

**CAMB-MVP**

**The Role of HIV Envelope Glycoprotein Induced Regulatory T Cells  
in Suppressing CD4+ T Cell Proliferation**

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## A. Specific Aims

Human immunodeficiency virus (HIV) infection is a global epidemic that affects millions of people. HIV envelope glycoprotein (Env) present on free virions and infected cells can target bystander CD4<sup>+</sup> T cells for suppression. Our studies have demonstrated that surface expression of Env on dendritic cells (DC) or 293T cells induces suppression of antigen-stimulated CD4<sup>+</sup> T cell proliferation, but does not lead to anergy, apoptosis or cell death. These CD4<sup>+</sup> T cells can phosphorylate key signaling mediators including MAPK, AKT, and STAT5a in a normal manner, but fail to up-regulate activation markers such as CD69, HLA-DR, and CD25. **Hence, even though Env induced suppression of CD4<sup>+</sup> T cell proliferation has been well established, the mechanism remains to be identified.** Recently, regulatory T cells (Treg) have been implicated to play a critical role in controlling the magnitude of immune response to chronic infection including HIV. Treg cells can exert their suppressive activity through cytokines IL-10 and/or TGF- $\beta$ . Interestingly, our preliminary experiments demonstrated that Env induces nearly a doubling in IL-10 production in the co-culture of Env-expressing 293T and PBMC. **I hypothesize that HIV Env, through direct interaction with Tregs, induces the generation of either an increased frequency or an enhanced activity of Treg cells that, in turn, suppress antigen-stimulated CD4<sup>+</sup> T cell proliferation.** In this proposal, I will pursue two specific aims to characterize the induction of Treg cells by Env *in vitro* as well as its physiological relevance in HIV-infected patients.

**Specific Aim 1: To determine if Env induces an increased frequency and/or enhanced activity of Treg cells and if induced Treg cells suppress CD4<sup>+</sup> T cell proliferation *in vitro*.** I will address this aim through the following sub-aims. **1)** To model the *in vivo* interaction of bystander T cells with Env present on free virions and infected cells during an antigen-specific response, an *in vitro* system will be established in which PBMC are co-cultured with Env-expressing 293T cells in the presence of anti-CD3 and IL-2; **2)** To determine if Env induces a higher frequency of Treg cells, flow cytometry will be used to phenotypically identify and enumerate Treg cells using CD4, CD25 and FoxP3 staining. In addition, ELISA and intracellular cytokine staining will be used for detection of IL-10 and TGF- $\beta$  to test if Env induces an enhanced activity of Treg cells. **3)** Based on our observations that Env-induced suppression is CD4, not co-receptor, dependent, I will determine whether Env induces Treg cells by signaling through T cell CD4 receptor using either soluble CD4 or Env lacking CD4 binding. **4)** To show

whether these Env-induced Treg cells suppress antigen-stimulated CD4<sup>+</sup> T cell proliferation, addition and subtraction cultures and IL-10 and/or TGF- $\beta$  neutralization studies will be performed.

**Specific Aim 2: To characterize the physiological relevance of Treg cells in HIV-infected patients.** PBMC from HIV infected patients with various viral loads (less than 100 copies to more than 100,000 copies/ml) will be collected for analysis in specific aim 2. **1)** To test the association of viral load with Treg cells, I propose that increased viral load (increased Env expression *in vivo*) correlates with either an increased frequency or an enhanced activity of Treg cells. The assays described in specific aim 1 will be used to measure the frequency and cytokine production of Treg cells in collected PBMC. **2)** The consistently heightened level of T cell activation during HIV infection inhibits or reduces the capacity of adaptive immune response to function efficiently. To assess the effect of Treg cells on high-level T cell activation, I hypothesize that a higher frequency or activity of Treg cells is associated with a lower level of T cell activation, which will be determined by mean fluorescence intensity (MFI) of CD38 staining on CD4<sup>+</sup> and CD8<sup>+</sup>T cells. **3)** Treg cells can suppress antigen specific immune response to allow a low level of pathogen persistence *in vivo*, which has been proposed to be favorable for the maintenance of long-term T cell memory. To test the functional impact of Treg cells in HIV infection, I hypothesize that Treg cells suppress the HIV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell response. PBMC with or without Treg cells will be stimulated with a pool of HIV Gag and control peptides. Effector response will be quantified using FACS.

## **B. Background and Significance**

Human immunodeficiency virus type-1 (HIV-1) infection is a global epidemic that affects millions of people. It is estimated that 4.9 million new infections occurred in 2005 (1). HIV infection causes a progressive decline in the number and function of CD4<sup>+</sup> T cells and eventually leads to the development of AIDS (2). On one hand, the observations that HIV infects CD4<sup>+</sup> T cells suggested that direct virus-mediated killing of CD4<sup>+</sup> T cells is a cause of progressive CD4<sup>+</sup> T cell loss during infection (3); On the other hand, in many patients only a small fraction of CD4<sup>+</sup> T cells appear to be infected and the degree of cell loss greatly exceeds the number of infected cells, which indicates that in addition to cytopathic effects of HIV

infection, virus-induced dysfunction of bystander T cells plays a major role in CD4<sup>+</sup> T cell depletion (4).

### **B-1. Suppression of bystander CD4<sup>+</sup> T cells by Env**

Among HIV proteins, Env has been demonstrated to play a predominant role in suppressing bystander CD4<sup>+</sup> T cells by multiple mechanisms (5, 6). Functional Env is a trimeric protein with each of its monomers possessing CD4 and co-receptor binding sites. In infection, Env trimers present on free virions and infected cells can cross-link the CD4 receptor, resulting in aberrant signaling in uninfected CD4<sup>+</sup> T cells that impairs normal TCR stimulation (7). Studies have shown that a fraction of uninfected T cells in peripheral blood as well as lymph nodes undergo apoptosis during HIV infection (8). Several pathways for delivery of proapoptotic signals to uninfected CD4<sup>+</sup> T cells have been reported, such as Fas-FasL, TNFR-TNF- $\alpha$  and DR4/DR5-TRAIL pathways (9-13). In addition to apoptosis, anergy has also been identified as a mechanism for Env induced suppression. Env suppresses CD4<sup>+</sup> T cells by inducing a non-proliferative state that could be overcome by addition of exogenous IL-2 (14).

In particular, our studies have demonstrated that surface expression of Env on dendritic cells (DC) and 293T cells induces suppression of antigen-stimulated CD4<sup>+</sup> T cell proliferation. These CD4<sup>+</sup> T cells can phosphorylate the key TCR and IL-2 signaling mediators including MAPK, AKT, and STAT5 $\alpha$  in a normal manner, but fail to up-regulate activation markers such as CD69, HLA-DR, and CD25. Apoptosis and IL-2 dependent anergy, the possible mechanisms reported in previous studies, were not observed. Hence, even though Env induced suppression of CD4<sup>+</sup> T cell proliferation has been well established, the mechanism remains to be identified.

### **B-2. Regulatory T cells and chronic infection**

Regulatory T cells (Treg) have been implicated to play a central role in controlling the magnitude of host T cell response to both self- and non-self antigens (15-17). Several types of CD4<sup>+</sup> Treg cells exist, some of which are induced in response to infectious challenge (so-called inducible Treg cells), and some of which are considered naturally occurring regulators and called natural Treg cells (18). Treg cells were thought to be generated both thymically and extrathymically and constitute a small fraction of circulating CD4<sup>+</sup> T cells in the adult human (19, 20), which are commonly identified by expression of high surface levels of IL-2Ra (CD25), as well as expression of the transcription factor FoxP3, as a key control gene in development and

function of Treg cells (21-24). Conventionally, Treg cells were assumed to be involved in regulating autoimmune response based on the early observations that the absence of Treg cells leads to severe autoimmune diseases both in mice (16, 17, 21, 23) and humans (25). However, there is now accumulating evidence that these cells are also actively engaged in negatively controlling immune response to chronic infection (26-31). For example, *Schistosoma* egg-induced Treg cells were shown to not only suppress the development of Th1 cells in mice, but also limit the magnitude of the dominant Th2 response (32). In the intradermal low-dose model of *Leishmania* infection, Treg cells suppress the adaptive immune response that potentially causes collateral tissue damage, leading to life-long low level of infection and the maintenance of memory effector cells (29). In addition, one of the latest studies has demonstrated that the induction of IL-10 producing Treg cells following infection is antigen specific and that this specific recognition is necessary for the function and survival of Treg cells (33). In particular, there have been several investigations focusing on identification of the potential role of Treg cells in chronic HIV infection. One of them demonstrated that HIV infection led to an increased frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells with regulatory activity in peripheral blood (34). CD4<sup>+</sup>CD25<sup>+</sup> T cells from HAART treated HIV<sup>+</sup> patients responded specifically to p24 antigen stimulation by expressing IL-10 and TGF- $\beta$ , two major suppressive cytokine produced by human Treg cells (35). Taken together, all these evidences support the idea that Treg cells, as a host-associated factor, might be involved in modulation of immune response to HIV which causes chronic infections in human.

Nevertheless, many questions remain to be answered regarding Treg cells in the context of HIV infection. First, can virus stimulate the induction of Treg cells in PBMC *in vitro*? If so, which viral gene products are involved in this process? Considering the observations in our studies previously described, is it possible that Env is able to induce Treg cells that, in turn, suppress autologous CD4<sup>+</sup> T cell proliferation? Second, how are the Treg cells induced? Does the virus or its gene products induce Treg cells through direct interaction with TCR, CD4 and/or co-receptors on Treg cells? Or does HIV infection cause disturbance in the cytokine environment that leads to induction of Treg cells? Moreover, the physiological relevance of Treg cells in HIV infected patient remains unclear. Is the frequency or activity of Treg cells associated with the virologic and immunologic parameters of patients such as viral load, level of T cell activation and HIV specific CD4<sup>+</sup> and CD8<sup>+</sup>T cell response? Is this functional impact of Treg cells

beneficial or detrimental to the disease progression and outcome? Therefore, it will be really interesting and useful to investigate in depth the induction of Treg cells by HIV *in vitro* as well as its physiological relevance in patients, since the manipulation of Treg cell function and number has high therapeutic potential.

### C. Preliminary Data

To investigate if Env induces either an increased frequency or an enhanced activity of Treg cells, we first examined the production of Treg cell effector cytokine IL-10 from the anti-CD3 stimulated PBMC exposed to Env. We found that Env induced nearly a doubling in IL-10 production by the stimulated PBMC at Day 1 and Day 2 compared to PBMC unexposed to Env. IL-10 was almost undetectable in un-stimulated PBMC (Figure 1). Importantly, the preliminary experiment for intracellular staining demonstrated that Env induced almost twice many FoxP3+CD4+ T cells to express IL-10 compared to those unexposed to Env (data not shown). These results strongly suggest that Env induces the activation of Treg cells in this co-culture system. Experiments on phenotypic and functional characterization of Treg cells will be described in detail in the following section.

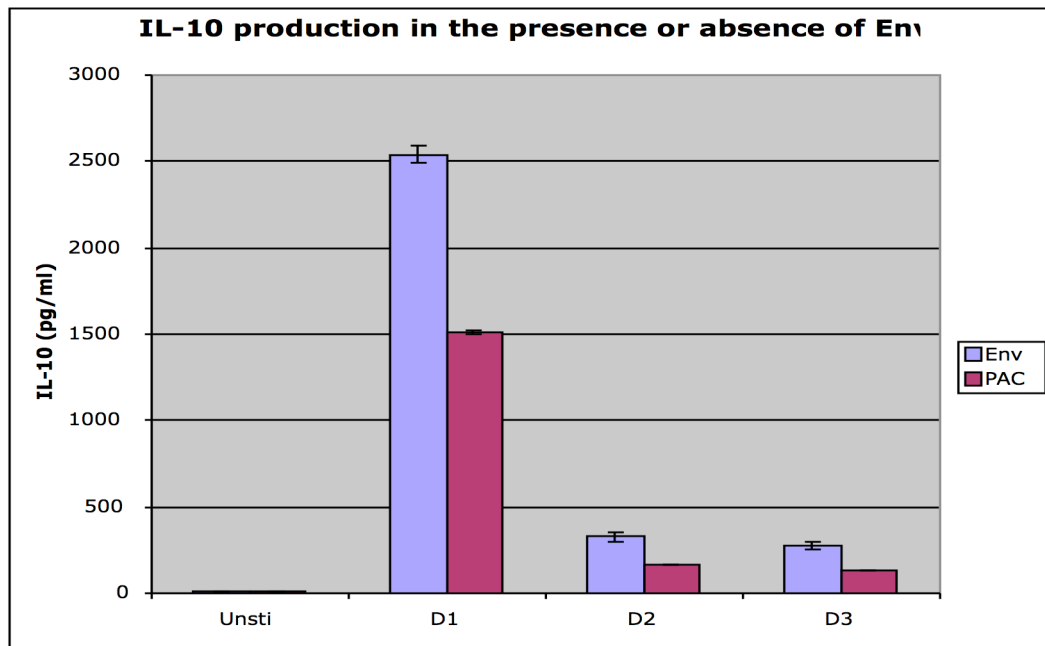


Figure 1. **R5-tropic HIV Envelope glycoprotein induces a significantly increased IL-10 production by the stimulated human PBMC.**  $2 \times 10^6$ /ml PBMC freshly isolated from healthy donor were co-cultured with Env expressing (Blue) or control (purple) 293T cells in the presence

of 0.5ug/ml soluble anti-CD3 and 10ng/ml IL-2. PBMC were harvested after overnight co-culture and re-suspended in fresh complete DMEM. Supernatants were collected at days 1, 2, and 3, respectively, after Env exposure. IL-10 was quantified using ELISA.

#### **D. Experimental Design and Methods**

**Specific Aim 1: To determine if Env induces an increased frequency and/or an enhanced activity of Treg cells and if induced Treg cells suppress CD4+ T cell proliferation *in vitro*.**

##### **Rationale**

As described previously, Env induces suppression of CD4+ T cell proliferation, but does not lead to apoptosis, anergy or cell death, which indicates that some “unknown” suppressive factors exist in the culture. It is known that Treg cells are the potent suppressor of T cell proliferation and can be induced for generation thymically as well as extrathymically. It has been shown that lymph node semi-mature DCs from untreated HIV infected patients are able to induce a large amount of CD4+ T cells to adopt phenotype of Treg cells (34), which is in line with the model that suboptimal activation of T cells is conducive to the induction of Treg cells (36). In our system, Env-suppressed CD4+ T cells can phosphorylate key TCR and IL-2 signaling mediators (STAT5a, MAPK and AKT) in a normal fashion, but fail to up-regulate activation markers such as CD69, HLA-DR and CD25, which is indicative of a suboptimal activation state (Paper submitted). **Thus, I hypothesize that Env exposure causes an increased frequency of Treg cells in PBMC that, in turn, suppress the antigen-stimulated CD4+ T cell proliferation (Appendix: Model 1).** In addition, it is likely that individual Treg cells vary in their functional activity according to activation state and suppressive cytokine secretion. Interestingly, our preliminary data have demonstrated that Env induces a doubling in IL-10 production in the co-culture of Env-expressing 293T and PBMC (Figure 1). **This raises another possibility that Env induces an enhanced activity of Treg cells instead of a higher frequency, or both (Model 2).** In specific aim 1, I will pursue five sub-aims to phenotypically and functionally identify Env-induced Treg cells.

#### **1.1 Establishment of *in vitro* system and T lymphocyte proliferation assay**

In HIV infection, Env present on free virions and infected cells can contact the uninfected CD4+ T cells for dysfunction. To model this *in vivo* interaction of Env and lymphocytes during

an antigen-specific response, an *in vitro* system will be established in which stimulated peripheral blood mononuclear cells (PBMC), as the source of CD4<sup>+</sup> T cells, are co-cultured with Env expressing 293T cells. Env-encoding mRNA will be used to transfect 293T cells for surface expression of functional Env, since mRNA-based transfection has high transfection efficiency (approximately 90%). For delivery to 293T cells, mRNA will be complexed to lipofectin, which is thought to protect mRNA from degradation and successfully deliver mRNA into cells with high efficiency. Fusion assays will be performed to verify the surface expression of Env on 293T cells after mRNA transfection. In each co-culture, 293T cells transfected with Poly-AC RNA will be used as negative control.

Four hours after 293T transfection, PBMC freshly isolated from healthy donors will be prepared for co-culture in the presence of soluble anti-CD3. In response to anti-CD3 stimulation, T cells produce endogenous IL-2 to drive the subsequent proliferation. Considering anti-CD3 causes the strong activation of T cells, exogenous IL-2 will be added into co-culture to sustain the high frequency of T cell proliferation. After overnight co-culture, PBMC will be harvested without disrupting the 293T cell monolayer and resuspended in fresh medium containing IL-2.

There will be two major experiments in each co-culture assay. First, at various time points (Days 1, 2, and 3) after Env exposure, PBMC as well as the supernatants will be collected to phenotypically and functionally identify Env-induced Treg cells, which I will describe in detail later. Second, CD4<sup>+</sup> T cell proliferation will be monitored using CFSE-labeled PBMC to verify the suppressive effect of Env in each co-culture. Briefly, fresh PBMC will be stained with CFSE for 8 minutes followed by co-culture with Env-expressing or control 293T cells in the presence of anti-CD3 and exogenous IL-2. At day 4 after Env exposure, PBMC will be harvested and stained for CD4. Proportion of proliferating CD4<sup>+</sup> T cells will be quantified using flow cytometry based on decreased CFSE intensity.

## 1.2 Phenotypic identification of Treg cells

CD4<sup>+</sup>CD25<sup>hi</sup>FoxP3<sup>+</sup> phenotype of T cells has been commonly utilized to identify Treg cells with suppressive function in human PBMC, which allows enumeration of Treg cells, by virtue of distinction from other CD4<sup>+</sup> T cells. To test the hypothesis that Env induces a higher frequency of Treg cells, flow cytometry will be used to enumerate Treg cells based on CD4, CD25 and intracellular FoxP3 multi-color staining.  $2 \times 10^6$  PBMC collected at various time points will first

be incubated with anti-Fc III/II receptor and normal mouse serum to reduce nonspecific antibody staining. Cells will then be washed and stained for surface markers such as CD4 (anti-CD4-FICT) and CD25 (anti-CD25-PE), followed by fixation, permeabilization, and intracellular FoxP3 staining (anti-FoxP3-APC). All incubations will be carried out on ice for 30 minutes in the dark during which the cell permeability will be maintained. The isotype IgG controls will be used to validate the intracellular staining. PBMC samples simply stained with either anti-CD4-FITC or anti-CD25-PE will be used to provide controls of setting proper compensation during cell acquisition. Unstimulated PBMC will also be prepared for staining to determine the baseline frequency of Treg cells.

Several important parameters will be considered during cell acquisition. First, in order to make statistically significant population frequency measurements, sufficiently large sample size will be acquired (at least 50,000 cells). Second, non-specific cell death will be equal among various PBMC samples, which will be determined by forward and side scatter panel as well as propidium iodine (PI) staining, a dye that binds to dsDNA and is commonly used to identify dead cells that loose membrane integrity. The data will be analyzed using Cell-Quest software. After gating on CD4<sup>+</sup> T cells, CD25<sup>+</sup>FoxP3<sup>+</sup> cells will be enumerated and the frequency will be compared between PBMC samples exposed to Env or not.

### **1.3 Cytokine production by Treg cells**

Similar to other T cells, activation via TCR is required for Treg cells to be functional and individual Treg cells vary in functional potential according to activation state and suppressive cytokine secretion. To test if Env induces an enhanced activity of Treg cells, the cytokine profile of Treg cells will be characterized using ELISA as well as FACS-based multi-color intracellular cytokine staining. First, IL-10 and TGF- $\beta$  in collected supernatants will be measured using quantitative ELISA. For each ELISA, normal Fetal Bovine Serum (FBS) blocking as well as sufficient washing is critical to minimize the background signal. Unlike IL-10, human soluble TGF- $\beta$  is secreted in an inactive form, and acid activation is usually required to trigger the conformational change that leads to recognition by capture and detection antibodies. Hence, before TGF- $\beta$  ELISA, supernatant will be incubated with 1N HCL for 60 minutes followed by neutralization of excess HCL with 1N NaOH. Serial dilutions of recombinant IL-10/TGF- $\beta$  will

be carried out to generate the standard curve. The normal FBS will be used to test background signal.

To determine the cellular source of IL-10 and TGF- $\beta$ , FACS-based multi-color staining for intracellular cytokine and surface markers will be performed. For detection of intracellular cytokines, it is necessary to block the secretion of cytokines with protein transport inhibitors during the last few hours of stimulation. PBMC will, therefore, be incubated for an additional 6 h with Brefeldin A, followed by anti-Fc III/II receptor blocking and surface marker staining (CD4 and CD25). Cells are then washed, fixed, permeabilized, and stained intracellularly for FoxP3 in combination with either anti-human IL-10 or anti-human TGF- $\beta$ . Experimental procedures such as staining, isotype/compensation controls, and cell acquisition will be the same as describe above. After gating on CD4<sup>+</sup> T cells, the frequency of CD25<sup>+</sup>FoxP3<sup>+</sup>IL-10<sup>+</sup> or CD25<sup>+</sup>FoxP3<sup>+</sup>TGF- $\beta$ <sup>+</sup> T cells will be quantified and compared among PBMC samples with or without Env exposure.

#### **1.4 Determine if the induction of Treg cells is Env specific**

Our studies have demonstrated that Env-induced suppression of CD4<sup>+</sup> T cell proliferation is CD4, not co-receptor, dependent, and that soluble CD4 (sCD4) abrogates the suppression (Paper submitted). To determine if Env specifically induces either a higher frequency or an enhanced activity of Treg cells by signaling through CD4 receptor, soluble CD4 (sCD4) will be added into co-culture to block the interaction of Env with membrane-bound CD4 receptor. To provide stronger evidence, 293T cells will be transfected for surface expression of CD4-dependent Env or its counterpart lacking CD4 binding and co-cultured with stimulated PBMC under the same experiment conditions described above. Western-blot will be performed to confirm the equal amount of Env expression. The effect of Env lacking CD4 binding or sCD4 on induction of Treg cells (frequency or cytokine production) as well as CD4<sup>+</sup> T cell proliferation will be characterized.

#### **1.5 Characterization of suppressive activity of Treg cells**

Treg cells can exert their suppressive function through either soluble factors or direct cell contact. To test if Env-induced Treg cells in this co-culture system suppress CD4<sup>+</sup> T cell proliferation, neutralization studies will first be performed in which anti-human IL-10 and/or

anti-human TGF- $\beta$  neutralizing antibodies, or control isotype IgG, will be added into co-culture. Moreover, to provide the direct evidence that Env-induced Treg cells suppress CD4<sup>+</sup> T cell proliferation, experiments with addition and subtraction cultures will be performed. Compared to conventional resting CD4<sup>+</sup> T cells, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells express markedly lower level of IL-7R (CD127) and fail to undergo proliferation in response to lymphopenia (37), which indicates that CD25<sup>hi</sup>CD127<sup>low</sup> is good way of isolating Treg cells. Hence, PBMC harvested from co-culture will be stained for CD4, CD25, and CD127. CD25<sup>hi</sup>CD127<sup>low</sup> T cell fraction will be removed from PBMC using FACS sorting. PBMC with or without Treg cells will be co-cultured with Env-expressing 293T cells in the presence of anti-CD3 and IL-2, and proliferation will be monitored as described above.

### **Expected results**

Treg cells can be induced from peripheral conventional CD4<sup>+</sup> T cells upon suboptimal activation. Thus in our system, anti-CD3 stimulated PBMC (lack of anti-CD28 co-stimulation), whether they are exposed to Env or not, should contain a high frequency of CD25<sup>+</sup>FoxP3<sup>+</sup> T cells as compared to un-stimulated PBMC. I expect that Env will have a synergistic effect with anti-CD3 to induce an even higher frequency of CD25<sup>+</sup>FoxP3<sup>+</sup> T cells in PBMC. It is possible that no difference in frequency of CD25<sup>+</sup>FoxP3<sup>+</sup> T cells will be observed between PBMC with or without Env exposure, which might indicate that Env induces an enhanced activity of Treg cells instead of a higher frequency. At the other extreme, if Env induces a lower frequency of CD25<sup>+</sup>FoxP3<sup>+</sup> T cells, I would argue that Treg cells, similar to other CD4<sup>+</sup> T cells, are targeted by Env for infection or suppression.

Preliminary experiments on the cytokine profile of co-culture have demonstrated that Env exposure induces a doubling in IL-10 production. Similarly, I expect that Env will induce an increased production of TGF- $\beta$ . No increase in TGF- $\beta$  may also be possible since the capacity of Treg cells to produce TGF- $\beta$  varies in different culture system and IL-10 is sufficient to cause suppression without TGF- $\beta$ . In human PBMC, both monocytes/macrophages and Treg cells can be the cellular sources of IL-10. Considering the big difference in IL-10 production before and after anti-CD3 stimulation (from undetectable to more than 2500 pg/ml), in combination with the preliminary observation that the majority of IL-10 was produced by T lymphocytes, I expect that in our system the major cellular source of IL-10 would be CD25<sup>+</sup>FoxP3<sup>+</sup> T cells. In addition, it

has been proposed that mature non-regulatory CD25<sup>-</sup> T cells in peripheral blood are induced to produce IL-10 after co-culturing with CD25<sup>+</sup> Treg cells, which is referred as “infectious tolerance” (38, 39). Therefore, it is possible that CD4<sup>+</sup>CD25<sup>-</sup> T cells produce some IL-10 and contribute in part to the IL-10 in the supernatants. The current data regarding TGF- $\beta$  production by Treg cells and its role in suppression remain conflicting. Therefore, in our system Env induces either increased production of TGF- $\beta$  by CD25<sup>+</sup>FoxP3<sup>+</sup> T cells or not, both of which are possible.

Since our studies have shown that Env-induced suppression is CD4, not co-receptor dependent, I expect that either transfection with Env lacking CD4 binding or addition of sCD4 will abrogate the induction of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells, resulting in decreased IL-10 and/or TGF- $\beta$  production and subsequent loss of the suppression of T cell proliferation. If not, Env might induce Treg cells in a pathway other than signaling through CD4 receptor. One of the plausible explanations is that Env causes alterations in the cytokine environment of the co-culture, which favors the induction of Treg cells. Finally, neutralization antibodies should abrogate the suppression of proliferation. If the suppression is cytokine-independent, the depletion of Treg cells from PBMC should circumvent suppression.

### **Limitations and Alternatives**

FoxP3 expression is essential for the development and function of Treg cells. In addition to FACS-based intracellular FoxP3 staining, RT-PCR to measure the FoxP3 expression from mRNA level will be a reasonable alternate. In brief, total RNA will be extracted from purified CD4<sup>+</sup> T cells, followed by reverse-transcription for cDNA synthesis. Real-time PCR will be established to amplify and quantify the FoxP3 mRNA, which will be normalized to CD4 or GADPH.

Currently, one of the biggest challenges for Treg cell research is that the specificity of the putative markers remains not entirely clear. Several markers utilized to identify Treg cells are also upregulated on recently activated T cells, and therefore any current immunophenotyping strategies for Treg cells have limitations. In addition to CD25 and FoxP3, Treg cells express markers such as GITR and CTLA-4, and it has been reported that FoxP3, in some cases, correlate with CTLA-4. In this regard, to best distinguish Treg cells from recently activated T cells, the supplementary experiments can be performed in which purified CD4<sup>+</sup> T cells from the

same co-cultures will be stained for CD25, FoxP3 plus candidate molecules like CTLA-4 or GITR to determine the degree to which these candidate molecules co-express with CD25<sup>hi</sup> and FoxP3. *In vitro* suppressive function assay will also be performed to verify which phenotype of cell population is most suppressive.

Finally, it has been reported that murine CD4<sup>+</sup>CD25<sup>+</sup> T cells show enhanced cytokine mRNA synthesis after activation instead of producing detectable amounts of cytokines. Therefore, an alternate experiment to determine IL-10 and/or TGF- $\beta$  expression by Treg cells in response to Env will be to test cytokine mRNA using RT-PCR.

## **Specific Aim 2: To characterize the physiological relevance of Treg cells in patients with chronic HIV infection.**

### **Rationale**

The physiological relevance of Treg cells in HIV infected patient remains unclear. In HIV infection a strong antigen-specific immune response appears to be beneficial in controlling viral replication (40, 41). However, the heightened pro-inflammatory response can also be harmful, which leads to activation-induced CD4<sup>+</sup> T cell depletion and progressive immunodeficiency possibly as the consequence of either immunologic exhaustion or inflammation-mediated damage to lymphoid tissue (42). It has been proposed that CD4<sup>+</sup> T cell loss during chronic phase of SIV/HIV infection is more directly related to the overall immune activation than the rate of virus replication. Considering the potent anti-inflammatory activity, Treg cells might act to balance the effector T cell response between active expansion and exhaustion. **Therefore, my overall hypothesis for specific aim 2 is that either an increased frequency or an enhanced activity of Treg cells following HIV infection is associated with the low level of T cell activation and the reduced antigen-specific T cell response in HIV infected patients.** I will pursue three sub-aims to test this hypothesis (*Rationales for each sub-hypothesis will be described in detail later*). PBMC from patients with various viral loads will be collected and cryopreserved for analysis.

### **2.1. Association between viral loads and Treg cells**

HIV-infected patients show various capacities to control viremia, and the viral loads can range from less than 100 copies/ml (Virologic elite controllers) to more than 100,000 copies/ml

(Virologic responders). The difference in capacity to control viremia reflects, to some extent, the variation of immunologic state of patients to HIV, in which Treg cells might play an important role. In addition, persistent pathogens are thought to promote the activation and expansion of antigen-specific Treg cells *in vivo*. **Hence, in accordance with specific aim 1, I propose that increased viral loads (increased Env expression *in vivo*) correlate with either an increased frequency or an enhanced activity of Treg cells in HIV infected patients.** PBMC and plasma samples will be obtained from 30 HIV-infected adults with viral loads ranging from less than 100 copies to more than 100,000 copies/ml as well as 15 HIV-uninfected adult controls. All the patients and non-infected individuals will have no history of, or currently, receiving any immune therapies, and no associated opportunistic infection and cancer. Viral load will be determined as plasma viral mRNA copies using RT-PCR. In brief, viral mRNA extracted from plasma will be subject to reverse transcription for cDNA synthesis followed by real time PCR amplification with HIV-1 *gag*-specific primers. In addition, the assays described in specific aim 1 will be used to measure the frequency and IL-10/TGF- $\beta$  production of Treg cells in PBMC from patients with given viral loads. The association between viral loads and Treg cells will be characterized using linear regression analysis.

## **2.2. Association between Treg cells and T cell activation**

HIV infection and replication occur preferentially in activated T cells. The pro-inflammatory effect of HIV infection causes a heightened level of immune activation, which has been shown to inhibit or reduce the capacity of adaptive immune response to function efficiently. It has been proposed that T cell activation may be the proximal event resulting in progressive immunodeficiency and CD4<sup>+</sup> T cell loss (43-45). **I hypothesize that during HIV infection, a higher frequency or activity of Treg cells is associated with a lower level of CD4<sup>+</sup> T cell activation.** Immune cell activation markers studied have included HLA-DR, CD25, and CD38. Of these, only CD38 has to date shown prognostic significance for HIV infection independent of that based on CD4<sup>+</sup> T cell counts. Upon activation, both CD4 and CD8 T cells up-regulate CD38. Therefore, PBMC from patients with a given frequency of Treg cells, which has been quantiated in aim 2.1, will be stained for CD4, CD8 and CD38, respectively. The mean fluorescence intensity (MFI) of CD38 staining on CD4<sup>+</sup> and CD8<sup>+</sup> T cells will determined as the readout for the activation level of T lymphocytes. Experimental procedures such as wash,

antibody staining, control, and FACS analysis will be carried out as described previously. The association between Treg cells and T cell activation will be analyzed using linear regression.

### **2.3. Functional impact of Treg cells on HIV-specific immune response**

In certain chronic infections, Treg cells have been proposed to suppress the antigen specific immune response to allow a low level of pathogen persistence *in vivo*, which is favorable for the maintenance of long-term T cell memory (29). In HIV infection, the emergence of virus-specific adaptive immune response after acute phase limits viral replication, but can not clear infection, resulting in a latent phase during which a low level of virus persistence exists. **Therefore, I hypothesize that Treg cells suppress antigen-specific CD4+ and CD8+ T cell responses in the context of chronic HIV infection.** The *in vivo* functional impact of Treg cells on antigen-specific immune response has been investigated in mice using antibody to deplete CD25+ Treg cells. In contrast to the mouse model, the anti-macaque CD25 that efficiently targets Treg cells for depletion in macaque remains unavailable. Therefore, functional impact of Treg cells on HIV-specific immune response will be tested *ex vivo*. PBMC with or without Treg cells (FACS sorting CD4+CD25<sub>hi</sub>CD127<sub>low</sub> as previously described) will be stimulated with a pool of Gag peptides (spanning the entire p55 Gag sequence) or the control peptides. Un-stimulated cells and super-antigen (TSST-1) stimulated cells will be used as negative and positive control, respectively. Stimulated cells will be incubated with in Brefeldin A for 6 hours before staining. Cells will then be fixed and permeabilized followed by incubation with corresponding anti-cytokine antibodies. The proportion of Gag-specific CD4+ and CD8+ T cells that express IL-2, IFN- $\gamma$ , IL-4, IL-10, or TNF- $\alpha$  will be quantified using FACS. The statistical difference between PBMC with or without Treg cells will be analyzed using one-way t test.

### **Expected Results**

Our *in vitro* studies have demonstrated that Env induces an increased IL-10 production. In addition, it has been shown that Treg cells undergo antigen-specific expansion *in vivo* in response to infection. Thus, I expect that patients with higher viral loads (higher Env expression *in vivo*) will have either a higher number or an increased cytokine production of Treg cells. If there no is apparent correlation between viral load and Treg cells, I will consider increasing the subject size. If viral load inversely correlates with Treg cells, the possible explanation is that

Treg cells, which have been shown to express CCR5, are the viral targets for infection and/or dysfunction, eventually resulting in Treg cell loss. Since Treg cells are potent suppressor of inflammatory response, a higher frequency or activity of Treg cells will be expected to be associated with a lower level of CD38 expression on both CD4+ and CD8+ T cells.

For the functional impact of Treg cells on HIV-specific T cell response, I expect that Treg cells in PBMC will cause a decrease in the proportion of gag-specific CD4+IL-2+IFN-r+ and CD8+IFN-r+ T cells. If super-antigen stimulated T cell proliferation is also suppressed, Treg cells might work in an antigen non-specific manner. Finally, if no apparent correlation between Treg cells and HIV-specific T cell response is found, two possible explanations can be proposed: 1) the factors that affect HIV-specific T cell response are highly complex during infection, and this complexity masks any impact that Tregs may have on this process *ex vivo*; 2) IFN-r production in response to HIV peptides may not be the best measure to assess Treg-mediated suppression of effector T cells, and I will propose an alternative system to test the gag-specific CD8+ T cell response by measuring Perforin-expressing CD8+ T cell proliferation and CTL activity.

### **Limitations and Alternative Approaches:**

Studies on *Leishmania* infected mice showed that parasite-specific Treg cells expand *in vivo* in response to infection and accumulate at the site of infection (45). In HIV infection, the majority of viral replication occurs in lymphoid tissues, which makes it possible that a fraction of Treg cells migrate to tissue lymphoid node where they exert site-localized immunosuppression. In this regard, in addition to PBMC, regional lymphoid tissues might be another appropriate compartment in which to accurately assess HIV-specific Treg cell frequency and activity. Therefore, lymphoid tissues will be collected from patients and stained for markers (CD25 and FoxP3), and the frequency of CD25+FoxP3+ will be measured using immunohistochemistry.

Depletion of Treg from PBMC offers a straightforward way of testing functional impact of Treg cells on HIV-specific immune response. However, this system has limitations in regard to recapitulating the *in vivo* interactions among Treg cells, CD4+ T cells, and CD8+ T cells during an antigen specific response. In HIV infection, the most potent protective immune response is CTL activity of effector CD8+ T cells in response to antigen stimulation plus helper signals from CD4+ T cells. Accordingly, to best model the *in vivo* interactions among these immune cells, the

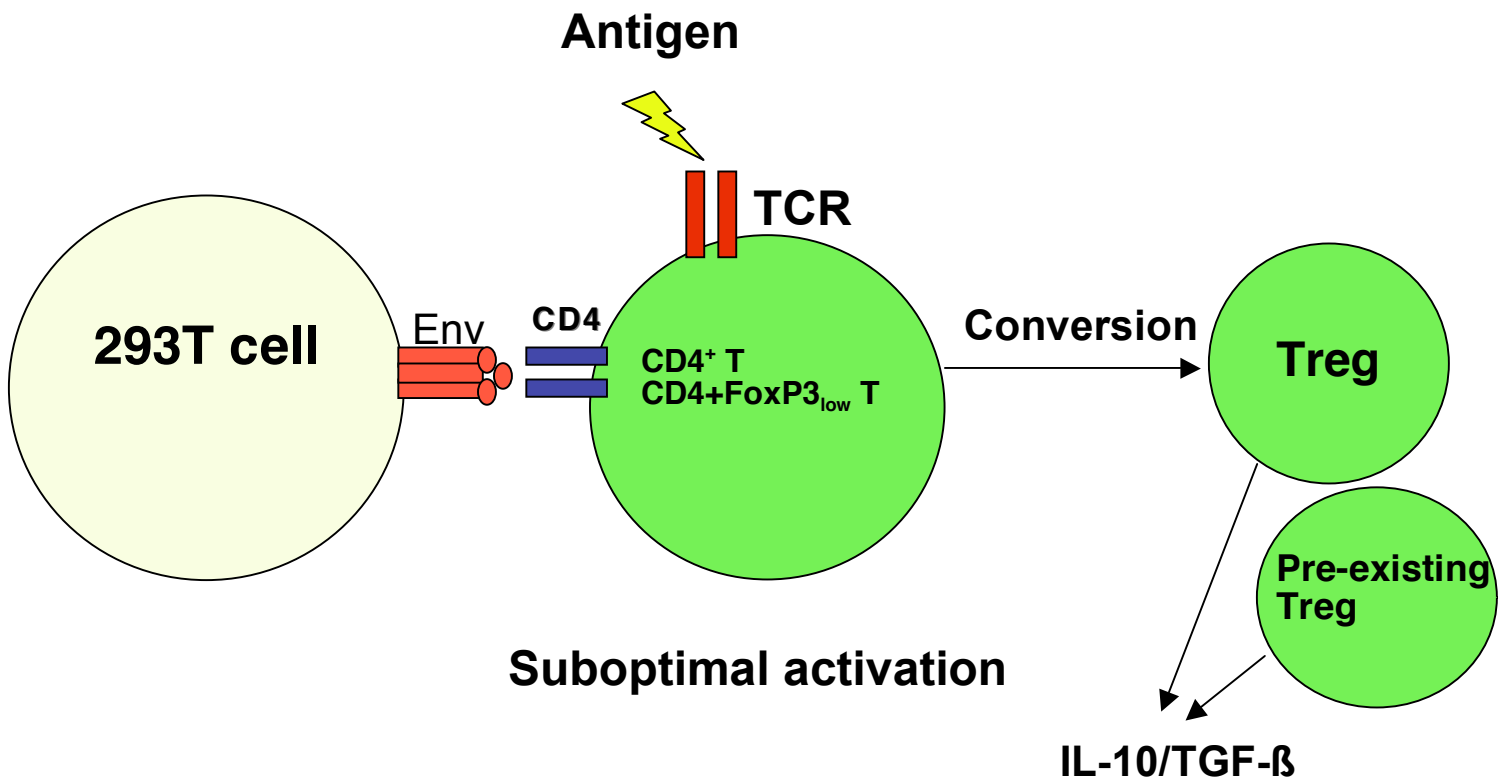
*ex vivo* suppression assay will be performed, in which sorted CD25<sup>+</sup>CD127<sup>low</sup> Treg cells, CD4<sup>+</sup> T cells and CFSE-labeled CD8<sup>+</sup> T cells will be co-cultured at various ratio in the presence of Gag-peptide pulsed allogeneic APCs. The CTL activity as well as proliferation of CD8<sup>+</sup> T cells will be measured. Ideally, CD25<sup>+</sup>CD127<sup>low</sup> Treg cells will suppress CTL activity and proliferation of CD8<sup>+</sup> T cells in a dose-dependent manner.

### **Summary and future directions**

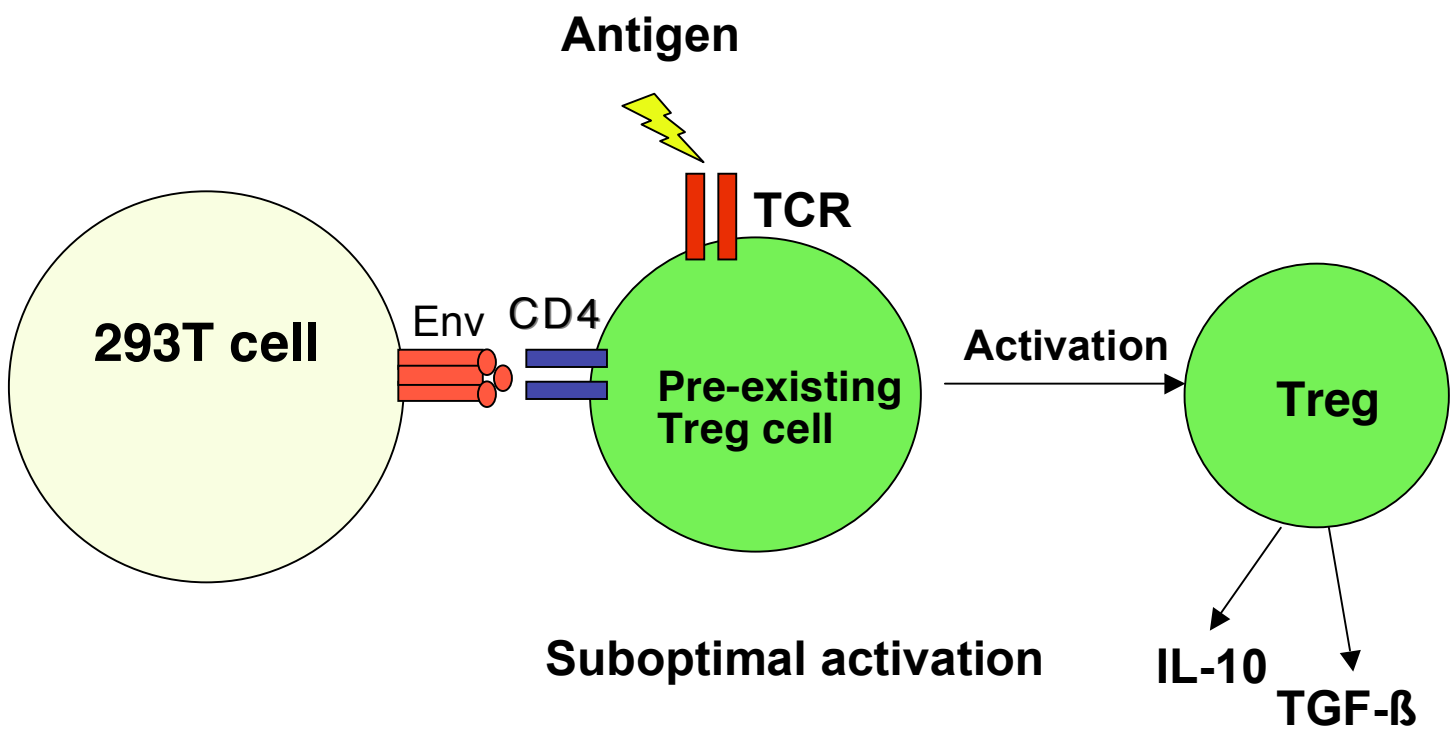
My proposal is to systemically characterize the induction of Treg cells by HIV Env *in vitro* as well as its physiological relevance in HIV infected patients. If the hypothesis is eventually proved to be correct, it will show the evidence that a HIV gene product, Env, induces Regulatory T cells directly, which helps understanding the molecular mechanism of the *in vivo* induction of HIV-specific Treg cells. However, my proposal cannot explicitly answer the question whether the induced Treg cells are beneficial or detrimental to HIV disease outcome. Therefore, the future studies will be centered on prognostic significance and therapeutic potential of Treg cells in HIV infection. The topics regarding the origins, immunophenotyping strategy, and the suppression mechanisms of the Treg cells are very interesting as well.

## Appendix

### Model 1: Env induces increased frequency of Treg cells in PBMC



**Model 2: Env induces enhanced activity of Treg cells in PBMC**



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