INTERFERON REGULATORY FACTOR 1 (IRF-1) DIRECTS IL-4/IL-13 INDUCED
DEATH OF NEONATAL TH1 CELLS

A Dissertation

presented to

the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

MINDY MARIE MILLER

Dr. Habib Zaghouni, Dissertation Supervisor

JULY 2017

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

INTERFERON REGULATORY FACTOR 1 (IRF-1) DIRECTS IL-4/IL-13 INDUCED
DEATH OF NEONATAL TH1 CELLS

presented by Mindy Marie Miller,

a candidate for the degree of Doctor of Philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Habib Zaghouani

Dr. Bumsuk Hahm

Dr. David Lee

Dr. Craig Franklin

Dr. Helen Braley-Mullen

Dr. Michael Sherman

ACKNOWLEDGEMENTS

First and foremost I must acknowledge my husband and best friend, Chris Miller, as he is a source of constant encouragement, motivation, and endless happiness. Chris, without you I am certain that this would not have been possible. I could not have chosen better traits in a partner and I thank God every day for you and for the way you value me as an equal. I can't wait to see what adventures the rest of our lives hold and I'm thrilled to soon be raising a child with you!

I would also like to thank my parents, Keturah and Mike Larum, for raising me to be a curious and inquisitive person. I am forever thankful for their encouragement and support throughout my education and for modeling to me what can be achieved through hard work and dedication. I hope to be half as good at parenting as you have been.

Whenever I have family and friends visit the lab I introduce them to my "lab family" and, while the moniker is cute, it is extremely accurate because the people with whom a lab space is shared truly become family. They witness and share some pretty joyful and also pretty rage-filled moments and its ok to express vulnerable emotions with them because it's the only way to cope with the tribulations of science. The people that I am lucky enough to include in my current family are: Lexy Cattin-Roy, Tobeckukwu Ukah, and Subhasis Barik. Lexina, our friendship extends far beyond lab life and I feel that anything I write here will feel reductive. That said, it's refreshing to have a close friend that I can also discuss science with and who interjects a scientific thought to normal everyday conversation. I hope you know how much I absolutely adore you and value our friendship. Tobe, I know that I can always rely on you to put things in perspective. I appreciate that you understand the value of things in life and know when something is not important enough to worry about. Sub, I will never forget the first day

you arrived in the US and were in complete culture shock. It's been fun showing you what life is like here and learning more about India than I ever imagined. Your scientific expertise has been critical in my success and so many of the skills I've gained were learned directly from you. I can't thank you enough.

The nature of a research lab is fluid and ever-changing and with each season brings new members in and out of the lab family. Some previous lab mates to whom I am forever grateful include Fiorella San Martin, Linda Rowland, Weirong Chen, Mermagya Dhakal, Xiaoxiao Wan, Jason Ellis, Marie-Therese Khairallah, and Jason Cascio, I have shared great morning conversation with each of you over a cup of Lakota coffee and your presence has made my stay in lab that much better.

Very special thanks go to my awesome committee members: Dr. Sherman, Dr. Lee, Dr. Hahm, Dr. Franklin, and Dr. Mullen. I always brag to fellow graduate students that I have the best committee. It's true. Dr. Sherman, thank you for initially inspiring me to pursue neonatal immunology. We've shared countless hours discussing science and life and your impact on my career has been profound. Someday I hope to mentor someone as you have mentored me. Dr. Lee, thank you for helping me navigate the challenging process of F31 grant writing and providing guidance as the director of graduate studies in my first few years of grad school. Dr. Hahm, your expertise in viral immunology and LCMV has been critical in my virus project. Dr. Franklin, I thoroughly enjoyed our discussions on the microbiome and the efforts in assembling the F31. Dr. Mullen, I always look forward to your input in journal club because you have never been afraid to speak your mind. I greatly admire that and aspire to do the same.

It is impossible to navigate the rigors of academia, focus on research and publishing, and complete coursework and teaching requirements without the help of administrative guidance. Fortunately, Jana Clark, Shelly Crawford, Kathy LaMere, and Karen Ehlert are always available to assist in whatever way they can and sometimes just to provide a listening ear during after a rough day. Equally important, Scott Greathouse has helped me numerous times resolve tech related issues. Thank you for your patience with my tech ignorance! Daniel Jackson in the CIC core has been instrumental in performing the cell sorting for these studies, providing assistance with flow cytometry, and being an incredibly good friend throughout the last 5 years. Your presence has enriched the lives of Chris and I immensely and I'm certain we will be life-long friends. Additionally, I'd like to thank Jelan Sultan not only for making sure our tools and glassware are sparkling clean and sterile, but for your friendship and kindness, warm smile, and genuine compassion. Immunology research would be impossible without access to animal models and I would like to specifically thank Angela Hall, Mina Guerrero, and Robin Cosby for their careful attention in maintaining my rodent colonies. Animal research is difficult but each of you demonstrates such compassion and kindness toward these animals and I am forever appreciative of the good care they receive.

A special thanks is awarded to Lakota Coffee which takes a large portion of my monthly income and, in return, provides me with the ability to function as a semi-normal human before 10AM. You are the true hero.

Last, yet most certainly not least, I must acknowledge my advisor and mentor, Habib Zaghouani. I will never forget the look on your face when, two weeks into my eight week rotation, I went to your office and announced that I'd like to join your lab.

You never promised that this endeavor would be easy. On the contrary, you prefaced our first meeting by saying that you would challenge me and that this would be difficult, but in the end I would undoubtedly be successful. You spoke the truth. But this experience has proven so much more than what I expected and it is because of your approach in mentoring students. Even though there were many difficult days and moments where I doubted myself and doubted you, I now realized you were intentionally molding me into a better scientist and a more critical thinker. There is nobody on Earth that wants to see me succeed more than you. Because of this, you exposed me not only to research, but to manuscript writing, grant writing and the entire NIH granting process, the review process, how to prepare for formal presentations, and how to deliver a succinct message. I feel well prepared for the next step in my scientific career, thanks to you.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS.....	ix
Chapter	
I. LITERATURE REVIEW.....	1
1. Introduction to Neonatal Immunity	2
2. Immune Cell Development.....	7
3. Innate Immunity in the Neonate	10
4. Adaptive Immunity in the Neonate.....	26
5. Neonatal Vaccinations	35
II. IL-4/IL-13 SIGNALING IN NEONATAL TH1 CELLS	38
1. Abstract	39
2. Introduction.....	40
3. Material and Methods	42
4. Results.....	54
A. HR signaling provokes neonatal Th1 cell apoptosis during secondary antigen challenge in two different strains of mice.....	54
B. Antigen and cytokine signaling are both required for neonatal Th1 apoptosis	58
C. HR signaling through STAT6 directs Th1 cell death.....	61
D. The intrinsic apoptotic pathway is active during neonatal Th1 cell death	65
E. STAT6-mediated IRF-1 induction directs apoptosis of neonatal Th1 cells.....	68
F. STAT6 binds to intron 3 in the IRF-1 gene to promote IRF-1 transcription in neonatal Th1 cells.....	71

G. IRF-1 and Bim co-immunoprecipitate and localize to the cytoplasm during neonatal Th1 apoptosis.....	74
H. Bim and IRF-1 do not bind directly in the yeast-two-hybrid assay.....	79
5. Discussion.....	82
III. THE ROLE OF THE IL-4/IL-13 HETERORECEPTOR IN NEONATAL VACCINATION	86
1. Abstract.....	87
2. Introduction.....	88
3. Material and Methods	90
4. Results.....	93
A. Neonatal peptide vaccination induces protective immunity against acute infection by lymphocytic choriomeningitis virus	93
B. The absence of the HR confers enhanced vaccine protection in neonatal mice	95
C. Increased vaccination efficacy of HR-deficient mice is not due to enhanced T cell responses after acute viral infection.	97
D. HR ^{-/-} neonates display enhanced T cell vaccine responses upon chronic LCMV Clone 13 infection	99
E. Inhibition of STAT6 signaling rescues T cell function and allows successful vaccination in neonatal mice.....	101
5. Discussion.....	104
REFERENCES	106
VITA.....	129

LIST OF FIGURES

Figure	Page
1.1 Estimated Global Neonatal Mortality Rates	4
1.2 Estimated Neonatal Deaths as Percentage of Under-Five Deaths	4
1.3 Murine and Human Dendritic Cell Populations.....	22
1.4 Neonatal CD4 T cell polarization	32
2.1 HR signaling provokes neonatal Th1 cell apoptosis during secondary antigen challenge in two different strains of mice.....	56
2.2 Antigen and cytokine signaling are both required for neonatal Th1 cell apoptosis.....	59
2.3 IL-4 and IL-13 activate STAT6 to induce neonatal Th1 cell apoptosis	63
2.4 Intrinsic apoptotic molecules are upregulated during neonatal Th1 cell death	66
2.5 Neonatal Th1 cell death relies on upregulation of IRF-1	69
2.6 STAT6 binds to intron 3 in the IRF-1 gene to promote IRF-1 transcription in neonatal Th1 cells	73
2.7 IRF-1 and Bim co-immunoprecipitate and localize to the cytoplasm during neonatal Th1 apoptosis	76
2.8 Examples of cellular localization of IRF-1	78
2.9 IRF-1 and Bim do not directly bind one another	80
3.1 Neonatal vaccination provides protection against later LCMV challenge in both HR ^{+/+} and HR ^{-/-} mice.....	94
3.2 HR ^{-/-} mice respond better to neonatal LCMV vaccination than HR ^{+/+} mice.....	96
3.3 The HR does not play a role in T cell responses to LCMV Armstrong infection after neonatal vaccination	98
3.4 HR ^{-/-} neonatal mice have enhanced Th1 vaccine responses upon chronic LCMV Clone 13 infection to support the generation of CTLs	100
3.5 Inhibition of STAT6 signaling at time of Clone 13 infection rescues protective vaccine responses in neonatal mice	102

LIST OF ABBREVIATIONS

Ag, antigen
Arm, LCMV Armstrong strain
BCG, Bacillus Calmette-Guerin vaccine
BFA, brefeldin A
BPI, bactericidal/permeability-increasing protein
cDCs, conventional DCs
CFA, complete Freund's adjuvant
CL13, LCMV Clone 13
CLP, common lymphoid progenitor
CMP, common myeloid progenitor
CTL, cytotoxic t lymphocyte
DC, dendritic cell
EAE, experimental allergic encephalomyelitis
GC, germinal center
GFP, green fluorescent protein
GP, glycoprotein
HBV, Hepatitis B virus
HR, heteroreceptor (IL-13R α 1/IL-4R α)
HSC, hematopoietic stem cell
IFA, incomplete Freund's adjuvant
IFN γ , interferon gamma
Ig, immunoglobulin
IL-, interleukin
ILC, innate lymphoid cell
IP, intraperitoneal
IRF-1, interferon regulatory factor 1
IV, intravenous
LCMV, lymphocytic choriomeningitis virus
LPS, lipopolysaccharide
LTi, lymphoid tissue inducer
MEP, megakaryocyte/erythroid progenitor
MPP, multipotent progenitor
MZ, marginal zone
NET, neutrophil extracellular traps
NK, natural killer
OVA, chicken ovalbumin
PAMPs, pathogen associated molecular patterns
pDCs, plasmacytoid DCs
pfu, plaque forming unit
PGN, peptidoglycan
PRRs, pattern recognition receptors
ROS, reactive oxygen species
RQ, relative quantity
SBE, STAT binding element

STAT, signal transducer and activator of transcription
TCR, T cell receptor
Tfh, T follicular helper cell
Th1, T helper type 1
Th2, T helper type 2
TLRs, toll-like receptors
TNF, tumor necrosis factor
Treg, T regulatory cell
TSLP, thymic stromal lymphopoietin
UTR, untranslated region

CHAPTER I
LITERATURE REVIEW

1. Introduction to Neonatal Immunity

The transition of a newborn from a sterile intra-uterine environment to the microbe-rich world we live in marks a unique immunological milestone. At this moment, an infant encounters both microbial friends and foes which will shape the health of the newborn throughout infancy and, potentially, well into adulthood. During the first few moments of extra-uterine life, the newborn will come into contact with a host of microbes that will colonize the skin and mucosal surfaces to establish commensal communities that ultimately benefit a newborn's health. At the same time, there is a heightened potential for pathogenic organisms to attack the infant host, especially given the immaturity of the immune system and lack of immunological memory. To better understand the neonatal immune system, it is necessary to consider the developmental events that shape this system before birth.

During gestation, it is critical that the fetus develops in a sterile environment as intra-uterine infections and the associated inflammation have been linked to infant mortality or preterm birth-related morbidity including neural injury such as intraventricular hemorrhage, white matter damage, and cerebral palsy¹, cardio-respiratory distress², and renal failure among many others³. In the first trimester, infection-induced tumor necrosis factor (TNF) and interleukin-1 β (IL-1 β) are associated with pre-mature labor and delivery⁴. Inflammatory responses are also linked to alloimmune reactions between the mother and fetus which may result in spontaneous abortion⁵. It is also believed that adaptive responses biased toward Th2 cells and away from Th1 ensures a successful pregnancy⁶.

To avoid these deleterious inflammatory responses, a physical barrier is required to protect the growing fetus -- namely, the placenta. While the placenta serves as an immunological barrier to maintain separation between the maternal and fetal immune systems and also providing barrier protection against invading microbes, it renders the infant immunologically naïve upon birth.

The lack of immunological memory, in combination with the immaturity of immune cells and tissues, contribute to high global infant mortality rates. It is estimated that, globally, 2.8 million infants die each year during the neonatal stage (the first 28 days of life) and that nearly a quarter of these deaths are directly due to infection⁷. While public health initiatives across the world have tremendously improved mortality rates over the past 25 years (Fig 1.1), the rate of progress is far behind the under-five survival (Fig 1.2). In fact, a closer look at this data reveals that the number of neonatal deaths as a percentage of all deaths that occur in children under the age of 5 is actually increasing⁸. This highlights an important gap in our understanding and treatment of neonatal diseases and calls for a greater push for neonatal immunity research.

Figure 1.1. Estimated Global Neonatal Mortality Rates. Estimates developed by the UN Inter-agency Group for Child Mortality Estimation and represented here as # of deaths per 1000 live births. Adapted from⁸.

Figure 1.2. Estimated Neonatal Deaths as Percentage of Under-Five Deaths. Estimates developed by the UN Inter-agency Group for Child Mortality Estimation. Adapted from⁸.

The scientific community's interest in neonatal immunity was sparked by seminal work by Sir Peter Medawar during the 1950's when he discovered that fetal exposure to antigen induces tolerance toward that antigen later in life⁹. It should be noted that this concept was predicted in 1949 by Australian scientist Frank Burnet¹⁰ and together Burnet and Medawar share a Nobel Prize. Fascinatingly, Medawar's research began as an endeavor to solve the problems involved with skin graft rejections seen in injuries sustained by soldiers in WWII. He, along with his postdoctoral fellow, Billingham, and graduate student, Brent, found that when cells from one mouse strain were injected into a fetal mouse of a different mouse strain the recipient would later grow into an adult capable of accepting a skin graft from the donor strain. This is in contrast to the phenomenon of rejection that occurs when a mouse is not exposed in early life. This concept became widely accepted and the neonatal period was considered a developmental window in which induction of immunological tolerance can be achieved. One can imagine the theoretical usefulness of this situation where later life ailments such as transplant rejection, autoimmunity, and allergy could all be avoided by careful administration of the antigen in early life. However, practicality and our failure to predict which future antigens to target have thus far precluded these therapeutic approaches.

As science progressed and neonatal immunity research blossomed in the 1990's, it became clear that the neonatal period represented more than just a state of tolerance and immune responses could be mounted. This notion came after the discovery of distinct T helper subsets in 1986¹¹ where evidence suggested that mice challenged with antigen during the neonatal period preferentially induce non-inflammatory T helper type 2 (Th2) responses later in life^{12, 13, 14, 15, 16, 17}. Surprisingly, in certain situations, T helper type 1

(Th1) responses could also be induced¹⁵. Such was the case when oral neonatal antigen exposure to myelin basic protein was shown to exacerbate later experimental autoimmune encephalomyelitis (EAE) disease progression¹⁸. However, Habib Zaghouani's lab demonstrated that a self-antigen immunoglobulin chimera given during the neonatal period was protective against the development of EAE¹⁹ which hints to the importance of antigen type and delivery in dictating immune outcomes, an idea that will be important in later sections of this document. What ultimately emerged from these neonatal studies is that Th2 responses, which are largely non-inflammatory, far outweigh the pro-inflammatory Th1 responses which bodes well with the protective effect of Medawar's transplant experiment and also provides insight into why neonatal vaccines often fail to protect against later infections.

If the scientific community is to address public health concerns involving neonatal morbidity and mortality it is critical to understand the unique immune environment of the neonate.

2. Immune Cell Development

All blood cells, including those of both innate and adaptive immunity, erythrocytes, and megakaryocytes, arise during the process of hematopoiesis. While it is appreciated that hematopoiesis occurs in the adult bone marrow, special consideration is taken to understand this process during neonatal development as hematopoiesis begins in the early fetal yolk sac, proceeds transiently through the liver, and finally settles in the bone marrow by term gestation. In early embryogenesis, it is thought that endothelial and hematopoietic cells both stem from the same cluster of mesoderm which presents, by three weeks of gestation, as thick primordial blood islands where the expression of CD34 is shared between both endothelial and hematopoietic cells in support of their common precursor^{20, 21}. Both erythroid and granulopoietic progenitors have been characterized in the yolk sac as early as 25 days of gestation, remain constant between day 35-50, and are completely gone by day 60^{22, 23}. However, the presence of these early progenitors in the yolk sac don't necessarily indicate functional differentiation, especially since the cells produced in the early yolk sac predominantly become primitive nucleated erythrocytes to synthesize embryonic hemoglobin²¹.

The transition from yolk sac hematopoiesis to other fetal organs initiates with the onset of cardiac beating/blood circulation and coincides with the switch from primitive erythroid production to definitive non-nucleated erythrocyte production, a designation defined by the type of hemoglobin synthesized by each cell^{24, 25}. Despite the stable production of primitive cells from the yolk sac between days 35-50, cardiac beating, and therefore fetal liver hematopoiesis, has been demonstrated to occur as early as three weeks of gestation although at very low frequency. The detection of rare CD34-

erythro/myeloid progenitors in the liver is thought to represent a first wave of hepatic colonization but that the more mature CD34+ progenitors enter during a second wave only after day 32^{20, 21}. Recent discoveries have identified that well after cardiac circulation is established, around 7-8 weeks the major source of hematopoietic stem cells (HSCs) is derived from endothelial cells of the aorta^{26, 27, 28} and that this population will seed the liver, thymus, and spleen where hematopoiesis continues until 7 months of gestation^{21, 29, 30}. Although hematopoiesis has been demonstrated in the bone marrow as early as 11 weeks³¹ the switch generally takes place at 7 months so that hematopoiesis occurs largely in the bone marrow by the time of birth.

The process of hematopoiesis generates all blood cells from HSCs which undergo stepwise differentiation into distinct cell subsets. In the late 1980's, Irving Weissman's group first isolated and began characterizing HSCs^{32, 33}. As HSCs are atop the hematopoietic hierarchy of development, they retain the unique ability to self-renew. In 1994, Weissman's group identified three distinct subsets of HSCs: Long-Term HSCs, Short-Term HSCs, and Multi-Potent Progenitors (MPPs)³⁴. Only the MPPs, which lack self-renewal capacity but retain multi-lineage potential as their name implies, will continue on to become one of two oligopotent cells: common lymphoid progenitors (CLP) or common myeloid progenitors (CMP)³⁵. CMPs can give rise to both megakaryocyte/erythrocyte progenitors (MEPs)³⁶ and also granulocyte/macrophage progenitors (GMPs) which will eventually fill the entire innate immune branch³⁷. CLPs, on the other hand, may become T cells, B cells, or innate lymphoid cells (ILCs)³⁸. It should be noted that dendritic cells (DCs) represent a unique group in that they have been

shown to arise from both CMPs and CLPs^{39, 40, 41}. The endeavor to understand the exact mechanism of commitment and differentiation of DCs is an ongoing process.

3. Innate Immunity in the Neonate

Innate immunity serves as the initial shield in defense against microbes while also activating and mobilizing the adaptive immune system. Since neonates have a developmental delay in adaptive immunity, their innate immunity branch must be able to compensate to ensure protection. As mentioned in the introduction, the fetal and neonatal immune systems must face three major physiological challenges: protection from microbial infection at the maternal-fetal interface, avoidance of inflammatory Th1 responses during gestation, and mediation of the transition to a microbe-filled extra-uterine life⁴². As the majority of these demands require physical separation of the infant from either mom or pathogen, special consideration must be given to physiological barriers as they pertain to innate immunity in addition to specific innate cell types.

Physiological Barriers of the Neonate

Skin.

As the human's largest organ, the skin serves as a primary barrier to defend against invading pathogens. Epidermal development begins early in embryogenesis and matures with gestational age⁴³. Before week 30 of gestation, the epidermis is weak and has a poorly formed stratum corneum (the outer most layer of skin) but by week 34 the epidermis is considered mature⁴⁴. Interestingly, in this same study, Evans and Rutter show that the postnatal environment significantly contributes to epidermal development as prematurely born infants will have skin maturation that resembles term infants within two weeks of being born, regardless of gestational age. The evolutionary trait for infants

to quickly develop a healthy skin barrier highlights just how important this function is for human health.

Another interesting feature of newborn skin is that it is much more alkaline than that of an adult. Whereas adult skin has pH of around 5.5⁴⁵, newborn skin averages a pH of 7 on day 1 of life but already starts acidifying by day 2⁴⁶. It is hypothesized that early life skin alkalinity may be due to the alkaline environment of amniotic fluid^{43, 47} and that skin acidification contributes to the maturation of barrier function⁴⁸ by influencing the composition of microbial flora⁴⁹. Skin acidification may also be influenced by the vernix caseosa, a waxy protective substance covering a newborns skin which is produced by fetal sebaceous glands during the last trimester of pregnancy. The vernix is composed mainly of water but contains proteins, lipids, and, importantly, antimicrobial peptides^{50, 51}.

Antimicrobial peptides are perhaps one of the most important constituents of the newborn skin barrier. Skin from neonatal mice and humans express various antimicrobial peptides such as cathelicidin, β -defensins, protegrins, bactericidal/permeability-increasing protein, S100 proteins, lactoferrin, lysozyme, and RNAses^{52, 53, 54}. While many skin and immune parameters are suboptimal in newborns, antimicrobial peptide levels are often increased during the perinatal period and may provide a compensatory mechanism for the developmental delay in other immune branches. In fact, gene expression levels of neonatal murine cathelicidin were 10- to 100-fold greater than in adult mice⁵². Similarly, protein levels of both lysozyme and lactoferrin were found to be greater in newborns immediately after delivery than in adults and these levels were not diminished after

bathing⁵⁵ which represents some level of permanency and not just an effect of the uterine environment.

Emerging from a relatively sterile intra-uterine environment to a world filled with microbes, it becomes necessary to allow rapid colonization of skin commensals while preventing colonization of pathogens. The newborn epidermis development, pH, and antimicrobial peptides mediate this transition to ensure health of the newborn.

Intestine.

Home to the vast majority of commensal bacteria, the intestines serve as a critical regulatory element for many aspects of human health such as immune system development, progression of multiple diseases, and, more recently appreciated, neural function. Newborns have traditionally been considered sterile at birth but this concept has recently been challenged by the detection of bacteria in meconium, the first stool to be passed after birth⁵⁶. To support the idea that these bacteria were contracted *in utero*, Ardisson et al. showed that these bacterial populations are similar to those found in amniotic fluid⁵⁷. Yet despite having the presence of some microorganisms, the overall microbial abundance and diversity of the newborn gut is low enough that the initial postnatal environment plays a pivotal role in defining and establishing the long-term microbial profile.

Two major events largely shape the microbial communities during development. The first event is the initial microbial exposure of the infant. For many, this would be the vaginal flora during delivery whereas others born by cesarean would be exposed to external sources and these infants demonstrate delayed colonization. Furthermore, these

different exposures have been linked to disparate microbial profiles⁵⁸. The second major colonization event occurs through the diet. Formula fed infants harbor *Bifidobacteria*, *Bacteroides* spp., and *Clostridium* spp. whereas babies fed breast milk have mostly *Bifidobacteria*⁵⁹. Diet plays the most profound role later in infancy during the transition from milk to solid food and, surprisingly, the emerging microbial composition remains fairly stable throughout life^{60, 61}.

Aside from harboring commensal microorganisms, the gut must be equipped to defend against pathogens and this function is largely tied to its physiological structure. There are considerable differences between mice and humans when assessing neonatal intestinal development. In general, humans are born with a much more mature gut than mice. The intestinal surface is characterized by a monolayer of polarized epithelial cells that comprise the only boundary between the “external” microbial content and “internal” host lumen⁶². Intestinal crypts are gland-like invaginations of the monolayer where various specialized cells reside deep within the crypt including mucus secreting goblet cells and anti-microbial secreting Paneth cells. In mice, crypts are not present until 12-15 days after birth during the weaning period⁶³ whereas humans display fully developed crypts by 19 weeks of gestation. Despite having complete gut architecture, human newborns still have a relatively “immature” gut since antigenic stimulation by the microbiota is required for full maturation⁶².

The gut barrier provides protection from pathogens by secreting mucus, defensins, and cathelicidins. The first line of defense is the mucus layer that provides physical separation of microbes and gut epithelium and is composed of mucin, the main structural component, and trefoil factors which aid in mucin polymerization--both of which are

produced by goblet cells⁶⁴. In humans, mucin mRNA is detected quite early at 12 weeks of gestation⁶⁵ and even preterm infants show rapid mucin production⁶⁶. This early protective mucus layer is likely to be related to gut maturity as neonatal mice which have a relatively immature gut show delayed production of mucin as goblet cells are not yet present⁶². A study that assessed antimicrobial peptides in human meconium found high levels of α -defensins HNP-1-2 and HD5 and the cathelicidin LL-37 which, since present at birth, may represent a defense system already in place to help control the establishment of gut microbiota and provide early life pathogen protection⁶⁷. Similarly, Mathias Hornef's group found that in very young mice there is an abundance of cathelicidins, particularly CRAMP, produced by epithelial cells to provide enteric protection in the early postnatal period and that cathelicidin production switches over to Paneth cells after crypt formation⁶⁸. Overall, this high and stable production of mucus, defensins, and cathelicidins in humans provides early life protection against pathogens. Importantly, special consideration must be taken when making comparisons between mice and human newborn intestinal studies.

Lung.

The lungs are one of the last critical organs to form during development and therefore the majority of premature infants have some problems associated with respiration. In fact, lung alveolarization occurs between 36 weeks of gestation and continues throughout the first 36 months of life⁶⁹. Since lung development is delayed it is no surprise that lung barrier function, largely provided by pulmonary surfactant, is also compromised in early life. Surfactant is comprised of 70-80% phospholipids, 10% protein, and about 10%

neutral lipids like cholesterol^{70, 71} and, along with barrier function, is critical for lung function as it reduces surface tension at the air/liquid interface preventing alveolar collapse⁷⁰. Newborns have lower levels of surfactant than adults, especially prematurely born infants who display respiratory distress syndrome, the prototypical disease of surfactant deficiency. This surfactant deficit in early life renders newborns susceptible to pulmonary infection.

Neonatal Innate Leukocytes

The innate immune cells include neutrophils, monocytes/macrophages, dendritic cells (DCs), natural killer cells (NKs), and other granulocytes such as basophils, eosinophils, and mast cells. A hallmark of innate cells is the way in which they sense foreign antigens. Each cell expresses a set of receptors that detect pathogen associated molecular patterns (PAMPs) which are molecules on pathogens that are not expressed by host cells. Such receptors include toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). The history of TLR discovery is rich in Nobel awards. Toll receptors were originally identified in 1985 in *Drosophila* as a determinant in dorsal-ventral polarity during embryogenesis⁷² and later appreciated to activate immune functions^{73, 74}. In 1997, Charles Janeway's group, with the then-postdoc Ruslan Medzhitov as first author, identified the first human TLR and the associated downstream NF- κ B mediated production of inflammatory cytokines as well as the ability of TLR signaling to upregulate co-stimulatory molecule B7.1⁷⁵. A year later, Beutler's group identified bacterial lipopolysaccharide (LPS) as the ligand for TLR4⁷⁶. After this, a cascade of discoveries eventually identified TLR1-TLR13, although only TLR1-10 are found in

humans where TLR1, 2, 4, 5, and 6 are found on the cell surface and function to recognize microbial membrane components and TLR3, 7, 8, and 9 are found in intracellular compartments and recognize intracellular microbial nucleic acids⁷⁷. Regarding neonates, the expression levels of TLRs and their accessory and adaptor proteins are developmentally linked with gestational age so that pre-maturely born infants express lower levels than term infants⁷⁸ but a term infant will express levels consistent with adults, albeit with reduced functional capacity⁷⁹. Specific functions of different innate cells of the newborn will be discussed below.

Neutrophils.

As the innate immune system is the main defense against newborn infections, neutrophils play a critical role as they are the first circulating cell type to respond to infection. However, neonates begin their lives at a disadvantage due to their low frequency of neutrophils. Neutrophils exist in three different pools: proliferative, circulating, and marginating³⁰. The proliferative pool resides in the bone marrow and comprises precursors that have the ability to multiply and restore peripheral neutrophil numbers. In the neonate, the proliferative pool is just 10% of that in an adult⁸⁰. During an infection, the proliferative pool of neutrophils is called to migrate to the periphery to aid in pathogen clearance and, because neonates have a limited number of these cells to begin with, they often develop neutropenia. This is particularly the case in neonatal sepsis where both cell number and the kinetics of the neutrophil response are compromised in newborns⁸¹. In an animal model, the neutrophil responses take 3-4 hours to initiate in a neonate whereas an adult can mount a response in about an hour⁸². Pre-maturely born

infants seem to be the most disadvantaged in this aspect as the proliferative capacity of progenitor cells increases rapidly with age until adult-like cell frequencies occur 4 weeks after birth⁸³.

The functional capacity of neutrophils relies on their ability to recognize PAMPs, utilize chemotaxis to migrate to sites of infection, extravasation into the tissue, and perform effector functions such as phagocytosis, degranulation, and release neutrophil extracellular traps (NETs). Neonatal neutrophils display some reduced function when compared to those from adults. While neonates and adults express similar levels of most TLRs, TLR4 expression is greatly reduced in the newborn and contributes to their inability to mount strong responses against LPS-harboring pathogens as evident by limited production of IL-1, IL-6, and TNF- α ⁷⁸. Even though the other TLRs are expressed at adult levels, PAMP signaling elicits reduced inflammatory cytokine responses during the neonatal period, indicating functional developmental deficits. Regarding chemotaxis, neonatal neutrophils display impairment compared to those from adults but this reduced ability subsides by 4 weeks of age. Specifically, both neonatal and adult neutrophils responded to 7 known chemotactic agents in the same relative order of potency (i.e. IL-8 was the strongest but GRO- β was the weakest) but the neonatal neutrophils migrated significantly less distance⁸⁴. The cause of reduced mobility is thought not to rely in the abundance or affinity of surface receptors, as they are expressed similar to adult, but instead signaling abnormalities stemming from low intracellular calcium mobilization⁸⁵. Once at the site of infection, neutrophils from neonates will encounter a greater challenge in extravasation as they display reduced expression and shedding of L-selectin⁸⁶ and diminished upregulation of CR3⁸⁷.

An important function of neutrophils is their ability to phagocytose microbial agents. Both neonatal and adult neutrophils are able to phagocytose both Gram-negative and Gram-positive bacteria at similar levels with a deficit only witnessed in preterm infants⁸⁸. Neutrophils must also degranulate and release microbicidal agents. While adult and neonatal neutrophils display similar abilities to release bactericidal/permeability-increasing protein (BPI), lactoferrin, and elastase, prematurely born infants display significant impairment^{89, 90}. Additionally, the concentration of lactoferrin within neonatal neutrophils is half of that measured in adult neutrophils⁹¹. Finally, the ability of neonatal neutrophils to produce NETs relies exclusively on the ROS-independent pathway in which exposure to pathogens activates complement, TLR2, or fibronectin^{92, 93} as they are completely defective in NET production through the traditional pathway which requires activation of NADPH oxidase and ROS production³⁰. Overall, neonates display some levels of impairment in neutrophil responses but these are all corrected relatively quickly after birth so that by one month of age adult-like responses can be mounted. Neutrophil impairment becomes a much larger problem in prematurely born infants as they lack the very first line of defense and display much higher rates of infection such as early onset sepsis and necrotizing enterocolitis.

Monocyte/Macrophage.

Monocytes are a circulating leukocyte that can differentiate into either macrophages or myeloid DCs. Monocytes can be classified as classical (CD14⁺ CD16⁻), inflammatory (CD14⁺ CD16⁺) or patrolling (CD14^{int} CD16⁺). While the literature agrees that frequencies of adult and neonatal classical monocytes are equivalent and express similar

expression of CD11c, CD80/CD86, CD163, and HLA-DR⁹⁴, some evidence suggests inflammatory monocytes are reduced in the neonate⁹⁵ or that neonatal patrolling monocytes are reduced⁹⁶. Furthermore, monocyte function has been shown to be relatively equivalent to that of adults. Placental monocytes cultured in vitro secreted lysozyme, lost peroxidase activity, and displayed 5'-nucleotidase activity at similar levels as monocytes from adult blood⁹⁷. The same study showed that both neonatal and adult monocytes were subject to similar infection and intracellular replication of *Toxoplasma gondii* and that either cell type could prohibit replication if pre-treated with supernatants from lymphocytes primed with *T. gondii*, indicating complete functional maturity of neonatal monocytes. Other experiments have shown that cord blood monocytes display adult-levels of adherence, chemotaxis, bactericidal activity, phagocytosis-associated chemiluminescence, production of superoxide, and generation of hydrogen peroxide⁹⁸. Moreover, in response to peptidoglycan (PGN), neonatal monocytes produce even higher levels of IL-12p70 and TNF than adult monocytes⁹⁴. Together, any immune deficits of the neonate are not likely to be contributed to the monocyte compartment.

Despite healthy monocytes in neonates, there does appear to be a deficiency in neonatal macrophage responses, particularly toward polysaccharide antigens. When stimulated with LPS, neonates failed to produce IL-1 β , IL-12, and TNF but instead made more IL-6 and IL-10 than adult counterparts⁹⁹. However, as previously described, neonates do not display high levels of TLR4 which could contribute to the suppressed LPS response.

DCs.

Dendritic cells may be the crux of neonatal immunity as they are critical in initiating an adaptive response yet show profound developmental delays. First described and named in 1973 by Ralph Steinman¹⁰⁰, dendritic cells function to take up, process, and present antigen to lymphocytes as well as produce cytokines which aid in this process. Most of the knowledge on dendritic cell development comes from murine studies so for this section, unless specifically stated otherwise, the information presented will be relevant to mice. In the murine neonate, dendritic cells first appear in the thymus by day 17 of embryonic development when thymocyte selection begins¹⁰¹. At birth, splenic DC frequency is extremely low, comprising just 0.2% of the total splenic cells and does not reach adult levels until 6 weeks of age. Interestingly, plasmacytoid dendritic cells (pDCs) represent a larger frequency (45%) of the DC population at one week of birth which eventually decreases to 20% by week 6 as the other DC populations expand^{101, 102}. pDCs, which express CD11c^{lo} produce large amounts of IFN in a short period of time, are considered the first line of defense against viral infections whereas conventional DCs (cDCs), which express CD11c^{HI} contribute to T cell induction and are critical in mounting a strong adaptive response¹⁰³.

Dendritic cells can be categorized based on the expression of surface markers such as CD11c, CD8, CD4, CD103, CD11b, and others as listed in Figure 1.3. Regarding cDCs found in lymphoid organs, the CD11c⁺ CD8⁻ subset is the first to emerge in the neonate followed by the CD11c⁺ CD8⁺ subset by day 6 or 7^{101, 104, 105}. The delayed emergence of the CD8⁺ subset has a major functional consequence as these cells contribute to the generation of Th1 responses through IL-12 production¹⁰⁴. IL-12p70,

comprised of IL-12p40 and IL-12p35 subunits, is one of the cytokines that, developmentally, takes the longest to reach adult levels upon TLR stimulation¹⁰⁶. This is thought to be due to defective transcription of the IL-12p35 subunit in neonates¹⁰⁷. Plasmacytoid DCs, on the other hand, show relatively little functional defect compared to adult pDCs^{101, 108}.

Human neonate DC studies rely on cord blood cells and cDCs present in the blood are considered to be immature cells that are transitioning from the bone marrow to peripheral tissue. The ratio of pDC:cDC of human cord blood is essentially reversed (3:1) to that found in adult blood (1:3)¹⁰⁹ and exist in a more immature state as evident by decreased expression of HLA-DR, CD80, CD86, and ICAM-1^{103, 110, 111}. Together, neonatal DCs show both developmental delays and functional defects, especially regarding their ability to produce IL-12 and generate a productive pro-inflammatory Th1-type response.

	MOUSE								HUMAN EQUIVALENT
DC Subpopulation	B220	CD4	CD11c	DEC205	Langerin	MHC II	PDCA-1	Siglec-H	
CD8+ cDC	-	-	+	+	-	+	-	-	CD11c ^{lo} , CD141 ⁺ , CD11b ⁻ , XCR1 ⁺
CD8- cDC	-	+/-	+	-	-	+	-	-	CD11c ^{hi} , CD11b ⁺ , CD1c ⁺
CD103+, CD11b-	-	-	+	+	+/- (*)	+	-	-	Similar subsets are suspected in Humans, see comments (*)
CD103+, CD11b+	-	-	+	+		+	-	-	
CD103-, CD11b+	-	-	+	+/-		+	-	-	
Langerhans Cells	-	-	int	+	+	+	-	-	CD1 ⁺ , CD207 ⁺ , HLA-DR ⁺ , CD11c ^{int}
pDCs	+	-	int	+	-	int	+	+	CD123 ⁺ , CD303 ⁺ , CD304 ⁺

Classical DCs in lymphoid organs	Classical DCs in non-lymphoid organs (mucosa)
Classical DCs in the skin	Non-classical DCs, plasmacytoid DCs

Figure 1.3. Murine and Human Dendritic Cell Populations. Comparison of different murine and human dendritic cell subpopulations based on cell surface markers. Source: https://www.biolegend.com/dendritic_cells.

Innate lymphoid cells.

The recently classified innate lymphoid cells (ILCs) comprise three subsets: ILC1 (includes NK cells), ILC2, and ILC3 (includes lymphoid tissue inducer cells, LT α). They mirror T helper cells in their transcriptional profile and cytokine production but differ in that they do not express a T cell receptor (TCR). ILC1s include NK cells, express T-bet transcription factor, and functionally parallel Th1 cells through production of IFN γ and TNF. ILC2s are similar to Th2 cells by way of Gata3 dependence and IL-13, IL-5, and IL-9 secretion. ILC3s are counterpart to Th17 cells, require ROR γ t, and make IL-17 and IL-22. Since these cells do not express specific antigen receptors, they are considered innate as the only stimulus required for cytokine production is receipt of the respective T helper cell polarizing cytokine. For example, IL-12 or IL-18 will cause an ILC1 to produce IFN γ or TNF whereas IL-25, IL-33, or TSLP will induce IL-13 or IL-5 secretion by ILC2s. With the exception of NK cells, ILCs have only been described in the last 10 years and relatively little is known on their function in neonatal immunity.

Neonates display higher numbers and percentages of NK cells in blood than adults¹¹² despite their lowered NK cell cytotoxicity and antibody-dependent cellular cytotoxicity^{113, 114}. However, enhanced cytotoxicity may be rapidly acquired by IL-15 which is produced by neonatal macrophages and monocytes¹¹⁵ which would suggest that neonatal NK cells are poised to quickly respond to viral infection if stimulated in the correct context.

Basophils/Mast cells/Eosinophils.

The hallmark cells of allergic responses, basophils, eosinophils, and mast cells are low frequency granulocytes that release immunomodulatory and often highly inflammatory granules, such as histamine, heparin, and leukotrienes, upon stimulation. Paul Ehrlich characterized each of these cells in the late 19th century early in his career with the discovery of mast cells included in his doctoral thesis work¹¹⁶. While basophils represent just about 1% of circulating blood cells, they are potent effectors of IgE-mediated immune responses. When multivalent antigens are recognized and bound by IgE, the Fc portion of IgE binds to the α chain of its high-affinity receptor, Fc ϵ R1, on the surface of basophils and mast cells. Aggregation of the receptor complex leads to Lyn phosphorylation of the β and γ subunits of Fc ϵ R1 which recruits Syk kinase and ultimately leads to cell activation and degranulation^{117, 118}. However, it is now appreciated that basophils function beyond IgE-mediated responses and produce appreciable quantities of both IL-4 and IL-13 cytokine^{119, 120}. Interestingly, there is also evidence that these cytokines influence basophil differentiation and function. Mice that lack the type II IL-4 receptor, through which both IL-4 and IL-13 can signal, have reduced frequency of basophils and basophil progenitors and their basophils were less likely to produce IL-4 upon TLR stimulation than wild type counterparts¹¹⁹. In the neonate, basophils are present and produce enough IL-4 to skew adaptive immune responses toward Th2 cells by signaling through the HR on DCs to dampen their IL-12 production. The reduced levels of IL-12 is enough to polarize naïve T cells toward Th1 but not enough to counter IL-13Ra1 upregulation on the Th1 cell which later serves as a death marker.

Mast cells are similar to basophils except they do not circulate and are instead tissue-resident, often found in blood vessels and epithelial surfaces¹²¹. Mast cells can be activated through IgE binding FcεR1, as with basophils, or through complement components C3a and C5a, and TLR ligands. Mast cells are found in neonates and can even be isolated from the umbilical cord. Surprisingly, both neonatal mast cells and basophils produce significantly more histamine than adult counterparts¹²² which may contribute to the prevalence of allergic responses in newborns. Prematurely born infants are prone to develop chronic lung disease of prematurity which is characterized by impaired alveolarization, lung scarring, and inflammation which are concurrent with, and likely due to, mast cell hyperplasia¹²³.

Eosinophils comprise ~1-5% of peripheral blood and while they serve important anti-helminth roles, they are highly involved in allergy associated inflammation such as asthma and eosinophilic oesophagitis¹²⁴. As neonates have increased type II cytokines and these cytokines are perpetuated by eosinophils, it is not surprising that newborns suffer from eosinophilic diseases. Up to 76% of prematurely born babies are reported to develop eosinophilia¹²⁵ which has been linked to the development of atopic diseases within the first 18 months of life^{126, 127}. Overall, neonates have no defect in granulocytes such as basophils, mast cells, or eosinophils which may contribute to adverse responses in the developed world.

4. Adaptive Immunity in the Neonate

The adaptive immune branch serves to provide specific and long lived protection against pathogens and serves an important role in vaccine responses. Unfortunately, this branch is compromised in neonates and doesn't fully mature to provide adult-like protection until later in life. The adaptive immune system is comprised of T lymphocytes and B lymphocytes which develop in the thymus and bone marrow, respectively. T cells mediate cellular immune responses by producing cytokines to enable phagocyte activation as well as performing cytotoxic functions to eliminate pathogen infected cells whereas B cells contribute to humoral immunity by generating antibodies which bind specific pathogens to neutralize and target them for destruction.

T Lymphocytes

T lymphocytes originate from precursor cells in the bone marrow which home to the thymus where they complete their development. During their stay in the thymus, T cells acquire their unique and vastly diverse T cell receptor through V(D)J recombination and positive and negative selection. These processes, while outside of the scope of this literature review, ensure that foreign antigen can be recognized by at least one of the T cells and that none of the T cells respond to self-antigens. In the human neonate, T cells arise fairly early in gestation and by delivery at term the frequency of T cells (19-26% of blood leukocytes) is similar to that of adults (24-30%) and the CD4:CD8 ratio of both newborns and adults is between 1.2:1 and 2:1¹²⁸. However, a study investigating prematurely born human infants found that the number of CD4 and CD8 T cells were markedly lower than in term infants¹²⁹ indicating that there is a developmental delay in T

cell emergence but that this defect is corrected by the time birth occurs at full term. In mice, however, T cell generation is delayed. In fact, the $\alpha\beta$ TCR isn't detectable until day 16-17¹³⁰ of the murine 20-day gestation with appreciable numbers of functional T cells emerging in the thymus around day 19¹³¹. For this reason young mice do not reach adult percentages of lymphocytes in the spleen until day 15 after birth and in the lymph nodes until 7 days of life¹³². The emergence of T cells from the thymus leads to lymphopenia-induced proliferation of naïve neonatal thymocytes which generates a peripheral T cell population that is CD44^{bright} and thus considered memory-like¹³³ which perhaps contributes to the differences in T cell function between adult and neonatal mice.

There are various subsets of CD4⁺ T helper cells: Th1, Th2, Th17, Tfh, and Tregs are the major types and are classified based on the cytokines they produce. With the exception of natural Tregs (nTregs), which are generated through strong TCR stimulation during thymic selection, each T helper subset is generated from naïve CD4 T cells in the periphery and their decision to polarize toward a specific subset depends on the surrounding cytokine milieu as dictated by the context of infection. Neonatal T cells preferentially polarize toward Tregs and Th2 which shift them away from the more pro-inflammatory Th1, Th17, and Tfh subsets¹³⁴ (Figure 1.4).

Evidence for the propensity of naïve neonatal T cells to polarize toward T regulatory cells instead of other cell types demonstrates that up to 70% of neonatal CD4⁺Foxp3⁻ thymocytes become Foxp3⁺ cells upon TCR stimulation as opposed to only 0.2% of adult thymocytes¹³⁵. Further investigation in the same study showed that TCR and costimulation were sufficient to drive neonatal Treg polarization even in the absence of exogenous TGF- β and IL-2 and that this capacity is a developmental trait that

disappears with age. It is interesting to note, however, that Foxp3-expressing nTregs are absent in the murine neonate until about day 4 of life and slowly increase in frequency to adult levels by day 21¹³⁶.

As with Tregs, neonatal naïve CD4 T cells also prefer to become Th2 cells. Unlike adult cells which require polarization and several rounds of cell division, freshly isolated murine CD4⁺ lymph node cells rapidly produce the Th2 cytokines IL-4 and IL-13 within 24 hours of stimulation¹³⁷. This rapid effector-like function is possible because the regulatory element, CNS-1, within the Th2 locus in T-lineage cells exists in a hypomethylated state, which enhances transcription, in neonates whereas adult CNS-1 is hypermethylated and thus transcription is repressed. The same group later defined the developmental window of CNS-1 hypomethylation as beginning in mid-gestation and continuing throughout the first week of life¹³⁸. This phenomenon has also been documented in human cord blood CD4 T cells where the chromatin in the IL-13 locus is maintained in an accessible configuration as determined by DNase I hypersensitivity and hypomethylation¹³⁹. Interestingly, the human IFN- γ locus in cord blood CD4 T cells is hypermethylated¹⁴⁰, indicating an intrinsic repression of Th1 cytokine responses in early life. While this neonatal Th2 bias is thought to serve an important role in maintaining pregnancy⁶, the absence of Th1, Th17, or Tfh cells impairs the protection of the neonate against pathogens.

It has long been reported that neonates have defective Th1 responses which explains the window of tolerance originally observed by Medawar and the susceptibility of neonates to infection. Even though this defect plays a critical role in the maintenance of pregnancy, it would benefit the newborn to be able to quickly adapt to extra-uterine

life and switch on the ability to generate Th1 cells. Decades of research have elucidated mechanisms in which the neonatal system can be manipulated in order to generate effective Th1 immunity. For example, injection of IL-12 can prevent Th2-dominant responses and reverse transplantation tolerance in neonatal mice¹⁴¹ which agrees with studies highlighting the importance of robust DC function in initiating Th1 responses¹⁰⁴. Certain early life viral infections, such as Cas-Br-E murine leukemia virus¹⁴² or a vaccinia-based viral vector¹⁴³, have also shown to induce protective Th1 responses upon later infection. Even though the default pathway for neonatal T helper polarization is toward Tregs and Th2 cells, it is possible to generate protective Th1 immunity given the appropriate context.

Until recently, functional studies involving murine neonates were restricted to analysis of the secondary response and, while this yields important information regarding how neonatal exposure affects health later in life, it doesn't really address how the immune system operates at the neonatal stage and how newborns can combat early life infection. The absence of primary response analyses stems from technical limitations associated with studying newborn mice and only modern laboratory techniques have afforded the ability to perform assessment of primary responses. These studies utilized a neonate-to-neonate adoptive transfer model and demonstrate that neonates do have the ability to mount balanced Th1/Th2 primary responses but that during a subsequent antigen challenge the Th1 cells die which leads to biased Th2 immunity¹⁴⁴. Interestingly, they found that the death of Th1 cells is due to expression of IL-13R α 1, which forms a cytokine receptor complex with IL-4R α through which both IL-4 and IL-13 can signal, on Th1 cells and that during the recall response, neighboring Th2 cells produce IL-4 and

IL-13 which induces apoptosis upon binding to their receptor on Th1 cells. Furthermore, the expression of IL-13R α 1 on Th1 cells is a developmentally controlled trait which manifests from birth through around day 6 of murine life¹⁰⁴. Studies that investigate the apoptotic pathway induced by IL-4 and IL-13 in Th1 cells may provide avenues in which preservation of Th1 cells is possible.

Much less is known on the development of neonatal Th17 cells. It is likely that differentiation is shunted away from the Th17 pathway and toward the Treg pathway due to the absence of pro-inflammatory cytokines. In the presence of TGF- β during T cell activation, a cell upregulates both Foxp3, the master transcriptional regulator for Tregs, and ROR γ t, the regulator for Th17 cells, but when cytokines like IL-6, IL-1 β , and IL-23 are absent, Foxp3 is able to preside and the cell will become a Treg instead of a Th17¹³⁴. Furthermore, the dominant Th2 pathway may also prevent Th17 differentiation as blockade of IL-4 during allogeneic cell transfer prevents tolerance and allows for the development of alloreactive Th17 cells¹⁴⁵. That said, certain adjuvants and infections are capable of stimulating a Th17 response in neonates such as the IC31 adjuvant¹⁴⁶ or infection with *Yersinia enterocolitica*¹⁴⁷ which, like Th1 cells, indicates that the correct stimulation conditions can induce protective immunity.

T follicular helper (Tfh) cells are critical for the generation of productive B cell responses and subsequent antibody production. Similar to Th1 and Th17 cells, it appears as though neonates have reduced capacity to generate Tfh cells but that they can be induced under certain circumstances. For example, IL-4 negatively regulates the generation of Tfh cells¹⁴⁸ which explains the relative paucity in neonates but adjuvants

with CpG oligonucleotides can overcome this defect and allow for robust germinal center responses and provide protective B cell responses¹⁴⁹.

Like CD4⁺ T helper cells, CD8⁺ cytotoxic T cells are also thought to be less effective in the neonate. A recent study investigating the transcriptional profile of human neonatal CD8 T cells found that they are transcriptionally distinct from their adult counterparts in that they have reduced expression of TCR and cytotoxicity related genes but upregulated genes involved in antimicrobial peptide generation and production of reactive oxygen species which led the authors to conclude neonatal CD8 T cells are innate-like¹⁵⁰. As with other cell types, specific circumstances license full adult-level cell activity. Congenital infection with *Trypanasoma cruzi* leads to a high frequency of pathogen-specific CD8 T cells in cord blood indicating a very early life ability to generate CD8 immunity¹⁵¹. Furthermore, in a murine model, adult level CD8 T cell responses can be induced in neonates using plasmid DNA vaccines¹⁵². Circumstances that induce protective CD8 T cell responses often correlate with protective Th1 immunity as well.

Figure 1.4. Neonatal CD4 T cell polarization. The relative propensity of neonatal naïve T cells to polarize toward a specific subset is indicated by the thickness of the arrow and is guided by the listed cytokines. Adapted from Debock et al, 2014.¹³⁴

B Lymphocytes

B cells are tasked with the important duty of generating pathogen specific antibodies to quell current and future infections. B cells arise in the bone marrow where they undergo V(D)J recombination to express a unique IgM B cell receptor. These cells are then released into the circulation as immature IgM^+IgD^- cells which finish their maturation into $\text{IgM}^{\text{lo}}\text{IgD}^{\text{hi}}$ mature B cells. Mature B cells circulate throughout lymphoid organs and migrate into the B cell follicle where the T-B interactions can occur, B cell differentiation takes place, and germinal centers are formed which support somatic hypermutation and isotype class switching eventually producing memory B cells and antibody-generating plasma cells. This process varies in mice, especially in regards to early life B cell development. For example, in mice, B cells are produced in the fetal liver and spleen until well into the first week of life when production switches to the bone marrow¹⁵³. Additionally, B cell follicles are not found in mice until 7-10 days after birth and germinal centers do not form until 3 weeks. For these reasons, productive humoral immunity is difficult to induce in early life.

It is also important to consider the difference between B cell subsets. B-1 cells are produced first in early life, largely reside in pleural/peritoneal cavities, do not self-renew, and do not require T-cell help as they do not undergo somatic hypermutation or develop memory. B-2 cells, however, are the typical marginal zone or follicular B cell that arises after birth, resides in secondary lymphoid organs, and requires T-cell help for somatic hypermutation and memory development. B-2 cells are the major players in vaccine responses and their absence in early life may help explain poor neonatal vaccination. Indeed, neonatal responses to vaccination are characterized by lower affinity and reduced

heterogeneity of IgG with a delayed onset and shorter duration of antibody production¹⁵⁴. This defect is age-dependent as the earlier an infant is immunized the worse their responses will be, regardless of maternal antibody¹⁵⁵. For this reason, the ideal vaccine schedule in young children consists of repeated injections of the vaccine to boost the response and perpetuate memory cells.

Some factors extrinsic to B cells also contribute to neonatal humoral immunity such as the presence of maternal antibody which can circumvent neonatal B cell responses. IgG antibodies that a fetus acquires through trans-placental maternal transfer are able to bind pathogens and direct them for destruction by innate cells. This is especially effective in protection from respiratory syncytial virus¹⁵⁶ and influenza¹⁵⁷. This process is so effective, lasting for 6 months, that pregnant mothers may receive booster shots in order to provide protection for their infants as is common with the Tdap vaccine to protect against tetanus, diphtheria, and pertussis. Despite the protection afforded by maternal antibody, there are negative consequences when considering the efficacy of neonatal vaccination as maternal antibodies may bind to vaccine antigens and mask their ability to generate protective immunity in newborns. This topic will be explored in greater detail in the following section.

5. Neonatal Vaccinations

It should be apparent by now that neonates have broad impairments in their immune function but that certain circumstances are able to evoke strong responses. The case is no different in the context of neonatal vaccinations. In general, vaccine responses are quite poor in early life and are characterized by failure to mount antigen specific memory to most antigens. This defect is age-related and a gradual increase in function occurs throughout postnatal development. However, it is possible in some situations to overcome the challenges associated with neonatal vaccinations and elicit protective immunity.

The cause for poor vaccine responses stems from both cellular immaturity, as discussed in detail in the preceding sections, and from physiological immaturity along with the presence of maternal antibody. Physiological immaturity is characterized by poor secondary lymphoid organ architecture that fails to generate B cell-mediated adaptive immune responses. The splenic marginal zone (MZ) is poorly developed at birth and does not fully form until 2-3 weeks in murine neonates and 1-2 years in humans^{158, 159}. Interestingly, a study that investigated post-mortem human spleens from very young infants/children found that the MZ was often completely missing in babies that succumbed to sudden infant death syndrome and/or infection¹⁶⁰. Furthermore, neonates display reduced germinal centers (GC)/germinal center reactions in both mice¹⁵³ and humans¹⁶¹ which is thought to be due to immature follicular dendritic cells which fail to attract B cells and nucleate the GC reaction¹⁵⁴.

The contribution of maternal antibody also undermines vaccine efficacy in early life. It is thought that maternal antibody present in the neonate may directly bind vaccine

antigens and mask them from neonatal immune recognition or through IgG binding to the FcγRIIB¹⁶². The latter type of suppression occurs when a maternal IgG binds to FcγRIIB on antigen-specific neonatal B cells crosslinks with an antigen-loaded BCR on neonatal B cells¹⁶³. When this occurs, the tyrosine based inhibitory motif of FcγRIIB impedes the tyrosine based activation of the BCR and renders the B cell unresponsive. Maternal antibody has been shown to inhibit responses from all vaccine types: live-attenuated, inactivated, subunit, and experimental vaccines¹⁶². Strategies to overcome maternal antibody hindrance of early life vaccination may include increasing dose of antigen and discerning the earliest effective time point at which protection is afforded.

Despite the disadvantage of the neonate to mount effective humoral immunity, there are, surprisingly, vaccines that work well in newborns. The Bacillus Calmette-Guerin (BCG) vaccine which protects against childhood tuberculous meningitis and miliary disease is a live attenuated *Mycobacterium bovis* vaccine and is routinely given to infants at birth. It is the most widely used vaccine in the world, yet is not generally used in the US which has relatively low rate of tuberculosis¹⁶⁴. The success of BCG is attributed to its ability to induce a Th1 response¹⁶⁵ which may be due to robust activation of DCs. Even more exciting is that when BCG is given alongside other vaccines, like Hepatitis B, it increases the efficacy of the partner vaccine, yet this does not hold true for all partner vaccines since there was no synergy seen when partnered with vaccines for tetanus and diphtheria¹⁶⁶. While it is not completely understood what makes BCG work so well in infants, it is clear that induction of protective immunity is possible in early life. Another effective vaccine given in infancy is for hepatitis B virus (HBV). The first dose of HBV vaccine is given at birth and followed by two boosters within the first six months

of life. As with many vaccines, boosters are often required, especially in early life to maintain protective titers. Such is the case with diphtheria-tetanus-acellular pertussis which is given at 2, 4, 6, and 15–18 months and Hib given at 2, 4, 6, and 12–15 months. For other vaccines, it is impossible to generate protective titers early in life and must be delayed until later, such as influenza which isn't given until 6 months of age or MMR and Varicella which are delayed until 12 months¹⁶⁷

Since certain vaccines do elicit protective immunity at birth, it remains high priority to determine the mechanism of action and to replicate it in other vaccines. Promising results have been obtained when using vaccines that focus on intracellular antigen delivery such as the case with a single cycle HSV-1¹⁶⁸ or an attenuated strain of *Listeria monocytogenes* which is an intracellular pathogen¹⁶⁹. Along these same lines, DNA vaccines also allow neonates to mount protective immunity. In this case, the vaccine consists of a DNA vector that encodes antigenic portions of pathogens so that the antigens are generated by the host and processed intracellularly. When neonatal mice were vaccinated with a DNA vaccine against LCMV they demonstrated long-lived protection that was mediated by both CD8⁺ T cells and IgG1 and IgG2a producing B cells¹⁷⁰. Furthermore, DNA vaccination is effective even in the presence of maternal antibody¹⁷¹. Approaches that include intracellular antigen delivery as well as potent adjuvants that aid in DC function may be the future key to establishing effective neonatal vaccinations.

CHAPTER II
IL-4/IL-13 SIGNALING IN NEONATAL TH1 CELLS

1. Abstract

It has long been observed that neonates have a bias toward Th2 immune responses, which explains the prevalence of allergic reactions, and relatively weak Th1 responses, allowing heightened susceptibility to microbial infection. Previous observations in a murine neonate-to-neonate transfer system indicate that, while Th1 cells actually arise alongside Th2 cells in the primary response, they undergo cytokine-mediated apoptosis during re-challenge, resulting in the Th2 bias. Oddly, there is a developmental window in neonates where their Th1 cells express the IL-4/IL-13 heteroreceptor (HR) and during subsequent antigen challenge, neighboring Th2 cells produce IL-4 and IL-13 cytokine which signals through the HR and causes apoptosis. Herein, we describe the mechanism by which these neonatal Th1 cells die. Specifically, IL-4/IL-13 signaling activates STAT6 transcription factor which binds to the Stat-binding element in IRF-1's third intron and acts as an enhancer to promote greater IRF-1 expression. IRF-1 then relocates from the nucleus to the cytoplasm where it joins Bim in a protein complex to trigger intrinsic apoptosis. STAT6 inhibition prevents IRF-1 upregulation and rescues the cells from apoptosis and, similarly, silencing of IRF-1 with shRNA just prior to cytokine treatment significantly reduced apoptosis. The elucidation of this novel IRF-1-mediated apoptotic pathway may provide avenues to block this signaling cascade, prevent Th1 apoptosis, and restore neonatal protection against microbes and perhaps even enable successful early life vaccination strategies.

2. Introduction

When the field of neonatal immunology was in its infancy, foundational studies described early life as a window of time in which tolerance can be induced to prevent later graft rejection⁹. In this case, tolerance was considered to be due to the inability of neonates to mount an immune response. Since the advent of more sophisticated tools used to dissect immune responses, it became clear that neonates do not lack the ability to respond; rather, their responses are biased toward Th2 cells and away from Th1 cells^{12, 13, 14, 15, 16, 17}. One of the critical deficits in neonatal immunity is the inability to mount strong inflammatory Th1 responses which are required for pathogen clearance and vaccine protection¹⁷². While it has long been thought that neonates simply don't have the ability to mount Th1 responses, we have previously shown that Th1 cells are able to arise alongside Th2 cells in the primary response but are susceptible to IL-4/IL-13 cytokine induced death in the recall response¹⁴⁴. This is rather unusual as Th1 cells were originally not thought to support IL-4 signal transduction¹⁷³ despite expressing the type I IL-4 receptor which is comprised of IL-4R α and the common γ chain. We found that only in neonates is the type II IL-4 receptor (referred to as HR, comprised of IL-4R α and IL-13R α 1 chains) expressed on Th1 cells: a feature that is developmentally controlled by IL-12^{104, 174}. Consequently, as murine neonates outgrow this developmental window and antigen challenge occurs after day 6 of life, they are capable of mounting Th1 secondary responses¹⁰⁴. This led us to question why signaling through the HR in neonatal Th1 cells results in cell death, especially that similar signal transduction does not lead to death in other cell types.

IL-4 and IL-13 are closely related Th2-associated cytokines critical for the development of both allergic and anti-helminth responses. Their receptors and signaling

mechanisms contain significant overlap. IL-4 cytokine first binds the IL-4R α chain which then either dimerizes with the common gamma chain or IL-13R α 1 to form either the type I or II signaling complex, respectively¹⁷⁵. IL-13, on the other hand, binds to IL-13R α 1 which then forms a dimer with either IL-4R α 1 or the decoy chain IL-13R α 2, a non-signaling receptor¹⁷⁶. Therefore, the type II IL-4R α 1/IL-13R α 1 HR represents the only infrastructure capable of mediating IL-4 and IL-13 signal transduction in neonatal Th1 cells. Ligand binding of this HR activates receptor-associated Janus kinases, particularly JAK1¹⁷⁷, JAK2^{178, 179}, and TYK2^{177, 180}, and downstream signal transducer and activator of transcription proteins, mainly STAT6¹⁸¹ but also STAT1¹⁸² and STAT3¹⁸³. How signaling through these molecules influences apoptosis has not been previously identified.

In this study we examined the mechanism of IL-4/IL-13 cytokine induced apoptosis of neonatal Th1 cells and uncovered a previously unknown IRF-1-mediated apoptotic pathway. Using primary murine Th1 cells, we show that engagement of IL-4 or IL-13 with the HR on neonatal Th1 cells during secondary antigen challenge leads to STAT6 activation and subsequent transcriptional activation of IRF-1 by STAT6. IRF-1 then translocates to the cytoplasm where it interacts with Bim in a protein complex to initiate the intrinsic apoptotic pathway.

3. Materials and Methods

Mice.

Balb/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). DO11.10/Rag2^{-/-} transgenic mice which express an OVA-specific TCR have been previously described¹⁸⁴. DO11.10/Rag2^{-/-} IL-13Ra1^{-/-} mice were generated by crossing Balb/c IL-13Ra1^{-/-} mice with DO11.10/Rag2^{-/-} mice where the Balb/c IL-13Ra1^{-/-} mice were generated in our laboratory as previously described by genetic deletion of exons 7, 8, and 9¹⁸⁵. IL-13Ra1^{+/+}-GFP mice, which express green fluorescent protein (GFP) under the IL-13Ra1 promoter were generated in our laboratory from in collaboration with GenOway (Lyon, France) as previously described¹⁸⁵. These mice were crossed onto the DO11.10 Balb/c background by speed congenics to produce DO11.10 IL-13Ra1-GFP reporter mice. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-II transgenic mice which express an OVA-specific TCR were a gift from Dr. Deyu Fang (Northwestern University, Chicago, IL). C57BL/6 IL-13Ra1^{-/-} mice were generated by crossing the Balb/c IL-13Ra1^{-/-} mice to the C57BL/6 background by speed congenics as previously described¹⁸⁵. C57BL/6 OT-II IL-13Ra1^{-/-} mice were generated by crossing C57BL/6 IL-13Ra1^{-/-} mice to OT-II mice. All mice were bred and maintained in the animal care facility for the duration of the experiments and all experimental procedures were performed according to the guidelines of the University of Missouri Animal Care and Use Committee.

Antigens.

OVA peptide (SQAVHAAHAEINEAGR) which encompasses amino acids 323-339 of the chicken ovalbumin protein was used as a source of antigen as it is immunogenic in both Balb/c and C57BL/6 mouse strains. OVAp was purchased from EZbiolab (Carmel, IN). Ig-OVA, an IgG2b molecule expressing OVAp within the heavy chain variable region has been previously described¹⁸⁶ and is produced in our laboratory.

Adoptive T cell transfer.

Total splenic cells (3×10^6) from 1-d-old DO11.10/Rag2^{-/-} or IL-13R α 1^{-/-} DO11.10/Rag2^{-/-} mice were transferred in sterile PBS into 1-d-old Balb/c mice by iv injection through the facial vein using a 30 gauge needle as previously described¹⁸⁶. To investigate this process in C57BL6 recipient mice, 1-d-old OT-II or IL-13R α 1^{-/-} OT-II mice were used as a source of donor cells. The next day, recipient mice were give ip 100 μ g Ig-OVA in sterile PBS.

Analysis of T cell responses.

Primary/ex vivo. Two weeks after T cell transfer and exposure to Ig-OVA, total splenic cells were briefly stimulated for four hours with 10 μ M OVAp and then stained for CD4 and KJ1-26 (to track DO11.10 cells) or CD4, V β 5, and V α 2 (to track OT-II cells). Subsequently, cells were fixed in 2% formaldehyde and permeabilized with 0.2% saponin and stained for intracellular IFN γ and IL-4. Flow cytometry analysis was performed on a Beckman Coulter CyAn (Brea, CA).

Recall. Two weeks after T cell transfer and exposure to Ig-OVA, total splenic cells were stimulated with 10 μ M OVAp for 48 hours and the amount of cytokine produced was quantified by ELISA whereas the number of cytokine producing cells was determined by ELISPOT (see below for details). Additionally, apoptosis of Th1 cells was assessed by Annexin V staining. Briefly, splenic cells were stimulated for 4 hours with 10 μ M OVAp and then given Brefeldin A at 10 μ g/ml for an additional 8 hours. Surface molecules were stained as described above to track DO11.10 or OT-II cells and then cells were resuspended in Annexin V binding buffer and PE-conjugated Annexin V was added and incubated for 15 minutes at RT. Cells were then washed, fixed, permeabilized, and stained for intracellular IFN γ as described above. Flow cytometry analysis was performed on a Beckman Coulter CyAn (Brea, CA).

Secondary. Two months after T cell transfer and exposure to Ig-OVA, the mice were challenged with 125 μ g OVAp emulsified in CFA. Ten days later, total splenic cells were stimulated with 10 μ M OVAp for 24 hours and the amount of cytokine produced was quantified by ELISA whereas the number of cytokine producing cells was determined by ELISPOT.

ELISA and ELISPOT.

ELISA. IL-4 and IFN γ production was measured using the standard BD biosciences protocol (San Jose, CA) using anti-cytokine antibodies with the following modifications: capture buffer, 0.1M NaHCO₃; blocking buffer, 3%BSA/PBS; ABTS as substrate. The OD450 was read on a SpectraMax 190 counter (Molecular Devices, Sunnyvale, CA) and

analyzed using SOFTmax PRO 3.1.1 software where cytokine concentrations are extrapolated from the linear portion of a standard curve.

ELISPOT. HA multiscreen plates (Millipore, Bedford, MA) were coated with capture antibody diluted in 1M NaHCO₃ overnight, washed 3x in PBS, and blocked with serum-containing media for 1 hour before stimulating 1×10^6 spleen cells/well with 10 μ M OVAp at 37°C for 48 hours. Plates were then washed thoroughly and secondary biotinylated antibodies diluted in 3% BSA/PBS were added and kept overnight at 4°C. The next day, plates were washed and incubated at 37°C for 1 hour with avidin peroxidase and then developed by washing and adding the AEC + hydrogen peroxide substrate buffer. Spots were imaged on a Cellular Technology Ltd Analyzer series 3B (Cleveland, OH) and counted using Immunospot software version 3.2 (Cellular Technology, Cleveland, OH).

Cell Sorting.

Splenocytes from 1-3 day old DO11.10 IL-13Ra1-GFP neonates were stimulated with 10 μ M OVAp for 20-24 hours. Cells were collected and washed to rid of free antigen, replated, and incubated for an additional 72 hours. Cells were collected again, stained with fluorescently labelled antibodies specific for CD4 and CD11c and HR⁺ neonatal Th1 cells were based on CD11c⁻CD4⁺GFP⁺. The CD11c⁻CD4⁺GFP⁻ population was sorted as HR⁻ control and CD11c⁺GFP⁺ cells were sorted as a source of neonatal DCs. Cells were sorted on a Beckman Coulter MoFlo (Brea, CA) and only sorts yielding >95% purity were used for experiments.

Analysis of T cell death.

After sorting purified populations, T cells were stimulated with 10 μ M anti-CD3e clone 145-2C11 and 1 μ M anti-CD28 clone 37.51 (referred to as “antigen”) with the addition of 5 units of either rIL-4 or rIL-13 for 24 or 48 hours. Cells were then collected and stained with 7-AAD for 10 minutes in the dark and immediately analyzed using a Beckman Coulter CyAn (Brea, CA).

Western blots.

Sorted HR⁺ CD4 T cells were stimulated with Ag and cytokine for the indicated amount of time and then collected, pelleted, and the supernatant removed. Cells were resuspended with ice cold RIPA buffer supplemented with protease and phosphatase inhibitors and kept on ice for 30 minutes with occasional agitation. The volume of sample was adjusted for equivalent cells/ml between stimulation groups. Samples were centrifuged at 12000rpm for 15 minutes and the supernatant transferred to a new tube and 4x loading buffer supplemented with 10% BME was added and the sample heated at 95°C for 5 minutes. Proteins were separated on a 4-12% SDS-PAGE NuPage gel and transferred to a PVDF membrane. The membranes were blocked in 5% BSA/TBST and incubated overnight with primary rabbit antibodies from Cell Signaling Technologies (Danvers, MA) diluted in 5% BSA/TBST. The next day, membranes were washed 4x 5 minutes each in TBST and then incubated at RT for 1 hour with secondary anti-rabbit-HRP antibody (CST, Danvers, MA). Membranes were washed again and LI-COR WesternSure Premium substrate (Lincoln, NE) was added just prior to imaging on a LI-

COR Odyssey Fc. Densitometry was analyzed with ImageStudioLite v5.2 software (Lincoln, NE).

Inhibitor Treatment

Sorted HR+ CD4 T cells were pretreated for one hour with small molecule inhibitors of STAT molecules before the addition of antigen and cytokines. The STAT1 inhibitor was 2.5 μ M Fludarabine (Selleckchem, Houston, TX), the STAT3 inhibitor was 100 μ M S3I-201 (Selleckchem, Houston, TX) and the STAT6 inhibitor was 200nM AS1517499 (Axon Medchem, Reston, VA).

RT-qPCR.

After stimulation, RNA was isolated from cells with Trizol extraction and ethanol precipitation. Quantitative RT-PCR was performed using the Power SYBR Green RNA-to-CT 1-Step Kit on a StepOnePlus thermocycler (both from Applied Biosystems, Foster City, CA).

shRNA silencing.

Three different 29mer shRNA molecules targeting murine IRF-1 were designed using the RNAi Central program offered by the Hannon Laboratory at Cold Springs Harbor Labs (http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA). Sequences were chosen that do not have homology with other regions in the murine genome. A scrambled non-specific sequence was also designed. Sequences were engineered to express the human U6 promotor as well as a loop containing the SmaI restriction site between the two

palindromic sequences. Full sequences containing restriction sites, promoter, shRNA palindromes separated by 8nt loop, and transcription end site are as follows (5'→3'):

#1:AAAGCGGCCGCTTGTGGAAAGGACGAAACACCTGCAGATTAATTCCAACC
AAATCCCAGGGACCCGGGACCCTGGGATTTGGTTGGAATTAATCTGCATTTTT
ATCGAT, #2:AAGCGGCCGCTTGTGGAAAGGACGAAACACCCATCGAGGAAGT
GAAGGATCAGAGTAGGAACCCGGGATCCTACTCTGATCCTTCACTTCCTCGAT
GTTTTTATCGAT, #3:AAAGCGGCCGCTTGTGGAAAGGACGAAACACCCACTGT
CACCGTGTGTCGTCAGCAGCAGTACCCGGGAACTGCTGCTGACGACACACGG
TGACAGTGTTTTTATCGAT Scrambled:AAAGCGGCCGCTTGTGGAAAGGACGA
AACACCGCGCACACCACATTAGTTCGTAGAACTAAACCCGGGATTAGTTCTA
CGAACTAATGTGGTGTGCGCTTTTTATCGAT. Ultramer oligos for each of these
full length sequences were purchased from IDT (San Jose, CA) and used as template in a
PCR amplification with the following primers:

Common Fwd: 5'-AAAGCGGCCGCTTGTGGAAAG-3'

#1Rev: 5'-ATCGATAAAAATGCAGATTAATTCCAACCAA-3'

#2Rev: 5'-ATCGATAAAAACATCGAGGAAGTGAAGG-3'

#3Rev: 5'-ATCGATAAAAACACTGTCACCGTGTG-3'

Scrambled Rev: 5'-ATCGATAAAAAGCGCACACCACATTAG-3'

These PCR products were T-A cloned into pUC19 SmaI site for propagation. Upon confirmation by Sanger sequencing using the common M13 primers, these constructs were individually cloned into the MSCV-IRES-Thy1.1 retroviral vector using NotI and ClaI restriction sites and retroviral particles were made by cotransfecting 293FT cells with the retroviral vectors along with pEco packaging vector using Lipofectamine 3000

(Invitrogen, Carlsbad, CA). Culture supernatants were used to spin transduce freshly sorted HR⁺ CD4 T cells. After 2.5 days, cells were stimulated with Ag and cytokine for 24 hours and IRF1 levels, as well as Annexin V staining, was assessed on Thy1.1⁺ transduced cells.

ChIP

After cell sorting and stimulation, cells (100,000 cells per stimulation group) were fixed and proteins crosslinked with 1% formaldehyde and 10% serum for 10 minutes shaking at RT. 0.125M glycine was added for 5 minutes to stop fixation. Cells were then washed twice and lysed for 10 minutes on ice with SDS lysis buffer supplemented with protease inhibitors. Samples were diluted to 400µl in 1x ChIP buffer (CST, Danvers, MA) plus protease inhibitors and sonicated on a Misonix 3000 sonicator (Power 5, 5 cycles of 15s “on” 60s “off”) with the tube submerged in a wet ice bath to produce chromatin fragments 150-500bp. Sonicated samples were clarified by centrifugation at 12,000rpm for 10 minutes and supernatant transferred to a new tube. To preclear the lysates, 30µl protein G magnetic beads (CST) were added and samples rotated at 4°C for 2 hours and after magnetic pelleting, chromatin samples were again transferred to a new tube. 5% of the sonicated chromatin was withheld and kept at -20°C to be used later in the percent input calculation. The remaining chromatin was incubated at 4°C rotating with either STAT6 antibody or rabbit IgG isotype overnight. The next morning, magnetic beads (CST) were added for an additional 2 hours and then pelleted, the samples washed 3 times in low salt buffer, 1 time in high salt buffer, and finally eluted in ChIP elution buffer (all buffers from CST SimpleChIP kit) for 30 minutes at 65°C with agitation. All samples

were proteinase K digested to reverse crosslinking and the DNA purified with ChIP DNA Clean and Concentrator kit (Zymo, Irvine, CA). The eluted and purified DNA was used as a template in PCR reactions to amplify specific STAT6 binding sites. The GRCm38.p5 murine genome assembly was used to assess binding sites and, specifically, the IRF-1 transcript ID: Irf1-001 ENSMUST00000108920.8 was chosen as this transcript is the largest and includes all of the regions in other transcripts. Binding sites were identified by assessing the entire IRF1 gene starting 2kb upstream of the transcription start site for the STAT6 consensus sequence TTC(N₃₋₄)GAA which yielded four sites: within the promoter (-1357 to -1365 from translation start site), exon (1570-1579), intron (1895-1903), or 3'UTR (6491 to 6499) regions of the IRF1 gene. Primers were designed to flank these regions amplify and are as follows (5'→3'): Promoter Fwd: CTTTCCAAGACAGGCAAGG, Promoter Rev: AACACTTAGCGGGATTCC, Exon Fwd: TCAGCCTTTTCCCATACTTG, Exon Rev: CGGAACAGACAGGCATC, Intron Fwd: TGCAGAATTACTGGAGCAG, Intron Rev: TTGAGAAAGGAAGATGGAAGTC, 3'UTR Fwd: GATCCTCAGGGAGAGCAG, 3'UTR Rev: TCAGAGGCAGGCAGAG. Real-time PCR reactions were performed using the SYBR Green Universal Master Mix (Applied Biosystems, Foster City, CA) and % Input was calculated as follows: % Input = 5% x 2^(C[t]5% Input sample - C[t]IP sample).

Co-Immunoprecipitation.

Sorted HR⁺ CD4 T cells were stimulated for 24 hours with Ag and IL-4 or IL-13 and then collected, pelleted, and supernatants removed. Cells were resuspended in ice cold RIPA buffer supplemented with protease and phosphatase inhibitors and kept on ice for 30

minutes with occasional agitation. Lysates were centrifuged at 12000 rpm for 15 minutes and supernatants transferred to a new tube. Samples were pre-cleared with rabbit IgG rotating at 4°C for 4 hours before protein G sepharose beads (Invitrogen, Carlsbad, CA) were added for an additional 2 hours and then centrifuged at 2000 rpm for 3 minutes and the cleared lysates transferred to a new tube. Lysates were incubated with anti-Bim or anti-IRF1 antibodies from CST (Danvers, MA) diluted in RIPA buffer overnight rotating at 4°C. The next morning, protein G bead slurry was added and rotating continued for 3 hours. Samples were centrifuged at 2000 rpm for 3 minutes and supernatant was removed and kept for an additional immunoprecipitation with beta-tubulin. Beads were washed 3x in wash buffer and eluted by heating beads in protein gel loading buffer supplemented with 10% BME at 95°C for 5 minutes. Proteins were detected by western blot as described above.

Confocal microscopy

Sorted HR⁺ CD4 T cells were stimulated with Ag and cytokine for 24 hours, collected, diluted to 200µl with PBS and spun onto glass slides using a StatSpin Cytofuge 2 (Iris Sample Processing, Westwood, MA) at 850rpm for 4 minutes. Slides were immediately placed into ice cold methanol for 20 minutes at -20°C and then stored dry at the same temperature. Slides were then rehydrated in PBS 3x for 5 minutes at RT and blocked in 5% BSA/PBS for 2 hours at 4°C. After washing in PBS, primary antibody (anti IRF1 from CST and Alexa647-conjugated anti Bim from Novus) diluted in 1% BSA/PBS was added and incubated at 4°C overnight in a moist chamber. The next day slides were washed 5x in PBS and incubated with secondary antibody (Anti rabbit Alexa555 from

CST) for 1 hour at 4°C. After washing 5x in PBS again, Fluoroshield mounting medium with DAPI (Abcam, Cambridge, UK) was added and slides were coverslipped and kept at 4°C until imaged. All images were acquired on a Leica TCP SP8 confocal microscope at 63x magnification and processed with LAS X software (Leica, Wetzlar, Germany).

Yeast-two-hybrid

Assessment of Bim and IRF1 interaction was performed using the ProQuest Two-Hybrid System (Invitrogen, Carlsbad, CA). To generate IRF-1, Bim, and Bcl-2 sequences, mRNA was isolated from a Balb/c mouse spleen, cDNA synthesized using RevertAid First Strand Synthesis cDNA kit (Thermo) and amplified by PCR using primers that include attB sites, Shine-Dalgarno, and Kozak sequences as follows (5'→3'):

IRF1 fwd: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAAC
CATGCCAATCACTCGAATGCGGATGA

IRF1 rev: GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATGGTGCACAAGGA
ATGGCCTGA

Bim fwd: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAAC
CATGGCCAAGCAACCTTCTGATGTAA

Bim rev: GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAATGCCTTCTCCATA
CCAGACGG

Bcl-2 fwd: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAAC
CATGGCGCAAGCCGGGAGAACAGGGT

Bcl-2 rev: GGGGACCACTTTGTACAAGAAAGCTGGGTTCCTTGTGGCCAGGTA
TGCACCC.

Entry clones were generated in pDONR plasmid by BP recombination using Gateway Technology Clonase II kit (Invitrogen) and sequences confirmed by automated sequencing. Bait and prey plasmids were then generated in pDest32 by LR recombination. MAV203 yeast cells were transformed with different combinations of bait and prey plasmids and transformants were assessed for protein-protein interaction by β -galactosidase assay, growth on plates lacking uracil, and growth on plates with 5FOA as described in the manual.

Quantitative liquid culture β -galactosidase assay was performed by growing the transformants in YPD medium until mid-log phase, recording the OD600, lysing the cells by rapid freeze/thaw cycles in Z buffer, adding ONPG at 37°C and measuring the elapsed time until yellow color developed as well as the OD420. β -galactosidase units were calculated by the following: $\text{units} = 1,000 \times \text{OD420} / (\text{time} \times \text{volume of cells} \times \text{OD600})$.

4. Results

A. HR signaling provokes neonatal Th1 cell apoptosis during secondary antigen challenge in two different strains of mice.

The primary response of neonates elicits balanced Th1 and Th2 immunity in both Balb/c and C57BL/6 mouse strains (Figure 2.1A). In order to investigate the murine primary response, a neonate-to-neonate adoptive transfer system was used^{104, 119, 144, 174, 186}. Briefly, spleen cells from either HR^{+/+} or HR^{-/-} DO11.10 OVA-specific TCR transgenic 1d-old mice were transferred i.v. to 1d-old Balb/c hosts or HR^{+/+} or HR^{-/-} OT-II OVA-specific TCR transgenic splenocytes were transferred i.v. to C57BL/6 neonatal mice. The next day, the neonatal recipients were given Ig-OVA and ex-vivo responses were measured 14 days later. In both Balb/c and C57BL/6 strains, equivalent frequencies of IFN γ -producing Th1 cells and IL-4-producing Th2 cells were detected by intracellular staining, regardless of whether the donor cells were from HR^{+/+} or HR^{-/-} mice (Figure 2.1A). However, analysis of the recall response indicates that, regardless of strain, mice that received HR^{+/+} cells have a shift toward Th2 responses with increased IL-4 production and diminished IFN γ responses compared to mice that received HR^{-/-} cells as detected by ELISPOT and ELISA (Figure 2.1B). This indicates that the HR, which is present only on Th1 cells during the neonatal stage¹⁴⁴, has an active role in suppressing Th1 responses. This skewing is still evident in the secondary response when neonatal recipients of transgenic T cells and Ig-OVA challenge are re-challenged with OVAp/CFA at 8 weeks of age (Figure 2.1C). A previous study indicated that the HR directs apoptosis of Th1 cells during a second antigen encounter¹⁴⁴. Here, we demonstrate that HR ablation reduces death of Th1 cells at the recall response and that this phenomenon also occurs in

C57BL/6 neonates (Figure 2.1D). Overall, the results indicate that neonates mount a primary response with balanced Th1/Th2 cells but that Th1 cells, which express the HR, undergo apoptosis during re-challenge which leads to the well-defined Th2-skewed immunity of neonates.

Figure 2.1. HR signaling provokes neonatal Th1 cell apoptosis during secondary antigen challenge in two different strains of mice. Neonatal Balb/c (left) or C57BL/6 (right) mice recipient of spleen cells from 1d-old HR $^{-/-}$ or HR $^{+/+}$ DO11.10 (given to Balb/c) or OT-II (given to C57BL/6) donor mice were given i.p. Ig-OVA the day after transfer and ex vivo (A), recall (B and D), and secondary (C) responses were analyzed. (A) 14 days after Ig-OVA injection, intracellular IFN γ and IL-4 production by CD4 $^{+}$ KJ1-26 $^{+}$ DO11.10 T cells (Balb/c) and by CD4 $^{+}$ V α 5 high V α 2 high OT-II T cells (C57BL/6) are shown as representative dot plots and combined results are presented from at least 3 independent experiments. Each bar represents mean $\pm$ SEM. (B) Recall IFN γ and IL-4 responses after in vitro stimulation with OVA $_p$ as measured by ELISPOT (left) and

ELISA (right). Each bar represents mean $\pm$ SEM from at least three independent experiments (C) Two months after spleen cell transfer and Ig-OVA injections, mice were challenged with OVAp/CFA and ten days later IFN γ and IL-4 responses were measured by ELISPOT (left) and ELISA (right) after in vitro stimulation with OVAp. Bars represent mean $\pm$ SEM from three independent experiments. (D) OVA-specific Th1 cell apoptosis was detected during the recall response by Annexin V staining on CD4⁺KJ1-26⁺IFN γ ⁺ DO11.10 cells (left) or CD4⁺ V α 5^{high} V α 2^{high} IFN γ ⁺ OT-II cells after in vitro stimulation with OVAp.

B. Antigen and cytokine signaling are both required for neonatal Th1 apoptosis.

To better understand how apoptosis of Th1 cells occurs through the HR, we utilized DO11.10 IL-13R α 1-GFP reporter mice to sort a pure population of HR-expressing neonatal Th1 cells that have already been activated with OVA_p in vitro. Since Th1 apoptosis occurs during the second antigen encounter and is dependent on the HR, we sought to determine the extent of TCR stimulation and IL-4 or IL-13 cytokine stimulation in apoptosis (Figure 2.2). The results indicate that when HR⁺ cells (GFP⁺) are stimulated with antigen, through anti-CD3 and anti-CD28 antibodies, in combination with either IL-4 or IL-13 cytokine stimulation for 24 hours, significantly more cells die as indicated by 7-AAD staining in the representative contour plots (Figure 2.2A). This is in contrast to unstimulated cells or cells that are treated with either antigen or cytokine alone. Conversely, when HR⁻ (GFP⁻) cells are stimulated in the same manner, they fail to undergo apoptosis as the 7-AAD staining levels are at background levels (Figure 2.2B). Combined results from three independent experiments confirm these findings and also demonstrate that even higher levels of apoptosis are evident after 48hr stimulation.

Figure 2.2. Antigen and cytokine signaling are both required for neonatal Th1 cell apoptosis. HR⁺ Th1 cells were generated by stimulating spleen cells from neonatal DO11.10 IL-13R α 1-GFP mice with OVA_p for four days and then sorting the CD4⁺

CD11c⁻ GFP⁺ population. These cells were then cultured for 24 hours in the presence or absence of 10 μ M anti-CD3/1 μ M anti-CD28 antibodies (Ag) with or without the addition of recombinant murine IL-4 or IL-13 (5U each) and cell death was measured by 7-AAD staining. Panel (A) shows apoptosis on sorted HR⁺ (GFP⁺) cells whereas (B) assesses the HR⁻ (GFP⁻) population. (C) Shows combined results after either 24h or 48h incubation time. Bars represent mean $\pm$ SEM of at least three independent experiments and the * above HR⁺ Ag+IL-4 and HR⁺ Ag+IL-13 bars represent significance both at the level of the HR (HR⁺ vs HR⁻) and within the HR⁺ samples they are significantly different from the other treatments (Ag, IL-4, and IL-13) but not from each other (Ag+IL4 vs Ag+IL13).

C. HR signaling through STAT6 directs Th1 cell death.

To further delineate the mechanism of apoptosis in neonatal Th1 cells, we began by looking at HR-proximal signaling events. Stimulation of murine cells with IL-4 or IL-13 has been shown to phosphorylate STAT1¹⁸², STAT3¹⁸³, and STAT6 (Reviewed in Hebenstreit et al.¹⁸⁷). Therefore, we began by assessing phosphorylation of these STAT molecules on sorted HR⁺ Th1 cells that were left unstimulated or stimulated with Ag with or without IL-4 or IL-13 cytokine. Figure 2.3A shows no significant induction of STAT1 at either Tyr701 or Ser727 or STAT3 at either Tyr705 or Ser727. However, STAT6 phosphorylation at Tyr641 was significantly induced upon Ag + IL-4 stimulation. It should be noted that Ag + IL-13 treatment resulted in an obvious band on the pSTAT6 western blot, the levels were not significant when compiled from multiple experiments. This is not surprising, though, as IL-13 has a much lower affinity for IL-13Ra1 (Kd ~30nM^{188, 189}) than IL-4 has toward IL-4Ra (Kd 20-300pM¹⁹⁰) which may contribute to the observation that IL-13 is less potent at inducing STAT6 phosphorylation than IL-4 in the A549 human alveolar epithelial cell line¹⁸⁹. In Figure 2.3A, STAT phosphorylation was measured by western blot and densitometry used to normalize the bands to total STAT levels. Results are compiled from multiple experiments and presented in the bar graphs above each blot. If phosphorylation of STAT6 is responsible for HR-mediated Th1 apoptosis, then blocking STAT6 through a chemical inhibitor should prevent the cells from dying. To test this premise, sorted HR⁺ neonatal Th1 cells were pre-treated with chemical inhibitors against STAT1, STAT3, and STAT6, or left untreated, and then stimulated with Ag and IL-4 or IL-13 for 48 hours (Figure 2.3B). The results demonstrate that blocking STAT1 or STAT3 fails to rescue Th1 cells from dying whereas STAT6

inhibition successfully protects Th1 cells as evident by minimal 7-AAD staining. Combined results from three independent experiments confirm these findings where only the STAT6 inhibitor treated cells have a significantly reduced percentage of 7-AAD⁺ cells (Figure 2.3C).

Figure 2.3. IL-4 and IL-13 activate STAT6 to induce neonatal Th1 cell apoptosis.

HR⁺ Th1 cells were sorted and stimulated with 10 μ M anti-CD3/1 μ M anti-CD28 antibodies (Ag) with or without the addition of recombinant murine IL-4 or IL-13. (A)

Cells were collected after 20min to assess phosphorylation levels of STAT1 and STAT3 or after 1h to measure STAT6 phosphorylation by western blot. The bars represent the mean $\pm$ SEM of three independent experiments and the * represents statistical significance compared to all other stimulation groups as analyzed by one-way ANOVA with Bonferroni posttest. (B) Sorted HR⁺ Th1 cells were pretreated with inhibitors against STAT1, STAT3, and STAT6 for one hour prior to stimulation. Apoptosis was measured by 7-AAD staining after 48h incubation. (C) Combined results from three independent experiments carried out as described in (B). Each bar represents the mean $\pm$ SEM of three independent experiments and the * represents statistical significance when compared to the no inhibitor group.

D. The intrinsic apoptotic pathway is active during neonatal Th1 cell death.

Apoptosis is thought to be initiated through either the intrinsic or extrinsic pathways. To define which of these pathways are active in neonatal Th1 cell death, we performed gene expression analysis of key molecules involved in either pathway (Figure 2.4). The results demonstrate that neonatal HR^+ (GFP^+) Th1 cells, but not adult Th1 cells, which do not express the HR, or neonatal DCs, which express the HR but do not die when cultured with IL-4/IL-13, upregulate expression of Bim, Bax, and Bak which are all involved in mitochondrial permeabilization preceding cytochrome c release in the intrinsic apoptotic cascade. This upregulation occurs only when cells are stimulated with Ag + IL-4 or Ag + IL-13 and not Ag alone indicating a direct role of the HR. Furthermore, there was a similar upregulation in FOXO3a, a transcription factor shown to promote Bim expression¹⁹¹ in the Ag + 4 and Ag + 13 treated HR^+ neonatal cells. In further support, these cells demonstrate lower Akt, which is a negative regulator of FOXO3a¹⁹². Considering extrinsic apoptosis, it has been reported that IRF-1, a transcription factor highly expressed in Th1 cells, mediates $\text{IFN}\gamma$ induction of TRAIL¹⁹³ as well as TCR induction of FasL¹⁹⁴. The results demonstrate that there was no significant upregulation of Fas/FasL or TRAIL/DR5 induced by IL-4 or IL-13 signaling but that IRF-1 was, surprisingly, upregulated in the neonatal Th1 cells (Figure 2.4). This upregulation was also seen at the protein level where sorted neonatal Th1 cells have a basal level of IRF-1 protein expression that is significantly upregulated in the presence of Ag + IL-4 and Ag + IL-13 (Figure 2.5A).

Figure 2.4. Intrinsic apoptotic molecules are upregulated during neonatal Th1 cell death.

HR⁺ (GFP⁺) CD4⁺ neonatal cells, were sorted and stimulated with 10 μ M anti-CD3/1 μ M anti-CD28 antibodies (Ag) with or without the addition of recombinant murine IL-4 or IL-13 for 24 hours. RNA was isolated and subjected to RT-qPCR of intrinsic (left column) or extrinsic (right panel) apoptotic molecules and transcription factors. CD11c⁺

neonatal DCs and adult IFN γ ⁺ CD4⁺ T cells were tested under the same conditions and used for control purposes. mRNA relative quantities (RQ values) were determined by normalizing Ct values to the beta-actin internal reference gene.

E. STAT6-mediated IRF-1 induction directs apoptosis of neonatal Th1 cells.

As IRF-1 levels were further incited by the addition of IL-4 and IL-13, we hypothesized that the cytokines were signaling through STAT6 to cause IRF-1 upregulation. Indeed, this proved to be the case as inhibition of STAT6 with a chemical inhibitor prevented IL-4 and IL-13-induced IRF-1 upregulation (Figure 2.5B). To assess whether IRF-1 plays a role in apoptosis of neonatal Th1 cells, we sought to silence IRF-1 during the Ag + cytokine exposure. Since IRF-1 is critical for Th1 polarization, we were not able to use an IRF-1-knockout mouse as a source of Th1 cells. Instead, we opted to engineer a retrovirus to deliver a cocktail of three IRF-1-shRNA molecules into the sorted Th1 cells prior to Ag + cytokine challenge (Figure 2.5C-D and Figure 2.6) allowing Th1 cells to develop but silencing IRF-1 during the challenge that normally induces apoptosis. Figure 2.5C demonstrates that the IRF-1-shRNA treatment significantly reduces IRF-1 protein levels in neonatal Th1 cells compared to cells that received nonspecific scrambled shRNA control. Furthermore, IRF-1 silencing rescued cells from apoptosis as measured by Annexin-V staining (Figure 2.5D). Together, these results highlight a novel role of STAT6-mediated IRF-1 induction in the death of neonatal Th1 cells.

Figure 2.5. Neonatal Th1 cell death relies on upregulation of IRF-1.

(A) HR^+ Th1 cells were sorted and stimulated with 10 μ M anti-CD3/1 μ M anti-CD28 antibodies (Ag) with or without the addition of recombinant murine IL-4 or IL-13 for 24 hours. Cell lysates were prepared and IRF-1 was measured by western blot. The bar graph shows densitometry of IRF-1 normalized to the β -tubulin loading control compiled from three independent experiments. (B) shows the level of IRF-1 as measured by

western blot and normalized to β -tubulin loading control after cells were pre-incubated with a STAT6 inhibitor (AS1517499) for one hour, or left untreated, and then stimulated with Ag (10 μ M anti-CD3/1 μ M anti-CD28) and either recombinant murine IL-4 or IL-13 for 24 hours. In (C), sorted HR⁺ Th1 cells were transduced with a retrovirus cocktail expressing three different shRNA molecules that target IRF-1 or a scrambled shRNA control and then incubated with antigen and cytokine as described in (A). After 24 hours cells were collected and analyzed for IRF-1 expression and apoptosis by Annexin V staining on Thy1.1⁺ transduced cells. Bars represent the mean $\pm$ SEM of at least three independent experiments where * is $p \leq 0.05$ and ** is $p \leq 0.01$.

F. STAT6 binds to intron 3 in the IRF-1 gene to promote IRF-1 transcription in neonatal Th1 cells.

The ability of IL-4 and IL-13 to induce, rather than inhibit, IRF-1 through STAT6 is rather surprising as previous reports show that IL-4-induced STAT6 antagonizes IRF-1 expression by competing with STAT1 for access to the STAT binding element (SBE) within the IRF-1 promotor¹⁹⁵. Alternatively, the trans-activation domains of STAT6 may directly inhibit IRF-1 transcription in the absence of STAT1 competition¹⁹⁶ but these studies were not performed in primary T cells. To decipher the influence of STAT6 in IRF-1 gene expression in murine neonates, we first identified regions in the murine IRF-1 gene where STAT6 could potentially bind based on the STAT6-binding consensus sequence TTC(N3-4)GAA¹⁹⁷. Figure 2.6A depicts the four sites containing this sequence comprising regions in the promotor, exon 3, intron 3, and the 3' UTR. Chromatin immunoprecipitation was performed to determine whether STAT6 binds to each of these sites in sorted neonatal Th1 cells stimulated with Ag and IL-4. Following precipitation of STAT6, only DNA from the SBE in the intron was able to significantly amplify over that bound by the nonspecific IgG control whereas no significant binding was detected in the promoter, exon, or 3' UTR (Figure 2.6B). To determine how cytokine stimulation affects STAT6 binding to the intron SBE, cells were stimulated with either Ag alone or in combination with IL-4 or IL-13 before chromatin immunoprecipitation. The results demonstrate that STAT6 is mobilized to the intron region only when cells were treated with antigen and cytokine together as a significant enrichment (% input) of STAT6 bound DNA is amplified from Ag + IL-4 and Ag + IL-13 treated cells versus Ag treatment alone (Figure 2.6C). Furthermore, IL-13 seems to be even more potent than IL-

4 at initiating this binding event despite the reduced ability of IL-13 to induce STAT6 phosphorylation (Figure 2.3A).

Figure 2.6. STAT6 binds to intron 3 in the IRF-1 gene to promote IRF-1 transcription in neonatal Th1 cells. (A) shows a schematic diagram of the murine IRF-1 gene locus. The stars in the promoter region (P), exon 3 (E3), intron 3 (I3), and the 3' UTR indicate the presence of the STAT6 binding site TTC(N3-4)GAA. In (B) and (C), sorted neonatal Th1 cells were stimulated for 40 minutes in the presence of antigen and IL-4 (B) or Ag with or without IL-4 or IL-13 (C) and subjected to chromatin immunoprecipitation with either anti-STAT6 antibody or IgG isotype control. The precipitated chromatin was then amplified by qPCR with primers to encompass the four putative binding sites (B) or just the intron region (C). Data are represented as percent input.

G. IRF-1 and Bim co-immunoprecipitate and localize to the cytoplasm during neonatal Th1 apoptosis.

The increase in IRF-1 during Ag and cytokine challenge didn't appear to regulate any of IRF-1's downstream targets in the extrinsic pathway such as TRAIL or FasL (Figure 2.4) so we hypothesized that IRF-1 plays a role in modulating the intrinsic apoptotic pathway. Prior reports demonstrate that a virally-encoded IRF-1 (HHV8 vIRF1) displays homology with cellular IRF-1^{198, 199, 200} and the two share several binding partners such as p300 and IRF-8^{201, 202}. Since HHV8 vIRF1 is known to bind and regulate the cellular localization of the intrinsic apoptotic molecule Bim²⁰³, we questioned whether murine IRF-1 may also bind Bim to initiate apoptosis of neonatal Th1 cells. To this end, sorted Th1 cells were left unstimulated or stimulated with either Ag or Ag + IL-4 or IL-13 and then subjected to immunoprecipitation. Upon immunoprecipitation with anti-Bim antibody, lysates from cells that had been stimulated with Ag + IL-4 and Ag + IL-13 displayed strong IRF-1 bands as detected by western blot (Figure 2.7A, upper panel), indicating that Bim and IRF-1 exist in a complex together. Control samples were immunoprecipitated and blotted with anti- β -tubulin. The reciprocal experiment also holds true where lysates prepared from Ag + cytokine treated cells were immunoprecipitated with anti-IRF-1 antibody and strong Bim signal was detected by western blot (Figure 2.7A, lower panel). In this case, however, Ag + IL-4 treatment appears to be stronger at inducing this interaction than Ag + IL-13. Interestingly, of the three isoforms of Bim, only BimEL was detectable. These findings then led us to consider the cellular localization of the Bim/IRF-1 protein complex as Bim usually exists in the cytoplasm and IRF-1 is active in the Th1 nucleus. Confocal microscopy was used to detect subcellular localization of IRF-1 and Bim in

cells treated with Ag with or without IL-4 or IL-13 (Figure 2.7B). Antigen treated cells, which do not undergo apoptosis, show Bim staining (green) in the cytoplasm whereas IRF-1 (red) is located within the nucleus (DAPI, blue). There is no co-localization when the images are merged. On the other hand, cells treated with Ag + IL-4 and Ag + IL-13 show that IRF-1 relocates to meet Bim in the cytoplasm and the merged image shows co-localization of Bim and IRF-1 (indicated by yellow color). Additionally, when cells were simultaneously treated with a STAT6 inhibitor, IRF-1 levels were reduced and the protein failed to localize to the cytoplasm, again supporting the role of IL-4 and IL-13 in IRF-1 upregulation and also highlighting the necessity of cytokine signaling in IRF-1/Bim interaction (Figure 2.7B). Over 100 cells were imaged from two independent experiments and data compiled to assess the localization of IRF-1 in each of these treatments where location is described as cytoplasmic, nuclear, or spread intermediately between the two (Figure 2.8 and Figure 2.7C). While there were cells that displayed intermediate localization in each of the treatments, the majority of cells treated with Ag + IL-4 or IL-13 displayed cytoplasmic IRF-1 localization. Conversely, in cells that were treated with only Ag or when Ag + cytokine treated cells were given STAT6 inhibitor, IRF-1 was found to be predominately localized to the nucleus. Together, these results demonstrate that IL-4 and IL-13 signaling in neonatal Th1 cells instigate IRF-1 relocation to the cytoplasm where it interacts in a complex with Bim.

Figure 2.7. IRF-1 and Bim co-immunoprecipitate and localize to the cytoplasm during neonatal Th1 apoptosis. (A) Shows co-immunoprecipitation (co-IP) of IRF-1 and Bim in lysates of neonatal Th1 cells left untreated or stimulated with Ag alone or in the presence of IL-4 or IL-13 for 24 hours. The upper blot shows lysates precipitated with anti-Bim antibody and blotted for IRF-1 whereas the lower blot shows lysates precipitated with anti-IRF-1 antibody and blotted for Bim. As a loading control, the same lysate stock used in the corresponding experiment was used to precipitate and blot for β -tubulin. (B) Representative confocal microscopy images of neonatal Th1 cells that were stimulated as described in (A) and probed for Bim and IRF-1. The bottom two rows

depict cells that were treated with a STAT6 inhibitor during stimulation. The scale bars represent 5 μm . (C) Compiled data from two independent experiments as described in (B) where the localization of IRF-1 is characterized as residing in the cytoplasm, intermediate, or nucleus (See Fig. 2.8 for examples). At least 100 cells were analyzed for each treatment.

Figure 2.8. Examples of cellular localization of IRF-1. Sorted neonatal Th1 cells were stimulated with Ag and IL-4 and stained with a fluorescently tagged antibody against IRF-1 (red). The left panel shows an example of nuclear localization by IRF-1 where the top is IRF-1 only and the bottom is IRF-1 and DAPI together. The middle panel shows intermediate IRF-1 localization where some protein is detected in the nucleus and some is detected in the cytoplasm. The right panel depicts cytoplasmic IRF-1 localization.

H. Bim and IRF-1 do not bind directly in the yeast-two-hybrid assay.

As Bim and IRF-1 appear to exist in a complex, we questioned whether they bind each other directly. To test this, we used the yeast-two-hybrid system. Three different isoforms of Bim were fused to the GAL4 DNA binding domain (bait) and IRF-1 was fused to the GAL4 DNA activation domain (prey) and transformed into yeast containing LacZ and URA3 reporter genes (Figure 2.9). When the transformants were assessed for beta-galactosidase activity, none of the Bim/IRF-1 combinations resulted in positive-interacting blue colonies which were evident in the positive controls Krev1/RalGDS-wt and Bcl2/BimEL and to a much lesser extent in the weak-positive control Krev1/RalGDS-m1 (Figure 2.9A, left column). Similarly, when grown on media lacking uracil, only the strong positive control Krev1/RalGDS-wt demonstrated healthy growth which was not seen in the other transformants (Figure 2.9A, middle column). The addition of 5FOA, a negative selection agent that kills cells expressing URA3, hampered the growth of the strong positive control but did not affect the others (Figure 2.9A, right column). When these transformants were grown in liquid culture, a quantitative beta-galactosidase assay revealed a similar trend where only the strong positive Krev1 control and Bcl2/BimEL control displayed enzymatic activity but not any of the other transformants (Figure 2.9B). These results strongly suggest that Bim and IRF-1 do not directly bind each other but likely exist in a complex with other proteins.

Figure 2.9. IRF-1 and Bim do not directly bind one another. The yeast two-hybrid system was used to assess the ability of different isoforms of Bim to bind IRF-1. Three different isoforms of Bim, as well as the positive control Bcl-2, were expressed as fusion proteins with the GAL4 DNA-binding domain (bait) whereas IRF-1 was fused with the GAL4 activation domain (prey). Both fusion proteins were expressed in MaV203 yeast strain containing LacZ and URA3 reporter genes downstream of the GAL4 DNA binding site. (A) Yeast transformants were assessed for interaction between the bait and prey proteins by replicate plating on different media. Positive interactions are indicated by

blue color in the beta galactosidase assay as well as the ability of the yeast strains to grow on media lacking uracil. The addition of 5FOA provides a method for negative counter selection of -URA transformants. The interactions between Krev1 and RalGDS were used as a positive control while the m1 mutation of RalGDS provided a weak positive interaction and m2 RalGDS serves as a negative control. An additional positive control included Bim and Bcl-2 which are known to interact. (B) Quantitative beta galactosidase assay was performed in liquid culture. Bars represent the mean $\pm$ SD of triplicate wells.

5. Discussion

The neonatal period represents a unique developmental transition where the *in utero* requirement for anti-inflammatory conditions contrasts sharply with the immediate demand for pro-inflammatory protection against pathogens upon birth. During pregnancy, Th2 cytokines are produced at the fetal-maternal interface and correspond to fetal survival⁶. The importance of maintaining an anti-inflammatory environment during gestation is further highlighted in studies showing T cells from peripheral blood in pregnant women are less capable of producing Th1 cytokines but more likely to make Th2 cytokines than in non-pregnant women²⁰⁴. Moreover, women with recurrent miscarriages have elevated Th1 levels that are similar to non-pregnant women. While this anti-inflammatory state is critical for the maintenance of pregnancy, it renders the infant susceptible to microbial infection immediately after birth.

Despite an intrinsic drive for neonatal naïve T cells to become Th2 cells, which has been attributed to epigenetic hypomethylation of the Th2 locus¹³⁷, we have demonstrated that neonates are indeed capable of mounting Th1 responses but that these Th1 cells undergo apoptosis during rechallenge¹⁴⁴. Understanding the mechanism behind this apoptotic pathway may highlight novel ways to prevent cell death and restore Th1 immunity to combat early life infection and confer enhanced vaccine protection. Herein, we define the signaling pathway whereby IL-4 and IL-13 cytokines utilize their shared heteroreceptor to divert antigen induced activation of neonatal Th1 cells toward cell death.

In previous studies we utilized the Balb/c strain of mice to investigate Th2 biased neonatal immunity^{104, 119, 144, 174}. To ensure that our findings were not an artifact of the mouse strain, we adapted the neonate-to-neonate transfer model¹⁸⁶ to the C57BL/6 strain where either HR^{+/+} or HR^{-/-} neonatal OVA-specific OT-II cells were transferred into neonatal C57BL/6 hosts to enrich the frequency of responding cells and provide a means of tracking whether the T cells became Th1 or Th2. Our findings corroborated the results from Balb/c mice where Th1 cells do develop in the neonate but they die during recall indicating that this phenomenon is not strain specific. Furthermore, we demonstrate that this process relies on both antigen and cytokine together to drive apoptosis.

Prior investigation of IL-4/IL-13 signaling pathways indicate that these cytokines have the potential to phosphorylate STAT1, STAT3, and STAT6. Indeed, our lab has demonstrated a pivotal role for both STAT6 and STAT1 phosphorylation downstream of the HR in lineage choice of early thymic progenitor cells (manuscript under review). However, in the context of neonatal Th1 cells, STAT6 is the only operative STAT molecule. Interestingly, even though IL-4 induced a much stronger phosphorylation signal than IL-13, there is no apparent difference in apoptosis at either 24 or 48 hours, indicating that a relatively weak IL-13 signal can still lead to significant apoptosis of Th1 cells. Furthermore, the chromatin immunoprecipitation assay suggests that antigen and IL-13 induces greater STAT6 binding to the intron region of IRF-1 than does antigen and IL-4, although the difference is not statistically significant. Again, this highlights how even a minimal STAT6 phosphorylation event by IL-13 can culminate in a significant biological process. There are likely differences in signaling kinetics and possibly even

minor deviations in the pathway induced by IL-4 versus IL-13 but these have not been identified.

While this study elucidates a critical role for IRF-1 in the intrinsic apoptosis pathway, the mechanism of IRF-1 relocation from the nucleus to the cytoplasm is still unknown. It is plausible that HR signaling induces a post-translational modification in IRF-1 that aids its nuclear export especially that IRF-1 can be phosphorylated, sumoylated, and ubiquitinated. Furthermore, it should be noted that even though Bim and IRF-1 do not display direct binding in the yeast-two-hybrid assay it is possible that a particular post-translational modification is not occurring in the yeast system which would allow direct binding.

Another interesting consideration is that the induction of intrinsic apoptosis is still not completely understood. It is clear, however, that apoptosis is regulated through the balance of pro- and anti-apoptotic factors, particularly the various Bcl-2 family proteins, at the mitochondrial membrane^{205, 206}. Our data indicates that Bim, a pro-apoptotic factor is upregulated during antigen and cytokine induced neonatal Th1 death. While this alone may have the potential to induce apoptosis, it is likely that IRF-1 is playing more of a direct role in tipping the scale toward apoptosis. Evidence for this is presented as the two proteins co-immunoprecipitate and localize together outside of the nucleus and shRNA silencing of IRF-1 reduces apoptosis. Perhaps IRF-1 allows Bim to be more potent or prevents Bcl-2 or other anti-apoptotic proteins to be less effective. Furthermore, the subcellular localization of Bim also plays a role in its function. In healthy cells Bim is sequestered to the microtubule-associated dynein motor complex and certain apoptotic stimuli cause Bim to release from the complex and move to the mitochondria where it is

able to overpower anti-apoptotic molecules such as Bcl-2 to initiate apoptosis²⁰⁷. However, in activated T cells Bim is constitutively associated with Bcl-2-related proteins on the mitochondria and not found on microtubules²⁰⁸. Therefore, IRF-1 may serve as a trigger to either promote Bim activity or inhibit Bcl-2 activity. Overall, this study provides new insight into the apoptotic pathway of neonatal Th1 cells and highlights a role for IRF-1 in the intrinsic apoptotic pathway.

CHAPTER III
THE ROLE OF THE IL-4/IL-13 HETERORECEPTOR IN
NEONATAL VACCINATION

1. Abstract

Neonatal immunity is characterized by poor Th1 responses which render the infant susceptible to microbial infection and non-responsive to vaccination. Efforts to understand poor Th1 immunity may offer insights to novel vaccine strategies. We have previously demonstrated that neonatal Th1 cells express the IL-4/IL-13 heteroreceptor (HR) which marks them for apoptosis upon later antigen encounter¹⁴⁴ and that the absence of the HR restores Th1 immunity¹¹⁹. Here, we extend these findings to the context of early life vaccination and provide evidence that the absence of the HR restores Th1 and CTL vaccine responses upon LCMV infection. Furthermore, the blockade of STAT6 signaling, which is downstream of the HR, similarly allows for improved T cell responses. Perhaps the key to unlocking vaccine potential in neonates resides in strategies that prevent HR signaling.

2. Introduction

Infections are common during early life and significantly contribute to neonatal morbidity and mortality. The lack of immunological memory, in combination with the immaturity of immune cells and tissues, contribute to high global infant mortality rates. It is estimated that, globally, 2.8 million infants die each year during the neonatal stage and that nearly a quarter of these deaths are directly due to infection⁷. While vaccination remains the forerunner of infectious disease prevention, such strategies often do not work well in neonates due to their limited Th1 responses and overall poor adaptive immune function¹⁵⁴. For this reason, many vaccines are delayed until later in life, such as influenza which isn't given until 6 months of age or MMR and Varicella which are delayed until 12 months¹⁶⁷. Even vaccines that are given earlier in infancy show reduced efficacy and several boosters are needed in order to provide sufficient protection as in the case of DTaP, Hib, and pneumococcal conjugate vaccine. Despite the physiological defects in neonatal immunity, some vaccines are able to illicit powerful responses, such as Bacillus Calmette-Guerin vaccine which is effective at birth¹⁶⁷. This highlights the ability of specific antigen/adjuvant combinations to prove useful in neonates and significant efforts in vaccine design are aimed at understanding this process along with understanding the function of neonatal immune cells.

Prior studies performed in our lab discovered a developmental phenomenon that renders Th1 cells, which are important for vaccine responses, susceptible to antigen induced death. Specifically, Th1 cells from neonatal mice express the type II IL-4 heteroreceptor (HR), comprised of IL-13R α 1 and IL-4R α , through which both IL-4 and IL-13 cytokines can signal¹⁴⁴. During recall, Th2 cells produce these cytokines which signal

through the HR on Th1 cells and lead to their death, thereby perpetuating the neonatal Th2 bias. Interestingly, this appears to be a developmental trait that is modulated by IL-12 as by day 6 of life there are sufficient IL-12-producing DCs to counter the upregulation of the HR on Th1 cells and prevent their apoptosis¹⁰⁴. Recent studies highlight the mechanism whereby IL-4/IL-13 induces apoptosis through a novel STAT6-IRF1 death pathway. It is currently unknown how HR expression on Th1 cells affects early life vaccination and if it is possible to restore vaccination capacity by interfering with the STAT6-IRF1 signaling pathway.

Mice that lack the HR have robust Th1 responses in both the primary and secondary responses¹¹⁹ so we hypothesized that HR-deficient mice would display enhanced responses to neonatal vaccination as compared to wild-type HR-sufficient mice. Herein, we report that HR^{-/-} mice have enhanced peptide vaccine responses to an acute viral infection induced by lymphocytic choriomeningitis virus (LCMV) Armstrong resulting in reduced viral titers as well as enhanced T cell responses when challenged with the chronic infection model LCMV Clone 13 as compared to HR-sufficient wild-type mice. Furthermore, this protective effect is likely due to a defective STAT6-IRF1 signaling pathway as in vivo inhibition of STAT6 signaling at the time of infection in HR-sufficient mice provides restored T cell responses. These findings provide evidence that poor vaccine outcomes during the neonatal period are due to HR signaling in Th1 cells and that blocking the signaling pathway may provide an avenue to restore cell function and enhance vaccination.

3. Materials and Methods

Mice.

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 IL-13Ra1^{-/-} mice were generated by crossing the Balb/c IL-13Ra1^{-/-} mice to the C57BL/6 background by speed congenics as previously described¹⁸⁵. All mice were bred and maintained in the animal care facility for the duration of the experiments and all experimental procedures were performed according to the guidelines of the University of Missouri Animal Care and Use Committee.

Antigens.

Lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) amino acid residues 33-41 (GP33, KAVYNFATC) is an H2-D^b restricted antigen recognized by CD8 T cells. GP 61-80 (GP61, GLKGPDIIYKGVYQFKSVEFD) is a CD4 I-A^b restricted antigen. Both peptides were obtained from EZBiolab (Carmel, IN) and used in immunization and ex vivo stimulation for analysis of T cell responses. LCMV strains Armstrong and Clone13 were propagated in lab and used to infect mice after immunization. Both virus strains were a gift from Dr. Bumsuk Hahm (University of Missouri, Columbia, MO).

Immunization and Infection.

Neonatal C57BL/6 and C57BL/6 IL-13Ra1^{-/-} mice were given a peptide vaccine consisting of 10µg each of GP33 and GP61 in 50% IFA (100µl volume) intraperitoneally (IP) at three days of age. 7-8 weeks later the mice were then infected with either 2x10⁵ PFU of LCMV Armstrong given IP or 2x10⁶ PFU LCMV Clone13 administered IV. In

the case of Armstrong infection, mice were sacrificed four days later and T cell responses as well as viral titers in spleen, kidney, liver, and lung were measured. Regarding Clone13 infection, serum was collected from mice weekly until sacrifice at 46 days post infection when T cell responses were measured along with virus titers from serum, spleen, kidney, liver, and lung.

Analysis of T cell responses.

Total splenic cells were stimulated for six hours with 1 μ g/ml GP33, 5 μ g/ml GP61, and BFA. Cells were then stained for CD4, CD8, GP33-specific tetramer, and GP61-specific tetramer to track antigen specific T cells. Subsequently, cells were fixed in 2% formaldehyde and permeabilized with 0.2% saponin and stained for intracellular IFN γ , TNF α , and IL-4. Flow cytometry analysis was performed on a BD LSR-Fortessa X-20 (Franklin Lakes, NJ). LCMV GP-specific tetramers were obtained through the NIH Tetramer Core Facility (Atlanta, GA)

Analysis of Viral Titers.

Viral titers were determined by plaque assay on Vero E6 cells (ATCC, Manassas, VA). Briefly, Vero cells were grown to confluency in 6-well plates and infected with various dilutions of homogenized tissue samples in culture media for 90 minutes at 37°C with gentle rocking every 15 minutes. Samples were then aspirated out of the wells and overlaid with agarose + EMEM and incubated at 37°C. 7 days later, samples were fixed with formaldehyde, the agarose overlay removed and stained with crystal violet to visualize plaques.

In vivo inhibition of STAT6.

Mice were given daily IP injections of the STAT6 inhibitor AS1517499 (Axon Medchem, Reston, VA) at a dose of 10mg/kg in a 60µl volume of 50% DMSO/PBS for 6 days total.

4. Results

A. Neonatal peptide vaccination induces protective immunity against acute infection by lymphocytic choriomeningitis virus.

As IL-4 and IL-13 induce apoptosis of neonatal Th1 cells, we questioned the functional consequence of HR signaling in neonatal vaccination. LCMV was chosen as the infection model because it is well-characterized and includes both acute and chronic strains. There are also many reagents and tools already developed in which to analyze the LCMV response such as tetramers that can be used to track antigen-specific T cells. The success of neonatal vaccination depends on the context of antigen type, dose, and adjuvant used and we therefore first sought to define a successful vaccine regimen that would allow analysis of the response. We chose a peptide vaccine comprised of both CD4 and CD8 epitopes to induce maximal response. Neonatal HR^{+/+} or HR^{-/-} C57BL/6 mice were given an IP peptide vaccine of LCMV GP33-41 and GP61-80 emulsified in IFA. Two months later the mice were challenged with 2×10^5 pfu LCMV Armstrong (Arm), which causes acute viral infection, and titers were measured 4 days later in spleen, kidney, liver, and lung. In both HR^{+/+} and HR^{-/-} mice, vaccination resulted in decreased viral titers in the spleen, kidney, liver and lung as compared to mice given a null vaccine which consisted of IFA alone (Figure 3.1). Each group consisted of 9 mice from two separate experiments. While results were statistically significant in the kidney and liver for the HR^{+/+} mice, the difference appeared to be greater in the HR^{-/-} mice in the spleen and liver and, most surprisingly, no virus could be detected in the lungs of vaccinated HR^{-/-} mice.

Figure 3.1. Neonatal vaccination provides protection against later LCMV challenge in both $HR^{+/+}$ and $HR^{-/-}$ mice.

Neonatal $IL-13R\alpha1^{+/+}$ ($HR^{+/+}$) or $IL-13R\alpha1^{-/-}$ ($HR^{-/-}$) C57BL/6 mice were immunized IP with 10 μ g each of LCMV GP33-41 and GP61-80 peptides in IFA (Vaccinated) or IFA alone (Nil) at 3 days of age. Seven weeks later, mice were infected IP with LCMV Arm at 2×10^5 pfu. Four days post infection, viral titers from various organs were quantified by plaque assay on Vero cells.

B. The absence of the HR confers enhanced vaccine protection in neonatal mice.

Following the immunization and infection model established above, we then sought to compare whether HR-deficiency affects neonatal vaccine protection against acute LCMV infection. Accordingly, three-day old HR^{+/+} or HR^{-/-} C57BL/6 mice were given an IP peptide vaccine of LCMV GP33-41 and GP61-80 emulsified in IFA. Two months later the mice were challenged with 2×10^5 pfu LCMV Arm and titers were measured 4 days later in spleen, kidney, liver, and lung. Results are combined from 9 mice per group from two different experiments. The results demonstrate that wild-type mice in which HR signaling is intact display significantly higher viral titers in the spleen and liver (Figure 3.2). Again, there were undetectable levels of virus in the lungs of HR-deficient mice which further supports the hypothesis that HR-deficiency will enhance vaccine responses.

Figure 3.2. HR^{-/-} mice respond better to neonatal LCMV vaccination than HR^{+/+} mice.

Neonatal IL-13R α 1^{+/+} (HR^{+/+}, shaded bars) or IL-13R α 1^{-/-} (HR^{-/-}, open bars) C57BL/6 mice were immunized IP with 10 μ g each of LCMV GP33-41 and GP61-80 peptides in IFA (Vaccinated) or IFA alone (Nil) at 3 days of age. Seven weeks later, mice were infected IP with LCMV Arm at 2x10⁵ pfu. Four days post infection, viral titers from various organs were quantified by plaque assay on Vero cells.

C. Increased vaccination efficacy of HR-deficient mice is not due to enhanced T cell responses after acute viral infection.

Given that HR-deficient mice have enhanced Th1 immunity relative to HR-sufficient mice¹¹⁹ and that HR-deficiency confers enhanced vaccine responses, we hypothesized that HR-deficient mice would display enhanced T cell responses. Further evidence for this hypothesis stems from our findings that signaling through the HR in neonatal Th1 cells leads to their apoptosis and therefore ablation of signaling capacity would spare Th1 cells from death and allow to them to respond accordingly. To test this hypothesis, we immunized three-day old HR^{+/+} or HR^{-/-} C57BL/6 mice with a GP33-41/GP61-80 peptide vaccine. Two months later the mice were challenged with 2x10⁵ pfu LCMV Arm and splenic T cell responses were analyzed four days after infection by surface and intracellular staining with flow cytometry. Results are combined from 9 mice per group from two different experiments. Surprisingly, there was no difference in the frequency of antigen-specific CD4⁺ or CD8⁺ T cells as measured by GP61 or GP33 tetramer staining, respectively (Figure 3.3, top panel). Furthermore, when we assessed intracellular cytokine production after brief ex vivo stimulation with BFA, we found that both the HR^{+/+} mice and the HR^{-/-} mice produced similar levels of IFN γ and TNF α in their CD4⁺ T cells (Figure 3.3, left side two bottom panels) and CD8⁺ T cells (Figure 3.3, right side two bottom panels). These results indicate that enhanced vaccine-mediated protection to the acute virus in HR^{-/-} neonates may occur through a mechanism independent of T cells.

Figure 3.3. The HR does not play a role in T cell responses to LCMV Armstrong infection after neonatal vaccination.

Neonatal IL-13R α 1^{+/+} (HR^{+/+}, shaded bars) or IL-13R α 1^{-/-} (HR^{-/-}, open bars) C57BL/6 mice were immunized IP with 10 μ g each of LCMV GP33-41 and GP61-80 peptides in IFA (Vaccinated) or IFA alone (Nil) at 3 days of age. Seven weeks later, mice were infected IP with LCMV Arm at 2x10⁵ pfu. Four days post infection, splenic cells were stimulated briefly with GP33/61 peptides in the presence of BFA, stained with GP33/61-specific tetramers and intracellular cytokines were detected by flow cytometry.

D. HR^{-/-} neonates display enhanced T cell vaccine responses upon chronic LCMV Clone 13 infection.

As the HR does not play a role in T cell responses to acute viral infection in immunized neonates we questioned whether the situation may be different in a chronic infection with LCMV Clone 13 (Cl13), especially since CD8⁺ CTLs rely on CD4⁺ T cell help during chronic infection²⁰⁹. To this end, we immunized neonatal HR^{+/+} or HR^{-/-} C57BL/6 mice with the same peptide vaccine used in the Armstrong studies: 10μg each of GP33 and GP61 peptides in IFA. Two months later, the mice were infected IV with 2x10⁶ pfu of LCMV Cl13 and splenic T cell responses were measured 46 days post infection. In contrast to infection with Armstrong, HR^{-/-} mice displayed increased frequency of GP61^{Tet+} CD4⁺ T cells relative to HR^{+/+} mice (Figure 3.4, upper left panel) indicating a heightened CD4 T cell response to vaccination. Furthermore, a greater percentage of CD4 T cells from HR^{-/-} mice produced IFNγ and TNFα (Figure 3.4, left panels) than their HR^{+/+} counterparts demonstrating an increased functional capacity afforded when HR signaling is non-functional. As CD4 T cells are critical for sustaining CTL responses during a chronic infection which ultimately leads to viral clearance, we also assessed the CD8⁺ T cell response. Indeed, HR^{-/-} mice have a greater frequency of GP33^{Tet+} CD8⁺ T cells as compared to those from HR^{+/+} mice (Figure 3.4, upper right panel). Yet, despite the enhanced frequency of antigen-specific CD8 responders, there was no difference between HR^{-/-} and HR^{+/+} regarding the functional capacity of CD8 cells to produce IFNγ or TNFα (Figure 3.4, right panels). It should be noted that this preliminary experiment included only two mice and therefore statistical analyses have not been applied.

Figure 3.4. $HR^{-/-}$ neonatal mice have enhanced Th1 vaccine responses upon chronic LCMV Clone 13 infection to support the generation of CTLs.

Neonatal IL-13R α 1^{+/+} ($HR^{+/+}$, shaded bars) or IL-13R α 1^{-/-} ($HR^{-/-}$, open bars) C57BL/6 mice were immunized IP with 10 μ g each of LCMV GP33-41 and GP61-80 peptides in IFA (Vaccinated) or IFA alone (Nil) at 3 days of age. Eight weeks later, mice were infected IV with 2x10⁶ pfu LCMV Cl13. At 46 days post infection, mice were sacrificed and splenic CD4⁺ T cell responses were measured after ex vivo stimulation with GP33/61 peptides. Data are compiled from 2 mice from a single experiment and therefore no statistics were performed.

E. Inhibition of STAT6 signaling rescues T cell function and allows successful vaccination in neonatal mice.

Previous studies (see Chapter II) have demonstrated that signaling through the HR underpins the function of Th1 cells during the neonatal period and that this signaling pathway, induced by both IL-4 and IL-13, proceeds through STAT6 activation. Indeed, silencing of STAT6 with a pharmacological inhibitor prevents Th1 cell apoptosis and should thus preserve Th1 responses in our vaccination model. As Th1 cell death occurs at the second antigen encounter, we sought to determine whether STAT6 inhibition at the time of infection would preserve Th1 responses. The same vaccination and infection strategy was utilized as in Figure 3.4 except a group of mice received daily IP injections of 10mg/kg STAT6 inhibitor (AS1517499) for 6 days total, beginning 3 days prior to infection and continuing 2 days after infection (Figure 3.5A). The results demonstrate that STAT6 inhibition does indeed preserve Th1 responses as indicated by the increased frequency and total number of GP61^{Tet+} CD4 T cells as compared to mice that did not receive the inhibitor (Figure 3.5B, upper panels). The increase in antigen-specific CD4 T cells also corresponded to an increase in their function as there were significantly increased frequencies of both IFN γ and TNF α producing cells, further indicating the restoration of Th1 responses when STAT6 signaling is nullified (Figure 3.5B, lower panels). Furthermore, the increase in CD4 responses also corresponded to an increase in GP33^{Tet+} CD8 T cells in frequency and number upon STAT6 inhibition (Figure 3.5C, upper panels) as well as an increase in TNF α ⁺ CD8 T cells but not IFN γ ⁺ CD8 T cells (Figure 3.5C, bottom panel). Again, this preliminary experiment includes just two mice and therefore no statistical analyses were performed.

Figure 3.5. Inhibition of STAT6 signaling at time of Clone 13 infection rescues protective vaccine responses in neonatal mice.

Neonatal IL-13R α 1^{+/+} (HR^{+/+}) C57BL/6 mice were immunized IP with 10 μ g each of LCMV GP33-41 and GP61-80 peptides in IFA at 3 days of age. Eight weeks later, mice were infected IV with 2x10⁶ PFU LCMV Cl13. Some of the mice received IP STAT6 inhibitor at 10mg/kg daily for 6 days beginning 3 days prior to infection and continuing 2 days after infection (Inh) while other did not (Nil). 46 days after infection mice were sacrificed and splenic T cell responses were measured after ex vivo stimulation with GP33/61 peptides.

5. Discussion

The transition of a newborn from a sterile intra-uterine environment to the microbe-rich world we live in marks a unique immunological milestone. At this moment, an infant encounters both microbial friends and foes which will shape the health of the newborn throughout infancy and, potentially, well into adulthood. During this neonatal period, the immune system is not yet fully functional. This functional defect manifests in poor inflammatory responses and impaired Th1 immunity while Th2-type responses are dominant. While such a bias is critical for fetal engraftment and maintenance of pregnancy^{4, 5, 6}, it renders the newborn susceptible to microbial infection and precludes the efficacy of early life vaccination. Herein, we demonstrate that IL-4/IL-13 heteroreceptor (HR) expression on neonatal Th1 cells contributes to poor vaccination that is common in early life. Genetic deletion of the HR, as well as interference with downstream signaling through STAT6 inhibition allow for restoration of adaptive immunity.

Initially, studies were performed using LCMV Arm as the infectious model to test whether early life vaccination was successful. Even though HR^{-/-} mice displayed significantly reduced viral titers in various organ there was no difference in the T cell responses to this acute virus. This makes sense in consideration of the findings by Matloubian, et al²⁰⁹ that demonstrate depletion of CD4 T cells does not affect the ability of CD8 T cells to completely clear an acute LCMV Arm infection. Since CD8 T cells are not known to express the HR, HR-deficiency had no effect on T cell responses. It is likely that HR^{-/-} mice display reduced viral titers due to the influence of the HR on other neonatal immune cells. The HR is expressed on most myeloid cells¹⁸⁵ and we have

previously shown that neonatal basophils, which express the HR and produce significant IL-4, diminishes the function of early life dendritic cells¹¹⁹. While studies using DC-ablated mice show that DCs are not required for clearance of LCMV Arm infection²¹⁰, the abundant IL-4 in HR^{+/+} mice as compared to HR^{-/-} mice may explain the discrepancy. With abundant IL-4, as is the case in the neonatal system in general, but especially in the HR^{+/+} mice¹¹⁹, it is possible that the entire immune response may be modulated so that virus is not cleared as efficiently as when there is less IL-4 like in HR^{-/-} mice.

The findings that HR-deficiency allows enhanced neonatal vaccination are exciting and highlight potential new areas to explore. While it is not clinically feasible to block STAT6 signaling, as blockade of this pathway must occur at the secondary response, i.e. at the time of infection, and it is impossible to know when exposure to virus will occur, our findings do solidify prior evidence that HR signaling stifles neonatal immunity and extends our knowledge that this is operative in an early-life vaccination context. Avenues which preserve Th1 immunity will likely prove fruitful in the endeavor to boost neonatal vaccine efficacy.

REFERENCES

1. Huleihel M, Golan H, Hallak M. Intrauterine infection/inflammation during pregnancy and offspring brain damages: possible mechanisms involved. *Reproductive biology and endocrinology : RB&E* 2004, **2**: 17.
2. Hallman M. Cytokines, pulmonary surfactant and consequences of intrauterine infection. *Biology of the neonate* 1999, **76**: 2-9.
3. Galinsky R, Polglase GR, Hooper SB, Black MJ, Moss TJ. The consequences of chorioamnionitis: preterm birth and effects on development. *Journal of pregnancy* 2013, **2013**.
4. Vitoratos N, Papadias C, Economou E, Makrakis E, Panoulis C, Creatsas G. Elevated circulating IL-1beta and TNF-alpha, and unaltered IL-6 in first-trimester pregnancies complicated by threatened abortion with an adverse outcome. *Mediators of inflammation* 2006, **2006**(4).
5. Makhseed M, Raghupathy R, Azizieh F, Omu A, Al-Shamali E, Ashkanani L. Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions. *Human reproduction* 2001, **16**(10): 2219-2226.
6. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunology today* 1993, **14**(7): 353-356.
7. UNICEF. Committing to Child Survival: A promise renewed-Progress report 2014. New York; 2014.
8. UNICEF. Levels and Trends in Child Mortality. New York; 2015.
9. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. *Nature* 1953, **172**(4379): 603-606.
10. Burnet FMF, F. The Production of Antibodies. 2nd edn. Macmillan: Melbourne, 1949.

11. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of immunology* 1986, **136**(7): 2348-2357.
12. Powell TJ, Jr., Streilein JW. Neonatal tolerance induction by class II alloantigens activates IL-4-secreting, tolerogen-responsive T cells. *Journal of immunology* 1990, **144**(3): 854-859.
13. Chen N, Field EH. Enhanced type 2 and diminished type 1 cytokines in neonatal tolerance. *Transplantation* 1995, **59**(7): 933-941.
14. Singh RR, Hahn BH, Sercarz EE. Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune deviation: implications for antibody vs. T cell-mediated autoimmunity. *The Journal of experimental medicine* 1996, **183**(4): 1613-1621.
15. Sarzotti M, Robbins DS, Hoffman PM. Induction of protective CTL responses in newborn mice by a murine retrovirus. *Science* 1996, **271**(5256): 1726-1728.
16. Forsthuber T, Yip HC, Lehmann PV. Induction of TH1 and TH2 immunity in neonatal mice. *Science* 1996, **271**(5256): 1728-1730.
17. Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* 1996, **271**(5256): 1723-1726.
18. Miller A, Lider O, Abramsky O, Weiner HL. Orally administered myelin basic protein in neonates primes for immune responses and enhances experimental autoimmune encephalomyelitis in adult animals. *European journal of immunology* 1994, **24**(5): 1026-1032.
19. Min B, Legge KL, Li L, Caprio JC, Pack CD, Gregg R, *et al.* Neonatal tolerant immunity for vaccination against autoimmunity. *International reviews of immunology* 2000, **19**(2-3): 247-264.
20. Tavian M, Hallais MF, Peault B. Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. *Development* 1999, **126**(4): 793-803.

21. Tavian M, Peault B. Embryonic development of the human hematopoietic system. *The International journal of developmental biology* 2005, **49**(2-3): 243-250.
22. Dommergues M, Aubeny E, Dumez Y, Durandy A, Coulombel L. Hematopoiesis in the human yolk sac: quantitation of erythroid and granulopoietic progenitors between 3.5 and 8 weeks of development. *Bone marrow transplantation* 1992, **9**: 23-27.
23. Huyhn A, Dommergues M, Izac B, Croisille L, Katz A, Vainchenker W, *et al.* Characterization of hematopoietic progenitors from human yolk sacs and embryos. *Blood* 1995, **86**(12): 4474-4485.
24. Peschle C, Migliaccio G, Lazzaro D, Petti S, Mancini G, Care A, *et al.* Hemopoietic development in human embryos. *Blood cells* 1984, **10**(2-3): 427-441.
25. Peschle C, Mavilio F, Care A, Migliaccio G, Migliaccio AR, Salvo G, *et al.* Haemoglobin switching in human embryos: asynchrony of zeta----alpha and epsilon----gamma-globin switches in primitive and definite erythropoietic lineage. *Nature* 1985, **313**(5999): 235-238.
26. Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* 2010, **464**(7285): 108-111.
27. Tavian M, Robin C, Coulombel L, Peault B. The human embryo, but not its yolk sac, generates lympho-myeloid stem cells: mapping multipotent hematopoietic cell fate in intraembryonic mesoderm. *Immunity* 2001, **15**(3): 487-495.
28. Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* 2010, **464**(7285): 112-115.
29. Luis TC, Killmann NM, Staal FJ. Signal transduction pathways regulating hematopoietic stem cell biology: introduction to a series of Spotlight Reviews. *Leukemia* 2012, **26**(1): 86-90.

30. Lawrence SM, Corriden R, Nizet V. Age-Appropriate Functions and Dysfunctions of the Neonatal Neutrophil. *Frontiers in pediatrics* 2017, **5**: 23.
31. Charbord P, Tavian M, Humeau L, Peault B. Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. *Blood* 1996, **87**(10): 4109-4119.
32. Muller-Sieburg CE, Whitlock CA, Weissman IL. Isolation of two early B lymphocyte progenitors from mouse marrow: a committed pre-pre-B cell and a clonogenic Thy-1-lo hematopoietic stem cell. *Cell* 1986, **44**(4): 653-662.
33. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science* 1988, **241**(4861): 58-62.
34. Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* 1994, **1**(8): 661-673.
35. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley interdisciplinary reviews Systems biology and medicine* 2010, **2**(6): 640-653.
36. Nakorn TN, Miyamoto T, Weissman IL. Characterization of mouse clonogenic megakaryocyte progenitors. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**(1): 205-210.
37. Pronk CJ, Rossi DJ, Mansson R, Attema JL, Norddahl GL, Chan CK, *et al.* Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell stem cell* 2007, **1**(4): 428-442.
38. Artis D, Spits H. The biology of innate lymphoid cells. *Nature* 2015, **517**(7534): 293-301.
39. Traver D, Akashi K, Manz M, Merad M, Miyamoto T, Engleman EG, *et al.* Development of CD8alpha-positive dendritic cells from a common myeloid progenitor. *Science* 2000, **290**(5499): 2152-2154.

40. Manz MG, Traver D, Akashi K, Merad M, Miyamoto T, Engleman EG, *et al.* Dendritic cell development from common myeloid progenitors. *Annals of the New York Academy of Sciences* 2001, **938**: 167-173; discussion 173-164.
41. Manz MG, Traver D, Miyamoto T, Weissman IL, Akashi K. Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood* 2001, **97**(11): 3333-3341.
42. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nature reviews Immunology* 2007, **7**(5): 379-390.
43. Oranges T, Dini V, Romanelli M. Skin Physiology of the Neonate and Infant: Clinical Implications. *Advances in wound care* 2015, **4**(10): 587-595.
44. Evans NJ, Rutter N. Development of the epidermis in the newborn. *Biology of the neonate* 1986, **49**(2): 74-80.
45. Schmid-Wendtner MH, Korting HC. The pH of the skin surface and its impact on the barrier function. *Skin pharmacology and physiology* 2006, **19**(6): 296-302.
46. Yosipovitch G, Maayan-Metzger A, Merlob P, Sirota L. Skin barrier properties in different body areas in neonates. *Pediatrics* 2000, **106**(1): 105-108.
47. Jauniaux E, Gulbis B. Fluid compartments of the embryonic environment. *Human reproduction update* 2000, **6**(3): 268-278.
48. Fluhr JW, Darlenski R, Taieb A, Hachem JP, Baudouin C, Msika P, *et al.* Functional skin adaptation in infancy - almost complete but not fully competent. *Experimental dermatology* 2010, **19**(6): 483-492.
49. Korting HC, Kober M, Mueller M, Braun-Falco O. Influence of repeated washings with soap and synthetic detergents on pH and resident flora of the skin of forehead and forearm. Results of a cross-over trial in health probationers. *Acta dermato-venereologica* 1987, **67**(1): 41-47.

50. Rissmann R, Groenink HW, Weerheim AM, Hoath SB, Ponc M, Bouwstra JA. New insights into ultrastructure, lipid composition and organization of vernix caseosa. *The Journal of investigative dermatology* 2006, **126**(8): 1823-1833.
51. Pickens WL, Warner RR, Boissy YL, Boissy RE, Hoath SB. Characterization of vernix caseosa: water content, morphology, and elemental analysis. *The Journal of investigative dermatology* 2000, **115**(5): 875-881.
52. Dorschner RA, Lin KH, Murakami M, Gallo RL. Neonatal skin in mice and humans expresses increased levels of antimicrobial peptides: innate immunity during development of the adaptive response. *Pediatric research* 2003, **53**(4): 566-572.
53. Kai-Larsen Y, Gudmundsson GH, Agerberth B. A review of the innate immune defence of the human foetus and newborn, with the emphasis on antimicrobial peptides. *Acta paediatrica* 2014, **103**(10): 1000-1008.
54. Battersby AJ, Khara J, Wright VJ, Levy O, Kampmann B. Antimicrobial Proteins and Peptides in Early Life: Ontogeny and Translational Opportunities. *Frontiers in immunology* 2016, **7**: 309.
55. Walker VP, Akinbi HT, Meinen-Derr J, Narendran V, Visscher M, Hoath SB. Host defense proteins on the surface of neonatal skin: implications for innate immunity. *The Journal of pediatrics* 2008, **152**(6): 777-781.
56. Madan JC, Salari RC, Saxena D, Davidson L, O'Toole GA, Moore JH, *et al.* Gut microbial colonisation in premature neonates predicts neonatal sepsis. *Archives of disease in childhood Fetal and neonatal edition* 2012, **97**(6): F456-462.
57. Ardisson AN, de la Cruz DM, Davis-Richardson AG, Rechcigl KT, Li N, Drew JC, *et al.* Meconium microbiome analysis identifies bacteria correlated with premature birth. *PloS one* 2014, **9**(3): e90784.
58. Gronlund MM, Lehtonen OP, Eerola E, Kero P. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *Journal of pediatric gastroenterology and nutrition* 1999, **28**(1): 19-25.

59. Fanaro S, Chierici R, Guerrini P, Vigi V. Intestinal microflora in early infancy: composition and development. *Acta Paediatr Suppl* 2003, **91**(441): 48-55.
60. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JJ, Knight R. Bacterial community variation in human body habitats across space and time. *Science* 2009, **326**(5960): 1694-1697.
61. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, *et al.* Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108 Suppl 1**: 4578-4585.
62. Tourneur E, Chassin C. Neonatal immune adaptation of the gut and its role during infections. *Clinical & developmental immunology* 2013, **2013**: 270301.
63. Hirano S, Kataoka K. Histogenesis of the mouse jejunal mucosa, with special reference to proliferative cells and absorptive cells. *Archivum histologicum Japonicum = Nihon soshikigaku kiroku* 1986, **49**(3): 333-348.
64. Sherman MP. New concepts of microbial translocation in the neonatal intestine: mechanisms and prevention. *Clinics in perinatology* 2010, **37**(3): 565-579.
65. Chambers JA, Hollingsworth MA, Trezise AE, Harris A. Developmental expression of mucin genes MUC1 and MUC2. *Journal of cell science* 1994, **107** (Pt 2): 413-424.
66. Schaart MW, de Bruijn AC, Schierbeek H, Tibboel D, Renes IB, van Goudoever JB. Small intestinal MUC2 synthesis in human preterm infants. *American journal of physiology Gastrointestinal and liver physiology* 2009, **296**(5): G1085-1090.
67. Kai-Larsen Y, Bergsson G, Gudmundsson GH, Printz G, Jornvall H, Marchini G, *et al.* Antimicrobial components of the neonatal gut affected upon colonization. *Pediatric research* 2007, **61**(5 Pt 1): 530-536.
68. Menard S, Forster V, Lotz M, Gutle D, Duerr CU, Gallo RL, *et al.* Developmental switch of intestinal antimicrobial peptide expression. *The Journal of experimental medicine* 2008, **205**(1): 183-193.

69. Copland I, Post M. Lung development and fetal lung growth. *Paediatric respiratory reviews* 2004, **5 Suppl A**: S259-264.
70. Veldhuizen R, Nag K, Orgeig S, Possmayer F. The role of lipids in pulmonary surfactant. *Biochimica et biophysica acta* 1998, **1408**(2-3): 90-108.
71. Nkadi PO, Merritt TA, Pillers DA. An overview of pulmonary surfactant in the neonate: genetics, metabolism, and the role of surfactant in health and disease. *Molecular genetics and metabolism* 2009, **97**(2): 95-101.
72. Anderson KV, Jurgens G, Nusslein-Volhard C. Establishment of dorsal-ventral polarity in the *Drosophila* embryo: genetic studies on the role of the Toll gene product. *Cell* 1985, **42**(3): 779-789.
73. Rosetto M, Engstrom Y, Baldari CT, Telford JL, Hultmark D. Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a *Drosophila* hemocyte cell line. *Biochemical and biophysical research communications* 1995, **209**(1): 111-116.
74. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* 1996, **86**(6): 973-983.
75. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997, **388**(6640): 394-397.
76. Poltorak A, Smirnova I, He X, Liu MY, Van Huffel C, McNally O, *et al.* Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. *Blood cells, molecules & diseases* 1998, **24**(3): 340-355.
77. Yoon HS. Neonatal innate immunity and Toll-like receptor. *Korean journal of pediatrics* 2010, **53**(12): 985-988.

78. Forster-Waldl E, Sadeghi K, Tamandl D, Gerhold B, Hallwirth U, Rohrmeister K, *et al.* Monocyte toll-like receptor 4 expression and LPS-induced cytokine production increase during gestational aging. *Pediatric research* 2005, **58**(1): 121-124.
79. Kollmann TR, Crabtree J, Rein-Weston A, Blimkie D, Thommai F, Wang XY, *et al.* Neonatal innate TLR-mediated responses are distinct from those of adults. *Journal of immunology* 2009, **183**(11): 7150-7160.
80. Christensen RD, Rothstein G. Pre- and postnatal development of granulocytic stem cells in the rat. *Pediatric research* 1984, **18**(7): 599-602.
81. Melvan JN, Bagby GJ, Welsh DA, Nelson S, Zhang P. Neonatal sepsis and neutrophil insufficiencies. *International reviews of immunology* 2010, **29**(3): 315-348.
82. al-Mulla ZS, Christensen RD. Neutropenia in the neonate. *Clinics in perinatology* 1995, **22**(3): 711-739.
83. Carr R, Huizinga TW. Low soluble FcRIII receptor demonstrates reduced neutrophil reserves in preterm neonates. *Archives of disease in childhood Fetal and neonatal edition* 2000, **83**(2): F160.
84. Fox SE, Lu W, Maheshwari A, Christensen RD, Calhoun DA. The effects and comparative differences of neutrophil specific chemokines on neutrophil chemotaxis of the neonate. *Cytokine* 2005, **29**(3): 135-140.
85. Weinberger B, Laskin DL, Mariano TM, Sunil VR, DeCoste CJ, Heck DE, *et al.* Mechanisms underlying reduced responsiveness of neonatal neutrophils to distinct chemoattractants. *Journal of leukocyte biology* 2001, **70**(6): 969-976.
86. Rebuck N, Gibson A, Finn A. Neutrophil adhesion molecules in term and premature infants: normal or enhanced leucocyte integrins but defective L-selectin expression and shedding. *Clinical and experimental immunology* 1995, **101**(1): 183-189.

87. Carr R. Neutrophil production and function in newborn infants. *British journal of haematology* 2000, **110**(1): 18-28.
88. Falconer AE, Carr R, Edwards SW. Impaired neutrophil phagocytosis in preterm neonates: lack of correlation with expression of immunoglobulin or complement receptors. *Biology of the neonate* 1995, **68**(4): 264-269.
89. Bektas S, Goetze B, Speer CP. Decreased adherence, chemotaxis and phagocytic activities of neutrophils from preterm neonates. *Acta paediatrica Scandinavica* 1990, **79**(11): 1031-1038.
90. Nupponen I, Turunen R, Nevalainen T, Peuravuori H, Pohjavuori M, Repo H, *et al.* Extracellular release of bactericidal/permeability-increasing protein in newborn infants. *Pediatric research* 2002, **51**(6): 670-674.
91. Ambruso DR, Johnston RB, Jr. Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. *The Journal of clinical investigation* 1981, **67**(2): 352-360.
92. Wang H, Wang C, Zhao MH, Chen M. Neutrophil extracellular traps can activate alternative complement pathways. *Clinical and experimental immunology* 2015, **181**(3): 518-527.
93. Byrd AS, O'Brien XM, Laforce-Nesbitt SS, Parisi VE, Hirakawa MP, Bliss JM, *et al.* NETosis in Neonates: Evidence of a Reactive Oxygen Species-Independent Pathway in Response to Fungal Challenge. *The Journal of infectious diseases* 2016, **213**(4): 634-639.
94. Sohlberg E, Saghafian-Hedengren S, Bremme K, Sverremark-Ekstrom E. Cord blood monocyte subsets are similar to adult and show potent peptidoglycan-stimulated cytokine responses. *Immunology* 2011, **133**(1): 41-50.
95. Pedraza-Sanchez S, Hise AG, Ramachandra L, Arechavaleta-Velasco F, King CL. Reduced frequency of a CD14⁺ CD16⁺ monocyte subset with high Toll-like receptor 4 expression in cord blood compared to adult blood contributes to lipopolysaccharide hyporesponsiveness in newborns. *Clinical and vaccine immunology : CVI* 2013, **20**(7): 962-971.

96. Prabhu SB, Rathore DK, Nair D, Chaudhary A, Raza S, Kanodia P, *et al.* Comparison of Human Neonatal and Adult Blood Leukocyte Subset Composition Phenotypes. *PloS one* 2016, **11**(9): e0162242.
97. Berman JD, Johnson WD, Jr. Monocyte function in human neonates. *Infection and immunity* 1978, **19**(3): 898-902.
98. Speer CP, Wieland M, Ulbrich R, Gahr M. Phagocytic activities in neonatal monocytes. *European journal of pediatrics* 1986, **145**(5): 418-421.
99. Chelvarajan RL, Collins SM, Doubinskaia IE, Goes S, Van Willigen J, Flanagan D, *et al.* Defective macrophage function in neonates and its impact on unresponsiveness of neonates to polysaccharide antigens. *Journal of leukocyte biology* 2004, **75**(6): 982-994.
100. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *The Journal of experimental medicine* 1973, **137**(5): 1142-1162.
101. Dakic A, Shao QX, D'Amico A, O'Keeffe M, Chen WF, Shortman K, *et al.* Development of the dendritic cell system during mouse ontogeny. *Journal of immunology* 2004, **172**(2): 1018-1027.
102. Sun CM, Fiette L, Tanguy M, Leclerc C, Lo-Man R. Ontogeny and innate properties of neonatal dendritic cells. *Blood* 2003, **102**(2): 585-591.
103. Willems F, Vollstedt S, Suter M. Phenotype and function of neonatal DC. *European journal of immunology* 2009, **39**(1): 26-35.
104. Lee HH, Hoeman CM, Hardaway JC, Guloglu FB, Ellis JS, Jain R, *et al.* Delayed maturation of an IL-12-producing dendritic cell subset explains the early Th2 bias in neonatal immunity. *The Journal of experimental medicine* 2008, **205**(10): 2269-2280.
105. Wu L, Liu YJ. Development of dendritic-cell lineages. *Immunity* 2007, **26**(6): 741-750.

106. Basha S, Surendran N, Pichichero M. Immune responses in neonates. *Expert review of clinical immunology* 2014, **10**(9): 1171-1184.
107. Goriely S, Vincart B, Stordeur P, Vekemans J, Willems F, Goldman M, *et al.* Deficient IL-12(p35) gene expression by dendritic cells derived from neonatal monocytes. *Journal of immunology* 2001, **166**(3): 2141-2146.
108. Vollstedt S, O'Keeffe M, Odermatt B, Beat R, Glanzmann B, Riesen M, *et al.* Treatment of neonatal mice with Flt3 ligand leads to changes in dendritic cell subpopulations associated with enhanced IL-12 and IFN-alpha production. *European journal of immunology* 2004, **34**(7): 1849-1860.
109. Borrás FE, Matthews NC, Lowdell MW, Navarrete CV. Identification of both myeloid CD11c+ and lymphoid CD11c- dendritic cell subsets in cord blood. *British journal of haematology* 2001, **113**(4): 925-931.
110. Encabo A, Solves P, Carbonell-Uberos F, Minana MD. The functional immaturity of dendritic cells can be relevant to increased tolerance associated with cord blood transplantation. *Transfusion* 2007, **47**(2): 272-279.
111. Hunt DW, Huppertz HI, Jiang HJ, Petty RE. Studies of human cord blood dendritic cells: evidence for functional immaturity. *Blood* 1994, **84**(12): 4333-4343.
112. Chin TW, Ank BJ, Murakami D, Gill M, Spina C, Strom S, *et al.* Cytotoxic studies in human newborns: lessened allogeneic cell-induced (augmented) cytotoxicity but strong lymphokine-activated cytotoxicity of cord mononuclear cells. *Cellular immunology* 1986, **103**(2): 241-251.
113. Dalle JH, Menezes J, Wagner E, Blagdon M, Champagne J, Champagne MA, *et al.* Characterization of cord blood natural killer cells: implications for transplantation and neonatal infections. *Pediatric research* 2005, **57**(5 Pt 1): 649-655.
114. Lin SJ, Yang MH, Chao HC, Kuo ML, Huang JL. Effect of interleukin-15 and Flt3-ligand on natural killer cell expansion and activation: umbilical cord vs. adult peripheral blood mononuclear cells. *Pediatric allergy and immunology : official*

publication of the European Society of Pediatric Allergy and Immunology 2000, **11**(3): 168-174.

115. Lee YC, Lin SJ. Neonatal natural killer cell function: relevance to antiviral immune defense. *Clinical & developmental immunology* 2013, **2013**: 427696.
116. Crivellato E, Beltrami C, Mallardi F, Ribatti D. Paul Ehrlich's doctoral thesis: a milestone in the study of mast cells. *British journal of haematology* 2003, **123**(1): 19-21.
117. Rivera J, Fierro NA, Olivera A, Suzuki R. New insights on mast cell activation via the high affinity receptor for IgE. *Advances in immunology* 2008, **98**: 85-120.
118. Gilfillan AM, Rivera J. The tyrosine kinase network regulating mast cell activation. *Immunological reviews* 2009, **228**(1): 149-169.
119. Dhakal M, Miller MM, Zaghoulani AA, Sherman MP, Zaghoulani H. Neonatal Basophils Stifle the Function of Early-Life Dendritic Cells To Curtail Th1 Immunity in Newborn Mice. *Journal of immunology* 2015, **195**(2): 507-518.
120. Falcone FH, Haas H, Gibbs BF. The human basophil: a new appreciation of its role in immune responses. *Blood* 2000, **96**(13): 4028-4038.
121. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. *The Journal of allergy and clinical immunology* 2010, **125**(2 Suppl 2): S73-80.
122. Damsgaard TE, Nielsen BW, Henriques U, Hansen B, Herlin T, Schiotz PO. Histamine releasing cells of the newborn. Mast cells from the umbilical cord matrix and basophils from cord blood. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* 1996, **7**(2): 83-90.
123. Veerappan A, Thompson M, Savage AR, Silverman ML, Chan WS, Sung B, *et al.* Mast cells and exosomes in hyperoxia-induced neonatal lung disease. *American journal of physiology Lung cellular and molecular physiology* 2016, **310**(11): L1218-1232.

124. Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease. *Nature reviews Immunology* 2013, **13**(1): 9-22.
125. Gibson EL, Vaucher Y, Corrigan JJ, Jr. Eosinophilia in premature infants: relationship to weight gain. *The Journal of pediatrics* 1979, **95**(1): 99-101.
126. Calbi M, Scarpellino B, Giacchetti L. Usefulness of neonatal eosinophil counts as a marker of atopy? *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* 1993, **4**(2): 86-88.
127. Juul SE, Haynes JW, McPherson RJ. Evaluation of eosinophilia in hospitalized preterm infants. *Journal of perinatology : official journal of the California Perinatal Association* 2005, **25**(3): 182-188.
128. Hannet I, Erkeller-Yuksel F, Lydyard P, Deneys V, DeBruyere M. Developmental and maturational changes in human blood lymphocyte subpopulations. *Immunology today* 1992, **13**(6): 215, 218.
129. Correa-Rocha R, Perez A, Lorente R, Ferrando-Martinez S, Leal M, Gurbindo D, *et al.* Preterm neonates show marked leukopenia and lymphopenia that are associated with increased regulatory T-cell values and diminished IL-7. *Pediatric research* 2012, **71**(5): 590-597.
130. Born W, Yague J, Palmer E, Kappler J, Marrack P. Rearrangement of T-cell receptor beta-chain genes during T-cell development. *Proceedings of the National Academy of Sciences of the United States of America* 1985, **82**(9): 2925-2929.
131. Snodgrass HR, Kisielow P, Kiefer M, Steinmetz M, von Boehmer H. Ontogeny of the T-cell antigen receptor within the thymus. *Nature* 1985, **313**(6003): 592-595.
132. Garcia AM, Fadel SA, Cao S, Sarzotti M. T cell immunity in neonates. *Immunologic research* 2000, **22**(2-3): 177-190.
133. Min B, McHugh R, Sempowski GD, Mackall C, Foucras G, Paul WE. Neonates support lymphopenia-induced proliferation. *Immunity* 2003, **18**(1): 131-140.

134. Debock I, Flamand V. Unbalanced Neonatal CD4(+) T-Cell Immunity. *Frontiers in immunology* 2014, **5**: 393.
135. Wang G, Miyahara Y, Guo Z, Khattar M, Stepkowski SM, Chen W. "Default" generation of neonatal regulatory T cells. *Journal of immunology* 2010, **185**(1): 71-78.
136. Fontenot JD, Dooley JL, Farr AG, Rudensky AY. Developmental regulation of Foxp3 expression during ontogeny. *The Journal of experimental medicine* 2005, **202**(7): 901-906.
137. Rose S, Lichtenheld M, Foote MR, Adkins B. Murine neonatal CD4+ cells are poised for rapid Th2 effector-like function. *Journal of immunology* 2007, **178**(5): 2667-2678.
138. Yoshimoto M, Yoder MC, Guevara P, Adkins B. The murine Th2 locus undergoes epigenetic modification in the thymus during fetal and postnatal ontogeny. *PloS one* 2013, **8**(1): e51587.
139. Webster RB, Rodriguez Y, Klimecki WT, Vercelli D. The human IL-13 locus in neonatal CD4+ T cells is refractory to the acquisition of a repressive chromatin architecture. *The Journal of biological chemistry* 2007, **282**(1): 700-709.
140. White GP, Watt PM, Holt BJ, Holt PG. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells. *Journal of immunology* 2002, **168**(6): 2820-2827.
141. Donckier V, Flamand V, Desalle F, Vanderhaeghen ML, de Veerman M, Thielemans K, *et al.* IL-12 prevents neonatal induction of transplantation tolerance in mice. *European journal of immunology* 1998, **28**(4): 1426-1430.
142. Fadel SA, Ozaki DA, Sarzotti M. Enhanced type 1 immunity after secondary viral challenge in mice primed as neonates. *Journal of immunology* 2002, **169**(6): 3293-3300.

143. Kovarik J, Gaillard M, Martinez X, Bozzotti P, Lambert PH, Wild TF, *et al.* Induction of adult-like antibody, Th1, and CTL responses to measles hemagglutinin by early life murine immunization with an attenuated vaccinia-derived NYVAC(K1L) viral vector. *Virology* 2001, **285**(1): 12-20.
144. Li L, Lee HH, Bell JJ, Gregg RK, Ellis JS, Gessner A, *et al.* IL-4 utilizes an alternative receptor to drive apoptosis of Th1 cells and skews neonatal immunity toward Th2. *Immunity* 2004, **20**(4): 429-440.
145. Debock I, Delbauve S, Dubois A, Petein M, Leo O, Goldman M, *et al.* Th17 alloimmunity prevents neonatal establishment of lymphoid chimerism in IL-4-deprived mice. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2012, **12**(1): 81-89.
146. Kamath AT, Rochat AF, Valenti MP, Agger EM, Lingnau K, Andersen P, *et al.* Adult-like anti-mycobacterial T cell and in vivo dendritic cell responses following neonatal immunization with Ag85B-ESAT-6 in the IC31 adjuvant. *PloS one* 2008, **3**(11): e3683.
147. Echeverry A, Saijo S, Schesser K, Adkins B. *Yersinia enterocolitica* promotes robust mucosal inflammatory T-cell immunity in murine neonates. *Infection and immunity* 2010, **78**(8): 3595-3608.
148. Debock I, Jaworski K, Chadlaoui H, Delbauve S, Passon N, Twyffels L, *et al.* Neonatal follicular Th cell responses are impaired and modulated by IL-4. *Journal of immunology* 2013, **191**(3): 1231-1239.
149. Mastelic B, Kamath AT, Fontannaz P, Tougne C, Rochat AF, Belnoue E, *et al.* Environmental and T cell-intrinsic factors limit the expansion of neonatal follicular T helper cells but may be circumvented by specific adjuvants. *Journal of immunology* 2012, **189**(12): 5764-5772.
150. Galindo-Albarran AO, Lopez-Portales OH, Gutierrez-Reyna DY, Rodriguez-Jorge O, Sanchez-Villanueva JA, Ramirez-Pliego O, *et al.* CD8+ T Cells from Human Neonates Are Biased toward an Innate Immune Response. *Cell reports* 2016, **17**(8): 2151-2160.

151. Hermann E, Truyens C, Alonso-Vega C, Even J, Rodriguez P, Berthe A, *et al.* Human fetuses are able to mount an adultlike CD8 T-cell response. *Blood* 2002, **100**(6): 2153-2158.
152. Zhang J, Silvestri N, Whitton JL, Hassett DE. Neonates mount robust and protective adult-like CD8(+)-T-cell responses to DNA vaccines. *Journal of virology* 2002, **76**(23): 11911-11919.
153. Adkins B, Leclerc C, Marshall-Clarke S. Neonatal adaptive immunity comes of age. *Nature reviews Immunology* 2004, **4**(7): 553-564.
154. Siegrist CA, Aspinall R. B-cell responses to vaccination at the extremes of age. *Nature reviews Immunology* 2009, **9**(3): 185-194.
155. Flanagan KL, Burl S, Lohman-Payne BL, Plebanski M. The challenge of assessing infant vaccine responses in resource-poor settings. *Expert review of vaccines* 2010, **9**(6): 665-674.
156. Ochola R, Sande C, Fegan G, Scott PD, Medley GF, Cane PA, *et al.* The level and duration of RSV-specific maternal IgG in infants in Kilifi Kenya. *PloS one* 2009, **4**(12): e8088.
157. Benowitz I, Esposito DB, Gracey KD, Shapiro ED, Vazquez M. Influenza vaccine given to pregnant women reduces hospitalization due to influenza in their infants. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2010, **51**(12): 1355-1361.
158. MacLennan IC, Bazin H, Chassoux D, Gray D, Lortan J. Comparative analysis of the development of B cells in marginal zones and follicles. *Advances in experimental medicine and biology* 1985, **186**: 139-144.
159. Martin F, Kearney JF. Marginal-zone B cells. *Nature reviews Immunology* 2002, **2**(5): 323-335.
160. Kruschinski C, Zidan M, Debertin AS, von Horsten S, Pabst R. Age-dependent development of the splenic marginal zone in human infants is associated with different causes of death. *Human pathology* 2004, **35**(1): 113-121.

161. Blanchard Rohner G, Snape MD, Kelly DF, John T, Morant A, Yu LM, *et al.* The magnitude of the antibody and memory B cell responses during priming with a protein-polysaccharide conjugate vaccine in human infants is associated with the persistence of antibody and the intensity of booster response. *Journal of immunology* 2008, **180**(4): 2165-2173.
162. Niewiesk S. Maternal antibodies: clinical significance, mechanism of interference with immune responses, and possible vaccination strategies. *Frontiers in immunology* 2014, **5**: 446.
163. Karlsson MC, Getahun A, Heyman B. FcγRIIB in IgG-mediated suppression of antibody responses: different impact in vivo and in vitro. *Journal of immunology* 2001, **167**(10): 5558-5564.
164. Andersen P, Doherty TM. The success and failure of BCG - implications for a novel tuberculosis vaccine. *Nature reviews Microbiology* 2005, **3**(8): 656-662.
165. Marchant A, Goetghebuer T, Ota MO, Wolfe I, Ceesay SJ, De Groote D, *et al.* Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination. *Journal of immunology* 1999, **163**(4): 2249-2255.
166. Ota MO, Vekemans J, Schlegel-Haueter SE, Fielding K, Sanneh M, Kidd M, *et al.* Influence of Mycobacterium bovis bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. *Journal of immunology* 2002, **168**(2): 919-925.
167. Demirjian A, Levy O. Safety and efficacy of neonatal vaccination. *European journal of immunology* 2009, **39**(1): 36-46.
168. Franchini M, Abril C, Schwerdel C, Ruedl C, Ackermann M, Suter M. Protective T-cell-based immunity induced in neonatal mice by a single replicative cycle of herpes simplex virus. *Journal of virology* 2001, **75**(1): 83-89.
169. Kollmann TR, Reikie B, Blimkie D, Way SS, Hajjar AM, Arispe K, *et al.* Induction of protective immunity to Listeria monocytogenes in neonates. *Journal of immunology* 2007, **178**(6): 3695-3701.

170. Hassett DE, Zhang J, Slifka M, Whitton JL. Immune responses following neonatal DNA vaccination are long-lived, abundant, and qualitatively similar to those induced by conventional immunization. *Journal of virology* 2000, **74**(6): 2620-2627.
171. Hassett DE, Zhang J, Whitton JL. Neonatal DNA immunization with a plasmid encoding an internal viral protein is effective in the presence of maternal antibodies and protects against subsequent viral challenge. *Journal of virology* 1997, **71**(10): 7881-7888.
172. Mohr E, Siegrist CA. Vaccination in early life: standing up to the challenges. *Current opinion in immunology* 2016, **41**: 1-8.
173. Huang H, Paul WE. Impaired interleukin 4 signaling in T helper type 1 cells. *The Journal of experimental medicine* 1998, **187**(8): 1305-1313.
174. Hoeman CM, Dhakal M, Zaghoulani AA, Cascio JA, Wan X, Khairallah MT, *et al.* Developmental expression of IL-12Rbeta2 on murine naive neonatal T cells counters the upregulation of IL-13Ralpha1 on primary Th1 cells and balances immunity in the newborn. *Journal of immunology* 2013, **190**(12): 6155-6163.
175. Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 receptor: signaling mechanisms and biologic functions. *Annual review of immunology* 1999, **17**: 701-738.
176. Hershey GK. IL-13 receptors and signaling pathways: an evolving web. *The Journal of allergy and clinical immunology* 2003, **111**(4): 677-690; quiz 691.
177. Welham MJ, Learmonth L, Bone H, Schrader JW. Interleukin-13 signal transduction in lymphohemopoietic cells. Similarities and differences in signal transduction with interleukin-4 and insulin. *The Journal of biological chemistry* 1995, **270**(20): 12286-12296.
178. Roy B, Bhattacharjee A, Xu B, Ford D, Maizel AL, Cathcart MK. IL-13 signal transduction in human monocytes: phosphorylation of receptor components,

association with Jaks, and phosphorylation/activation of Stats. *Journal of leukocyte biology* 2002, **72**(3): 580-589.

179. Murata T, Noguchi PD, Puri RK. IL-13 induces phosphorylation and activation of JAK2 Janus kinase in human colon carcinoma cell lines: similarities between IL-4 and IL-13 signaling. *Journal of immunology* 1996, **156**(8): 2972-2978.
180. Murata T, Obiri NI, Puri RK. Human ovarian-carcinoma cell lines express IL-4 and IL-13 receptors: comparison between IL-4- and IL-13-induced signal transduction. *International journal of cancer* 1997, **70**(2): 230-240.
181. Hou J, Schindler U, Henzel WJ, Ho TC, Brasseur M, McKnight SL. An interleukin-4-induced transcription factor: IL-4 Stat. *Science* 1994, **265**(5179): 1701-1706.
182. Brunn GJ, Falls EL, Nilson AE, Abraham RT. Protein-tyrosine kinase-dependent activation of STAT transcription factors in interleukin-2- or interleukin-4-stimulated T lymphocytes. *The Journal of biological chemistry* 1995, **270**(19): 11628-11635.
183. Orchansky PL, Kwan R, Lee F, Schrader JW. Characterization of the cytoplasmic domain of interleukin-13 receptor-alpha. *The Journal of biological chemistry* 1999, **274**(30): 20818-20825.
184. Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* 1990, **250**(4988): 1720-1723.
185. Haymaker CL, Guloglu FB, Cascio JA, Hardaway JC, Dhakal M, Wan X, *et al.* Bone marrow-derived IL-13Ralpha1-positive thymic progenitors are restricted to the myeloid lineage. *Journal of immunology* 2012, **188**(7): 3208-3216.
186. Li L, Legge KL, Min B, Bell JJ, Gregg R, Caprio J, *et al.* Neonatal immunity develops in a transgenic TCR transfer model and reveals a requirement for elevated cell input to achieve organ-specific responses. *Journal of immunology* 2001, **167**(5): 2585-2594.

187. Hebenstreit D, Wirnsberger G, Horejs-Hoeck J, Duschl A. Signaling mechanisms, interaction partners, and target genes of STAT6. *Cytokine & growth factor reviews* 2006, **17**(3): 173-188.
188. Andrews AL, Holloway JW, Puddicombe SM, Holgate ST, Davies DE. Kinetic analysis of the interleukin-13 receptor complex. *The Journal of biological chemistry* 2002, **277**(48): 46073-46078.
189. LaPorte SL, Juo ZS, Vaclavikova J, Colf LA, Qi X, Heller NM, *et al.* Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell* 2008, **132**(2): 259-272.
190. Lowenthal JW, Castle BE, Christiansen J, Schreurs J, Rennick D, Arai N, *et al.* Expression of high affinity receptors for murine interleukin 4 (BSF-1) on hemopoietic and nonhemopoietic cells. *Journal of immunology* 1988, **140**(2): 456-464.
191. Gilley J, Coffey PJ, Ham J. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *The Journal of cell biology* 2003, **162**(4): 613-622.
192. Hay N. Interplay between FOXO, TOR, and Akt. *Biochimica et biophysica acta* 2011, **1813**(11): 1965-1970.
193. Park SY, Seol JW, Lee YJ, Cho JH, Kang HS, Kim IS, *et al.* IFN-gamma enhances TRAIL-induced apoptosis through IRF-1. *European journal of biochemistry* 2004, **271**(21): 4222-4228.
194. Chow WA, Fang JJ, Yee JK. The IFN regulatory factor family participates in regulation of Fas ligand gene expression in T cells. *Journal of immunology* 2000, **164**(7): 3512-3518.
195. Ohmori Y, Hamilton TA. IL-4-induced STAT6 suppresses IFN-gamma-stimulated STAT1-dependent transcription in mouse macrophages. *Journal of immunology* 1997, **159**(11): 5474-5482.

196. Goenka S, Youn J, Dzurek LM, Schindler U, Yu-Lee LY, Boothby M. Paired Stat6 C-terminal transcription activation domains required both for inhibition of an IFN-responsive promoter and trans-activation. *Journal of immunology* 1999, **163**(9): 4663-4672.
197. Schindler U, Wu P, Rothe M, Brasseur M, McKnight SL. Components of a Stat recognition code: evidence for two layers of molecular selectivity. *Immunity* 1995, **2**(6): 689-697.
198. Li M, Lee H, Guo J, Neipel F, Fleckenstein B, Ozato K, *et al.* Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor. *Journal of virology* 1998, **72**(7): 5433-5440.
199. Moore PS, Boshoff C, Weiss RA, Chang Y. Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science* 1996, **274**(5293): 1739-1744.
200. Russo JJ, Bohenzky RA, Chien MC, Chen J, Yan M, Maddalena D, *et al.* Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proceedings of the National Academy of Sciences of the United States of America* 1996, **93**(25): 14862-14867.
201. Burysek L, Yeow WS, Lubyova B, Kellum M, Schafer SL, Huang YQ, *et al.* Functional analysis of human herpesvirus 8-encoded viral interferon regulatory factor 1 and its association with cellular interferon regulatory factors and p300. *Journal of virology* 1999, **73**(9): 7334-7342.
202. Jacobs SR, Damania B. The viral interferon regulatory factors of KSHV: immunosuppressors or oncogenes? *Frontiers in immunology* 2011, **2**: 19.
203. Choi YB, Nicholas J. Bim nuclear translocation and inactivation by viral interferon regulatory factor. *PLoS pathogens* 2010, **6**(8): e1001031.
204. Reinhard G, Noll A, Schlebusch H, Mallmann P, Ruecker AV. Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. *Biochemical and biophysical research communications* 1998, **245**(3): 933-938.

205. Shamas-Din A, Kale J, Leber B, Andrews DW. Mechanisms of action of Bcl-2 family proteins. *Cold Spring Harbor perspectives in biology* 2013, **5**(4): a008714.
206. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nature reviews Cancer* 2002, **2**(9): 647-656.
207. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Molecular cell* 1999, **3**(3): 287-296.
208. Zhu Y, Swanson BJ, Wang M, Hildeman DA, Schaefer BC, Liu X, *et al.* Constitutive association of the proapoptotic protein Bim with Bcl-2-related proteins on mitochondria in T cells. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(20): 7681-7686.
209. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *Journal of virology* 1994, **68**(12): 8056-8063.
210. Hilpert C, Sitte S, Matthies A, Voehringer D. Dendritic Cells Are Dispensable for T Cell Priming and Control of Acute Lymphocytic Choriomeningitis Virus Infection. *Journal of immunology* 2016, **197**(7): 2780-2786.

VITA

Mindy Marie Miller was born on October 16th, 1987 in Denver, Colorado as the younger of two children and only daughter to Michael and Keturah Larum. She attended Lakewood High School where she was involved in music performance playing violin and piano in high school ensembles as well as various youth orchestras and symphonies in the Denver area. She graduated a semester early in December 2005 with a 4.0 GPA. After spending a year working in the defense industry in an electronics lab and teaching violin and piano, she realized how much she missed school and decided to continue her education in biology at Western State Colorado University. There she fell in love with science and graduated with a BA degree in biology, emphasis in cellular biology, and a minor in music performance with a 4.0 GPA. Ten days after graduation she married her best friend, Chris Miller, and the two moved to Prescott, Arizona where Chris was finishing his engineering degree. After taking a year off to climb mountains, explore the southwest, and truly enjoy life, Mindy and Chris moved to Columbia, Missouri where Mindy would pursue her PhD in immunology under the guidance of Dr. Habib Zaghouani.