGNALING IN TREGS

Introduction

The observation of robust TNF expression by both cord blood and adult Tregs suggests an important function for this cytokine. Recent reports disagree about the role of TNF-α signaling on Tregs; some reports suggest that TNF-α selectively activates and expands Tregs via tumor necrosis factor receptor 2 (TNFR2) while others indicate that TNF-α inhibits the suppressive function of human Tregs. Furthermore, inhibition of TNF results in the onset or exacerbation of autoimmunity in some patients. However, expression of TNF by human Tregs has not been reported. For these reasons we decided to investigate the function of TNF produced by Tregs.

Effect of TNF on Treg Differentiation

TNF is reported to inhibit phosphorylation of Foxp3, therefore reducing suppressive function of Tregs in rheumatoid arthritis (RA). However, other reports demonstrate a requirement for TNF signaling via TNFR2 on Tregs for maintenance of highly suppressive Tregs during inflammation. TNFR2+ Tregs reportedly upregulate Foxp3 and proliferate in response to TNF, suggesting a role of TNF in activation and expansion of Foxp3+ Tregs. Furthermore, interaction of transmembrane TNF (mTNF) with TNFR2 has been shown to block differentiation of Th17 cells in favor of Treg differentiation. To test the impact of TNF on
differentiation of Tregs, we used a neutralizing antibody against TNF to block TNF signaling during differentiation of cord blood Tregs, expecting to see a reduction of Foxp3^+CD25^+ Tregs in cultures treated with anti-TNF if TNF is important to Treg generation. However, we observed no difference in differentiation of CD4^+ or CD8^+ Tregs from cord blood treated with anti-TNF, and isotype control antibody (mIgG1), or those left untreated (Fig. 7). Although only one concentration of anti-CD3 is shown above, concentrations as high as 25 µg/ml and as low as 40 ng/ml also had no effect on frequency of Tregs generated in vitro. Specifically, no change in
total Foxp3 frequency was observed in CD4+ or CD8+ Tregs between conditions. Furthermore, no change in Foxp3+CD25+ phenotype was observed. These results suggest TNF does not affect cord blood Treg differentiation during induction in vitro.

**Effect of TNF on Suppressive Activity of Tregs**

TNF signaling has been linked to alterations in suppressive activity of Tregs, however discordant results prompted us to investigate the impact of TNF inhibition on in vitro suppressive activity of human Tregs. We tested the effect of TNF neutralization on suppression of naïve T cell proliferation using a standard suppression assay. Induced Tregs from cord blood or adult Tregs isolated from adult peripheral blood were mixed (1:1) with naïve T cells (adult CD3+CD45RA+ FACS sorted after collection from peripheral blood) labeled with CFSE to track cell division. Cells were stimulated by anti-CD3 antibody and APCs (CD3- FACS sorted after isolation from peripheral blood), and after 5 days proliferation of naïve T cells was determined by flow cytometric analysis of CFSE dilution. Naïve T cells and APCs were isolated from the same donor as Tregs in experiments with adult Tregs. To discern between Tregs and naïve T cells, Tregs were labeled with Cell Trace Violet (CTV) and removed from analysis by gating out CTV+ cells. As a control for viability of naïve T cells, naïve T cells were stimulated with anti-CD3 and APCs in the absence of Tregs. Both cord blood derived Tregs and adult peripheral blood Tregs potently suppressed proliferation of naïve T cells, however no difference was observed between cells treated with anti-TNF antibody and those treated with the isotype control (Fig. 8). Neither cord blood nor adult Tregs exhibited a decrease in suppressive activity when TNF was inhibited, however a slight increase in suppression was observed. This small increase in suppression may be due to reverse signaling of mTNF when bound to anti-TNF, thus
activating Tregs and mildly increasing suppression. Together these results suggest that *in vitro* suppression of naïve T cell proliferation is not decreased by TNF inhibition.

**Figure 8. Effect of TNF inhibition on suppressive activity of cord blood and adult Tregs.** Whole cord blood derived Tregs or adult Tregs isolated from peripheral blood were tested for suppressive activity in the presence or absence of anti-TNF antibody. CFSE dilution in labeled naïve T cells was determined by flow cytometry after 5 days.

**Adult Foxp3⁺ Tregs Express Membrane TNF**

TNF is expressed as a type II transmembrane protein and must be cleaved by TACE to produce the soluble form. Both membrane and soluble forms of TNF are active and can signal via TNF receptors, however each form exhibits specific activity. Soluble TNF signals more readily via TNFR1, while mTNF has a greater affinity for TNFR2. TNFR1 contains an intracellular death domain and signals for cell cycle arrest and apoptosis, while TNFR2 lacks a death domain and activates NF-κB to promote growth and proliferation. Due to the different effects mediated by the two different forms of TNF, we investigated which cells in adult peripheral blood express mTNF.
Total adult PBMCs were collected and stimulated *in vitro* with PMA and ionomycin. Expression of mTNF was detected by surface staining followed by analysis by flow cytometry. We found that expression of mTNF was limited to CD4^+^CD25^+^ cells (Fig. 9A). In contrast, CD4^+^CD25^-^ cells did not express mTNF. Human T cells transiently express CD25 when activated, but Tregs constitutively express high amounts of CD25^{36}. To test whether Tregs express mTNF, Tregs were isolated from PBMCs by magnetic sorting and stimulated. Expression of mTNF on conventional T cells was compared by magnetic sorting for CD4^+^CD25^-^ cells followed by stimulation. To ensure mTNF expression by Foxp3^+^ cells, both enriched populations were stained for TNF along with co-staining for CD4, CD25, and Foxp3 and analyzed by flow cytometry. We found that mTNF expression was indeed limited to a group of CD4^+^CD25^-^Foxp3^+^ cells, and found no mTNF expression on stimulated CD4^+^CD25^-^Foxp3^-^ cells (Fig. 9B). Although expression was low, greater than 50% of Foxp3^-^ T cells express detectable mTNF. These results demonstrate mTNF is preferentially expressed by Tregs in comparison to conventional T cells.

TNF is traditionally considered a Th1 cytokine, so as a control we assessed production of the signature Th1 cytokine IFN-γ by Tregs. Total expression of TNF and IFN-γ were assessed by intracellular staining with Foxp3 costaining. We found that Foxp3^-^ Tregs expressed TNF when stimulated but did not express any IFN-γ (Fig. 9C). We also compared expression of TNF and IFN-γ between Foxp3^-^ Tregs and conventional T cells. Total CD4^-^CD25^-^Foxp3^-^ T cells expressed IFN-γ and TNF, however Foxp3^-^ T cells did not express IFN-γ (Fig. 9D).
Figure 9. Expression of TNF by human Foxp3+ Tregs
Adult peripheral blood T cells were stimulated by PMA plus ionomycin for 4 hrs in the presence of monensin (details described in methods). The expression of cytokines was determined by intracellular staining or surface staining and analysis by flow cytometry. (A) Expression of the membrane form of TNF (mTNF) by CD4+CD25+ (right) and CD4+CD25− T cells (left). Representative data from 4 donors. Dashed lines indicate unstimulated samples and solid lines indicate stimulated samples (B) Expression of mTNF by CD4+CD25+Foxp3+ Tregs (left) or by CD4+CD25−Foxp3− conventional T cells (right). A representative data set from three donors. (C)
Expression of total TNF (upper panels) and IFN-y (lower panels) by unstimulated (left panels) or stimulated (right panels) Foxp3+ Tregs by intracellular staining. A representative data set from three samples. (D) Frequencies of TNF (left) or IFN-y (right) expressing cells among CD4+CD25+Foxp3+ (Foxp3+) cells and CD4+CD25−Foxp3− (Foxp3−) cells. TNF (n=6), IFN-y (n=3). * p<0.05 (student t test). (E) Expression of TNFR1 and TNFR2 on human Tregs by flow cytometry. (F) Expression of mTNF (n=3) and total (intracellular) TNF (n=5) by mouse CD4+ T cells. Representative data sets from 2 experiments. Foxp3+ Tregs (left) were gated first on live CD4+CD25+ cells, while Foxp3- T cells (right) were gated first on live CD4+CD25− cells. Dashed lines indicate unstimulated samples and solid lines represent stimulated samples.

The anti-inflammatory effect of TNF has been described in mice as well as humans, so we investigated whether TNF is expressed on mouse Tregs126. We isolated total splenic CD4+ T cells from C57BL/6 mice and stimulated as previously described. Total TNF expression was assessed by intracellular staining after stimulation in the presence of monensin while mTNF expression was assessed by surface staining after stimulation without monensin. We found similar expression of total TNF by Foxp3+ and Foxp3− T cells, however Foxp3+ T cells expressed a greater amount of mTNF (Fig. 9F). Some Foxp3− T cells expressed detectable mTNF as well, however expression was lower than in Foxp3+ cells. These data demonstrate that Foxp3+ Tregs preferentially express the membrane form of TNF in both mice and humans, suggesting that anti-inflammatory roles for TNF described in mouse and man may be related to mTNF expression by Tregs.

Membrane TNF signals preferentially through TNFR2 and promotes survival and growth rather than death. To investigate the possible outcomes of TNF signaling by Tregs, we assessed the expression of TNF receptors by adult peripheral blood Tregs. In agreement with literature, we found that Foxp3+ T cells express TNFR2 but not TNFR1 (Fig. 9E). These data suggest that mTNF may signal Tregs for survival and proliferation via TNFR2 in an autocrine manner.
**Function of Treg-derived TNF**

To test the hypothesis that mTNF signals Tregs for survival and proliferation in an autocrine manner, we developed an assay to test survival of adult Tregs in response to TNF inhibition. Tregs were isolated from peripheral blood, labeled with CFSE and stimulated *ex vivo* with plate-bound anti-CD3 and soluble CD28 in the presence of anti-TNF neutralizing antibody or isotype control. Importantly, IL-2 is considered a powerful growth factor for Tregs and is required for their survival *in vitro* \(^\text{20}\). Therefore, to test the impact of TNF on Treg survival independent of IL-2, we did not add exogenous IL-2. Furthermore, we added a neutralizing antibody against IL-2 to block any endogenous IL-2 production. As a control for cell viability we included Tregs stimulated in the presence of IL-2. After 5 days, survival was assessed by counting live cells. In the presence of TNF inhibition, survival was significantly reduced compared to untreated cells (Fig. 10). Furthermore, increasing concentration of anti-TNF reduced survival of Tregs. Specifically, 5 µg/ml and 25 µg/ml anti-TNF significantly reduced survival in comparison to untreated control in a concentration-dependent manner. While reduced survival of Tregs treated with 1 µg/ml anti-TNF did not achieve significance, 2 donors exhibited a reduction in survival, suggesting that this concentration is the threshold for effect in this assay. Importantly, the isotype control did not affect cell survival in comparison to untreated cells.

To confirm the role of cell proliferation and apoptosis in overall cell number after 5 days of stimulation, we analyzed CFSE dilution by flow cytometry and stained with annexin V to detect phosphatidyl inositol, a marker of apoptosis when exposed on outer leaflet of cell membranes. In the untreated and isotype controls, annexin V staining was positive in a small fraction of cells (~20%), indicating few cells undergoing apoptosis (Fig. 11, upper panel).
However, addition of 1 µg/ml anti-TNF increased annexin V$^+$ cells (~50%) and higher concentrations resulted in the majority of cells exhibiting an apoptotic phenotype (~80%). Cells treated with IL-2 did not bind annexin V and increased in size (data not shown). These data demonstrate that inhibition of TNF increases apoptosis, suggesting a role for TNF in survival of Tregs in the absence of IL-2.

To test the role of TNF in Treg proliferation \textit{in vitro}, CFSE dilution was measured by flow cytometry (Fig. 11, lower panel). About half of all untreated and isotype control-treated cells proliferated in response to stimulation (~50%) as indicated by dilution of CFSE. In contrast, fewer cells proliferated in anti-TNF-treated conditions, with almost total abrogation of cell division in the highest concentration of anti-TNF (~7% cells divided). Together, these data demonstrate that Tregs fail to proliferate and undergo apoptosis during \textit{ex vivo} stimulation in the presence of TNF inhibition.
Figure 10. Treg survival in response to TNF inhibition. Tregs isolated from peripheral blood were stimulated by plate-bound anti-CD3 and soluble anti-CD28 in the presence of anti-TNF for isotype control antibody for 5 days. Cell survival was calculated by comparing cell number of treated samples to untreated control. Statistical analysis by paired T-test (*p<0.05, ***p<0.001) with bars representing mean and standard deviation.
**Figure 11. Effect of TNF neutralization on apoptosis and cell proliferation.** Tregs were labeled with CFSE (bottom) prior to stimulation to track proliferation. After 5 days culture with anti-TNF, cells were stained with Annexin V (top) to detect apoptotic cells. Data representative of 3 independent experiments with 5 donors total.

**Discussion**

TNF drives inflammation but also contributes to anti-inflammatory immune responses. Studies in humans and mice demonstrate TNF mediates autoimmune inflammation, but inhibition of TNF also results in onset or exacerbation of autoimmunity\(^{124}\). Multiple cells types can produce TNF and the pleiotropic effects of this cytokine are dependent upon the type of cell and receptor engaged. Discordant studies of TNF function on immune homeostasis, along with the identification of TNF expression by cord blood and adult Tregs prompted us to investigate the function of Treg-derived TNF.

Several studies implicate TNF signaling in the differentiation of Tregs, however we did not observe any effect of TNF inhibition during cord blood Treg induction. The conditions used for ex vivo Treg induction in our lab may override any impact of TNF on Foxp3 expression. We provide exogenous IL-2 to drive Treg differentiation, raising the possibility that IL-2 potently
induces Tregs and negates any effect of TNF signaling. Furthermore, TNFR2 signaling is implicated in expansion of Foxp3+ cells, and Tregs induced from cord blood do not express TNFR2 (data not shown). Therefore, expression of TNFR2 may be important to mouse and adult human Treg generation. However, we have not directly tested this possibility.

Contradictory studies also report the effects of TNF on suppressive activity of Tregs, however we did not observe any effect on suppression of naïve T cell proliferation when TNF was blocked \textit{in vitro} \textsuperscript{127, 129}.

Our data demonstrate that Tregs express the membrane form of TNF, which is reported to selectively signal via TNFR2 and results in growth and survival rather than apoptosis\textsuperscript{116}. To test the possibility that mTNF promotes survival and proliferation by autocrine signaling, we purified Tregs from adult peripheral blood and stimulated them in the absence of IL-2 but in the presence or absence of TNF inhibition. Strikingly, we found that TNF inhibition reduces survival of Tregs in the absence of IL-2 \textit{in vitro} and demonstrated an increase in apoptotic phenotype along with abrogation of proliferation. These results support the hypothesis that mTNF provides autocrine signals to Tregs to promote survival. However, the impact of TNFR2 in this phenomenon is correlative. Lack of commercially-available blocking antibodies against TNFR2 necessitate the use of genetic approaches to determine the role of TNFR2 in pro-survival mTNF signaling. In the future, we aim to knock out or knock down TNFR2 in primary human Tregs in order to test the involvement of this receptor. In addition, the expression of TNFR2 and mTNF by mice provides a possible \textit{in vivo} model system to test TNF signaling in Tregs. While TNFR2 and TNF knockouts exist in mice, the pleiotropic nature of this cytokine necessitates a Treg-
specific knockout. Future work will aim to knock out TNF or TNFR2 in Foxp3+ T cells in mice to assess the importance of Treg-derived TNF signaling in vivo.

The presence of the membrane form of TNF on adult Tregs suggests decreased shedding of soluble TNF, which may account for the differences in the form of TNF expressed by Tregs in comparison to conventional T cells. The membrane form of TNF must be cleaved by the metalloprotease TNF alpha converting enzyme (TACE) in order to produce the soluble form\textsuperscript{115}. Therefore, decreased TACE activity may account for the increased expression of mTNF on Tregs. TACE forms dimers in cell membranes under resting conditions and is inhibited by the tissue inhibitor of metalloproteinase 3 (TIMP-3) by direct association with the cytoplasmic domain of TACE\textsuperscript{183}. Activation of ERK or the p38 MAP kinase pathway results in a shift from TACE dimers to monomers, relieving inhibition by TIMP-3 and resulting in activation of TACE\textsuperscript{183}. Tregs exhibit decreased ERK activation after TCR signaling and therefore may exhibit decreased active TACE and increased TACE dimers associated with TIMP-3\textsuperscript{184}. Decreased active TACE may account for the increase in the membrane form of TNF, as active TACE is required to cleave TNF to the soluble form. Inhibition of TACE may prove effective in controlling inflammation by reducing shedding of soluble TNF. Specifically, modulation of TIMP-3 activity in macrophages or T cells may be an alternative to TNF inhibition in the treatment of inflammatory diseases. Future work will aim to modulate shedding of TNF by T cells through modulation of TACE activation.
Our data demonstrate that antigen receptor stimulation of cord blood T cells results in
differentiation of Tregs with distinct phenotype. Cord blood-derived Tregs express a pattern of
chemokine receptors and surface antigens that is distinct from adult peripheral blood Tregs.
Furthermore, production of pro-inflammatory cytokines demonstrates functional differences
between cord blood-derived Tregs and adult tTregs. These data suggest unique roles for fetal
and neonatal Tregs in contrast to adult. Fetal and neonatal immune responses are initiated and
regulated much differently than in adults, raising the possibility that Tregs regulate both
tolerance and inflammation during development and early life and represent an adaptation to the
challenges faced by newborns.\textsuperscript{185}

Distinct chemokine receptor expression by cord blood Tregs suggests that they migrate
differently in response to chemokines. While adult Tregs express high levels of CCR4 and
CCR6 and low levels of CCR7 and CXCR3, we observe the opposite in cord blood-derived
Tregs. In contrast, we observed low CCR4 and CCR6 expression and elevated CCR7 and
CXCR3. These data suggest that cord blood Tregs preferentially traffic to sites of Th1
inflammation and secondary lymphoid tissues rather than taking up residence in skin or other
epithelial tissues.\textsuperscript{186} Furthermore, cord blood-derived Tregs express a profile of surface antigens
that contrasts with adult Tregs. Expression of surface receptors which modulate T cell activation
and tolerance provide further evidence for multifunctionality of cord blood Tregs. In particular,
CD26 expression distinguishes cord blood from adult Tregs. While this enzyme facilitates a wide variety of functions, genetic deficiency of one substrate, adenosine deaminase, results in severe combined immune deficiency (SCID) and death\(^\text{159}\). Therefore, CD26 function on Tregs may be crucial to maintenance of immune homeostasis in neonates but not adults. Future work will seek to determine the role of CD26 activity in neonatal and fetal immune homeostasis.

Despite stable expression of Foxp3 and potent suppressive activity, cord blood Tregs express multiple pro-inflammatory cytokines. Strikingly, we observe production of IL-8 and GM-CSF in response to stimulation. IL-8 potently recruits and activates neutrophils to sites of infection and GM-CSF induces activation and differentiation of monocytes and macrophages\(^\text{174,178}\). IL-8 is considered a prototypic cytokine during infection in neonates, raising the possibility that cord blood Tregs initiate immune responses in peripheral tissues by recruiting neutrophils to the site of infection\(^\text{105}\). GM-CSF production by Th17 cells is linked to neuroinflammation in MS, however we do not observe IL-17 expression in cord blood Tregs\(^\text{177}\). Instead, we observe a group of Foxp3\(^+\) cells that express IL-8 and GM-CSF, along with TNF. These data provide evidence that cord blood Tregs may be able to activate innate immune responses while suppressing adaptive immunity.

The tolerogenic immune status of infants in dependent upon multiple mechanisms, and not solely due to Treg-mediated suppression. Th1 responses are harmful to placental development, and during fetal and neonatal life a bias exists toward Th2 immunity rather than Th1\(^\text{185}\). While the precise mechanisms of this phenomenon remain poorly understood, we demonstrate that the Th1-polarizing cytokine IL-12 reduces proliferation in response to antigen stimulation, ultimately resulting in cell death. This response may be a means to control
expansion of Th1 cells and prevent complications during pregnancy, however this hypothesis has not been tested. In addition, we observe expression of TIM-3 on Tregs, a receptor linked to inhibition of Th1 immune responses in mice\textsuperscript{162}. These data suggest a role for cord blood Tregs in maintenance of tolerance via inhibition of Th1 T cell immunity. Furthermore, the induction of highly inflammatory Th17 cells is reported to develop months after birth\textsuperscript{139}. In agreement, we observe no difference in Treg generation in Th17-polarizing conditions \textit{in vitro}, along with a lack of Th17 generation. The differences in T cell differentiation in cord blood suggest markedly different adaptive immune responses during pregnancy and early life compared with adult immunity.

Suppression of T cell proliferation by cord blood Tregs is comparable to adult Tregs, however unlike tTregs, cord blood-derived Tregs express pro-inflammatory cytokines known to potently activate innate immunity. Furthermore, chemokine receptor expression suggest cord blood-derived Tregs migrate to peripheral tissues and lymph nodes to control Th1 inflammation. Together, these results demonstrate the possibility that multifunctional Tregs from cord blood suppress adaptive immune responses while regulating innate immunity in peripheral tissues.

Surprisingly, both cord blood and adult Tregs express TNF when stimulated. The pro-inflammatory functions of TNF are well described, however recently the anti-inflammatory functions of this pleiotropic cytokine are beginning to be elucidated. Inhibition of TNF is effective in reducing inflammation in patients with RA and Crohn’s disease, however some patients display onset or exacerbation of autoimmune inflammation after treatment\textsuperscript{124}. TNF is also reported to impact suppressive activity of Tregs and induce proliferation of Tregs via TNFR2\textsuperscript{127, 134}. We did not observe any impact of TNF inhibition on suppressive activity or
differentiation of Tregs. However, in the absence of IL-2, inhibition of TNF reduced survival of Tregs and reduced proliferation. Furthermore, increasing concentration of anti-TNF antibody upregulated apoptosis as indicated by increased phosphatidylserine on the outer leaflet of cell membranes. We demonstrate that a similar frequency of Tregs and conventional T cells express TNF, however Foxp3+ T cells preferentially express the membrane form of TNF. TNF is reported to promote Treg expansion and survival via TNFR2, indicating that autocrine TNF signaling may be blocked during inhibition of TNF. Our data support the hypothesis that Tregs require TNF signaling for survival in the absence of IL-2. In peripheral tissues, where IL-2 concentrations are low, Tregs may depend on autocrine TNF to maintain stability and survival. Therefore, inhibition of TNF in patients may result in inadvertent reduction in Treg frequency in peripheral tissues. Subsequently, lymphocyte infiltration and proliferation may drive inflammation observed in some patients due to a paucity of Tregs. In the future, we aim to investigate the impact of TNF inhibition on Treg homeostasis in the periphery. Furthermore, modulation of TACE activity may be efficacious in controlling inflammation as an alternative to TNF inhibition. Future work will investigate the effect of TACE activity modulation in inflammation.
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VITA

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