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The Development of the Human Adaptive Immune System and Its Role in Promoting Immunological Tolerance

Author
Mold, Jeffrey Eron

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The Development of the Human Adaptive Immune System and Its Role in Promoting Immunological Tolerance

by

Jeffrey Eron Mold

DISSERTATION

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Acknowledgments

First and foremost, I would like to thank my wonderful parents Dr. James W. Mold and Sandra Mold. Few people are as lucky as I have been in life to have two parents who have sacrificed so much for their children. Without their unconditional love and support I could never come as far as I have. They taught me the value of questioning the status quo and made me believe that my opinion mattered. More importantly they never doubted my ability to succeed and have been there for me through all of the difficult periods of my life. If I grow up to be half as happy and successful as they are I will consider myself to be extremely fortunate.

Although I had not considered a career in science prior to the latter half of my undergraduate studies, it did not surprise many of my family members that I chose this career path. Most of my childhood was spent exploring creeks and forests for wildlife. Some my fondest memories involved collecting and breeding turtles with my grandparents, James and Nelda Mold, in the woods of Durham, North Carolina. Looking back it was clear from a very early age that I was destined to be in the life sciences. I am eternally grateful to my parents and grandparents for knowing this long before it became apparent to me, and for encouraging and guiding me on this career path.

My initial intent upon entering the University of Michigan, Ann Arbor was to pursue studies in film and creative writing. Almost purely by chance I enrolled in my first undergraduate level biology course in my second semester of my freshman year, which I found to be incredibly engaging and prompted me to consider biology as a major. However, had it not been for my undergraduate research experiences in the lab of Dr.

Dennis “Keith” Bishop, I very well could have lost interest in biology and would certainly not have chosen to pursue Immunology. Keith and his lab manager Sherri Chan recognized that I was a capable research technician and provided me with an incredible amount of support throughout the latter half of my undergraduate education. Through their help and support I was able to explore life as a young scientist and make an informed decision about pursuing graduate schools. For this reason I will always cherish the time that I spent working with Keith and Sherri.

As is likely the case with many young graduate students, I first arrived in San Francisco full of excitement and vigor but with almost no idea about what I intended to study. My first year was spent exploring the city of San Francisco and trying to acclimate myself to my new environment, while pursuing coursework and also trying to find a laboratory environment that suited my personality. During one of my laboratory rotations an older graduate student suggested that I contact Dr. Joseph “Mike” McCune about potentially working in his lab. Mike and I agreed to meet at a Café in the marina district to discuss rotation projects and I was immediately taken by his approach to scientific discussion and his appreciation for “outside of the box” ideas. We had several more meetings like this and I eventually joined the lab to pursue studies related to serum factors that could inhibit HIV infectivity in human beings. I will always remember these informal discussions with Mike as one of the more enjoyable times in my graduate career.

Both Mike and I were taking a fairly large risk having me join the lab when I did. When Mike agreed to take me on as his student, he was in the process of preparing to leave for a year long sabbatical to Paris, France, which meant that I would be without a

mentor for the first year of my Ph.D. thesis project. Fortunately, Mike believed in my ability to work independently and I was young and naive enough to agree. In the period that Mike was gone I managed to completely change the focus of my research from HIV virology to developmental immunology. This was a very bold move for many reasons and I surely would not have been successful had it not been for the support I received from an incredibly talented post-doctoral student name Jakob Michaëlsson in the neighboring laboratory run by Dr. Douglas Nixon. Both Jakob and Doug provided the mentorship that I needed during Mike's absence and were instrumental in the generation of all of the data presented in this thesis. When Mike returned I was already preparing to publish the first series of data from my studies on human T cell function and he provided his enthusiastic support for me to continue this project as my thesis project.

I consider my time as a graduate student to be one of the happiest times of my life. While I knew that I was making the correct decision when I enrolled as a Ph.D. student in biomedical sciences, my time at UCSF has only strengthened my thoughts on this matter. In addition to Mike, Jakob, and Doug I have had the immense privilege to be surrounded by countless numbers of brilliant and enthusiastic students and faculty members. My thesis committee members, Drs. Susan Fisher, Matthias Wabl, and Warner Green have been incredibly supportive of my often unconventional approach to science and I consider myself lucky to have had their unwavering support throughout my thesis work. Through collaborative efforts with the Blood Centers of the Pacific I have also been blessed with the opportunity to work with Drs. Michael Busch, Tzong Hae Lee, Marcus Muench, and William Reed who helped to promote my enthusiasm about my project and were always eager to give support.

I have witnessed many post-doctoral students and graduate students come and go from the McCune laboratory in my time as a student. In the first year in the lab, Dr. Jean-Francois Poulin, Ann-Marie Cruz, and Mary Beth Hanley all helped me to better myself both as a scientist and person. In the later years I came to greatly appreciate the unique personalities of several other members of the lab. Dr. Paul Baum was always available for late night discussions about my project and long-term plans concerning my future as a scientist. Drs. Marc Schweneker and Kristen Ladell were also there to provide support and to give me the hard truth when needed, and I am sure our paths will cross again in the future. Finally, I have to acknowledge the friendship and support of Drs. Bittoo Kanwar, Trevor Burt, and Louise Swainson as well as my fellow graduate students Corey Miller, Jana Broadhurst, and Rebecca Botelho, who have made my final years in the lab some of the most enjoyable times of my life. I feel so fortunate to be excited to come to work every morning and all of these individuals make that possible.

Finally, I have to acknowledge Jennifer Hicks, who was there for me for nearly the entirety of my graduate work. When we first met, I had begun to lose my enthusiasm and love of science. My project had reached a stalling point and I was questioning whether I was fit to be a good scientist. Jen's unwavering love and support throughout the last four and half years of my graduate career were invaluable in helping me to maintain the confidence needed to complete my thesis work. Looking back at my time in San Francisco I am certain that it would have been far less enjoyable had Jen not been a part of my life. Jen has been my best friend, my confidant, and my muse and I will always be indebted to her for her patience, wisdom, and understanding. I am without doubt a better person for having had the privilege of having her in my life.

Contributions to Presented Work

All of the work presented in this thesis was done under the supervision of my graduate mentor Dr. Joseph M. McCune. The work presented in both Chapters 2 and 3 was also done with the help of Dr. Douglas F. Nixon who is credited as a co-author on both of the publications that arose from the work. Dr. Jakob Michaëlsson was a co-first author of the work presented in Chapter 2 and many of the experiments described were done in direct collaboration with him. For the rest of the studies, I will briefly describe the contributions that co-authors and collaborators have made to the work below.

Chapter 2 was published as Michaëlsson J, Mold JE, McCune JM, and Nixon DF. (2006) Regulation of T cell responses in the developing human fetus. *J Immunol*. **176(10)**: pp. 5741-8. Dr. Michaëlsson and I agreed to pursue this work after I made the initial observation that T cells bearing a phenotype consistent with regulatory T cells were overrepresented in fetal tissues. Dr. Michaëlsson and I continued to work in close collaboration on this project and on the initial experiments that were published with the data presented in Chapter 3. For this reason we are listed as co-first authors on the data published from Chapter 2 and he is the 2nd author listed for the data published from Chapter 3.

Chapter 3 includes the data that made up the bulk of my graduate work and was published as Mold JE, Michaëlsson J, Burt TD, Muench MO, Beckerman KP, Busch MP, Lee TH, Nixon DF, and McCune JM. (2008) Maternal Alloantigens Promote the Development of Tolerogenic Fetal Regulatory T cells *In Utero*. *Science.* **322(5907)**: pp. 1562-5. In addition to the invaluable help provided by Dr. Michaëlsson, Dr. Trevor Burt and Dr. Tzong Hae Lee played a particularly important role in helping with experimental

procedures published in this study. Dr. Burt aided in experimental design and in the collection of all samples relevant to the studies done with samples taken from children and their families to measure T_{Reg} cell effects on anti-maternal and paternal MLR responses in young children. Dr. Lee was instrumental in performing all of the experiments to address the presence of maternal microchimerism in fetal tissues. Additional help was obtained from Dr. Marcus Muench who aided in the collection of fetal tissues and from Dr. Karen Beckerman who had acquired the data on umbilical cord blood chimerism presented in supplementary figure 2 of this publication.

Chapter 4 is in the process of being completed for publication. Dr. Christopher Barker and Linda Ta at the Gladstone Genomics Core provided invaluable help with study design and microarray experiments. Dr. Yuanyuan Xiao was responsible for microarray data analysis. Dr. P'ng Loke and Jackie Leung performed additional microarrays for thymocytes from fetal and infant thymuses, and contributed to the data analysis for these array experiments. Initial fluoescence assisted cellular sorting was done at the Gladstone Institutes of Virology and Immunology by Dr. Marty Bigos and C.K. Poon. All subsequent sorting was done at the Division of Experimental Medicine by me. The experiments involving the SCID-hu Thy/Liv mice were done by the lab of Dr. Cheryl Stoddart, who kindly donated her time to help with experimental design and with the development of a protocol for engrafting HSC into mouse cohorts generated in her lab. Jose Rivera, Sofiya Galkina, Galina Kosikova, and Mary Beth Moreno provided excellent technical assistance for these experiments.

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The Development of the Human Fetal Adaptive Immune System and Its Role in Promoting Immunological Tolerance

By Jeffrey Eron Mold

Abstract

The developing immune system has long been recognized to be uniquely prone to tolerance induction. Historical observations in rodent models, suggest that the tolerogenic properties of the fetus and neonate are linked to the immaturity of the adaptive immune system at this stage of development. However, the adaptive immune system of larger mammals undergoes considerable maturation during fetal development and is not thought to be amenable to tolerance induction at this time. There have, however, been clinical observations that suggest that the human fetus may be subject to tolerance induction under certain conditions, though very little is known about the mechanisms underlying such tolerance. This is in part attributable to a general deficit of knowledge concerning the functional properties of the developing immune system in human beings.

We characterized fetal T cells at early stages of T cell development (10-20 gestational weeks (g.w.)). Our analysis revealed that a high frequency of fetal CD4+ T cells bore the phenotype of regulatory T cells (T_{Reg}). Analysis of neonatal cord blood and adult peripheral blood and lymphoid tissues revealed that fetal tissues contained greater frequencies of T_{Reg} than neonates or adults.

These observations, coupled with the clinical findings that certain individuals showed tolerance to non-inherited maternal HLA molecules in a transplant setting, led to

the hypothesis that fetal T_{Reg} could play a role in suppressing T cell responses to maternal alloantigens. A survey of lymph node specimens revealed that maternal cells could be detected within the developing fetal tissues. Fetal T cells were highly responsive to alloantigens yet fetal T_{Reg} were capable of suppressing fetal T cell responses to maternal alloantigens. Stimulation of naïve fetal T cells with alloantigens led to the differentiation and expansion of T_{Reg} cells, providing a mechanism for how tolerance could be achieved following fetal exposure to foreign antigens in humans.

Ongoing studies have revealed that the tolerogenic properties of the developing fetal immune system may be rooted in changes that occur within the hematopoietic stem cell (HSC) compartment. These findings provide novel insights into the development of immunological tolerance in human beings.

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CHAPTER 1

Introduction

A History of Immunological Tolerance

Paul Erlich introduced the concept of *horror autotoxicus* in the early 1900's to describe the general unwillingness of an organism's immune system to mount a response to host-derived, or "self," antigens (1). Despite evidence to the contrary (2), the view that the immune system was incapable of attacking self-tissues remained widely accepted throughout the early 1900's. Recognition that autoimmunity could cause disease in humans slowly dawned in the mid-1900's with the discovery of autoantibodies that could be linked to specific diseases (3-5). Thus, it became clear that tolerance to self-antigens was not a *de facto* feature of the immune system and that it could be lost in certain situations, resulting in autoimmunity. Implicit in this understanding was the fact that tolerance may also be gained to foreign substances under the appropriate conditions. In fact, developmental biologists were frequently taking advantage of the tolerogenic nature of developing organisms for the purpose of grafting tissues onto embryos (6). Yet, it took startling realization, by Ray D. Owen in 1945, that Free Martin cattle could share a circulatory system without any signs of immunological rejection, to promote the recognition that tolerance could be generated to foreign substances in the context of a developing organism (7). What would follow was an inspired series of experiments initiated by Sir Frank Macfarlane Burnet and Sir Peter Medawar that would shape the modern view of immunology.

As a transplant surgeon, Medawar sought to understand tolerance from the clinical perspective as a means for promoting allograft acceptance. Ironically, the first experimental demonstration of true acquired immunological tolerance came as a failed attempt by Medawar to design a test for determining whether calf twins were fraternal or

identical (6). Medawar reasoned that identical twins would be fully transplantable whereas genetically distinct fraternal twins would reject a skin graft from their siblings (8-10). This series of experiments serendipitously coincided with the publication of Owen's seminal work on the intermingling of the circulatory system in fraternal cattle twins, in which Owen noted yet declined to expand upon the immunological considerations implied within his findings (7). Additionally, Burnet had recently published his theory about self versus non-self discrimination, which held that the immune system's capacity to distinguish between antigens native to the host and all foreign substances was rooted in the development of the immune system itself (11). Burnet's theory on the development of immune responses postulated that exposure to antigen at a stage prior to the development of a mature immune system would result in tolerance regardless of whether the antigen was derived from the host or a foreign substance. Medawar's intial experiments on fraternal cattle twins thus represented the first actual experimental proof of acquired immunological tolerance. Later, along with Leslie Brent and Rupert Billingham, Medawar would famously demonstrate that experimental exposure to foreign cells could result in lasting tolerance in mice (12) leading to his sharing the Nobel Prize for Medicine in 1960 along with Burnet.

The introduction of the theory of acquired immunological tolerance provided a launching board for tackling several previously enigmatic phenomena. Perhaps the most intriguing natural case of immunological tolerance was that which arose during normal pregnancy (reviewed in ref. 13). Medawar noted in his Nobel lecture that, "Tolerance makes one think anew about the special relationship that holds between a mammalian mother and her unborn young " (10). He went on to differentiate the rodent models of

tolerance from the human, suggesting that the length of gestation and the permeability of the placenta is likely to differ between species and, as such, may contribute to differences in the frequency of fetal exposure to maternal antigens. Nevertheless, Medawar proposed several possible mechanisms that might account for maternal/fetal tolerance, focusing on the role of the maternal immune responses in relation to foreign, non-inherited paternal alloantigens expressed by fetal tissues: "(a) the anatomical separation of foetus from mother; (b) the antigenic immaturity of the foetus; and (c) the immunological indolence or inertness of the mother." As these still remain the most viable explanations for maternal/fetal tolerance after more than 50 years of research, I shall focus on addressing what is known at this stage and conclude with several small revisions based on work performed in the context of this thesis.

Clinical Support for Tolerance During Pregnancy

There is little doubt that the placenta serves an important function as a barrier between fetal and maternal circulations (reviewed in ref.14). Placentation and the establishment of the fetomaternal circulatory system within the placenta are highly complex processes, involving numerous steps, many of which are still topics of intense investigation (15). There is clear evidence that the placenta evolved in part to prevent widespread intermingling of fetal and maternal blood (16). However, it is becoming increasingly clear that the placental barrier is itself imperfect. Small numbers of fetal cells can be detected in the maternal circulation during pregnancy and appear to persist for some time thereafter in a process termed fetal microchimerism (17). Likewise, maternal cells can frequently be detected in fetal tissues and may persist long after birth

in a process known as maternal microchimerism (18). There has been significant speculation about the origins, cellular makeup, and consequences of fetal and maternal microchimerism during pregnancy and much remains unknown (19).

Because of the wide diversity of cell types detected in maternal tissues, both during and after pregnancy, it has been suggested that fetal stem cells or progenitor populations may be responsible for initiating fetal microchimerism (20). In addition, there is evidence demonstrating that microchimeric cells are found at elevated frequencies in sites of tissue injury even decades after pregnancy, suggesting that a long-lived population of fetal cells may persist in the mother and mobilize in pathological conditions (21). While there is some consensus that fetal stem cells are likely to contribute to the microchimeric populations identified in maternal tissues, the exact nature of the stem cell populations is uncertain. Several studies have reported detection of fetal hematopoietic stem cells (HSC) in maternal blood during and after pregnancy (21-23). However, the presence of additional non-hematopoietic cell types suggests that a more primitive stem cell population with greater multi-lineage potential, or that multiple distinct populations of lineage-committed stem cells, are involved (21). The possibility that fetal HSC may contribute to microchimerism is of specific interest because of the well-recognized differences between fetal and adult HSC populations (24), a point that will be further discussed in later sections.

The observation that fetal cells may persist in the mother long after birth has several important clinical implications, most notably because the fetal cells likely express non-inherited paternal HLA molecules they are potential targets for maternal immunity (17, 25). That these cells can be maintained for long periods in the mother suggests some

form of tolerance has been evoked. Yet, it seems that some autoimmune disorders arise in the context of fetal microchimerism (17) and it has been suggested that small populations of fetal cells could promote an allogeneic immune response, prompting attacks on otherwise healthy maternal tissues. The majority of studies have relied on either fluorescence in situ hybridization (FISH) or polymerase chain reaction (PCR) analysis of biopsy samples from patients versus healthy unaffected controls (related or in some cases unrelated) (26). With the exception of systemic sclerosis, where numerous studies have found increased quantities of fetal microchimerism in affected patients, the results are still unclear, and the link between the presence of microchimerism and the increased incidence of autoimmunity is still somewhat disputed.

Reciprocally, there have been several interesting reports detailing increased rates of maternal microchimerism in the tissues of children with a range of autoimmune disorders (27-30). Recently, one group reported an increase in the frequency of maternal microchimerism in children with Type 1 Diabetes (31). While at face value this would appear to indicate that maternal microchimerism could lead to autoimmunity, the authors concluded that the majority of microchimeric cells were in fact islet β cells, suggesting that maternal cells were playing a beneficial role by contributing to tissue repair. This conclusion was reached because maternal cells were rarely found to be of hematopoietic origin, ruling out the possibility of maternal anti-fetal immunity. Maternal islet β cells were also not found to be elevated in the period immediately following diagnosis, suggesting that the onset of autoimmunity was not associated with the presence of non-inherited maternal alloantigens. It remains unknown whether the persistence of fetal or maternal microchimerism is associated with tolerance. However, the fact that cells of

hematopoietic origin can be identified in individuals long after birth is suggestive of a tolerance mechanism.

The historical view that tolerance can be induced upon fetal exposure but not after the full maturation of the immune system would suggest that maternal, but not fetal, microchimerism would be associated with the development of tolerance. Clinically, there is some evidence that this may in fact be the case. Before the widespread use of erythropoietin in patients experiencing kidney failure, people on the waiting list for a kidney transplant were forced to receive multiple rounds of blood transfusions. The repeated exposure to foreign HLA molecules over the course of this therapy left patients with a reduced panel of potential HLA mismatches. A screening of suitable mismatches in a subset of European kidney transplant patients revealed the surprising finding that among the rare HLA types that did not elicit a response in a primary mixed leukocyte reaction (MLR) there was a highly significant enrichment of non-inherited maternal HLA types (32). The authors speculated that this may be the result of tolerance induced *in utero* to maternal alloantigens, thus representing the first human example of actively acquired immunological tolerance described by Billingham, Brent, and Medawar in mice 30 years prior.

In the years after this observation, a landmark study was published which retrospectively addressed the rate of kidney transplant rejection in patients that received sibling transplants that had either mismatch for a single non-inherited maternal HLA type (NIMA) or a single inherited paternal HLA type (IPA) (33). The results of this study were striking: patients who received a kidney with a NIMA mismatch showed a reduced incidence of chronic rejection and significantly greater graft survival at both 5 and 10

years post transplant. Interestingly, an increased rate of acute rejection was also noted in the NIMA-mismatched patients, suggesting the possibility that fetal or neonatal exposure may be able to prime both tolerance and immunity to NIMA.

Not surprisingly, the opposite effect has been reported when the mother receives a transplant bearing IPA-mismatched HLA molecules (34,35). Evidence of antibodies directed against paternal HLA antigens can be found in 15-30% of women who have been pregnant (36). There are also reports, in both mice and humans, that multiparity is associated with the priming of cytotoxic lymphocytes with specificity for minor histocompatibility antigens (37,38). In line with these observations, clinical differences have been reported with regards to graft survival when the donor is the father or one of the offspring (34). Several reports have documented increased rates of rejection among husband to wife kidney transplants as compared with wife to husband transplants (39,40). While not all studies addressed whether the women were previously pregnant, those that did found a significant negative relationship between prior pregnancy and graft survival, suggesting that exposure to IPA may prime the maternal immune system. In a somewhat surprising twist, the rates of rejection for offspring-mother transplants are not reportedly increased when there was a mismatch for paternal HLA antigens (41,42). In one study, it appeared that there might even be a slight benefit to receiving a transplanted kidney from one's offspring (42). This has been attributed to a greater chance of the offspring having shared HLA antigens as well as the possibility of residual fetal microchimerism. Regardless of the mechanism involved, there appears to be enough evidence that some clinics have instituted policies against transplanting female recipients with organs that bear mismatches for paternal HLA types (34).

While the maternal immune system may be immunized against IPA expressed on fetal cells that cross the placental barrier, there is no evidence to suggest that this is a common cause of clinically relevant maternal anti-fetal immune responses during pregnancy. On the contrary, during pregnancy there appears to be a shift in the maternal immune response such that a more tolerogenic response may be favored (reviewed in ref. 43). It has long been recognized that ~70% of women who suffer from the autoimmune disease rheumatoid arthritis resolve inflammation during pregnancy (44). This shift appears short-lived, however, and many women regain their symptoms shortly after birth. Interestingly, this was originally proposed to occur as a result of a shift in steroid hormones and played a role in the discovery of corticosteroids, for which Philip Hench shared the Nobel Prize in 1950. More recently, however, a direct role for steroid hormones has been dismissed in favor of a shift in the cytokine profile of lymphocytes and the nature of the immune response (45,46). The precise mechanisms for how rheumatoid arthritis (RA) is controlled during pregnancy remain unknown, yet recent findings about the mechanisms controlling maternal tolerance to fetal alloantigens may shed new light on this subject.

Mechanisms of Tolerance During Pregnancy

In addition to the premise that the placenta prevents maternal/fetal crosstalk, Medawar postulated two additional hypotheses for explaining tolerance during pregnancy. Both of these centered around the ability of the maternal immune system to sense and to respond to fetal antigens. Many lines of evidence suggest that fetal antigens are capable of eliciting an immune response from the more “mature” adult immune

system. Maternal immunity to fetal antigens does, however, appear to be regulated during pregnancy. In studies employing mice that expressed a transgenic T cell receptor (TCR) specific for paternal alloantigens, there was a significant change in the frequency and reactivity of transgenic T cells during pregnancy (47). This was dependent upon mating with males expressing the foreign HLA type, as the frequency of transgenic T cells was not altered in syngeneic matings. Moreover, the shift in T cell frequency was associated with reduced immunity to tumors bearing the paternal HLA type, suggesting a dominant form of tolerance. The observed "tolerance" towards paternal HLA antigens was also short-lived and immunity was restored shortly after pregnancy not unlike what has been observed for maternal autoimmune disorders.

A potential breakthrough for understanding the tolerogenic properties of pregnancy on the maternal immune system was made after the tryptophan catabolizing enzyme indoleamine 2,3 dioxygenase (IDO) was found to be highly expressed by the placenta (48). IDO was a known immunosuppressive enzyme that had previously been shown to exert potent affects on T cell function in both mice and humans (reviewed in ref. 49). Pharmacologic inhibition of IDO during pregnancy led to immunologic rejection of fetuses in allogeneic, but not syngeneic, matings in mice (48). This was found to be T cell dependent, suggesting that IDO either subverts or converts T cell immunity away from a harmful inflammatory response. How IDO accomplishes this feat remains largely unknown, although both tryptophan depletion and the production of cytotoxic tryptophan metabolites (kynurenines) have been suggested as possible mechanisms (49). An interesting link between the role of IDO in maintaining

immunologic tolerance and regulatory T cells (T_{Reg}) has also been suggested in recent years however the precise mechanisms involved are still somewhat unclear (50).

A role of T_{Reg} in controlling immune responses and maintaining peripheral tolerance has long been postulated and more recently clarified. Initially referred to as "suppressor T cells," the putative population of T cells that blocked immune responses fell from favor after numerous failures to identify any specific markers (51). In the mid-1990's the concept of a regulatory T cell was rejuvenated by the discovery that expression of the IL-2 receptor α chain (CD25) could demarcate a subset of CD4+ T cells in mice under homeostatic conditions (52). Further, it was demonstrated that transfer of T cell populations that were depleted of these CD25+ T cells led to the development of spontaneous autoimmunity within multiple different organs. It should be noted that, prior to this seminal observation, CD25 had been exclusively thought of as a marker of T cell activation, making earlier observations about the origin and function of CD4+CD25+ T cells less clear than initially reported. In fact, initial studies analyzing T cell activation states during pregnancy described an elevated frequency of CD25+CD3+ T cells throughout the course of normal pregnancy and decreasing after birth (53). This was taken to mean that fetal antigens were promoting the activation of maternal lymphocytes. In retrospect, it seemed equally likely that pregnancy was associated with the expansion or induction of T_{Reg} cells in the maternal peripheral immune system. This alternative explanation was in fact confirmed in a recent study where syngeneic or allogeneic matings were analyzed after depletion of CD25+ cells (54). After depletion of CD25+ T cells, the allogeneic conceptuses were universally rejected by the maternal immune system, whereas the syngeneic conceptuses were spared. Interestingly, the expansion of

maternal T_{Reg} cells was observed in both syngeneic and allogeneic matings, prompting the hypothesis that changes due to pregnancy rather than alloantigens from the fetus are responsible for the increased frequencies of T_{Reg}. Since the publication of this study, a series of reports have shown a similar expansion of T_{Reg} cells in human pregnancy (55,56).

While extensive reviews exist about the origins of T_{Reg} and their putative roles in controlling immunity to both self and foreign antigens (57, 58), I will attempt to briefly summarize some of the more pertinent and well-established features of these cells here. Perhaps the single most important breakthrough in the characterization of T_{Reg} cells has been the finding that the transcription factor, Forkhead Box P3 (FoxP3), is a master regulator of T_{Reg} differentiation and function (59). Mutations in the gene encoding FoxP3 are widely recognized to result in spontaneous and often lethal autoimmunity in both mice and humans (60). A population of CD4+CD25+FoxP3+ T_{Reg} is present in the thymus, suggesting that some T_{Reg} are a distinct population of T cells that develops through alternative pathways from conventional effector T cells (61). However, FoxP3 can also be induced in conventional T cells upon activation in the presence of the growth factor TGFβ (62). These "induced" T_{Reg} can theoretically be specific for any antigen in the host periphery, including foreign antigens, whereas the thymically-derived "naturally occurring" T_{Reg} are presumably specific for self-antigens presented in the thymus (57). T_{Reg} are capable of blocking "effector" functions of conventional T cells, including proliferation, cytotoxic activity, and cytokine secretion. How they accomplish this feat is not well understood and numerous mechanisms have been implicated, including the

secretion of immunosuppressive molecules and the engagement of receptors involved in dampening immune activation (58).

Several additional, non-exclusive mechanisms have been implicated in maintaining maternal tolerance during pregnancy. These include the action of uterine natural killer (NK) cells, expression of non-classical HLA molecules by fetal trophoblasts, the action of complement proteins, and the effects of immunomodulatory cytokines and signaling molecules, such as galectin-1 (13). Obviously maintaining tolerance to the allogeneic fetus is critical for the survival of any species that relies on sexual reproduction. It is accordingly not surprising that a wide array of mechanisms exist to accomplish this feat.

Development of the Fetal Immune System

While there is a vast literature on maternal tolerance to fetal antigens during pregnancy, relatively little is know about the functional properties of the developing fetal immune system (reviewed in Chapter 2, Remington and Klein *Infectious Diseases of the Fetus and Newborn Infant* 6th edition, Elsevier press). Medawar did not give much attention to the immune system of the fetus in his seminal studies on tolerance, as he viewed the fetal immune system as immature or undeveloped. In fact the immaturity of the fetal immune system was the basis for Medawar's initial experiments to generate tolerance. The view that fetal immunity is dysfunctional, or "immature," has largely been maintained through the years, despite some good evidence to the contrary. In large part, this can be attributed to significant differences in the rate of development of the immune system across different species. Inbred mice, which have been the primary model

organism for studying immune function since the time of Medawar, do not show any signs of peripheral T cell colonization prior to birth and thus are truly immunologically "immature" during fetal development (63). In stark contrast, the much less well studied human immune system displays substantial development of both the adaptive and innate immune system as early as the beginning of the second trimester of fetal development (64). Even still, the pervasive view in the medical literature is that the immune system is immature prior to birth, despite relatively little evidence regarding the functional properties of the fetal immune system in organisms where development of the immune system begins *in utero*, including human beings.

The development of the human immune system was intensely studied in the mid-1900's, primarily through the identification and characterization of different hematopoietic cell types within the fetal tissues (65). In recent years, there have been very few attempts to build on these early descriptions and modern immunological techniques have not been fully applied to understanding human immune function *in utero*. Thus, there is a significant body of work detailing the appearance and organization of different immunological subsets in the human fetus but a dearth of functional information about fetal immunity. This could in part explain the somewhat contradictory view that the immune system in the developing human fetus is immature, despite having all of the necessary components to mount immune responses. Not surprisingly, the fundamental features of hematopoiesis are well conserved between mice and humans, despite the vast differences in the development of mature lymphocyte subsets. A substantial amount is known about the earliest stages of hematopoiesis, which will not be

discussed in this brief review. Instead the focus will be on the development of mature immune cells and the establishment of the peripheral immune system.

The development of the human immune system begins in the yolk sac at ~19 days gestation with the appearance of CD34+ progenitors and the formation of blood islands comprised of primitive erythroid cells (reviewed in ref. 64). Hematopoiesis is evident in this region until ~50-60 days gestation, at which point blood begins to circulate from the yolk sac to the intraembryonic tissues, carrying the hematopoietic stem cells to the liver. Throughout the latter half of the first trimester and the second trimester, the fetal liver serves as the primary hematopoietic organ and is responsible for the generation of the first wave of immune cells. Lymphocytes and myeloid-derived antigen presenting cells (APCs) appear at roughly the same time between 9 and 10 gestational weeks (g.w.). Consistent with these observations, colonization of the thymus by lymphoid progenitors occurs shortly prior to this time at ~7-8 g.w. Peripheral lymphoid tissues first develop between 11 and 12 g.w., although organized B cell follicles do not appear until much later, typically after 20 g.w. (65). Throughout the second trimester, the peripheral lymph nodes and spleen appear fairly disorganized relative to adult lymphoid tissues. The spleen contains patches of lymphocytes and dendritic cells surrounding lymphatic vessels and lymph nodes generally consist of scattered T cells surrounding larger dendritic cells arranged throughout the parenchyma. Non-lymphoid tissues such as the skin and mucosal sites like the lung and gastrointestinal tract also contain scattered lymphocytes and APCs (66).

Based on the expression of defined surface antigens, the earliest immune cells to colonize the peripheral tissues appear to be “naïve” (67). Several populations of dendritic

cells (DCs) have been identified in the fetus, with many expressing high levels of MHC class I and II surface antigens (67,68). DCs and Langerhan's cells in the skin have been found to be fully competent at promoting activation of adult T cells in a mixed lymphoycte reaction (MLR) (67). Likewise fetal B cells have been shown to produce both IgM and IgG after stimulation *in vitro*, although the ability to generate other classes of antibodies appears restricted prior to birth (69). Large numbers of NK cells are found in different fetal tissues with many belonging to the CD16+CD56- "immature" class of NK cells (70). Evidence for NK cell cytotoxicity has been found during fetal development, suggesting that fetal NK cells are functional. Finally, the T cell compartment of the fetus is comprised of both CD4+ and CD8+ T cells, with the majority expressing CD45RA and other "naïve" T cell markers (71). There have been reports, however, of T cells bearing a more "activated" phenotype based on the expression of CD45RO, CD95, and CD25 (67). The fact that this population of cells expresses high levels of CD25 and is predominantly found in the CD4+ T cell compartment suggests that they may be T_{Reg} cells, although at the time this was not known.

Ontogeny of the Functional Adaptive Immune Response

The functionality of fetal lymphocytes has been a somewhat contentious issue, with a range of views existing in the literature. Modern medical texts support the view that fetal T cell responses are reduced or delayed compared to adults. This view is largely supported by the available literature concerning clinical outcomes following fetal or neonatal infections. However, the mechanisms underlying reduced fetal T cell immunity have not been fully established and it remains unknown whether this is due to intrinsic

deficits in T cell function or to extrinsic factors, including environmental differences or differences in APC function (72). As will be discussed later, there is strong evidence that T cell immunity can develop at early stages *in utero* under certain conditions, suggesting that fetal T cells themselves are more competent than often considered.

A great deal of research on the development of the immune system in humans has focused on the functionality and phenotype of cells in the umbilical cord blood (UCB) or neonatal blood. As a result, much of what is known about immunological competence during early stages of development does not actually concern the response of the fetal immune system as it exists during the earliest stages of hematopoiesis. Several important distinctions may account for differences between fetal and neonatal immune responses. First, unlike the fetus, the newborn is undergoing massive antigenic challenge as it enters a world that is no longer protected from microbial organisms, which rapidly colonize the tissues. Colonization of the neonate by foreign microbial organisms surely has profound impact on the phenotype and function of peripheral immune cells, making direct comparisons between neonatal and fetal immunity difficult (72, 73). Cord blood is likely to still be sterile, yet the peripheral immune cells in the circulation at this time have had many weeks to acclimate themselves to the host tissues, which may impact their phenotype and function as well. Hormonal changes are also likely to occur in the latter stages of fetal development leading up to birth, which may alter immune responses (74). Thus, while the study of neonatal immunity is particularly important for understanding neonatal infections and vaccination strategies, it may not have direct relevance to understanding immunological tolerance and the specific role that the fetal immune system plays during development.

Prior to the generation of antibodies specific for human T cell antigens, studies of fetal lymphocyte function were restricted to measuring proliferative responses and cytolytic T lymphocytes (CTL) responses to polyclonal stimulation, often in the form of an MLR or with non-specific activators such as phytohemagglutinin (PHA) (75,76). These early reports documented the appearance of responsive lymphocyte populations in fetal tissues as early as 5 g.w. (liver) (77) and within the thymus at 10-11 g.w. (78). Since no T or B cells are present in the periphery prior to 9-10 g.w. (79), it can be concluded that the earliest responses documented in the liver are not from lymphocytes. Nonetheless the seminal studies on fetal immune function concluded that immune responses were, for the most part, intact as early as the initial stages of immunological development. There is still some doubt about the potential of fetal T cells to mediate cytotoxicity (80). Some reports have shown reduced capacity for CTL generation *in vitro* (75), while others have provided evidence for the *in vivo* priming of CTL responses in the context of infection *in utero* (82-84). These discrepancies may be explained by critical differences in the nature of the stimulation used and environmental differences between *in vitro* culture systems and the lymphoid environment *in utero*. More recent evidence gained from studying the UCB blood of infants exposed to different pathogenic agents *in utero* has supported the view that CD8+ effector responses, including those mediated by CTL, can occur during fetal development (82).

One commonly held view about fetal and neonatal immunity is that T cell responses are biased towards humoral, or T helper type 2 responses (Th2), rather than T helper type 1 (Th1) immunity (85). Th2 responses are characterized by the secretion of IL-4, IL-5, and IL-13, which have all been implicated in the regulation of B cell

responses, whereas Th1 responses typically are characterized by the production of interferon-γ (IFNγ) and the generation of CTL, which are particularly important for viral immunity (86). The reason for the view that fetal and neonatal immunity are Th2 biased comes primarily from a landmark publication demonstrating that the reduced immunity observed in neonatal mice was associated with an absence of Th1 immune responses (87). At the time of this publication, Th2 immunity was considered by some to be associated with reduced inflammation and in some cases tolerance, perhaps accounting for the initial desire to examine Th2 immunity in the neonate (88). While there are many reports confirming a Th2 bias in neonatal humans, the only studies that have examined T helper cell priming in the fetus point to an absence of Th2 type cytokines and a robust predominance of IFN responses, suggesting a Th1 bias (89). Still, relatively little is known about the capacity for fetal T cells to generate distinct populations of effector T cells, suggesting more is needed to be done before any conclusions can be made regarding the Th1/Th2 bias of the fetus. One report has demonstrated that fetal T cells, like UCB T cells, do show a marked proliferative response to exogenous IL-4 *in vitro*, which may indicate that Th2 immunity may significantly alter fetal T cell function in a way that is distinct from that seen in adults (90).

Experimental and Clinical Evidence for Fetal Immune Function

As highlighted in the previous section, there is relatively little known about the requirements for fetal T cell activation and the function of fetal T cells in human beings. Perhaps the best evidence for fetal T cell involvement in the generation of immune responses comes from historical studies aimed at understanding broader questions about

fetal immune responses to different challenges at different gestational stages (65). Many of these studies were performed using sheep as a model organism since the development of the fetal immune system in sheep is more similar to that of the human than the development of the mouse immune system. Because these studies were carried out prior to the advent of methods for detecting specific cellular components of the immune response, the primary read out is largely restricted to pathological assessment of immunity. Nonetheless, the findings are particularly relevant to understanding the nature of fetal immunity as well as to the potential role of the fetal immune system in promoting tolerance.

Noted historian Arthur M. Silverstein performed the seminal studies aimed at understanding fetal immunity. In a series of reports entitled "Fetal response to antigenic stimulus I-IV," Silverstein and colleagues first addressed the competence of the fetal immune system (91-94). These studies were published shortly after the surge of studies aimed at defining the tolerogenic properties of the fetus prompted by Medawar's seminal discoveries, and challenged the widely held view that the fetus existed in an "immunologically null" state throughout development. Notably, several reports preceding this work had documented the appearance of different forms of immunity (delayed type hypersensitivity, anaphylaxis) in humans (95), guinea pigs (96), and in cattle (97) providing an impetus for further investigation into fetal immune function.

Silverstein's findings revealed a far more complex process of immunological development in the fetus than was previously assumed. Rather than existing in either an immunologically "null" state or a "mature" state, the fetal immune system appeared to gradually mature *in utero*. This was demonstrated when different forms of antigen were

found to elicit immune responses at different stages of development. For example, when bacteriophage ϕX174 was injected into fetal sheep prior to 40 days gestation, an antibody response could be measured whereas allograft rejection was not observed prior to 85 days gestation, and immunity to *Salmonella typhosa*, diptheria toxoid, and Bacillus Calmette-Guérin vaccine was not observed until well after birth (65). These differences may reflect several unique features of the fetal immune response that are discussed in more detail below.

One interesting aspect of the fetal immune response to allogeneic organ transplantation was that rejection appeared to occur in the absence of any overt signs of plasma cell generation or antibody production (92,93). Fetal B cell responses have been found to show significant differences from adult B cell responses, potentially reflecting differences in the types of B cell found in the fetus and adult (98). Since Silverstein's initial studies were performed prior to the discovery of discrete T cell subsets and the development of assays to measure T cell function, the detection of immune responses to antigens introduced during fetal development relied on the measurement of antibody responses. Thus, a failure to detect immunity could result from differences in the nature of the antibody response or specific differences with respect to T cell contributions in generating antibody responses. In mice, the B-1 subset of B cells has been found to be the primary subset present during fetal development and shows reduced affinity for many foreign antigens and a heightened sensitivity for immunoglobulins, self-antigens, and bacterial polysaccharides (98). While a human (or sheep) equivalent of B-1 B cells has not been fully demonstrated, it is likely that such a population exists and thus may contribute to alterations observed with respect to antibody responses across development.

Characterization of the ontogeny of the immune system in fetal sheep provides some additional clues about the differences in immune responses across fetal development. The thymus in the fetal lamb is first colonized at ~45 days gestation and evidence of peripheral T lymphocytes first appears shortly thereafter, at approximately 60 days gestation (99). In this time frame, allogeneic organ transplantation is fully permissible with complete tolerance observed (65,92,93). However, at ~75-80 days gestation there is a change that results in the onset of acute rejection. There is an appreciable increase in the numbers of circulating T cells at this time and, since acute rejection of organ transplants is known to be a T cell-mediated phenomenon, it seems reasonable to assume that the pool of fetal T cells is at least partially responsible for rejection. A similar window for graft acceptance in fetal sheep has been documented for allogeneic and xenogeneic HSC transplants (100). In humans, a specific role for T cells in mediating rejection of allogeneic HSC has been assumed, based on successful cases of intrauterine stem cell transplantation in the setting of severe-combined immunodeficiency diseases (SCID) where T cells, but not B or NK cells, are absent (101).

Another interesting finding regarding the fetal T cell repertoire was recently noted in studies of lymphocyte recirculation and lifespan in fetal sheep. Cahill and colleagues assessed T cell turnover during fetal development in comparison to neonatal or adult life by administering ^{3}H-thymidine intravenously and cannulating efferent lymphatic vessels to measure T cell turnover (102). High rates of T cell recirculation were noted as the majority of labeled cells fell off in a linear fashion during the 7-day cannulation period. In addition, the authors noted a fairly constant, low-level of detection of ^{3}H-thymidine after the rapid decrease (~12.5%) which was interpreted as representing the thymic output

of newly developed T cells. Together, these observations prompted the conclusion that fetal T cells are a long-lived population of cells that is rapidly recirculating throughout the fetal tissues. Additional experiments to address the rate of T cell turnover suggested that fetal T cells are not rapidly dividing. The most striking findings of this study were that the T cells present in the fetus were rapidly, and almost completely, replaced by newly formed T cells in the period shortly following birth. These newly formed cells showed high rates of turnover and greater retention in the peripheral lymphoid tissues suggesting that they may be functionally distinct from their fetal counterparts. While the authors could only speculate on the significance of this observation, they noted that it may reflect a specific role for fetal lymphocytes in establishing peripheral tolerance to self-antigens. This is consistent with findings in mice that demonstrated a particular role for neonatal T cell trafficking to the skin in the generation of peripheral immune tolerance (103).

Intrauterine infections also offer a clinical and experimental model for assessing immune function in the fetus. Passive infection of fetal mice with lymphocytic choriomeningitis virus (LCMV) by transplacental passage from the infected mother results in the absence of disease (11,65). As LCMV pathogenesis is likely due in part to the chronic nature of the infection and the inflammatory damage that results, tolerance may in fact provide protection against disease in the setting of LCMV infection. In fact, this was initially postulated by Burnet and Fenner, in their historic text, "*The Formation of Antibodies,*" published in 1949 (11). A similar scenario may exist in the setting of chronic HIV infection as well as other chronic viral infections such as hepatitis C (HCV) where inflammation has recently been recognized to contribute to disease pathogenesis

(104). In both HIV and HCV infection, the rate of intrauterine transmission is far lower than might be anticipated based on viral loads circulating in the mother during pregnancy, perhaps reflecting the protective nature of the fetal immune response (105,106). Evidence in support of this hypothesis can be found in studies demonstrating the presence of primed T cell populations in healthy, uninfected infants and children born to HIV+ mothers (107). Tolerance has also been reported as depletion of endogenous T_{Reg} cells can increase the frequency of cells responding to HIV+ antigens in exposed, uninfected infants (108). Most studies, however, have focused merely on determining whether evidence of antigenic priming can be found in newborn blood. This has now been well documented in many settings including congenital CMV infection, toxoplasmosis, and even in a setting of passive immunization with influenza vaccines (82-84, 109). Despite many clinical studies, there still remains very little known about the role of the fetal immune system in responding to and controlling infections. Furthermore, a specific role for tolerance to pathogens has not been elucidated at this time.

In the studies presented in this thesis, we have examined the functional properties of fetal lymphocytes from mid-gestation human fetuses. We have applied modern immunological techniques to revisit unanswered questions about the phenotypic and functional properties of T cells at the earliest stages of lymphocyte colonization of the human periphery. Finally, we have addressed the role of the fetal immune system in the generation of peripheral tolerance with a particular emphasis on the role of T_{Reg}. Our findings shed new light on the importance of the fetal immune system in promoting peripheral tolerance and offer new insights into how the fetal immune response may be used to promote tolerance to foreign antigens in human beings.

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CHAPTER 2

Regulation of T cell responses in the developing human fetus

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* co-first authors

Abstract

Although human T cells enter the peripheral lymphoid tissues early during fetal development, the adaptive immune system in the fetus has largely been regarded as functionally immature and unresponsive to stimulation. Here we show that depletion of fetal $CD4^+CD25^{high}$ T regulatory (T_{Reg}) cells, which are present at high frequency in fetal lymphoid tissues, results in vigorous T cell proliferation and cytokine production *in vitro*, even in the absence of exogenous stimulation. Analysis of $CD4^+$ and $CD8^+$ T cell populations revealed a large subset of cells that expressed the early activation antigen, CD69. We show that this population represents a subset of highly reactive fetal T cells actively suppressed by fetal $CD4^+CD25^{high}$ T_{Reg} cells during development. These findings indicate that fetal T cells are, in the absence of $CD4^+CD25^{high}$ T_{Reg} cells, highly responsive to stimulation and provide evidence for an important role for $CD4^+CD25^{high}$ T_{Reg} cells in controlling T cell responses *in utero*.

Introduction

Phenotypic and functional differences are known to exist between the fetal and the adult immune systems (1-6). Billingham, Brent, and Medawar initially advanced the concept that antigen presentation *in utero*, as opposed to antigen presentation in the adult, normally leads to tolerance instead of adaptive immunity (2). Thereafter, the notion that fetal immune responses are functionally distinct from adult immune responses has been widely accepted (1, 6). This view has since been modified by observations that the fetus can generate vigorous B and T cell responses to foreign antigens under certain circumstances, e.g., after transplacental spread of infectious agents (7-9). These observations, by extension, support the hypothesis that an active form of immune suppression, rather than a passive functional deficit, exists during fetal development.

$CD4^+CD25^{high}$ T_{Reg} cells suppress a broad spectrum of immune responses (10-14) and have recently been described to exist in the human fetus, both in the cord blood and in fetal lymphoid organs (15-18). In cord blood, the frequency of $CD4^+CD25^{high}$ T_{Reg} cells is higher (approximately 12% of $CD4^+$ T cells) at gestational week 25 than at birth, when it approximates that found in healthy adult peripheral blood (18). In the thymus, the frequency of $CD4^+CD25^{high}$ T_{Reg} cells is maintained at an intermediate level (mean 8% of CD4 single positive thymocytes) throughout gestation (16, 17). We hypothesized that fetal $CD4^+CD25^{high}$ T_{Reg} cells play an important role in maintaining a general state of immune suppression during fetal development. Our findings reveal that the frequency of $CD4^+CD25^{high}$ T_{Reg} cells is higher in fetal lymph nodes (LN) than that observed in adult LN and suggest that these $CD4^+CD25^{high}$ T_{Reg} cells play an important role in the suppression of fetal T cell responses during development.

Results

Comparison of fetal and adult $CD4^+CD25^{high}$ T_{Reg} cells in lymphoid tissues.

As the thymus becomes a functional organ of T cell production between the 7th and 16th gestational week (g.w.) of human development, $CD4^+$ and $CD8^+$ T cells move into the periphery (19). The frequency of $CD4^+CD25^{high}$ T cells was assessed in mesenteric lymph nodes (mLN) and in spleen at varying gestational ages. Extending the findings of recent reports (16, 17), a large fraction of $CD3^+CD4^+$ T cells in both organs expressed high levels of CD25 by g.w. 16 and 20 (Figs. 1a-b). Similar frequencies of $CD4^+CD25^{high}$ T cells could be detected in mLN at g.w. 12 (Fig. 1a), whereas no T cells could be detected at that time in the fetal spleen. Analysis of fetal thymus at time points from g.w. 12 through 20 revealed that high levels of CD25 were expressed by approximately 8% of $CD3^{high}CD4^+$ single positive thymocytes, similar to that previously reported in both fetal and neonatal thymus samples (16, 17). To assess the frequency of $CD4^+CD25^{high}$ T cells in fetal LN in comparison to adult LN, we performed a direct analysis of adult and fetal lymphoid tissues. The frequency of $CD4^+CD25^{high}$ T cells was lower in the adult mesenteric and inguinal LN (range 0.7-6.8%, n=4 donors, 12 total LN) compared to fetal mLN, and comparable to frequencies found in adult PBMC (Fig. 1a and 1b).

Scurfin, a transcription factor encoded by the *FOXP3* gene, is considered to be a specific marker for $CD4^+CD25^{high}$ T_{Reg} cells (20). As examined by immunohistochemistry and immunofluorescent microscopy, $scurfin^+$ cells were found to be scattered throughout the parenchyma of fetal mLN (Fig. 1c) as well as in the medulla

of the fetal thymus (data not shown). Scurfin^{+} cells could also be found in adult LN, albeit at a significantly lower frequency than in fetal mLN (Fig. 1d and 1e). In the fetal mLN, scurfin^{+} cells were CD4^{+} (Fig. 1f) but not CD8^{+} (Fig. 1g). An average of 87% of the fetal CD25^{+} cells were found to be scurfin^{+} (Fig. 1h), and occasional CD25^{+} scurfin^{-} cells (Fig. 1i, arrow) were also observed. Conversely, approximately 90% of the scurfin^{+} cells in fetal mLN were CD25^{+}, with relatively few CD25^{-} scurfin^{+} cells identified in each section analyzed (Fig. 1j, arrow). The presence of a small fraction of CD25^{-} scurfin^{+} cells in the fetal mLN is consistent with findings in mice, in which a small fraction of scurfin^{+} cells in the lymph nodes was found to not express CD25 (21). Flow cytometric detection of scurfin expression was performed on fetal mLN cells to confirm the frequencies of CD4^{+}CD25^{high} and CD4^{+} CD25^{-} T cells expressing scurfin. In accordance with the immunofluorescent microscopy results, the majority of scurfin^{+} cells were found to express CD25 (~80%), with a small fraction of CD25^{-} cells expressing low to moderate levels of scurfin (Fig. 1k). Additionally, intracellular staining by flow cytometry confirmed that scurfin expression was predominantly restricted to the CD4^{+} T cell compartment of the fetal mLN (data not shown).

To confirm that the fetal CD4^{+}CD25^{high} T cells were able to suppress T cell responses to a known antigen, functional studies were carried out *in vitro*. After stimulation of CD25^{-} cells (mLN-derived cells depleted of CD4^{+}CD25^{+} T cells) with Staphylococcus enterotoxin B (SEB), a large proportion of fetal CD4^{-}CD25^{-} and CD4^{+}CD25^{-} T cells produced IFN-□ (Fig. 1l, left and right panel, respectively). Cytokine production was suppressed upon admixture of autologous CD4^{+}CD25^{+} T cells at a ratio

Figure 1

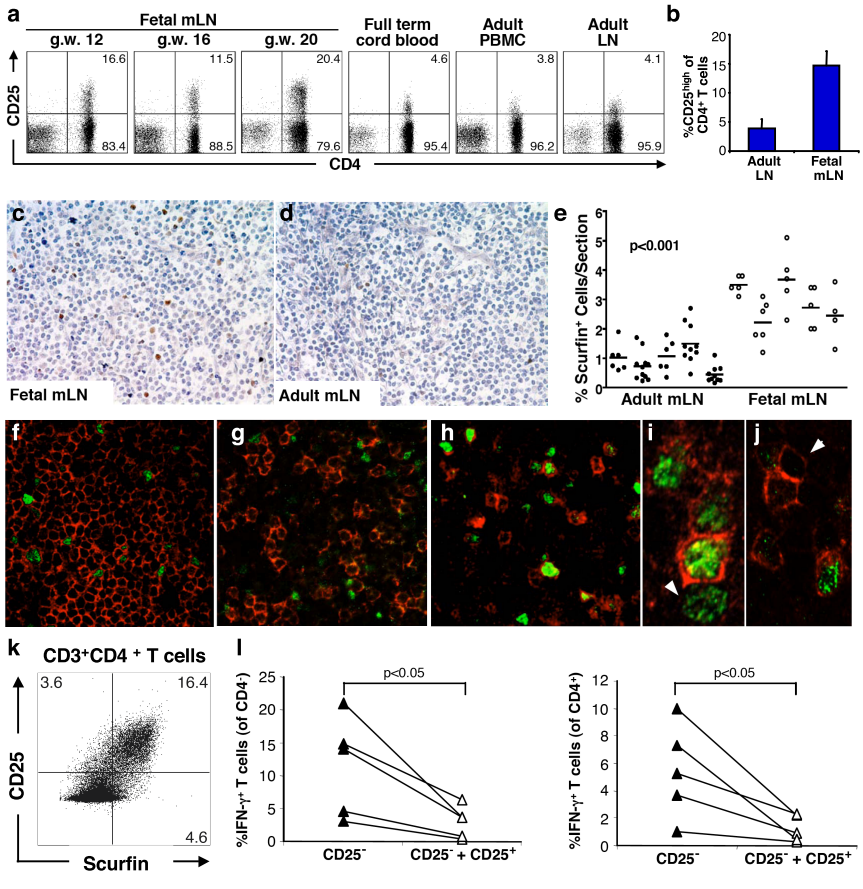

Figure 1. CD4+CD25high T cells are abundant in fetal but not adult lymphoid tissue. (a) Cell surface expression of CD25 on CD3+ T cells from fetal mLNs (mLN) (g.w. 12, 16, and 20), full-term cord blood, adult PBMC, and adult mesenteric LN, assessed by flow cytometry. The numbers in the upper right quandrant indicate %CD25high cells out of CD4+ T cells. (b) Mean percentage of CD25high cells of CD3+CD4+ T cells from adult mesenteric and inguinal LN (n=12, 4 donors) and fetal mLN (n=17, g.w. 20). Error bars indicate 1 std. dev.. (c) Fetal and (d) adult mLN stained with anti-scurfin antibody. Scurfin+ cells are dark in color (40x magnification). (e) Measurement of scurfin+ cells in sections of adult (black circles) and fetal (white circles) (g.w. 20) mLN stained with anti--scurfin antibody. Five adult donors and five fetal donors are shown. Each circle represents the frequency of scurfin+ cells from one 40x field such as those shown in (c) and (d). Horizontal bars indicate the mean frequency of all fields counted for each donor. The p-value in the graph indicates a statistically significant difference in the frequency of scurfin+ cells between adult and fetal LN, as tested by a Mann-Whitney rank-sum test. Immunofluorescent staining on fetal mLN (g.w. 20) for (f) CD4 (red) and scurfin (green) and (g) CD8 (red) and scurfin (green) reveals intracellular scurfin expression by CD4+ cells (63x magnification) but not by CD8+ T cells. (h) CD25 (red) co-localizes with scurfin (green) (63x magnification). (i) Example of a CD25+scurfin+ cell (arrow). (j) Example of a CD25-scurfin+ cell (arrow). (k) Scurfin and CD25 expression by CD3+CD4+ T cells isolated from 20 gw. fetal mLN measured by intracellular flow cytometry. (l) Expression of IFNg by CD4+ T cells (left panel) and CD4+ T cells (right panel) from fetal mLN depleted of CD25+ T cells (CD25+ cells) after stimulation with SEB. CD25+ cells stimualted alone (closed triangles) and CD25+ cells stimulated after admixture with autologous CD4+CD25+ T cells at a 1:3 ratio (open triangles, 10^5 CD4+CD25+ T cells mixed with $3x10^5$ CD25- cells). Statistical significance was tested with a two-tailed Student's paired t-test.

of 1:3 ($1x10^5$ $CD4^+CD25^+$ T cells and $3x10^5$ $CD25^-$ cells) (Fig. 1l), whereas addition of the same number of autologous $CD25^-$ cells had no effect (data not shown). The data above demonstrate that fetal mLN contain a large population of T cells that are phenotypically and functionally similar to "naturally occurring" $CD4^+CD25^{high}$ T_{Reg} cells. Interestingly, the frequency of such cells in the developing human fetus is much higher than that observed in the adult human.

Acquisition of effector function by fetal T cells in the absence of $CD4^+CD25^{high}$ T_{Reg} cells.

Several studies have proposed that changes in the frequency of T_{Reg} cells might play a determinant role in regulating the balance between activation and tolerance within a specific lymphoid environment (14, 22, 23). In tumor draining LN of adult humans, an increase in the frequency of $CD4^+CD25^{high}$ T_{Reg} cells correlates with a decrease in the capacity to mount a productive immune response (23). We reasoned that the high frequency of $CD4^+CD25^{high}$ T_{Reg} cells in fetal LN might establish an environment favoring suppression over activation and that removal of the $CD4^+CD25^{high}$ T_{Reg} cells *in vitro* might reveal potentially autoreactive T cells. To test this possibility, the proliferation of fetal mLN cells was measured using a CFSE dilution assay. In the presence of $CD4^+CD25^{high}$ T_{Reg} cells, unstimulated $CD4^+$ and $CD8^+$ mLN T cells proliferated only to a small extent (Figs. 2a, upper and lower panels, respectively). After depletion of $CD4^+CD25^{high}$ T_{Reg} cells, however, $CD4^+$ and $CD8^+$ T cells in cultures of mLN cells proliferated vigorously (Fig. 2b, upper and lower panels, respectively). By contrast, no $CD4^+$ or $CD8^+$ T cell proliferation could be detected in unstimulated cultures of adult LN, either before or after depletion of $CD4^+CD25^{high}$ T cells (Fig. 2c and 2d,

Figure 2

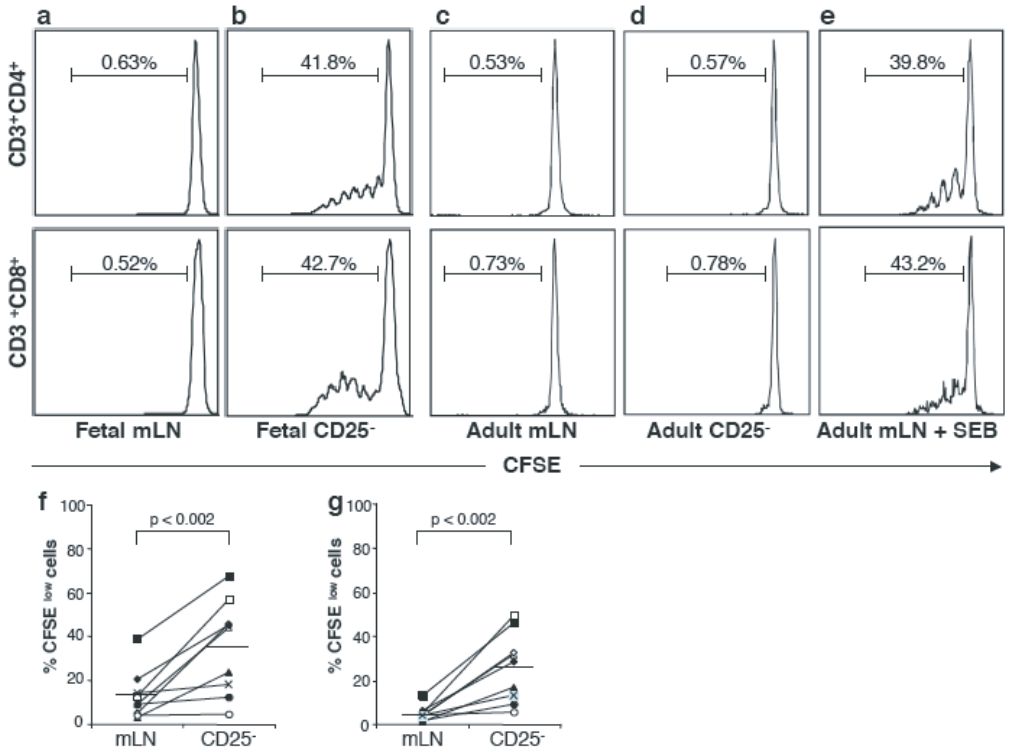

Figure 2. Proliferation of fetal CD25- T cells in the absence of exogenous stimulation is controlled by fetal CD4+CD25high TReg cells. Proliferation of CFSE-labeled fetal CD4+ T cells (top panel) and CD8+ Tcells (bottom panel) in unstimulated cultures of (a) unseparated fetal mLN cells, (b) fetal mLN depleted of CD25+ cells, (c) unseparated adult mLN cells, and (d) adult mLN cells depleted of CD25+ cells. (e) Adult mLN cells stimualted with SEB. The data shown in c-e are representative of four separate experiments involving 13 lymph nodes (4 mesenteric, 9 inguinal LNs) from four different healthy donors. (f) Frequency of CFSElow CD4+ T cells in unstimulated cultures of unseparated fetal mLN cells (left) and mLN cells depleted of CD25+ cells (right). (g) Frequency of CFSElow CD8+ T cells in unstimulated cultures of unseparated fetal mLN cells (left) and mLN cells depleted of CD25+ cells (right). Results from 8 different experiments involving 8 different donors are shown. The difference in T cell proliferation between unseparated mLN and mLN depleted of CD25+ cells was statistically significant, as tested by a student's paired t-test.

upper and lower panels, respectively). As expected, $CD4^+$ and $CD8^+$ T cells in cultures of adult LNs proliferated vigorously in response to SEB (Fig. 2e, upper and lower panel, respectively), demonstrating that the adult T cells were capable of proliferating in response to a known antigen. A summary of the frequency of dividing fetal $CD4^+$ and $CD8^+$ T cells ($CFSE^{low}$ cells) in the presence or absence of $CD4^+CD25^{high}$ T_{Reg} cells from eight different donors is depicted in Figs. 2f and 2g, respectively. These data indicate that fetal $CD4^+CD25^{high}$ T_{Reg} cells suppress the proliferation of both $CD4^+$ and $CD8^+$ T cells in cultures of unstimulated fetal mLN cells.

Fetal T cell proliferation in the absence of $CD4^+CD25^{high}$ T_{Reg} cells is not dependent on IL-2 or IL-7.

The proliferation of fetal mLN T cells observed in the absence of $CD4^+CD25^{high}$ T_{Reg} cells could be driven by cytokine-mediated homeostatic mechanisms. T cells adoptively transferred into neonatal lymphopenic mice undergo multiple rounds of division and gain the capacity to become effector cells upon antigen stimulation in an IL-7 dependent manner (24), and IL-7 and IL-2 are known to be regulators of neonatal T cell homeostasis and proliferation (25). Most fetal T cells in mLNs, with the exception of the $CD4^+CD25^{high}$ T_{Reg} cells (data not shown), were found to express high levels of the high affinity IL-7Rα (CD127). To determine whether IL-2 and/or IL-7 were responsible for the observed proliferation in cultures of unstimulated fetal mLN T cells depleted of $CD4^+CD25^{high}$ T_{Reg} cells, neutralizing antibodies to each cytokine were added to the assay. Compared to the levels of $CD4^+$ and $CD8^+$ T cell proliferation observed after depletion of $CD4^+CD25^{high}$ T cells ($CD25^-$ control) (Fig. 3b, upper and lower panels,

Figure 3

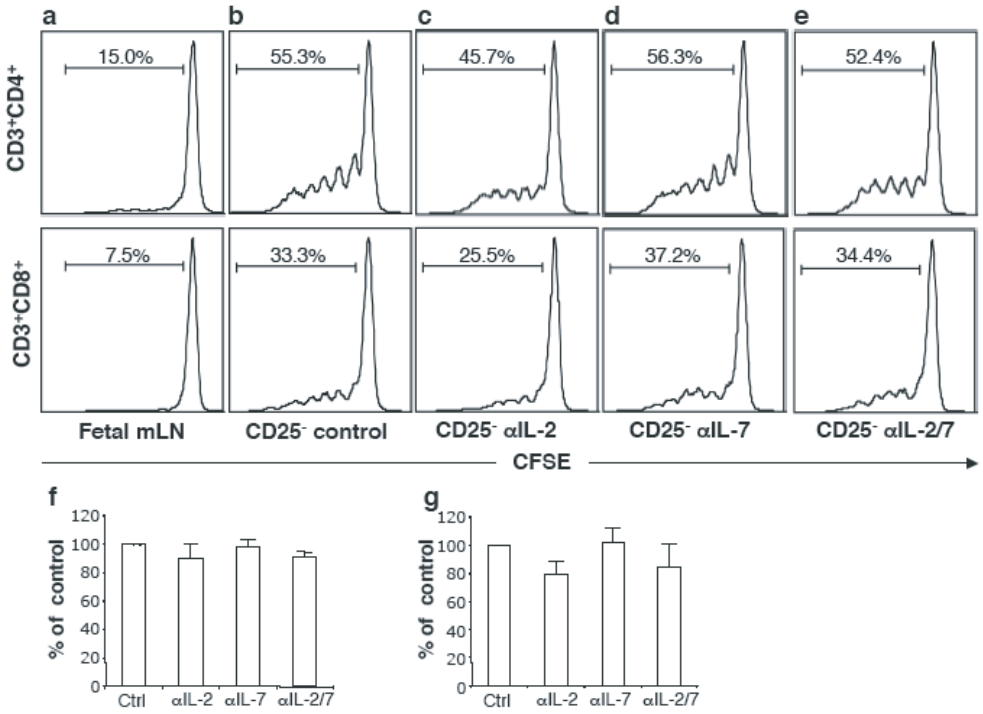

Figure 3: Proliferation of CD25- T cells in the absence of exogenous stimulation is independent of IL-2 and IL-7. Proliferation of CFSE-labeled CD4+ T cells (top panels) and CD8+ T cells (bottom panels) measured in cultures of (a) unseparated fetal mLN cells, (b) mLN cells depleted of CD4+CD25+ T cells (CD25- cells) without neutralizing mAbs (control), (c) CD25- cells with anti-IL-2, (d) CD25- cells with anti-IL-7, and (e) CD25- cells with a combination of anti-IL-2 and anti-IL-7 mAbs. The cells were cultured for 4 days in R15 medium alone. The antibodies were added to the cultures at the start of the assay at a concentration of 5 ug/ml. (f) Proliferation of CD4+ T cells and (g) CD8+ T cells expressed as percent of proliferation in cultures of fetal mLN depleted of CD25+ cells (Ctrl) after addition of antibodies to IL-2 and IL-7, either alone or in combination. The average of three experiments is shown. Error bars indicate one standard deviation.

respectively), the addition of neutralizing antibodies to IL-2 had only a minor effect in reducing the proliferation of both $CD4^+$ and $CD8^+$ T cells (Fig. 3c, upper and lower panels, respectively) and the addition of neutralizing antibodies to IL-7 had no effect (Fig. 3d, upper and lower panels, respectively). $CD4^+$ and $CD8^+$ T cell proliferation in cultures with both anti-IL-2 and anti-IL-7 antibodies was similar to that observed in cultures with anti-IL-2 alone (Fig. 3e, upper and lower panels, respectively). The effect of IL-2 and IL-7 neutralization on $CD4^+$ and $CD8^+$ T cell proliferation in three independent experiments is summarized in Figs. 3f and 3g, respectively. Proliferation is depicted as a percentage of the proliferation seen in cultures of $CD25^-$ fetal mLN cells without addition of antibodies (control). Our data suggest that IL-2 and IL-7 induced homeostatic mechanisms are not responsible for the proliferation of fetal $CD4^+$ and $CD8^+$ T cells following depletion of the $CD4^+CD25^{high}$ T_{Reg} cells from fetal mLN cultures.

A population of fetal T cells expressing CD69 proliferates and secretes IFN-γ in the absence of $CD4^+CD25^{high}$ T_{Reg} cells.

If fetal mLN T cells are not proliferating as a result of homeostatic cytokine-driven mechanisms, it is possible that they are being activated by antigens present in the periphery of the developing fetus. In mice, for instance, there are reports of increased frequencies of T cells bearing autoreactive TCRs during the initial period of T cell colonization of the peripheral lymphoid tissues (26). This time frame is comparable to the 2^{nd} trimester of human fetal development, when T cell colonization of the periphery occurs (5, 19). To determine whether a population of autoreactive human T cells might exist *in utero*, we first examined mLN and splenic T cell populations for the expression

of CD69, a cell surface protein transiently upregulated within hours of T cell receptor stimulation. Expression of CD69 was consistently detected on a subset of $CD25^{-}CD4^{+}$ and $CD25^{-}CD8^{+}$ T cells examined directly *ex vivo* (Fig. 4a), both in mLN (left panels) and in spleen (right panels). To test the possibility that the $CD69^{+}$ T cell population represented T cells subject to regulation by fetal $CD4^{+}CD25^{high}$ T_{Reg} cells, the proliferation of unstimulated fetal $CD4^{+}$ and $CD8^{+}$ T cells was measured in cultures of unseparated mLN cells (Fig. 4b, upper and lower panels, respectively), in cultures of mLN cells depleted of $CD4^{+}CD25^{high}$ T_{Reg} cells ($CD25^{-}$ cells, Fig. 4c, upper and lower panels, respectively), and in cultures of mLN cells depleted of both $CD4^{+}CD25^{high}$ T_{Reg} cells and $CD25^{-}CD69^{+}$ cells ($CD25^{-}CD69^{-}$ cells, Fig. 4d, upper and lower panels, respectively). As shown in previous figures (Fig. 2 and Fig. 3), depletion of $CD4^{+}CD25^{high}$ T_{Reg} cells from cultures of fetal mLN cells resulted in proliferation of a substantial fraction of both $CD4^{+}$ and $CD8^{+}$ T cells (Fig. 4c). When $CD25^{-}CD69^{+}$ T cells were removed from the cultures of $CD25^{-}$ fetal mLN cells, the levels of T cell proliferation (Fig. 4d) decreased to those seen in whole mLN cultures containing $CD4^{+}CD25^{high}$ T_{Reg} cells (Fig. 4b). This observation suggests that it is the $CD25^{-}CD69^{+}$ T cell population that is normally suppressed by $CD4^{+}CD25^{high}$ T_{Reg} cells. This notion is further supported by the vigorous proliferation of $CD25^{-}CD69^{+}$ T cells observed in experiments where sorted, CFSE-labeled $CD25^{-}CD69^{+}$ cells were admixed with unlabeled $CD25^{-}CD69^{-}$ cells at a 1:1 ratio (Fig. 4e).

Figure 4

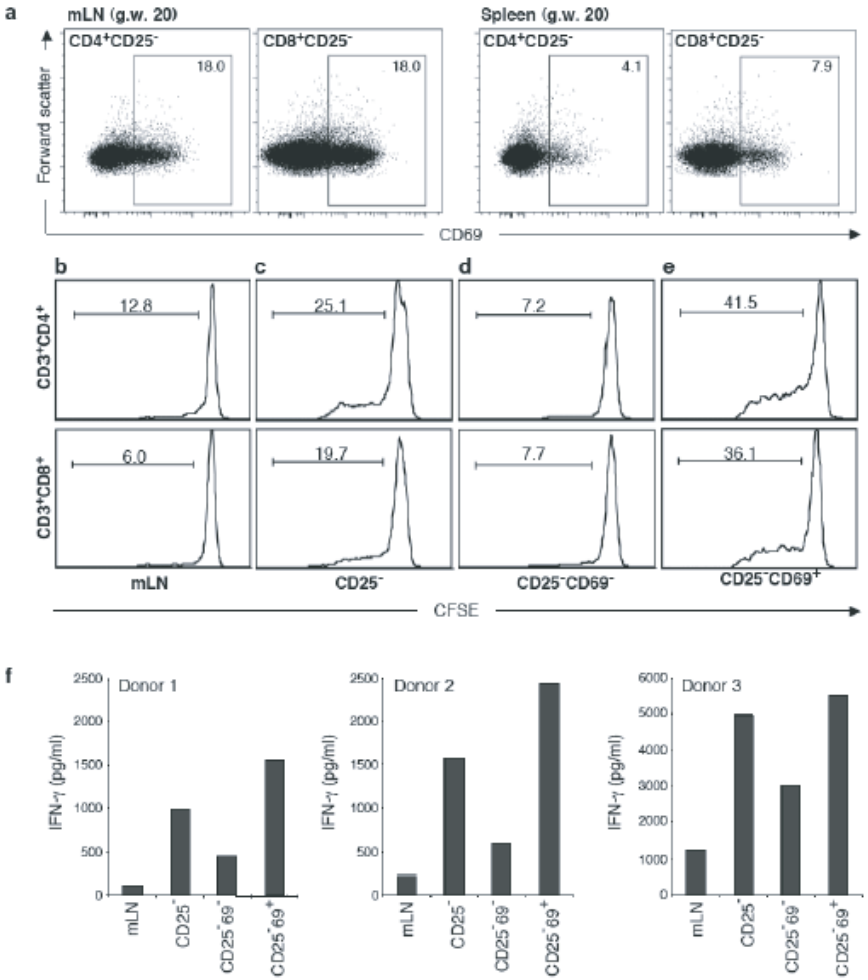

Figure 4: Fetal CD69+CD25- T cells proliferate and produce IFN-gamma in the absence of CD4+CD25high TReg cells. (a) Subpopulations of CD4+CD25- and CD8+CD25- T cells in fetal mLN (left panels) and fetal spleen (right panels) express the early T cell activation marker CD69. Cells shown are gated on CD3+CD25- cells and the numbers in the gate indicate the percentage of CD69+ cells. (b-e) Proliferation of CD4+ (upper panels) and CD8+ (lower panels) T cells was measured in CFSE dilution assays using flow cytometry after 4 days of culture in medium alone. The numbers above each bar indicate the percentage of CFSElow (divided) cells. Data from one representative donor of three are shown. (b) Proliferation of CFSE-labeled T cells in cultures of unseparated mLN cells. (c) Proliferation of CFSE-labeled T cells in cultures of CD25- cells. (d) Proliferation of CFSE-labeled T cells in cultures of mLN cells depleted of CD4+CD25high T cells and CD25-CD69+ cells (CD25-CD69- cells). (e) Proliferation of CFSE-labeled T cells in cultures of CD25-CD69+ cells mixed with unlabeled CD25-CD69- cells at a 1:1 ratio. Unlabeled CD25-CD69- cells were excluded from the analysis based on their lack of CFSE. (f) IFN-gamma concentration measured in the supernatant of unstimulated cultures of unseparated mLN cells, CD25- cells, CD25-CD69- cells and CD25-CD69+ cells mixed with CD25-CD69- cells at a 1:1 ratio. The supernatants were collected after 4 days of culture. Three individual donors are shown.

These data indicate that the $CD4^+CD25^-CD69^+$ T cells represent a population of T cells that has been activated in response to antigens present in the fetal tissues *in utero,* and that this population is actively suppressed from proliferating by the large fraction of $CD4^+CD25^{high}$ T_{Reg} cells present in fetal lymphoid tissues. Importantly, we could not detect similar proliferation in the absence of exogenous stimulation in cultures of adult LNs depleted of $CD4^+CD25^{high}$ T cells, despite the presence of a large fraction of $CD69^+$ T cells in adult LNs (Figs. 2c-d and data not shown). The frequency of $CD4^+$ and $CD8^+$ T cells expressing CD69, like the frequency of fetal $CD4^+CD25^{high}$ T_{Reg} cells, was fairly consistent from donor to donor, implying that the underlying mechanism(s) accounting for the apparent activation of these cells are not likely attributable to aberrant antigen exposure within a given fetus.

Although CD69 is generally considered to be associated with TCR stimulation and early events leading to T cell activation, it is possible that IL-2 and IL-7 independent events not associated with the acquisition of T cell effector functions may trigger proliferation of the $CD25^-CD69^+$ T cell subset. To test this possibility, we measured secreted IFN-γ concentrations in the supernatants of 4-day cultures of unseparated fetal mLN, $CD25^-$ cells, $CD25^-CD69^-$ cells, and $CD25^-CD69^+$ T cells mixed with $CD25^-CD69^-$ cells at a ratio of 1:1 (Fig. 4f). In three separate donors, the concentration of IFN-γ was low in cultures of unseparated fetal mLN cells and substantially increased upon removal of $CD4^+CD25^{high}$ T_{Reg} cells. IL-10 was measured simultaneously and was consistently detected only at very low levels in cultures of mLN, with only small increases upon depletion of $CD4^+CD25^{high}$ T_{Reg} cells (data not shown). Depletion of

$CD69^+$ cells from the $CD25^-$ cells resulted in a decrease in IFN-γ secretion while re-addition of $CD25^-CD69^+$ cells with $CD25^-CD69^-$ cells at a 1:1 ratio led to an increase in IFN-γ secretion to levels greater than those seen in cultures of $CD25^-$ cells (Fig. 4f). These findings demonstrate that the large population of fetal $CD4^+$ and $CD8^+$ T cells that is activated *in utero* acquires effector functions (e.g., IFN-γ secretion) after removal of fetal $CD4^+CD25^{high}$ T_{Reg} cells *in vitro*.

Both $CD69^+$ and $CD69^-$ $CD4^+CD25^{high}$ T_{Reg} cells suppress T cell proliferation.

To investigate differences in the activation status of $CD4^+CD25^{high}$ T_{Reg} cells in relation to their suppressive function, we measured expression of CD69 and GITR (a candidate phenotypic marker of T_{Reg} cells) by flow cytometry. Approximately half of the $CD4^+CD25^{high}$ T_{Reg} cells were found to express CD69 and expression of GITR was found almost exclusively within the $CD69^+$ fraction of fetal $CD4^+CD25^{high}$ T cells (Fig. 5a, left panel). As indicated by the lower expression levels of CD45RA and CCR7, these $CD4^+CD25^{high}CD69^+$ T cells (Fig. 5a, lower, right panel) have a memory/effector phenotype, consistent with the possibility that they have been activated *in utero*. It was recently suggested that $CD4^+CD25^{high}$ T_{Reg} cells acquire suppressive function against autoantigens after activation in the periphery, as measured by CD69 and GITR expression (16). This is an attractive hypothesis and is consistent with the above data (Fig. 4) showing that CD69 expression on $CD25^-$ T cells reflects their ability to proliferate and to secrete cytokines such as IFN-□. To determine whether both the $CD69^+$ and the $CD69^-$ $CD4^+CD25^{high}$ T_{Reg} cells could suppress T cell responses, the ability of

Figure 5

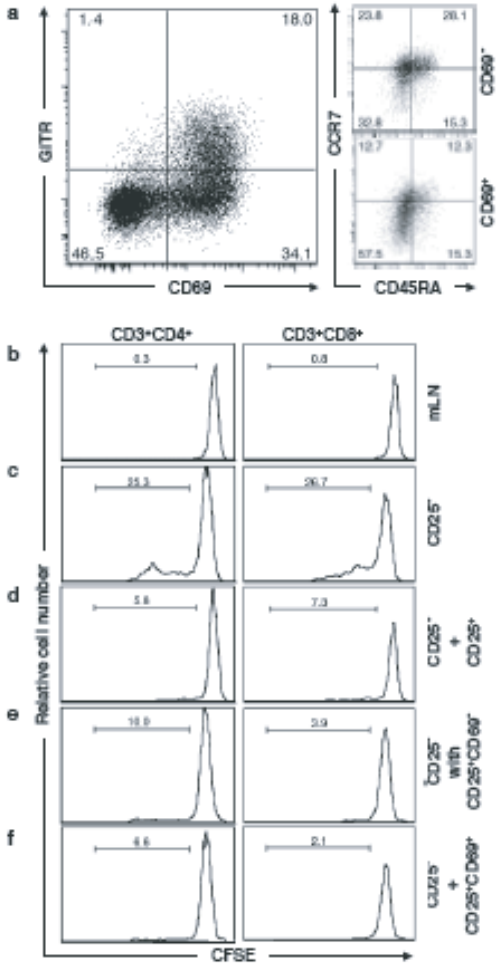

Figure 5: Both CD69+ and CD69- CD4+CD25high TReg cells suppress T cell proliferation. (a) Freshly isolated fetal mLN cells (g.w. 20) stained with anti-GITR and anti-CD69 antibodies. Cells shown are gated on CD3+CD4+CD25high cells within the lymphocyte gate. GITR is almost exclusively expressed by CD4+CD25+CD69+ T cells (left panel). CD3+CD4+CD25highCD69- cells (upper right panel) express higher levels of CD45RA and CCR7 compared to CD3+CD4+CD25highCD69+ cells (lower right panel). (b-e) Proliferation of CD4+ T cells (left panels) and CD8+ T cells (right panels) was measured in CFSE dilution assays. Data from one representative donor are shown. (b) Proliferation of CFSE-labeled T cells in cultures of unseparated mLN cells. (c) Proliferation of CFSE-labeled T cells in cultures of mLN cells depleted of all CD4+CD25high T cells (CD25- cells). (d) Proliferation of CFSE-labeled T cells in cultures of CD25- cells depleted of CD4+CD25highCD69+ T cells (CD25- with CD25+CD69-). (e) Proliferation of CFSE-labeled T cells in cultures of CD25- cells with CD4+CD25highCD69+ T cells added back at a 1:4 ratio. (f) Average frequency of CFSElow CD4+ T cells (left panel) and CD8+ T cells (right panel) in cultures of CD25- mLN cells, in cultures of CD25- mLN cells with CD4+CD25highCD69- T cells and in cultures of CD25- mLN cells with CD4+CD25highCD69+ T cells. Three independent experiments with three different donors were included in the analysis. No statistically significant difference in suppression by CD69- and CD69+ CD4+CD25high T cells could be detected.

each population to suppress spontaneous proliferation of fetal mLN T cells *in vitro* was tested. In cultures of mLN cells depleted of the $CD69^{+}CD4^{+}CD25^{high}$ T_{Reg} cells only ($CD25^{-}$ with $CD25^{+}CD69^{-}$; Fig. 5e), proliferation was similar to that observed in cultures of unseparated mLN cells (Fig. 5b). Conversely, addition of the sorted $CD69^{+}CD4^{+}CD25^{high}$ T_{Reg} cells suppressed proliferation of $CD25^{-}$ T cells to a level similar to that observed in cultures of unseparated mLN cells (Fig. 5f). Thus, the expression of CD69, and hence GITR, did not correlate with enhanced suppressive activity, as both $CD69^{-}$ and $CD69^{+}$ $CD4^{+}CD25^{high}$ T_{Reg} cells suppressed fetal T cell proliferation to a similar extent (Fig. 5e-f).

DISCUSSION

We show here that human fetal T cells exist in a dynamic balance between activation and quiescence, and not in a passive state of inactivity. Further, our data indicate that this balance is regulated by the presence of a large population of $CD4^{+}CD25^{high}$ T_{Reg} cells, as removal of this population resulted in substantial T cell proliferation and IFN-γ production. A majority of the T cells that spontaneously underwent proliferation and produced IFN-γ upon removal of the $CD4^{+}CD25^{high}$ T_{Reg} cells could be identified directly *ex vivo* by the expression of the early activation marker, CD69. These findings indicate that fetal T cells are, in the absence of $CD4^{+}CD25^{high}$ T_{Reg} cells, highly responsive to stimulation and provide evidence for an important role for $CD4^{+}CD25^{high}$ T_{Reg} cells in maintaining peripheral T cell tolerance *in utero*.

The conclusions of this report are corroborated by clinical findings from patients with the inherited disease IPEX (immunodysregulation, polyendocrinopathy, enteropathy,

X-linked syndrome) who have mutations in the *FOXP3* gene and, therefore, are thought to have deficiencies in $CD4^{+}CD25^{high}$ T_{Reg} cell development and/or function (27). Severe cases of IPEX have been described in which the disease manifests itself *in utero,* causing the affected newborn to present with insulin-dependent diabetes and an inability to tolerate oral feeding due to severe chronic inflammation of the gastrointestinal tract at the time of birth (28). In conjunction with these clinical findings, our data suggest that the elevated frequencies of $CD4^{+}CD25^{high}$ T_{Reg} cells observed in the fetal periphery may play an essential role in the regulation of potentially devastating activation of self-reactive T cells during fetal development. Since only a subset of patients diagnosed with IPEX show severe signs of autoimmunity at birth, it seems likely that both genetic differences and environmental differences might play an important role in determining the temporal progression and severity of autoimmune disease seen in these patients (29).

Our finding that depletion of $CD4^{+}CD25^{high}$ T_{Reg} cells from adult mLN cultures did not lead to T cell proliferation, as seen after depletion of $CD4^{+}CD25^{high}$ T_{Reg} cells from fetal mLN cultures, suggests that the fetal and adult adaptive immune systems exhibit significant differences with respect to the requirements for maintenance of peripheral tolerance. The high levels of proliferation and IFN-γ production observed in the absence of $CD4^{+}CD25^{high}$ T_{Reg} cells in fetal mLN cultures reveal an increased frequency of potentially autoreactive T cells during fetal development, as has been reported in mice (26). Such an increase could be explained by several different mechanisms including: incomplete central tolerance during early thymic development (26), a competitive advantage for self-reactive T cells in the fetal periphery due to lymphopenia (30), or intrinsic differences in activation requirements/thresholds of fetal

and adult T cells. Another important consideration is that the human fetus may be exposed to maternal alloantigens during development via cross-placental trafficking of maternal cells or proteins. In mice and humans, there is now evidence to suggest that maternal $CD4^{+}CD25^{high}$ T_{Reg} cells play an important role in controlling maternal alloreactivity to the developing fetus (22). A better appreciation of the immunological environment during fetal ontogeny should benefit future efforts to understand the development of autoimmune diseases and the establishment of peripheral tolerance in humans.

Methods

Fetal tissue and adult blood: Fetal tissue (mesentery, spleen, and thymus) and matched maternal blood was obtained from Advanced Bioscience Resources (Alameda, CA). Adult blood from healthy individuals was collected in heparinized vacutainer tubes (BD Biosciences, Franklin Lakes, NJ) after informed consent. Fresh LNs from adults were obtained from the National Disease Research Interchange (NDRI). Paraffin-embedded adult LNs were verified (by Dr. Walter E. Finkbeiner of the Department of Pathology, UCSF, San Francisco General Hospital) to be non-inflamed and non-cancerous by hematoxylin and eosin staining. Cord blood was obtained from the Department of Obstetrics, Gynecology, and Reproductive Sciences at San Francisco General Hospital, after informed consent from women giving birth to full term babies. Mesenteric lymph nodes were dissected from the fetal mesentery. LN, thymus, and spleen were incubated with 0.2 mg/ml collagenase B (Roche Diagnostics, Alameda, CA) in R15 medium [RPMI 1640 (MediaTech, Herndon, VA) supplemented with 15% fetal calf serum (FCS)

(Gemini BioProducts, Woodland, CA), 2 mM L-glutamine, 10 mM Hepes, and 100 U/ml penicillin/streptomycin (Invitrogen, Grand Island NY)] for 1h at 37ºC and processed into single cell suspensions by passing the cells through a 40 μM cell strainer (BD Falcon, Bedford, CA). PBMC from adult and full-term cord blood were isolated using Ficoll-Hypaque PLUS (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation and washed in R15 medium prior to phenotypic analysis or functional assays. When necessary, red blood cells were lysed by incubation with ACK lysis buffer (Quality Biological, Gaithersburg, MD). In some cases, fetal mLNs and thymus were frozen or formalin fixed (10% Neutral Buffered Formalin) and embedded in paraffin for tissue sections (see below).

Antibodies and flow cytometry: For phenotypic analysis, blocking, and cell sorting by flow cytometry, the following antibodies were used: anti-CD3-FITC, anti-CD4-PerCP, anti-CD4-Pacific Blue, anti-CD4-PE, anti-CD25-PE, anti-CD25-APC (clone M-A251), anti-CD8-APC, anti-CD8-Pacific Blue, anti-CTLA-4-PE, anti-CD45RA-FITC, anti-CCR7-PE-Cy7, anti-IFN-□-APC, (all from BD Biosciences, San Diego, CA), purified anti-IL-2 (clone 5334.21), purified anti-IL-7 (clone 7417.111), anti-GITR-FITC (R&D Systems, Minneapolis, MN), anti-CD69-APC, anti-CD4-PE-Cy7 (CalTag, Burlingame, CA) and anti-CD127-PE (Beckman Coulter, Miami, FL). For phenotyping, the cells were incubated with the relevant antibodies diluted in PBS-1% BSA for 30 min on ice, followed by two washes with PBS-1% BSA, and then fixed in 1% paraformaldehyde. The panel of antibodies used for phenotyping in Fig. 1a was anti-CD3-FITC, anti-CD25-APC, and anti-CD4-PerCP. Anti-TCR V□ antibodies were from Beckman Coulter Biotech. Intracellular detection of scurfin was performed using anti-FoxP3-APC (clone

PCH101) and the accompanying staining kit provided by eBioscience, in accordance with the manufacturer's protocol (eBioscience, San Diego CA, USA). In all assays with more than 4 colors, anti-mouse Ig compensation particles (BD CompBeads, BD Biosciences) single stained with each of the antibodies were used as compensation controls for software based compensation using FlowJo software (Tree Star). For 4-color flow cytometry, the samples were analyzed on FACSCalibur (BD Biosciences). For 5-8-color flow cytometry, we analyzed samples on a LSR II flow cytometer (BD Biosciences) modified from the standard configuration by the addition of a 150 mW green (532 nm) diode laser, and the upgrade of the blue and red lasers to 100 mW and 25 mW respectively. The green diode was used for the excitation of all the PE tandem conjugates. All data were analyzed using FlowJo software (Tree Star).

Tissue sections and confocal microscopy: Fetal thymus or mLN were frozen in Tissue Tek OCT (Sakura Finetek, Torrance, CA) and sectioned into 6-8 μm thick sections for immunofluorescent staining. For all immunohistochemistry, fetal and adult mLN were first fixed in 10% buffered formalin and embedded in paraffin. For immunofluorescence (IF), the antibodies used were mouse anti-human CD4 (Dako Cytomation, Carpinteria, CA), mouse anti-human CD8 (DakoCytomation), mouse anti-human CD25 (Novus Biologicals, Littleton, CO), and goat anti-human FoxP3 (scurfin) (AbCam, Cambridge, MA). Immunohistochemical detection of scurfin was performed using a rabbit anti-human scurfin polyclonal antibody (AbCam). For IF microscopy, sections were fixed in cold acetone and blocked with TRIS buffered saline (TBS pH 8.5) containing 5% human AB serum. The sections were then incubated for 1 hour with combinations of mouse anti-human CD4 (1:200 dilution), mouse anti-human CD8 (1:200 dilution) or mouse anti-

human CD25 (1:200 dilution), and goat anti-human scurfin (1:50 dilution) diluted in TBS. The sections were washed in TBS/Tween for 5 minutes and incubated with anti-mouse Ig Cy3 (1:500 dilution, Jackson ImmunoLabs, West Grove, PA) and anti-goat Alexa488 (1:200 dilution, Molecular Probes, Eugene, OR) for an additional 45 minutes. The slides were mounted with Gel/Mount (Biomedia Corp., Foster City, CA) and analyzed using a Zeiss LSM5100 confocal microscope. Data were processed with the Zeiss LSM software package. Formalin fixed, paraffin-embedded tissues were cut into 5 μm thick sections and de-paraffinized with Histosol followed by rehydration in graded ethanol. The sections were incubated with hydrogen peroxide (3% in PBS) followed by a protein block (Dako Cytomation) for 30 minutes at room temperature. The sections were incubated overnight at 4^{o} C with a rabbit anti-scurfin polyclonal antibody (1:2000 dilution). After primary incubation, the sections were washed in TBS/Tween (0.5% Tween) and incubated for 45 minutes with a mouse anti-rabbit secondary antibody (1:200 dilution, Dako Cytomation) followed by washing with TBS/Tween and a 45-minute incubation with streptavidin-conjugated horse radish peroxidase (1:200 dilution, Dako Cytomation). The sections were developed with DAB (Dako Cytomation) and counterstained with Gil's hematoxylin (Sigma) followed by a final dehydration step and mounted for analysis. $Scurfin^{+}$ cell frequencies were assessed by counting 40x magnification images onto which a grid was applied and comparing cells staining positive for scurfin as a fraction of total cells per image.

Separation of $CD25^{+}$ T cells by magnetic beads: Magnetic beads were used for separation of $CD25^{+}$ and $CD25^{-}$ cells in the experiments shown in Figs. 1- 3. $CD25^{+}$ T cells were separated from $CD25^{-}$ cells by MACS CD25 microbeads (Miltenyi Biotech,

Auburn, CA), according to the manufacturer's instructions. Typically, the $CD25^+$ fraction contained >90% $CD4^+$ T cells, of which >85% were $CD25^{high}$. Staining by intracellular flow cytometry for scurfin demonstrated that <1% of the remaining $CD4^+$ T cells were $CD25^+scurfin^+$.

Sorting by flow cytometry: Sorting by FACS was used for experiments shown in Figs. 4 and 5. mLN from g.w. 20 were processed as described above to obtain a single cell suspension and stained with anti-CD25-PE, anti-CD69-APC, and anti-CD4-PE-Cy7. Sorting was performed on a BD DIVA flow cytometer. A lymphocyte gate was set on the basis of forward and side scatter. To control for potential non-specific effects of sorting, 10^6 cells were sorted based on the lymphocyte gate alone ("unseparated mLN cells"). Total mLN cells were sorted to deplete $CD4^+CD25^{high}$ cells from total mLN cells (termed $CD25^-$ cells). Similarly, total mLN cells were sorted to isolate $CD4^+CD25^{high}CD69^+$ cells from the remaining mLN cells (termed $CD25^-$ with $CD25^+CD69^-$ cells). Finally, $CD4^+CD25^{high}$ cells and $CD25^-CD69^+$ were separated from total mLN cells, and the remaining cells (termed $CD25^-CD69^-$ cells) were collected.

Cytokine flow cytometry: Cells from mLN depleted of $CD25^+$ cells ($CD25^-$ cells) by MACS beads (see above) were labeled with 0.5 μM CFSE (Molecular Probes). The CFSE labeled $CD25^-$ cells, alone or admixed with non-labeled $CD4^+CD25^+$ T cells or non-labeled $CD25^-$ cells, were stimulated with 5 μg/ml Staphylococcal enterotoxin B (SEB) (Sigma-Aldrich) or with R15 medium alone, and then incubated for 24 h. Brefeldin A (Sigma-Aldrich) was added at a final concentration of 5 μg/ml for the last 6 h of incubation. After stimulation the cells were harvested, incubated with anti-CD4-PE

(BD BioSciences), washed twice, fixed in 1% paraformaldehyde, and permeabilized with FACS Permeabilizing Solution (BD BioSciences) for 20 min prior to being stained with anti-CD3-PerCP and anti-IFN-□-APC (BD BioSciences), and then analyzed on a FACSCalibur flow cytometer (BD Biosciences). In the analysis of IFN-γ producing cells, we gated on the $CFSE^+$ cells, in order to exclude cells added back to the assay from the analysis.

Proliferation assays: In the analysis of proliferation in the absence of exogenous stimulation (Figs. 2-5), the responder cells (unseparated mLN, $CD25^-$ cells, $CD25^-CD69^-$ cells and $CD25^-CD69^+$ cells) were labeled with 1 μM CFSE. For unseparated mLN cells, $CD25^-$ cells and $CD25^-CD69^-$ cells, $3x10^5$ responder cells were cultured in 96-well U-bottom plates in R15 medium. To analyze the proliferation of $CD25^-CD69^+$ cells (Fig. 4e), $2x10^5$ CFSE labeled $CD25^-CD69^+$ cells were cultured with $2x10^5$ non-labeled $CD25^-CD69^-$ cells. After 4 days of culture, cells and supernatants (the supernatants were analyzed for IFN-□ concentration, as described in the section below) were harvested. The harvested cells were stained with anti-CD3-ECD, anti-CD4-PE-Cy7, anti-CD8-Pacific Blue, and ethidium monoazide (EMA) (Molecular Probes). EMA was cross-linked to DNA in dead cells by 8 minutes light exposure, prior to washing in MACS buffer, fixation in 1% paraformaldehyde and analysis on a LSR II flow cytometer (BD Biosciences). BD CompBeads single-stained with the respective antibodies and cells single-stained with EMA and CFSE were used for software-based compensation with FlowJo software (Tree Star). All analyses were performed using FlowJo software (Tree Star). Non-labeled cells added back to the assays were excluded from the analysis on the

basis of lack of CFSE fluorescence. The frequency of $CFSE^{low}$ cells was used as a measurement of total T cell proliferation. In proliferation assays using anti-IL-7 and anti-IL-2 antibodies (Fig. 3), CFSE-labeled mLN cells depleted of $CD4^{+}CD25^{+}$ T cells by magnetic beads ($CD25^{-}$ cells) were cultured for 4 days in R15 medium alone or together with neutralizing antibodies recognizing IL-2 and IL-7. The antibodies were added to the cultures at the start of the assay both separately and in combination at a concentration of 5 μg/ml. The concentration used for each neutralizing antibody in these assays was roughly 5-50x higher than that required for complete neutralization of recombinant IL-7 and IL-2 as described in the manufacturer's protocols. Proliferation was analyzed as described above.

Measurement of IFN-□ concentration in culture supernatants.

Cell culture supernatants from the proliferation assays in Fig. 4 were collected after 4 days of culture. The IFN-□ concentration was measured using cytokine bead arrays (BD Biosciences), according to the instructions from the manufacturer. Data were collected on a FACSCalibur and analyzed using FlowJo software (Treestar).

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CHAPTER 3

Maternal Alloantigens Promote the Development of Tolerogenic Fetal Regulatory T Cells *In Utero*

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Abstract

Although the developing fetal immune system can be tolerized against alloantigens, the mechanism(s) underlying such tolerance remain unclear. Here, we show that FoxP3 is induced in a large fraction of human fetal T cells responding in vitro to allogeneic cells. Amongst these T cells are $CD4^{+}CD25^{high}FoxP3^{+}$ regulatory T cells (T_{Reg}) that suppress responses to unshared maternal alloantigens. We provide evidence that such T_{Reg} are likely induced by the presence of maternal cells in the fetus that persist in the circulation after birth in most, if not all, individuals. Our findings suggest a mechanistic basis for understanding fetal immunity in humans and establish a conceptual framework for clinical strategies aimed at generating tolerance to foreign antigens *in utero*.

Over fifty years have passed since Billingham, Brent, and Medawar first advanced the concept that "actively acquired immunologic tolerance" in the mouse occurs as a result of fetal exposure to foreign antigens (1). There have since been numerous reports suggesting that the transfer of foreign antigens (including proteins, parasites, and even cells) from the mother to the fetus is a common occurrence, not an anomaly (2, 3, 4). In light of these findings, a closer examination of how the fetal immune system recognizes and responds to such antigens is warranted.

Temporal differences in the development of the adaptive immune system vary significantly between species (5). Newborn mice show few signs of peripheral T cell colonization (6) whereas, in the human fetus, peripheral lymphoid tissues are populated by T cells as early as 10 gestational weeks (g.w.) (7). It is accordingly not clear whether *in utero* tolerance induction would occur upon fetal exposure to foreign antigens in the human as it does in the mouse (8). In fact, not much is known about the functional properties of the fetal human immune system: some reports suggest that it is functionally deficient while others indicate that fetal immune responses to pathogens and vaccines are intact (9-12). In two independent clinical studies (13, 14), specific tolerance towards non-inherited maternal HLA antigens (NIMA) was observed in organ transplant recipients, consistent with the possibility that fetal exposure to NIMA may promote lasting tolerance in humans (1).

In certain circumstances, e.g., severe combined immunodeficiency disease (15), maternal cells cross the placenta and engraft into human fetal tissues *in utero*, resulting in "maternal microchimerism" (4). Because the human fetal immune system may be

functionally responsive against NIMA during the first trimester, we wished to understand whether such microchimerism was the exception or the norm. Lymph nodes (LN) were isolated from the mesentery of 18 fetal products of conception (POC) at 18-22 g.w. and analyzed for the presence of maternal DNA (16). Maternal microchimerism was observed in 15/18 LN samples (Table 1, Fig. S1), with a frequency (0.003-0.83%) comparable to that reported in non-lymphoid organs from human fetal specimens, neonates, and healthy adults (17, 18). Analysis of the cellular composition of maternal cells in neonatal cord blood revealed a predominance of hematopoietic cells (Fig. S2).

To determine whether human fetal T cells are responsive against alloantigens, fetal (~20 g.w.) lymphocytes from spleen or LN (Figs. 1A, S3) were labeled with the dye carboxy-fluorescein diacetate succinimidyl ester (CFSE) and co-cultured with irradiated antigen presenting cells (APCs) from the peripheral blood of a single healthy adult donor (19). After five days in this mixed leukocyte reaction (MLR), substantial proliferative responses were observed for both $CD4^+$ and $CD8^+$ fetal T cells (Fig. 1A), prompting the question: if fetal T cells respond so vigorously against alloantigens in vitro, would they not also respond against NIMA expressed by maternal cells that have moved into fetal LNs *in utero*?

We recently reported that, compared to adults, elevated frequencies of regulatory T cells (T_{Reg}) are found in human secondary fetal lymphoid tissues (20). Because T_{Reg} are known to regulate maternal immunity to fetal alloantigens (21), we reasoned that fetal T_{Reg} may suppress fetal immune responses against invading maternal cells. Depletion of T_{Reg} resulted in a highly significant increase in proliferation of fetal T cells against maternal APCs, but only a slight increase against unrelated APCs (Fig. 1B-D). An

Figure 1

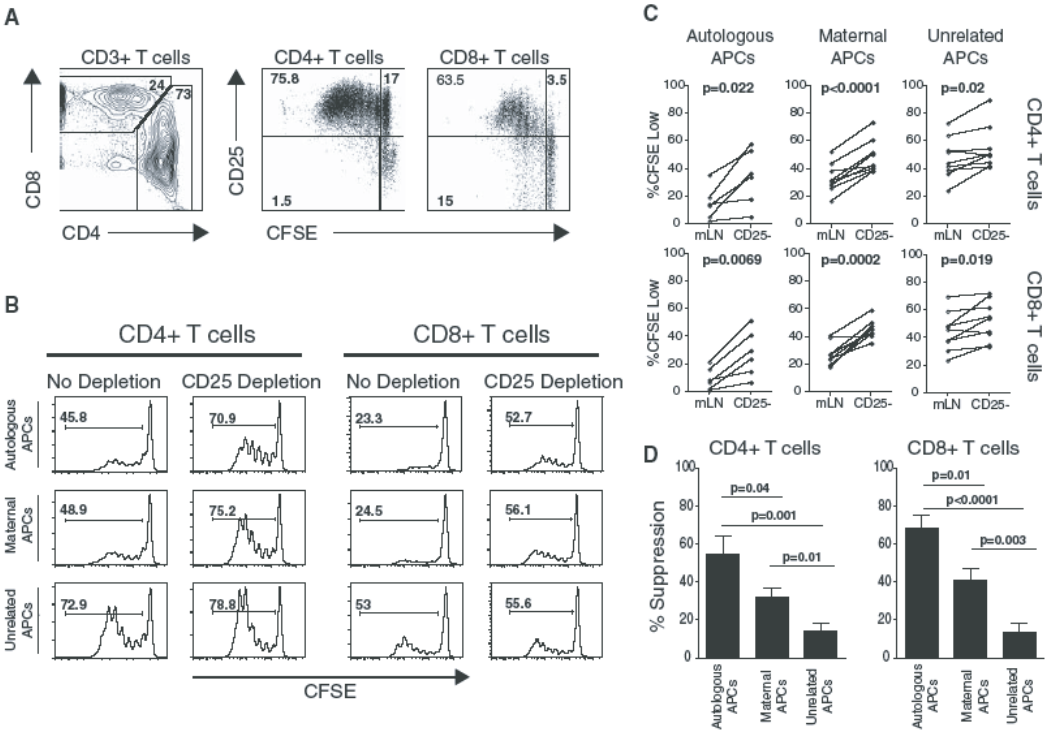

Fig. 1. Fetal T_{regs} suppress fetal T cell responses to maternal alloantigens. (**A**) Fetal T cell proliferation after stimulation with allogeneic APCs from an unrelated donor for 5 days (3:1 ratio of fetal lymphocytes:allogeneic APCs). (**B**) Proliferative responses to autologous, maternal, or unrelated APCs after a 5-day MLR. Histograms depict proliferation in the presence (no depletion) or absence (CD25 depletion) of fetal T_{regs}. CFSE, carboxy-fluorescein diacetate succinimidyl ester. (**C**) Summary of all experiments addressing fetal T cell proliferative responses to autologous ($n = 6$), maternal ($n = 9$), or unrelated ($n = 9$) APCs in the presence or absence of fetal T_{regs}. Statistical significance was determined by paired Student's t test. mLN, mesenteric lymph nodes. (**D**) Comparison of T_{regs} suppression against autologous, maternal, or unrelated APCs.

increase in proliferation was also noted when autologous APCs were used as stimulators, suggesting that T cell responses to "self" antigens are suppressed by fetal T_{Reg}. These data indicate that fetal T cells are not inherently deficient at responding to maternal alloantigens; rather, their function is actively suppressed by a large pool of fetal T_{Reg}.

Natural T_{Reg} originate in the thymus and are specific for "self" antigens presented by thymic epithelial cells (22). We found no difference in the frequency of $CD4^+$ $FoxP3^+$ T_{Reg} between the fetal and infant thymus (Fig. S4). By contrast, the frequency of T_{Reg} in peripheral lymphoid organs changes markedly during the course of gestation, falling from ~15-20% of total $CD4^+$ T cells at 12-20 g.w. to ~3-7% at birth (23). We reasoned that this change in frequency might reflect a greater propensity of naïve fetal T cells to differentiate into T_{Reg} in response to stimulation. To test this, we depleted fetal LNs (and spleen; Fig. S5A) of $CD25^+$ T_{Reg} and stimulated the remaining cells with irradiated APCs from an unrelated donor. Following a five-day culture period, many of the dividing fetal T cells had upregulated FoxP3 (Fig. 2A). To further characterize the kinetics of this response, a parallel analysis of FoxP3 upregulation was performed following stimulation of fetal or adult T cells (depleted of T_{Reg}) with a single unrelated donor (Figs. 2B, S5, S6). While both fetal and adult T cells displayed similar patterns of activation during the first 2 days of stimulation (Fig S5, S6), fetal T cells showed greater signs of activation thereafter (Figs. 2B, S5, S6). Sustained expression of FoxP3 is a necessary feature of $CD4^+CD25^{hi}$ T_{Reg} in both mice and humans. While adult T cells can express FoxP3 following stimulation (22), we found that most activated adult T cells were $CD25^+FoxP3^-$ after 6 days of stimulation. Expression of FoxP3 by fetal T cells was maintained over the

course of the stimulation period, with ~50% of fetal CD4+ T cells expressing both CD25 and FoxP3 by day 6 (Fig. 2B).

Environmental cues play a central role in determining T cell differentiation pathways during an immune response. To address whether fetal lymphoid tissues are enriched for cytokines that might favor T_{Reg} differentiation during T cell activation, cytokine gene expression patterns were evaluated in fetal (n=5) and adult (n=4) LNs (Fig. 2C). As anticipated, adult LNs had elevated levels of interleukins and interferons compared with fetal LNs. Analysis of TNF family members revealed more variable expression patterns, with transcripts for some genes (e.g., OX40L, CD70, and APRIL) being higher in adult LNs while others (e.g., LTα, LTβ, TRANCE) were more highly expressed in fetal LNs. TNF family members more highly expressed by fetal LNs were predominantly those important for LN organogenesis in mice (e.g., LTα, LTβ, TRANCE, and TNFα) (24). Interestingly, many TGFβ family members were more highly expressed in fetal LNs, including bone morphogenic proteins (BMP) 1-5, 7, and 8B, growth and differentiation factors (GDF) 2, 3, 10, and 11, as well as TGFα and TGFβs. High expression of various TGFβ family members in developing LNs is consistent with the role that members of this family play in embryonic developmental pathways (25). TGFβ signaling is known to induce FoxP3 upregulation during T cell activation and to be critical for the differentiation of T_{Reg} during an immune response. To test whether TGFβ signaling was required for FoxP3 upregulation during fetal T cell responses, we measured FoxP3 expression in a 5-day MLR in the presence or absence of a TGFβ inhibitor (SB 431542) and found that, indeed, inhibition of TGFβ signaling resulted in a large reduction in FoxP3 upregulation by fetal T cells (Fig. 2D).

Figure 2

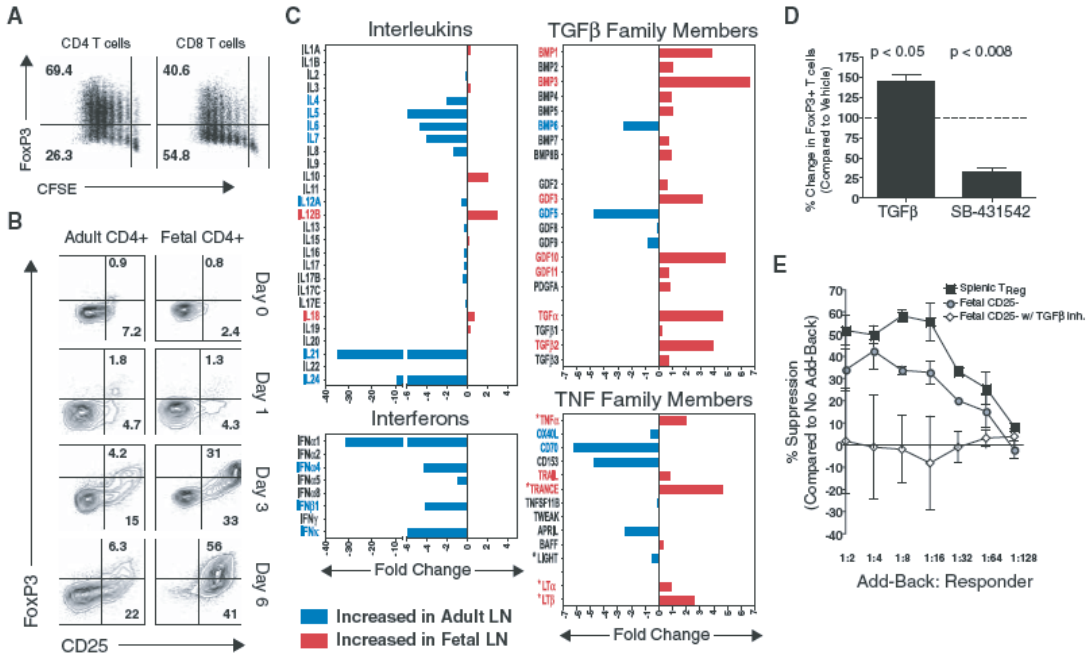

Fig. 2. Fetal T cells differentiate into T_{regs} upon stimulation with alloantigens. (**A**) Fetal T cells depleted of CD25+FoxP3+ cells were stimulated for 5 days with unrelated APCs, and FoxP3 expression was measured in proliferating T cells. (**B**) Kinetic analysis of CD25 and FoxP3 up-regulation by adult and fetal CD4+ T cells after stimulation with alloantigens. (**C**) Fold difference in cytokine mRNA expression in normal adult ($n = 4$) and fetal ($n = 5$) LNs. Genes found to be significantly different are labeled in red (fetal) or blue (adult) ($P < 0.05$, unpaired Student's t test). Asterisks denote TNF family members involved in organogenesis. (**D**) Inhibition of TGFβ signaling by addition of the activin receptor–like kinase inhibitor, SB-431542 (1 uM), which blocks FoxP3 up-regulation by fetal T cells stimulated with unrelated APCs. (**E**) TGFβ-dependent acquisition of suppressive function after stimulation of fetal T cells with alloantigens. Error bars indicate SD observed in three separate experiments.

Since FoxP3 can be induced in some activated T cells that are not functionally suppressive (22), we determined whether FoxP3 expression following fetal T cell activation was associated with the acquisition of suppressive function. Primary MLRs were performed with fetal CD25-depleted LN cells, with or without TGFβ inhibition; endogenous splenic $CD25^{hi}$ T_{Reg} served as a positive control. After a 7-day culture period, fetal CD25-depleted LN cells and splenic $CD25^{hi}$ T_{Reg} were tested for function in a conventional add-back assay. Fetal LN cells which had upregulated FoxP3 following stimulation with adult allogeneic APCs were found to be functionally suppressive whereas those activated in the presence of the TGFβ inhibitor lacked the ability to suppress T cell proliferation (Figs. 2E, S7). While the endogenous pool of fetal splenic $CD4^{+}CD25^{hi}$ T_{Reg} appeared to have increased suppressive function (Fig. 2E), this may reflect a greater percentage of $FoxP3^{+}$ cells within this population: ~30-50% of $CD4^{+}$ T cells from fetal LN had upregulated $FoxP3^{+}$ after 7 days of stimulation in vitro whereas ~50-80% of splenic "endogenous" T_{Reg} were $FoxP3^{+}$ after 7 days in culture. (Fig. S7B).

The above studies indicate that suppressive $FoxP3^{+}$ T_{Reg} are generated against NIMA *in utero*. Given previous reports indicating that NIMAs are better tolerated than non-inherited paternal alloantigens in the setting of adult solid organ transplantation (13, 14), we tested the possibility that $FoxP3^{+}$ T_{Reg} generated against NIMA *in utero* might persist after birth. MLRs were performed utilizing lymphocytes of children (ages 7-17 years old) to measure T cell proliferation in response to maternal or paternal alloantigens, with or without prior depletion of T_{Reg} (Figs. 3A-C). Some children demonstrated T_{Reg} suppression against maternal alloantigens but not against paternal alloantigens (Figs. 3B, C) or autologous APCs (Fig. S8). A parallel analysis of maternal and paternal T cell

Figure 3

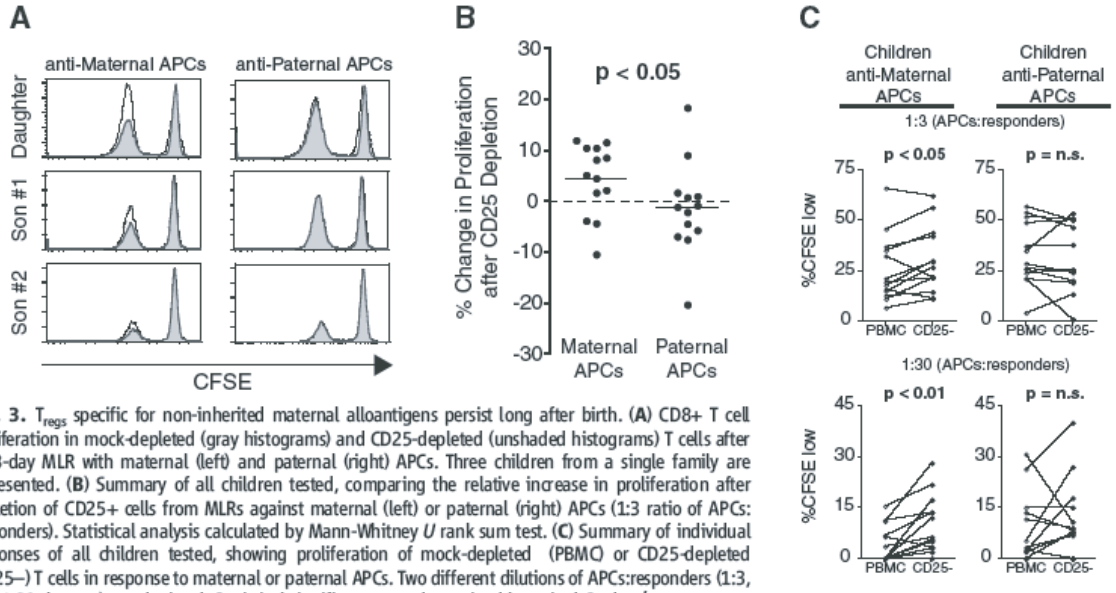

Fig. 3. T_{regs} specific for non-inherited maternal alloantigens persist long after birth. (**A**) CD8+ T cell proliferation in mock-depleted (gray histograms) and CD25-depleted (unshaded histograms) T cells after an 8-day MLR with maternal (left) and paternal (right) APCs. Three children from a single family are represented. (**B**) Summary of all children tested, comparing the relative increase in proliferation after depletion of CD25+ cells from MLRs against maternal (left) or paternal (right) APCs (1:3 ratio of APCs: responders). Statistical analysis calculated by Mann-Whitney *U* rank sum test. (**C**) Summary of individual responses of all children tested, showing proliferation of mock-depleted (PBMC) or CD25-depleted (CD25−) T cells in response to maternal or paternal APCs. Two different dilutions of APCs:responders (1:3, top; 1:30, bottom) are depicted. Statistical significance was determined by paired Student's *t* test.

responses directed against their childrens' alloantigens revealed that maternal T_{Reg} with specificity for their childrens' alloantigens persist long after birth as well (Fig. S8H). These preliminary findings indicate that T cell tolerance to alloantigens perceived *in utero* may, in some cases, be maintained after birth through the establishment of long-lived T_{Reg}, as has been reported for B cell tolerance (13).

It has long been recognized that central deletion of autoreactive T cell clones is an important mechanism for generating immunological tolerance. Here, we provide evidence that the fetal peripheral adaptive immune system can rapidly generate functionally suppressive T_{Reg}, providing another mechanism by which the fetus can establish tolerance to foreign and self-antigen present during development *in utero*. While this study has focused on fetal tolerance to maternal alloantigens, there is no reason to believe a priori that the fetal immune system would respond differently to other antigens encountered *in utero*, including "self" antigens, food antigens, and antigens associated with infectious agents carried by the mother. Further investigation into these areas is likely to provide important insights about the treatment of fetal disease, the development of tolerance to self and foreign antigens in humans, the establishment of strategies to induce antigen-specific tolerance during fetal development, and the pathogenesis of maternal-to-child transmission of pathogens, such as HIV, *in utero*.

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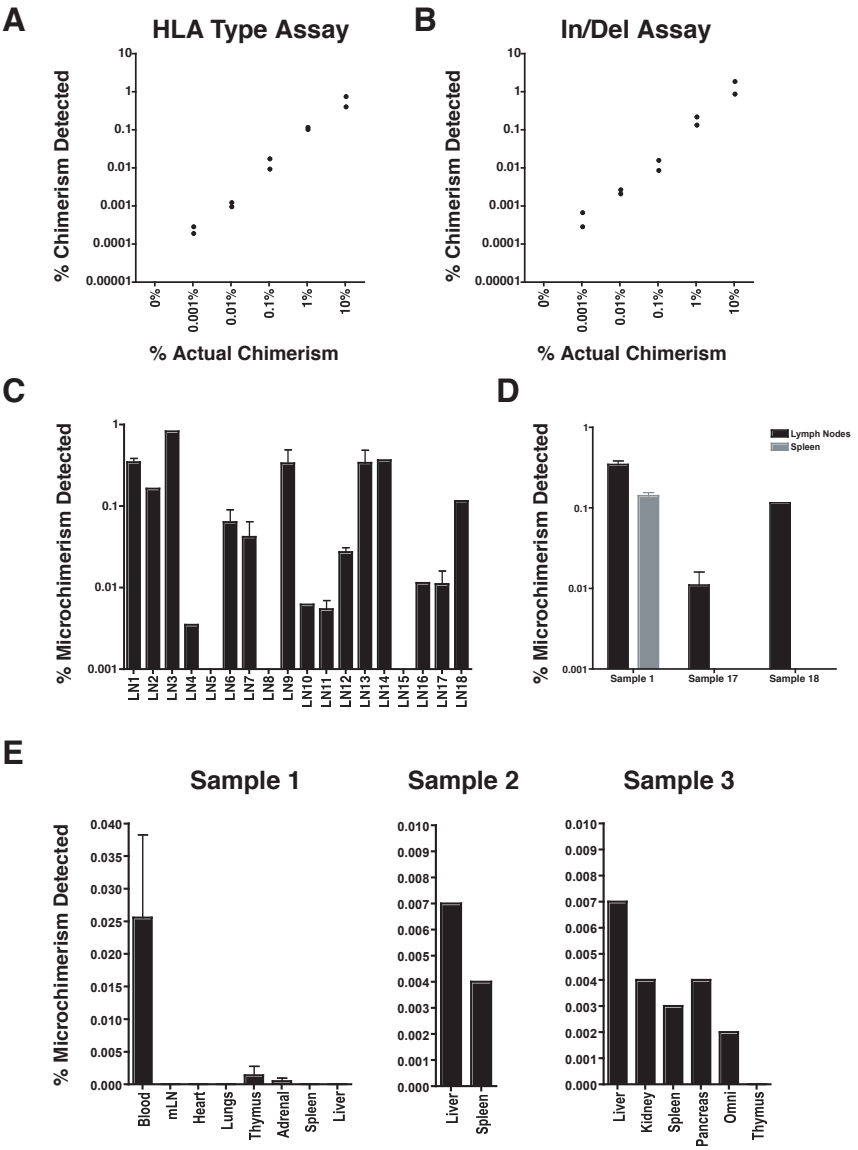
Table 1. Maternal Microchimerism in Fetal Lymph Nodes

Fetal mesenteric lymph nodes (18-22 g.w.) were analyzed for levels of maternal microchimerism using two separate assays (16). Informative HLA types and/or insertion/deletion polymorphisms are listed for each donor. "None" refers to situations where no informative HLA type or polymorphisms were identified.

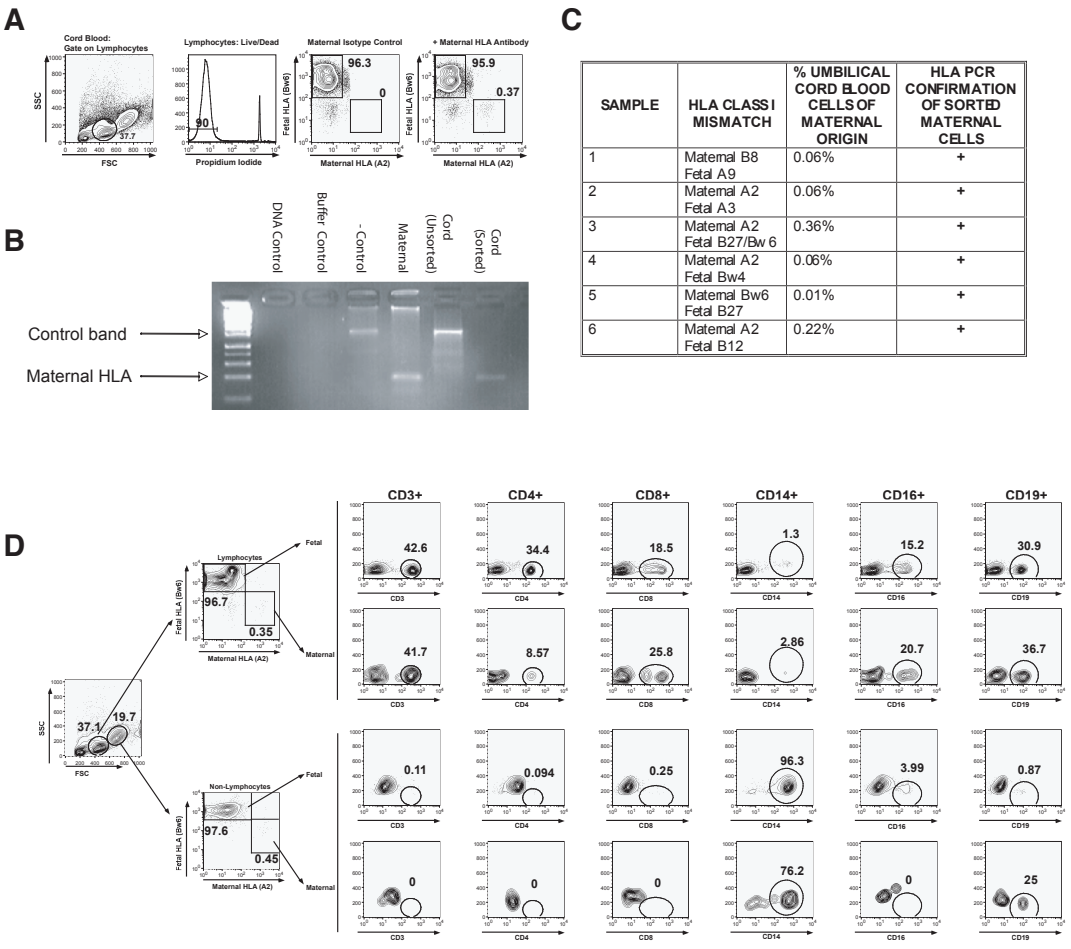
Sample Number	HLA Type/ In-Del Marker	% Microchimerism (HLA Type)	% Microchimerism (In/Del)
1	DR13/SO10	0.3860%	0.3080%
2	None/SO3		0.1640%
3	DR11/None	0.8260%	
4	DR4/None	0.0035%	
5	None/None		
6	DR9/SO7B	0.0370%	0.0906%
7	DR1/SO6	0.0650%	0.0190%
8	DR13/None	Neg.	
9	DR7/SO8	0.1780%	0.4934%
10	SO6/None	0.0062%	
11	None/ SO9		0.0070%
	SO10		0.0039%
12	DR1/SO4B	0.0312%	0.0234%
13	None/ SO9		0.4869%
	SO11		0.1933%
14	DR1/None	0.3663%	
15	DR15/SO3	Neg.	Neg.
16	DR11/None	0.0114%	
17	DR15/SO3	0.0161%	0.006%
18	DR15/None	0.1158%	

Fig. S1



Supplementary figure 1. Detection of maternal microchimerism in the human fetus. Microchimerism assays were tested for accuracy by performing blinded experiments to detect the presence of chimerism in PBMCs (Donor A) spiked with PBMCs from an unrelated donor (Donor B) at concentrations ranging from 0.001% - 10%. Both (**A**) HLA-based and (**B**) Insertion/Deletion-based (In/Del) detection assays showed highly reproducible results. Note that both methods underestimate the actual level of chimerism, indicating that the actual level of microchimerism in the fetal samples may be higher. Points represent two separate samples of PBMCs from Donor A spiked with identical numbers of PBMCs from Donor B. Total numbers of cells used for testing microchimerism assays were consistent with the numbers of cells tested for fetal donors (~2-3 million cells). (**C**) Graphical representation of data from Table 1 in the main text (LN5 had no informative alelle and LN8 and LN15 did not have detectable levels of chimerism). Error bars represent range of chimerism detected using HLA- or In/Del-based assays. (**D**) Analysis of maternal microchimerism in paired spleens and LNs from three donors. Only one spleen showed detectable levels of microchimerism, which was considerably lower than that detected in LNs. The LNs are less likely to come in contact with maternal cells during tissue harvesting since they are embedded in layers of connective tissue (omentum) within the mesentery. The experiment thus suggests that detection of microchimerism in LNs is not due to maternal contamination during the harvesting of the fetal tissues. (**E**) Organs from three intact products of conception were dissected and tested for the presence of maternal microchimerism. All three samples had detectable microchimerism, although only one was tested for chimerism in the LNs (which were negative). All three samples were very carefully processed to ensure that fetal tissues were not exposed to maternal cells during tissue harvesting. Blood analyzed in Sample 1 was obtained directly from the interior chamber of the heart.

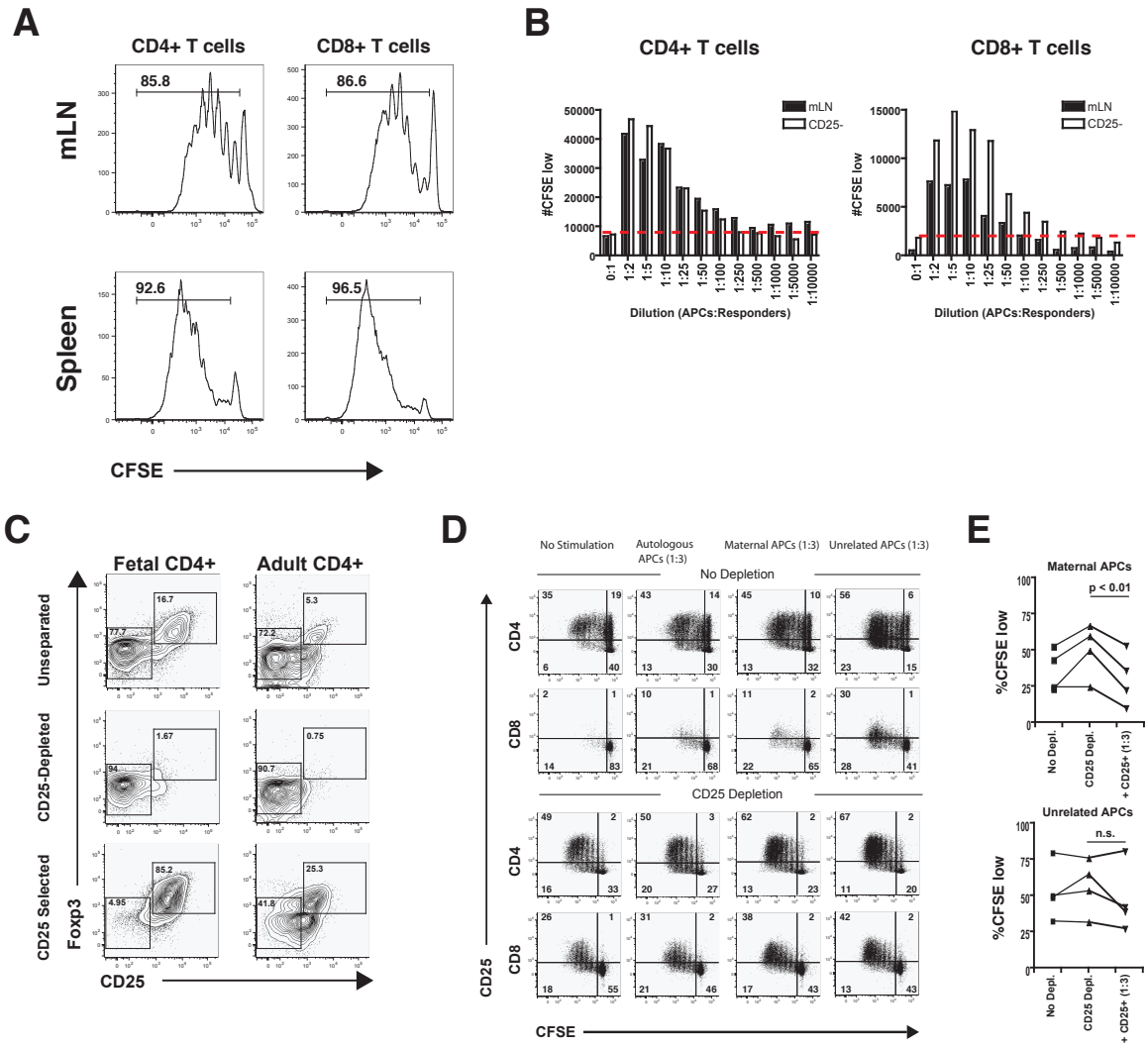
Fig. S2



SAMPLE	HLA CLASS I MISMATCH	% UMBILICAL CORD BLOOD CELLS OF MATERNAL ORIGIN	HLA PCR CONFIRMATION OF SORTED MATERNAL CELLS
1	Maternal B8 Fetal A9	0.06%	+
2	Maternal A2 Fetal A3	0.06%	+
3	Maternal A2 Fetal B27/Bw 6	0.36%	+
4	Maternal A2 Fetal Bw4	0.06%	+
5	Maternal Bw6 Fetal B27	0.01%	+
6	Maternal A2 Fetal B12	0.22%	+

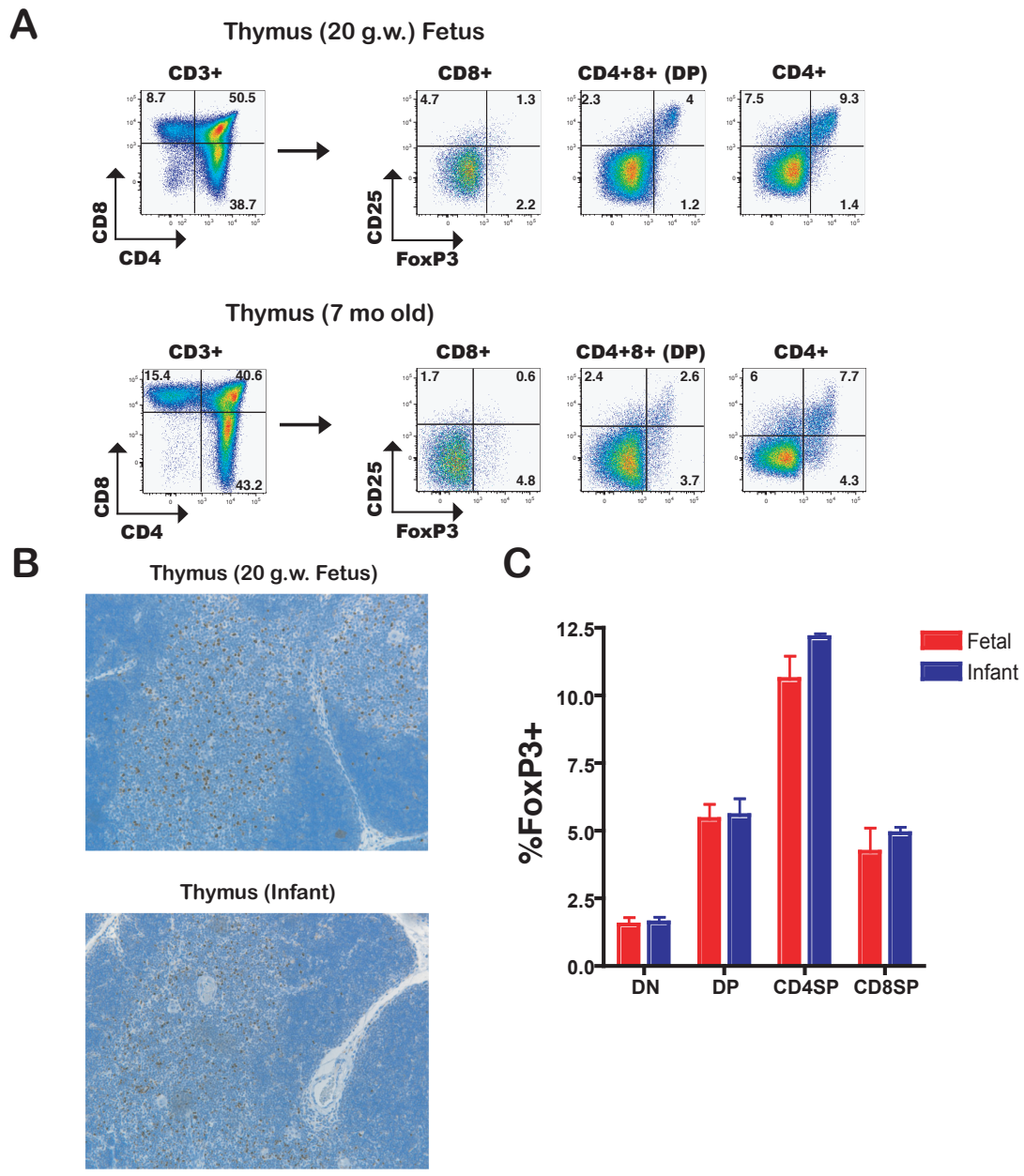
Supplementary figure 2. Maternal cells are present in full-term umbilical cord blood (UCB) and are represented by different lineages of immune cells. (**A**) Representative example of labeling and gating strategy used to detect unique non-inherited maternal- (HLA-A2 in this case) and paternally-inherited fetal (HLA-Bw6) HLA Class I types on the surface of UCB cells, including isotype control for maternal HLA Class I type to ensure specific staining. (**B**) The above strategy was used to sort and collect maternal cells from UCB, which were then lysed and analyzed for maternal HLA Class I alleles by PCR. A representative DNA gel is shown, demonstrating the presence of an appropriately-sized band representing amplification of the unique maternal allele which is present in maternal blood cells used as a positive control (Maternal) as well as in sorted maternal cells from UCB. The band was not detected in unsorted UCB, in DNA from cells known not to possess the maternal allele (- control), in PCR amplification reaction lacking added DNA (DNA control), or with lysis buffer added (Buffer control). (**C**) Summary table of six UCB samples tested detailing the percentage of live cells that were positive for maternal HLA Class I antigen and that were verified to be of unique maternal HLA Class I type by PCR. (**D**) Representative flow cytometry plots of fetal and maternal cells, demonstrating a different distribution of immune cells within these populations. Total cells were divided into lymphocyte and non-lymphocyte gates based on forward and side scatter characteristics to account for size and autofluorescence characteristics. As expected, $CD14^+$ monocytes were found primarily in the non-lymphocyte gate, while most other cell types were detected in the lymphocyte gate, including cells positive for CD3, CD4, CD8, CD16 (likely predominantly NK cells), and CD19. Numbers in gates refer to percentage of the parent population that stained positively for a given marker.

Fig. S3



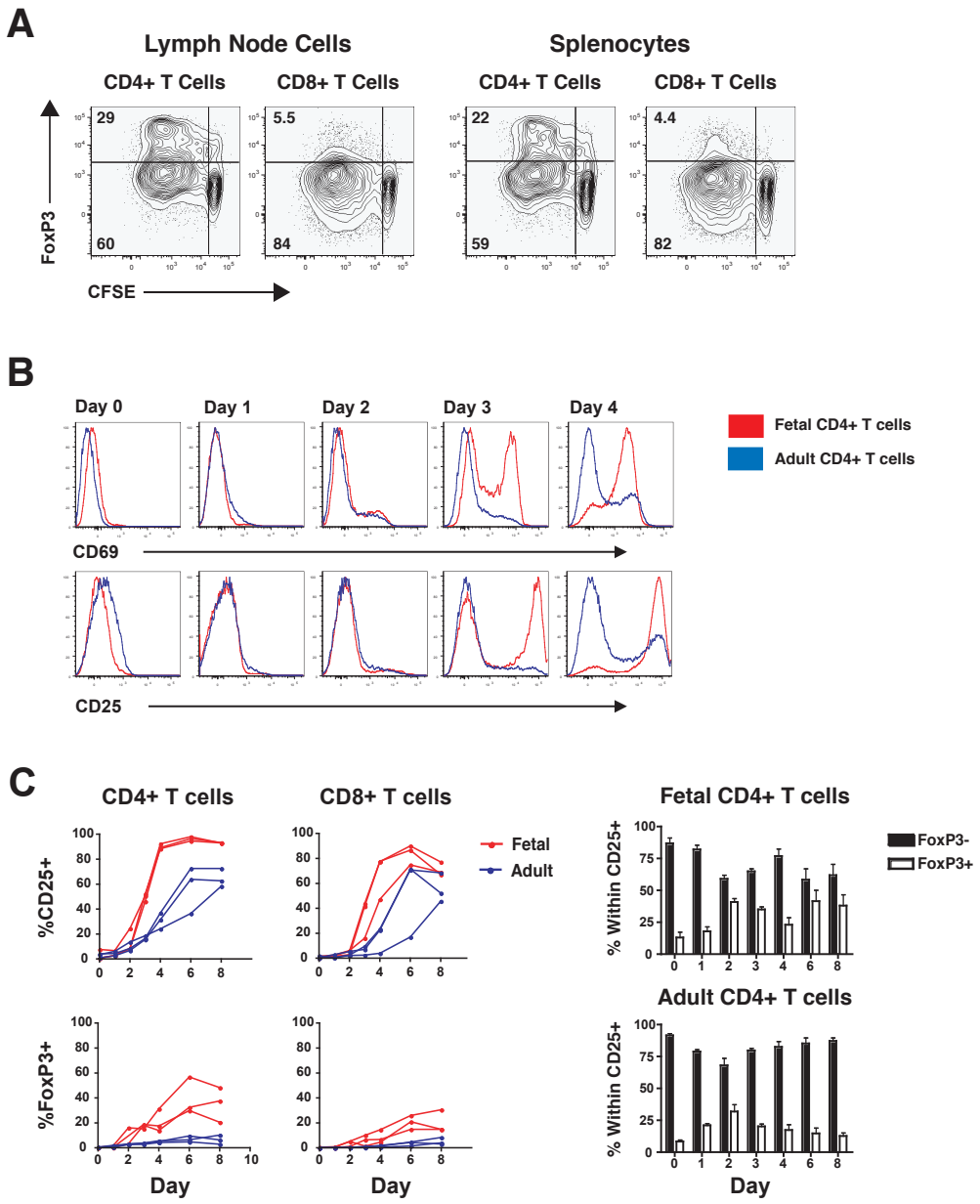
Supplementary figure 3. Fetal T cells from mLN and spleen are highly responsive to stimulation with alloantigens from unrelated donors. (**A**) Total lymphocytes from the spleen and mLN were isolated and stimulated in vitro with allogeneic APCs at a 3:1 ratio of fetal responders to allogeneic APCs. T cells from both LN and spleen were highly responsive to stimulation in primary MLRs (day 5) as measured by CFSE dilution. **(B)** To better illustrate the potential of fetal T cells to respond to alloantigens, fetal mLN cells were stimulated with allogeneic APCs from an unrelated donor at a range of dilutions. Proliferation was measured in terms of the number of cells that had divided. A substantial increase in the number of proliferating cells was observed at higher concentrations of allogeneic APCs and proliferative responses above background (0:1) were observed at ratios as low as 1:100. (**C**) Representative example of purities for fetal LNs or adult PBMCs after positive selection for $CD25^+$ cells with magnetic beads. CD25 selection (and depletion) was efficient in fetal tissues due to the high expression of CD25 by fetal T_{Reg} and the absence of cells expressing intermediate levels of CD25 typically found in adult PBMCs (shown in the bottom right panel). (**D**) Proliferation of fetal $CD4^+$ and $CD8^+$ T cells after stimulation with different sources of APCs (no stimulation, autologous APCs, maternal APCs, or unrelated APCs), before (top) and after (bottom) depletion of $CD25^+$ T_{Reg} cells. $CD4^+CD25^+$ T_{Reg} cells appear to proliferate in the undepleted LNs (top panels). After depletion of $CD4^+CD25^+$ T_{Reg} cells, responding $CD4^+CD25^-$ T cells were found to upregulate high levels of CD25. Removal of proliferating $CD4^+CD25^+$ T_{Reg} cells may lead to an underestimate of suppressed $CD4^+$ T cells, as proliferating T_{Reg} cells are not observed after CD25 depletion. (**E**) Add-back of $CD4^+CD25^+$ T_{Reg} cells to cultures at a 1:3 ratio was found to suppress anti-maternal responses to a greater extent than unrelated APCs (n=4). Statistical significance was determined by paired Student's t-test.

Fig. S4



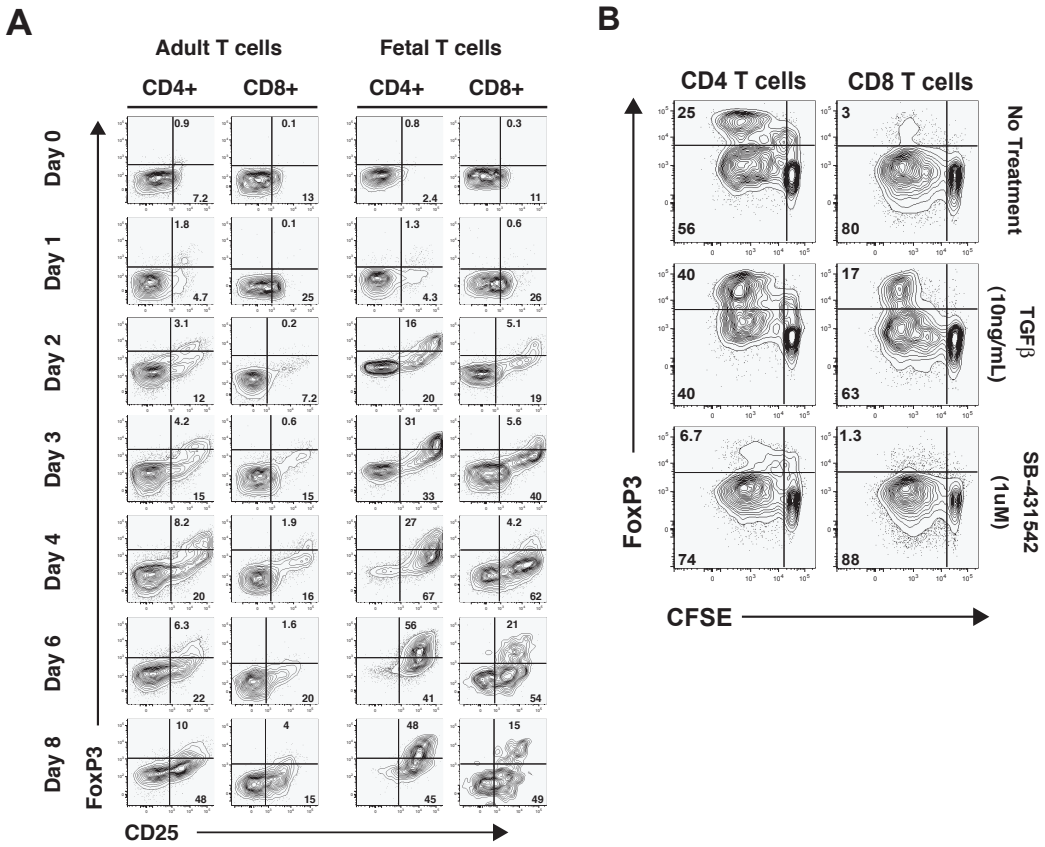
Supplementary figure 4. Fetal and infant thymuses have comparable frequencies of FoxP3$^+$ T cells. (**A**) Representative flow cytometry plots from a 20 g.w. fetal thymus and 7 month-old infant's thymus. All cells are gated on the CD3$^+$ fraction to exclude immature thymocytes (which are predominantly FoxP3$^-$). Similar frequencies of FoxP3$^+$ cells are found in the 20 g.w. fetal thymus and in the infant's thymus, with the highest frequencies of FoxP3$^+$ cells found in CD4SP thymocytes. (**B**) Immunohistochemistry of fetal and infant thymus for FoxP3$^+$ cells. FoxP3$^+$ cells are confined to the medullary region of both fetal and infant thymus, with rare cells present in the cortical regions. One significant difference regards the general absence of Hassall's corpuscles in the fetal thymus. Hassall's corpuscles begin to appear during fetal development and have been reported as early as 16 g.w. but are not prominent before 24 g.w. Recently, Hassall's corpuscles were reported to play a critical role in the generation of FoxP3$^+$ T_{Reg} cells by secreting thymic stromal lymphoprotein (TSLP), leading to the activation of neighboring dendritic cells (6). Our findings that FoxP3$^+$ medullary thymocytes are detectable at 12-14 g.w., before Hassall's corpuscles develop, and the relative consistency of FoxP3$^+$ T cell frequency throughout development, argue that additional factors may contribute to FoxP3$^+$ T_{Reg} development in the thymus. (**C**) The frequency of FoxP3$^+$CD3$^+$ T cells in fetal (n=5) and infant (n=2) thymuses was measured by flow cytometry. No difference was detected for any of the CD3$^+$ thymocyte subsets.

Fig. S5



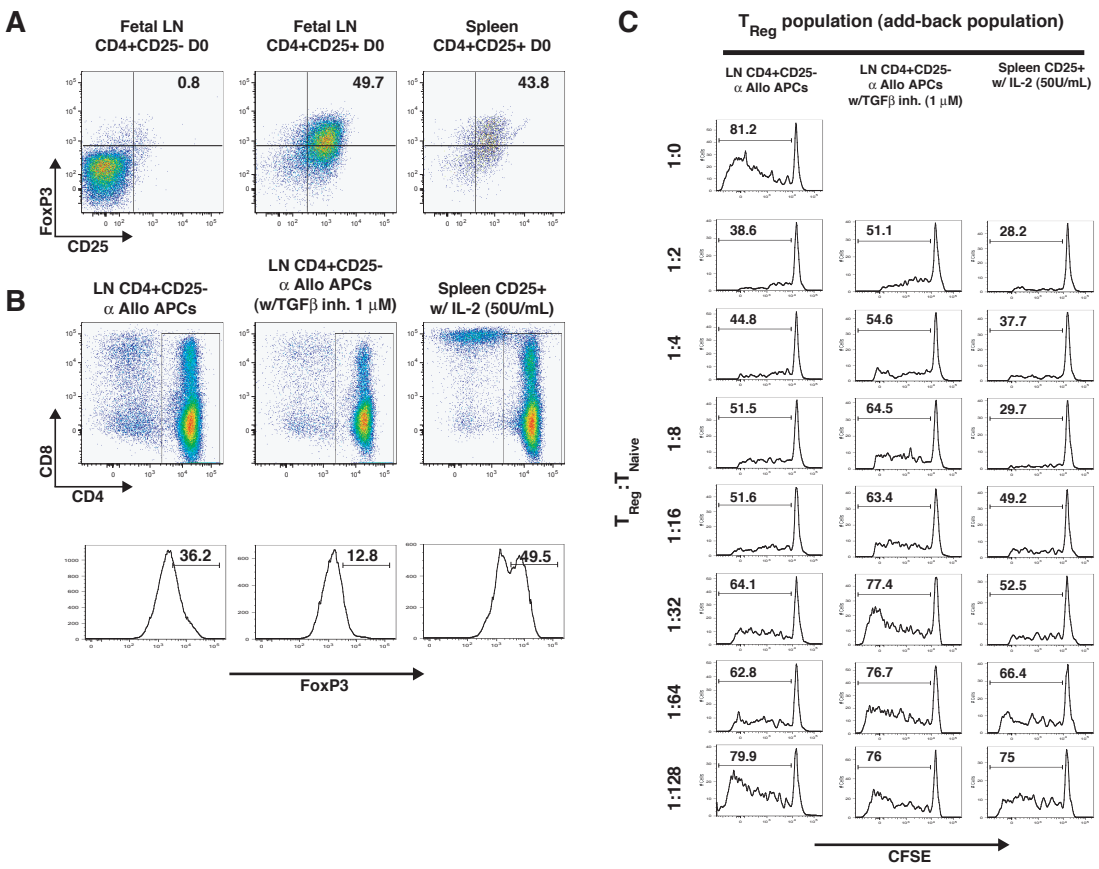
Supplementary figure 5. Upregulation of FoxP3 after stimulation of fetal T cells with allogeneic APCs. (**A**) Fetal LN cells and splenocytes were cultured with a single unrelated allogeneic donor in a 5-day MLR. FoxP3 expression was determined in $CD4^+$ and $CD8^+$ T cells in relation to proliferation (CFSE dilution). (**B**) Comparison of activation markers on fetal and adult $CD4^+$ T cells after stimulation with allogeneic APCs. No differences were observed for fetal or adult $CD4^+$ T cells in the first two days of a primary MLR. However, on the third day of stimulation, a massive expansion of $CD69^+CD25^{bright}$ cells was detected amongst the fetal T cells. After four days of stimulation, almost all fetal $CD4^+$ T cells were $CD69^+CD25^{bright}$ whereas only a small fraction of adult T cells were $CD69^+CD25^{bright}$. (**C)** Upregulation of CD25 and Foxp3 by fetal or adult T cells as a function of time (3 fetal and 3 adult donors are shown). Within the activated, $CD25^+$ population of cells, there is a greater fraction of $FoxP3^+$ cells in fetal vs adult $CD4^+$ T cells.

Fig. S6



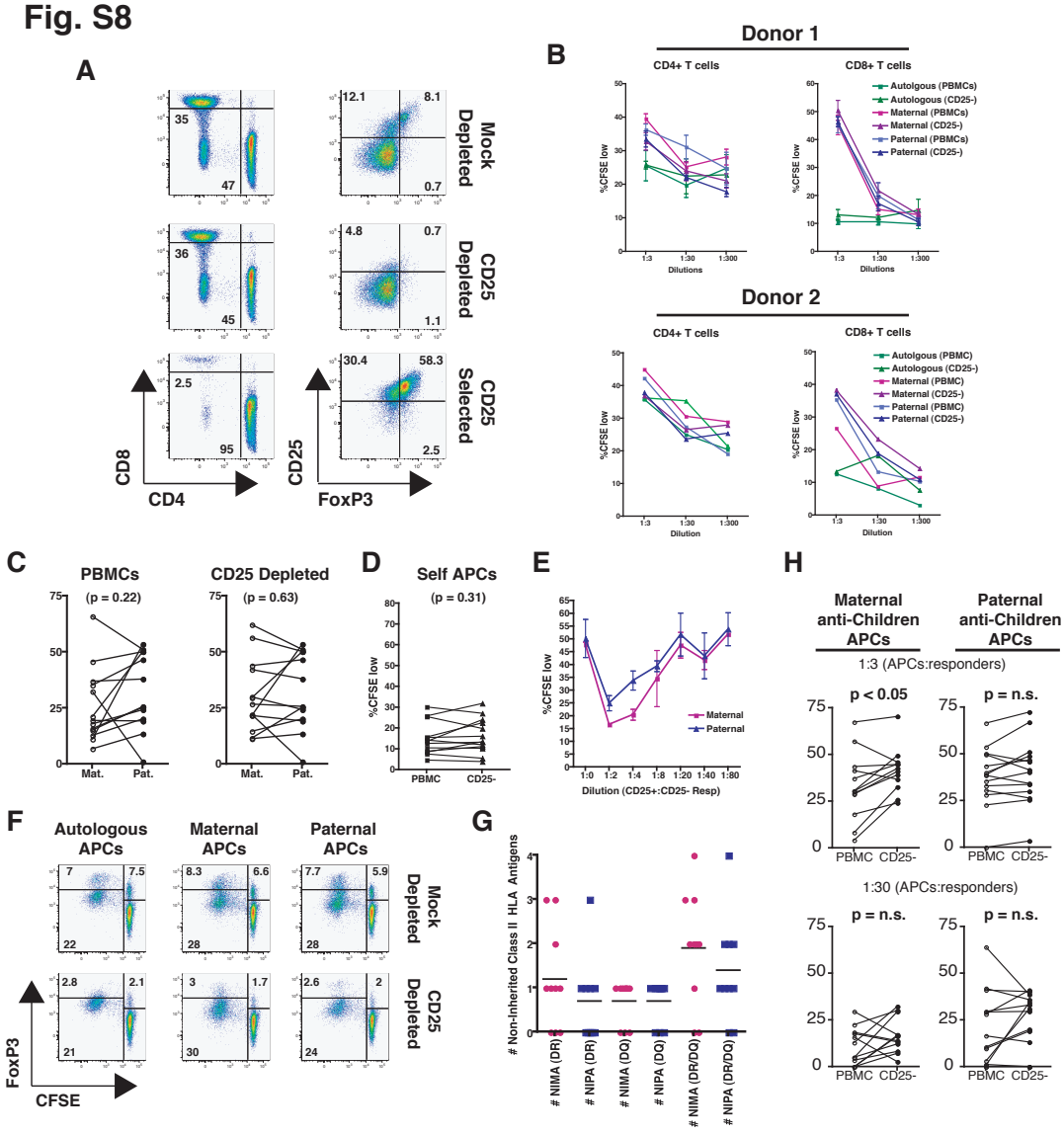
Supplementary figure 6. (**A**) Kinetic analysis of adult and fetal $CD4^+$ and $CD8^+$ T cell activation following stimulation with APCs from a single unrelated donor. CD25 and FoxP3 expression are shown at different timepoints. Both fetal $CD4^+$ and $CD8^+$ T cells upregulated FoxP3 but $CD4^+$ T cells appear to express higher levels of both CD25 and FoxP3. (**B**) Representative flow cytometry plots demonstrating the ability of TGFβ to enhance FoxP3 expression by fetal T cells cultured with allogeneic APCs and that inhibition of TGFβ signaling greatly abrogates FoxP3 upregulation (as summarized in Fig. 2E).

Fig. S7



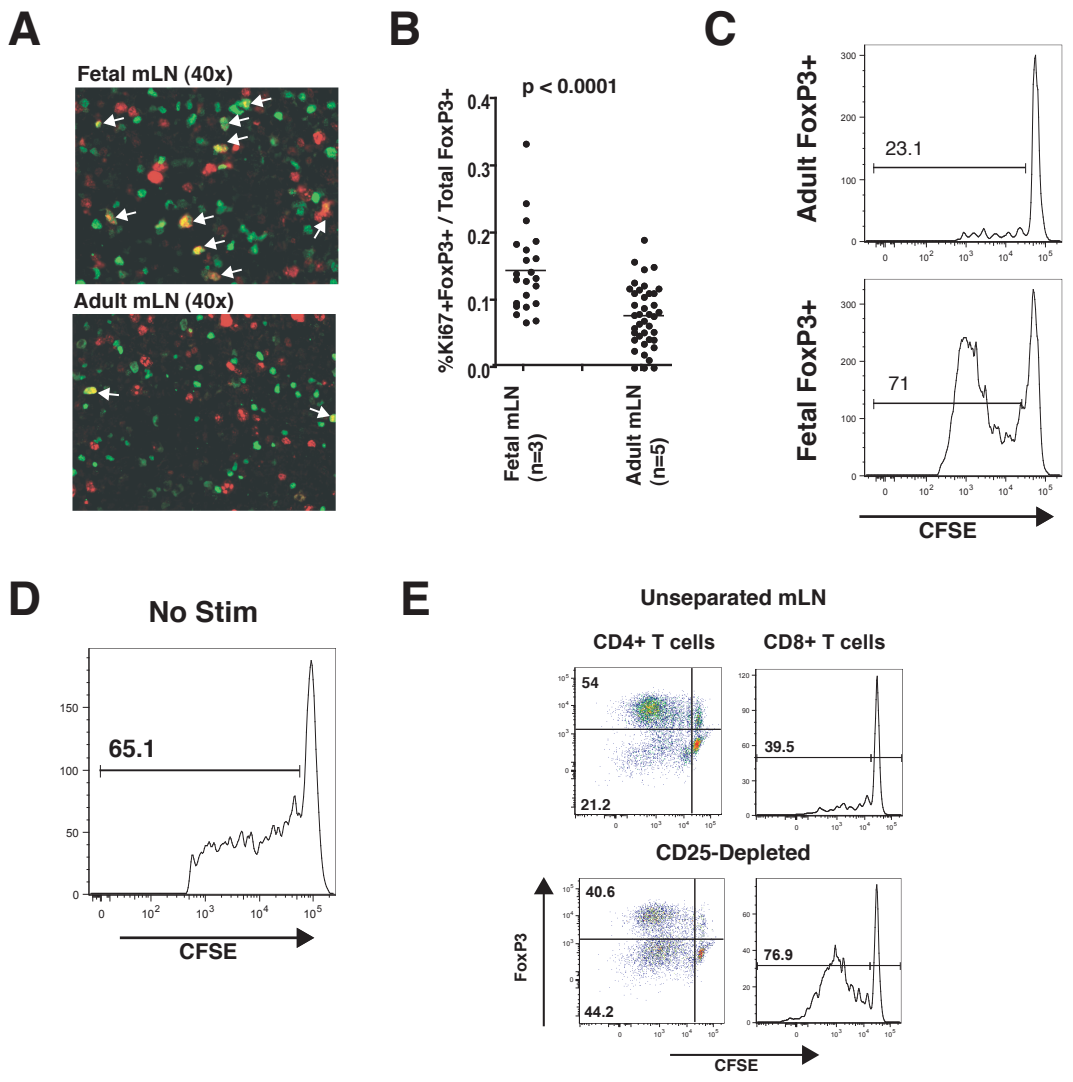
Supplemental figure 7. Acquisition of suppressive properties by fetal T cells following stimulation with unrelated alloantigens *in vitro*. (**A**) Representative purities for fetal mLN $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ (positively selected/depleted T_{Reg}), and splenic $CD4^+CD25^+$ T_{Reg} on day 0. FoxP3 (y-axis) vs CD25 (x-axis) expression is shown. (**B**) Expression of FoxP3 by fetal T cells stimulated for 7 days with unrelated allogeneic APCs with or without addition of TGFβ inhibitor (1 uM) SB-431542. Splenic $CD4^+CD25^+$ T_{Reg} cultured in the presence of 50U/mL IL-2 for 7 days in parallel. Top panels show CD4 vs CD8 expression. Fetal LN cells were depleted of $CD8^+$ T cells on day 0 to avoid potential problems with interpreting CFSE dilution by CFSE-labeled $CD8^+$ cells responding to alloantigens in add-back assays. (**C**) Individual histograms depicting CFSE-dilution (proliferation) of labeled fetal $CD8^+$ splenocytes in add-back suppression assay.

Fig. S8



Supplementary figure 8. Additional children-versus-parents MLR data. (**A**) Sort purity after mock depletion (with anti-biotin beads) or depletion of CD25$^+$ cells from PBMCs. Typical CD3$^+$ T cell fractions are shown for depleted and positively-selected PBMC fractions. Contamination of CD25$^{+/-}$FoxP3$^-$ cells within the positively-selected fraction varied significantly between donors, making add-back suppression assays difficult to interpret. (**B**) Dilutions of autologous, maternal, or paternal APCs for 8-day MLRs. CD4$^+$ T cell proliferation was more variable within the lower ranges of allogeneic APCs and had higher background, as determined by autologous MLRs (green lines). CD8$^+$ T cell proliferation was more consistent and was therefore chosen as the measure for detecting suppression. The 1:3 dilution was chosen because it provided adequate stimulation to promote CD8$^+$ T cell proliferation that was greater than that observed against autologous APCs. (**C**) CD8$^+$ T cell proliferation against maternal or paternal APCs using mock-depleted PBMCs as responders. There were no significant differences in the frequency of children's CD8$^+$ T cells responding to maternal or paternal APCs in mock depleted (p=0.22) or CD25-depleted (p=0.63) MLRs. (**D**) Depletion of CD25$^+$ T cells does not affect background proliferation observed in autologous MLRs. (**E**) Example of an add-back assay using CD25$^+$ cells depicted in panel A, where minimal FoxP3$^-$ cells were present. Duplicate assays performed on separate 96-well plates revealed a significant increase in suppression directed against maternal APCs, as compared with paternal APCs, despite no apparent difference in maximal response. (**F**) CD4$^+$ T cell proliferation in relation to FoxP3 expression for mock- and CD25 depleted PBMCs. Significant expansion of FoxP3$^+$CD4$^+$ T cells was observed against autologous, maternal, and paternal APCs. After depletion of FoxP3$^+$ T cells, there was a substantial decrease in the frequency of FoxP3^{bright}CD4$^+$ T cells. (**G**) Comparison of the number of non-inherited HLA DR and HLA DQ (or both) antigens from mother or father shows that there was a comparable level unshared Class II antigens for both. (**H**) Maternal, but not paternal, CD8$^+$ T cell proliferation is increased in response to their children's allogeneic APCs after T_{Reg} removal. Two different ratios of responders:stims are shown (1:3 top, 1:30 bottom).

Fig. S9



Supplemental figure 9. Fetal T_{Reg} cells are are actively proliferating *in vivo* and *in vitro*. (**A**) Representative image of fetal mLN (20 g.w.) or adult mLN stained for FoxP3 (green) and the nuclear antigen Ki67 (red) which is upregulated during cellular division. Cells that co-stain for FoxP3 and Ki67 appear yellow. (**B**) Quantification of $FoxP3^+Ki67^+$ cells in relation to tal $FoxP3^+$ cells in fetal (3 separate donors represented with multiple 40x fields examined) or adult (5 separate donors with >5 40x fields/LN counted) mLN. Fetal mLN had significantly more $FoxP3^+$ cells that display signs of active cellular division in vivo. Statistical analysis performed by Student's t-test (unpaired). (**C**) Purified adult or fetal $CD25^+$ T cells stimulated in vitro with unrelated allogeneic APCs for 5 days. Adult $CD4^+FoxP3^+$ T cells were primarily non-responsive whereas fetal $CD4^+FoxP3^+$ T cells were highly responsive. Representative of at least three separate experiments. (**D**) Purified fetal $CD25^+$ T cells were isolated from mLN (20 g.w.), labeled with CFSE, and cultured for 5 days with autologous, unlabeled mLN cells. Proliferation of $FoxP3^+$ cells was measured by CFSE-dilution. Fetal $CD4^+FoxP3^+$ cells were highly proliferative in the absence of stimulation. (**E**) Representative example fetal T cell responses in the absence of exogenous stimulation (as in Figs. 1B, C, and S3D). The spontaneously proliferating cells are predominantly $CD4^+FoxP3^+$ T_{Reg} cells. Depletion of $CD25^+$ cells resulted in the robust exapansion of a new population of $FoxP3^+$ T cells.

Supporting Online Material

Materials and Methods

Isolation and preparation of human tissues. All of the human tissue that was obtained for evaluation in this study was obtained with approval from and under the guidelines of the UCSF Committee on Human Research. Fetal tissues (mesenteric lymph node, spleen, and thymus) from 12-24 gestational week specimens and matched maternal blood samples were obtained from Advanced Bioscience Resources and San Francisco General Hospital. Infant thymus (7 months – 2 years) was obtained from normal donors undergoing thoracic surgery at Moffitt Hospital (University of California, San Francisco). Peripheral blood mononuclear cells (PBMCs) were isolated from healthy adults and children by density centrifugation over a ficoll-hypaque (Amersham Biosciences, Piscataway, NJ) gradient. Umbilical cord blood (UCB) was obtained in acid citrate dextrose or heparin sodium tubes by sterile cordocentesis. PBMCs from maternal and cord blood were isolated by ficoll-hypaque (Amersham Biosciences, Piscataway, NJ) gradient and cryopreserved using commercially-prepared freezing medium (IGEN; Origen, Inc., Gaithersburg, MD). Fetal lymph nodes, spleen, and thymus were processed as previously described (1). All tissues were extensively washed with sterile phosphate buffered saline (PBS) (> 40 mL per wash and > 5 washes per specimen) prior to harvesting cells. Whole fetal mesenteries were washed in PBS prior to isolation of individual LNs, which were scattered throughout the omentum. A typical fetal mesentery yielded 20-30 LNs and ~10-20 million mononuclear cells. Fetal spleen and thymus were processed by mechanical dispersion in sterile PBS (2% HI FBS) followed by incubation in 0.2 mg/ml collagenase B (Roche Diagnostics, Switzerland) for 1 hour at 37°C. In some

cases, red blood cells were removed by additional density centrifugation over a ficoll-hypaque gradient after collagenase digestion.

Analysis of maternal microchimerism. Detection of maternal microchimerism was performed as previously described (2-5). Briefly, whole cell suspensions were prepared from processed fetal tissues and from maternal blood samples, and cell pellets were frozen at ~3-5 million cells per tube. DNA was isolated from frozen cell pellets. Maternal and fetal HLA types (HLA-DR based assays) and insertion/deletion profiles (In/Del assay) were determined. Fetal samples were then analyzed for informative maternal alleles (i.e., maternal polymorphisms not present in the fetus) by quantitative allelespecific real-time polymerase chain reaction (qPCR) for the presence of minute amounts of maternal DNA. The fetal samples were also amplified by qPCR using primers specific to HLA-DQ alpha to determine the concentration of total DNA in each specimen. We estimated the concentration of total DNA and minor-type DNA (i.e., maternal cells) in the fetal samples by comparison of sample cycle thresholds to those from parallel amplification of 10-fold serial dilutions of standards with known cell count. We divided the concentration of maternal cell DNA by the concentration of total sample DNA to obtain the frequency of maternal cells in each fetal sample.

Cell preparation and antibody labeling. For flow cytometry analysis, cells were washed in flow cytometry staining buffer (PBS with 2% FBS and 2 mM EDTA) and incubated on ice in the presence of labeled antibodies for 45 minutes. Stained cells were then washed twice in MACS buffer and fixed in 2% paraformaldehyde. Antibodies used for phenotyping lymphocytes included: anti-CD3 Alexa Fluor 700 (SP34-2; BD Pharmingen, San Diego, CA), CD4 ECD (Clone T4; Beckman Coulter Inc. Fullerton, CA), CD8 PE-

Cy5.5 (Clone 3B5; Caltag/Invitrogen, Carlsbad, CA), CD25 PE-Cy7 (Clone M-A251; BD Pharmingen), CD69 APC-Cy7 (Clone FN50; BD Pharmingen), and FoxP3 allophycocyanin (Clone PCH101; eBioscience, San Diego, CA). All cells were stained with a live/dead marker (Amine-Violet/Pacific Blue; Invitrogen) to exclude dead cells from the analysis. FoxP3 staining was carried out according to the manufacturer's protocol with slight modifications. Cells were washed twice in MACs buffer after staining with primary antibodies, re-suspended in FoxP3 fixation/permeabilization buffer (eBioscience), and then incubated for 1 hour at 4°C. Cells were washed twice in FoxP3 permeabilization buffer (eBioscience) and stained with FoxP3 APC in FoxP3 permeabilization buffer for 1 hour on ice. Cells were then washed 3 times in FoxP3 permeabilization buffer, and data were acquired with an LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Treestar, Ashland, OR).

Magnetic separation of cells. For *in vitro* assays involving cell depletion or selection, cells were washed in flow staining buffer and separated into different tubes based on selection criteria. For CD25 depletion/isolation, cells were incubated with anti-CD25 mAb directly conjugated to magnetic beads (Miltenyi CD25 Microbeads II) for 30-45 minutes at 4°C . Labeled cells were washed with flow staining buffer and run through magnetic columns (MS Columns, Miltenyi Biotec). The unbound fraction was kept as the CD25 negative (CD25-) fraction and the cells that were retained in the column were isolated later as the CD25 positive (CD25+) fraction, enriched in TReg. For mock depletions, cells were processed in parallel and incubated with anti-biotin magnetic beads (Miltenyi) and isolated following the same parameters (no flow through was collected). For APCs, whole PBMCs were incubated with biotin-conjugated anti-CD3 (BD

Bioscience) and anti-CD56 (BD Bioscience) for 30 minutes on ice, and washed in flow staining buffer followed by incubation with anti-biotin magnetic beads (Miltenyi), according to the manufacturer's protocol. Labeled cells were washed once more in flow staining buffer and passed over magnetic columns (MS columns). The flow-through was kept as APCs (CD3 and CD56 depleted, i.e., enriched for monocytes, B cells, macrophages, and dendritic cells). All cells purified by magnetic separation were monitored for purity by comparing them against unfractionated PBMCs using a standard phenotyping panel (listed above in *Cell preparation and antibody labeling*). Sorted cells were counted with a hemacytometer using trypan blue to determine the number of live cells, and re-suspended in appropriate buffer.

Mixed leukocyte reactions and in vitro proliferation assays with CFSE. For proliferation assays, cells were first labeled with the dye carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Invitrogen). CFSE labeling was carried out by incubating cells in 1 μM CFSE in PBS at 37°C for 10 minutes, followed by three washes in RPMI with 10% FBS. Labeled cells were added to 96-well U-bottom plates at a concentration of 300,000 cells/well in 200 ul of RPMI culture media [10% HI FBS, 2 mM L-Glu, 100 U/mL penicillin/streptomycin (Invitrogen Life Sciences)]. For allogeneic/autologous MLRs, unlabeled APCs (CD3-CD56- lymphocytes) were irradiated (6000 rads from a cesium source) and 100,000 cells were added to wells for a final ratio of 1:3 (100,000 APCs per 300,000 responders). For all TReg add-back assays, 200,000 CFSE-labeled responder lymphocytes were incubated with a range of dilutions of CD25+ cells (positively-selected fraction) for 5 days. Stimulated cells were collected at various times and supernatants were stored for cytokine analysis. Cells were then washed in MACs

buffer and prepared for flow cytometry analysis by incubation with appropriate antibodies. Typically, the same flow cytometry panel that was used for phenotyping [CD3 Alexa 700, CD4 ECD, CD8 PE-Cy5.5, CD25 PE-Cy7, CD69 APC-Cy7, and an amine reactive dye (live/dead violet fixable dead cell stain kit (Invitrogen)] was used for CFSE assays. Unstimulated cultures were prepared in parallel and "background" levels of proliferation were measured and subtracted from final results**.** For *in vitro* MLRs where CFSE was not used (e.g., the kinetic experiment in Fig. 2), a similar set up was employed with the exception of the antibody panel used. For these experiments, anti-CD62L FITC was used in place of CFSE. Flow cytometry data were acquired on an LSRII and analyzed with FlowJo software.

Suppression assay with induced fetal TReg. Suppression assays for induced fetal TReg were performed as follows. Fetal mLN and spleens were obtained from 18-22 g.w. specimens. On day 0, fetal mLN were depleted of CD8+ T cells and CD25+ T cells by positive selection using miltenyi magnetic beads against CD8 and CD25. Fetal splenocytes were depleted of CD25+ cells by positive selection using miltenyi magnetic beads against CD25. All cells were taken for analysis of purity by flow cytometry to ensure appropriate depletion of indicated cell populations. Fetal CD25- splenocytes were resuspended in freezing medium (90% HI FBS, 10% DMSO) on ice and stored in liquid N2 during the primary stimulation of fetal CD8-CD25- LN cells. Fetal CD8-CD25- LN cells were stimulated with irradiated APCs from an unrelated donor in the presence or absence of a TGFβ inhibitor (SB-45312; 1uM) for 7 days. Fetal CD25+ splenocytes were cultured in parallel with recombinant human interleukin 2 (50U/mL) (added at days 0, 3, and 5). After 7 days in culture the stimulated fetal CD8-CD25- LN cells and fetal CD25+

splenocytes were enumerated and monitored for FoxP3 expression by flow cytometry. Fetal CD25- splenocytes were thawed, rested for >4 hours in fresh RPMI media (10% FBS, L-Glu, Pen/Strep) and counted using Trypan blue to determine cell death and yield after thawing. Fetal CD25- splenocytes were then labeled with CFSE and cultured in 96-well U-bottom plates at a concentration of 200,000 cells/well with 50,000 irradiated allogeneic APCs (same donor used to stimulate fetal LN cells). Pre-stimulated fetal CD8-CD25- LN cells and CD25+ splenocytes were added at different ratios and cultured with fetal CFSE+ CD25- splenocytes for 5 days. After 5 days proliferation was determine by flow cytometry (staining panel: CD3-Alexa 700, CD4-ECD, CD8-PE Cy5.5, CD25-PE Cy7, FoxP3-APC, Violet amine reactive live/dead marker-PB, and CFSE). Percent Suppression determined based on the following calculation: % Suppression = 1-[(%CFSElow (total LN cells)) / (%CFSElow (CD25-depleted))] x 100. Statistical significance determined by unpaired Student's t-test.

Analysis of cytokine gene expression patterns in fetal and adult LNs. For adult LNs, total RNA was obtained from normal donors (26, 27, 29, 34 years old; all male) (Biochain, Hayward, CA). Fetal LNs (19-22 g.w.) were isolated as described above, and whole LNs were dispersed in lysis buffer (RNeasy kit Qiagen) and processed according to the manufacturer's protocol (Qiagen Inc. Valencia, CA). Cytokine gene expression patterns were determined by polymerase chain reaction (PCR)-based gene expression profiling of total adult and fetal LN RNA, using a pre-designed set of primers specific for a range of normal cytokines (General Cytokine Array, SuperArray Bioscience Corp. Frederick, MD). Preparation and analysis of samples was carried out according to the manufacturer's protocols. Briefly, total RNA concentration was measured, and RNA

purity and integrity were verified using a bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse transcription was carried out to generate cDNA (SuperArray RT-PCR kit) and SYBR green-based quantitative real-time PCR was performed on samples (SuperArray, SYBR Green RT-PCR master mix). RT-PCR was run with an ABI 7700 (Applied Biosystems, Foster City, CA) and data were analyzed using a Microsoft Excel platform designed by the manufacturer (SuperArray, General Cytokine Kit – Human).

Immunohistochemistry. Thymus sections were stained for FoxP3 as previously described (17). In brief, deparaffanized thymus sections from fetal and infant donors were incubated for 1 hour at room temperature with rabbit anti-FoxP3 (1:500 Ab #Ab10563, Abcam, Cambridge, MA). Detection was performed with the Dako Envision secondary detection system (Dako, Denmark), which employs a pre-diluted anti-rabbit antibody preparation that is directly conjugated to a horseradish peroxidase (HRP) polymer. 3,3'-diaminobenzidine (DAB) was used to develop slides, and images were acquired with a Leica DM 6000 microscope (Leica Microsystems, Wetzlar, Germany) and Image-Pro 5.1 software package (Media Cybernetics, Silver Springs, MD).

HLA Class I typing and immunophenotyping of umbilical cord blood (UCB) and maternal PBMC. Maternal and UCB PBMC were labeled with anti-HLA Class I haplotype-specific (A2/28, A3, A9, B8, B12, B13, B27, B57/58, Bw4, Bw6 and Class I positive control) mAbs (One Lambda Laboratories, Canoga Park, CA) that were directly fluorescein isothiocyanate (FITC)-conjugated by the manufacturer, conjugated with activated B-phycoerythrin (PE) using the Prozyme Phycolink B-Phycoerythrin Conjugation Kit (Prozyme, San Leandro CA), biotinylated, or unconjugated. Unlabeled primary mAbs were detected with allophycocyanin (APC)-conjugated goat anti-mouse

IgG (Molecular Probes, Inc., Eugene, OR) and biotinylated antibodies were detected using streptavidin-APC (SA-APC) or streptavidin-PE (SA-PE). For more extensive immune phenotyping of maternal and fetal cells in UCB, FITC-conjugated mAbs directed against CD3, CD4, CD8 , CD14, CD16, and CD19 were used in conjunction with PE or APC-labeled HLA antibodies. In all samples, propidium iodide (PI) was used as a marker of dead and dying cells. Flow cytometry was carried out on a FACSCan (Becton Dickinson), and data were acquired using CellQuest software (Becton Dickinson) and analyzed using FlowJo software (Tree Star, Inc).

Sorting and genetic phenotyping of fetal and maternal populations in UCB. UCB samples were double labeled with FITC-labeled mAb directed against maternal HLA and a biotinylated mAb directed against the common fetal HLA, which was then detected with SA-APC or SA-PE. Labeled cells were incubated with PI and sorted on a FACSVantage cell sorter (Becton Dickinson). Cells identified as PI-negative (live), fetal HLA Class I negative, and maternal HLA Class I positive were collected, and assayed for the maternal HLA Class I allele by PCR, which was carried out using oligonucleotide primers specific for HLA-A2, -A3, -A9, -B8, and –B17 according to the manufacturer's instructions (One Lambda Laboratories). Briefly, cell lysate was mixed with commercially prepared and optimized PCR cocktail (D-mix; One Lambda Laboratories) and Taq-polymerase (Boehringer-Mannheim Laboratories) with standard cycling parameters in a GeneAmp PCR System 9700 (Applied Biosystems). PCR products were analyzed on a 5% agarose gel stained with ethidium bromide.

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CHAPTER 4

Evidence for a Developmental Switch that Accounts for the Appearance of Distinct T Cells During Fetal Development and Adult Life

Abstract

Stem cells in the developing fetus are known to be different from those found in the adult. Changes in the hematopoietic stem cell compartment between fetal development and adult life have also been found to account for differences in the developmental potential of these cells. Here, we provide the first evidence that distinct HSC populations in the human fetal liver and in the adult bone marrow generate distinct populations of α/β T cells. These findings challenge the conventional view that the development of the immune system is a linear process, and have broad clinical implications for HSC therapies and for understanding differences in immune function at different stages of development.

Introduction

The mammalian adaptive immune system is traditionally thought to develop in a linear and stepwise manner. In this view, the first B and T cells to appear in the periphery are naïve and only after exposure to antigen do they achieve functional maturity. Thus, the developing fetal and neonatal immune system has been primarily considered to be "immature." This model of immune development has contributed to the prevailing view that fetal and neonatal immunity is compromised, a belief that is well backed by observations in both animal models and in clinical settings (1). However, a growing body of evidence suggests that the developing immune system is more active than generally thought. Fetal immune responses to intrauterine infections have been documented in several studies and maternal vaccinations have been shown to elicit fetal immune responses (2-5). Perhaps the best evidence for fetal immune function is the observation that severe multi-organ autoimmune disorders can arise as early as 30 gestational weeks in humans suffering from the inherited genetic disease IPEX (immunodysregulation, polyendocrinopathy, X-linked syndrome) (6). These observations suggest a greater complexity underlying the maturation of the adaptive immune system.

Several interesting changes have been described within the T cell compartment throughout fetal development. Fetal CD4+ T cells, in particular, show substantial heterogeneity with respect to surface antigens typically associated with activation and maturation status (7). A substantial fraction of fetal CD4+ T cells express the CD45RO surface antigen, consistent with a mature, memory T cell phenotype. By contrast CD45RO is virtually absent on newborn CD4+ T cells (Fig 1A). Fetal T cells also express very high levels of the surface antigen CD38, which is often considered to be a

marker of activation in adult T cells (Fig 1B). Because CD38 is differentially expressed during thymic maturation of T cells, it may be that high levels of CD38 expression reflect the fact that many fetal T cells are likely to be recent thymic emigrants (8). Expression of the high affinity IL-2 receptor, CD25, has also been described on a subset of fetal CD4+ T cells (7). We have since demonstrated that these CD25+ cells represent fetal regulatory T cells (T_{Reg}) (Chapter 1) that appear to play a role in suppressing autoimmunity and immunity to maternal alloantigens (Chapters 2 and 3). Interestingly, the frequency of CD4+CD25+ T_{Reg} cells in the fetus declines progressively throughout fetal development, reaching the relatively low adult frequencies at birth (9).

Functionally, fetal T cells are thought to be poor at responding to antigens. There is a wealth of evidence documenting reduced capacity for neonatal T cells to respond to stimulation *in vitro* (reviewed in ref. 1). By contrast, the functional properties of fetal T cells have not been well studied and cord blood T cells are often taken to represent fetal T cells at all stages of development. Because of the obvious phenotypic changes within the fetal T cell compartment throughout development (1,7,9), it is likely that many differences may exist between those T cells at early stages of gestation (10-20 g.w.) and those present at birth. The few reports that have considered the functional properties of mid-gestation fetal T cells have found that they are fully capable of mediating proliferative responses in a mixed leukocyte reaction (MLR) (10-12). In fact, we have found that fetal T cells are exquisitely sensitive to alloantigens in a primary MLR, with dramatic proliferation occurring within the first 5 days of stimulation (Fig 1C).

Because mice lack conventional α/β T cells during fetal development, larger mammals (e.g., sheep) have been employed to study the functional properties T cells at

Fig. 1

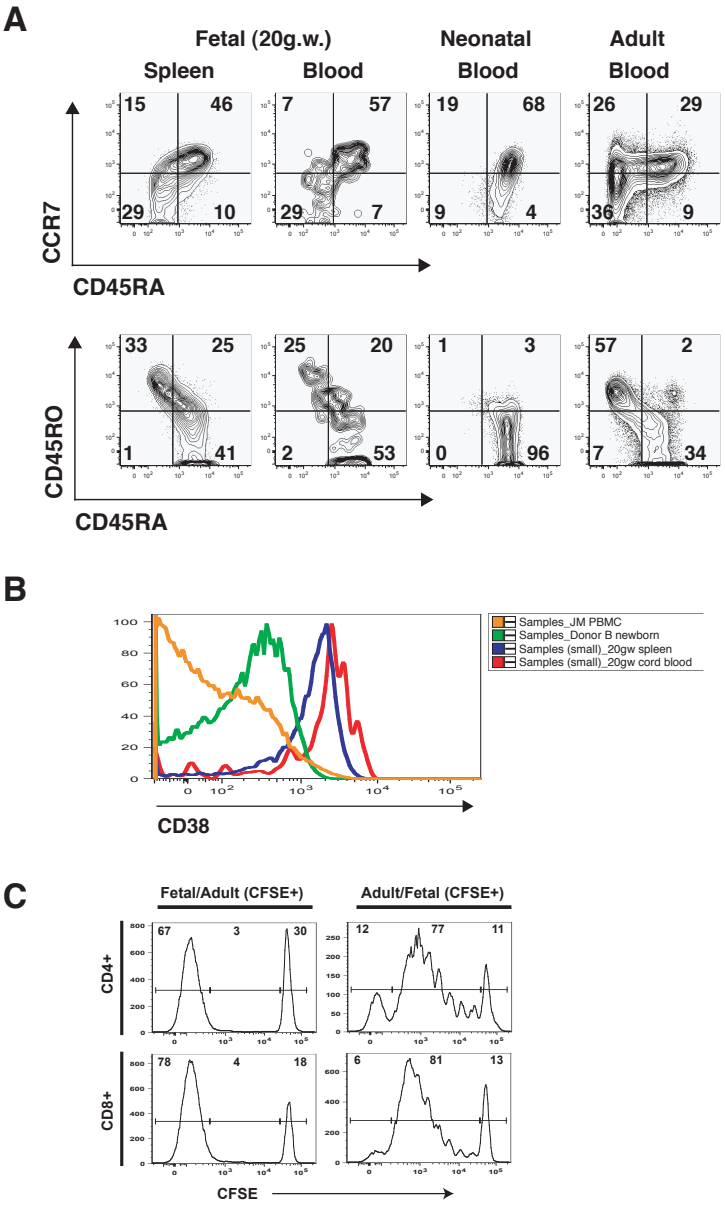

Figure 1. Phenotype and function of fetal versus adult CD4+ T cells. A) Comparison of naive and memory markers on fetal (20g.w.), neonatal (1 day post-birth), and adult (>20y.o) CD4+ T cells. Both splenocytes and cord blood T cells are depicted from the same fetal specimen. B) CD38 expression is highly elevated on fetal CD4+ T cells, but not neonatal or adult CD4+ T cells. C) Proliferation of adult (left panels) or fetal (right panels) CD4+ and CD8+ T cells in response to stimulation with unlabeled fetal (left) or adult (right) mononuclear cells. Fetal T cells undergo substantially more division in the 5-day culture period. Labeled cells are depicted on the left and unlabeled cells are depicted on the right side of the histogram.

this time (13-17). The ability of sheep to mount immune responses during fetal development has been documented for almost fifty years (18); little, however, is known about the specific functions of different lymphocyte populations during the generation of an immune response. Interestingly, fetal sheep are found to undergo a gradual shift with respect to their ability to mount immunity to different antigens (reviewed in ref. 18). Whereas some antigens, such as ferritin or bacteriophage lambda, could elicit antibody responses at the onset of lymphocyte development (d60-80), others such as *Salmonella typhosa* and diphtheria toxoid generated no response until after birth. Whether this can be explained by the appearance of distinct waves of lymphocyte populations with differential capacity for responding to antigens, or by changes in the way that the fetal immune system is able to sense these different antigens remains unknown. A more recent study has shown that a fundamental shift can be seen in the nature of the T cell pool around the time of birth, wherein the vast majority of fetal lymphocytes are lost and rapidly replaced by a new population of T cells (19). This observation hints at the possibility that the lymphocytes in the fetus may not be the same as those found in an adult.

Results

To determine whether fetal α/β CD4+ T cells were fundamentally distinct from their adult counterparts, we performed microarray analysis on highly purified populations "naïve" T cells or T_{Reg} isolated from 18-22 g.w. fetuses and healthy adults (25-35 y.o) (Fig 2A). Our results suggest that there is a dramatic difference in the gene expression program between fetal and adult T cells (Fig 2B). Amongst the most highly differentially-

Fig. 2

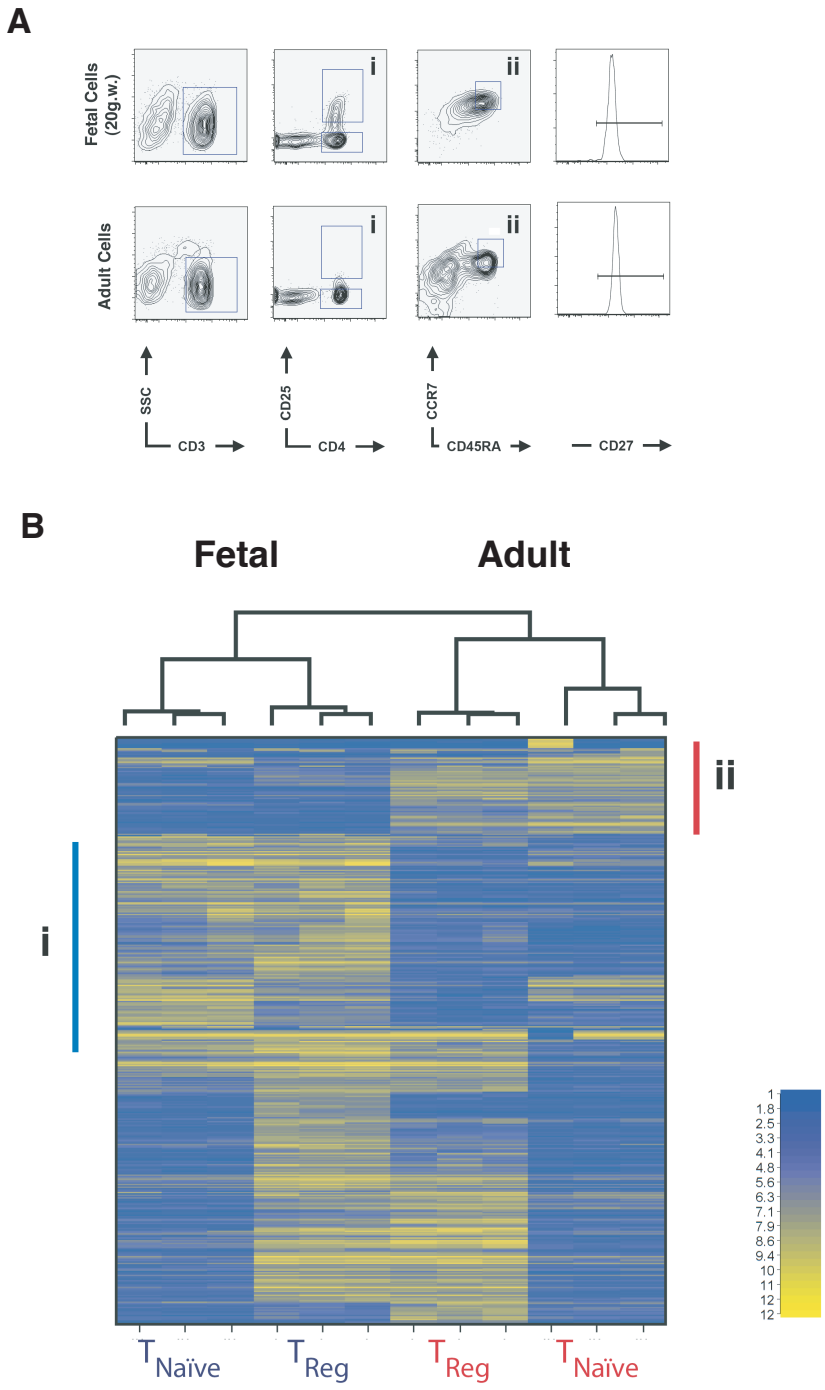

Figure 2. Sorting parameters and microarray results. A) Sorting phenotypes for fetal (top) and adult (bottom) CD4+CD25^{high} Tcells (box i) or CD4+CD45RA+CCR7+CD27+ naive T cells (box ii). B) Microarray results for 3 fetal and 3 adult donors for each cell type. Blue line (i) denotes genes that are highly expressed in fetal naive T cells and fetal T_{Reg} as compared to adult T cells. Red line (ii) denotes genes that are highly expressed in the adult naive T cells and adult T_{Reg} as compared to fetal T cells.

expressed genes, we were able to identify substantial numbers of genes that were expressed specifically by fetal (Fig 2B, 3A) or adult T cells (Fig. 2B, 3B). The genes identified for both the fetal and adult T cell populations spanned a wide range of functions and suggest that fetal and adult T cells may be much more different than previously assumed. Interestingly, many genes identified as being specifically expressed by fetal T cells (e.g., TIPARP, ELK3, CDCA7, BCAT1, PRDM16, FAS, NOXA, WEE1, BIRC3, BCL2L11, RAB25) are known to be involved in cell cycle regulation and apoptosis, consistent with our observation that fetal T cells are hyperproliferative and prone to cell death after stimulation in vitro (Chapter 3, Fig S5 and data not shown).

We were also able to identify a small subset of genes expressed by both fetal naïve and T_{Reg} cell populations and also found to be expressed in adult T_{Reg} but not in adult naïve T cells (Fig 3C). Because we previously had found that fetal T cells appear to exhibit a predilection towards adopting a T_{Reg} cell fate after stimulation (12), it is possible that certain genes in this group may be involved in regulating T_{Reg} cell differentiation. The transcription factor, FoxP3, which is believed to be a master regulator of T_{Reg} differentiation and function, was recently found to act downstream of additional unknown factors, indicating that some of the major determinants of T_{Reg} cell fate remain unknown (20). Therefore, the data set generated from these arrays may prove valuable in identifying FoxP3-independent factors that control T_{Reg} fate in humans.

To validate our array results, we performed real-time quantitative polymerase chain reaction (qPCR) on sorted populations of naïve fetal or adult T cells from additional donors. We selected three genes found to be highly expressed in either fetal (BCL11A, BCAT1, ROBO1) or adult (KLF9, SERPINB6, TRPC1) T cells (Fig 4A). The

Fig. 3A

Fetal T cell genes (within top 500 most DE genes)

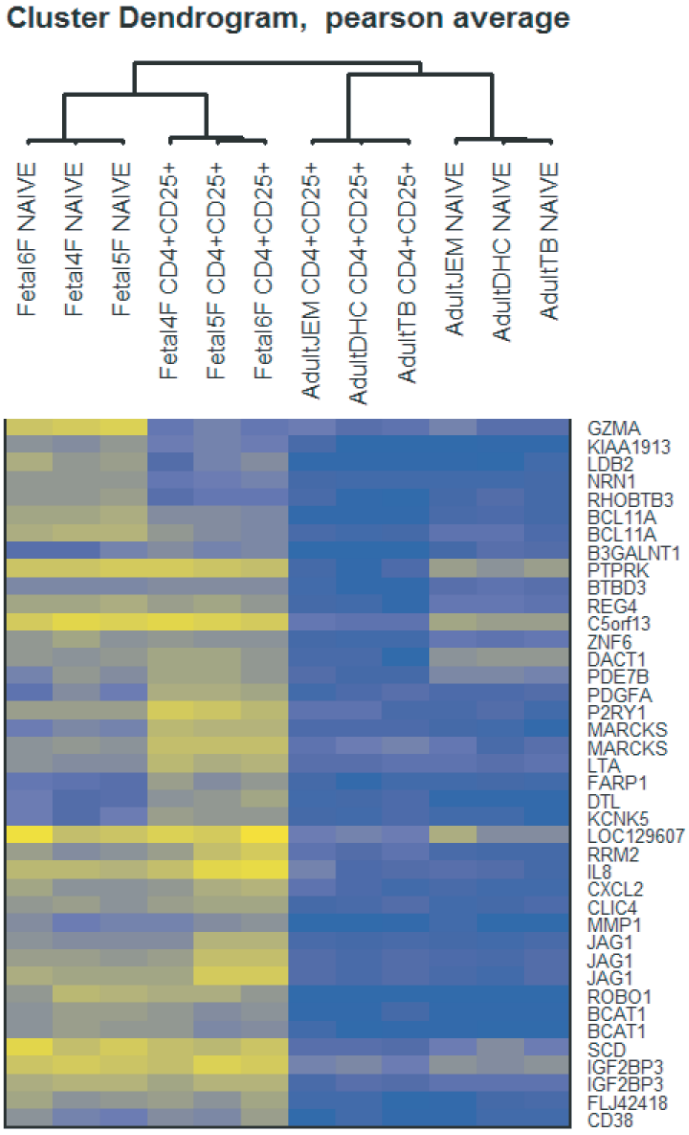

Figure 3A. Gene list for "fetalt" specific genes identified in preliminary analysis. ROBO1, BCAT1, and BCL11A were chosen for subsequent verification by qPCR (figure 4).

Fig. 3B

Adult T cell genes (within top 500 most DE genes)

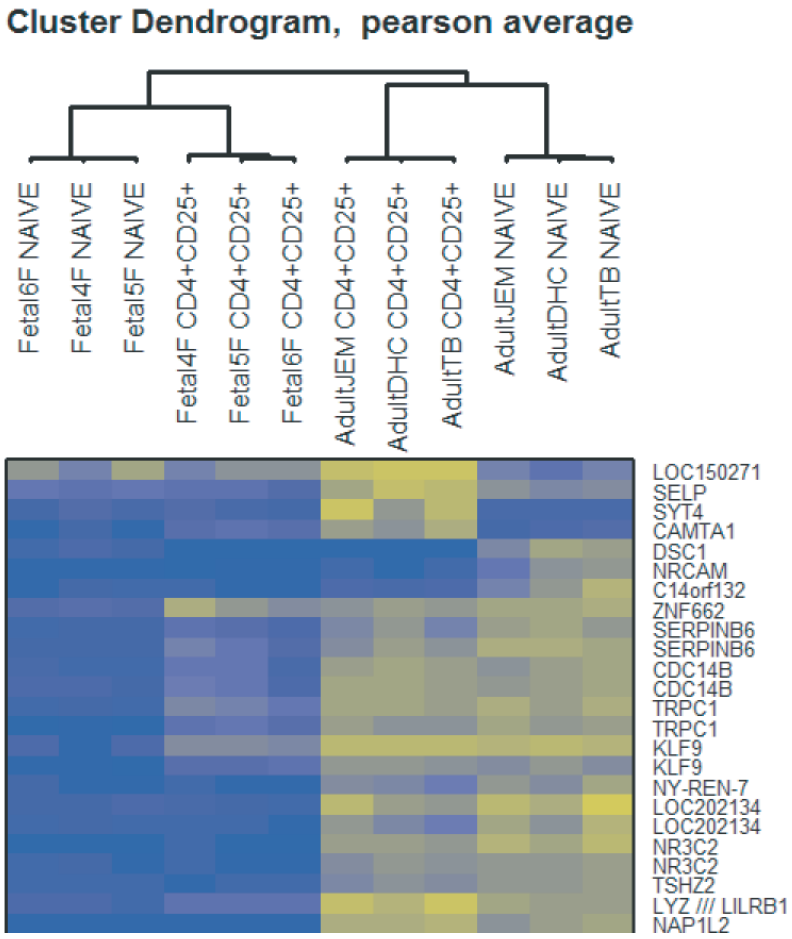

Figure 3B. Gene list for "adult" specific genes identified in preliminary analysis. TRPC1, SERPINB6, and KLF9 were chosen for subsequent verification by qPCR (figure 4).

Fig. 3C

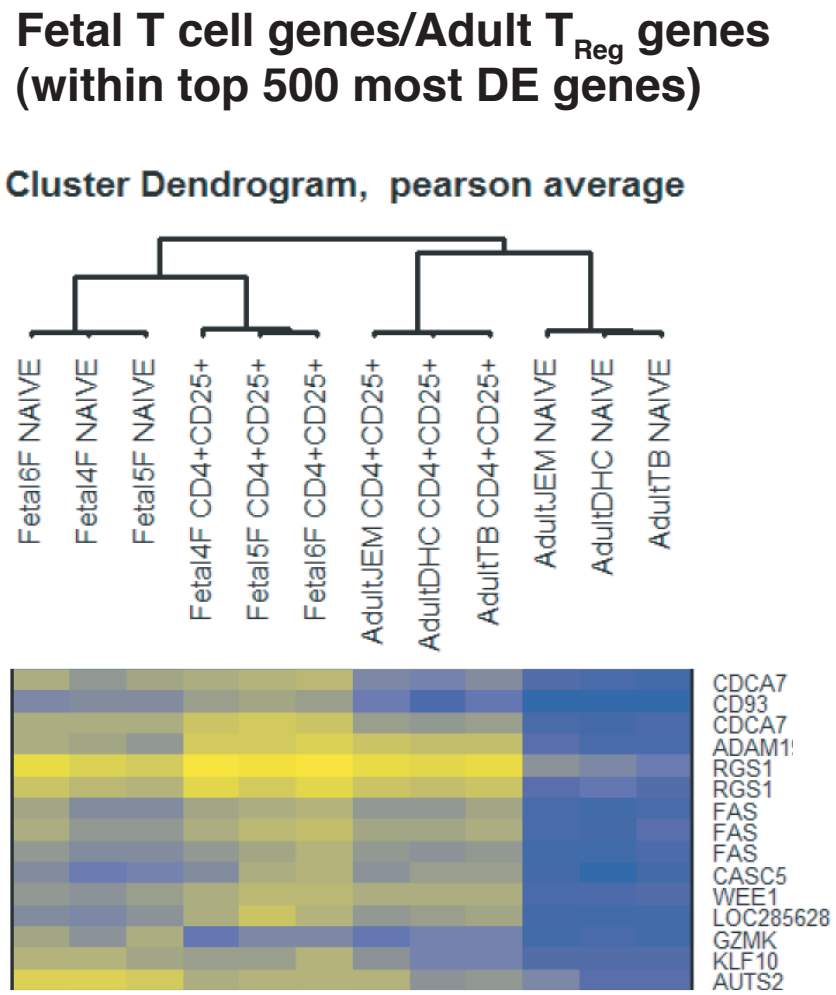

Figure 3C. Gene list for "fetal naive/T_{Reg} and adult T_{Reg}" specific genes identified in preliminary analysis.

Fig. 4

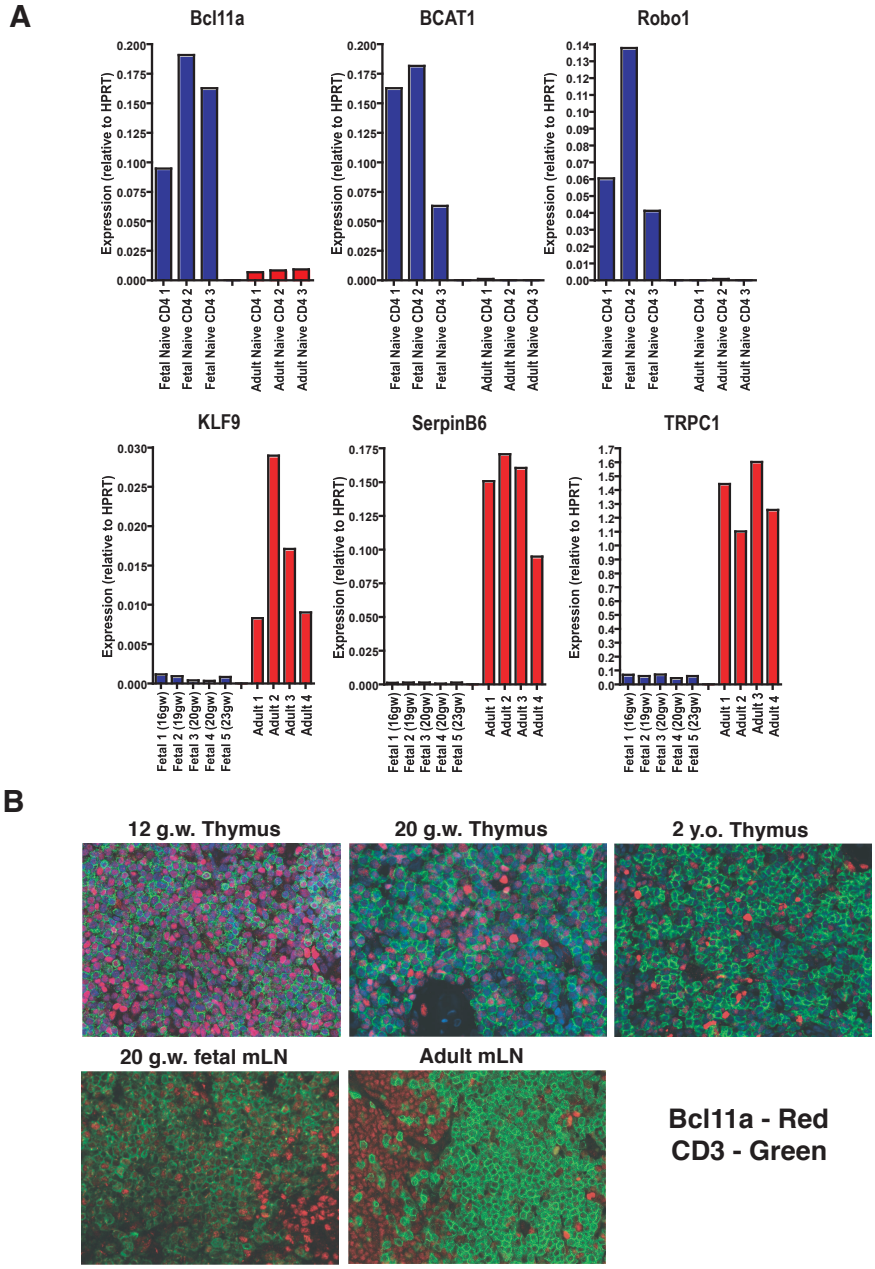

Figure 4. Gene expression verification and analysis of BCL11A expression in fetal and adult lymphoid tissues. A) Genes identified from microarray experiment in figure 2 and 3 were verified by performing quantitative real-time PCR on fetal and adult T cells sorted from separate donors. B) BCL11A protein expression was measured in fetal thymus (12g.w. and 20g.w.) and infant thymus (2y.o.) as well as in fetal and adult lymph nodes. Expression of BCL11A by medullary CD3+ thymocytes decreases throughout gestation and is largely absent at birth. BCL11A is expressed in CD3+ cells in fetal LN but is restricted to CD3- cells (presumably B cells) in adult lymph nodes.

expression of these genes was very consistent with the array results and most of these genes were exclusively found in either adult or fetal T cell populations. Additional genes (e.g., PTPRK, SYT4, CLIC4, NRN1) have since been confirmed (data not shown). Because of the vast array of differences observed in the gene expression profiles of what were otherwise phenotypically similar populations of CD4+ T cells, we hypothesized that these cells may in fact represent unique lineages of α/β T cell.

The existence of distinct lineages of lymphocytes in fetal and adult life has been previously reported in mice (21-24). In the mouse, there are very few if any α/β T cells in the periphery prior to birth (25). There is, however, peripheral colonization in the fetal mouse by a population of T cells that specifically express the Vγ3/δ1 T cell receptor. These cells are known as dendritic epidermal T cells (DETC) because of their distinctive shape and preferential localization in the skin (21,22). Vγ3/δ1 DETC were initially identified as the earliest mature T cells found in the fetal thymus after the first wave of thymic colonization by hematopoietic stem cells (HSC) at embryonic day 10.5 (E10.5) (21,22). Several additional waves of thymic colonization have been characterized in the mouse, with each successive population of precursor cells replacing the previous population (26). DETC appear to be specifically generated from the earliest wave of precursor cells (22). These cells are then completely replaced by a new population of precursor cells, whose appearance coincides roughly with birth and which gives rise to predominantly α/β T cells, the primary T cell population found in the thymus and in the periphery throughout adult life. Thus, mice undergo a similar change in T cell repertoire at or near birth, as was described in fetal sheep, with a primary difference being that fetal

mouse lymphocytes appear to predominantly be of the Vγ3/δ1 DETC lineage, making them easily distinguishable from their adult counterparts.

Much like the fetal T cell populations found in mice, fetal B cell populations can be easily discriminated from adult B cells on the basis of a unique surface receptor profile and tissue tropism (23). These cells have been designated B-1 B cells and are uniquely characterized by their co-expression of surface IgM and CD5 (27). B-1 B cells are relatively enriched in the adult peritoneal cavity, where it is thought that they may play a role in anti-bacterial host defense (28). While the developmental origins of B-1 B cells has long been debated, recent findings strongly suggest that these cells are specifically generated by a fetal lymphocyte precursor that is not present after birth (24).

While initial experiments strongly suggested that both Vγ3/δ1 DETC and B-1 B cells represented distinct populations of fetal lymphocytes, there remained questions about the development of each cell type and whether they required a fetal environment or were generated by a distinct fetal HSC population. This question has since been resolved for both populations with a series of studies demonstrating that fetal, but not adult, HSC populations are specifically capable of generating both cell lineages (24, 29). However, Vγ3/δ1 DETC were found to require both a fetal thymic environment as well as fetal HSC (29), whereas B-1 B cells could be generated in adult mice if provided with fetal HSC (30). It is unknown what changes occur in the fetal and adult thymus that regulate the changes in lymphocyte differentiation, but there is histological evidence that substantial changes occur with respects to the antigen presenting cell compartment in the fetal thymus during the initial stages of T cell maturation (31).

In addition to changes in the lymphocyte populations between fetal and adult life, there are also substantial changes that occur with respect to the erythrocyte compartment throughout fetal development (32). Fetal erythrocytes (or F cells) are readily identifiable by the expression of fetal hemoglobin, which differs from adult hemoglobin with respect to the affinity for which it binds to oxygen (32, 33). After birth, F cells are almost completely replaced by adult erythrocytes, although a small fraction of F cells remains in most (34). Differences in the frequency of F cells after birth are in part related to polymorphisms in the gene BCL11A, which contributes to differences in the level of expression of different BCL11A isoforms (35). BCL11A was recently found to play a role in the transcriptional regulation of adult hemoglobin genes, suggesting that it plays a critical role in regulating the switch between fetal and adult hemoglobin (36). At this point it remains unclear whether the switch from fetal to adult erythrocytes is regulated at the level of the stem cell or by environmental signals (37), although there is some evidence that certain signals can promote fetal hemoglobin expression in adults (38).

Developmental changes that occur within the hematopoietic compartment have been well described for many years (39). However, with the exception of the examples provided here, there has been relatively little interest paid to the differentiation potential of fetal and adult HSC. Much of our knowledge instead relates to the specific functional properties of the HSC populations at different stages of development. For example, it has long been appreciated that fetal HSC have substantially greater capacity for self-renewal than adult HSC, which are generally considered to be quiescent by comparison (40). Gene expression studies on highly purified fetal and adult HSC populations have revealed many differences in the molecular signatures of these populations, congruent with the

observed functional differences (41). Such studies led to the identification of a transcription factor, Sox17, which plays a specific role in fetal but not adult HSC, confirming the unique identity of these two populations (42). Deletion of Sox17 in mice resulted in embryonic lethality, with a noticeable loss of hematopoiesis in the yolk sac and fetal liver. Expression of Sox17 was found to be restricted to fetal and neonatal HSC and conditional deletion of Sox17 in adult mice resulted in no changes in the hematopoietic compartment. A specific role for Sox17 in the maintenance of fetal and neonatal HSC populations was thus revealed. However, the mechanisms underlying the importance of Sox17 remain unknown as do the factors governing expression of Sox17 in fetal/neonatal HSC.

One possible factor that may contribute to differences in the function and developmental potential for fetal and adult HSC relates to environmental signals that differ within different tissues (43). Fetal HSC are primarily found in the developing liver where they reside from ~6-20 g.w. in humans (44) and ~E11-16 in mice (39). After this time there is a dramatic decline in HSC numbers in the liver, coincident with the appearance of large numbers of HSC in the developing bone marrow (45). This process is dependent upon specific chemoattractant signals and is most likely triggered by the development of suitable niches for HSC migration, as HSC appear to be constantly present in the circulation throughout the time that they are found in the fetal liver (45). For this reason, some have speculated that different signals in the fetal liver and bone marrow may contribute to the changes in HSC function. It has been proposed, for instance, that Wnt signaling molecules predominate in the fetal liver whereas Notch signaling is favored in the bone marrow (46). Whether a change in these signaling

pathways can account for differences in HSC function remains unclear. The observation that both the fetal liver and fetal bone marrow, but not adult bone marrow, can give rise to Vγ3/δ1 DETC suggests that additional factors not associated with tissue tropism may contribute to HSC function during fetal and adult life.

Based on the wealth of evidence that fetal and adult HSC have distinct differentiation potential in mice, we hypothesized that differences that we had observed with respect to the function and molecular signature of fetal and adult T cells may result from differences in the HSC populations contributing to these cells. This hypothesis is consistent with the developmental stage that we had examined, as fetal T cells present in 18-22 g.w. fetal tissues are likely to have been derived from fetal liver HSC. In humans, the fetal liver is believed to be the primary site of hematopoiesis until ~20 g.w. , when HSC function becomes clearly observable in the fetal bone marrow.

To test this hypothesis, we utilized the SCID-hu Thy/Liv model for human hematopoiesis (47). This model has been used successfully in the past to generate mature T cells from both fetal and adult HSC (48). Because these mice are generated with human fetal thymic fragments, they have the added benefit of controlling for differences between fetal and adult tissues. Thus, any observed differences are likely to be directly linked to the origin of the HSC and not to the presence of different environmental signals. By identifying donors that express unique HLA markers, we are able to specifically distinguish T cells derived from donor HSC from the endogenous thymic T cells. While true HSC populations are defined in humans as CD34+CD38lo/-CD90+c-kit+Lineage- cells (49), we decided to use a less stringent method for purification and included all CD34+ cells. CD34 expression is mainly restricted to HSC and progenitor populations

within the hematopoietic compartment, and is not found on mature T cells, making it unlikely that contaminating peripheral T cells could be introduced into the thymic implants. We chose to use a less stringent purification strategy so that we could maximize the yield of HSC/progenitors, as both populations are relatively rare in adult bone marrow. Also, the intent of these experiments was not to define quantitative differences in hematopoiesis, but to identify qualitative differences in the potential of fetal or adult HSC populations to generate distinct lymphocyte lineages. Thus, we wished to include all potential populations of HSC/progenitor present in fetal or adult tissues. In this manner, we would not be likely to exclude unique populations of progenitors that expressed different surface phenotypes between fetal and adult life.

Isolation of fetal liver, fetal bone marrow, and adult bone marrow CD34+ cells was performed by magnetic selection for CD34+ cells, as described previously (50). HLA typing was done by flow cytometry on CD34- cells that remained after selection, using a panel of HLA antibodies (A2, A3, A9, A11) (Fig 5A, B). Selected CD34+ cells were cryogenically stored until a suitable (HLA-mismatched) cohort of SCID-hu Thy/Liv mice could be generated. The SCID-hu Thy/Liv cohort was HLA typed and determined to have a mismatch allowing identification of mature T cells derived from fetal or adult HSC populations (Fig 5B). As expected, fetal liver was enriched for “true” CD34+CD38lo/-c-kit+ HSC populations whereas both fetal and adult bone marrow contained a greater proportion of CD34+CD38+ progenitors, although both contained “true” HSC as well.

For the first experiments, we injected either fetal liver or adult bone marrow HSC populations directly into the thymic implants in SCID-hu Thy/Liv mice. The implants were allowed to mature for 18 weeks prior to injection and the mice were irradiated (250

Fig. 5

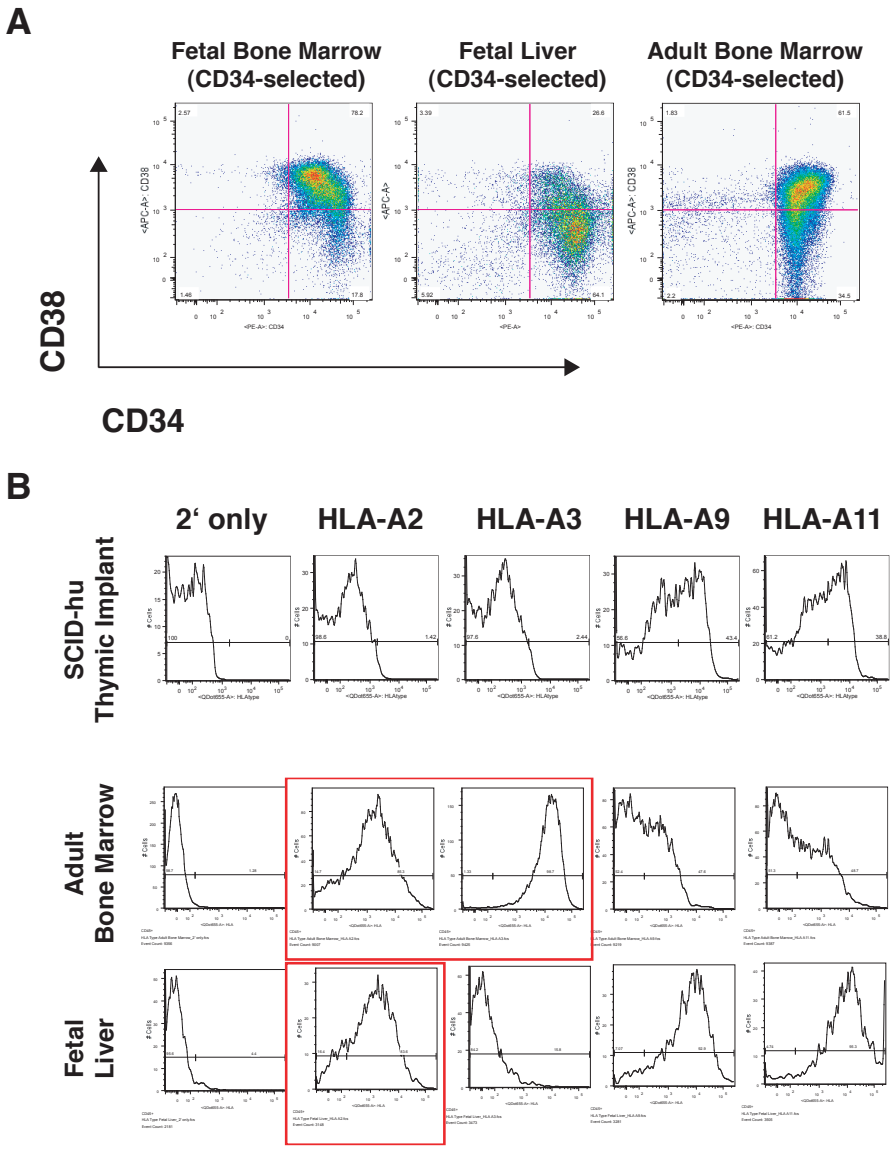

Figure 5. Phenotype of purified CD34+ HSC populations from fetal liver, fetal bone marrow, and adult bone marrow. A) Flow plots are depicted for all live cells and display the relative contributions of progenitors (CD34+CD38+) and “true” HSC (CD34+CD38- cells at different ages and in different tissues. Fetal liver and bone marrow are from the same 19g.w. donor and adult bone marrow are from a 25y.o. healthy donor. B) HLA type for SCID-hu thy/liv implant (top) and adult bone marrow or fetal liver/bone marrow HSC populations. HLA A2/A3 are suitable mismatches for adult bone marrow HSC and HLA-A2 is a suitable mismatch for the fetal HSC populations.

rads) immediately prior to injection of HSC, to deplete endogenous mature thymocyte populations and provide space for the engrafted HSC (Fig 6A, B). Three groups of mice were used: mock injected (5 mice), fetal liver HSC injected (5 mice; 250,000 cells/mouse), and adult bone marrow HSC injected (5 mice; 250,000 cells/mouse). The mice were monitored for 7 weeks, at which point they were sacrificed and the implants were harvested for analysis of T cell populations (Fig 6C). Whether differences in tissue localization contribute to differences in HSC function remains an important question. In subsequent experiments, we have included fetal bone marrow HSC to address whether these cells differ from the fetal liver HSC. These data, however, are not yet available.

Analysis of T cell populations within the thymic implants revealed that both fetal liver and adult bone marrow cells were able to support thymopoiesis. Fetal liver cells gave rise to a greater number of cells, likely reflecting both the fact that the fetal liver is enriched for "true" HSC with the potential for self-renewal and the fact that fetal HSC are generally known to have higher rates of reconstitution (42). Nonetheless, we were able to detect both fetal and adult HSC-derived thymocyte populations within the double negative (DN), double positive (DP), and both mature CD4+CD3+ (SP4) and CD8+CD3+ (SP8) populations (Fig 7A). Analysis of CD4+CD25+ thymic T_{Reg} populations revealed the surprising finding that only fetal liver CD34+ HSC were capable of generating thymic T_{Reg} (Fig 7A, B). Previous analyses of fetal thymus and infant thymus revealed no difference in the frequency of FoxP3+ cells; however, there was a slight difference with respect to the expression of CD25 within this population (12). No analysis of T_{Reg} frequencies or phenotype in the adult human thymus has been published and it is possible that the T_{Reg} seen in the infant thymus consist of some cells that are

Fig. 6

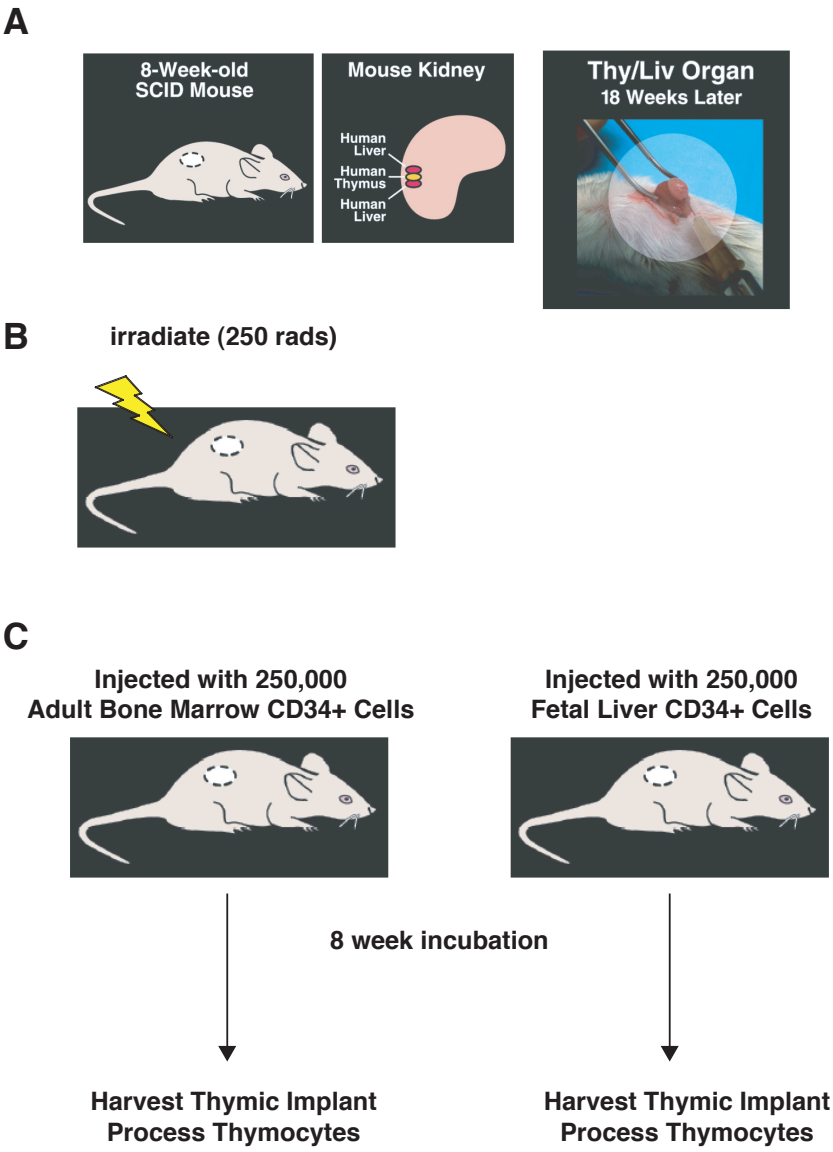

Figure 6. Construction of SCID-hu Thy/Liv mice and protocol for injecting HSC. A) method for implantation of fetal liver/thymus tissue under the kidney capsule of SCID mouse. B) Irradiation of mice prior to HSC injection. C) Injection protocol for fetal or adult HSC.

Courtesy of Dr. Cheryl Stoddart

Fig. 7

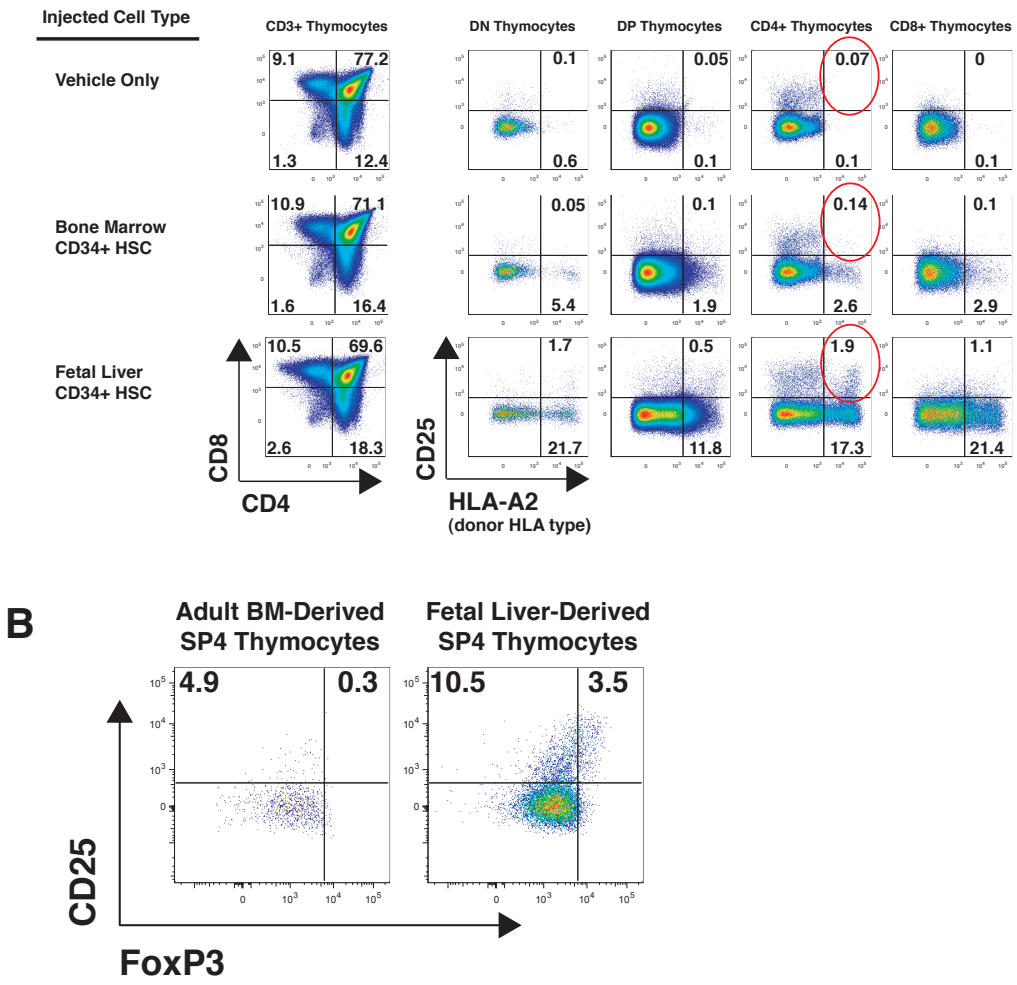

Figure 7. Thymocyte phenotype in SCID-hu Thy/Liv implants after HSC injection. A) Evidence for reconstitution of SCID-hu thymic implants by adult or fetal HSC. Top row demonstrates "vehicle only" controls which had no evidence of HLA-A2+ cells. HLA-A2+ cells are present in all thymocyte populations shown for adult and fetal HSC injected implants. Red circle indicates CD4+CD3+CD25+ thymic T_{Reg} compartment. Greater frequency of CD4+CD3+CD25+ T_{Reg} are seen in thymic implants injected with fetal HSC. B) FoxP3 expression was detected in CD4+CD3+CD25+ derived from fetal HSC but not seen in the CD4+CD3+ T cells derived from adult HSC. Experiments are representative of 3 separate experiments and 3-5 different SCID-hu implants from each experiment.

derived from fetal liver HSC that remain in the initial period after birth. Recent evidence points to a distinct pool of thymic progenitors with the potential to generate T_{Reg} (51). Thus, it is possible that fetal liver HSC may be more likely to generate a progenitor with T_{Reg} potential than adult HSC derived from adult (or fetal) bone marrow. If true, this would have significant implications for bone marrow transplantation.

The primary goal of these studies was to identify the molecular signatures of T cells derived from fetal and adult HSC. Our initial experiments revealed that there are in fact significant differences in the gene expression profiles of SP4 thymocytes derived from fetal or adult HSC in the context of the SCID-hu Thy/Liv thymic implant. These results are still pending, however, and a complete analysis and comparison is currently underway (data not shown). Of note, the gene BCL11A (which specifically was found in fetal peripheral T cells) was identified as being ~5-6 fold higher in SP4 thymocytes derived from fetal liver HSC. As mentioned previously, BCL11A is involved in regulating the switch between fetal and adult erythrocytes. The specific expression of BCL11A by fetal T cells suggests that BCL11A may play a larger role in determining differences between fetal and adult hematopoiesis.

We have also analyzed the gene expression profiles of SP4 thymocytes from fetal (19 and 20 g.w.) and infant (1 and 2 y.o) thymuses isolated directly ex vivo (Tables 1, 2). Here we were able to identify a number of differentially-expressed genes, including some that were identified as different for fetal HSC and adult HSC derived SP4 thymocytes (Tables 1, 2: genes that were also identified in preliminary screen of fetal versus adult HSC are highlighted in bold). Subsequent analysis of these gene sets will hopefully allow

the identification of genes that are consistently found to be high in fetal or adult T cells, regardless of whether they are found in the thymus or periphery.

Conclusions

At this point, several interesting conclusions can be made from these experiments. First, there are striking differences in gene expression by fetal and adult T cells that are likely to reflect significant differences in the functional properties of these cells. Previously, we observed that fetal T cells were prone to upregulate FoxP3 after stimulation with alloantigens (12). We also found that fetal HSC appear to be more capable of generating FoxP3+ SP4 thymocytes, perhaps reflecting a greater tendency of fetal T cells to adopt a T_{Reg} fate following stimulation. If true, this may explain why the fetal immune system appears to be uniquely prone to tolerance induction. A predisposition towards T_{Reg} development by T cells produced from fetal liver HSC is also backed by the observation that T_{Reg} numbers are high in the fetus (during the period when fetal HSC are located in the liver) and decline throughout the period when the hematopoietic function of the bone marrow becomes more evident (9). Whether there are differences in the developmental potential of fetal HSC that are found in the liver or bone marrow at similar stages of development remains a topic of interest.

Both Vγ3/δ1 DETC and B-1 B cells are thought to recognize and respond to self-antigens (28, 52). Since the fetus is generally considered to be shielded from most foreign antigens, it follows that populations of lymphocytes arising in this period may manifest preference for self-antigens. We found that fetal T cells in humans are also highly prone to spontaneous proliferation after removal of T_{Reg}, perhaps implicating self-antigens as a

source of stimulation (53). One interesting difference that has been described for fetal T and B cells in mice concerns the expression of terminal deoxynucleotidyl transferase (TdT), which is involved in adding nucleotides to junctional regions within the B and T cell receptors during lymphocyte maturation to increase the diversity of these receptors (54). We also noted that TdT was reduced in SP4 thymocytes in fetal thymus and in SP4 thymocytes derived from fetal HSC. In mice, enforced expression of TdT in fetal B cells was found to prevent the development of prototypical B-1 cells with specificity for self and bacterial antigens (55). Likewise, genetic ablation of TdT in mice was found to result in a reduction in T cell diversity that is thought to be due in part to increased rates of positive selection (56). Paradoxically, this may result in the generation of promiscuous T cell clones that may be capable of reacting with more self-antigens or MHC molecules. We have found that fetal T cells in human beings are highly responsive to alloantigens with far more cells responding than what is seen with adult T cells. This may be due to reduced diversity of the fetal T cell pool, leading to an increase in the ability of individual T cells to respond to unique antigens. In the case of allogeneic MHC there is already a relatively large fraction of T cells capable of responding to individual foreign MHC in adults, so reducing the threshold for allorecognition could result in a large number of T cells being able to respond to a given alloantigen (57).

The existence of a unique lineage of T cells during fetal development raises important questions about the nature of fetal immunity and the role of the fetal immune system in generating tolerance to self and foreign antigens. The fetal periphery is a rapidly evolving environment. Thus, the initial colonization of the fetal tissues by lymphocytes may pose a threat to the well being of the fetus should any self-antigen

trigger an inflammatory response. One possibility that we have considered through the course of these studies is that the first wave of fetal lymphocytes may play a fundamental role in conditioning the fetal periphery for the development of a "mature" immune system armed with the potential to promote inflammatory immune responses. In this view, the first wave of fetal T cells enters the newly developing peripheral lymphoid tissues and is directed to become T_{Reg} cells with specificity for any antigens that are presented in these sites. When the second wave of lymphocytes (derived from either fetal or adult bone marrow HSC) enters these tissues, there is already an intact mechanism to prevent immunity to any antigens found in the fetal tissues. Conceptually, this would make sense, as almost all antigens present in the fetus under normal conditions are self-antigens. Our observations regarding fetal tolerance to maternal alloantigens may thus represent an accidental, bystander phenomenon rather than a fundamental role of the fetal immune system.

This notion of a "layered" immune system has been proposed in the past (58). The initial identification of B-1 B cells and Vγ3/δ1 DETC T cells, coupled with the demonstration that a fetal progenitor was responsible for the generation of these cells, led to the suggestion that the immune system may not mature in a linear manner and instead may develop in layers. In this model, the successive waves of thymic colonization represent distinct forms of hematopoiesis which each give rise to different types of T cell. While this model for hematopoiesis has not been widely accepted, it would seem that our data fit very well within this framework for understanding the development of the immune system.

The idea of a layered immune system also helps to explain the stepwise acquisition of immunological competence described in fetal sheep and explains several enigmatic aspects of fetal infections (1). If the first wave of lymphocytes is promoting tolerance, fetal infections as this stage could give the appearance of failing to elicit immunity when in fact they are promoting tolerance to antigens present on the invading organism. Tolerance to microbial organisms has been described in the setting of fetal infection in both mice and humans (18,59,60). A layered immune system also explains why fetal immunity is compromised, despite the apparent generation of antigen-specific effector T cells. If the infectious agent were to gain access to the fetus at a time when both tolerogenic and "mature" T cell populations were present at similar proportions in the fetal periphery (e.g., during the latter part of the 2nd and throughout the 3rd trimester), both tolerogenic and effector T cell responses may be generated, with the former playing a dominant role to block protective immunity.

These findings raise some interesting and important questions about the nature of the fetal immune system. Fetal and neonatal infections pose a serious health risk in many parts of the world and understanding better ways to develop vaccines represents an important goal for the health care community. Moreover, the possibility that distinct lymphocyte populations may be able to contribute to tolerance versus effector immune responses suggests that it may be possible to manipulate the immune system to trigger a tolerogenic response to self or foreign antigens in children and adults. This would be a major advance for the treatment of autoimmune disorders and for aiding in transplant tolerance. Understanding the unique features underlying the generation and functional

properties of fetal and adult T cell populations would thus represent an important step towards achieving these goals.

Materials and Methods

Isolation of fetal and adult lymphocyte populations.

Fetal tissues were obtained and lymphocytes were isolated as described previously (Chapters 1 and 2). Fetal cord blood was isolated by manually dissecting umbilical cords from 19-20g.w. fetal specimens. Umbilical cords were subjected to rigorous washing with sterile phosphate buffered saline (PBS) to exclude any contaminating maternal lymphocytes. Adult peripheral blood and neonatal blood was also obtained as described in previous sections (Chapter 2).

Flow cytometry and cell sorting

For standard phenotyping cells were incubated with fluorescently labeled antibodies in cold MACS buffer (PBS w/2% fetal bovine serum (FBS) and 2mM EDTA) and data were collected with an LSRII flow cytometer (Becton Dickson). Data analysis was performed with the FlowJo software package (Treestar). For cell sorting, fetal lymph node (LN) and adult peripheral blood cells were isolated and incubated overnight at 37°C in RPMI media with 5% FBS. This pre-incubation was done to normalize fetal and adult cell populations and to reduce the chances that tissue-specific genes would be identified. Cells were stained in MACs buffer with antibodies specific for naïve (CD45RA, CCR7, CD27) and regulatory (CD25) T cell markers. Peripheral T cells were sorted on a FACS DIVA (BD) cell sorter. Sort purity was verified as >99% pure and cells were pelleted and were immediately frozen at -80°C. For SCID-hu Thy/Liv and fetal/neonatal thymus arrays, cells were stained immediately after isolation and sorted with a FACS ARIA

(BD). Sort purity was verified and cells were suspended in TRIZOL LS and stored at -80C for RNA acquisition at a later time.

RNA isolation and PCR

RNA for the initial microarray experiments on peripheral T cell populations was isolated with the Stratagene RNA isolation kit (Stratagene, Absolutely mRNA purification kit). Purity and quality was verified by a Nanodrop and Agilent bioanalyzer prior to preparation for microarray. RNA was then amplified (Nugen Ovation kit), fragmented, and labeled with biotin. Sorted cells from the SCID-hu Thy/Liv studies were processed for RNA using TRIZOL/Chloroform extraction and RNA was purified with RNeasy columns (Qiagen). Again, purity and quality was verified by Nanodrop and Agilent Bioanalyzer. RNA amplification for these experiments was performed with the Nugen FFPE amplification kit due to low yield. Sorted cells from fetal/neonatal thymus microarray experiments were also processed using TRIZOL/Chloroform extraction. In these experiments microarray analysis was done with custom designed gene array chips by competitive hybridization (courtesy of Dr. P'ng Loke). For quantitative real-time PCR, cDNA was generated by reverse transcription (Stratagene RT kit) and gene expression was determined using commercially available primers (Applied Biosystems Assay on Demand) labeled with FAM/TAMRA fluorescent probes. PCR was performed on a ABI7700 high performance RT-PCR machine (Applied Biosystems).

Hematopoietic stem cell isolations

Fetal liver was obtained and processed to select for CD34+ HSC as described previously (50). In brief, fetal liver was washed rigorously in sterile PBS and then cut into small fragments. These fragments were re-suspended in RPMI media containing 1 mg/mL collagenase/dispase (Roche) and 1 U/mL DNAse type I (Roche), and incubated at 37°C for 1 hour with periodic agitation. After 1 hour, the remaining fragments were removed and the suspended cells were subjected to density centrifugation to remove red blood cells (Ficoll Hypaque). Cells were harvested from the monolayer and incubated on ice for 1 hour with magnetically labeled CD34 antibodies (Miltenyi Bioscience CD34 isolation kit). CD34+ cells were then isolated by positive selection (Miltenyi LS columns). For fetal bone marrow, femurs and tibias were dissected and marrow was mechanically dissociated by scraping the interior. Adult bone marrow was obtained from healthy donors and mononuclear cells were isolated by density centrifugation (AllCells Inc.). Fetal and adult bone marrow HSC were isolated from re-suspended marrow by positive selection as described above.

SCID-hu Thy/Liv Studies

SCID-hu Thy/Liv mice were constructed as described previously (47, 61). The thymic implant was allowed to grow for 18 weeks so as to provide a suitable organ for stem cell injection. Prior to injection, the mice were irradiated to deplete endogenous thymocytes (Cesium source, 250 rads)(62). Immediately after irradiation, HSC populations were injected directly into the thymic graft in 50 μl of RPMI (250,000 cells/50 μl). Mice were monitored for 7 weeks at which time the thymic grafts were isolated and processed to

obtain thymocytes. Cells were immediately stained and analyzed or sorted by flow cytometry.

Microarray analysis and data analysis

Microarray analysis of peripheral T cells and SCID-hu thymocytes was done with the help of the Gladstone Genomics Core Facility. Affymetrix Human Genome U133 plus 2.0 were used for both peripheral T cell studies and for SCID-hu Thy/Liv studies. Data analysis was done by the Bioinformatics Core of the UCSF Clinical and Translational Science Institute (CTSI). Microarray analysis of fetal and infant thymocytes was done separately. For these experiments, RNA was then amplified for microarray analysis using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion). The 19 g.w. or 20 g.w. fetal thymic SP4 samples were labeled with Cy5 dye and hybridized against the Cy3-labeled 1 y.o or 2 y.o. infant thymic SP4 samples on Human Exonic Evidence-Based Oligonucleotide (HEEBO, Invitrogen) microarrays printed in-house at the UCSF Center for Advanced Technologies. The HEEBO oligos have been designed using an exon-centric approach to detect both constitute and alternatively spliced exons and represent at least one transcript from every gene that has been identified or confidently predicted in the human genome. Arrays were scanned using a GenePix 4000B scanner and GenePix PRO version 4.1 (Axon Instruments/Molecular Devices). The Spotreader program (Niles Scientific) was used for array gridding and image analysis. The resulting data files were uploaded to Acuity version 4.0 (Molecular Devices), where the raw data was log transformed and filtered for high quality spots. The results were exported into Excel as tables.

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Table 1. Genes Increased in Fetal SP CD4+CD3+ Thymocytes

Gene Name	Gene Function	Log_2 Expression 19 g.w. Fetal SP4/ 2 y.o. Infant SP4	Log_2 Expression 20 g.w. Fetal SP4/ 1 y.o. Infant SP4
HBG2	Oxygen Transport	5.2	3.64
HBG1	Oxygen Transport	4.98	2.85
CDC2	Mitosis	3.56	2.132
DLG7	Microtubule Stabilization	3.46	0.93
HIST1H3G	Chromatin Stabilization	2.7	1.43
CENPK	Mitosis	2.66	1.252
PSPHL	Phosphorylation/Signaling	2.66	1.01
S100B	Cell Survival	2.64	1.55
AURKA	Mitosis	2.58	0.9
TSPAN6	Cell Growth/Adhesion	2.49	2.332
IGF2BP3	Cell Growth/ Differentiation	2.49	1.022
SLC18A2	Transport of Cytosolic Monoamines	2.3	1.742
MTHFD2	Mitochondrial Metabolism	2.27	1.032
IGFBP2	Growth/Differentiation	2.14	1.75
HPGD	Prostaglanding Signaling	2.1	1.52
LOC285965	Unknown	2.1	2.0
PTTG3	Growth/Differentiation	2.0	0.9
ACOT7	Fatty Acid Metabolism	2.0	1.26
PLAG1	Transcriptional Regulation	1.99	1.39
OAS1	RNA Degradation/ Host Viral Defense	1.97	1.182
GZMA	Protease/ Apoptosis/ Host Defense	1.84	1.612
LDHA	Metabolism	1.75	1.2
GINS2	Mitsosis	1.72	1.23
AIF1	Growth/Migration	1.71	1.28
ZAK	Signaling/Phosphorylation/ TGFβ Signaling	1.66	1.15
NME2	Signaling/ Potassium Transport	1.56	1.39
PASK	Signaling	1.55	1.03
CSDA	Transcriptional Regulation	1.48	0.412
PLN	Signaling	1.34	1.43
CTSC	Protease/ Activation of GZMA	1.3	1.34
RHOBTB3	Signaling/ Vesicle Transport	1.12	1.23

Table 1. Genes Increased in Infant SP CD4+CD3+ Thymocytes

Gene Name	Gene Function	Log_2 Expression 2 y.o. Infant SP4/ 19 g.w. Fetal SP4	Log_2 Expression 1 y.o. Infant SP4/ 20 g.w. Fetal SP4
BZRP	Benzodiazepine Receptor	2.38	2.2
ARL7	Vesicle Traffic/ Endocytosis	2.0	1.33
SPINK2	Serine Protease Inhibitor	1.98	0.94
LYZ	Host Defense/ Protein Degradation	1.77	1.598
CD1A	Antigen Presentation	1.7	0.88
HLA-DRA	Antigen Presentation	1.41	1.37
SPOCK	Adhesion/Proliferation	1.36	1.01
SERTAD2	Proliferation	1.35	1.18
HLA-DP1	Antigen Presentation	1.3	0.92
IL-16	Chemokine	1.31	1.14
PNRC1	Signaling/ Hormonal Regulation	1.3	1.1
UBL3	Protein Regulation	1.2	1.13
HSPA1A	Cell Survival/ Stress Responses	1.19	1.06
TBXAS1	Thromboxane Synthesis	1.1	1.39
CBX7	Gene Silencing/Chromatin Organization	1.01	1.1
ARNT	Transcriptional Regulation	0.95	1.17
NIN	Mitosis	0.94	1.418
EGR2	Signaling/ Anergy	0.93	0.94

CHAPTER 5

Conclusions

In these studies we have addressed the functional features of developing fetal T cells in human beings. In the process we have described a novel form of dominant peripheral immune tolerance that is apparent at the onset of development of the fetal adaptive immune system. Based on our findings we concluded that a large fraction of T_{Reg} cells which are present in the developing fetal lymphoid tissues play a role in suppressing immunity during development (Chapter 2). Building on existing clinical and experimental data we formulated the hypothesis that one role for fetal T_{Reg} might be to prevent potentially harmful fetal anti-maternal immunity (Chapter 3). The basis for this hypothesis is that a growing body of evidence suggests that maternal cells frequently enter the fetal tissues throughout development (1-3). Considering our findings that fetal T cells were highly competent at responding to alloantigens, it seemed likely that one consequence of maternal microchimerism would be the activation of the fetal adaptive immune system. However our preliminary findings suggested that T cell responses to maternal alloantigens were reduced relative to responses to unrelated donors. Upon removal of the endogenous fetal T_{Reg} cell pool we were able to document a significant increase in fetal anti-maternal T cell responses suggesting that fetal T_{Reg} could block fetal anti-maternal immunity. This result was consistent with previous findings demonstrating that fetal γ/δ T cell responses were generated against maternal alloantigens *in utero* (4). Thus it would appear that activation of fetal T cell populations by maternal alloantigens is a common occurrence in the context of normal human pregnancy.

One of the more interesting observations taken from our studies concerns the many differences that we noted in our comparisons of fetal and adult T cell populations

(Chapter 4). The phenotype and function of fetal lymphocyte populations had not been well studied in the modern era of immunological research and much of what is known about fetal immunity was based on very basic studies performed more than 20 years ago. Since that time there have been many advances regarding our understanding of the immune system and the techniques available for scrutinizing biological systems. Paradoxically it is possible that fetal adaptive immune function has been so poorly studied because modern advances in biological techniques have resulted in the adoption of the mouse model as the primary model for addressing questions about immunology. Mice are a poor model for studying the development of the human adaptive immune system, owing to substantial differences in the rate at which each develops (5,6). Through the use of modern techniques we have been able to demonstrate striking differences in the molecular signature of fetal and adult T cell populations that were not immediately obvious from basic phenotyping by flow cytometry.

Our preliminary observations regarding the phenotypic and functional features of fetal and adult T cell populations led us to revisit some historical findings about the nature of the fetal immune response. The observations made by Silverstein and Colleagues in fetal sheep, bear particular importance in leading me to my final conclusions (7). The many eloquent studies published by Silverstein in the 1950's and 1960's became apparent to me after coming across a commentary published by Silverstein in the journal *Science* in the late 1990's (8). Silverstein took issue with several papers in which the author's "purported to overthrow the modern tenants of immunology" by demonstrating that they could achieve T cell activation in response to foreign antigens in neonatal mice (9-11). Silverstein's frustration with the modern view

that fetal (and in this case neonatal) immunity was generally compromised was well founded. He and many others had long since proven that many organisms including guinea pigs, opossums, sheep and human beings were capable of mounting immunity to foreign antigen during fetal development (reviewed in ref. 7). The real question that remained wasn't whether the fetus could mount an immune response, but rather what kind of immune responses can the fetus mount and what accounts for the appearance of immunologic maturity? What was clear from the studies of Silverstein and Colleagues was that immunologic competence was not an all or none phenomenon and that development of a functional immune response appeared in a gradual, step-wise manner.

Some interesting clues about the nature of the fetal immune response can be gleaned from the recent characterization of a large population of T_{Reg} cells in the fetal tissues and circulation (Chapter 2). Initially thought to be activated T cells, these newly defined T_{Reg} exhibit many of the classical features of adult T_{Reg} including the expression of CD25 and of the transcription factor FoxP3 as well as the ability to suppress T cell proliferation and cytokine production (12-14). However mid-gestation T_{Reg} exhibit some notable differences from T_{Reg} found in the UCB at birth, including the expression of the memory marker CD45RO rather than the naïve CD45RA isotype (12-15). Fetal T_{Reg} also appear to be prone to proliferation *in vitro* and *in vivo*, whereas adult T_{Reg} are generally thought to be quiescent *in vitro* (Chapter 3, Fig. S9). The frequency of T_{Reg} in UCB was found to decline in a linear fashion leading up to birth, at which point the frequency of T_{Reg} within the total CD4+ T cell compartment is similar to adult levels of ~5-8% (16). Because of the well defined role of T_{Reg} in promoting peripheral tolerance to self antigens it seemed likely that elevated frequencies of T_{Reg} in the fetus may be essential for

reducing unwanted T cell activation in the rapidly developing (and changing) fetal tissues. Clinical evidence supports this hypothesis as some patients with severe forms of the genetic disease IPEX, resulting from mutations in FoxP3, manifest severe autoimmunity *in utero* resulting in eventual demise (17). These clinical findings underscore both the functional potential of the fetal immune system and the importance of T_{Reg} in regulating fetal immunity.

The finding that elevated T_{Reg} in the fetus may play an important role in suppressing "unwanted" T cell responses is perhaps not surprising though it does raise many interesting questions about the nature of the fetal immune system. The most obvious question concerns the mechanisms underlying the generation of these T_{Reg} and what accounts for the change in frequency leading up to birth. Several well known factors have been described which are capable of promoting the development of T_{Reg}. Among these TGFβ stands out as the best characterized inducer of T_{Reg} (18). We found that TGFβ family members were elevated in fetal lymphoid tissues and that inhibition of TGFβ signaling could block FoxP3 upregulation caused by stimulation of naïve fetal T cells with alloantigens (Chapter 3). Stimulation of fetal T cells also led to a TGFβ-dependent acquisition of suppressive function, suggesting that FoxP3 upregulation led to development of regulatory properties. This last point deserves some notice in light of recent findings that polyclonal activation of adult naïve CD4+ T cells in the presence of TGFβ could lead to acquisition of FoxP3 protein expression in the absence of suppressive function (19).

Thus our *in vitro* studies suggested a potential role for TGFβ in driving the induction of T_{Reg} cells in fetal tissues. The hypothesis that T_{Reg} may be induced in the

periphery, rather than generated during thymic maturation, was also supported by our findings that T_{Reg} frequencies were relatively stable in the fetal and neonatal thymus (Chapter 3, Figure S6) whereas peripheral T_{Reg} frequencies declined significantly in the same timeframe (16). Moreover we found evidence of significantly greater frequencies of FoxP3+ cells that were actively proliferating in fetal versus adult LN samples suggesting either an increase in the rate of expansion of the existing T_{Reg} cell pool or the acquisition of FoxP3 during T cell activation in the periphery (Chapter 3, Figure S9).

Because of the vast number of potential self-antigens that fetal T_{Reg} may recognize, we were forced to consider more tractable sources of antigen to probe the question of whether peripheral T_{Reg} induction could occur during fetal development. It was for this reason that we chose to study maternal alloantigens as a potential source of antigen. The decision to study the effect of maternal alloantigens was based on both experimental and clinical observations regarding the apparent tolerance that many individuals exhibit towards non-inherited maternal HLA types (20,21). Our hypothesis that fetal T_{Reg} could suppress fetal anti-maternal T cell immunity proved true and led to the serendipitous observation that fetal T cells were highly responsive to alloantigens in a primary MLR. This was particularly evident when comparing fetal T cell proliferation in the first 4-5 days after stimulation with cells from a single unrelated donor with adult naïve CD4+ T cell proliferation (Chapter 3, Figure 1, Figure S6).

In an effort to establish the basis of the functional discrepancies that we observed for fetal and adult T cells, we performed global gene expression analysis on sorted naïve CD4+ fetal and adult T cells (Chapter 4, Figure 2). Sorted fetal and adult CD4+CD25^{high} T_{Reg} cells were also analyzed in parallel. The results of our gene expression analysis

revealed striking differences in the gene signature of fetal and adult T cells that was conserved between both naïve CD4+ T cells and T_{Reg} cells. We still have yet to address specific roles for any of the identified genes in regulating fetal versus adult T cell function. A preliminary assessment of the most highly differentially expressed genes revealed a large number of the genes expressed by fetal T cells that have been previously implicated in cellular proliferation and regulation of apoptosis. Among the more notable genes identified was BCL11A which has recently been implicated in determining the frequency of erythrocytes expressing fetal hemoglobin (F cells) in the adult circulation (22). A specific role for BCL11A in regulating the expression of fetal and adult hemoglobin expression has since been uncovered. BCL11A has also been identified as a critical factor regulating the developmental potential of HSC implicating BCL11A as an important transcriptional regulator at multiple stages of hematopoiesis (23). Whether BCL11A plays a more general role in balancing fetal and adult hematopoiesis remains unclear and is a topic of ongoing investigation.

Our findings raise several questions that have not been answered but remain active areas of investigation. We limited our studies to α/β CD4+ T cells however it is not unreasonable to assume that distinct lineages of all immune cells might exist throughout development. As previously mentioned there have been reports that distinct B cell (24) and γ/δ T cell lineages (25,26) are derived from fetal HSC in mice though these findings have yet to be reproduced in human beings. Therefore, while constituent cells of the fetal and neonatal immune system are typically regarded as immature versions of those found in the adult, a second possibility to consider is that these cells are in fact different forms of the same type of cell.

Another intriguing possibility that we have considered is that HSC present in the adult liver might retain features of fetal HSC. If this were true then it may offer some insight into the tolerogenic properties of liver allografts (27). The establishment of microchimerism by passenger HSC in transplanted livers is a well-documented phenomenon and has been shown to play a critical role in promoting tolerance in the setting of liver transplantation (28). The recent description of a female patient who developed complete hematopoietic chimerism following receipt of a male liver transplant underscores the potential of the liver as a site of hematopoiesis after birth (29). Further the absence of overt GVHD or rejection of the donor liver in this patient despite removal of immunosuppressive therapy one year after the transplant suggests that the liver HSC population might provide a suitable means of generating tolerance. An alternative explanation for the observed differences between bone marrow HSC and liver HSC in the context of transplantation could be that bone marrow preparations typically are less pure and may contain resident mature lymphocytes that are the source of GVHD responses. Ongoing clinical efforts have focused on deriving highly purified populations of HSC to exclude the potential for these responses (30).

A major unanswered question raised by our findings concerns the nature of the immune response to foreign agents at different stages of development. A growing number of pathogens are recognized to gain access to the fetus during pregnancy including viral and bacterial pathogens as well as more complex parasites such as *Toxoplasma gondii* and *Plasmodium falciparum* (reviewed in ref. 31). Intrauterine and neonatal infections are often associated with significantly greater pathologies than infection in a healthy adult. The general absence of an immune response is a reasonable explanation for the observed

severity of fetal and neonatal infections by organisms that are typically cleared by the adult immune system. This is not inconsistent with our model, where rather than an absence of fetal immunity to infection a tolerogenic response could be formed. This latter explanation may be more consistent, however, with several different clinical findings. First, as mentioned previously, patients with IPEX show massive inflammation *in utero*, providing evidence that an active immunosuppressive mechanism involving FoxP3+ T_{Reg} is responsible for controlling fetal T cell immunity (17). Second, there are several reports that have demonstrated the existence of pathogen-specific T cells in the UCB of fetuses infected or exposed to pathogens *in utero* (32-34). Thus it would appear that T cell immunity can be acquired to foreign pathogens during fetal development. Similar findings have been documented regarding NK cell maturation in the setting of fetal infection (35) as well as antibody production and isotype switching by B cells during fetal infections (36).

Perhaps the most intriguing cases of fetal infection are those that occur in settings where pathology is not observed. Chronic viral infections such as Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV) are typically poorly dealt with by the adult immune system. Alternatively, these viral infections are rarely found to affect the fetus and many children born to untreated infected mothers show no signs of infection *in utero* (reviewed 31). A growing body of evidence suggests that exposure may still occur in the absence of infection suggesting that virus can enter the fetal tissues but may not establish active infection in many cases (33). This is not surprising as the viral burdens associated with chronic retroviral infections are generally very high with large amounts of viral particles detectable in the circulation. Considering that there is likely to

be some intermingling of the fetal and maternal circulation during pregnancy the chances of fetal exposure would appear quite high. Yet vertical transmission during pregnancy is relatively rare prompting the question, does the fetal immune system control these types of infections such that the fetus doesn't become chronically infected?

There is some precedent for this hypothesis from classical studies in murine models of lymphocytic choriomeningitis virus (LCMV) infection (17). Pathogenic LCMV infection occurs with particular strains of LCMV that are capable of establishing chronic infections (37). Thus the disease associated with LCMV is linked to the chronic nature of the infection suggesting that chronic inflammation resulting from the maintenance of viremia over a long period of time could account for the pathogenic nature of the infection. However, mice that are exposed to LCMV *in utero* become chronically infected yet fail to develop signs of disease (17,38). This unexpected result challenged the traditional view that a mature immune responses is necessary for the host to defend itself from viral infections. In the case of chronic infections it appears that a tolerogenic response may prove beneficial for the host, as it would prevent the potentially harmful consequences that arise from prolonged inflammatory responses.

Whether a similar situation exists for HIV and Hepatitis viruses remains unknown. There is some evidence that intrauterine transmission can prove lethal for HIV infection but this appears to happen very rarely with most pathogenic infections occurring at birth or from breastfeeding (31). A recent study examining whether uninfected infants born to HIV+ mothers showed signs of exposure during fetal development suggests that immunologic exposure is likely to occur frequently (33). In these exposed uninfected neonates there was additional evidence that T_{Reg} may have been generated to suppress T

cell immunity to HIV antigens. Similar findings have been made for other intrauterine infections, however many of these studies were limited by small numbers of patients and more data is clearly needed (39).

The development of immunologic maturity in human beings has continued to be a major field of study. The window for which vaccinations can provide protection is not well established and understanding the mechanisms underlying successful vaccination strategies is an important clinical consideration. Our work has provided a new explanation for the differences that are seen between the developing and the mature adaptive immune system. If these findings hold true, it could have a great impact on our understanding of the mechanistic basis of immunological maturity. Moreover it sheds new light on the development of immunological tolerance in human beings. The development of techniques for generating immunological tolerance to foreign cells and even to pathogens is one of the primary goals of modern immunology. This will be necessary for a range of clinical settings including solid organ transplantation, embryonic stem cell transplantation, and the treatment of autoimmune disorders.

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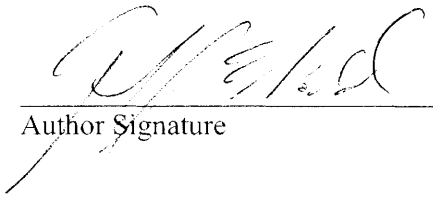

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