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Research Proposal
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Project Summary

Cytokines play an important role during the immune response since they mediate messages between immune cells that regulate immune responses. During viral infection, Th1, Th2, and pro-inflammatory cytokines play important roles in mediating antibody and cell mediated responses, and the inflammatory response. A proper balance of these cytokines is necessary for pathogen clearance, and cytokine imbalances may lead to the formation of an environment favorable to viral persistence, leading to chronic infection. Some cytokines regulate other cytokine secretions in complex ways, and often possess capabilities to promote viral persistence and progression. For example, IL-10 has been shown to mediate several cytokines, such as Th1, Th2, and pro-inflammatory cytokines, in ways that enhance or suppress HIV progression. Since HIV creates imbalances in each of these key cytokine classes, it may serve as an effective model to study how the IL-10 mediated cytokine pathway directs the immune response against chronic viral infection.

AIM 1: Identify candidate cytokines specific to IL-10 mediated pathways that differ in concentration between HIV^+ individuals who are resistant or susceptible to HIV progression. SIV infections in rhesus macaques (RMs) and sooty mangabeys (SMs) will be used as model systems for characterizing various cytokines and viral load. Cytokines will be obtained from the whole blood where the red blood cells are removed. In vivo cytokine levels will be quantified using enzyme-linked immunosorbent assay (ELISA) and viral load will be measured by reverse-transcriptase polymerase chain reaction (RT-PCR).

AIM 2: Test the behavior of candidate cytokines from AIM 1 in HIV^+ individuals who are susceptible or resistant to HIV progression. Peripheral blood mononuclear cells (PBMCs) cultured in vitro from SMs and RMs will be used to measure cytokine levels. Antibody blockades specific to the candidate cytokines will be used to neutralize cytokine cell binding so they cannot initiate cell responses, and the subsequent effects of the cytokine blockade on the behavior of other IL-10 pathway cytokines will be analyzed. Changes in cytokine levels will be measured using ELISA, and SIV load will be measured using (RT-PCR).

Background

Function of Cytokines in the Immune System

Cytokines are proteins secreted by immune cells, such as T lymphocytes, B lymphocytes and macrophages, to mediate communication between immune cells. Messages between an immune cell and a target immune cell are delivered via cytokine binding to specific receptors on the target cell to initiate signal transduction, thereby altering the gene expression of the target cell[16]. Thus, cytokines activate, enhance, or suppress a variety of immune cell functions, including inflammatory responses, cell-mediated responses, antibody-mediated responses, immune cell proliferation, and other cytokine secretions. A cytokine often possesses pleiotropic properties where a single cytokine affects target cells in a variety of ways. Others have synergistic properties where the combination of two or more cytokines has an additive effect on the target cell, or antagonistic properties where one cytokine blocks the action of another cytokine. It is possible for a cytokine to simultaneously possess all of these properties, and effect cytokine secretions and immune cell functions in several ways. Collectively, the binding of several cytokines to a variety of immune cells creates a complex web of synergistic, antagonistic, and pleiotropic effects on immune cell functions and other cytokine secretions to produce an overall effect on the immune system.

Interleukin-10 (IL-10) embodies the complexity a single cytokine can have through its mediation of several immune cell functions. IL-10 exhibits its pleiotropic properties by simultaneously inhibiting and stimulating different classes of T helper cells to downregulate cell-mediated immune responses, and upregulate antibody-mediated responses[20]. Different kinds of cytokines work in conjunction with IL-10 to enhance these effects, while other cytokines suppress IL-10's functions by direct inhibition of cells that secrete IL-10 or the inhibition of a cytokine secretion within an IL-10 initiated cascade.

The behavior of cytokine secretions during viral infection, such as the HIV infection, is complex and requires careful study to understand fully. IL-10 is an example of a cytokine that is known to mediate several immune cell functions; however, its effect on other cytokine secretions during chronic HIV infection is not well understood. Through a better understanding IL-10 mediated cytokine pathways, this study aims to develop HIV as a model system for understanding the role of IL-10 in the immune response against viral infection.

Th1, Th2, and Pro-inflammatory Cytokines

Th1, Th2, and pro-inflammatory cytokines play an important role during viral infections. Th1 and Th2 cytokines secreted by Th1 and Th2 cells ultimately initiate cell-mediated and antibody-mediate responses, respectively[6,10]. Pro-inflammatory cytokines induce a physiological response that recruits immune cells to the site of infection for pathogen removal. Table 1 summarizes the source and functions of some major pro-inflammatory and Th1/Th2 cytokines. Ideally, these cytokines mediate immune cell

functions to create an environment that is favorable to the removal of the viral infection, however, the viral pathogen often creates an imbalance in these cytokines to favor viral survival, resulting in harmful pathological effects and chronic infection. Determining how Th1, Th2, and pro-inflammatory cytokines interact with each other through immune cell binding would enhance the understanding of how cytokine imbalances are created and their effects on immune responses during chronic viral infection. Additionally, by understanding the role of these cytokines, effects of other cytokines (such as IL-10) on Th1, Th2, and pro-inflammatory cytokine secretions can be characterized more fully.

Table 1. Cytokine sources and functions. *More functions may exist. Only functions relevant to the study were listed.

Cytokine	Th1	Th2	Macrophages	Function*
IL-1	-	-	+	Pro-inflammatory; induces IL-6 expression
IL-12	-	-	+	Induces Th1 differentiation into CTLs; induces IFN-γ production
IL-2	+	-	-	Activate T cells; induces T cell proliferation
IFN-γ	+	-	-	Inhibits viral replication; represses Th2 proliferation; promotes phagocytosis by macrophage activation
TNF-β	+	-	+	Promotes phagocytosis by marcrophage activation; pro-inflammatory
IL-4	-	+	-	Promotes Th2 cell differentiation; promotes B cell proliferation and differentiation
IL-5	-	+	-	Promotes synthesis of IgA by B cells
IL-6	-	+	+	Promotes B cell proliferation and antibody secretion
IL-10	-	+	+	Inhibits Th1 cytokine production by inhibiting antigen presentation by marcrophage; represses inflammatory cytokines, stimulates B cell activation and survival

Th1 and Th2 Cytokines and their Role in Immune System Regulation

A proper balance of Th cell cytokines is needed because an imbalance can lead to the overactivation or underactivation of immune cells to create an environment that favors viral persistence, and a harmful immunopathology on the body. An understanding of the level of Th1 and Th2 cell cytokine secretions during chronic viral infection, and their effects on the viral pathogen and the body would provide insights into how cytokines, such as IL-10, ultimately affect the levels of Th1 and Th2 cytokines and the immune response.

Cytokine secretions by $CD4^+$ T helper cells ($CD4^+$ Th cells) is particularly important because of $CD4^+$ Th cells' role in regulating both cell-mediated and antibody-mediated immunity[6,10]. $CD4^+$ Th cells can be divided into two subsets, Th1 and Th2. The Th1 subset is associated with many cell-mediated responses, while the Th2 subset directs mostly antibody-mediated responses. For example, Th1 secretes interferon- γ (IFN-γ) and other cytokines that promote $CD8^+$ cytotoxic lymphocyte (CTL) proliferation, a cell-mediated response directed against viral infections[3,10,24]. In addition, IFN-γ suppresses the proliferation of Th2 cells, which helps the immune system shift its focus from antibody-mediated responses to cell-mediated responses. The Th2 subset possesses cytokines that promote antibody-mediated responses, which eliminates a broader range of foreign material, such as foreign proteins, bacteria, toxins, parasites, and viruses. This type of pathogen elimination is accomplished by antibody (produced by B cells) binding to the foreign material so that it is marked for phagocytic removal. Th2 cytokines, such as IL-10, promote antibody-mediated responses by activating B cells, increasing antibody production, and suppressing Th1 cells[3,10,24].

Pro-inflammatory Cytokines and their Role in Immune System Regulation

Pro-inflammatory cytokines initiate inflammatory responses against foreign invasion by activating lymphocytes, macrophages, and other immune cells. Upon recognition of foreign antigen, different kinds of immune cells (primarily monocytes and macrophages) secrete pro-inflammatory cytokines, which act as chemotactic factors to attract lymphocytes and macrophages to the site of infection, and activate other immune cells so that there is a more aggressive response to the foreign material. In excess, pro-inflammatory cytokines can lead to tissue wasting and the overactivation of lymphocytes so that they are less responsive to viral antigen[6]. Chronic viral infection is often associated with chronic inflammation that causes these harmful effects on the immune response. IL-10 is an important factor to the regulation of the inflammatory response since it inhibits antigen presentation and pro-inflammatory secretions through the deactivation of macrophages, ultimately leading to the downregulation of the inflammatory response. The characterization of pro-inflammatory cytokines secretions in association with IL-10 secretions would provide a better understanding of these cytokines' behavior during chronic viral infection.

HIV Infection Models and their Relation to Cytokine Secretions

The Human Immunodeficiency Virus (HIV) is a retrovirus that infects $CD4^+$ T cells and marcrophages, thereby effecting Th1, Th2, and pro-inflammatory cytokine secretions. Therefore, HIV serves as a model to test the understanding of IL-10's role in effecting Th1, Th2, and pro-inflammatory cytokine secretions during chronic viral infection. HIV infection is characterized not only by a decline in

the number of $CD4^+$ T cells, but also a reduction in the $CD4^+$ T cells' ability to serve as helper cells to other lymphocytes[25]. HIV promotes the formation of inhibitory receptors on $CD4^+$ T cells, thus deactivating $CD4^+$ T cells and causing a decline in $CD4^+$ T cell function[3]. In addition, increasing the number of inhibitory receptors results in the $CD4^+$ T cells being more susceptible to apoptosis, or programmed cell death, leading to a decline in $CD4^+$ T cell numbers. This phenomenon, know as T cell exhaustion, intensifies as HIV viral load increases. Another characteristic of HIV infection is the non-specific hyperactivation of B cells. Much of B cell hyperactivation can be attributed to the chronic over secretion of pro-inflammatory cytokines. Since HIV-induced B cell hyperactivation is non-specific, the ability of infected individuals to mount defenses against specific pathogens is hindered[25]. Two models of chronic HIV infection attempt to explain the basis of these characteristics of HIV infection. Both models attribute the persistence of HIV infection to cytokine imbalances (pro-inflammatory cytokines in one model, and Th1/Th2 cytokines in the other) that lead to a poor immune response. In both models, IL-10 is involved in the cytokine pathways that affect the Th1/Th2 and pro-inflammatory cytokine imbalances.

Th1→Th2 Shift Model of HIV Infection

The Th1→Th2 shift model of HIV infection contends that as an HIV infection progresses, cytokine secretions shift from Th1 cells to primarily Th2 cell cytokine secretions (Figure 1). The model claims that this cytokine imbalance leads to poor regulation of the immune system and the progression of HIV[3,10]. Studies that demonstrate the Th1→Th2 shift observe decreases in the Th1 cell cytokines (e.g. IL-2 and IFN-γ) and increases in the Th2 cytokines (e.g.IL-4 and IL-10)[3,10]. Th1 cytokines are associated with several cell-mediated immune responses, most importantly in HIV, the initiation of $CD8^+$ CTL proliferation. Therefore, this decrease in Th1 cytokines is detrimental to HIV clearance by $CD8^+$ CTLs, resulting in HIV progression. IL-10 can potentially enhance this effect by inhibiting macrophage cytokine secretions that increase Th1 cell expression. Additionally, the upregulation of Th2 cytokines plays a large role in the suppression of T cell activation since many Th2 cytokines inhibit Th1 cytokines that promote cell-mediated immune responses.

The Th1→Th2 shift model is also consistent with the observation of B cell hyperactivation during HIV infection, since Th2 cells promote B cell activation through the secretion of their cytokines. Furthermore, the model is consistent with the finding of increased IgE production by B cells during HIV infection. Particular HIV domains overstimulate IgE production by Th2 activated B cells, resulting in reduced production of other antiviral antibodies specific to HIV epitopes[3,10].

Pro-inflammatory Cytokine Model to HIV Infection

Another model for HIV infection focuses on the link between pro-inflammatory cytokines and the progression of HIV (Figure 2). Three major pro-inflammatory cytokines, IL-1, IL-6, and TNF-α, are overproduced by macrophages during HIV infection, although other proinflammatory cytokines also play a role during HIV infection[1,9,10,12]. Each of these cytokines is shown to increase HIV replication alone and in synergy with one another in peripheral blood mononuclear cell (PBMC) cultures. According to the model, the production of pro-inflammatory cytokines creates a positive feedback loop that enhances HIV replication[6]. HIV infection of macrophages induces the secretion of pro-inflammatory cytokines, increasing HIV replication and the infection of more macrophages, which then increases the release of more pro-inflammatory cytokines. The increase of HIV replication by pro-inflammatory cytokines is well characterized for TNF-α. TNF-α activates a transcription factor, NF-κB, which binds to the transcription initiation site of HIV, thereby increasing HIV expression[1,6,9]. IL-1 and IL-6 increase HIV replication by a similar mechanism, as well as by post-translational mechanisms[1,6]. IL-10 can potentially suppress HIV progression through the inhibition of TNF-α and other pro-inflammatory secretions by macrophage.

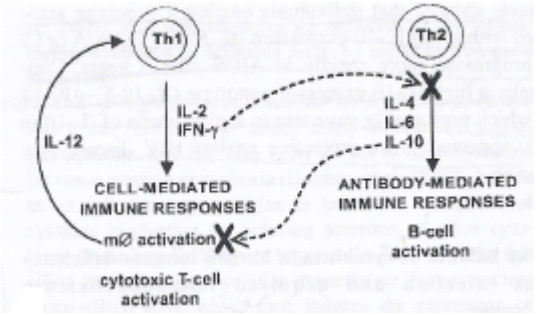

Figure 1. The Role of IL-10 in the Th1→Th2 Shift HIV Infection Model. Solid lines indicate activation and dashed lines indicate inhibition. Borrowed from Breen 2002[6].

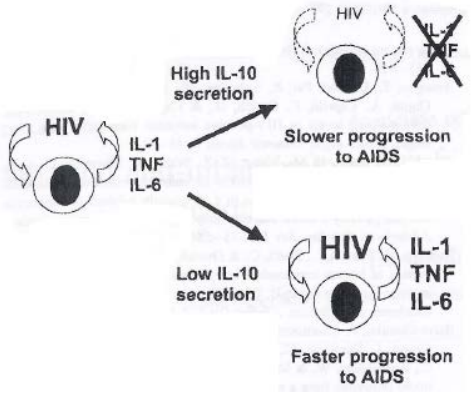

Figure 2. The Role of IL-10 in the Pro-inflammatory HIV Infection Model. Modified from Breen 2002[6].

The Role of IL-10 during HIV Infection

Interleukin-10 (IL-10) is an immunoregulatory cytokine that is secreted primarily by Th2 cells, but also by monocytes, macrophages, and B cells[20]. Through the regulation of Th1 and Th2 cell responses, IL-10 influences immune responses in innate immunity, and both the cell-mediated and antibody-mediated arms of adaptive immunity. Functions of IL-10 include inflammation suppression through inhibition of macrophage antigen presentation, and the suppression of pro-inflammatory cytokines (e.g. IL-1, IL-6, and TNF-α) secreted by Th1 cells[6,20]. IL-10 also acts to suppress cell-mediated

immune response through the activation of Th2 cytokines that prevent Th1 cell secretions IL-2 and IFN-γ[12]. The lack of IL-2 and IFN-γ secretions limits $CD8^+$ CTL activation and differentiation. In addition, IL-10 promotes antibody-mediated responses by acting on Th2 cells to stimulate B cell activation and prolong B cell survival[12]. Several studies associate chronic HIV infection with heighten IL-10 levels compared to non-infected or post-infected individuals, although the overall effect of increased IL-10 levels on HIV progression is debatable. IL-10 initiates and suppresses many immune responses during chronic HIV infection, some that promote HIV progression and others that inhibit HIV progression. Therefore, depending on how IL-10 cytokine pathways are regulated, IL-10 can ultimately enhance or suppress the progression of HIV.

IL-10 plays a complex role during HIV infection because it possesses properties that favor both HIV progression and HIV repression. The anti-HIV properties of IL-10 include the suppression of pro-inflammatory cytokines, which otherwise act to create a favorable environment for HIV progression. The anti-inflammatory role of IL-10 fits in well with the pro-inflammatory model for HIV infection since increased IL-10 expression is often associated with decreased pro-inflammatory cytokine levels, resulting in the inhibition of HIV progression (Figure 2). Contrarily, IL-10 favors HIV progression by inhibiting the secretion of anti-viral Th1 cytokines (through macrophage inactivation), and the inducing the secretion of Th2 cytokines that cause B cell overstimulation. IL-10's influence on Th1 and Th2 cytokines is consistent with the Th1→Th2 shift model of HIV infection (Figure 1). Because of IL-10's bifunctionality, research on IL-10's overall effect on HIV infection and its relation to the HIV infection models has produced contradictory results.

Support for Th1→Th2 Shift Model and IL-10 as a HIV Infection Enhancer

Several studies show that IL-10 ultimately increases viral load in CD4 and CD8 T cells in vitro during chronic HIV infection. Brockman et al. measured plasma IL-10 protein and IL-10 mRNA from cultures of peripheral blood mononuclear cells (PBMCs) of HIV^+ patients. They found that increased IL-10 levels (protein or mRNA) was associated with higher viral loads in $CD4^+$ and $CD8^+$ T cells, and suppressed HIV specific CD4 and CD8 T cell responses[8]. An IL-10 blockade with an anti-IL-10 antibody was shown to decrease IL-10 levels, resulting in lower viral loads and a restoration of CD4 and CD8 T cell responses. This relationship suggests that IL-10 plays an important role in HIV persistence. Elrefai et al. found similar results by removing $CD8^+$ T cells that secreted high levels of IL-10 from the PBMCs of HIV^+ patients. They showed that removal of IL-10 positive $CD8^+$ T cells resulted in increased Th1 function (indicated by an increase of IL-2 levels), which directs cell-mediated responses against viral infections[13]. Buonaguro et al. found more support for IL-10 as a HIV enhancer by demonstrating that

when PMBCs of HIV^+ patients are exposed to HIV proteins, Th2 cytokine production (IL-6, IL-10, TNF-α) is induced, while Th1 cytokine production is suppressed (IFN-γ)[10].

Finally, several studies show that the HIV tat protein (HIV regulatory protein) induces IL-10 to suppress the immune system[14,17,18,19]. Gupta et al. supports IL-10's role further by showing that tat immunosuppression is not observed in IL-10 deficient mice, suggesting that IL-10 plays a role in promoting HIV persistence[15]. Each of these studies supports that Th1→Th2 shift model of HIV infection, since IL-10 is acting as a Th2 cytokine that suppresses anti-viral Th1 functions (Figure 1).

Support for the Pro-Inflammatory Model and IL-10 as a HIV Repressor

Although there are several studies showing IL-10's immunosuppressive properties, many in vitro studies using similar methodologies show that IL-10 decreases viral loads and replication rates in HIV-infected cells during chronic infection. Bento et al. used a similar methodology as Brockman et al., but found IL-10 reduced, rather than increased, viral replication in T cells[4]. Additionally, Bento et al. neutralized IL-10 with anti-IL-10 antibodies, and found that HIV replication increased in T cells, which is opposite to the findings of Brockman et al. and Elrefai et al. Bento et al. also found that upon the neutralization of IL-10, pro-inflammatory cytokine secretions increased. Similar results were found in an in vivo study by Pott et al; they found decreases in pro-inflammatory cytokine levels when IL-10 was injected into HIV^+ patients[23].

Furthermore, many studies analyzing IL-10 gene promoter polymorphisms found IL-10 promoter polymorphisms that associate with higher IL-10 secretions correlate negatively with infection risk and viral load, while low producing IL-10 polymorphisms have the opposite effect[5,7,11,21,22]. Cumulatively, these studies support the pro-inflammatory model of HIV infection; IL-10 acts to suppress pro-inflammatory cytokines to inhibit HIV replication and decrease viral load (Figure 2).

Overlapping of the Th1→Th2 Shift Model and the Pro-inflammatory Model

Studies demonstrate that IL-10's function during HIV infection is multifaceted, and that its effects on HIV and the immune system are complex. IL-10 can potentially have positive or negative effects on the control of HIV infection; it reduces immunopathology by preventing chronic inflammation, but also downregulates cell-mediated responses essential to viral elimination. When IL-10 is in low concentrations or absent, chronic inflammation occurs, which can be detrimental to viral control if HIV is not removed during the acute phase of infection. Therefore, the elimination of HIV appears to depend on effective regulation by IL-10, where initial pro-inflammatory mechanisms are allowed to remove the pathogen and then the inflammation is suppressed by IL-10 before the pathology of chronic inflammation

occurs. In addition, it is important that IL-10 properly regulates B cell activation since overstimulation of B cells indirectly by IL-10 results in an ineffective antibody response to HIV.

Most studies only show evidence for one HIV infection model, and only produce results that show IL-10's affects on HIV through one pathway (Th1/Th2 cytokines or pro-inflammatory cytokines). However, a few studies provide evidence for both models and demonstrate involvement of IL-10 in both pathways. Andrade et al. showed that upon an increase in IL-10 levels (PBMCs from HIV^+ patients were used), IL-10 was allowed to induce pro-inflammatory cytokine secretions (e.g.TNF-α), but not Th1 cytokine secretions (e.g. IFN-γ)[2]. In other words, IL-10's influence on immune cell cytokine secretions was inhibited in the pathway that leads to increased viral replication (IL-10 suppression of Th1 cytokines) and allowed to function in the pathway that decreased viral replication (IL-10 suppression of pro-inflammatory cytokines). In this way, Il-10's functions were expressed and inhibited in a way that optimizes the clearance of HIV. These observations are not possible if each of the models is considered alone, however, considering both models concurrently is consistent with these results.

Recent studies suggest that neither the Th1→Th2 shift nor pro-inflammatory model alone can adequately describe the role of cytokine pathways and IL-10 during HIV infection. Consideration of both models simultaneously better describes the results seen in the study by Andrade et al. For the HIV models to overlap, there must be another variable(s) involved in regulating IL-10 (Figure 3). In high concentrations, IL-10 normally suppresses Th1 cytokines. However, in the study by Andrade et al., Th1 levels remained high despite high IL-10 levels, indicating that another cytokine somewhere in the Th1 pathway is affecting the Th1 cells. The direct inhibition of IL-10 is not consistent with the results because macrophages would not be allowed to be deactivated by IL-10, resulting in pro-inflammatory cytokine secretion. The reactivation of macrophages by another cytokine so that IL-12 can be released to activate Th1 cells is not possible either, because pro-inflammatory cytokines would be secreted by the macrophage. Therefore, IL-12, or some other Th1 activating cytokine, must come from a source other than macrophages (Figure 3). This way, IL-10 would be allowed to suppress pro-inflammatory cytokines through macrophage deactivation, but would not have to use the macrophage activation as a source of IL-12 for Th1 cell activation. The purpose of this study will be to identify candidate cytokines that reactivate the suppressed portion of this IL-10 mediated pathway so that IL-10 can inhibit pro-inflammatory cytokine secretions by macrophages, but not suppress Th1 functions.

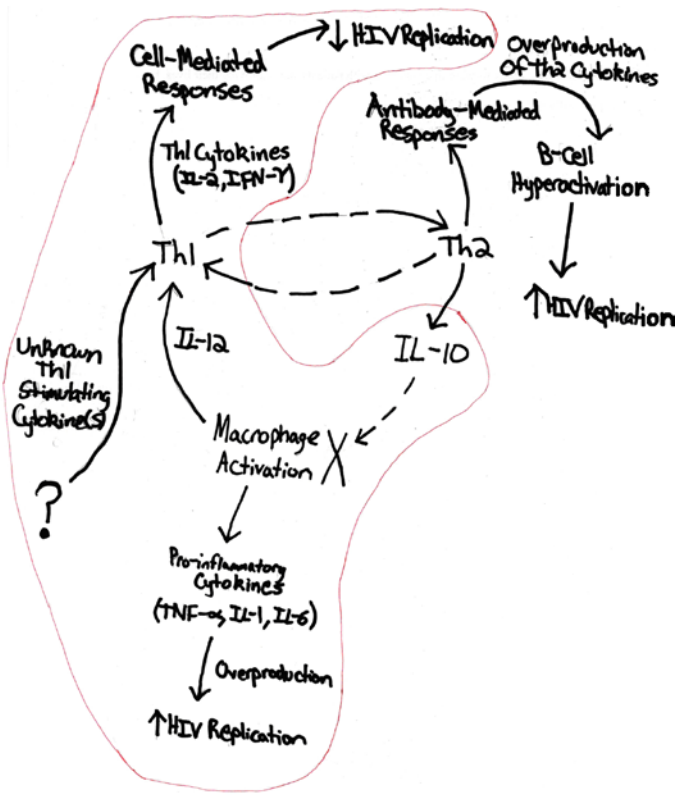

Figure 3. Overlap of Th1→Th2 shift and Pro-inflammatory HIV Models. Outlined in red are the IL-10 affected pathways that influence pro-inflammatory and Th1 cytokines. Solid lines indicate activation and dashed lines indicate inhibition.

Significance

The role of cytokine pathways during chronic HIV infection is extremely complex, and there is much to be understood about how certain cytokine profiles enhance or restrain HIV progression. Recent studies reveal that current HIV infection models do not sufficiently explain the behavior of cytokines during HIV infection. More research needs to be conducted to determine the validity of a newly proposed model that incorporates two HIV infection models, which attribute imbalances in pro-inflammatory and Th1/Th2 cytokines to the persistence of chronic HIV infection. IL-10 will play an essential role in newly proposed models since its regulation through various cytokine pathways can affect pro-inflammatory and Th1/Th2 secretions, and affect the immune response in several ways. The purpose of this proposal is to test the validity of the proposed HIV model by determining what cytokines affect IL-10 mediated pathways. Specifically, there will be a focus on cytokine pathways downstream of IL-10 induction since IL-10 affects both pro-inflammatory and Th1 cytokines downstream of its induction. Ultimately, this study will provide a better understanding of IL-10's role in cytokine pathways during chronic HIV infection, and may lead to the development of HIV infection as a model system for exploring IL-10's role in the immune system.

Experimental Design and Methods

AIM 1: Identify candidate cytokines specific to IL-10 mediated pathways that differ in concentration between HIV^+ individuals who are resistant or susceptible to HIV progression. SIV infections in rhesus macaques (RMs) and sooty mangabeys (SMs) will be used as model systems for characterizing various cytokine levels and viral load. In vivo cytokine levels will be quantified from whole blood using enzyme-linked immunosorbent assay (ELISA) and viral load will be measured by reverse-transcriptase polymerase chain reaction (RT-PCR).

AIM 2: Test the behavior of candidate cytokines from AIM 1 in HIV^+ individuals who are susceptible or resistant to HIV progression. Peripheral blood mononuclear cells (PBMCs) cultured in vitro from SMs and RMs will be used to measure cytokine levels. Antibody blockades specific to the candidate cytokines will be used to neutralize cytokine cell binding so they cannot initiate cell responses, and the subsequent effects of the cytokine blockade on the behavior of other IL-10 pathway cytokines will be analyzed. Changes in cytokine levels will be measured using ELISA, and SIV load will be measured using RT-PCR.

Experiment to Address AIM 1

Rationale

An analysis of the SIV resistant and SIV susceptible cytokine profiles will give insight into how IL-10 mediated pathways are optimized to inhibit viral progression, or how IL-10 mediated pathways are imbalanced to favor HIV progression. Candidate cytokines identified in AIM 1 will be tested in AIM 2 to determine their behavior during viral infection, and see if the candidate cytokines fit into current models of HIV infection.

Hypothesis

Since SMs and RMs differ in their resistance to SIV progression, RMs will likely possess a cytokine profile that supports SIV progression and SMs will possess a cytokine profile that resists SIV progression.

Approach

To characterize cytokine profiles in individuals resistant and susceptible to HIV progression, a non-human primate model system will be used [5]. SIV^+ *Macaca mulatta*, commonly know as rhesus macaques (RMs), will act as individuals susceptible to SIV progression, and SIV^+ *Cercocebus atys*, commonly known as sooty mangabeys (SMs), will act as individuals resistant to SIV progression

(Regional Primate Research Center of Emory University). SIV^- RMs and SMs will be used as controls. Pro-inflammatory cytokines (TNF-α, IL-1, IL-6), Th1 cytokines (IL-2, IFN-γ), IL-12, and IL-10 will be quantified, since all of these cytokines are downstream of IL-10 induction and are involved in current HIV models. IL-12 will be of specific interest since it may be involved in increasing expression in Th1 cytokines when IL-10 is inhibiting macrophage cytokine secretion (Figure 3). Additionally, IL-3, IL-7, IL-8, IL-9, IL-13, IL-17 IFN-α, and TGF-β will be measured in attempt to identify candidate cytokines for AIM 2.

For cytokine quantification, first whole blood will be drawn from SIV^+ and SIV^- RMs and SMs, and red blood cells will be removed by lysis, as described previously[23]. Indirect antibody ELISA will be used to quantify cytokine levels[8,10,13,23]. Aliquots will be taken from the serum and placed into wells of the polystyrene plate so that each cytokine can be tested. Then, the wells will be washed with PBS-Tween to remove unbound cytokines. Primary antibodies (BD Biosciences) will be added to each well, where the antibody is specific to an epitope on the particular cytokine. A primary antibody (diluted in PBS-Tween) specific to a different cytokine will be added to separate wells so cytokine levels can be tested individually. A second wash with PBS-Tween will be done to remove excess antibody. Then, a secondary antibody (BD Biosciences) with a conjugate peroxidase attached is added so that it binds to the primary antibody specifically. A third wash with PBS-tween will be added to remove excess secondary antibody. The substrate solution containing ABTS (2,2-azinoadi(3-ethylbenzthiasolinesulfonic acid)), citrate-phosphate buffer, and 30% hydrogen peroxide will be added so that the peroxidase can react to create a color change (BD Biosciences). The intensity of color will reflect the concentration of cytokines in the serum. Cytokine levels will be quantified using an absorbance microplate reader (Molecular Devices) to measure the absorbance of the wells.

The viral load of SIV^+ and SIV^- SMs and RMs from blood samples will be measured using RT-PCR (Applied Biosystems)[8,23]. Viral RNA will be removed from blood plasma and treated with reverse transcriptase (Applied Biosystems) to create cDNA from the viral RNA. The PCR is then used to amplify the cDNA and the resulting DNA products are hybridized with specific oligionucleotides (Applied Biosystems). A probe with an attached enzyme (Applied Biosystems) makes the cDNA visible so that it can be quantified. A standard curve of the PCR reaction and a copy number calculation must be made to determine the amount of viral RNA present in the original sample.

Expected Results and Interpretations for Pro-inflammatory, Th1, and IL-12 Cytokine Levels in SM and RM Individuals

I expect cytokine levels for pro-inflammatory cytokines, Th1 cytokines, and IL-12 will differ between SM, RM, and control (SIV^- SMs and RMs) individuals because of differences in SIV susceptibility. In SM individuals, I expect Th1 cytokines and IL-12 to be elevated relative to the controls and RM individuals. Additionally, I expect pro-inflammatory cytokine levels to be lower relative to RMs. I expect this cytokine profile to be associated with decreased viral load. I will interpret the association of this cytokine profile with decreased viral load to be indicative of an individual with cytokine profile that favors SIV resistance. The elevation of Th1 cytokines initiates cell-mediated responses, which is needed for SIV resistance, and IL-12 is needed to activate Th1 cells to secrete their cytokines. So it is appropriate to see these cytokines at higher levels in SIV resistant individuals. Excessive levels of pro-inflammatory cytokines are associated with increased viral load, so a decrease in these cytokines matches what would be expected in SM individuals resistant to SIV progression.

An opposite trend in cytokine levels is expected in RM individuals, who are susceptible to SIV progression. Decreased Th1 cytokine and IL-12 levels, and increased pro-inflammatory cytokine levels are expected relative to SMs and the controls. I expect these cytokine levels to be associated with increased viral load.

If these trends in cytokine levels are seen, it will show that the non-human primate model for HIV infection is reliable since the trends SIV infection produce are consistent with what is predicted by current HIV infection models (Figure 3). Additionally, it will provide further evidence for the role of Th1 cytokines, pro-inflammatory cytokines, and IL-12 in IL-10 mediated pathways during an immune response against viral infection.

Expected Results and Interpretations of IL-10 Cytokine Levels in RM and SM Individuals

I expect that IL-10 will be elevated in the SMs, but decreased viral load will only be seen with high IL-10 levels if an additional factor in adequate concentrations regulates the IL-10 pathway after macrophage deactivation (Figure 3). This unknown factor, possibly IL-12, will most likely be associated with the SM profile, but not the SIV susceptible RM profile. This is because proper regulation of IL-10 pathway by an additional factor allows for the elevation of Th1 cytokine levels necessary for SIV resistance, while also allowing for the inhibition of macrophages by IL-10 to suppress pro-inflammatory secretions (Figure 3).

The RM profile can produce an IL-10 cytokine profile that promotes SIV progression in two ways. First, IL-10 may be elevated, thus reducing pro-inflammatory cytokines (favorable to SIV suppression), but the additional factor will fail to activate Th1 cells to secrete their cytokines that favor

SIV resistance. Another possibility is that IL-10 is in low levels so that it does not deactivate pro-inflammatory cytokine secretions by macrophages, which is favorable to SIV progression. However, macrophages would be allowed to secrete IL-12 to induce Th1 cell secretions favorable to SIV resistance. Although in each of these situations there is some cytokine behavior favorable to SIV resistance, there is also cytokine behavior that favors SIV progression, which I expect will ultimately lead to increased viral loads due to cytokine imbalances.

Expected Results and Interpretations of the Levels of Candidate Cytokines in SM and RM Individuals

I expect that candidate cytokines that affect IL-10 mediated pathways associated with SIV resistance in SMs will be identified by an increase or decrease in a particular cytokine level relative to the level of the same cytokine in the control and RMs. Candidate cytokines associated with SIV progression will be identified in the same manner, but the cytokine levels of the RMs will be compared to the cytokine levels in SMs and the control. I will interpret differences in the cytokine levels to be reflective of SIV resistant or progressive state of the individual.

Limitations

One limitation of this experiment is the possibility that several of candidate cytokines will behave differently in SM and RM individuals. This would make it difficult to determine how each of the candidate cytokines are related to each others' functions and the functions of other cytokines in the IL-10 mediated pathway. Another possibility is that none of the cytokines from the list of candidate cytokines will behave differently between SM and RM individuals. In this case, a new list of candidate cytokines would have to be tested to determine which factor(s) are contributing to the IL-10 mediated pathway.

Another limitation of this experiment is that it does not identify the source of the candidate cytokines. This would have to be addressed in a further study so that cells that secrete the candidate cytokines could be incorporated into the HIV models.

Experiment to Address AIM 2

Rationale

Performing antibody blockades on candidate cytokines associated with increased/decreased viral loads would confirm their overall effect on SIV suppression or progression, and their influence on cytokines in IL-10 mediated pathways. The behavior of candidate cytokines may then be able to be incorporated into the HIV infection model for understanding IL-10 mediated pathways during the immune response.

Hypothesis

Antibody blockades on a candidate cytokine may affect the candidate cytokine's influence on the levels or other IL-10 pathway cytokines, which will be identified by an increase or decrease in the levels of IL-10 pathway cytokines. These changes in IL-10 pathway cytokine levels due to the candidate cytokine blockade may result in a shift from an SIV resistant state to a SIV progressive state, or vice versa (as measured by SIV load).

Approach

Antibody blockades of cytokines will be conducted to disable the cytokine's function so that its affect on other cytokines in the IL-10 mediated pathway can be determined. As in AIM 1, the non-human primate model will be used, expect PBMCs will be cultured in vitro to measure cytokine levels. Cytokine knockouts will be conducted on pro-inflammatory cytokines (TNF-α, IL-1, IL-6), Th1 cytokines (IL-2, IFN-γ), IL-12, and IL-10. Pro-inflammatory cytokines will be neutralized individually and as a group, as will Th1 cytokines. Additionally, IL-12 and IL-10 cytokines will be knocked out. Candidate cytokines identified in AIM 1 will also be neutralized to determine their roles in the IL-10 mediated pathway.

Whole blood will be drawn from SIV^+ and SIV^- RMs and SMs, and red blood cells will be removed by lysis, as described previously[23]. PBMCs will be isolated by density centrifugation (Ficoll-Histopaque; Sigma Aldrich), and cultured in RPMI 1640 media (Life Technologies), as previously described[8,10]. Cultures will be made for each cytokine or set of cytokines that are to be tested. PBMCs will be pulsed with HIV-VLPs (R&D Systems) for 16 hours to induce cytokine secretions. Cytokine levels will then be measured before the cytokine blockade by ELISA, as described in AIM 1. Cytokines will be blockaded by adding antibodies (R&D Systems) specific to the cytokine of interest into the culture (antibodies will be diluted in PBS-Tween). ELISA will be used (as described in AIM 1) to measure the change in cytokine levels in the PBMC cultures relative to levels present before blockade treatment. Viral load will be measured as described in AIM 1, except samples will be taken from the in vitro PBMC cultures rather than directly from whole blood.

Expected Results and Interpretations for Cytokine Blockades of SM and RM Individuals

I expect that the inhibition of a candidate cytokine that is associated with high Th1 cell secretions in SM , RM, and control individuals (SIV^- SMs and RMs) will result in lower Th1 secretions and higher viral load in comparison to cytokines not treated with a blockade. An opposite result is expected with candidate cytokines associated with lower levels of Th1 cytokines are blockaded. More dramatic changes in Th1 cytokine levels are expected in SMs since they will likely have higher concentrations of Th1

cytokines than RMs prior to the blockade. Blockades of IL-12 are expected to decrease Th1 cytokine levels and increase viral load since they are associated with higher Th1 levels.

Knockouts of Th1 cell cytokines individually or collectively are expected to increase viral load in SM, RM, and control individuals, however these effects are expected to more dramatic in SMs since their cytokine profile is suspected to be associated with higher Th1 levels before the blockade. Knockouts of pro-inflammatory cytokines are expected to decrease viral load in RM, SM, and control individuals, however this effect is expected to be more dramatic in RMs since they will probably have higher initial pro-inflammatory levels.

An IL-10 knockout is expected to increase pro-inflammatory cytokine levels in SM, RM, and control individuals, but not influence Th1 cytokines relative to pre-blockade cytokine levels. I expect that this will lead to an increase in viral load since pro-inflammatory cytokines will be expressed highly, but I expect the increase to not be as high as a knockout effecting Th1 cytokines. More dramatic pro-inflammatory cytokine decreases in RM individuals are expected since they have higher initial concentrations of pro-inflammatory cytokines. It is possible that an IL-10 knockout may not increase pro-inflammatory levels significantly if a candidate cytokine reduces pro-inflammatory secretions.

I will interpret these results as a confirmation of the role of various cytokines in the IL-10 mediated pathway, rather than simply correlating levels of cytokine secretions. Knockouts of cytokines that produced decreased Th1 levels or increased pro-inflammatory levels can potentially shift a SIV resistant SM cytokine profile to a profile favorable to SIV progression. Likewise, it is possible to shift a SIV progressive cytokine profile to a SIV resistant profile through an IL-10 knockout, but only if an outside cytokine is able to regulate the resulting increase in pro-inflammatory cytokines. Ultimately, I hope to confirm the role of candidate cytokines in the IL-10 mediated cytokine pathway so that role of the IL-10 mediated pathway in HIV infection can be better understood.

Limitations

The primary limitation of this experiment is that it is done in vitro rather than in vivo, since the behavior of cytokines may be different in vitro. A comparison of cytokine levels in the cultures and in vivo will be done to determine the degree cytokine level differences. Also, as stated in the limitations in AIM 1, it is possible that many candidate cytokines will play a part in the IL-10 mediated cytokine pathway, thus complicating the analysis of each cytokines effect. Additionally, the cellular source of the cytokines will not be known in this study, which limits the understanding of how candidate cytokines interact in the IL-10 mediated cytokine pathway. A future study would address the cellular source of the identified candidate cytokines.

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