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Proteins Research Proposal
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Effects of Aberrant Self-Lipid Antigen Presentation by Autoimmune B Lymphocytes on iNKT Cell Homeostasis

Background

The Human Immune System

Humans are exposed to pathogens on a daily basis through ingestion, inhalation, and contact. The human immune system is a remarkable model of biochemical machinery that protects the body from such infectious agents by eliminating and destroying the majority of invading organisms. Proper functioning of this system is essential to survival. Absence or malfunctioning of the immune system renders the body defenseless against biotic and abiotic factors, including the body itself, which could prove lethal. It is the body's natural defense mechanism against invading foreign organisms, such as bacteria, viruses, fungi, and other organisms, that weaken or kill the host. The immune system is divided into two major components: the innate and adaptive systems, which function in tandem to provide sufficient protection of the body from invading organisms.

The adaptive immune system is the body's mechanism by which rapid, specific responses are generated against previously encountered pathogens that invade the body again [13]. However, this system develops following birth as exposure to pathogens occurs. Also, because exposure and generation of a specific response to an antigen must occur, the adaptive immune system takes longer to respond than the innate immune system. Following first exposure, however, the adaptive immune system remembers the antigen and launches a faster, stronger response during the next encounter. The adaptive immune system, like the innate immune system, is comprised of different component cells, including B and T lymphocytes [13].

The innate immune system is present in the human body from birth and does not need to learn from exposure to an invading organism. It provides a widespread, nonspecific response to many types of pathogens due to an ability to recognize a limited number of antigens present on the invading agents [5]. However, the innate immune system retains no memory of encounters with pathogens and, therefore, offers no lasting protection against future invasions. Innate immunity is conducted by several types of white blood cells, most notable among them being the natural killer cells [5]. A subset of these cells, termed invariant natural killer T (iNKT) cells also serve functions in the adaptive immune system, where they recognize antigens on the surface of antigen presenting cells (APC), but still incite a general, nonspecific response as in the innate immune system.

Natural Killer Cells and Subclasses

Natural killer (NK) cells are an innate immune cell type that develop in the lymph nodes and become fully functional following formation [5]. These cells provide a robust immune response by recognizing surface antigens on infected and cancerous cells and by subsequently releasing enzymes or other cytotoxic substances that damage the outer membrane of infected or cancerous cells, effectively killing them [5]. A type of these cells that share properties of both innate natural killer cells and acquired T lymphocytes are the iNKT cells.

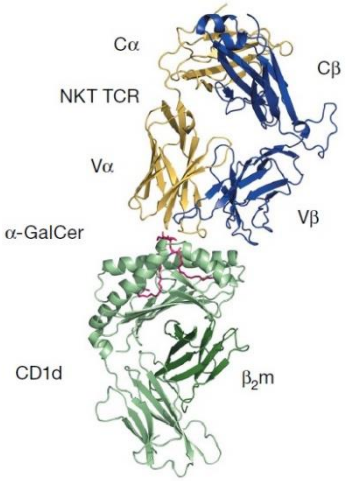

Figure 1. iNKT cell TCR [4]

iNKT cells are technically a subset of T lymphocytes that express surface receptors characteristic of both NK cell and T lymphocyte lineages [13]. iNKT cells develop in the thymus and undergo positive and negative selection during maturation to distinguish self from non-self, eradicating any self-reactive cells

that may have developed. These immune cells express a T-cell receptor (TCR) on their cell surface similar to conventional T lymphocytes [4]. iNKT cells are designated as a type I NKT cell and earned their name because they express a TCR whose structure in humans contain semi-invariant Vα24-Jα18 and Vß11 chains (Figure 1). However, iNKT cells do not recognize and react with classical major histocompatibility complex (MHC) class I or class II molecules that present peptide antigens, like other T cells do. Rather, iNKT cells interact with MHC-like molecules such as the glycoprotein CD1d, that present lipid or glycolipid antigens to elicit an immune response [13]. Similar to NK cells, though, these cells do not possess the capacity for memory unlike adaptive immune cells [5].

The other main subset of NKT cells, deemed type II or variant natural killer T (vNKT) cells, possess a more diverse array of TCRs on their surfaces. vNKT cells have been shown to function as a cross-regulator of type I NKT cells. During instances of autoimmunity, iNKT cells have been shown to contribute to pathogenesis while vNKT cells perform a protective role. vNKT cells modify and induce a dormant state known as anergy in iNKT cells to preserve the body during this state of immunological distress [8]. Here, the focus will be on type I NKT cells that specifically function in tandem with B lymphocytes *in vivo* to produce an effective immune response.

B lymphocytes

B cells are immune cells that function as part of the adaptive immune system providing a humoral response. The humoral response of the adaptive immune system refers to an antibody mediated response. Upon activation, B cells produce antibodies against a certain antigen, marking them for destruction. B cells are so named because they develop in bone marrow [16]. These immune cells differ from innate immune cell types in that they express surface antigen

receptors and can recognize a nearly infinite number of different antigens. These cells serve as an antigen-presenting cell (APC) to T cells utilizing MHC and MHC-like molecules, and in response, produce antibodies when activated [3].

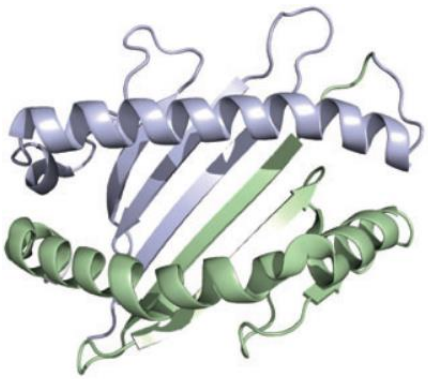

Figure 2. Structure of the classical class I MHC molecule, HLA-2 [1]

Major Histocompatibility Complex (MHC) Molecules

The major histocompatibility complex (MHC) is a family of proteins responsible for antigen presentation on the surface of APCs, such as B lymphocytes, for recognition by specific T cells. MHC molecules exist in four types. There are the classical MHC I and MHC II molecules as well as the non-classical MHC family of proteins, all of which are encoded in the MHC region on human chromosome 6. The fourth type comprises MHC class I-like molecules encoded by genes outside the MHC coding region. Class I MHC proteins are comprised of two semi-parallel α domains, α1 and α2, each contributing one helix and four strands of the ß-sheet underneath (Figure 2). Class II MHC molecules are similar to class I molecules, however, they possess a second transmembrane domain and lack an invariant chain. Class II MHC proteins contain an α1 and ß1 domain with different numbers of ß strands and orientation of the α-helix in contrast to class I MHC proteins. The structure of non-classical MHC proteins is nearly identical to class I MHC molecules, but they possess variations dictated by their diverse biochemical functions [1]. Here the focus will be the MHC class I-like proteins that possess the classical MHC fold yet are not encoded in the MHC region of chromosome 6.

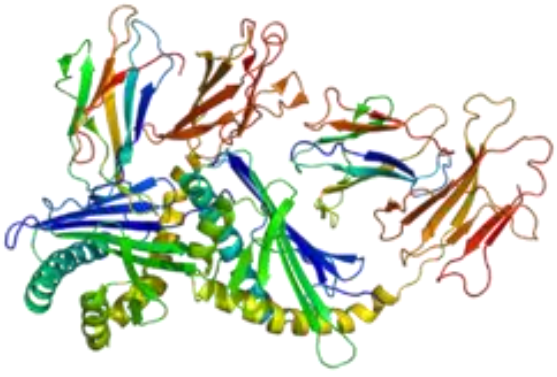

Figure 3. Crystal structure of human CD1d [4]

B lymphocytes commonly utilize these transmembrane proteins for antigen presentation to other immune cell types. Specifically, the CD1 family of proteins is integral for the interaction between iNKT and B cells.

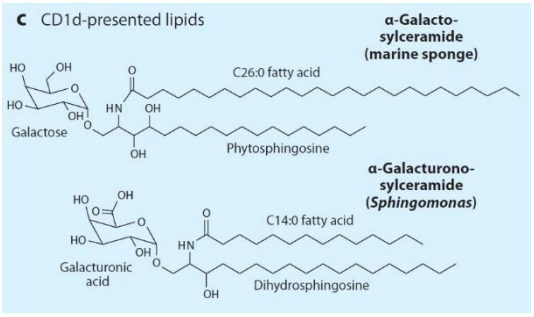

Figure 4. Structure of CD1d lipid antigens [1]

The CD1 Family

The CD1 family is a class of monomorphic MHC class I-like lipid antigen-presenting glycoproteins whose structure resembles the classical MHC fold with slight variations [1]. The CD1 family in humans contains three groups and five isoforms of these glycoproteins. Group I contains isoforms CD1a-c, Group II consists of CD1d, and Group III is comprised of CD1e [21]. CD1 molecules present lipid antigens via a different mechanism than classical MHC peptide-presenting molecules. CD1 molecules contain two large hydrophobic pockets that bury the tails of the lipids being presented, exposing solely the hydrophilic polar head group outward from the binding groove for recognition by other T cells [1]. The crystal structure of these proteins is known (Figure 3). Here, the focus will be on CD1d because the TCR of iNKT cells is specific to this glycoprotein, which only recognizes and elicits a response to lipid antigens presented by this molecule. The known lipid antigens presented by CD1d are α-galactosylceramide and α-galacturonosylceramide (Figure 4) [7]. B lymphocytes express high levels of CD1d on their cell surfaces, making them key antigen-presenting cells for recognition by iNKT cells [8].

B Cell Lipid Antigen Presentation

B cells are key APCs in the adaptive immune system. A diverse array of mechanisms have been uncovered that B cells utilize to take up and process antigens from invading pathogens for presentation. The B cell receptor (BCR) is a transmembrane receptor protein located on the

cell surface of B lymphocytes that is comprised of a membrane-bound immunoglobulin isotype and CD79, a signal transduction molecule bound to the immunoglobulin by two disulfide bridges [3]. Uptake of exogenous lipid antigens, specifically α-galactosylceramide, by the BCR has been shown to stimulate the lymphocytes and enhance presentation of such antigens to iNKT cells in a CD1d-dependent manner, eliciting a more effective response [3].

A second mechanism used by B cells for lipid antigen presentation is a pathway mediated by apolipoprotein and low density lipoprotein receptor (LDL-R). Previous work has shown that exogenous lipids bound to apolipoprotein-E1 (ApoE1) are more efficiently delivered to and taken up by B cells for presentation to iNKT cells [2]. LDL-R is the main surface receptor of ApoE1 on B cells and helps internalize lipid antigens. The interaction between ApoE1 and LDL-R has been shown to be necessary for efficient antigen uptake by B cells. Lipid antigens bound to the related protein ApoE2 are not presented by B cells because ApoE2 does not recognize LDL-R, indicating a dependence on ApoE1 [2]. In fact, B cells activated by other immunoglobulins have demonstrated a pattern of upregulation of LDL-R to further enhance antigen capture and processing. Following antigen uptake, the LDL-R presents the antigen to intracellular CD1d for processing, loading, and presentation on the B cell surface [2]. This evidence further demonstrates the dependence of B lymphocytes on CD1d glycoproteins for effective antigen presentation to iNKT cells.

iNKT Cell TCR and B Cell CD1d Interaction

B lymphocytes and iNKT cells interact via costimulation, where binding of the TCR to the lipid antigen constitutes the first signal. However, a response cannot be generated unless a second signal is received from the B cell [4]. The recruitment of iNKT cells by B cells is dependent upon the interaction of CD1d and the TCR of iNKT cells. Following uptake,

processing, and presentation of lipid antigens by LDL-R and CD1d respectively, the TCR of iNKT cells must recognize and bind to CD1d for activation of iNKT cells to occur [2]. The molecular interaction that occurs between the two components has been largely elucidated. The iNKT TCR has been found to form van der Waals and electrostatic interactions using residues mainly on its CDR2ß and CDR3α loops with CD1d. However, the specificity of the TCR is conferred by 6 amino acid residues unique to the α1 and α2-helices of CD1d among other CD1 family members [10]. These residues are Ser76, Asp80, Glu83, Lys86, Met87, and Asp151 [4]. The CDR3ß loop of the NKT TCR was later shown to play a critical role in the positive and negative modulation of iNKT TCR recognition of CD1d and its presented antigens. The dileucine motif (ßL95 and ßL96) at the tip of the CDR3ß loop makes direct contacts with L145 and K148 on CD1d. This forms a cap over the hydrophobic region on CD1d encompassing the residues A152, V149, K148, L145, and M87 [10].

Previous work has shown that iNKT cells display high affinities for CD1d-presented lipid antigens, including glycolipids and sphingolipids [6]. Here, the focus will be on endogenous self-lipids, which are synthesized within the B lymphocyte, and processed and presented via CD1d. Strong evidence exists suggesting that the natural ligand of iNKT cells is the endogenous lipid isoglobotrihexosylceramide (iGb3). The product of the *Hexb* gene, ß hexosaminidase b, is a lysosomal glycosphingolipid degrading enzyme that converts iGB4 to iGB3. Suppression of this gene results in insufficient concentrations of iGb3 for presentation, resulting in a lack of iNKT cell recruitment [18]. Following recognition of CD1d by iNKT TCR, iNKT cells become activated. These cells rapidly proliferate which is evident by an increased production of cytokines such as interleukin-2, interleukin-4, and interferon-γ. [13]. It was previously thought that iNKT cells undergo apoptosis following activation, but recent work has shown that these

cells downregulate expression of their TCR. This renders them anergic for a short duration allowing them to endure in peripheral tissues [6]. Knowing the mechanisms behind B and iNKT cell interactions in nondisease states helps further the understanding of these interactions and will be critical to understanding their differences in autoimmune disease conditions.

Development of Autoimmunity with B Lymphocytes

The failure of the human immune system to distinguish between self and non-self has proven extremely detrimental as evidenced by the variety of autoimmune diseases known to date. A plethora of factors and conditions must be met to ensure developing immune cells acquire a tolerance for the body's own tissues and cells. During B lymphocyte development in the bone marrow, the immature cells must enter into what is known as the germinal center [9]. Here, the B cells differentiate and become tolerant of the self. iNKT cells have been shown to play a pivotal role in the positive and negative selection of these developing B cells. Prior to entering the germinal center, iNKT cells interact with immature B cells via CD1d-TCR coupling that selectively reduces the number of autoreactive B cells that reach the GC [14]. Later work has shown that autoreactive B cells express higher levels of CD1d on their surface than healthy B cells, which aids in detection by iNKT cells. Following detection, the iNKT cells inhibit autoantibody production by secreting cytokines to further activate normal B lymphocytes [15].

iNKT cells recognize and respond to endogenous self-lipids, resulting in activation and proliferation. One study found that the enzyme, α-galactosidase A, is essential for continuous degradation of endogenous self-lipid antigens. Deficiencies in α-Gal A resulted in exorbitant amounts of self-antigen presentation and caused a reduction in iNKT cell numbers via apoptosis due to overstimulation [7]. A previous study found that the unique sequences in the CDR3ß loop of the NKT TCR encoding a hydrophobic motif promote self-association with CD1d.

The autoreactivity of the iNKT cells may be due in part to the inherent affinity between CD1d and NKT TCR, which results in the recognition of a broad range of CD1d-restricted self-antigens, including iGB3 [10]. Later work determined that chronic signaling by Toll-like receptor 7 (TLR7) expressed on B cells is sufficient for the induction of a type of autoimmune B cell known as age-associated B cells (ABCs). These ABCs have been demonstrated to be autoreactive, autoantibody producers, and deficient in Fas [11, 12].

Fas and Autoreactive B Cells

Fas, also known as CD95, is a member of the TNF-R family encoded by the *Fas* gene that is expressed on the surface of B cells. When bound to Fas ligand (FasL), apoptosis of the Fas positive cell is induced [9]. In the case of autoimmune B cells, Fas expression plays a key role in germinal center B cell homeostasis. Research has found that Fas-mediated apoptosis is essential for negative selection of autoreactive and other defective B cells in the GC. Previous work has shown that the cytotoxic activity of iNKT cells is dependent on the interaction between Fas and FasL. Therefore, Fas deficient B cells evade negative selection in the GC due to the lack of apoptosis [9]. Most recently, research has found that Fas-deficient B cells in mice are autoreactive and present abberant self-lipids in a CD1d-dependent manner to iNKT cells [12]. Presentation of these self-lipids results in hyperactivation and a radical reduction in population size of iNKT cells due to apoptosis. These are indicated by an increased level of cytokine production, specifically IL-2, IL-4, IL-13, and IFN- γ. Additionally, iNKT cells express higher levels of caspase-3, which is indicative of greater rates of apoptosis and cell turnover. The work has also shown that the autoimmune B cells are ABCs that possess an altered lipidome. Specifically, iGb3 was expressed on B cell surfaces at a higher concentration. Unlike the aberrant self-lipids, the identity of which remains undiscovered, iGB3 was unaltered [12].

Significance

The immune system's inability to distinguish its host from foreign pathogens and organisms carries severe consequences in the form of autoimmunity. Autoimmune diseases, such as systemic lupus erythematosus, type 1 diabetes, and multiple sclerosis [12], are the result of this incapacity and can be some of the most difficult diseases to treat effectively. The incidence and prevalence of these disorders is high (1 in 31 Americans) and on the rise, which is sufficient to necessitate the development of effective treatments to provide patients with the best quality of life.

Although antigens can take many forms, such as peptides, lipids, etc., lipid antigens are more commonly involved in autoimmune conditions. The identity of autoimmune self-lipids is still undetermined and more work is required to determine if these altered lipids are also present in B cells classified as autoimmune due to other genetic mutations or deficiencies. A full understanding of the mechanism behind autoimmune B cells and development, self-lipid antigens, and influence on other immune cell types may provide invaluable insight into possible therapies that could either greatly alleviate or possibly cure many autoimmune diseases. This proposal seeks to determine if other genetic mutations, aside from *Fas*, in B cells produce similar autoimmune responses, specifically aberrant presentation of self-lipids.

Individualized medicine is becoming more popular, and possible, in the medical field in recent years. Knowledge of a patient's genome may provide more effective diagnoses and subsequent treatments. Understanding the genetic component of autoimmunity in B cells may be useful in generating patient and/or disease specific treatments for autoimmune disorders that may provide lasting treatment, or even a cure.

Experimental

Project Goal

The goal of this research is to further elucidate the mechanism of action of autoimmune B cells upon iNKT cells that results in their hyperactivation and reduced population size. Previous research has shown that autoimmune B cells were characteristically deficient in the *Fas* gene, which allowed them to avoid negative selection during maturation in the germinal center and thus proliferate within the body. It was found that autoimmunity was caused in part by the presentation of aberrant self-lipids synthesized within B cells [12]. Autoimmune B cells hyperactivate iNKT cells, which leads to higher rates of apoptosis and an overall reduction in population size. This means more autoreactive B cells may be allowed to mature and proliferate throughout the body due to decreased negative selection, as well as diminished numbers of iNKT cells available to fight future infections.

This work seeks to determine if other genetic deficiencies result in similar autoimmune phenotypes, specifically in B cells, that produce equivalent detriments on iNKT cells. In addition, previous work has yet to identify the aberrant self-lipid(s) responsible for the diminution of iNKT cells, so this work seeks to characterize such molecules. By gaining a complete understanding of the genetic components involved in autoimmunity in B cells and the products, such as aberrant lipids, that enact erroneous immune responses, the development of treatments to eradicate autoreactive cells and replenish the body with healthy ones may be possible.

Specific Aim #1

To identify potential candidate genes that, when knocked down, may produce similar autoimmune behavior in B cells.

Rationale

Currently, *Fas* is the only gene known to be deficient in autoimmune B cells. Knowledge of normal B cell function and the full array of genetic lesions that might cause aberrant expression of self-lipid antigens could prove useful in developing more effective therapeutic treatments via gene therapy. Based on the deleterious gene responsible for the autoimmune behavior, disease and/or patient specific treatments could possibly be developed.

Experiment 1a

Hypothesis

I hypothesize that the high-throughput genetic knockdown and screen will identify at least one other genetic mutation that produces a phenotype similar to *Fas*-deficient B lymphocytes.

Approach

A primary mouse splenic B cell line will either be cultured or purchased commercially. Additionally, a library of potential gene candidates will be purchased for use in a massively parallel, high-throughput genetic screen and knockdown experiment. The genetic screen will be completed using robotics. The knockdown will utilize purchased synthetic RNAi to specifically silence each candidate gene in the library. Small interfering RNA (siRNA) molecules will be exogenously transformed into subcultures of the B cell line, targeting a specific gene. The cell cultures will be incubated. A negative control of normal, healthy B cells will also be transformed with a scrambled RNAi sequence. After a sufficient growth period, potential autoimmune candidates will be selected based on phenotype deviations from healthy B cell cultures as indicated by increased levels of B cell costimulatory molecules specific to iNKT cells [12]. The nucleic acid will be recovered from the abnormal cell populations for further testing.

Expected Results, Interpretations, and Limitations

I expect the genetic screen to identify B cell populations expressing an autoimmune phenotype similar to *Fas*-deficient B cells due to other genetic deficiencies. I will interpret these results as consistent with my hypothesis. The next step would be to test the effect of these mutants on iNKT cell homeostasis relative to that of *Fas*-deficient B cells. If no other mutant phenotypes are identified, I would conclude that *Fas* is a key gene in normal B cell functioning and the sole contributor to autoimmunity development in these cells.

A major limitation to this experiment is time and cost. The technology necessary to perform a knockdown and screen of this magnitude is expensive and fairly difficult to acquire. A second limitation is that the presence of other mutant phenotypes does not specifically describe the mechanism by which it disrupts normal function. For example, a mutant phenotype, when cultured with iNKT cells, may produce no abnormal or adverse effects on the iNKT cells.

Experiment 1b

Hypothesis

I hypothesize that identified mutant B cell phenotypes derived from other genetic deficiencies than *Fas* will cause similar hyperactivation, proliferation, and apoptosis of iNKT cells.

Approach

The mutant B cell populations will be cocultured with CFSE-labeled C57BL/6 iNKT cells for 3 days as well as bromodeoxyuridine (BrdU) [12]. Flow cytometry will be employed to detect tagged iNKT cells and quantify population levels by the percentage of cells that incorporated BrdU, which is indicative of cell replication, to observe the effect of the mutated B cells on iNKT cell homeostasis.

Expected Results, Interpretations, and Limitations

I expect the mutant B cells expressing a similar phenotype to *Fas*-deficient B cells to overactivate iNKT cells, causing a reduction in their population size due to increased proliferation and turnover. I will interpret this result as supportive of my hypothesis and conclude that these mutations function in autoimmune conditions via a similar mechanism to *Fas*-deficient B cells. If no abnormal or adverse effects are observed on iNKT cells, I will conclude that the mutations do not play a role in the mechanism of interaction between B and iNKT cells *in vivo*, but may be involved in other immune cell type interactions with B lymphocytes.

A limitation to this experiment is that the result will not definitively demonstrate that presentation of aberrant self-lipids is the cause of the change in iNKT cell homeostasis. Therefore, characterization of the lipidome, as described in experiment 2, must be performed to confirm the mechanism employed by the mutant B lymphocytes.

Specific Aim #2

To identify and characterize the altered self-lipid(s) synthesized in autoimmune B cells and to test their influence on healthy B lymphocytes and iNKT cells.

Rationale

Knowledge of the identity of the aberrant self-lipid would prove useful in not only understanding, but also treating autoimmune disorders of this nature. One could develop a method for targeting the specific lipid using a CD1d antibody. Then, one could selectively block presentation of the aberrant lipid molecules, which may help alleviate some, if not all, effects of

the autoimmune disorder. In addition, one could also either disrupt synthesis of such lipids or attempt to replace them with normal molecules.

Experiment 2a

Hypothesis

I hypothesize that immuno-isolation of CD1d and LC-MS will produce evidence of the presence of aberrant self-lipid(s) in *Fas*-deficient B cells.

Approach

Healthy ($Fas^{+/+}Cd19^{Cre/+}$) and $Fas^{f/f}Cd19^{Cre/+}$ mouse B lymphocyte cultures will be utilized from experiment 1a. The B cells will be subjected to a protease to cleave all surface membrane proteins from the cell. The target protein would be the extracellular domain of CD1d that presents the aberrant lipids. The solution would be run on an affinity column bound with CD1d antibodies to isolate the fragments. The isolated CD1d-lipid fragments would be suspended in an ice-cold methanol:tricine:chloroform mixture to separate the lipids from the CD1d [12]. The lipids would remain in the chloroform layer which would be blown dry with liquid nitrogen. The remaining lipid extract would be reconstituted in the specific liquid chromatography (LC) solvent for analysis by LC-MS. Deviations in ionization pattern and structural differences from the healthy B cells in autoimmune B cell lipid populations would indicate the presence of aberrant self-lipids. The lipids would then be identified by comparison of ionization pattern to healthy B cell self-lipids.

Expected Results, Interpretations, and Limitations

I would expect to see abnormal ionization patterns for lipids extracted from autoimmune B cells relative to healthy B cells. I would interpret this result as supportive of my hypothesis and that these aberrant self-lipids are synthesized in autoimmune B cells deficient in the *Fas* gene.

A limitation to this experiment is it would not indicate whether the identified aberrant lipids identified are involved in the mechanism of B and iNKT cell interaction that disrupts the iNKT cell homeostasis within the cell. Therefore, the isolated aberrant lipids would need to be introduced into healthy B cells to determine if they are sufficient to induce autoimmune behavior.

Experiment 2b

Hypothesis

I hypothesize that introduction of previously isolated aberrant self-lipids into healthy B lymphocytes will cause similar iNKT cell hyperactivation and increased turnover upon presentation.

Approach

The aberrant lipids isolated in experiment 2b will be tested on healthy B cells. Using a lipisome vesicle, the aberrant lipid molecules will be introduced to healthy B cells for uptake or surface loaded onto CD1d for more efficient presentation. Following an incubation period, the aberrant lipid containing B cells will be cocultured with CFSE-labeled iNKT cells as in experiment 1b for three days. The number of iNKT cells remaining will be quantified via flow cytometry to determine if the introduction of the aberrant lipid into healthy B cells is sufficient to induce autoimmunity and increased apoptosis of iNKT cells.

Expected Results, Interpretations, and Limitations

I expect to see a reduction in iNKT cell population size and hyperactivation and proliferation. I will interpret this result to support my hypothesis and determine the cause of autoimmunity being rooted in the presentation of altered self-lipid antigens by B lymphocytes.

A limitation of this experiment may be effectively introducing the self-lipid into the B cell or surface loading it onto CD1d. Also, presentation of the lipid may be indistinguishable from presentation of other endogenous lipids already synthesized in the healthy B lymphocytes.

Summary of Experiments

I have proposed two experiments to further understand the effects of autoimmune B cells on iNKT cell homeostasis. First, a massively parallel, high-throughput genetic knockdown experiment to determine if other genetic lesions aside from *Fas* result in autoimmune development in B lymphocytes. Second, isolation and characterization of aberrant self-lipids in autoreactive B cells via affinity chromatography and LC-MS to determine if the presentation of such aberrant lipids is sufficient to induce autoimmunity in healthy B cells and disrupt iNKT cell homeostasis. I have considered the results of these experiments and possible interpretations of them in addition to the limitations inherent to performing these experiments.

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'On my honor, I pledge that I have not given, received, nor tolerated others' use of unauthorized aid in completing this work.'