SPECIFIC AIMS

The development of the mammalian immune system is typically thought to occur in a linear fashion, from immaturity to maturity as a function of antigen exposure. Previous findings in birds and in mice, however, indicate that this view is oversimplified. Thus, in these species, the developing immune system appears to be "layered"[1] in a manner that is independent of antigen exposure, beginning as a multilineage fetal system that is replaced by an anatomically and biologically distinct multilineage system after birth.[2,3] If so, then developmentally ordered and unique hematopoietic stem/progenitor cells (HSPC) could give rise to distinct lymphocyte lineages at different stages of development.

In ongoing experiments, we have found that such immune system "layering" occurs in humans. Our preliminary data show that: (1) the human fetus can mount a vigorous immune response to exogenous antigens;[4] (2) the human fetal immune response to exogenous antigens can be actively suppressed by antigen-specific Tregs;[4,5] (3) fetal Tregs are derived from a fetal-specific lineage of T cells; (4) fetal and adult HSPCs give rise to distinct populations of T cells; and (5) fetal HSPC-derived T cells show an enhanced ability to generate Tregs during thymic maturation and upon exposure to foreign antigens *in vitro*.[6] These data suggest that the human immune system is comprised of two distinct waves: one generated from a "fetal" HSPC that exists *in utero* in the fetal liver and bone marrow, and another generated from a superseding "adult" HSPC that resides in the bone marrow at later time points. The former gives rise to an immune system that is prone to deliver a tolerogenic response to foregin antigens. The latter gives rise to an immune system that is more likely to generate an immunoreactive responses (*e.g.,* one including cytotoxic T cells and neutralizing antibodies).

Given these findings, we hypothesize that physiologic layering of immune system ontogeny leads to a normal range in the ratio of fetal- to adult-type T cells at birth, with some neonates exhibiting a higher fraction of fetal T cells than others; and that those with a high ratio of fetal/adult T cells will generate predominant Th2 responses to routine childhood immunizations.

These hypotheses will be addressed in the experiments of the following Specific Aims:

Specific Aim 1. To determine the normal range of fetal to adult T cells in the umbilical cord blood of the full term neonate.
In these experiments, comprehensive phenotypic, transcriptional, and functional analyses will be carried out on umbilical cord blood (UCB) mononuclear cells from a total of 200 normal full-term deliveries, obtained over an 18-month time frame from the Human Cord Blood Bank of the UCSF Clinical and Translational Sciences Institute, from Dr. Elizabeth Shpall of the University of Texas M.D. Anderson Cancer Center, and as part of a prospective study to be carried out with Dr. Shannon Thyne of the Child Health Center at San Francisco General Hospital (SFGH). Naïve T cells in these samples will be studied to determine the ratio of fetal/adult T cells (T_F/T_A) and the relationship of this ratio to naïve T cell function.

Specific Aim 2. To determine whether those full-term neonates with a high ratio of fetal/adult T cells are more likely to generate a Th2-polarized immune response to routine childhood immunizations.
Under the auspices of an existing protocol that has been approved by the UCSF Committee on Human Research protocol and in collaboration with Dr. Shannon Thyne, 50 full-term infants will be followed from birth through 12 months. Cord blood samples obtained from each of these newborns will be examined for the T_F/T_A ratio and this ratio will be related to the response of the newborn to hepatitis B vaccination.

We anticipate that this study will reveal normal variation in the ratio of fetal to adult T cells at birth and that such variability in this ratio will be directly related to – and possibly causal of – a Th2 skew that results in a poor response to childhood vaccines *and* a heightened predisposition to childhood infections and to atopic disorders. If so, then modalities aimed at changing this ratio more towards the adult lineage at birth may provide benefit to a substantial number of newborns.

RESEARCH STRATEGY

(a) SIGNIFICANCE The developing human immune system faces a balancing act that must be carefully timed. On the one hand, it must tolerate the presence of the surrounding mother and her non-inherited maternal alloantigens (NIMA) or otherwise risk the potential of engaging a fatal "graft vs. host" disease. On the other hand, novel antigens must be recognized as foreign when encountered after birth, triggering a vigorous adaptive immune response (*e.g.*, with cytolytic T cells and neutralizing antigens) against them. Otherwise, the newborn will be susceptible to diseases caused by multiple infectious agents.

In ongoing experiments, we have obtained preliminary data (see below) indicating that this switch from a fetal-type to an adult-type immune response is dependent upon the stage-specific appearance of distinct multilineage hematopoietic stem/progenitor cells (HSPC).[6] Thus, *in utero*, hematopoiesis in the first and second trimester is largely sustained by a fetal-type HSPC that gives rise to tolerogenic Tregs; later (and perhaps as early as the third trimester), an adult-type HSPC instead gives rise to immunoreactive T cells. The timing of this switch coincides with birth and normally allows the newborn to move from a stance of tolerance to one of active defence against all foreign antigens. In this manner, the "immune privileged" aspect of mammalian pregnancy is preserved while the ability of the newborn to fight infections is also permitted.

Nonetheless, infection remains a leading cause of death and morbidity in newborns. Not only are neonates susceptible to more severe forms of disease caused by human pathogens such as herpes simplex virus 1, respiratory syncytial virus, *Bordetella pertussis* and *Staphylococcus aureus*), they are also subject to serious infection by microbial entities that are commensal flora in adults. For example, even after implementation of intensive screening and prevention practices, the estimated rate of Group B Streptococcal sepsis in the first week of life is 0.34 per 1000 live births, resulting in 60-70 deaths per year.[7] In addition to the immediate impact of neonatal illness and death, the long-term disability resulting from these infections represents a profound public health burden.[8] Premature infants, in particular, are predisposed to more severe infections from all pathogens and can also succumb to fatal infection by microbes that infrequently cause severe disease in adults, such as *Staphylococcus epidermidis.*[9] Compared with adults and older children, newborns produce less, and generally less effective, antibody in response to most immunizations. They are also less able to generate T cells that mediate effective antimicrobial responses.[10-15] Together, these deficiencies render the neonate a vulnerable target for a host of invading pathogens.

If the switch to an "adult-type" immune system is incomplete or overly slow after birth, two other problems may also arise. First, the neonate may respond less well to immunizations provided during the first months of life, either generating low levels of an effective response or polarized features of a non-effective response.[10,11,16-25] Secondly, those neonates that are most likely to develop atopic disorders after birth are also those who are most likely to generate suboptimal (and/or strong Th2-type) response to vaccination.[21,26] Since fetal Tregs may suppress Th1-type (or other) immune responses to vaccines in a manner that is different than adult Tregs, we speculate that strong Th2 polarization of childhood responses to vaccines may in part be due to a higher than normal proportion of fetal Tregs at birth.

In the studies of this proposal, we hypothesize that the immune system "layering" that is necessary for effective *in utero* development and postnatal protection of the human fetus occurs at a dissimilar pace in different individuals, predisposing some at birth to less effective immune responses to childhood immunizations.

(b) INNOVATION Previous experiments have demonstrated that similar "layering" of the immune system can occur in avian and murine models.[1-3] In these species, however, the timing and/or anatomic constraints are entirely different. In particular, the murine immune system develops at a markedly different pace than does the human immune system, *e.g.*, with very few Tregs detectable until three days after birth[27] as compared to the late 1st trimester in the human. This study is innovative in two respects: this is the first time that human immune system layering has been studied *in utero* and at birth; in addition, we have identified and validated a set of genes that are uniquely expressed in fetal or adult T cells, allowing us to quantitatively and qualitatively study the kinetics of the two populations as a function of time. The proposed research has the potential to improve prevention (through improved vaccine strategies) and treatment of neonatal infection (by providing a better understanding of normal human fetal immune development), and should teach us how the developmental state of the fetus and newborn affects their ability to respond to pathogens or vaccines.

(c) RESEARCH PLAN We propose to test the hypothesis that the immune system of the human newborn is comprised of two distinct hematopoietic lineages, one derived from a multilineage HSPC that resides in the fetal liver and bone marrow, and another from an HSPC that begins to function later in pregnancy and that supplants the fetal lineage thereafter. The former lineage is endowed with tolerogenic T cells that allow the fetus to co-exist with the mother (including her NIMA and other foreign antigens circulating with her); the latter lineage is instead comprised of T cells that are more likely to develop effector functions against novel antigens. The basis for this hypothesis is found by precedent in avian and murine models as well as by way of our largely unpublished Preliminary Data:

The human fetus can mount a vigorous immune response to exogenous antigens. Although the human fetal and neonatal adaptive immune systems are often described as "immature" (*i.e.,* dysfunctional or ineffective at mounting a response to antigenic challenge), there is substantial evidence that immune responses can develop at or before birth in species such as sheep and nonhuman primates (but, interestingly, not mice) (reviewed in refs.16 and 28). To determine whether human fetal T cells are responsive against alloantigens, fetal (~20 g.w.) lymphocytes from spleen or lymph node (LN) were labeled with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with irradiated antigen-presenting cells (APCs) that had been isolated from the peripheral blood of a single healthy adult donor. After five days substantive proliferative responses were observed for both $CD4^+$ and $CD8^+$ fetal T cells (Fig. 1). This finding raised the question: if fetal T cells respond so vigorously to alloantigens *in vitro*, do they not also respond to NIMA expressed by maternal cells that have moved into fetal LNs *in utero*?

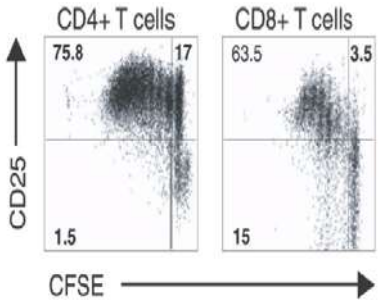

Figure 1. Representative plot of CD4+ and CD8+ fetal T cell proliferation after stimulation with allogeneic APCs from an unrelated donor for 5 days (3:1 ratio of fetal lymphocytes: allogeneic APCs)

The human fetal immune response to exogenous antigens can be actively suppressed by antigen-specific Tregs. We recently demonstrated that human fetal secondary lymphoid tissues contain significantly higher frequencies of $CD4^+CD25^{high}$ Tregs than those of adults.[4] Because Tregs have been shown to regulate maternal immunity to fetal alloantigens,[29] we reasoned that fetal Tregs might also play a role in suppressing fetal immune responses against invading maternal cells. To test this hypothesis, fetal LN cultures were either depleted or "mock-depleted" of Tregs before stimulation with self (autologous), maternal, or unrelated allogeneic APCs. Depletion of Tregs resulted in a highly significant increase in proliferation of $CD4^+$ and $CD8^+$ T cells responding to autologous or maternal APCs, but only a slight (yet statistically significant) increase in proliferation of those responding to unrelated APCs (Fig. 2). These data indicate that fetal T cells are not inherently deficient at responding to maternal alloantigens; rather, they are actively suppressed by fetal Tregs.

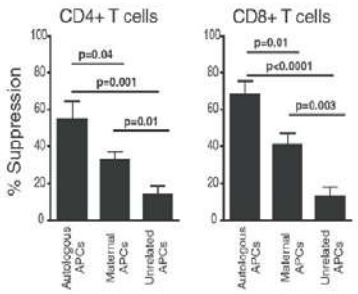

Figure 2. Comparison of fetal T_{Reg} cell suppression against autologous, maternal, or unrelated APCs determined based on the following calculation: % Suppression = 1-[(%CFSElow (total LN cells))/(%CFSElow (CD25-depleted LN cells))]

The frequency of Tregs 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.[30] To test the hypothesis that such a change in frequency reflects a greater propensity of fetal naive $CD4^+$ and $CD8^+$ T cells to differentiate into Tregs in response to stimulation, fetal LN cells were depleted of $CD25^{high}$ Tregs and stimulated *in vitro*. After a five-day primary mixed lymphocyte reaction (MLR), a significant fraction of fetal, but not adult, $CD4^+$ and $CD8^+$ T cells had divided and upregulated FoxP3 expression to high levels (Fig. 3A, B).

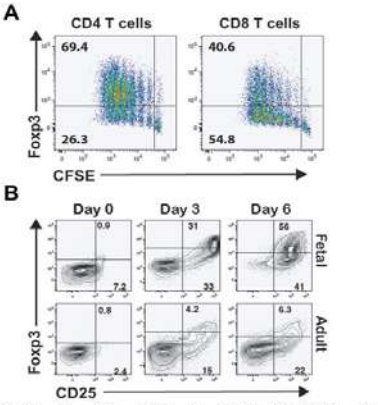

Figure 3. (A) Fetal T cells depleted of CD25+Foxp3+ cells were stimulated for 5 days with unrelated APCs and Foxp3 expression measured. (B) CD25 and Foxp3 upregulation by adult and fetal T cells depleted of CD25+ Foxp3+ cells at day 0, as a function of time after stimulation.

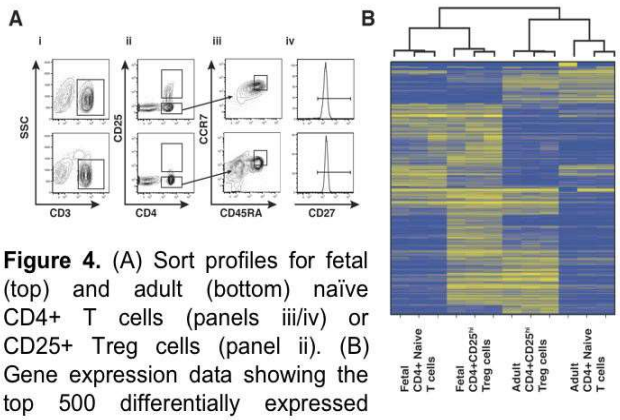

Figure 4. (A) Sort profiles for fetal (top) and adult (bottom) naïve CD4+ T cells (panels iii/iv) or CD25+ Treg cells (panel ii). (B) Gene expression data showing the top 500 differentially expressed genes and clustering

Fetal Tregs are derived from a fetal-specific lineage of T cells. The above studies revealed profound differences in function between fetal and adult T cells that had otherwise indistinguishable phenotypes. To determine whether such differences are intrinsic to the T cell lineages found during these stages of ontogeny, $CD4^+CD25^{high}$ Tregs and $CD4^+CD45RA^+CCR7^+CD27^+$ naïve T cells from fetal and adult samples were sorted with a FACSDiVA (Fig. 4A). Microarray analysis (Fig. 4B) identified thousands of genes whose expression levels in adult and fetal $CD4^+$ naïve T cell populations differed significantly (P<0.05) and in a highly consistent manner between donors, including NOG, GZMA, and RGS1 were highly expressed (20-55 fold greater) by fetal cells whereas NAP1L2, NR3C2, and SYT4A were highly expressed by adult cells.

Fetal and adult HSPCs give rise to distinct populations of T cells. In avian and mouse models, there is strong evidence that fetal HSPCs give rise to unique subsets of lymphocytes that cannot be generated from adult HSPCs and that immune system "layering"[1] occurs during ontogeny.[2,3] To test whether a similar situation exists in humans, we performed a series of experiments in which fetal HSPCs from fetal liver and BM (18-22 g.w.) and adult BM samples were injected directly into the human Thy/Liv organ of the SCID-hu Thy/Liv mouse.[31] Following a 7-8 week maturation period, we were able to identify mature thymocyte populations derived from each HSPC population, based on the expression of a unique HLA type (typically HLA-A2 or A3) expressed by the donor (source of HSPCs) but not by the recipient thymic implant.[31,32] We isolated mature $CD3^+CD4^+CD8^-CD25^-$ thymocytes from thymic implants injected with fetal liver, fetal BM, or adult BM-derived HSPCs by FACS (FACS Aria), and performed microarray analysis on each population (Fig. 5A). We found that both HSPC populations from fetal liver and BM gave rise to identical populations of $CD4^+$ thymocytes on the basis of gene expression, with no differentially-expressed genes between them (Fig. 5B). By contrast, adult BM-derived HSPCs gave rise to $CD4^+$ thymocytes that showed substantial differences in gene expression patterns compared to each population of fetal HSPC-derived thymocytes (Fig. 5B: 1243 and 1162 differentially-expressed genes versus fetal liver and fetal BM, respectively). These data are consistent with the hypothesis that the developmental stage of HSPCs is in part responsible for the differences seen in peripheral T cell compartments in the fetus and adult, and that layering occurs during the ontogeny of the human immune system.

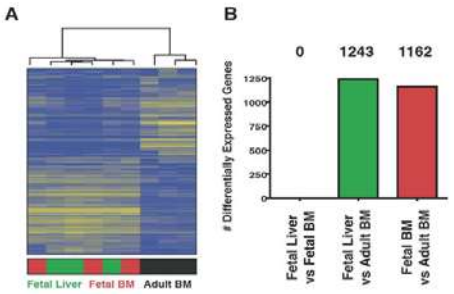

Figure 5. (A) Clustering of the top differentially expressed (DE) genes by CD4+ thymocytes from fetal liver HSPC (green), fetal BM HSPC (red), and adult BM HSPC (black). (B) Total number of DE genes (>2-fold difference expression difference and p<0.05) for each thymocyte population.

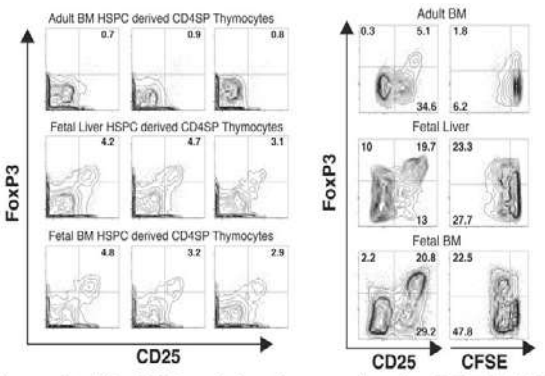

Figure 6. (A) CD25 and Foxp3 expression by CD3+CD4+CD8- thymocytes from adult BM, fetal liver, or fetal BM (3 SCID-hu thymic implants/group). (B) Expression of CD25 and Foxp3 (left) and proliferation (CFSE-dilution, right) following stimulation of CD3+CD4+CD8- thymocytes with allogeneic APCs for 7 days *in vitro*.

Fetal HSPC-derived T cells show an enhanced ability to generate Tregs during thymic maturation and upon exposure to foreign antigens *in vitro*. $CD4^+CD25^+Foxp3^+$ Tregs can be generated during thymic maturation or following activation of peripheral T cells. Some evidence indicates that Tregs may arise from a committed progenitor that is distinct from conventional T cell precursors.[33] We observed that fetal HSPC-derived thymocyte populations contained significantly greater frequencies of Tregs than those derived from adult HSPCs (Fig. 6A). In accordance with what we observed in peripheral fetal and adult T cell populations, we also noted that fetal HSPC-derived thymocytes were highly responsive to stimulation with allogeneic APCs and showed a propensity to differentiate into $FoxP3^+$ Tregs (Fig. 6B).

In sum, the above Preliminary Data indicate that the fetal

immune system is derived from a HSPC that gives rise to tolerogenic Tregs while the adult HSPC is more likely to give rise to immunoreactive T effector cells. At this point, we have very little information about the relative balance of these two compartments at birth. It is also not clear whether and to what extent variations in this balance may impact upon the response of the neonatal immune system to novel antigens, including those associated with routine vaccines or with environmental allergens. The experiments of this proposal are designed to explore these questions.

Specific Aim 1. To determine the normal range of fetal to adult T cells in the umbilical cord blood of neonates at birth

Hypothesis. Physiologic layering of the human immune system during ontogeny leads to a normal range in the ratio of fetal to adult-type T cells at the time of birth, with some neonates born with a more tolerogenic immune system than others.

Rationale. As described in the above Preliminary Data, the human fetal immune system is poised to generate a tolerogenic Treg response upon stimulation, an attribute that is conferred by an HSPC that resides within the fetal liver and bone marrow. After birth, bone marrow-derived HSPC give rise instead to immunoreactive T cells with a reduced propensity to generate Tregs. Teleologically, such "layering" of the immune system would appear to be consistent with, and possibly necessary for, maintenance of the semi-allogeneic state of pregnancy and, reciprocally, for the generation of an active immune response against foreign (*e.g.,* infectious agents) after birth. Similar stage-specific waves of distinct hematopoietic progenitors have also been described in avian and murine models.[1-3] A key question that remains unanswered is the following: is there inter-individual variation in the rate at which the fetal-type hematopoietic system is replaced by the adult-type system over time? In this Aim, we propose to determine whether and to what extent such variability may exist at the time of birth. Given known transcripts that uniquely identify tolerogenic fetal T cells (T_F) and immunoreactive adult T cells (T_A), the normal range of these two T cell subpopulations in the umbilical cord blood will be determined.

Experimental Approach. Comprehensive phenotypic, transcriptional, and functional analyses will be carried out on umbilical cord blood (UCB) mononuclear cells from a total of 200 normal full-term deliveries. Over an 18-month time frame, 75 of these samples will be obtained on a recharge basis from the Human Cord Blood Bank of the UCSF Clinical and Translational Sciences Institute (see attached letter from Dr. William Balke), 75 will be obtained on a collaborative basis from Dr. Elizabeth Shpall of the University of Texas M.D. Anderson Cancer Center (see attached letter), and 50 will be obtained as part of a prospective study to be carried out with Dr. Shannon Thyne of the Child Health Center at SFGH (see attached letter). Initial studies will focus on naive T cells obtained by a combination of ficoll hypaque gradient enrichment and FACS sorting; excess cells will be viably cryopreserved in liquid nitrogen for future experiments that may interrogate other subpopulations of cells. The following assays will be carried out:

1. Phenotypic analysis of T cell populations. The frequency of various T cell populations in the cord blood will be analyzed using standard markers of naïve $CD4^+$ T cells ($CD45RA^+CD27^+CCR7^+$), memory/effector T cells ($CD4^+CD45RO^+CD95^+HLA\text{-}DR^+$), and Tregs ($CD4^+CD25^{high}FoxP3^+CD127^{low}$).[5] Absolute numbers will be quantified using TRUcount tubes (BD).
2. Transcriptional analysis of naïve T cell populations. Phenotypically-pure naïve $CD4^+$ T cells will be obtained by sort purification on a FACSAria (BD) and subjected to qRT-PCR assay to detect transcripts (transcript specific to cell-type Z denoted as ${}_tZ$) that are unique to fetal naive T cells (*e.g.,* NOG, GZMA, and RGS1; ${}_tT_F$) or to adult naïve T cells (*e.g.,* NAP1L2, NR3C2, and SYT4A; ${}_tT_A$) as well as transcripts for house-keeping genes that are equivalently expressed in fetal and adult naive T cells (*e.g.,* the β chain of the T cell receptor or HPRT; denoted by ${}_tT_X$). Each transcript will be quantitated in replicate and three standardized ratios of fetal/adult T cell transcripts (F/A-T) will be calculated based on the formula $({}_tT_F/{}_tT_X)/({}_tT_A/{}_tT_X) = ({}_tT_F/{}_tT_A)$. The three ratios will be F/A-T1 (NOG/NAP1L2), F/A-T2 (GZMA/NR3C2), and F/A-T3 (RGS1/SYT4A), and the mean of these ratios will be used to represent the fetal/adult T cell ratio (T_F/T_A).
3. Functional analysis of T cell populations. To test whether UCB T cells upregulate FoxP3 and adopt a Treg phenotype upon activation with alloantigens,[5] naïve $CD4^+$ T cells will be isolated by FACS and stimulated with allogeneic adult APCs plus or minus concurrent stimulation with cross-linking antibodies against CD3 and CD28. This type of stimulation reliably leads to Treg differentiation from naive $CD4^+$ T_F (see Figure 3, Preliminary Data). Prior to stimulation, the cells will be labeled with CFSE for determination of proliferation. After six days of stimulation, the cells will be harvested and each T cell subpopulation will

be measured for proliferation (CFSE dilution) and for Treg differentiation (FoxP3 upregulation) by flow cytometry. Standard types of cytokine response (Th1, Th2, Th17, and Th22) will be measured by cytokine production after six days of differentiation *in vitro* by carrying out intracellular cytokine flow cytometry (CFC) for the following cytokines: IL-2, INF-γ, TNF-α, IL-4, IL-17, and IL-22. To test whether cord blood Tregs are better able to suppress Th1 vs. Th2 responses, $CD4^+$ naïve T cells will be sort-purified from a healthy adult (or cord blood) donor and cultured under Th1 or Th2 conditions in the presence of anti-CD3/anti-CD28. Th1 and Th2 polarization will be measured by cytokine production after six days of differentiation *in vitro*. These cells will subsequently be labeled with CFSE and cultured in the presence of different quantities of sort-purified $CD4^+CD25^+$ Treg from allogeneic adult blood or cord blood in the presence of anti-CD3/CD28. Suppression of Th1 or Th2 cells will be measured by inhibition of proliferation (by CFSE dilution) and suppression of cytokine secretion (by cytokine flow cytometry) after a six-day culture period.

Interpretation of Results. The experiments described in this Aim extend observations that we have made in human fetal and adult samples to a much larger number of samples of human cord blood. We anticipate that, at term, there will be a normative range of fetal- and adult-type Tregs and HSPCs in cord blood, representing variable kinetics by which layering of immune system ontogeny proceeds in different individuals. The relative frequency of fetal versus adult T cell-specific transcripts ($_tT_F/_tT_A$) will be taken as a measurement of the actual fetal/adult T cell ratio (T_F/T_A). Thus, we will make the assumption that $_tT_F/_tT_A = T_F/T_A$. In those cases in which the fetal/adult T cell ratio (T_F/T_A) is high, it is predictable that cord blood naïve T cells will be more likely to upregulate FoxP3 upon stimulation and that a predominant tolerogenic response to antigen will ensue.

Potential Problems and Alternative Approaches. Given our existing data sets and the techniques that have already been established in the lab, the experiments of this Aim should be relatively straightforward. Though it is highly unlikely, it is possible that there will be no significant variability in the T_F/T_A ratio in full-term newborns. This would be an interesting finding, suggesting that the fetal-to-adult T cell transition occurs earlier during the third trimester of pregnancy and is complete at birth. Should this be the case, we will shift out attention to human premature infants and to nonhuman primates, each of which can be studied during the timeframe of the third trimester.

Specific Aim 2. To determine whether those full-term neonates with a high ratio of fetal/adult T cells are more likely to generate a Th2-polarized immune response to routine childhood immunizations.
Hypothesis. Infants with a high ratio of fetal/adult T cells will generate predominant Th2 responses to routine childhood vaccinations.

Rationale. In human neonates, T cell responses are often characterized by deficient Th1 responses,[16-19] a reduced capacity to induce T cell memory,[20] a high frequency of IgE and IgG4 production,[21] a skewed Th2 response,[22-24] and even the induction of hyporesponsiveness.[25] Such responsiveness to routine childhood vaccines has been found to vary within populations, possibly as a result of genetic and/or environmental factors[26,34,35] and is in part magnified by the formulation of vaccines with the Th2-polarizing adjuvant, alum.[36] In addition, those neonates that are most likely to develop atopic disorders after birth are also those who are most likely to generate suboptimal (and/or strong Th2-type) response to vaccination.[21,26,37] Since fetal Tregs may suppress Th1-type (or other) immune responses to vaccines in a manner that is different than adult Tregs, we speculate that strong Th2 polarization of childhood responses to vaccines may in part be due to a higher than normal proportion of fetal T cells at birth.

Recombinant Hepatitis B (HepB) vaccine is routinely given at birth in the United States and provides an ideal opportunity to investigate an *in vivo* response to antigenic stimulation in the newborn. The immune response to HepB vaccine is well studied and is characterized by a meager (10%) seroconversion rate with the first dose at birth.[38] Subsequent vaccine doses are then observed to yield a substantial antibody response.[39] The primary neonatal $CD4^+$ T cell response to HepB vaccine is characterized by both Th1 and Th2 cytokine production; interestingly, however, the HepB-specific memory $CD4^+$ recall response consists of robust Th2 cytokine production at one year of age.[40]

Experimental Approach. The experiments of this Aim will be carried out under the auspices of a study that has been approved by the UCSF Committee on Human Research protocol (H6325-26775. The effects of regulatory T cells on the development of the pediatric immune system; McCune PI). In a prospective study design, 50 pregnant women will be enrolled prior to delivery. Exclusion criteria will include previous/current

HepB infection ($HepBsAg^+$), other immunomodulatory infections detected by prenatal screening (HepC, HIV), or plans for the use of cord blood for alternative purposes (*e.g.*, banking). With assistance from collaborators in the SFGH Child Health Center (see attached letter of collaboration from Dr. Shannon Thyne), cord blood will be obtained from these deliveries and each of the 50 infants will be followed with blood draws at 6 and 12 months. In all cases, infants will have received routine childhood immunizations, including those against HepB at birth, 1-2, and 6-12 months of age. UCB and infant peripheral blood will be processed by ficoll hypaque and concurrent purification of $CD4^+$ cells by negative selection (RosetteSep, StemCell). These cells will be tested for the following parameters over time:

1. The fraction of T_F vs. T_A in the umbilical cord blood. This will be carried out using the qRT-PCR-based approach described in Aim 1 to determine the ratio of fetal/adult T cells (T_F/T_A).
2. Analysis of the cellular immune response against HepB vaccine. Established flow cytometric assays will be used to assess the fraction of $CD4^+CD25^{high}FoxP3^+CD127^{low}$ Tregs among $CD3^+CD4^+$ T cells at each time point. These fractions will be converted to absolute numbers using TruCount tube (BD) analysis of whole blood counts prior to $CD4^+$ cell isolation, as described.[41] In addition to quantitative phenotyping, the ability of circulating Tregs to suppress the proliferation of HepB-specific responder $CD4^+CD25^-$ T cells will be assessed using well-established methods.[5,41,42] UCB or peripheral mononuclear cells that have been depleted of $CD25^+$ cells (or mock depleted) will be stimulated with (1) polyclonal activators (cross-linking antibodies against CD3 and CD28), (2) soluble HepB antigen, and (3) peptides corresponding to HepB. Cytokine production in $CD4^+$ cells will be assessed by CFC, as described above in Aim 1, to determine whether antigen-specific stimulation yields a response that is predominantly Th1 or Th2 in type.
3. Levels of circulating Immunoglobulin (Ig) isotypes generated against HepB vaccine. Established ELISAs will be used to quantitate circulating levels of vaccine-induced IgG1, IgG2, IgG3, IgG4, and IgE relative to total Ig.

Interpretation of Results. This study will relate the ratio of fetal to adult naïve T cells (T_F/T_A) to a number of immune parameters associated with routine childhood immunizations. In particular, it will be of interest to know whether a high T_F/T_A ratio is associated with a higher propensity towards a less effective "immature" response (*e.g.,* a Th2-predominant cytokine response to vaccine antigens and the predominance of less-mature, antigen-specific IgG1, IgG3, and IgE compared to IgG2). Infants will be studied both cross-sectionally as well as prospectively, and we anticipate that the T_F/T_A ratio and measures of immaturity (especially a bias towards a Th2 response) will decrease with age in tandem. The ability of Tregs to suppress antigen-specific responses against HepB will also be measured. Given the hypothesis that T_F in the newborn generate tolerance to antigens they encounter by becoming Tregs, more robust HepB-specific Treg suppression would be expected in those children with a higher T_F/T_A ratio.

Statistical Analysis. These data will be analyzed in consultation with biostatisticians in the Biostatistics Consultation Service, associated with the UCSF CTSI (see attached letter of support from Dr. Peter Bacchetti). The sample size that has been chosen will detect a 35% difference in cytokine production, with a study power of 0.8 and significance level of 0.05. The statistical approach will use an ANCOVA-type linear model approach for each outcome variable. Some data may be longitudinal as well as cross-sectional and we will include random effects to account for within-subject correlation. We will explore approaches that treat age as continuous, in particular, the Laird-Ware repeated measures model. If the measures exhibit strong skewness or outlying values, we will attempt to transform the outcome (*e.g.,* log-transformation) to mitigate these issues. If this is not adequate, we will compare the groups using non-parametric (*e.g.,* rank based) methods.

Potential Pitfalls and Alternative Approaches. The primary challenge in this study is that of recruiting and retaining 50 patients over an 18-month period. Given the experience of our collaborator, Dr. Shannon Thyne, we believe that this goal is attainable. The Birth Center at SFGH delivers 1250-1300 infants yearly, all of whom are under the care of the Division of Neonatology, in which Dr. Burt (an MD investigator in the PI's lab who will be working on this study) is an attending physician. He will therefore have the ability to recruit patients (under our CHR-approved protocol) as they are admitted to the hospital in labor. Furthermore, in previous studies carried out through the Birth Center, members of the McCune lab have successfully recruited similar numbers of patients in a period of approximately one year. Approximately 80% of infants born at SFGH receive their primary care in the Child Health Center at SFGH. If by three months of recruitment, we are not meeting expected goals, this study will be extended to the Birth Center at UCSF, which has a similar delivery rate and where we also have CHR approval to recruit.