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Lymphoid Hematopoiesis and the Role of B-cells in Transgenic Mouse Model of Sickle Cell Disease

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Introduction
Sickle-cell disease (SCD) is the most common genetic blood disorder, affecting millions of people worldwide (1.2, 1.22). The disease results from a point mutation of glutamic acid to valine in hemoglobin, the oxygen carrying molecule in erythrocytes (1.3). This mutation causes red blood cells (RBCs) to stiffen into a sickled shape during de-oxygenation (1.3). Circulation of sickled cells affects the vessels and associated tissues through which they flow. The harmful mutation is kept in circulation because it confers some resistance to malaria when in the heterozygous state (1.3). Issues like vaso-occlusion, occurring when sickled RBCs block blood vessels, anemia, or a lack of hemoglobin in the blood, and raised hemolytic rate, or RBC lysis, are common in SCD due to blockages and subsequent low oxygen levels in circulation (1.3). The amount of debris in the blood that must be filtered out is drastically increased as a result of sickled RBC hemolysis. Amassed hemolytic particles and circulatory system blockages contribute to the formation of splenomegaly, or palpable enlargement of the spleen (1.4).

One of the main functions of a healthy spleen is to filter the blood, remove old erythrocytes, and act as an immune center (1.5). In order for the spleen to function properly, it needs to possess the appropriate cells, namely B and T cells, and internal structure, which is organized into red pulp, where the blood is filtered; white pulp, which consists of lymphocytes; and a marginal zone in between (1.5). With SCD, the spleen does not possess this orderly structure (1.2). As a result, there is a decrease in baseline immunity associated with the disease, specifically infection with *Streptococcus pneumoniae* (1.2). Anything that is present in or travels through a SCD spleen is subject to its hostile environment caused by issues such as hypo-splenism, splenic sequestration, and overall decreased functionality (1.4).
In 1983, a new subset of innate-like B cells known as the B-1 cells were discovered in mice by the Herzenberg lab (1.6). This group of researchers is one of the leading laboratories in the fields of genetics and immunology. They have been curious about the development, function, and life of the B-1 cells since their discovery (1.7, 1.8). This new B cell subset is present mainly in the peritoneal and pleural cavities as well as the spleen (1.6, 1.9). B-1 cells have been shown to develop earlier and have different stem cell origins than B-2 cells during growth, theorized as either antigen-driven differentiation or following a layered immune system model with distinct lineages/progenitors, (1.17), and secrete self- and poly-reactive antibodies that form the natural antibody reserve in the absence of exposure to foreign antigen (1.6). Naïve B-1 cells are present in the spleen and are sent to the peritoneal cavity upon activation (1.6). To get to the spleen in a developed person, these IgM+ cells are first produced in the bone marrow (1.17).

In mice, the B-1 cells can be recognized as either B-1a (CD5+) or B-1b (CD5-). The B-1a cells are important first responders to pathogenic infection that produce natural low affinity antibodies independently of B cell receptor (BCR) specificity which react to self-antigens and help to clear apoptotic cells (1.6, 2.6, 2.27). Data has shown that these cells are mostly derived from the fetal liver (0.1, 1.6). They then travel to the bone marrow where they develop without T cell help, (4), traffic to their niche in the spleen and are sent to the peritoneal cavity upon activation with antigen (2.27, 2.30). It is unclear if the seemingly self-maintained population of B-1a cells have a correlation to adult bone marrow hematopoietic stem cells (HSCs) (1.6). B-1b cells are uniquely T cell independent when it comes to their antibody production and have a long-term effect after bacterial infection (1.6). Both B-1a and B-1b cells have been shown to
have particular roles in developing immunity to *S. pneumoniae* infections (2.5). **Interestingly, in SCD B-1a cells have been shown to be consistently diminished in the spleen, (1.2), but not the peritoneal cavity of adult mice.** A decrease in the number of B-1a cells is likely a contributor to the increased susceptibility to infection associated with SCD (1.2).

The development of the immune system is an essential part of fetal growth. In SCD, where components of the immune system do not function properly, it begs the question as to what point in development this issue begins? Because of the intimate interactions between the different systems of the body during development, it is crucial to draw parallels between different fields in order to arrive at cohesive explanations for these anomalies. HSC niche development and the correlations between the development of the neuroendocrine system and immune system could provide crucial information to explain this aspect of SCD and will be discussed in Chapters 1 & 2. SCD may cause a hostile environment for developing HSCs and/or may have side effects within the neuroendocrine system, both of which could affect whether or not the immune system develops properly.

Based on published data, I predict that B-1a cell numbers are lower in the SCD spleen because of the hostile environment the cells have to develop in, rather than it being an innate defect in the HSCs themselves. This hypothesis is the basis of the project conducted in Chapter 3 in an attempt to discern whether the B-1a cell diminution associated with SCD is an issue with generation or activation. As HSCs progress from their multi-potent state to differentiated B-1 cells, development aspects like their induction and trafficking may be affected by the associated symptoms of SCD. Experimentation has been done to determine whether or not there is an innate defect in the adult bone marrow HSCs in terms of their ability to populate the lacking B-
1a cell niche in the SCD spleen (Ch. 3). Another method currently being utilized to gain more insight regarding the B-1a cell diminution is BCR sequencing of the B-1a cells in the peritoneal cavity. This method will reveal if the seemingly normal population of B-1a cells in the SCD peritoneal cavity are more monoclonal, insinuating that the population of cells was derived from a smaller number of precursors sent from the spleen. The results I have obtained with the Szczepanek lab are bringing us closer to finding out the cause and implications of the B-1a cell diminution in SCD.

The reason behind looking into the B-1a cell diminution in SCD is its potential effect on the immune potential of those patients. As stated, patients with SCD have an increased susceptibility to infection, especially with *S. pneumoniae* (1.2). Prevnar-13, the vaccine against *S. pneumoniae* in children, has also been shown to have decreased efficacy long-term in SCD mice (1.22; Appendix). The mechanism of the vaccine is unclear, but if B-1 cells have been shown to play a role in defending against *S. pneumoniae* infection, (2.5), and have been shown to be diminished in SCD, (1.2), then maybe the vaccine functions through a pathway involving these cells. We set out to determine which B cell subsets respond to Prevnar-13 in SCD and wild-type (WT) mice (Chapter 4) to see if there is a connection between the B-1a cell population and the vaccine that could affect the decreased efficacy.
CHAPTER 1

Review: Hematopoietic Stem Cells in Sickle Cell Disease
HEMATOPOIETIC STEM CELL DEVELOPMENT and EFFECTS IN VIVO

Hematopoiesis is seen as a “probabilistic stochastic process,” (1.11), that is evolutionarily conserved and cycles through different areas of the mammal, going from the yolk sac to the aorta-gonad mesonephros (AGM) region to the fetal liver and then finally to the bone marrow (1.10, 1.11). Hematopoietic stem cell (HSC) numbers reach a maximum in the AGM region directly prior to their presence in the fetal liver (1.12). The development of these cells has a strong spatial and temporal correlation with endothelial cell development, leading some to predict that the two cell types may arise from the same precursor cell (1.11). As the cells migrate through these various embryonic niches, (1.14), they differentiate to different degrees and move down distinct developmental pathways based on the context of inputs received, varying from cell-cell interactions to cytokine signals (1.11). Even before HSCs commit to becoming a certain cell type heterogeneity is seen between the stem cell fractions, making them difficult to isolate in high purity (1.13). They differ in properties such as surface markers, developmental potential, and cell cycle status depending on their age and their localization, whether it be in the fetal liver or the bone marrow (1.10).

In order to maintain cell replaceability, HSCs need to reproduce to yield cells moving further down the differentiation pipeline as well as stem cells with the capacity for self-renewal. Their function may depend on their localization in specific bone marrow niches, which can tend to be very hypoxic (1.10, 1.16). Long term (LT) reconstituting HSCs are present in adult animals as a pool of renewable stem cells (1.11). Most adult HSCs in the bone marrow are quiescent but will undergo lymphopoiesis to maintain homeostasis, although their division is rare (1.10, 1.14). These multipotent cells are functionally influenced by Wnt/β-catenin and
Notch-Delta signaling pathways, where prolonged Wnt signaling could actually cause stem cell exhaustion, (1.10), and Notch signaling induces the transcription of Runx1 for HSC activation (1.11). HSCs have the capacity to produce more differentiated cell types; the developmental pathways interwoven with lymphoid cells and their progenitors will be the focus of this review. Lymphoid lineages can be derived from the LT reconstituting cells known as definitive HSCs (1.11). Differentiation into lymphocytes occurs once the HSCs have migrated to the fetal liver (1.10). Important genetic factors will assist in the driven differentiation of specific lineages, an example being Runx1 HSC activation to form megakaryocytes and lymphocytes (1.10).

B and T lymphocytes are generated from a shared multipotent progenitor but are derived from their own committed precursor cell lines and signaling cascades (1.10). B cell activity is seen as early as 8-9 dpc in certain tissues (1.12). This time designation provides a relative idea of just how early HSC migration to the fetal liver occurs because the cells do not differentiate until they reach this point. Movement of cells to populate certain tissues may occur by means of the circulatory system and by direct migration through tissue (1.10). The circulatory system plays a crucial role the life cycle of HSCs and how their daughter cells affect surrounding tissue. In SCD, HSCs have been shown to trigger remodeling of the perivascular niche of the fetal spleen (1.19). Due to the altered circulation present in SCD, the conformational change of the niche could cause the HSCs to have a problem with proliferation, which could ultimately be the reason that B-1a cells are diminished in SCD. The blood supplies cells with necessary molecules such as oxygen and glucose (1.15). During vertebrate embryogenesis, it was shown that blood flow is also a pertinent player in HSC formation (1.15). North et. al. depicted a directed correlation between the blood flow and number of HSCs
present. Compounds shown to modify blood flow, such as nitric oxide (NO), affected HSC induction after the embryo had developed a heartbeat, but NO donors regulated the number of HSCs with or without blood circulation (1.15). NO has been established as a direct regulator of vascular tone to increase vessel diameter, and thus increase the passage of total blood volume (1.15). NO has now been pointed as factor in hematopoietic cluster formation and as a key component of HSC production from the vascular niche after it was shown to be produced in the endothelium of the AGM region upon interaction with blood flow (1.15). Increased vessel diameter also directly corresponded to an increase in Runx1 expression, an important factor in the formation of functional HSCs (1.15). If circulation ceased, HSC formation was impaired (1.15). This link between blood flow in the developing embryo and the development of HSCs could be a crucial point when thinking about immune cell development in SCD because abnormal blood flow is characteristic of the disease.

A burning question that has been hovering around the field of developmental biology is concerning the purpose of circulation and a beating heart at early stages in development when diffusion is sufficient for oxygenation of developing tissue at the embryonic level (1.15). It may be possible that blood flow is necessary to carry and deliver molecules and signals needed for stem cells to differentiate properly, like VEGF, (1.14), and if blood flow is impaired, an issue with activation and proper develop of the cells might be seen. When stem cells move towards blood vessels, they are often on the road to differentiation (1.16). HSC niches in the bone marrow tend to be very hypoxic. This low oxygen concentration in the bone marrow regulates primitive hematopoiesis by preserving the stem-like character of HSCs (1.16). The blood of a patient with SCD has lower oxygen levels comparatively because of the inability of sickled cells
to carry oxygen productively (1.3). A difference between the stem cell niche in the bone marrow and the blood vessels that HSCs migrate toward is their oxygen level, so it may be possible that the stem cells are not properly stimulated to differentiate in areas like the spleen in SCD. Even after progenitors are committed, in order for maturation and proliferation to be successful, a certain level of physiological oxygen concentration needs to be present (1.16).

The relationship between the embryo and the mother from which it is receiving all of its nutrients can sometimes be overlooked. The placenta actually contains HSCs and other multipotential progenitors, demonstrating the significance of the mother’s circulatory system for the embryo’s development, especially in establishment of HSCs (1.14). The umbilical cord allows HSCs to migrate between the developing embryo and the placenta for interaction with the mother’s circulatory system (1.18). Once HSCs arrive at the fetal liver, they undergo varied levels of expansion (1.18). This critical step in development could be impaired in SCD because of possible pathology in the spleen even at early stages (Figure 3-3).

SCD has high levels of associated inflammation, as described, leading to increased inflammatory signaling. It was noted that genes linked to innate immunity and inflammatory signaling are enriched simultaneously in developing HSCs (1.20). This connection between inflammation and innate immunity could lead towards uncovering why B-1a cells are diminished in SCD. Inflammatory signaling is present during embryogenesis, notably in the AGM region, and helps to regulate HSC development, often promoting survival and proliferation of the stem cells (1.20). Innate immune cells secrete inflammatory cytokines as a method of maintaining hematopoietic homeostasis so that the immune system is ready to defend the body at any point. HSCs can actually respond directly to bodily harm, such as pathogenic
infection and endogenous ligands (1.20). In general, increased immune activation has the potential to impair renewal of HSCs and lead to their exhaustion (1.21). Chronic activation of the immune system in SCD could therefore have a cascade of negative effects.

DISCUSSION

Immune cell development is a finely intertwined process that incorporates many other body systems. When so many components are involved, there are many aspects that can be regulated, but that also means there are many areas where error can accumulate. After becoming familiar separately with the literature surrounding B cell development and SCD, I hypothesize that the functionality of immune cells will be altered and/or decreased in SCD because of the hostile environment that the cells have to develop and be activated in. The main alternative to this prediction is that decreased immune cell function is resultant from an innate defect in the hematopoietic stem cells responsible for the defective immune cells in SCD.

To indicate whether or not the stem cells are defective themselves in SCD, competitive experiments between SCD and WT cells could be conducted. One experiment would be to create mixed bone marrow chimeric SCD:WT mice via bone marrow adoptive transfers in lethal irradiated mice. This would reveal how the hematopoietic stem cells in a WT and SCD patient may compare in their ability to generate differentiated immune cells. Ratios of SCD to WT immune cells comparable to the ratios of stem cells implanted supports the hypothesis that there is nothing innately wrong with the hematopoietic stem cells in SCD. The cell ratios would
most likely have to be measure via analysis using Flow Cytometry. I have conducted this experiment and the results are presented in Chapter 3.

In the event that there is an issue with the generation of the B cells in SCD because of an innate problem with the hematopoietic stem cells, I would expect there to be a more monoclonal population of differentiated B-1a cells in the peritoneal cavity because the B-1a cells are diminished in the spleen but not the peritoneal cavity of SCD mice even though these immune cells traffic from the spleen to the peritoneal cavity (unpublished). If there are diminished B-1a cells in the spleen, where do the cells in the peritoneal cavity come from? Is it possible that the cells that are present would be leaning towards a more monoclonal population? In other words, are there a limited number cells being produced and sent to the peritoneal cavity that then divide to fill in the niche that is meant to be filled with these B-1a cells in SCD? This question of monoclonality can be tested using BCR sequencing of B-1a cell DNA from the peritoneal cavity.

As stated, an associated symptom of SCD is increased susceptibility to infection. It is possible that issues with hematopoietic cell trafficking and development in SCD are the cause. Children with SCD are commonly put on prophylactic penicillin to prevent them from contracting an infection with S. pneumoniae. Being prescribed a constant dose of a bactericidal agent would kill both the bad and good bacteria present in the system, possibly causing a slew of additional adverse side effects. Research with the microbiomes of specific diseases has become popular, so it would be interesting to look into how this issue with hematopoietic stem cell activation or generation cascades into an amplified range of other issues.
Altered components of the circulatory system associated with SCD may provide the knowledge necessary to explain why B-1a cells are diminished in SCD. In SCD, a physical change in the shape of the HSC perivascular niche could affect how the cells are activated. Many of the signals needed to keep HSCs in the pluripotent state or send them to be differentiated are contained within the specific stem cell niche. If the HSC perivascular niche in the fetal liver, arguably one of the most critical organs in HSC development, is altered in SCD, the activation and differentiation of these cells could be highly compromised. The circulatory system is also critical for transporting oxygen throughout the body, no matter what stage of development. HSCs have a dichotomy in the oxygen levels of their niche and the blood to which they migrate. In SCD, the decrease in oxygen carriers in the blood shortens the gap between the oxygen levels in the niche and in circulation. If HSCs sense the difference in the oxygenation of their environment as a signal to differentiate, then their potential activation could be affected in SCD as a result.

As stated above, I am currently leaning towards the idea that the activation of HSCs is impaired in SCD due to the resultant hostile in environment of the disease. Inflammation plays a key role in hematopoietic developmental signaling and well as HSC activation in an adult. In some cases, it promotes HSC proliferation. With the degree of inflammation and immune activation that occurs in the SCD, the HSCs have a high chance of reaching the point of exhaustion, stripping them of their ability to do their job and reform the appropriate differentiated immune cells in the body. As inflammation is studied and HSCs themselves are found to be able to defend the body against damage in SCD, whether it be from an exogenous agent or an endogenous agent, it is becoming clear the amount of tasks that must be carried
out by this critical form of stem cells. Is it possible that all of the tasks delegated to HSCs are split up like a pie chart? If one of the segments grows, another segment must shrink to compensate. Relatively, is it possible that because the HSCs are busy defending against inflammation and increase infection susceptibility in SCD, their potential to activate and differentiate declines?

By conducting experiments, labs around the globe are hoping to find out just what effect SCD has on the immune system in the host. I am hoping that the experiments that I have designed are telling and will provide a clue into this search for an answer.
CHAPTER 2

Review: Neuroendocrine Control over Lymphoid Hematopoiesis applied to Sickle Cell Disease
INTRODUCTION

Lymphoid hematopoiesis is the branch of immune cell development that stems from a common lymphoid progenitor and results in the production of B cells, T cells, and Natural Killer (NK) cells (2.3,2.7). B cells develop from hematopoietic stem cells (HSCs) in the bone marrow following parturition and in the fetal liver before, and mature in both the bone marrow and the spleen (2.3,2.4,2.7). To mature, they go through commitment, Ig repertoire formation, and cellular selection (2.4). This whole process is highly regulated and yields two main subsets known as the B-1 and B-2 B cells (2.4). B cells as a whole constitute the adaptive humoral response (2.4). The B-1 cells are involved in the early response to pathogens, releasing natural polyreactive antibodies in a more innate-like manner (2.5,2.6). T cells are also formed in the bone marrow but mature in the thymus, where self-reactive cells are eliminated (2.3). Many subsets of T cells are generated to act in the adaptive cell-mediated response, with the major groups being helper T cells and cytotoxic T cells (2.3). NK cells, which function much less specifically than B and T cells, also develop and mature in the bone marrow (2.3,2.7). NK cells are members of the innate immune response (2.3).

The bidirectional communication between the neuroendocrine and immune systems has been demonstrated as a crucial component of their individual operations (2.1). Both afferent and efferent pathways have been discovered between structures that make up these systems once they are matured (2.2). These interconnections are manifested through different types of signaling, including antibody, antigen, electrical, and hormonal (2.2). Signals from the neuroendocrine system to the immune system provide immunoregulation and enhance immunospecificity, or the presence of high affinity cells, (2.2), but how early does this inter-
system communication begin? It is not a coincidence that the immune and endocrine systems develop in a near parallel manner during early ontogeny (2.2). Many hormones and endocrine factors have an effect on the development of the immune system, specifically lymphoid hematopoiesis, from the onset of development, (2.2), (see Figure 2-1). In cases, like sickle cell disease, (SCD), where the immune system functionality is dampened, in order to determine the cause of the problem, it is important to take into account the components of the immune system in addition to any processes that have a regulatory effect. This review will focus on the effect of the neuroendocrine system on the immune system and its development. Correlations will also be drawn to SCD and how the dampened immune response may be able to be attributed to an endocrine disruption.
Figure 2-1: Besedovksy outlines the ontogenetic interactions between the immune and endocrine systems (2).
LITERATURE REVIEW

Lymphoid Hematopoiesis follows a series of events including differentiation, maturation and activation, trafficking and programmed apoptosis (2.3,2.7). Apoptosis ensures that lymphocytes only express function antigens and not self- or auto-reactive ones (2.18). Apoptotic cascade factors are necessary for differentiation of HSCs into lymphoid and myeloid lineages (2.18). Excessive pro-apoptotic factors during instances of stress can be detrimental to the immune system as well. Hormones can have stimulatory or inhibitory effects on these processes to help achieve the end goal of a mature immune system by maintain developmental balance. Many lymphoid cells and their progenitors possess hormonal receptors (2.13). Proliferation of T cells and production of antibodies by plasma cells are hormone dependent (2.13). Without the hypothalamus, NK cells have minimal activity and blast transformation is diminished (2.13). These effects and more are due to specific hormone effects on the immune cells throughout the body. Non-stimulated immune cells are more easily influenced by hormones so it is plausible to think that hormones would have prominent effects on progenitor and other early cells (2.2). Major hormones that have been discovered as having effects on the immune system and its development in normal and stressful situations will be discussed below.

STRESS IN THE IMMUNE SYSTEM

Stress was defined by Hans Seyle in 1974 as “the non-specific response of the body to any demand imposed up on it” (2.24). Stress induces neuroendocrine hormone production, specifically glucocorticoids and catecholamines (2.24). Normally, glucocorticoids are immunomodulatory and anti-inflammatory but under stress conditions they become
immunosuppressive (2.24). With their increased levels, these hormones reduce NK cells activity, lymphocyte proliferation, mitogenic blast transformation, lymphocyte populations by apoptosis, and antibody production (2.1,2.8,2.11,2.13,2.24). Long-lasting stress responses early in ontogenesis could cause homeostatic imbalances and leave an effect that is difficult to reverse (2.14).

**GROWTH HORMONE**

Growth Hormone (GH) is a peptide hormone that is traditionally known to be released by the somatotrophs of the anterior pituitary’s *pars distalis* (2.1,3,2.8). Its production is pulsatile and primarily nocturnal (2.1). GH secretion by the pituitary is regulated positively by growth hormone releasing hormone (GHRH) and negatively by somatostatin (GH-inhibiting hormone), both of which are released by the hypothalamus (2.1,2.3,2.9). GH binding to its receptor (GHR) causes dimerization of the GHR and a signaling cascade via the JAK-STAT pathway (2.8,2.9) ([Fig. 2-2](#)). Upon binding of GH, a site for JAK-2 to bind is created on the GHR (2.8,2.9). JAK-2, being a tyrosine kinase, phosphorylates its own tyrosine residues to provide a binding site for STAT via its SH2 domain (2.8,2.9). JAK-2 will also phosphorylate the tyrosine residues on STAT to activate the proteins and continue the cascade towards controlling gene transcription (2.8,2.9). GH can also cause recruitment of focal adhesion kinase (FAK), Ras and Raf, phosphatidylinositol-3 (PI-3) kinase, Akt/protein kinase B, MAPK, phospholipase C (PLC), PKC and others to JAK-2 for multiple pathways to be activated depending on the cell type (2.8). The effects of GH in pro-B cells and HSCs are mediated by the PI-3-kinase/Akt pathway and via the NF-κB transcription factor (2.8) ([Fig. 2-2](#)). Bcl-2, an anti-apoptotic protein, is expressed in
pro-B cells and HSCs by this pathway (2.8). Many cytokines also signal through these pathways so there is speculation of cross-talk between GH and different cytokines (2.9). The human GHR has been shown to have no sequence homology to other known kinases in its area of the cytoplasmic membrane, (2.9), so if cross-talk were to be occurring it would need to be downstream of the initial binding. Supporting the idea of cross-talk, it was shown that inflammatory cytokines, such as IL-6 or TNFα, are able to stimulate feedback mechanisms that also effect GH (2.9). Also, GH can provide negative feedback for prolactin (PRL) because it signals through JAK-STAT and insulin-like growth factor (IGF-I) and because it uses a receptor tyrosine kinase (RTK), both of which will be discussed later on (2.9).

**GH EFFECT ON IMMUNE CELLS**

GH has a widespread effect on the body’s systems including the immune system. NK, T and especially B cells, express the GHR and act as targets for GH (2.8,2.9,2.11). GH can also be produced by cells of the spleen, bone marrow, and thymus (2.8,2.12). GHRH and somatostatin have also been detected in human lymphocytes (2.12). Consequentially, autocrine/paracrine signaling of GH has been proposed within the lymphoid tissue, (2.8), including bone marrow, spleen, thymus, lymph nodes, and tonsil (2.12). The local production could be compensating for a lack of this apparently crucial endocrine hormone (2.12). In general, GH promotes cell cycle progression and is a negative regulator for apoptosis, but it also has specific effects on immune tissues (2.8). GH is one of the hormones capable of increases the immune response and it does so in part enhancing MHC reactions (2.12,2.13). It is proportionate in circulation to the numbers of B and T cells (2.13). In mice defective in the production of GH, (Snell dwarf mice or
hypophysectomized mice), lymphocyte development and functioning were less than optimal, especially with NK and T cells, and thymic atrophy was observed leading to immunodeficiency (2.8,2.11,2.13,2.23). These phenotypic abnormalities were able to be rescued by exogenous GH administration, (2.8,2.11), causing an increase to thymocyte, T cell and B cell numbers as well as the size of the thymus, liver and spleen (2.7,2.13) (study rev. in Clark 1997). In another study, a deficiency in GH lead to decreased amounts of B cell precursors (2.23). Even though this was the case, GH itself is not an obligate thymopoietic or B-lineage immunoregulatory hormone, (2.7), because many of its functions are carried out by induction of IGF-I (See IGF-I). GH promotes T cell proliferation by stimulating DNA-synthesis and somatostatin inhibits mitogen induced T cell proliferation (2.8,2.13). GH has also been shown to have the capacity to reverse CD4+ and CD8+ T cell deficiencies (2.10) (See IGF-I).

**GH ROLE IN STRESS RESPONSE**

Stress causes changes to the body that may be harmful to its systems. GH and IGF-I are two of the hormones that may be able to counteract negative immunoregulatory signaling during stress manifested by glucocorticoids, (2.7,2.12), by exerting its own protective immunoregulatory effect (2.8). Stress conditions induce apoptosis and GH is able to partially protect immune cells from this detrimental outcome (2.8). Inflammatory cytokines also cause stress on the body (2.9). SOCS, (suppressor of cytokine signaling), regulates cytokine levels in immune tissues (2.9). Like STAT in the GH signaling cascade, SOCS also has a SH2 domain (2.9). Proteins produced by the GH cascade are therefore able to interact with SOCS, stimulating its production and providing GH with another way to protect immune tissue from damage (2.9).
drawback of the presence of SOCS is that if levels are too high, it could cause stunting of the immune system’s development by blocking cytokines (2.9). Because the cascades of SOCS and GH are interrelated, SOCS is able to feedback inhibit GH to keep the levels of both GH and SOCS in check (2.9).

Figure 2-2: Jeay shows GH effects on pro-B cells. (8)
INSULIN-LIKE GROWTH FACTOR – I

Insulin-like growth factor - I (IGF-I) is a protein hormone that is produced following GH stimulation in the liver and immune tissues, namely the thymus (2.3,2.7,2.12). Macrophages are the greatest sources of IGF-I in immune tissues (2.10). Many of the effects of GH are achieved through induction of IGF-I, (2.7), which includes growth and cell number increase as evidenced by ‘growth factor’ in its full name. IGF-I is found bound to its designated binding proteins (IGFBPs) when in circulation. The IGF-I receptor (IGF-R) is a RTK that also binds insulin (2.3,2.12) (Fig. 2-3). It proceeds through the Shc/Grb2/MAPK pathway as well as the IRS/Akt/PI-3-kinase pathway (2.15) (Fig. 2-3).

IGF-I EFFECT ON IMMUNE CELLS

GH and IGF-I can stimulate general immune cell function (2.1,2.7). It was shown that together they can increase thymic, splenic, and bone marrow cellularity by both enhancing survival and inducing proliferation of the lymphoid cells (2.1,2.7,2.10,2.12). IGF-Rs have been found on erythrocytes, monocytes, B lymphocytes and T lymphocytes and in particularly high quantity on NK cells and CD4+ T-helper cells (2.15). GH and IGF-I can induce clonogenesis of T lymphoblasts, (2.10), and antigen-responsive B and T cells, increasing the number of responsive cells (2.7). In cases of thymocyte deficiency, the combination of GH and IGF-I can provide repair (2.14). GH and IGF-I are able to cooperate with cytokines, like GM-CSF, to help promote hematopoiesis (2.1). These cytokines, those involved in inflammation, GH and IGF-I mutually regulate each other to ensure that the developmental processes remain balanced (2.1,2.7). They are also able to increase the release of IgG and IgE, which are antibodies necessary for the
humoral immune response (2.10). IGF-I is able to indirectly initiate immunoglobulin gene rearrangement and expression of antibodies like IgM as well by acting as a differentiation signal for pro-B cells to mature into pre-B cells (2.10,2.12). With IGF-I present, B cells showed enhanced antibody response as well (2.12).

IGF-I is recognized as an important survival, proliferation, and differentiation factor for HSCs (2.10,2.15). IGF-I also functions by preventing apoptosis in HSCs (2.10,2.15). An increase in IGF-I could lead to elevated lymphoid hematopoietic progenitor cell activity or number in the bone marrow (2.10). Conversely, a deprivation of IGF-I can lead to HSC apoptosis (2.10). Because IGF-I requires IGFBPs to remain present in circulation, HSCs will actually secrete these binding proteins to ensure that there is enough IGF-I present (2.12). IGF-I produced by bone marrow stromal cells is a potent B lymphopoietic factor as well (2.15). It acts to suppress cell death signals sent to pro-B cells in conjunction with R-Ras (2.15). IGF-I works together with IL-7 to stimulate proliferation of pro-B cells (2.15). IGF-I has also been shown to stimulate CD4+ T-helper cells and splenic B cells to proliferate (2.15).
Figure 2-3: Zumkeller shows the IGF receptor schematic and a portion of its associated signal cascade. (15)
**PROLACTIN**

Prolactin (PRL) is a protein hormone with many functions and is best known as being released from the anterior pituitary (2.3,2.7). The PRL receptor (PRL-R) is similar to the GHR in that it dimerizes in the membrane upon binding and signals through the JAK-STAT cascade (2.3). Because of this similarity, human GH is able to bind and activate the PRL-R (2.7).

**PRL EFFECT ON IMMUNE CELLS**

PRL is a hormone, like GH, that increases the immune response (2.12,2.19). Snell dwarf mice, which lack PRL, show flaws with their lymphocyte development and function (2.1). PRL-R is expressed in peripheral blood lymphocytes, thymocytes, HSCs, stromal cells of the bone marrow and thymus, and cells that mediate natural immunity, including NK cells, (2.7,2.9), but is most prominent in B cells (2.12). PRL stimulates antibody production by B cells, (2.13), but is not required for B cell development (2.7). This hormone can stimulate lymphocyte proliferation but is not an obligate thymopoietic hormone (2.7). Also, PRL administration does not affect the activity of CD4-/CD8- thymocyte populations, meaning it may not have a hand in the entire developmental process (2.7). Because of the presence of the PRL-R on HSCs, PRL may have a direct or indirect effect on early lymphoid hematopoiesis (2.7).

**PRL ROLE IN STRESS RESPONSE**

Stress causes immunosuppressive/anti-inflammatory signals and apoptosis of lymphoid cells due to excess glucocorticoids (2.7). Glucocorticoids bind to cytoplasmic receptors (GR) (2.7). The newly formed complex recognizes its DNA motif, glucocorticoid response element
(GRE), which activates STAT-5 (2.7). Upon activation, STAT-5 binds the glucocorticoid-GR complex and inhibits GRE as a negative feedback (2.7). Simultaneously, STAT-5 is required for the PRL response and ends up enhancing the PRL cascade (2.7). By these means, PRL may be able to counteract the effects of stress in immune tissues (2.7). Therefore, stress induces release of PRL, which is speculated to assist in maintaining the immune system (2.7). In tissues like the skeletal muscles and the liver where glucocorticoids are necessary to mediate metabolism, PRL-R are not common so that the response is not dampened (2.7). The immune tissue expression of PRL-R with GR protects it from negative manifestations of chronic stress, like the degeneration of the immune response (2.7).

**THYROID HORMONES**

Thyroid hormones (TH), T₃ (triiodothyronine) and T₄ (thyroxine), are amino acid–derived hormones produced in the thyroid gland following stimulation by thyroid stimulating hormone (TSH) from the anterior pituitary (2.7). The function of THs is to regulate protein expression and cell cycle progression, but it is also known to act on the development and function of the immune system (2.7,2.13). The TH receptor (THR) is a nuclear receptor that is bound to a corepressor in its resting state and represses DNA regions known as thyroid response elements (TREs) (2.16,2.17). Upon binding of T₃, a conformation change in the THR that displaces the corepressor and recruits a coactivator to activate transcription of the TREs (2.16,2.17) (**Fig.2-4**).
**TH EFFECT ON IMMUNE CELLS**

Even though THs may stimulate thymocyte proliferation, it has been shown that THs are not necessary for thymopoiesis (2.7). THR is aboundantly found on lympho-hematopoietic cells (2.7). Instead of T cells, TH is needed for primary B cell development (2.7). If TH is not present, the numbers of B lineage cells in the bone marrow were significantly decreased due to decreased survival and lower rate of proliferation (2.7). A lack of TH also causes depressed humoral and cell-mediated immunity as well as involution of the spleen and lymph nodes (2.7). In line with the previous statements, hypothyroidism is correlated with arrested T and B lymphopoiesis (2.7,2.14). Not much is known about the effect of TH on NK cells, but it has been shown that TSH increases NK cell numbers in circulation (2.1). TH is may also play a role in counteracting negative immunoregulatory signals and the negative effects of stress but this has yet to be described (2.7).
Figure 2-4: Dayan and Panicker provide a schematic for the cascade leading up to TH action. (17)
ARGININE VASOPRESSIN

Arginine vasopressin (AVP) is a peptide, neurohypophyseal hormone that is produced in the hypothalamus and released into the blood by the posterior pituitary (2.14, 2.19). It is released as early as prenatal ontogenesis (2.14). AVP is known for its antidiuretic function and as a vasoconstrictor, but it should also be considered a neuroendocrine regulator of the immune and inflammatory responses (2.19).

AVP EFFECT ON IMMUNE CELLS

AVP works together CRH to cause the release of adrenocorticotropic hormone (ACTH) from the pituitary (2.19). ACTH stimulates the release of corticosteroids from the adrenal glands which function to inhibit cytokines, so AVP indirectly decreases inflammation (2.19). The receptor of AVP is also known to be present on inflammatory cells, human peripheral blood mononuclear cells, and splenic lymphocytes so it can have a direct effect (2.19). AVP can modulate the fever response and increase the power of primary antibody responses (2.19). In the event that corticosterone levels are low with high AVP levels, inflammation is favored and T-helper cell development is enhanced (2.19). This has been shown in cases of chronic inflammatory disease where AVP levels are high in the plasma and hypothalamus (2.19). For the opposite case where there is an AVP shortage, thymic and splenic involution are accelerated, lymphocyte numbers in the blood decrease, and general immunity is depressed (2.14).
**SEX HORMONES**

The major sex hormones are the steroid hormones estrogen and testosterone (2.3, 2.20-22). Both estrogen receptors (ER) and androgen receptors (AR) are nuclear receptors that affect gene activation (2.3, 2.20-22).

**SEX HORMONES’ EFFECT ON IMMUNE CELLS**

ERs and ARs are expressed on progenitor and mature immune cells, (2.1), as well as accompanying stromal cells (2.22). Their steroid hormones therefore have the capacity to affect the development and function of these cells (2.1). In comparison with males, females, who have higher innate levels of estrogen, have more pronounced humoral and cell-mediated immune responses, (2.21), especially during their reproductive years (2.1). Sthoeger summarized these alterations to include elevated immunoglobulin levels, general decreased susceptibility to infection, increased antibody formation following immunization, and graft rejection rate differences (2.21). Both enhancing and suppressing effects are observed upon addition of estrogen and testosterone to a culture of peripheral blood mononuclear cells (PBMCs), consisting of B, T, and NK cells as well as monocytes (2.21). Very early lymphoid progenitor cells have indeed been shown to be controlled in part by estrogen, showing that estrogen it participates in bone marrow development and acts as a negative regulator of lymphopoiesis (2.14, 2.20). Lymphoid progenitor cells were depleted in mice treated with estrogen, meaning that the progenitors for T, B, and NK cells were specifically targeted (2.20). Estrogen and testosterone also have the potential to increase B cell differentiation of PBMCs, (2.21), most likely by depleting the pro-B cell populations (2.20). This was tested in part by
measuring the frequency of B220+ cells after estrogen treatment (2.20). Likewise, estrogen deficiency was correlated with T cell loss and a decrease in inflammatory cytokine levels (2.22). Gonadotropin-releasing hormone (GnRH), which is released from the hypothalamus and indirectly stimulates production of sex steroid hormones, has also been shown to regulate immunity during ontogenesis (2.14). Prior to birth, if GnRH signaling is blocked, thymocytes are unable to proliferate or become activated by antigen (2.14). As current research stands, it seems as though estrogen enhances mature immune cells and suppresses lymphoid progenitor cells.

**APPLICATION TO SICKLE CELL DISEASE**

Many stresses are associated with sickle cell disease (SCD) (2.25). The sickled red blood cells can cause issues like vaso-occlusion, anemia, increased hemolysis, and splenomegaly (2.25). This stress could be a cause of the increased susceptibility to infection seen associated with the disease (2.27,2.29). As aforementioned, glucocorticoids become immunosuppressive under stress conditions (2.7,2.24). One large stress inducer in SCD is a zinc (Zn) deficiency (2.28,2.29). Zn is critical for many enzymes and Zn-dependent transcription factors (2.29). Zn deficiency leads to quickly diminished humoral and cell-mediated responses and lymphopenia in circulation as a result of B and T cell precursor losses in the bone marrow, 40-80% (2.29). Zn deficiency induces glucocorticoid production and glucocorticoid-mediated apoptosis, disallowing normal levels of lymphopoiesis and specifically depleting the B cell compartment (2.29). Zn supplementation to SCD patients increased multiple components of the immune
system and decreased oxidative stress, infection incidence, and generation of inflammatory cytokines (2.28).

During development, the hormones in milk could be enough to allow the production of normal lymphoid cells, (2.23), but when this natural supplementation is no longer available, neuroendocrine hormone effects could serve as the immunoregulators necessary to push for normal immune homeostasis because all of the major hormones discussed played a role in stimulating the immune system (GH, IGF-I, PRL, TH, AVP, and sex steroids). But importantly, the stresses caused by SCD could begin prior to birth because it is a genetic disease and cause homeostatic imbalances with these hormones (2.14). The interwoven nature of the neuroendocrine system with immune system is promising for unraveling the workings of the immune systems in SCD and looking into these connections could yield treatment ideas for the manifestations of SCD.

DISCUSSION

The focus of this review was the unidirectional communication from the neuroendocrine system to the immune system with a focus on development. The bidirectionality of the communication between the two systems should not be overlooked but was outside of the scope of this review. Cytokines and hormones produced by immune cells themselves have just as important of a role regulating the neuroendocrine system as the reverse. A lack of antigenic challenge was correlated with not only underdevelopment in the lymphoid tissue but also an altered endocrine state (2.2).
The interconnectivity of the immune and neuroendocrine systems provide potential avenues for intervention in cases where one is not functioning properly, like with SCD. Each hormone discussed in this review had an immunostimulatory effect, whether it be direct or by negatively regulating immune dampeners. Also, each hormone aside from AVP was shown to have a positive effect on lymphoid hematopoiesis at some point during development. These hormones and others in addition to new factors, like microRNAs, (2.34), should have their levels tested in certain disease states to discover possible supplementation therapies. Additional studies need be conducted to conclude where hormones fit in when thinking of cell lineages and developmental differentiation. Even though there is evidence for the necessity of some hormones in early development, more controlled cases need to run to be sure of the entire effect.
CHAPTER 3

Personal Research: B-1a Cell Diminution in Sickle Cell Disease Spleen — An Issue of Generation or Activation
INTRODUCTION

It has been shown that B-1a cells are diminished in the spleens of transgenic SCD mice (1.2). B-1 cells play a role in developing immunity to *Streptococcus pneumoniae* and that patients with SCD have a decreased baseline immunity, especially to *S. pneumoniae* (1.2, 1.6). Is it possible that the diminution in B-1a cells is one of the reasons behind the decreased immunity in SCD? If this is a possible effect of the diminution, are there other effects as well? In order to eventually determine the potential effects of the B-1a cell diminution associated with SCD spleen, we sought to find out why the B-1a cells are diminished in the first place. We tested different aspects of their development to try and determine when they are affected. This is to solidify if the B-1a cell diminution is an issue of generation of the cells from early on or from activation in the hostile environment of SCD. Because their developmental pathway goes from the fetal liver to the bone marrow to the spleen and then to the peritoneal cavity and occasionally back to the spleen, (1.6), we decided to conduct tests for the capacity of the HSCs in the adult bone marrow to produce B-1a cells and the B-1a cell numbers in their other main terminal niche, the peritoneal cavity. Based on published data and our previous data, we hypothesized that B-1a cells and diminished in the SCD spleen because they have to develop in a hostile environment rather than there being an innate defect in the stem cells. In terms of trafficking, B-1 cells appear to traffic from the spleen to the peritoneal cavity during development (1.2, 1.6). Also, if the cells are exposed to antigen only in the peritoneal cavity then they will remain and proliferate but if that antigen exposure is accompanied by inflammation in the peritoneal cavity, the cells will travel to the spleen to become plasma cells.
Given this information and our previous data, we expected to see a similar diminution of B-1a cells associated with the peritoneal cavity as with the spleen in SCD.

**MATERIALS AND METHODS**

**Animal Research Ethics Statement**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Connecticut (protocol #A14-029). Following irradiation, mice were weighed daily and monitored for signs of distress. Mice that were determined to exhibit moderate to severe clinical signs were considered for euthanasia via an overdose of carbon dioxide followed by cervical dislocation.

**Mice**

Two to three month-old mice harboring knock-ins of the human alpha globin transgene, along with either a normal human beta globin or sickle beta globin transgene [B6;129-Hbatm1(HBA)Tow Hbbtm2(HBG1,HBB)Tow/Hbbtm3(HBG1,HBB)Tow/J; stock number 013071] were purchased from Jackson Laboratories. Mice homozygous for the normal human beta globin transgene are henceforth referred to as wild-type (WT) mice and those homozygous for the sickle beta globin transgene are referred to as SCD mice.

Two to three month-old wild-type C57BL/6 congenic mice [B6 CD45.1, PepBoy; stock number 002014] were purchased from Jackson Laboratories. These mice carry the differential Ptprcα pan leukocyte marker, and will be referred to as CD45.1 mice.
**Irradiation**

Irradiation was conduction on the WT recipient mice using a 137-cesium source (gamma radiation levels at time of use: 11,576 R/hr) for 8 min each (1543.47 R total exposure). The literature has shown doses of greater than 700 R to successfully ablate the bone marrow, (3.2), which was the intended purpose of the irradiation in this experiment. Mice were weighed prior to irradiation and were monitored and weighed daily for two weeks afterwards, which is the approximate duration of potential irradiation sickness (Figure 3-1). Irradiation sickness causes dehydration, difficulty eating dry food, and weight loss. The mice were given wet food, which was changed daily and allowed them to eat more easily. If the mice achieved their weight prior irradiation and no longer showed clinical signs then the transfer was deemed a success.

Portions of protocol adopted from previous work by Cui and Duran-Struuck (3.1,3.2).

**Figure 3-1:** Weight vs. days post adoptive transfer – Data from one mouse for each group (experimental & control) displayed to show the weight loss the then regain by the end of the two week irradiation sickness period.
Bone Marrow Extraction

Bone marrow contained hematopoietic stem cells was collected from the femurs of donor CD45.1 and SCD mice and pooled respectively (WT marrow was used in place of SCD for control mice). The femoral extraction protocol was extrapolated from multiple well established techniques (3.3-3.6). Both end of the femur were removed using a scalpel and forceps on a hard surface, being careful not to crack the bone in any other places. Bone marrow was then pushed through the femur using sterile PBS loaded into a 1 mL syringe with a 25 gauge needle into a 1.5 mL microcentrifuge tube. To ensure the majority of the cells are released from the cavity, the needle was also run around the inside of the cavity. Once pooled, the cell suspension was run through the needle a few times to ensure that the trabeculae were broken up and the cells were released into suspension.

Chimera Development

Bone marrow cells were counted using a hemocytometer and Trypan Blue to ensure only live cells were counted. The 9:1 chimera (CD45.1:SCD) was made using the acquired numbers and suspension volumes (and CD45.1:WT for controls). The chimera was then spun down at 300 rcf for 5-10 min and resuspended with sterile PBS at a \(5 \times 10^6-1 \times 10^7\) cells/100 µL concentration with the appropriate number of doses (100 µL/recipient) for slight more than the desired number of recipients. The cells were kept on ice. Each dose was removed at the time of injection, making sure not to flick the syringe and kill the cells and that there is no air in the syringe.
Adoptive Transfer by Tail Vein Injection

Recipient WT mice were given approximately 1-2 hours to rest after the irradiation. For the adoptive transfer tail vein injection, (bone marrow transplantation), each mouse was dealt with individually. The mouse is loaded into the restrainer slowly to ensure not to induce excessive stress or cause shock. Once in the restrainer and it has been confirmed that the mouse has enough room to breathe, apply Lidocaine gel to the tail (numbing agent and helps to make the vessels more visible) and locate the most visible vessel. If available, a heat lamp can help to help dilate the vessels. Pinch the tail directly over the chosen vessel to apply pressure prior to the injection to again make the vessel more visible. These steps are especially important when doing tail vein injections on darker colored mice. With a 25 gauge needle on a 1 mL syringe, hold the bevel up, shallowly insert the needle into the vessel, and slowly inject the chimeric suspension. If resistance is felt, the needle is not in the vessel and will need to be repositioned. As soon the injection is completed, remove the mouse from the restrainer to reduce the chance of shock and apply pressure to the injection site. Recipient mice were monitored for four weeks to allow the transferred cells to reconstitute the irradiated immune system. Refer to Figure 3-2 for full timeline of the experiment.

Figure 3-2: Mouse model of bone marrow chimera adoptive transfer
Peritoneal and Splenic Lymphocyte Collection

After four weeks, the mice were euthanized. Lymphocytes were collected from the peritoneal cavity by peritoneal lavage and from the spleen of each mouse. Peritoneal lavage was conducted using 5 mL of sterile PBS. Lavage samples with visible blood in them could not be used. Spleen samples were made into single cell suspensions using the Miltenyi gentleMACS tissue dissociator and the majority of red blood cells were removed from the suspension using a brief treatment with red blood cell lysis buffer.

Flow Cytometry

Lymphocytes were differentially stained for distinguishing markers. The general B cell marker used was CD19 and was associated with the fluorophore APC. The general B-1 cell marker used was CD43 and was associated with the fluorophore PE-Cy7. B-1a cells were distinguished from B-1b cells using CD5, which is unique to B-1a cells and was associated with the fluorophore FITC. Samples and FMOs were made to ensure gating would be accurate. Samples were analyzed on FlowJo using the BD Biosciences LSRFortessa X-20 Cell Analyzer in the University of Connecticut Flow Cytometry Facility.

RESULTS

Initial experiments showed very interesting results. When samples were taken from the spleen and peritoneal cavity of WT and SCD mice, we saw the diminished B-1a cell population in the spleen, consistent with the literature. More importantly though, we saw that B-1a cells are
not diminished in the peritoneal cavity of SCD mice, (Figure 3-3), when compared to their WT counterparts.

**Figure 3-3:** Flow Cytometry separating B cell subsets in the Spleen and Peritoneal Cavity – Naïve B-1a cells of the spleen are diminished in SCD mice, but activated B-1 cells are still present in the peritoneum. **(A)** WT spleen: B-1a cells present in WT spleen; **(B)** WT peritoneum: B-1a cells present in WT peritoneum; **(C)** SCD spleen: B-1a cells diminished in SCD spleen; **(D)** SCD peritoneum: B-1a cells present in SCD peritoneum. Gating strategy was stepwise, selecting the lymphocyte population from the FSC vs SSC scatter plot and then sub-gating B cells off of that using CD19-APC. Being displayed are CD43-PE-Cy7 vs CD5-FITC scatter plots. The double positive (DP) gate denotes the B-1a cells. The CD43 gate denotes B-1b cells, which are CD43+ but CD5-. Lastly, the double negative (DN) gate denotes other, which includes the B-2 cells because it was sub-gated off of the CD19+ population. All gating was based off of single stain FMOs.
Figure 3-4: B-1a cells show normal counts in the peritoneal cavities of SCD mice – (A) SCD and (B) WT (control) mice. The percentages of B-1a cells in the peritoneal cavity of the SCD mice were consistent with the range seen in the WT mice. Shown are plots from individual mice to represent each group. For each flow plot, the following key applies:

- [   ] Lymphocytes
- [   ] B cells
- [   ] B-1 cells

To test the feasibility of the hypothesis that the B-1a cells are diminished because their developmental process has to occur in a hostile environment, a short experiment was conducted to see how early the manifestations of SCD begin to be apparent. Histologic staining was done on P0 mouse embryo organs that had been micro-dissected in RPMI media. The spleen showed a potentially distinct phenotype (Figure 3-5). It appears that the SCD spleen section is redder that the WT, leading to the speculation that sickled red blood cells may have...
already started to accumulate in the spleen at this time point. If this is the case, it could be possible that the phenotypic changes associated with SCD occur early enough to affect hematopoietic stem cell (HSC) development.

![Figure 3-5: Neonatal (P0) Histology (hematoxylin and eosin stain) – (A) WT; (B) SCD: More blood appears to be present in the spleen, but this representation is not able to show whether or not the organization of the SCD spleen is still intact at the P0 stage.]

Another way we went about answering the question of activation versus generation was by studying the capacity of HSC’s in the adult bone marrow to form each B cell subset. Mixed bone marrow chimera were formed from 9 parts congenic WT CD45.1 bone marrow to 1 part SCD CD45.2 bone marrow from donor mice (same process for controls with 9 WT CD45.1: 1 WT CD45.2; n=2). From there, the chimeras were adoptively transferred via tail vein injection into WT CD45.2 recipients (n=3) who had been sub-lethally irradiated to ablate their bone marrow. Once the recipients’ cells had been reconstituted, approximately 4 weeks post-transfer, splenic cells were collected and stained for analysis by flow cytometry (CD19-APC, CD43-PE-Cy7, CD5-FITC, CD45.1-PE). Analysis of the data showed that the transferred bone marrow chimera was able to maintain the 9:1 ratio of WT CD45.1: SCD CD45.2 for each B cell subset (B-1a, B-1b, B-2)
The ratios were not exactly 9:1 but were within the bounds of error shown by the control samples (± 4.8%). Peritoneal lavages using PBS were also done to collect the cells of the peritoneum but the results were inconclusive and more tests will need to be done to clarify the data.

**Figure 3-6**: Analysis revealing consistent B cell ratios of SCD and WT cells in the spleen – Histograms gated to show CD45.1+ and CD45.1– in each B cell subset of experimental and control spleen samples. **(A) Experimental: 9:1 (CD45.1:SCD) into WT** – The progenitor cells from the SCD bone marrow were able to maintain the 9:1 ratio for each cell subset as shown in the flow cytometry plots. **(B) Control: 9:1 (CD45.1:WT) into WT** – The control’s ratios were within ± 4.8% of the 9:1 ratio of cells initially injected. The experimental data varied from an exact ratio of 9:1, but the variation was within the confidence interval confirmed by the control. For each flow plot, the following key applies:

- [ ] B-1a cells  
- [ ] B-1b cells  
- [ ] B-2 cells
DISCUSSION

From the experiments conducted in this paper, we are getting closer to figuring out the cause/s and effect/s of the B-1a cell diminution in SCD. Looking at the B-1 cells’ pathway during development, data showing that B-1a cells are diminished in the SCD spleen but not the SCD peritoneum is very interesting. If the B-1 cells do indeed have to travel from the spleen to their niche in the peritoneal cavity where they wait to encounter antigen, how can they be sent from a diminished source and not be diminished in their final destination (Figure 3-3 & 3-4)? Is it possible that a smaller number of cells are initially sent to the peritoneal cavity from the spleen and then that set of cells divides to fill up the designated niche, making the B-1a cell population a more monoclonal population? Or could it be that the appropriate cells are sent to the peritoneal cavity before they are completely mature, leaving the cells in the peritoneal cavity nonfunctional? To test the clonality of the B-1a cells in the peritoneum, we have started to develop a protocol to prepare samples for BCR sequencing. We theorize that if the population of B-1a cell population in the SCD peritoneum is more monoclonal that that of the WT, then the B-1a cells populate the peritoneal cavity niche by expanding from a smaller set of starting cells. If there is a set niche for the B-1a cells in the peritoneal cavity and a smaller number of cells are sent from the spleen initially because the B-1a cells are diminished in the spleen, then when they expand to fill the niche the cell population would be more monoclonal in nature. If the B-1a cells of the SCD peritoneal cavity are polyclonal compared to the WT, then it is possible that the cells bypass the spleen because of the disrupted architecture and go straight to the peritoneal cavity? If the cells do not first go through the spleen, they are not able to become activated and therefore would be nonfunctional in the peritoneal cavity (see Chapter 4). We are
hoping to complete this BCR sequencing soon to be able to complete this story to an even greater degree.

What is shown here about when SCD manifests itself in the spleen is just scratching the surface (Figure 3-5). Different time points of these dissections and histologic staining should be done to see when there begins to be a difference in the splenic architecture. In addition it may be useful to look at different time points through immunohistochemical staining for each of the immune cells that are supposed to be present in the spleen as was shown in Szczepanek’s 2012 publication, (3.7), to really get an idea for the starting point of the disrupted niche. By discovering when the disease manifests itself in organs like the spleen, that time point can be potentially correlated to a stage downstream in hematopoiesis. The B-1a progenitor cells can then be studied at that time point to see if that is when the issue with their diminution begins. From the results in Figure 3-6, which show that the 9:1 ratios were maintained by the SCD and WT HSC’s within each B cells subset, we concluded that the HSC’s in SCD adult bone marrow have the same capacity to proliferate and differentiate into each B cell subset as the HSC’s in the WT adult bone marrow. This does not necessarily prove that there is not an innate issue with the fetal stem cells that reside in the fetal liver and are responsible for the initial formation of the B-1 cells, (0.1), but it does point towards the SCD B-1a diminution not being a result of an innate issue with the stem cells capable of forming the B-1a cells. If the SCD HSCs are completely capable of forming B-1a cells in a WT environment, what is stopping them from completing the B-1a cell niche in the spleen in the SCD environment? Also, were the B cells formed from the HSCs in the experiment displayed in Figure 3-6 functional? Future work will need to continue to focus on identifying the mechanism by which B-1a cells become diminished
in SCD mice in an effort to understand how this diminution effects the baseline immunity of patients with SCD.
CHAPTER 4

Publication in Review: Peritoneal B-1b and B-2 B-cells Confer Long-term Protection After Vaccination with Prevnar-13 and are Defective in Sickle Cell Disease Mice
Peritoneal B-1b and B-2 B-cells Confer Long-term Protection After Vaccination with Prevnar-13 and are Defective in Sickle Cell Disease Mice

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ABSTRACT

Long-term immunity after pneumococcal conjugate vaccine (Prevnar-13) inoculation is impaired in sickle cell disease (SCD) mice. We sought to determine which B-cell subsets confer long-term immunity after Prevnar-13 vaccination in wild-type (WT) mice, but are defective in SCD mice.

We vaccinated donor WT and SCD mice three times at three week intervals with Prevnar-13.

Fourteen weeks later, $5 \times 10^4$ cells of isolated peritoneal B-1a, B-1b, and B-2 cells were harvested from donors and intraperitoneally transferred to Rag -/- recipients. A week later recipients were intraperitoneally challenged with $10^3$ CFU of *Streptococcus pneumoniae* (serotype 3). Recipient mice that received either B-1b or B-2 B-cells from WT mice survived challenge, whereas mice that received B-1a cells died. Recipient mice that received B-1a, B-1b, or B-2 cells from SCD mice died after challenge. Both B-1b and B-2 cells appear to confer long-term immunity after Prevnar-13 vaccination, yet neither subset functions properly in SCD mice.

Keywords

Prevnar, PCV, *Streptococcus pneumoniae*, pneumococcal, B-cell, sickle cell
INTRODUCTION

Sickle cell disease (SCD) is a hematological condition that ultimately impairs immunity and leaves affected individuals susceptible to severe infections. The encapsulated bacterium, *Streptococcus pneumoniae* (i.e. the pneumococcus), causes many of these infections and invasive pneumococcal disease (IPD) has been found to be 10–100 times more prevalent in children with SCD than the general population [4.1]. The induction of anti-pneumococcal polysaccharide antibodies is the immunological correlate of protection for *S. pneumoniae* vaccines, yet waning anti-pneumococcal antibody titers have been observed in children with SCD after vaccination with the pneumococcal polysaccharide-conjugate vaccine Prevnar-13 [4.2, 4.3]. Furthermore, some pneumococcal isolates obtained from infected SCD patients in the years since the licensure of Prevnar match serotypes found in the vaccine, indicating that these individuals have insufficient post-vaccination immunity [4.4].

We previously characterized aberrant baseline immunity and dysregulated responses to a model vaccine in transgenic SCD mice [4.5, 4.6], which led us to speculate about the efficacy of clinically relevant vaccines in these mice. Using a prime-boost approach with Prevnar-13, we showed that pneumococcal-specific IgG, IgG3, and IgM titers spike in vaccinated SCD mice following inoculation but rapidly wane thereafter, even after booster shots are given. Importantly, we also showed that these same mice are not protected when they are challenged with a type-matched strain of *S. pneumoniae* three months after boosting, whereas control mice are protected [4.7]. These data indicate that SCD mice lack long-term protective B-cell responses after Prevnar-13 vaccination.
Previous work in mice indicates that a subset of T-cell independent B-cells found in the peritoneal cavity, called B-1a cells, are responsible for “natural immunity” against *S. pneumoniae* infection, while the B-1b subset provides protection after vaccination with pneumococcal polysaccharides. Conventional T-cell dependent B-2 cells have little contribution to protective immunity in either of these cases [4.8]. The pneumococcal polysaccharide-conjugate vaccine Prevnar was created such that T-cell dependent B-cell responses could be induced in children under 2 years of age, as they do not have very strong T-cell independent B-cell responses. Unfortunately, no one has previously identified which B-cell subset(s) responds to Prevnar in mice. We sought to better understand this basic question so that we could identify which B-cell subset(s) is dysfunctional and prevents long-term immunity after Prevnar-13 vaccination in SCD mice.

**METHODS**

**Mice.** Donor mice were two to three month-old male and female mice harboring knock-ins of the human alpha globin transgene, along with either a normal human beta globin or sickle beta globin transgene [B6;129-*Hba*tm1(HBA)Tow-*Hbb*tm2(HBG1,HBB #)Tow/Hbbtm3(HBG1,HBB)Tow/J; Jackson Labs stock number 013071] [4.9]. Mice homozygous for the normal human beta globin transgene are henceforth referred to as “wild-type” (WT) mice and those homozygous for the sickle beta globin transgene are referred to as SCD mice. Recipient mice were two to three month-old male and female mice homozygous for a knockout of the Rag 1 gene (Rag -/-) [B6.129S7-*Rag1*tm1Mom/J; Jackson Labs stock number 002216]. These mice were selected from a colony
that we maintain at the University of Connecticut, using IACUC approved methods (protocol # A14-029).

**Vaccination.** Donor mice were vaccinated as previously described [4.7]. Mice were rested for 14 weeks after the second boost was administered, then they were humanely euthanized by CO₂ overdose immediately prior to peritoneal lavage.

**B-cell harvesting, subset selection, and adoptive transfer.** Peritoneal leukocytes were harvested via lavage. After washing and resuspension, cells were counted using a hemocytometer and Trypan Blue dye. 4*10⁷ cells were then mixed with a cocktail of anti-NKp46-Biotin and B-1a cell biotin antibodies (which includes anti-CD5/APC antibodies) at 1:4 dilutions in MACS buffer, allowed to incubate for 10 minutes with anti-biotin microbeads, and then run over a magnetic LD column to deplete non-B-cells (Miltenyi Biotec, Cambridge, MA). B-1a cells were then positively selected using anti-APC microbeads at a 1:4 dilution and a MS column. The flow-through from the previous step was then mixed with CD43 microbeads at a 1:90 dilution in MACS buffer and run over a MS column to positively select for B-1b cells. B-2 cells remained in the flow-through from the previous step. Preparations were approximately 80-95% pure, as determined by flow cytometry. After selection, cells were washed and resuspended in B-cell transfer buffer [citation (Zuccarino-Catania)], and 5-6*10⁴ cells were intraperitoneally transferred to Rag-/- recipient mice (controls included mice sham-transferred with transfer buffer alone). After adoptive transfers were completed, live cell counts were conducted on the stocks to ensure recipient mice received the appropriate concentration of live cells.
**Bacteria and infection.** The A66.1 *Streptococcus pneumoniae* strain, which expresses pneumococcal polysaccharide serotype 3 (PPS3), was slowly thawed from -80°C, diluted in Todd-Hewitt broth and kept on ice. Mice were intraperitoneally infected one week after cell transfer with $1 \times 10^3$, $1 \times 10^4$, or $1 \times 10^5$ CFU of bacteria in 200ml of Todd Hewitt broth (or sham-infected with broth alone as a control). After infections were completed, stock bacterial solutions were grown overnight on blood agar plates to ensure that infected mice received the appropriate dose of live bacteria. Infected mice were monitored for signs of distress or illness, and mortality was recorded.

**Statistics.** Mortality curves were assessed for statistical differences using the log-rank/Mantel-Cox test. Significance was determined using $p < 0.05$ as a cutoff. Statistics were calculated using Prism version 6 software (GraphPad, La Jolla, CA).

**RESULTS**

**Peritoneal B-1b and B-2 cells confer long-term protection from pneumococcal infection after Prevnar-13 vaccination, both of which are defective in SCD mice**

All Rag-/- recipient mice that were challenged with $1 \times 10^4$ or $1 \times 10^5$ CFU succumbed to infection by day 2 post-infection, regardless of group. All recipient mice that received a sham-transfer of cells and were challenged with $1 \times 10^3$ CFU of bacteria died by day 2 post-infection, whereas those that were sham-transferred and sham-challenged lived for the 10 day duration of the study. All recipient mice that received either B-1b or B-2 B-cells from WT mice survived $1 \times 10^3$ CFU challenge, whereas all mice that received B-1a cells from WT mice died by day 2 post-
infection. All recipient mice that received B-1a, B-1b, or B-2 cells from SCD mice died from infection with $1 \times 10^3$ CFU of the bacteria (Figure 4-1). In order to determine if B-1a cells from WT donor mice have a relative defect in protecting recipient mice from pneumococcal infection (when compared to B-1b and B-2 B-cells), we transferred $2.5 \times 10^5$ B-1a cells to Rag -/- recipients (5 times more cells than other recipients) and intraperitoneally challenged them with $1 \times 10^3$ CFU of bacteria, with the same control groups described above. These mice still succumbed to pneumococcal infection (data not shown), indicating that B-1a cells are not involved in long-term protection after Prevnar-13 vaccination.

**DISCUSSION**

The cellular correlates of protection have been well elucidated in mice for pneumococcal polysaccharide vaccines, which seems to rely heavily on peritoneal B-1b cells in both young and aged mice [4.8, 4.10]. Unfortunately, this vaccine suffers from weak immunity in both very young and elderly people, as well as many groups with various chronic diseases. The development of Prevnar has helped to resolve many of the problems associated with immunization with plain polysaccharide vaccines. This is presumably accomplished through the addition of T-cell help, which should expand the repertoire of responding B-cells (to include conventional B-2 cells), but this has never been explicitly shown in mice. In this work we found that this presumption is indeed true, as peritoneal B-2 cells from Prevnar-13 vaccinated WT donor mice (14 weeks after boosting) were capable of conferring protection from pneumococcal infection in Rag -/- recipient mice. Interestingly, we also found that B-1b cells are
capable of protecting recipient mice from infection, indicating that this vaccine can stimulate B-cells in both a T-dependent and T-independent manner. B-1a cells from Prevnar-13 vaccinated WT donor mice were not capable of conferring long-term protection from infection, even when 5 times more cells are transferred to recipient mice.

While Prevnar has been shown to overcome immune deficiencies in many groups with chronic conditions, efficacy is not always equivalent between these groups and the general population, or between transgenic mice and WT littermates. The lack of long-term protection from infection after multiple inoculations with Prevnar-13 in SCD mice is concerning, as it indicates that there is a lack of development of both B-cell memory and long-lived plasma cells when these mice are vaccinated. Our current finding that neither B-1b nor B-2 cells from vaccinated SCD donor mice can protect Rag-/- recipient mice from pneumococcal challenge (whereas these same cells from WT donors do) helps to enlighten us as to why the long-term efficacy of Prevnar-13 is so poor in SCD mice. Understanding what gene pathways are aberrant in these cells throughout the vaccination model will provide insight as to the potential molecular mechanism(s) that is responsible for dysfunctional immunity to Prevnar-13. Future research should also focus on understanding the functionality of “equivalent” cells in SCD patients, namely CD19⁺CD20⁺CD27⁺IgM⁺CD43⁺CD5⁺/- B-cells [4.11, 4.12].
**Contributions of the authors:** SMS designed the experiments, CC and SMS performed the experiments, CC and SMS analyzed the data, and CC and SMS wrote the manuscript.

**Conflict of Interest:** The authors declare no conflicts of interest.

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Figure 4-1. B-1b and B-2 cells, but not B-1a cells, confer protection in WT mice after vaccination with Prevnar-13, but are defective in SCD mice.

WT and SCD donor mice were vaccinated three times with Prevnar-13. B-cells were harvested from the peritoneum, subsets were selected using magnetic beads, and 5-6*10^4 cells from each subset were transferred to Rag-/- recipient mice. Recipient mice were intraperitoneally challenged with 1X10^3 CFU of A66.1 and mortality was assessed. p = 0.01 for all three datasets, as determined by the log-rank/Mantel-Cox test.
Discussion and Conclusions
The significance of the research conducted on the immune capacity in SCD is much greater than just obtaining the results. Patients, especially children, who are suffering from SCD not only have to deal with the direct manifestations of the sickled red blood cells circulating throughout their body, but they also have to be careful about infections as they are highly susceptible. With the results that are being obtained, a story will eventually come together to explain why there is a decreased immune capacity associated with SCD and potentially how to treat it so that patients can lead more normal lives. Here I have conducted research in different disciplines that I can apply to SCD. Szczepanek showed that B-1a cells are diminished in the spleens of SCD transgenic mice. I became interested in this cell type, wondering at what point in its development it is affected by the disease. The B-1a cells have been shown to specifically help to mount a response against *S. pneumoniae* infection so it made sense to look at these cells as a potential factor in the increased susceptibility to infection by this specific bacteria. I was recruited to help complete experimentation for the publication included as Appendix, where we concluded that the poor long-term efficacy of Prevnar-13 in SCD mice is associated with an inability to sustain pneumococcal polysaccharide-specific antibody titers. We predict that this may be correlated to the lack of long-lived plasma cell generation in the SCD mice and a lacking B cell memory from the vaccine, even after boosting. So in addition to the decreased baseline immunity in SCD, there is decreased efficacy of the vaccine that is meant to help protect against of the main bacteria that infects patients with SCD. If normal protection against *S. pneumoniae* is facilitated by the B-1a cells, we thought it may be possible that Prevnar-13 acted through this cell type as well, as it did not work in the disease model of SCD where the B-1a cells are diminished.
With this background about SCD, B-1a cells, Prevnar-13, and *S. pneumoniae* we decided to formulate a few different angles to look at this topic. I wanted to take a look at the interactions between the different body systems as they develop to see if there were any other factors that could be affecting the B-1a cells. First I looked at hematopoiesis and how SCD may affect it (*Chapter 1*). Congestion of the circulatory system occurs in SCD and could cause issues with trafficking of cells as well as signaling molecules. HSCs niches have a specific environment that keeps the HSCs pluripotent. If the niche for these cells in the bone marrow or the fetal liver or a niche for a more specific immune stem cell in the spleen is disrupted, as it may be due to the circulation of injurious sickled cells or the lack of proper architecture in the SCD spleen, then the microenvironment needed by the stem cells for proper maintenance, activation, and differentiation could disappear entirely. Once component of the normal circulatory system that is drastically different in the HSC niche is the level of oxygen. If the HSC’s use this oxygen gradient as a sort of signaling to know when to become activated, then in SCD where there is much less circulating oxygen this signaling could also be disrupted and HSC source could become compromised. In both of these possible cases, a common issue that could be occurring is cell exhaustion. If the HSCs are activated for too long or by too many different signals then maybe they lose some of their functioning capacity. That same idea could apply to differentiated immune cells as well. Maybe they are too busy trying to clear the body of hemolytic particulates and apoptotic debris that their sensitivity to invading pathogens like *S. pneumoniae* is decreased. These ideas would interesting to investigate in the future.

The other mayor developmental process I wanted to look into with the premise of comparing it to the stressful environment of SCD was the neuroendocrine system (*Chapter 2*).
The bidirectional communication between the neuroendocrine system and the immune system during development was particularly appealing because of the effect of stress on each of the systems. Stress in SCD is caused by things like vaso-occlusion, anemia, increased hemolysis, and splenomegaly. In addition, a Zn deficiency has been found which increases the levels of stress even (supplementation decreases oxidative stress, infection incidence, and generation of inflammatory cytokines). In addition it causes decreased immunity, lymphopenia in the bone marrow, and increased glucocorticoid production. I discovered that under stressful conditions, glucocorticoids actually become immunosuppressive by increasing apoptosis and decreasing lymphopoiesis, especially in the B cell compartments. With the levels of stress associated with SCD, glucocorticoids that are produced would most likely take on this role. In addition to stress-related immunity, there could also be a correlation between signaling and the circulatory congestion in SCD. Is it possible that hormone levels and/or their signaling pathways are affected by this, disallowing crucial signaling molecules to stimulate processes like lymphoid hematopoiesis, movement, and proliferation? Generally the body is working properly if homeostasis is maintained so if certain sources are being blocked then imbalances could arise and cause improper signaling. Also, supplementation hormones from a mother’s milk could be enough to maintain the necessary immunostimulatory signals to counterbalance the immunosuppressive signals that are caused by the disease.

After familiarizing myself with the field and learning about the many things that could be affecting the immune system in SCD, I was able to formulate and conduct my own experiments to try and discern the causes and effects of the decreased baseline immunity in SCD (Chapter 3 & 4). We saw that HSCs from the adult bone marrow in SCD and WT have equal
capacity to form each of the B cell subsets in the spleen. With these results we have evidence that the diminution is not an innate issue with the adult stem cells. Further experimentation should be done using fetal stem cells to see if the results are consistent. But if there are fewer B-1a cells in the SCD spleen then why are the fully capable HSCs not producing more cells? To gain more evidence that the B-1a cell diminution of SCD is not an issue of generation, the induction of HSCs/FSCs and how they development into B-1 cells must be studied in depth.

In terms of the peritoneal cavity, we found that even though B-1a cells are diminished in the SCD spleen, their numbers in the peritoneal cavity are unchanged from those of WT (see Chapter 3, Discussion). One of our hypotheses about the B-1a cells in the SCD peritoneal cavity is that a small group of seeding cells are initially sent from the smaller group of cells in the spleen and then when the cells reach the peritoneal cavity, their niche is undisturbed, potentially unlike their niche in the spleen, so they proliferate to fill up the niche. If this were the case then the B-1a cell population in the peritoneal cavity of SCD mice would be a more monoclonal population in terms of their VDJ combinations, which we are currently testing using BCR sequencing. Our other hypothesis is that the cells that end up in the peritoneal cavity bypass the spleen entirely because of the improper architecture. Because the cells need the spleen to become activated, this explanation would render the cells nonfunctional in the peritoneal cavity. This hypothesis was tested by the experimentation in Chapter 4.

With the method that was used to test the functionality of the peritoneal B cells, we were unable to see if the B-1a cells were more or less functional in the SCD than the WT because neither the SCD nor the WT vaccinated donor B-1a cells conferred protection from S. pneumoniae infection. In fact, none of the B cell subsets from the vaccinated SCD donor mice
were able to confer protection from infection with *S. pneumoniae* in Rag-/- mice. So, even though the B cells number in the peritoneal cavity of SCD mice appear normal, the cells are not functional in conferring protection from infection after vaccination. This could be a reason why SCD mice lack long-term protection from infection after Prevnar-13 vaccination (Appendix). If those cells are nonfunctional in the peritoneal cavity, there is a good chance that they are also unable to form a memory of the pathogen. Although we were not able to learn much about the functionality of the peritoneal B-1a cells in SCD from this experiment, we were able to learn more about Prevnar-13. Until now, it was unknown which of the B cell subsets responds to Prevnar-13 vaccination. The vaccine was presumed to work through T cell help which we were able to show because the WT peritoneal B-2 cell transfers were able to confer protection from *S. pneumoniae* infection in Rag -/- recipient mice. The B-1b cells from the vaccinated donor mice were also capable of protecting against infection, meaning this vaccine works in both a T cell dependent and independent manner. So even though the B-1a cells are thought to be important in mounting a response against *S. pneumoniae* infection, they are seemingly not recruited in response to Prevnar-13 vaccination in WT or SCD mice. The mechanism of this vaccine in the WT and SCD model needs to be studied in more depth.


2.9. Redelman, D. et al. (2008). Neuroendocrine hormones such as growth hormone and prolactin are integral members of the immunological cytokine network. Cellular Immunology 252:111-121.


APPENDIX

Publication: Poor Long-Term Efficacy of Prevnar-13 in Sickle Cell Disease Mice Is Associated with an Inability to Sustain Pneumococcal-Specific Antibody Titers
RESEARCH ARTICLE

Poor Long-Term Efficacy of Prevnar-13 in Sickle Cell Disease Mice Is Associated with an Inability to Sustain Pneumococcal-Specific Antibody Titers

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ABSTRACT

Background
One of the most common causes of morbidity and mortality in children with sickle cell disease (SCD) is infection with the pneumococcal bacterium (Streptococcus pneumoniae). Unfortunately, the polysaccharide-conjugate vaccine appears to be less effective in individuals with SCD when compared to the general population. We sought to better understand the relative efficacy of pneumococcal vaccination in a SCD mouse challenge model.

Methods
Transgenic control and SCD mice were monitored for mortality after intranasal pneumococcal infection or pneumococcal vaccination with Prevnar-13 and type-matched challenge. Anti-pneumococcal antibody titers were measured by ELISA and opsonophagocytosis was measured in vitro.

Results
Mortality after pneumococcal infection was similar between control and SCD mice. However, after three intramuscular polysaccharide-conjugate vaccinations, all control mice were protected following high-dose intranasal infection, whereas 60% of SCD mice died. Anti-pneumococcal antibody titers showed initial IgG and IgM responses in both groups, but waning titers were observed in the SCD group, even after boosting.
assayed in vitro, serum from SCD mice 13 weeks after a second booster shot maintained little to no ability to opsonize pneumococci, while serum from control mice sustained a significantly higher capacity opsonization. Thus, it appears that SCD mice do not maintain antibody responses to pneumococcal polysaccharides after Prevnar-13 vaccination, thereby leaving them susceptible to mortality after type-matched infection.

Conclusion
Our results emphasize the need to better understand the correlates of immune protection in SCD so that pneumococcal vaccines can be improved and mortality reduced in this susceptible population.

Introduction
Sickle cell disease (SCD) is a complex hematological disorder that has a dramatic effect on immunity and resistance to infection. The current case-fatality rate within this population due to infection (when not adjusted for age) stands at approximately 14% [1]. Children with SCD are at a particularly high risk for infection with the encapsulated bacterium, Streptococcus pneumoniae (i.e. pneumococcus), which is presumably due to altered splenic architecture and function [2]. A life-threatening condition arises if the bacterium becomes invasive and causes bacteremia (known as invasive pneumococcal disease, or IPD). IPD has been found to be 10–100 times more prevalent in children with SCD than the general population [3] and is even twice as likely in individuals with sickle cell trait [4, 5]. Given the extreme morbidity and mortality associated with pneumococcal infection in the SCD population, current clinical guidelines dictate that these patients be placed on prophylactic penicillin at approximately 4 months of age and adhere to a strict regimen of pneumococcal vaccination.

Cases of splenectomy have demonstrated that the spleen is a crucially important organ in protection from IPD in both humans [6] and mouse models [7]. Previous vaccination appears to be sufficient to maintain antibody titers in many cases of splenectomy; however, retention of memory B-cells is adversely affected [8]. Furthermore, while it is agreed upon by most in the field that anti-pneumococcal titers are induced in children with SCD shortly after vaccination, it has been reported that titers may not be maintained long-term after vaccination with the unconjugated pneumococcal polysaccharide vaccine [9], indicating that these children may have defects in the generation of memory B-cells and/or long-lived plasma cells. Protection from IPD has been demonstrated to rely heavily on the presence of “memory IgM B-cells” (human) or “B-1a B-cells” (mouse) [10, 11]. These cells produce antibodies that target carbohydrate moieties commonly found on encapsulated bacteria. The presence of a functional spleen has been shown to be essential to the survival of these cells [12]. Interestingly, we have previously shown that splenic architecture is disrupted in transgenic SCD mice and B-1a B-cells are dramatically reduced in number in the spleens of these mice [13]. Hence, it is likely that the generation of a robust plasma cell and memory B-cell response is essential to thwart recurrent pneumococcal infection, and a lack thereof may be responsible for increased susceptibility in children with SCD who lack splenic function and normal numbers of memory IgM B-cells.

Since the introduction of the use of prophylactic penicillin and the newer pneumococcal polysaccharide-protein conjugate vaccine Prevnar in children with SCD, hospitalization associated with infection from this pathogen has been reduced three-fold [14] and infection has been concomitantly reduced to approximately one-third of its previous level [15]. Unfortunately,
this still leaves room for improvement in treatment and therapies to combat infection by this pathogen in children with SCD. Given the strict adherence to pneumococcal vaccination in SCD patients at many hematology clinics, this phenomenon is surprising and vaccine failure may be to blame for some of these cases. While little is known about the ability of Prevnar to specifically protect from type-matched infection in SCD patients, we do know that the 23-valent pneumococcal polysaccharide vaccine has been shown to have little to no efficacy in SCD patients in some reports, even after administering a booster dose [16, 17]. Hence, the efficacy of pneumococcal vaccination does not appear to be as high in children with SCD when compared to the general population.

Immune dysregulation in the transgenic SCD mouse model has recently become apparent. We have shown that disrupted splenic architecture is prevalent at a young age in these mice, as are aberrations in the distribution of lymphocyte populations, cytokines/chemokines, and antibody classes [13]. Further changes in immunity have been noted after animals received a vaccination with ovalbumin and the adjuvant aluminum hydroxide (OVA/alum). These vaccinations resulted in high IgE titers, further dysregulation of cytokines/chemokines/antibodies, and a notable increase in the levels of IL-1β and IL-6 in bronchoalveolar lavage fluid of the SCD mice [18]. Given our previous findings that immunity is dysregulated in the SCD mouse model, we hypothesize that immunity is impaired in SCD and drives the reduced pneumococcal vaccine efficacy that has been clinically observed in this population. Herein we describe the immunogenicity and efficacy of the pneumococcal polysaccharide-conjugate vaccine Prevnar-13 in the SCD mouse model to address the above hypothesis.

Materials and Methods

Animal Research Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at Albany Medical College (protocol #14–04003; Metzger) and the University of Connecticut (protocol #A14-029; Szczepanek). During experimental infections, mice were weighed daily and checked for signs of distress. Mice that were determined to exhibit moderate to severe clinical signs (i.e. severely reduced mobility or persistent recumbency) were considered for euthanasia via an overdose of the anesthetic sodium pentobarbital followed by cervical dislocation (conducted by staff trained in the use of the technique).

Mice

Two to three month-old female mice harboring knock-ins of the human alpha globin transgene, along with either a normal human beta globin or sickle beta globin transgene [B6;129-Hba<sup>tm1(HBA)Tow</sup> Hbb<sup>tm2(HBG1,HBβ1)Tow</sup> /Hbb<sup>tm3(HBG1,HBβ)Tow</sup>/J; stock number 013071] were purchased from Jackson Laboratories (Bar Harbor, ME). Mice homozygous for the normal human beta globin transgene are henceforth referred to as “control” mice and those homozygous for the sickle beta globin transgene are referred to as SCD mice. Serum for ELISA was obtained via retro-orbital bleed. Five to eight mice were used for all experiments. All mice were maintained and utilized at the Albany Medical College Animal Facility.

Bacteria

The A66.1 <i>S. pneumoniae</i> strain, which expresses pneumococcal polysaccharide serotype 3 (PPS3), was cultured at 37°C in Todd-Hewitt broth until mid-log phase (using BSL-2
precautions), washed and resuspended in fresh broth containing 15% glycerol, and stored at −70°C until use.

Infection

Infection was induced in naïve, unvaccinated mice intraperitoneally anesthetized with ketamine HCl (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Phoenix Scientific, St. Joseph, MO) in PBS and inoculated intranasally with a dose of 5 X10⁴ CFU of A66.1 pneumococci in 50 μl of Ringer’s solution. Mice were weighed daily, monitored for signs of distress or illness, and mortality was recorded. Lungs were removed from mice that died during infection and were fixed overnight in 10% formalin, embedded in paraffin and sectioned, and H&E stained using standard protocols of the CT Veterinary Medical Diagnostic Laboratory (CVMDL) at the University of Connecticut. Resolution of S. pneumoniae in the lungs was performed using a Gram stain of paraffin-embedded tissues. Histologic evaluation of lung lesions was conducted by a board-certified veterinary pathologist from the CVMDL.

Vaccination and Challenge

Mice were intramuscularly inoculated in the hindlimbs with Prevnar-13 (Wyeth Pharmaceuticals, Collegeville, PA) with a dose containing 0.22 μg of each polysaccharide. This vaccine includes PPS3. The mice were boosted twice, tested for serum anti-PPS3 antibodies, and challenged according to the schedule outlined in Fig 1. Challenge of vaccinated mice was conducted via intranasal inoculation of 1X10⁶ CFU of A66.1 pneumococci in 50 μl of Ringer’s solution. Mice were weighed, monitored for signs of distress or illness, and mortality was recorded.

ELISA

PPS3-specific antibody titers were measured using ELISA. 96 well Polysorp Nunc-Immuno plates (Nalge Nunc International, Rochester, NY) were coated with 50 μL of PPS3 antigen (2 μg/mL, ATCC, Manassas, VA) and incubated at 4°C overnight. Plates were then washed three times with PBS (Life Technologies, Carlsbad, CA) containing 0.05% Tween 20 (Sigma) and then blocked with 5% fetal calf serum (Hyclone Laboratories, Inc., Logan, UT) at room temperature for 1 hr. Serial dilutions of serum in blocking buffer were added to the plates, which were then incubated at room temperature for 2 hr. After five washes with PBS-0.05% Tween20, 50 μL of biotin-conjugated goat anti-mouse IgG or IgM antibody (SouthernBiotech, Birmingham, AL) was added, and the plates were incubated at room temperature for 1 hr. After six washes, 50 μL of Streptavidin-HRP (Biosource/Life Technologies, Carlsbad, CA) was

![Fig 1. Mouse model of pneumococcal vaccination and infectious challenge.](image-url)

2–3 month old mice were bled, and serum was collected prior to initial priming with Prevnar-13 on week 0. Mice were boosted on weeks 3 and 5 with the same vaccine. Mice were again bled on weeks 4, 9, and 14. Mice were then challenged with the virulent A66.1 strain of S. pneumoniae on week 18 and mortality was assessed for 16 days after challenge.

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Prevnar-13 Vaccination in SCD Mice
added and the plates were incubated at room temperature for 30 mins. After seven washes, 50 μL of TMB Peroxidase substrate solution was added. 50 μL of 1.8 N Sulfuric acid was used to stop the reaction. Finally, the absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT). Dilutions corresponding to 50% maximal binding were used to calculate serum titers.

Opsonophagocytic Killing Assay

The protocol used is adapted from Romero-Steiner et al. [19]. Mouse macrophage J774a.1 cells obtained from ATCC (Manassas, VA) were thawed and cultured according to manufacturer recommendations. J774a.1 cells were then added to 96-well round bottom plates at a concentration of 4 x 10^5 cells/well and incubated with a pneumococcal cells (A66.1) at a concentration of 2 x 10^5 cells/well, diluted from a stock of a known concentration (using CFU counts on blood agar plates). Serum was collected from Prevnar-13 vaccinated control and SCD mice 13 weeks after their third vaccination, which was then diluted 1:2 in assay buffer. Mouse complement (Rockland Antibodies and Assays, Limerick, PA) was added at 10 μL/well (890 μg). Following final incubation, a 10 μL aliquot was diluted 1:10 in Todd-Hewitt Broth and 20 μL was plated on to blood agar plates and incubated for 18 hr at 37°C. Colonies were counted and compared to a no serum control. Data are expressed as the percentage of phagocytosis over no serum control.

Statistics

Mortality curves were assessed for statistical differences using the log-rank/Mantel-Cox test. Statistical differences in body weight over time were determined using repeated-measures ANOVA. Differences in antibody titers were determined separately at each time-point for between group comparisons using a two-tailed t-test, while within group comparisons across time points were determined by One-way ANOVA. Opsonophagocytosis between groups was compared using a two-tailed t-test. Significance was determined as \( p < 0.05 \). Statistics were calculated using Prism version 6 software (GraphPad, La Jolla, CA).

Results

Mortality and Histopathology After Pneumococcal Infection

Initial pilot studies with non-transgenic wild-type mice of the same genetic background as the SCD mice identified 5X10^4 CFU as the approximate LD50 for intranasal A66.1 pneumococcal infection (data not shown) and this dose was subsequently used in the SCD mouse model. Overall mortality after eight days of infection was not different in both SCD (n = 8) and control mice (n = 8). Although SCD mice tended to die earlier than control mice (Fig 2a), there was no statistical difference in the mortality curves between the two groups. Similarly, there was no statistical difference between the groups in weight lost after infection (Fig 2b).

In this study, lungs from infected control mice (Fig 3a) were much more severely inflamed than those from SCD mice (Fig 3b), with notable perivascular and peribronchial infiltrates and pleuritis in the control mice that was very mild to absent in SCD mice. Pneumococcal bacteria were visually present in both the blood and attached to the surface of the lung tissue in the control mice (Fig 3c), whereas they were only observed in the blood of SCD mice (Fig 3d). Thus, lung colonization was associated with lung lesions in control mice that died from infection, but mild lung lesions were associated with the visual presence of the bacteria only in the blood of SCD mice that died from infection.
Fig 2. Differential mortality kinetics of control and SCD mice after infection. 2–3 month old mice were infected intranasally with $5 \times 10^4$ CFU of A66.1 S. pneumoniae and mortality (A) and weight loss (B) were measured. No statistical differences were observed for either parameter. White boxes = SCD, black boxes = controls.

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Fig 3. Histopathology and Gram staining of lung tissues in mice that succumb to intranasal pneumococcal infection. H+E staining of lung tissue from control (A) and SCD (B) mice after they succumbed to pneumococcal infection. Gram stain of the same lung tissue in control (C) and SCD (D) mice. Wide arrows point to pneumococci in blood vessels and narrow arrows point to pneumococci colonizing the surface of the lung tissue.

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Antigen-specific Antibody Titers and Functional in vitro Opsonophagocytosis of Pneumococci After Vaccination with Prevnar-13

After intramuscular priming and boosting with Prevnar-13 (as indicated in Fig 1), serum anti-PPS3 total IgG, IgG3, and IgM antibody titers were measured by ELISA. Initial anti-PPS3 total IgG and IgG3 titers increased within 4 weeks in both control (n = 5) and SCD (n = 5) mice (Fig 4), which is in keeping with clinical findings of induced antigen-specific titers shortly after vaccination in people with SCD. These titers continued to significantly increase (p < 0.05) in the control animals after booster shots were administered, as was expected. Conversely, anti-PPS3 total IgG and IgG3 titers significantly waned to near naïve levels in SCD mice at the 9 and 14 week time points, even with repeated boosting (p < 0.05). Not surprisingly then, the anti-PPS3 total IgG and IgG3 titers were significantly higher in control mice when compared to SCD mice at the 9 and 14 week time points (p < 0.01 and p < 0.05, respectively). Control mice trended towards an increasing anti-PPS3 IgM response (p = 0.08) over time, whereas SCD mice induced a strong initial anti-PPS3 IgM response (week 4) that again waned to near naïve levels thereafter (p < 0.05). These findings are in line with clinical observations that anti-pneumococcal antibody titers are not maintained long-term in people with SCD.

Functional analysis of the long-term antibodies that are induced by three shots of Prevnar-13 were determined using an in vitro opsonophagocytic killing assay (Fig 5). Exogenous mouse complement was added to the assay to ensure that differences between groups for macrophages to phagocyte pneumococci were the result of differences in antibody titer and/or quality, and not due to differences in complement. Serum from control mice (n = 3) maintained a significantly higher (p < 0.05; mean = 64% over no serum control) ability to opsonize pneumococci than serum from SCD mice (n = 3, mean = 35% over no serum control) 13 weeks after a third shot with Prevnar-13. Notably, pooled serum from unvaccinated control and SCD mice was not much different in its capacity to opsonize pneumococci (mean = 32 and 26, respectively) than vaccinated SCD mice. These findings are in line with the nearly naïve anti-PPS3 titers observed in SCD mice after three injections with Prevnar-13 and indicate that the waning antigen-specific titers observed in SCD mice are functionally relevant.

Mortality from Infectious Challenge After Vaccination with Prevnar-13

We used a challenge dose of A66.1 that is approximately two orders of magnitude higher than the LD50 dose in unvaccinated mice to ensure that observed survival is associated with vaccination. Mice were intranasally given 1X10^6 CFU of A66.1 pneumococci (PPS3, a serotype found in Prevnar-13) approximately three months after the last vaccine boost was administered. All control mice (n = 8) survived challenge with virulent PPS3; however, 60% of SCD mice (n = 5) died after infection (Fig 6, p = 0.01). Hence, the vaccine appears to have little long-term efficacy in the SCD mouse model, which correlates with the waning antibody titers observed in these animals at the time of challenge.

Discussion

The importance of pneumococcal vaccination in children with SCD cannot be overemphasized due to the devastatingly high morbidity and mortality associated with infection by this pathogen. While the pneumococcal polysaccharide-protein conjugate vaccine Prevnar has dramatically reduced infectious burden in children with SCD, far too many cases of pneumococcal infection still occur every year in this group and little is known as to why this is the case. We found in our infection model that mortality for both control and SCD mice were similar, which was surprising given the dramatically increased vulnerability of children with SCD to
Fig 4. Pneumococcal-specific antibody titers after vaccination. PPS3-specific total IgG (A), IgG3 (B), and IgM (C) antibody titers were measured by ELISA in Prevnar-13 vaccinated 2–3 month old control and SCD mice at weeks 0 (before vaccination), 4, 9, and 14. * = p < 0.05 and ** = p < 0.01 for between groups comparisons at the same time point (as determined using a two-tailed Student’s T-test) and † = p < 0.05 for within group comparisons across time points (as determined using a One-way ANOVA). For IgM comparisons between groups, p = 0.07 and p = 0.08 at weeks 4 and 9, respectively. For IgM comparisons within the control group, p = 0.08. White boxes = SCD, black boxes = controls.

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pneumococcal infection. Furthermore, Miller et al. previously reported that SCD mice are hyper-susceptible to pneumococcal infection [20]. However, this study followed mice for a much shorter duration (72 hours), and our results agree with their findings up to this time point as we observed a delay in mortality in the control mice when compared to SCD mice.

**Fig 5. in vitro opsonophagocytosis of pneumococci 13 weeks after boosting with Prevnar-13.** Serum from vaccinated mice (or pooled serum from naïve mice) was mixed with exogenous mouse complement and A66.1 S. pneumoniae and added to cultures of mouse macrophages in vitro. Mixtures were then streaked out on blood-agar plates, incubated at 37°C overnight and viable pneumococcal colonies were counted. Data are reported as the percentage of pneumococcal colonies that were not phagocyted by macrophages when compared to “no serum control” wells. $p < 0.05$ as determined by a t-test. Comparisons to naive mice were not performed as those samples were pooled.

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**Fig 6. Prevnar-13 vaccination does not protect SCD mice from pneumococcal infection.** Mice were vaccinated and challenged according to the schedule outlined in Fig 1. Mice were intranasally challenged with 1X10⁶ CFU of A66.1 and mortality was assessed measured. $p = 0.01$, as determined by the log-rank/Mantel-Cox test. White boxes = SCD, black boxes = controls.

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early in infection. Interestingly, SCD mice that died from pneumococcal infection had mostly mild lung lesions, and pneumococci could only be found in the blood when tissues were Gram stained. This was in contrast to control mice that died from infection, which exhibited moderate to severe lung lesions and pneumococci could be observed in both the blood and along the surface of the lung tissue. While requiring further investigation before any definitive conclusion can be reached, given the relative delay in the time to death in control mice and the relative lack of lung lesions/colonization in SCD mice, it appears that SCD mice may experience IPD earlier in infection and do not require lung colonization for this to occur, whereas control mice do. Such an explanation would be in line with the in vivo bioimaging data presented by Miller et al. [20], which showed that pneumococci remain in the lungs of WT mice 24 hours after infection, but IPD occurs within this same time period in SCD mice.

Given our finding of the overall lack of a difference between the groups in terms of susceptibility infection, we were further surprised when the SCD mice differentially succumbed to infectious challenge after vaccination. It is important to note that the challenge dose after vaccination that we used is two logs higher than the dose used during the infection study (which killed more than half of the mice in both groups). Thus, mice would likely require a protective immune response to be induced from vaccination in order to survive this high challenge dose, and protective antibody responses were only maintained in the control mice at the time of challenge. These findings correlated with an in vitro opsonophagocytic killing assay conducted using serum from control and SCD mice 13 weeks after their second booster shot with Prevnar-13, which showed that control mice maintain functional antibody titers, but SCD mice do not. This data has clinical relevance as a recent study in children with SCD demonstrated their inability to sustain anti-PPS3 pneumococcal responses one year after Prevnar-13 vaccination, thus potentially leaving vaccinated children susceptible to infection [21]. This lack of vaccine “take” has important clinical implications and warrants further investigation into the cellular and molecular mechanisms driving this failure.

It has been observed that the administration of antibiotics enhances the deposition of complement components on the surface of *S. pneumoniae*, thereby enhancing protective phagocytic responses in conjunction with specific anti-pneumococcal antibodies [22, 23, 24]. It has also been previously shown in SCD patients that have reduced opsonization due to defects in the alternative complement pathway, which acts in conjunction with impaired anti-pneumococcal antibody responses to further inhibit opsonization [25]. It is therefore possible that the co-administration of penicillin and pneumococcal vaccination may be acting synergistically to improve vaccine efficacy in people with SCD. We measured the plasma levels of the complement protein C3 in control and SCD mice, but both groups had normal C3 levels and were not statistically different from each other (data not shown). However, it may still be possible to increase pneumococcal vaccine efficacy in the SCD mouse model through the co-administration of penicillin with Prevnar-13 vaccination.

One potential culprit for lack of protection after vaccination and infectious challenge observed in our model is the well-documented splenic dysfunction and loss of memory IgM/B-1 B-cells found in SCD (which are important for generating anti-polysaccharide antibodies). We have previously shown that these important cells are reduced in proportion in the spleens of SCD mice when compared to controls and correlate with reduced baseline levels of total serum IgM and subclasses of IgG [13]. In this study, we found that serum anti-PPS3 IgG3 titers waned after vaccination, and this subclass of IgG is associated with responses to polysaccharide antigens and is protective against *S. pneumoniae* infection [26]. Additionally, antibody titers increased in SCD mice after initial vaccination, but then waned after subsequent booster shots and did not recover. These findings indicate that responses from short-lived plasma cells remain intact in SCD mice; however, long-lived plasma cells do not appear to be generated...
and/or sustained. Plasma cell longevity has been associated with up-regulation of anti-apoptotic genes [27], suggesting these as intriguing targets for further study in B-cells from people and mice with SCD. Other molecules involved in the long-term differentiation and survival of plasma cells (such as APRIL or Blimp-1) would be worth investigating as well [28, 29]. We should also note that Carter et al. [30] found that using pneumococcal proteins as vaccine antigens also did not confer protection to SCD mice upon infectious challenge (whereas some of the control mice survive). This raises the possibility that SCD mice have immunological impairments in addition to B-1 cell deficiencies.

In addition to the lack of generation of long-lived plasma cells in young SCD mice after pneumococcal vaccination, the absence of a booster response indicates that B-cell memory is also not generated. If such a lack of B-cell memory induction is also observed in SCD patients, this would warrant the need for more frequent vaccination to ensure that protective antibodies are maintained. However, the safety of more frequent vaccination has never been tested in children with SCD and should be approached used with caution. Indeed, we have previously found that vaccination may yield adverse effects in SCD mice [18]. Furthermore, the phenomenon of B-cell exhaustion has recently been described [31], and it is possible that increasing the frequency in which this population is vaccinated could further contribute to the problem.

Pneumococcal infection is an enormous problem in children with SCD, and the utilization of prophylactic strategies to prevent infection is essential to prolong the duration and quality of life in this population. Disturbingly, there have been reports that pneumococcal vaccines may not be as efficacious in children with SCD when compared to the general population. We utilized a SCD mouse model of pneumococcal vaccination and infection to study this problem and found that the polysaccharide-conjugate vaccine Prevnar-13 actually has very poor efficacy in these mice. The reasons for incomplete protection from infection appear to correlate with their inability to maintain polysaccharide-specific antibody titers. The potential candidates for this problem are a lack of the generation and/or maintenance of long-lived plasma cells and/or memory B-cells (likely of B-1 origin). Understanding which correlates of protective immunity are lacking in SCD mice will be essential so that targeted therapeutics can be developed to allow for achievement of long-term immunity after vaccination and subsequent reduction of morbidity and mortality in children with SCD.

Supporting Information

S1 Text. Raw data in S1 Text.

Author Contributions

Conceived and designed the experiments: SMS ERS RST BA DWM. Performed the experiments: SMS SR KR CC SS. Analyzed the data: SMS SR AJA SJB SS ERS. Contributed reagents/materials/analysis tools: SMS RST BA DWM. Wrote the paper: SMS SR.

References


