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Acquired immune deficiency syndrome (AIDS), caused by Human Immunodeficiency Virus (HIV) is the most devastating global health problem. Long Term Non Progressors (LTNP) are seropositive individuals that have delayed progression to AIDS. Potent anti-HIV $CD8^+$ T cell responses are associated with LTNP's; therefore, an effective cytotoxic T cell (CTL) response is vital for the immune system to control HIV. Human leukocyte antigen (HLA) B14 and HLA B27 types are commonly present in LTNP's.

2B4 (CD244) is expressed on NK cells, monocytes, basophils, eosinophils, and a small population of $CD8^+$ T cells. Although the expression of $2B4^+CD8^+$ T cells has been shown to increase during HIV disease progression, the role of $2B4^+$ $CD8^+$ T cells has not been established. Studying the differences in activation and cytotoxic activity of $2B4^+$ and $2B4^-$ $CD8^+$ T cells will expand our knowledge of this receptor in T cells, which has not been fully addressed in the past. HIV infected patients could benefit from immunotherapy efforts using the knowledge we gain from 2B4 studies in T cells. In addition, the information gathered from this study can be used for *in vivo* studies of this receptor in natural HIV infection.

The immunomodulatory effects of Corticotrophin releasing hormone and Epinephrine on $2B4^-$ and $2B4^+$ $CD8^+$ T cells during activation and the effects on

cytotoxicity have not been published to date. Knowledge about their effects will allow us to learn the function of these hormones that are released due to stress during immune regulation in HIV disease.

FUNCTIONAL ROLE OF $2B4^+$ $CD8^+$ T CELLS AGAINST HIV EPITOPES

THESIS

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Acquired Immune Deficiency Syndrome…...……...(AIDS)

Ankylosing Spondylitis...……..…….(AS)

Antigen Presenting Cells……..……(APC)

β_2-Microglobulin……..(β_2M)

B Lymphoblastoid Cell Lines……(BLCL)

Brefeldin A……..(BFA)

Central Nervous System..……(CNS)

Corticotropin-Releasing Hormone……..(CRH)

Cytotoxic T cell……...(CTL)

Epinephrine……...(EPI)

Exposed Uninfected……...(EU)

Group-Specific Antigen…….(gag)

Highly Active Anti Retroviral Therapy……...(HAART)

Human Immunodeficiency Virus…….(HIV)

Human Leukocyte Antigen…….(HLA)

Interferon-gamma Enzyme Linked ImmunoSorbent Assay…….(IFN-γ ELISA)

Long Term Non Progressors…….(LTNP)

Magnetic Activated Cell Sorting…….(MACS)

Major Histocompatibility Complex……(MHC)

Mature Dendritic Cells……...(mDC)

Mean Fluorescence Intensity……(MFI)

Monocyte Conditioned Media……...(MCM)

Natural Killer…….(NK)

Peripheral Blood Mononuclear Cells…….(PBMCs)

Signaling Lymphocytic Activation Molecule……..(SLAM)

SLAM Adaptor Protein……..(SAP)

Sodium Chromate…….(^{51}Cr)

CHAPTER I

INTRODUCTION

1.1 Human Immunodeficiency Virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS)

HIV infection is one of the most destructive pandemics in recorded history. About 5 million new HIV cases occur worldwide each year. In 2007, it was reported that 6800 people became infected with HIV and over 5700 people died of AIDS each day (UNAIDS, 2007).

HIV possesses many genes that allow it to infect, evade and replicate in a host. The group-specific antigen (gag) gene in HIV codes for many structural proteins needed for the viron itself. Specifically we are interested in the p17 and p24 gag region which makes up the protective matrix and viral capsid respectively. It is a highly conserved region of the viral genome which means that mutations occurring in this region are rare. This region is important because it was previously shown to activate a more potent $CD8^+$ T cell response towards HIV (Johnson et al. 1991).

HIV infects $CD4^+$ T cells, monocytes and macrophages through interactions with the CD4 molecule and other co receptors. HIV is a retrovirus, so once inside the cell it uses reverse transcriptase to convert its RNA genome into double stranded DNA. This DNA then enters the nucleus and becomes integrated into the genome of the host cell,

thus creating the provirus. This provirus remains latent until the cell is activated by the immune system, after which the virus is able to exploit the host cell's processes to create the proteins necessary to replicate and multiply. Infected individuals generate about one to ten billion virus particles per day (O'Brian et al. 2004). HIV eventually causes AIDS by depleting the $CD4^+$ T cell population, thus weakening the immune system and allowing opportunistic infections to occur. Since it was first recognized on December 1, 1981, AIDS has claimed more than 25 million lives (UNAIDS, 2007).

1.2 Current Treatments and Failures in HIV Vaccine Creation

Currently the most effective treatment for HIV/AIDS is Highly Active Anti Retroviral Therapy (HAART). These HAART drugs are a combination of 3 or more antiretrovirals. They usually consist of reverse transcriptase inhibitors that will inhibit the replication of the virus, or protease inhibitors that inhibit the protease activity that is needed by the virus during replication (O'Brian et al. 2004). This combination of drugs only slows the course of the disease, meaning that eventually the disease will progress to AIDS. There is no vaccine or cure for HIV/AIDS, infection with HIV being 90% fatal (O'Brian et al. 2004).

Over 20 years of research has yielded no successful vaccines. There are several problems with vaccine creation. First, HIV uses error prone reverse transcriptase in its replication cycle, this leads to a high mutation rate. This actually benefits the virus by helping it stay one step ahead of the immune responses towards it. Secondly, one

infected individual can have more than one strain of HIV detected in their body, thus confusing the immune responses. Another major problem is HIV infects $CD4^+$ T cells and this results in the destruction of any memory $CD4^+$ T cells that were induced by a successful vaccine. Without functional memory $CD4^+$ T cell secreting cytokines the memory $CD8^+$ T cells may lose their cytotoxic function (O'Brian et al. 2004). A $CD8^+$ T cell response is essential in preventing the virus from taking over the immune system and controlling the progression to AIDS.

1.3 Long Term Non Progressors and Human Leukocyte Antigen B27

An effective cytotoxic T cell (CTL) response is essential for the immune system to control HIV (Schneidewind et al. 2008, Uyl et al. 2004). There are two groups of individuals that have a unique relation to HIV and AIDS progression. One of these groups comprises of Exposed Uninfected (EU) individuals, who remain seronegative regardless of repeated exposures to HIV. The second group contains seropositive individuals that are capable of containing HIV infection and delaying the progression to AIDS (Imami et al. 2002). Many studies have shown that a potent anti-HIV $CD8^+$ T cell response is connected with these two groups (Schneidewind et al. 2008, Uyl et al. 2004).

Human Leukocyte Antigen (HLA) is the Major Histocompatibility Complex (MHC) in humans. It consists of MHC class I and class II antigen presenting receptors

that are expressed on a variety of cells, including immune cells. In particular HLA class I receptors are classified as A, B or C and present foreign antigens from the cytoplasm. HIV is an intracellular pathogen, therefore its proteins are frequently presented on HLA class I.

HLA B27 is a type of class I molecule that has a role in delayed HIV progression (Uyl et al. 2004). Many LTNP's possess HLA B27 and several studies have shown that individuals that express it have an overall low severity of disease during viral infection. Although HLA B27 is more commonly known for its association with autoimmune diseases, such as ankylosing spondylitis (AS), it has been studied extensively in the course of HIV disease and progression to AIDS. In autoimmune disorders, it has been hypothesized that HLA B27 is the source of autoimmunity and AS is the cause of molecular mimicry (Khan et al. 2007). While many hypotheses have been proposed for this reaction, the most recognized is connected to *Klebsiella pneumoniae* (Ebringer et al. 2007). With respect to HLA B27 and its association with the course of HIV infection, it involves high affinity binding of conserved regions of HIV epitopes, more efficient presentation and thus activation of $CD8^+$ T cells. In other words, HLA B27 is able to present epitopes of the virus that are shared among different strains and present it with complex stability (Brooks et al. 1998), as well as cause heightened activation of killer T cells that can kill more viral particles, allowing the immune systems of infected individuals to keep up with viral replication for long periods of time although they cannot completely rid their bodies of the virus.

1.4 2B4 (CD244) Receptor and its role in HIV

2B4 (CD244) is expressed on all natural killer (NK) cells, basophils, eosinophils, monocytes and some populations of $CD8^+$ T cells. In human NK cells, this receptor usually acts as an activator of cytotoxic function and IFN-γ secretion, although it has been found to act as an inhibitory receptor when expressed at high levels, mainly due to the amount of receptor-ligand interactions and signaling molecules available to cause the activation or inhibition (Chlewicki et al. 2008). This phenomenon has not been shown in relation to 2B4 expression on T cells. CD48 is the physiological ligand for 2B4 and its interaction causes cell proliferation when induced between NK cells or with nearby T cells (Chlewicki et al. 2008). Uniquely, 2B4 expressing NK cells or T cells do not need recognition of MHC-pathogen peptide complexes to induce activation of killing (Mathew et al. 1993).

SLAM adaptor protein (SAP) is an intracellular protein expressed in NK and T cells. It is believed to be part of the intracellular signaling that leads to the activation of 2B4. After 2B4 interacts with CD48 on the extracellular surface of the NK cell or T cell and the target cell, phosphorylation of the tyrosine-based motif in the cytoplasmic tail occurs and the SAP and Src family kinase Fyn become recruited intracellularly (Assarsson et al. 2005). To highlight the importance of the SAP molecule, many studies have looked at the immunological reactions in X-linked lymphoproliferative patients. People that are affected by this disease have a mutated SAP gene which in turn causes an absence or dysfunctional form of this protein (Assarsson et al. 2005). In these patients,

2B4 and CD48 are present, but upon binding of these receptors there is no functional SAP molecule to start the intracellular cascade. Through these studies it is apparent that this signaling molecule is important for the activating function of 2B4.

$2B4^+$ $CD8^+$ T cells represent a population of activated or memory T cells (Altvater et al. 2009), usually about 50% or less of the total amount of the whole $CD8^+$ T cell population (Assarsson et al. 2005). Rey et al (2006) concluded that the acquirement of 2B4 on $CD8^+$ T cells, along with CD160, is a marker of highly cytotoxic T lymphocytes. Little is known about this receptor's functional role in HIV and the progression to AIDS. Interestingly the levels of $2B4^+CD8^+$ T cells have been shown to increase during the course of HIV disease (Peritt et al. 1999), while the expression of the CD48 ligand is downregulated in HIV infected cells (Ward et al. 2007). During HIV infection, NK cells have been shown to upregulate 2B4 (Ostrowski et al. 2005), but have lowered killing activity of HIV infected cells, despite the infected cell downregulation of MHC molecules that leave them susceptible to NK cell killing. This drop in killing may be due to CD48 downregulation (Ward et al. 2007) and can possibly play a role in T cell killing activity as well.

1.5 Corticotropin-Releasing Hormone (CRH) and Epinephrine (EPI): Role in HIV

The central nervous system (CNS) and immune system have the ability to regulate each other. The CNS can alter immune responses by using hormones (Webster et al. 2002). Corticotropin-Releasing Hormone (CRH) is normally secreted by the

hypothalamus in response to stress. Intriguingly, it has also been shown that T lymphocytes can also produce and secrete this hormone (Ekman et al. 1993), as well as express CRH receptors (Baigent et al. 2000). The function of this hormone in T cells and the immune system has not yet been fully described, but the fact that it is secreted by these immune cells could possibly mean that it has a role in stress and immune regulation.

Epinephrine (EPI) is released by the adrenal glands in response to stress also. It is very similar to Norepinephrine in its stress inducing "fight or flight" responses, and it is actually synthesized from Norepinephrine. Epinephrine suppresses the immune system during times of stress and causes heart rate to increase along with digestion to decrease, which is why it is commonly used to treat anaphylaxis.

Stress and depression play a key role in HIV disease progression to AIDS. It can even affect the decline in $CD4^+$ T cell counts during progression of HIV (Leserman et al. 2003) which will eventually lead to the development of AIDS. There have been reports that two or more stressful life events is associated with a three-fold increase risk of $CD4^+$ percent decline in HIV infected patients (Leserman et al. 2003). Depression could be due to hormone imbalances, and CRH or EPI could have a role in this. If found to have a role, these hormones could be a useful focus in therapy to slow the $CD4^+$ T cells count decline and increase the survival of patients with HIV.

1.6 Significance

HIV infection is regulated by many different types of immune responses and can be affected by many different factors. Among these immunomodulatory factors, we are interested in studying genes that limit viral progression, the role of $2B4^+$ $CD8^+$ T cells and the affects of stress hormones in HIV progression.

HLA B27 has been shown to be present in long term non progressors, individuals that have slowed progression of HIV to AIDS as compared to individuals that do not possess these alleles (Uyl et al. 2004). It has been suggested that the resistance caused by these MHC class I subtypes are due to a strong CTL response against the p24 gag proteins (Schneidewind et al. 2008). By taking advantage of this population's ability to combat the virus we can expand our treatment methods.

Studying the differences in activation and cytotoxic activity of $2B4^+$ and $2B4^-$ $CD8^+$ T cells will expand our knowledge of this receptor in T cells, which has not been fully addressed in the past. HIV infected patients could benefit from immunotherapy efforts using the knowledge we gain from 2B4 studies in T cells. For instance, if found that the 2B4 receptor in $CD8^+$ T cells represents the population of T cells that can either effectively kill more virus particles or hinder killing of infected cells, we can use this knowledge to try to induce upregulation or downregulation of this receptor in HIV seropositive individuals in an attempt to extend HIV patient's lives. In addition, the

information gathered from this study can be used for *in vivo* studies in natural HIV infection.

The immunomodulatory effects of CRH and EPI on $2B4^-$ and $2B4^+$ $CD8^+$ T cells during activation and the effects on cytotoxicity have not been published to date. Knowledge about their effects will allow us to learn the function of these hormones during immune regulation in HIV disease.

The central hypothesis for this study is multiple HLA alleles have the ability to recognize specific constrained HIV antigens that can stimulate an effective CTL response regulated by the expression of 2B4 and influenced by the presence of neuroendocrine hormones.

CHAPTER II

MATERIALS AND METHODS

2.1 Identification of HLA B27 Supertype Donors and Cell Lines

Initial screening of subjects was done by obtaining buccal swabs from 40 healthy volunteers from different racial/ethnic groups, for analyzing their HLA (human leukocyte antigen) types, with prior approval from UNTHSC IRB. These samples were HLA typed at Tepnel Lifecodes, Stamford, CT. Four volunteers were identified as having HLA B27 and B14. Blood samples were obtained from these donors and the respective B Lymphocyte Cell Lines (BLCL) were generated at the tissue culture facility at UNC Lineberger Comprehensive Cancer Center. BLCL-1 expresses class I HLA B14 and B44. BLCL-2 expresses class I HLA B14 and B8. BLCL-3 expresses class I HLA B27 and B44. BLCL-4 expresses class I HLA B27 and B8. All cell lines were cultured in RPMI 1640 supplemented with 10%FBS and antibiotic.

2.2 Conserved HIV Peptides

Synthetic HIV peptides were selected using the Los Alamos database. These peptides were chosen based on known affinity for HLA B14 and B27. All peptides are from conserved regions of the HIV protective matrix and capsid structural protein, p17 and p24 group specific antigens (gag) respectively. Synthetic peptides were synthesized by

Celtek Peptides, Nashville, TN. Ten conserved HIV peptides including escape variants and one influenza virus peptide were used and listed in table 1.

2.3 B Lymphoblastoid Cell Line Peptide Binding Assays

BLCL's ($1x10^6$ cells) were treated for 90 seconds with 100µl of cold citrate phosphate buffer (pH2.8, .26M citric acid and .12M sodium phosphate) containing 1%BSA and 3µl/ml β_2-microglobulin (β_2M) for 90 seconds on ice after which 1.5ml of cold sodium phosphate dibasic buffer (pH9.0, .20M sodium phosphate) containing 1%BSA, 3µl/ml β_2M and 10µl/ml peptide was added to neutralize. Some BLCL's remained untreated as controls. The BLCL's were cultured in wells overnight with 3µl/ml β_2M and with or without 100µM peptide in 1 ml AIM V medium at 4˚C. Some wells received Brefeldin A (BFA) to inhibit the cell surface expression of new MHC class I molecules. After incubation, they were harvested and processed for flow cytometric analysis with unconjugated mouse anti-human β_2M antibody (TU99, BD Pharmingen).

2.4 Monocyte induced Differentiation to Dendritic Cells

Whole blood was obtained from the same donors who had previously donated blood to prepare the BLCL, on day 0 and day 10. PBMC's were purified by ficol separation using a histopaque gradient. Monocytes were isolated by magnetic activated cell sorting (MACS) on day 0 using a monocyte isolation kit (Miltenyi Biotec) or by allowing

adherence to a polyethylene plate for 3 hours at 37˚C. These monocyte derived dendritic cells were obtained from whole blood on day 10 by cell adherence as previously described (Zarling et al. 1999). Monocytes were differentiated into dendritic cells by culturing for 10 days in the presence of 800 U/ml recombinant human GM-CSF and 500 U/ml recombinant human IL-4 (R&D systems, Minneapolis, MN). Every 2 days 100 μl of old media was replaced with 300 μl of new media and cytokines added in similar concentrations. Monocyte conditioned media (MCM) was added for dendritic cell maturation on day 7 at a 50% concentration. MCM was made as previously described (Zarling et al. 1999). Briefly, PBMC's were depleted of T cells by CD3 MACS using a FITC isolation kit (Miltenyi Biotec). Human gamma globulin coated bacterial plates were made just prior to layering of T cell depleted PBMC's by swirling plate for 1 minute and washing once with PBS. Adherence of cells by incubation for 3 hours in 5ml 4+ RPMI with 10% human AB serum and removal of nonadherent cells occurred. Immediately 10ml of the same medium was added and the plate was incubated for no more than 24 hours. Media was isolated via centrifugation and kept in -80˚C until used.

2.5 Dendritic Cell Presentation and $CD8^+$ T Cell Activation

Whole blood was taken on day 10, purified by ficol separation using a histopaque gradient. $CD8^+$ T cells were isolated by MACS using a $CD8^+$ T cell kit (Miltenyi Biotec). Separation into $2B4^-$ and $2B4^+$ $CD8^+$ T cell populations was performed via 2B4

monoclonal antibody (C1.7) and cytopeia influx cell sorter (UNTHSC Flow Cytometry Core Facility). Monocyte-derived matured dendritic cells (mDC) after 10 day incubation were cultured with 50 μg/ml of each peptide and 3μg/ml β_2M in AIM V medium for 2-4 hours at 37˚C. After peptide pulsing, mDC's were treated with 100 μg/ml mitomycin C, immediately washed and resuspended into 4+RPMI with 10% human AB serum added. $CD8^+$ T cells that were resuspended into the same medium were layered onto the DC's with a final volume of 1.5ml. IL-7 (10ng/ml) was added to the cultures on day 1 and IL-2 (10 units/ml) was added on day 2. Some wells received Corticotrophin-Releasing Hormone (CRH) at a concentration of $2x10^{-4}$ or Epinephrine (EPI) at a concentration of 10^{-7} in addition to peptide stimulation. They were cultured for 8 days at 37˚C in a CO_2 incubator. After the first stimulation new monocyte-derived dendritic cells were added in a similar fashion and cultured for another 8 days. If the cells became too dense in the media, 200μl of old media was removed and replaced by 400μl of new media with cytokines already added in the same concentrations as above. After each stimulation 300 μl of supernatant was harvested from each well and an IFN-γ ELISA was performed.

2.6 Cytotoxicity Assay Comparison of $2B4^-$ and $2B4^+$ $CD8^+$ T Cells

After two stimulations with APC's, $CD8^+$ T cells were harvested from each well, counted and similar amounts of each were made, using the least amount in any given well as the limiting amount used for the rest of this experiment. For making target cells, BLCL's

that were discussed in chapter 2.1 were used. The type of BLCL used (1,2, 3 or 4) was correlated with the donor used in all of the previous experiments listed above. Briefly, the BLCL's were pulsed with a concentration of 50μg/ml of each of the individual peptides overnight in AIM-V medium at 37˚C prior to performing this assay. The number of target cells was dependent on the ratios needed for the cytotoxicity assay and the least number of effector $CD8^+$ T cells in any given well after 16 days of coculture with APCs. After pulsing overnight, these BLCL's were harvested separately from each well (each were pulsed with different peptides, although only pulsed with peptides that were the same as used for activation, some were not pulsed with peptides that served as the negative control, resuspended into 4+ RPMI with 10% human AB serum and incubated for 90 minutes at 37˚C with sodium chromate (^{51}Cr) at a concentration of 1/5 chromate to 4/5 medium. After the 90 minute incubation these cells were washed 3 times with 1ml medium and resuspended in RPMI such that the same amount of BLCLs could be distributed in 100μl of medium for each well of a standard 96 well U bottom plate. During the above incubation of BLCLs, effector $CD8^+$ T cells were used to make serial dilutions such that there would be triplicate wells with effector to target ratios of 20:1, 5:1 and 1:1. There were also wells made for each different peptide types with target cells only in medium only (to get a background release of chromium) and in lysis buffer (to give the measurement of the highest lysis possible). After the effector and target cells were distributed into the plates, they were incubated for 4 hours at 37˚C, cells were spun down and supernatants were added to 2ml scintillation cocktail fluid in a standard 7ml

polyethylene scintillation vial with cap (VWR) and run overnight on the scintillation counter. Percent specific lysis was calculated using the equation [(Mean cpm release in experimental sample)-(mean cpm of spontaneous release) / (mean cpm of maximal release)-mean cpm of spontaneous release)] X 100.

CHAPTER III

IDENTIFYING MHC CLASS I STRUCTURALLY CONSTRAINED HIV EPITOPES THAT BIND DIFFERENT HLA ALLELES

This aim essentially begins with establishing the peptide binding assay technique and elucidating the binding affinities of each HIV peptide. Although already established in several publications (Zarling et al. 1999, Tsai et al. 1998, Celis et al. 1994, Van der Berg et al. 1995), this experiment is unique to each cell line and requires meticulous standardization. The basis of this aim is to remove the self peptide from the MHC class I receptor and exogenously add a conserved synthetic HIV peptide (previously designed using the Los Alamos HIV database, see Table 3.2), all of which have known affinity towards HLA B27 or HLA B14. These immortalized B cell lines or B lymphoblastoid cell lines (BLCL's, see Table 3.1) were created from donor blood that was HLA typed to be of the B27 and B14 allele. Another benefit of these experiments is to ascertain structurally constrained epitopes that have not yet been identified as being able to bind these HLA alleles, such as epitopes known to bind to B14 but not shown for B27, or vice versa.

Our lab previously screened 40 HIV negative blood donors and found that four possessed the HLA B27 supertype, having either HLA B27 or HLA B14 class I expression. The blood samples from these donors were sent to the tissue culture facility

at UNC, Chapel Hill, NC for preparing the BLCL's (see Table 3.1). Known HIV epitopes and escape variants were previously chosen from the Los Alamos HIV database and publications based on their structurally constrained nature and ability to bind to HLA B27 superfamily, these were commercially synthesized for this study (Celtek Peptides, Nashville, TN). These epitopes were examined for their abilities to bind to MHC class I, in particular HLA B14 and B27, and form stable complexes.

This technique begins with striping BLCLs with citric acid buffer (pH 2.8) for 90 seconds to remove the self peptide from the MHC class I. A consequence of removing this peptide is the disassociation of β_2-microglobulin (β_2M) from the receptor due to instability (Sugawara et al. 1987). To neutralize the pH, sodium phosphate buffer with HIV peptides and exogenous β_2M was added immediately after stripping. Theoretically, these peptides will have affinity for these HLA types and bind in the binding cleft, and then the β_2M added will also bind and stabilize the complex. Only the HIV epitopes that were shown to bind to these HLA types were used in the subsequent experiments.

RESULTS

The citric acid stripping standardization in figure 3.4 gives an idea of how much self peptide stripping occurs during different stripping times and pH changes. For this particular cell line (BLCL-3) we chose pH 2.8 and 120 second stripping times because it removed a similar percentage of peptide as pH 2.8 at 180 seconds. At pH 2.8 incubation

for 180 seconds a significantly higher number of cells were non viable as compared to the same pH with treatment of 120 seconds.

The flow cytometry peaks represented in figure 3.5a show the negative and positive controls for figure 3.5b. Figure 3.5b, A and B show that BLCL-2 and BLCL-4 have binding affinity towards HIV peptide DE10-1. Previously, DE10-1 was shown to bind to HLA B14 (Jones et al. 2004, Wagner et al. 1999). Although we have shown slight binding to HLA B27, the binding could be explained by the similar peptide structure between HLA B14 and B27. We have also shown binding of BLCL-4 to IK-9 and IY-11 (figure 3.5b, C and D). This finding has been reported by others (Goulder et al. 2001, Gahery-Segard et al. 2003). All of the above figures appear to show only a small amount of binding as compared to the positive control (shown in figure 3.5a). A plausible explanation for this finding is evident when examining figure 3.4. At 120 seconds and pH 2.8 we see that about 50% of the MHC class I molecules are stripped of their self peptide. In addition, we are stripping HLA class I A, B and C types, but only adding back peptides that should theoretically bind to HLA B. If HLA class I A, B and C each make up about 33% of the total MHC class I molecules expressed on a given cell, then for HLA class I B we should hope to get near this percentage for efficient binding.

CELL LINE NAME	HLA ALLELES
BLCL-1	**B14, B44**
BLCL-2	**B14, B8**
BLCL-3	**B27, B44**
BLCL-4	**B27, B8**

Figure 3.1 HIV negative blood donor HLA Alleles used in study

NAME	EPITOPE SEQUENCE	BINDING	REGION OF VIRUS
FL-8	**F L K E K G G L**	**INFLUENZA PEPTIDE**	
DE10-1	**D R F Y K T L R A E**	**B14**	p24 gag 166-174
DE10-2	**D R F Y K I L R A E**	**Escape Variant**	
GL-8	**G H Q A A M Q M L**	**B38, B3901 and B1510**	p24 gag 61-69
GY-9	**G L N K I V R M Y**	**B15 and B62**	p24 gag 137-145
IK-9	**I R L R P G G K K**	**B27**	p17 gag 19-27
IY-11	**I L G L N K I V R M Y**	**B7, presumed to bind B27**	p24 gag 135-145
KK10-1	**K R W I I L G L N K**	**B27**	p24 gag 131-140
KK10-2	**K R W I I M G L N K**	**Escape Variant**	
KK10-3	**K T W I I L G L N K**	**Escape Variant**	
KK10-4	**K T W I I L M G L N K**	**Escape Variant**	

Figure 3.2 HIV conserved epitopes and escape variants used in study

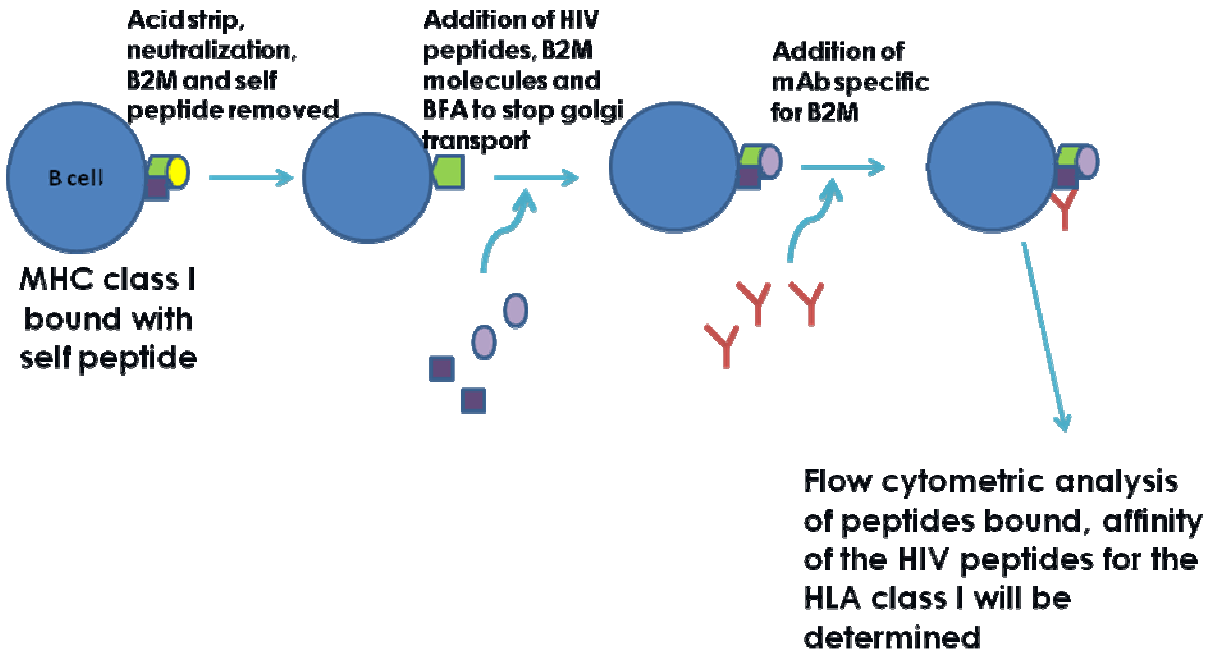

Figure 3.3 Overview of peptide binding assay scheme

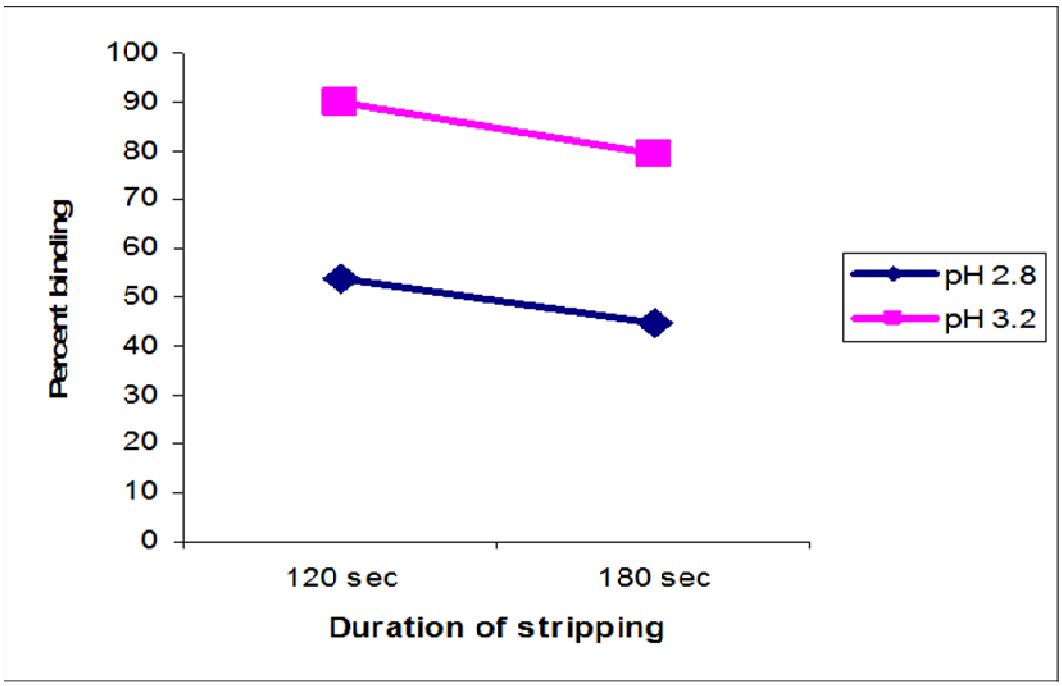

Figure 3.4 Citric Acid MHC class I stripping standardization

BLCL-3 (HLA B27) used to show a comparison of different pH and citric acid stripping times. Percent binding is the amount of self peptide left bound after citric acid treatment. pH is the citric acid solutions pH measurement. Longer stripping time seems more efficient, but can possibly be detrimental to the viability of the cells.

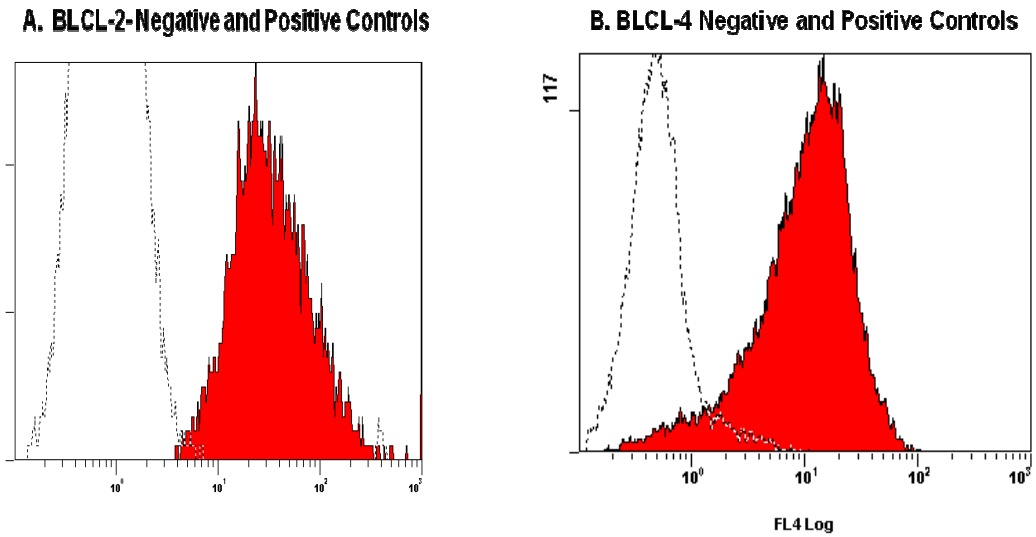

Figure 3.5a BLCL-2 (HLA-B14) and BLCL-4 (HLA-B27) has binding ability to DE10-1, IK-9 and IY-11 HIV peptides.

A. Hollow peak is non-stripped BLCL-2, isotype control. Red peak shows positive control with no stripping and β2m antibody.

B. Hollow peak is non-stripped BLCL-4, isotype control. Red peak shows positive control with no stripping and β2m antibody.

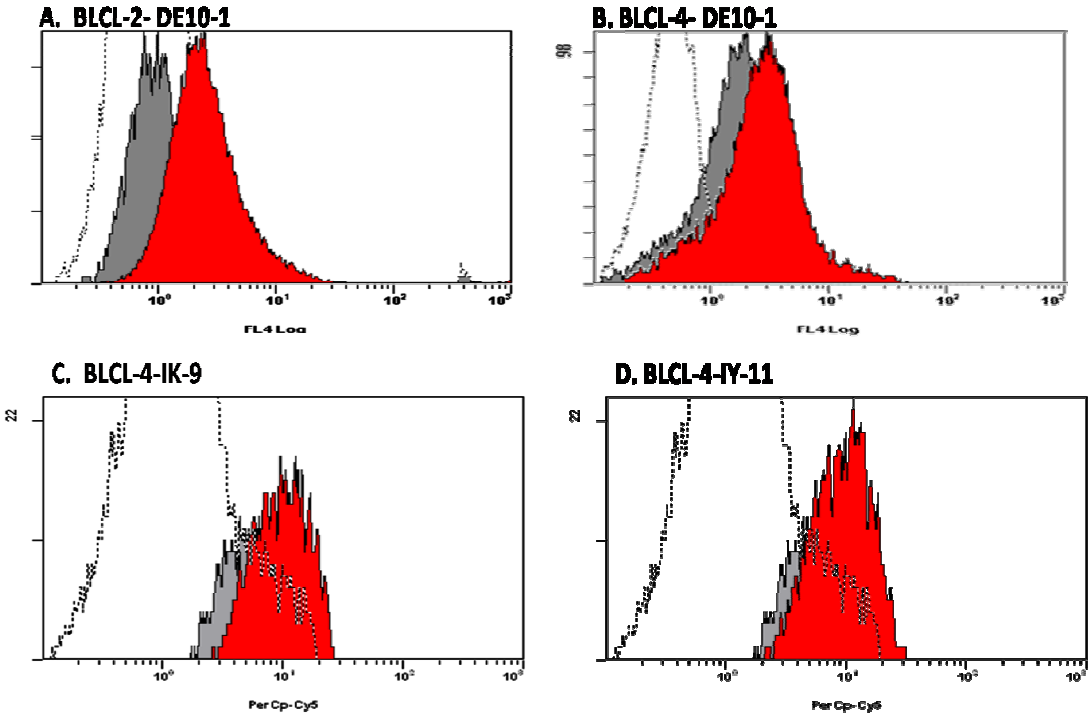

Figure 3.5b BLCL-2 (HLA-B14) and BLCL-4 (HLA-B27) has binding ability to DE10-1, IK-9 and IY-11 HIV peptides.

A. Hollow peak is non-stripped BLCL-2, isotype control. Grey peak is acid stripped BLCLs with β_2m antibody. Red peak shows binding of DE10-1 peptide.

B. Hollow peak is non-stripped BLCL-4, isotype control. Grey peak is acid stripped BLCLs with β_2m antibody. Red peak shows binding of DE10-1 peptide.

C. Hollow peak is non-stripped BLCL-4, isotype control. Grey peak is acid stripped BLCLs with β2m antibody. Red peak shows binding of IK-9 peptide.

D. Hollow peak is non-stripped BLCL-4, isotype control. Grey peak is acid stripped BLCLs with β2m antibody. Red peak shows binding of IY-11 peptide.

CHAPTER IV

EVALUATING THE ABILITY OF STRUCTURALLY CONSTRAINED HIV EPITOPES AND CORRESPONDING ESCAPE VARIANTS TO INDUCE CYTOKINE SECRETION OF $2B4^-$ AND $2B4^+$ $CD8^+$ T CELLS IN THE PRESENCE AND ABSENCE OF NEUROENDOCRINE HORMONES

To establish the differences in $2B4^-$ and $2B4^+$ $CD8^+$ T cell cytokine secretion we primed them with the HIV peptides used in the previous chapter. This required several experiments with culturing to standardize the viability and function of $CD8^+$ T cells for 28 days (Figure 4.1). Briefly, Peripheral blood mononuclear cells (PBMCs) were drawn from any of the four HIV negative donors that were used to create the HLA B27 and HLA B14 BLCLs from the previous chapter. The monocytes were depleted by negative selection using a Magnetic Activated Cell Sorting (MACS) isolation kit (Miltenyi Biotec, Auborn, CA) and cultured for 10 days with cytokines to induce differentiation to mature dendritic cells (mDC). These mDCs were used as antigen presenting cells (APC), after peptide pulsing with HIV peptides, by coculturing with $2B4^+$ and $2B4^-$ $CD8^+$ T cells separately. Subsequently, another round of monocyte differentiation to mDC occurred and these were used for a restimulation of the same $CD8^+$ T cells. To compare the expression of $CD8^+$ T cell cytokine secretion by these APC's we performed an Interferon-gamma Enzyme Linked ImmunoSorbent Assay (IFN-γ ELISA). Previously, it has been shown that the expression of $2B4^+$ $CD8^+$ T cells increases as HIV-AIDS disease

progresses (Peritt et al. 1999), but the upregulation of the 2B4 receptor in $2B4^{-}$ $CD8^{+}$T cell population during disease progression or after activation with HIV peptides has not been explored. To reveal if this, in fact, is occurring we analyzed these cells for the increased expression of 2B4 using flow cytometry after each of the two stimulations with APCs.

RESULTS

Figures 4.4a and 4.4b represent the donor that BLCL-3 was created from, heterogeneously possessing HLA B27 and HLA B44. In figure 4.4a, after the first round of APC stimulation and 8 days of coculture, the HIV peptides seem to inhibit $2B4^{+}$ $CD8^{+}$ T cell secretion of IFN-γ when compared to $2B4^{-}$ $CD8^{+}$ T cells following the first APC stimulation. However, activating in the presence of CRH or EPI causes greater IFN-γ secretion in $2B4^{+}$ $CD8^{+}$ T cells, as well as increases in IFN-γ secretion in $2B4^{-}$ $CD8^{+}$ T cells to similar levels as the $2B4^{+}$ population. Figure 4.4b shows IFN-γ secretion after the second round of APC stimulation and 16 days of coculture with the peptides listed on the graph. This data suggests that $2B4^{+}$ $CD8^{+}$ T cells secrete more IFN-γ than $2B4^{-}$ $CD8^{+}$ T cells following the second APC stimulation and 16 days of coculture.

Figures 4.5a, 4.5b, 4.6a and 4.6b represent the donor that BLCL-4 was created from, heterogeneously possessing HLA B27 and HLA B8. Figures 4.5a and b show the IFN-γ secretion of $CD8^{+}$ T cells after APC stimulation with the listed peptides. $2B4^{+}$

$CD8^+$ T cells show more secretion of IFN-γ than $2B4^-$ $CD8^+$ T cells following the first APC stimulation and 8 days of coculture. Conversely, it also shows the IFN-γ levels after the addition of CRH and EPI. This data indicates that in this heterogeneous individual CRH and EPI causes the $2B4^-$ population to secrete similar amounts of IFN-γ due to the addition of either hormone, this correlates with the other B27 donor previously discussed. Figures 4.6a and 4.6b also show the IFN-γ secretion of $CD8^+$ T cells after APC stimulation with the same listed peptides in the previous figures. This data suggests that $2B4^+$ $CD8^+$ T cells still secrete more IFN-γ after 16 days of coculture, but the amount of cytokine secreted by these cells cultured in the presence of EPI start to decrease as compared to peptide activation alone or CRH added to the culture.

Figures 4.7a and 4.7b show the mean fluorescence intensity (MFI) of 2B4 expression in $2B4^-$ and $2B4^+$ $CD8^+$ T cells analyzed by flow cytometry. The donor was heterogeneous for HLA B27, B8. After the first stimulation and 8 days of incubation with APCs pulsed with the listed peptides, 2B4 expression was upregulated in both $2B4^-$ and $2B4^+$ $CD8^+$ T cells, although expression was upregulated more in the $2B4^+$ $CD8^+$ T cells. The same result occurred after the second stimulation and 16 days of coculture with APCs. CRH and EPI was added to some of the cultures but in most cases it did not cause greater expression of 2B4 as compared to cultures without these hormones.

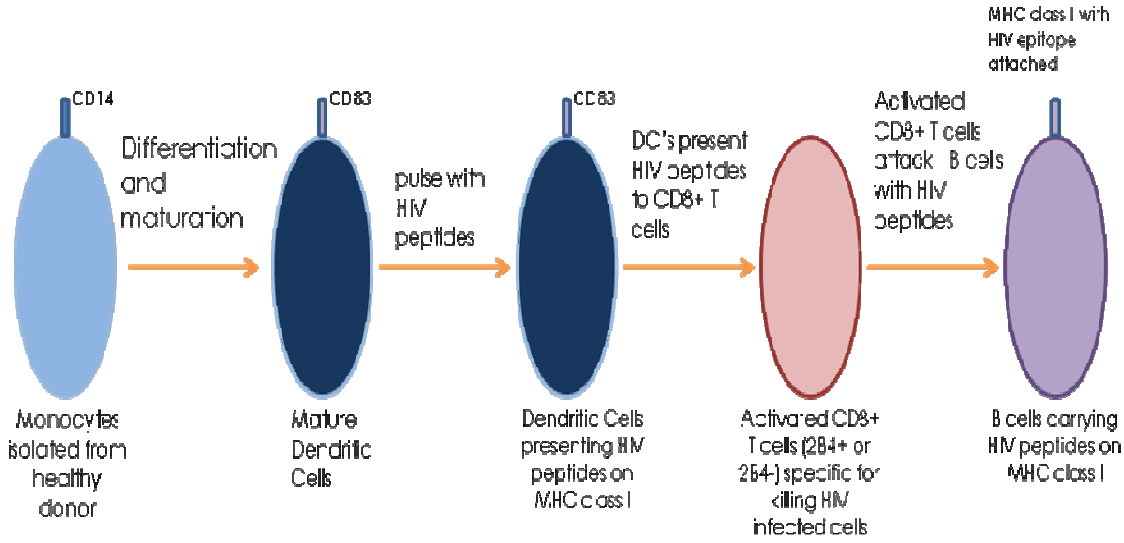

Figure 4.1 Overview of experiments

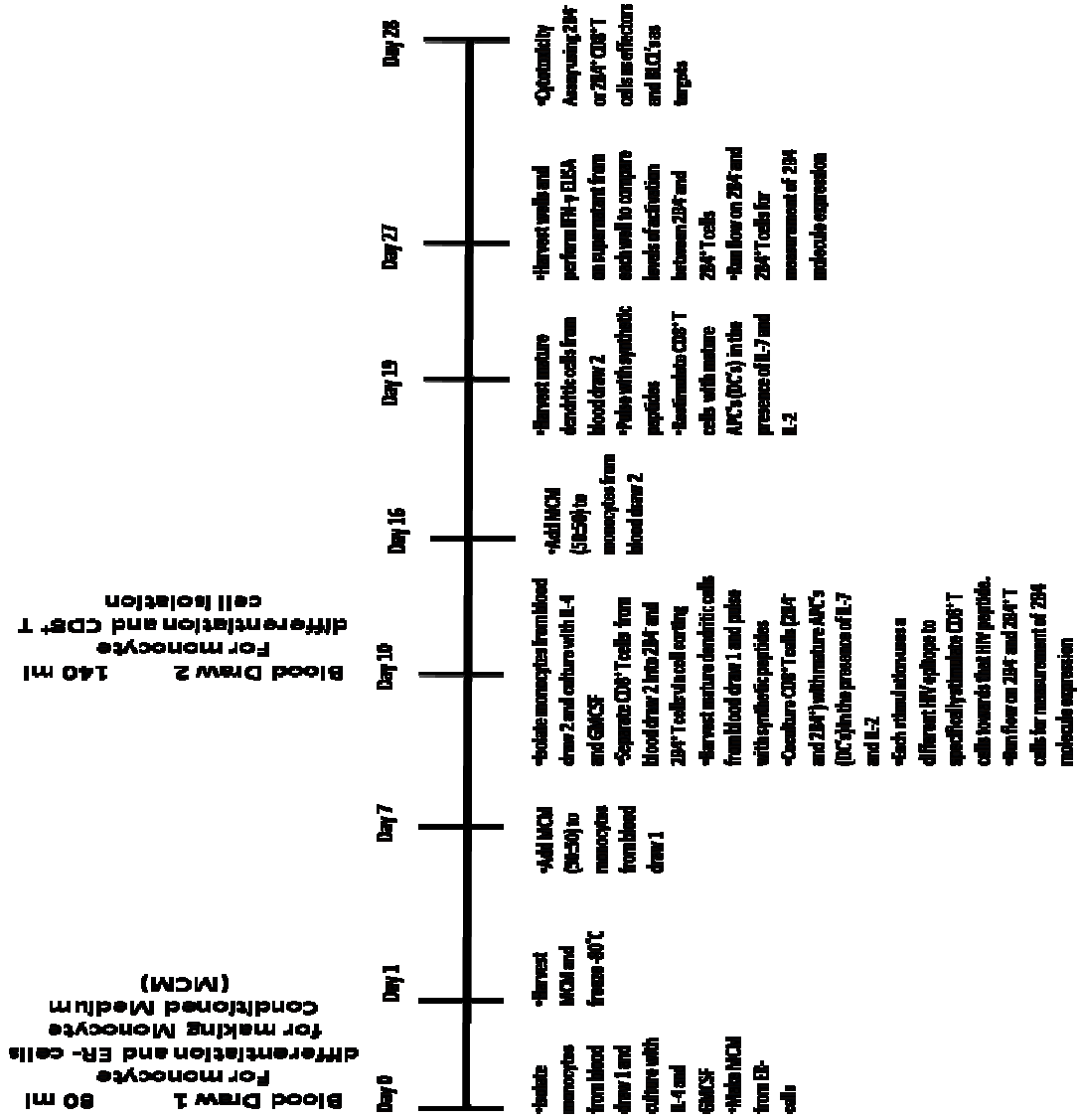

Figure 4.2 Overview of experimental timeline

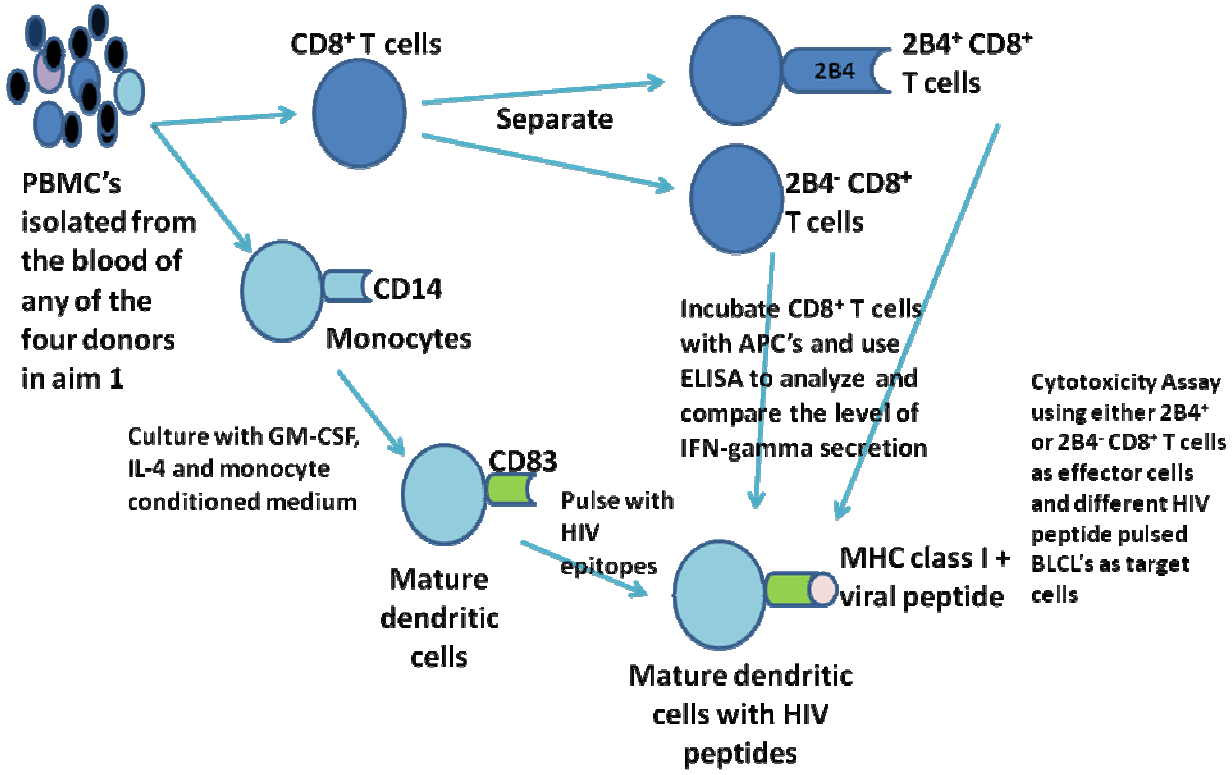

Figure 4.3 Overview of HIV epitope presentation and CD8+ T cell activation

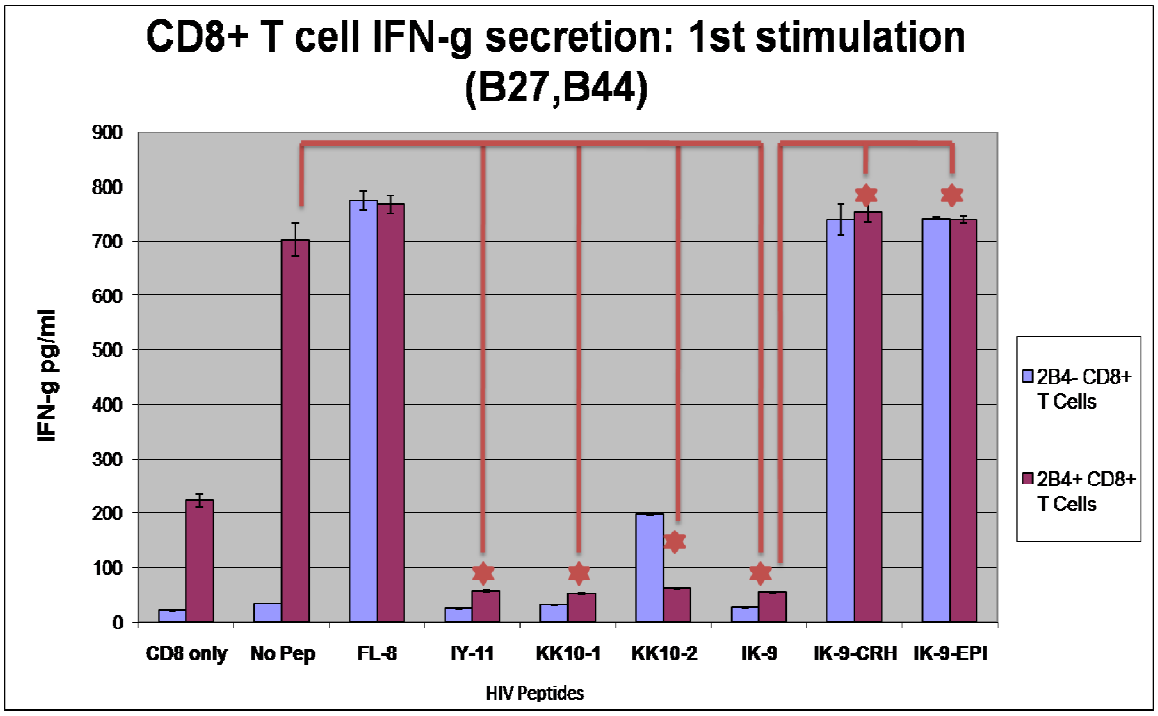

Figure 4.4a In heterogeneous HLA B27, B44 donor, HIV peptides inhibit $2B4^+$ $CD8^+$ T cell secretion of IFN-γ as compared to $2B4^-$ $CD8^+$ T cells following the first APC stimulation, however, activation in the presence of CRH or EPI causes increases and similar IFN-γ secretions in $2B4^-$ and $2B4^+$ $CD8^+$ T cells.

$2B4^+$ and $2B4^-$ $CD8^+$ T cells primed with APC's that present HIV epitopes. Data shown is IFN-γ ELISA after first stimulation with APC's, approximately 8 days after initial stimulation with APC's. Cells from this experiment are from an HLA B27 donor (BLCL-3). (*, $P<.05$; t test)

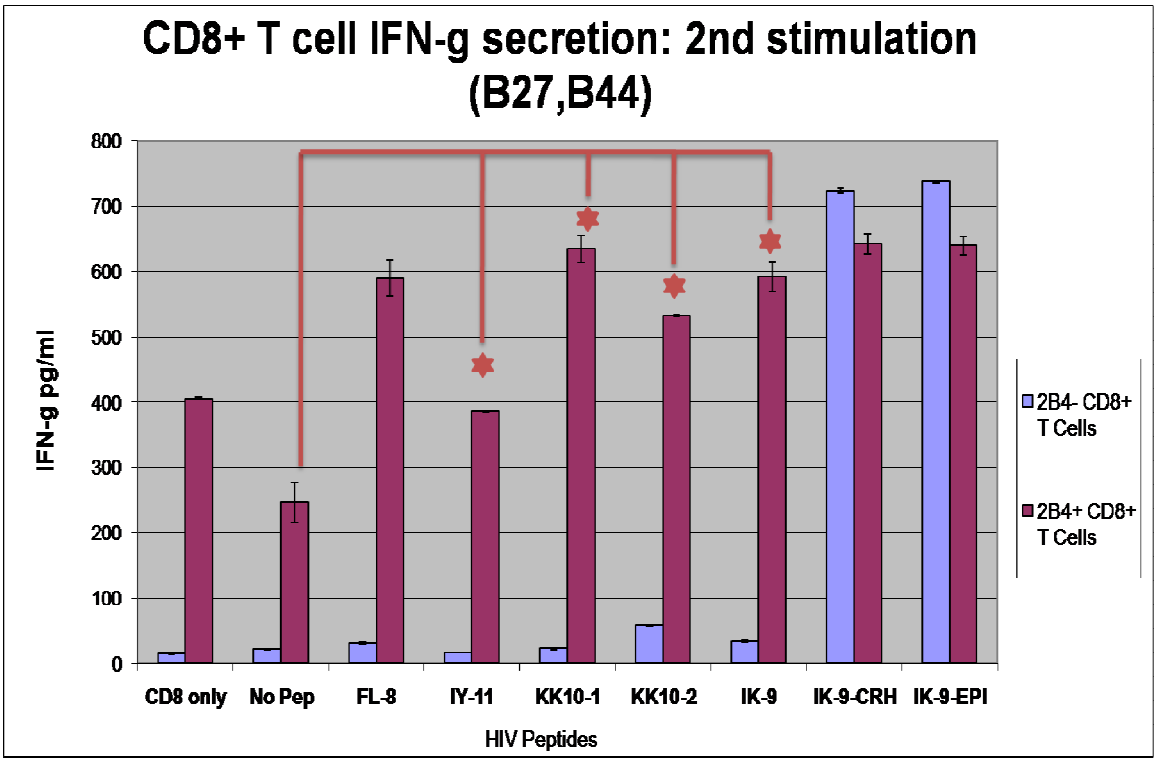

Figure 4.4b In heterogeneous HLA B27, B44 donor, $2B4^+$ $CD8^+$ T cells secrete more IFN-γ than $2B4^-$ $CD8^+$ T cells following the second APC stimulation.

$2B4^+$ and $2B4^-$ $CD8^+$ T cells primed with APC's that present HIV epitopes. Data shown is IFN-γ ELISA after second stimulation with APC's, approximately 16 days after initial stimulation with APC's. Cells from this experiment are from an HLA B27 donor (BLCL-3). (*, P<.05; t test)

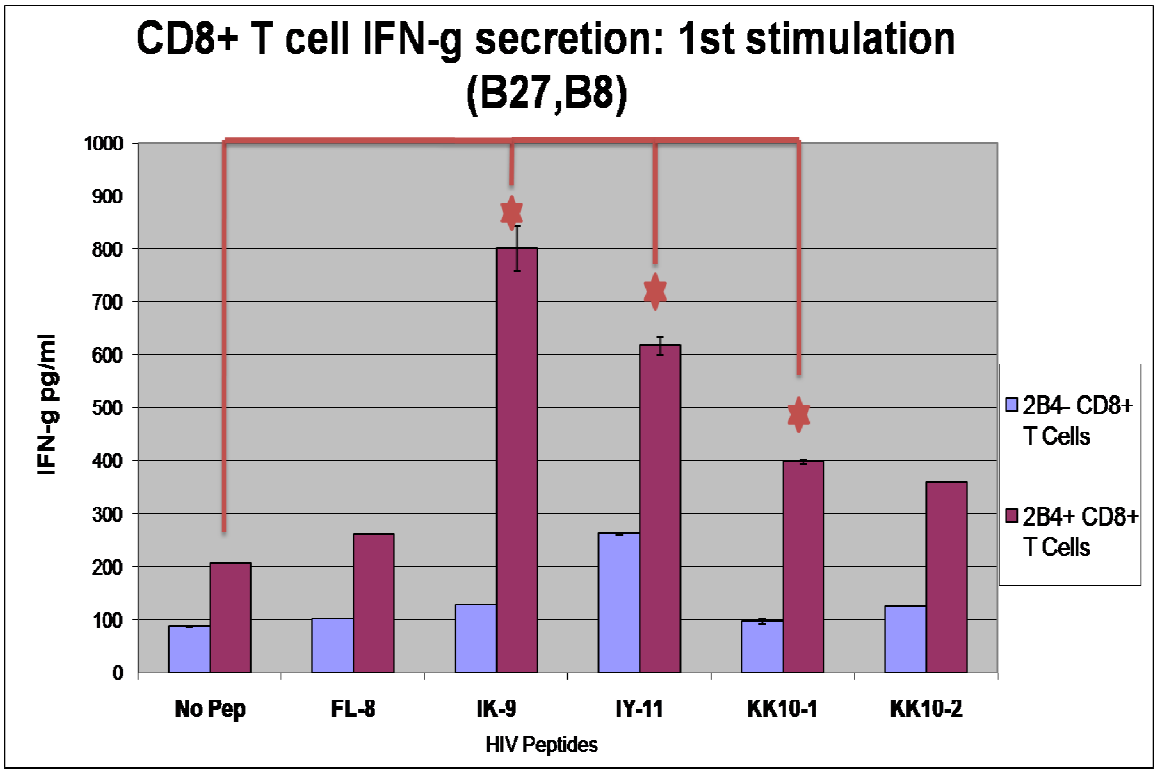

Figure 4.5a In heterogeneous HLA B27, B8 donor, $2B4^{+}$ $CD8^{+}$ T cells secrete more IFN-γ than $2B4^{-}$ $CD8^{+}$ T cells following the first APC stimulation.

A. $2B4^{+}$ and $2B4^{-}$ $CD8^{+}$ T cells primed with APC's that present HIV epitopes. Data shown is IFN-γ ELISA after second stimulation with APC's, approximately 8 days after initial stimulation with APC's. Cells from this experiment are from an HLA B27 donor (BLCL-4). (*, $P<.05$; t test)

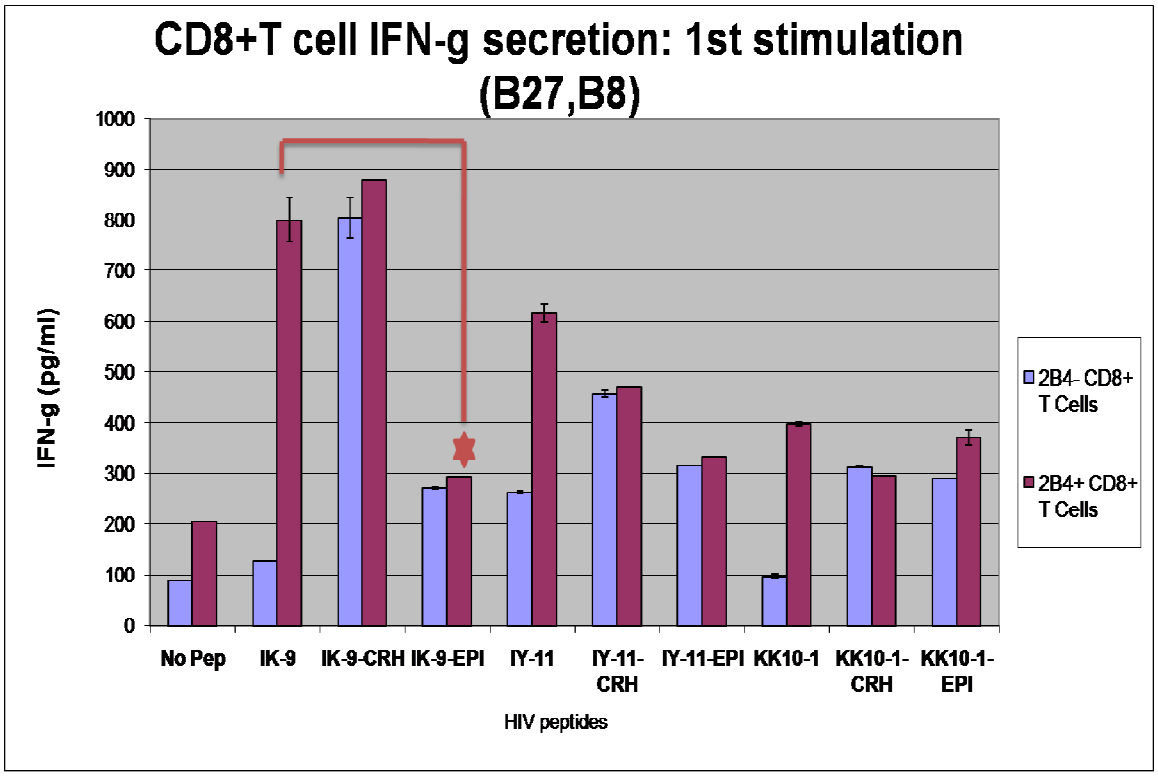

Figure 4.5b In heterogeneous HLA B27, B8 donor, $2B4^+$ $CD8^+$ T cells secrete more IFN-γ than $2B4^-$ $CD8^+$ T cells following the first APC stimulation, however, CRH and EPI cause similar secretion of IFN-γ in $2B4^-$ and $2B4^+$ $CD8^+$ T cells.

B. $2B4^+$ and $2B4^-$ $CD8^+$ T cells primed with APC's that present HIV epitopes. Data shown is IFN-γ ELISA after first stimulation with APC's, approximately 8 days after initial stimulation with APC's. Cells from this experiment are from an HLA B27 donor (BLCL-4). (*, P<.05; t test)

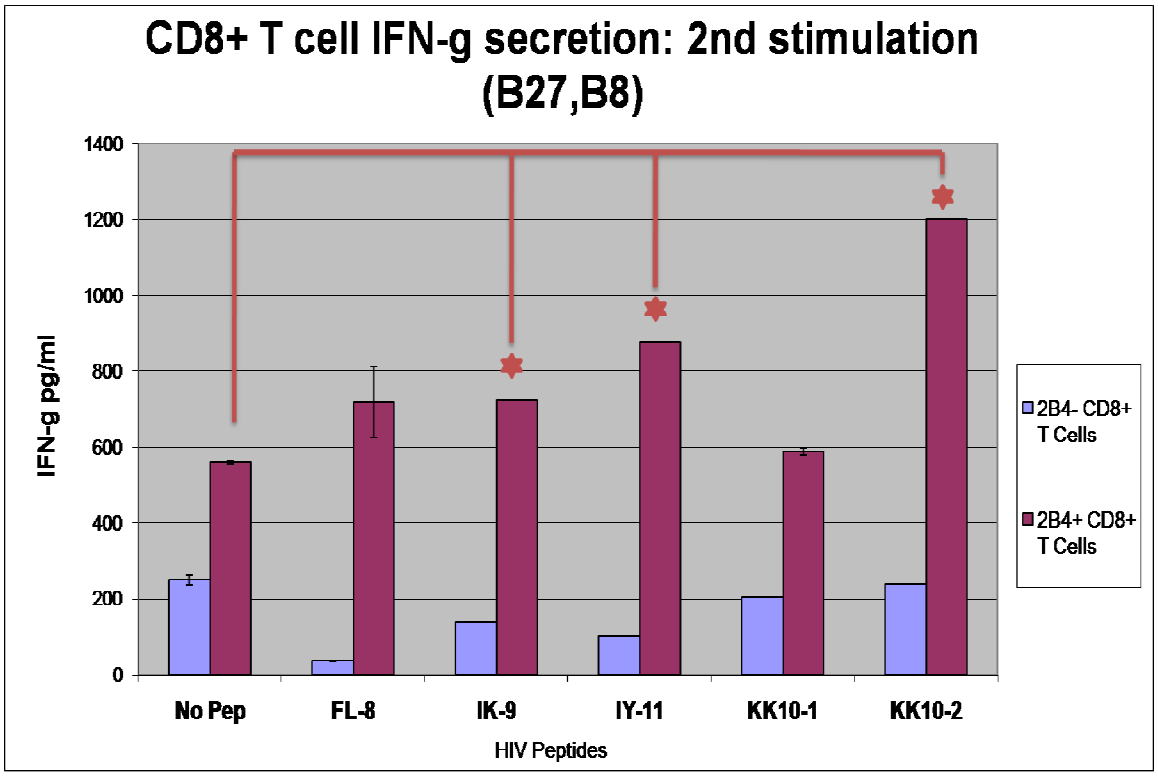

Figure 4.6a In heterogeneous HLA B27, B8 donor, $2B4^{+}$ $CD8^{+}$ T cells secrete more IFN-γ than $2B4^{-}$ $CD8^{+}$ T cells following the second APC stimulation.

A. $2B4^{+}$ and $2B4^{-}$ $CD8^{+}$ T cells primed with APC's that present HIV epitopes. Data shown is IFN-γ ELISA after second stimulation with APC's, approximately 16 days after initial stimulation with APC's. Cells from this experiment are from an HLA B27 donor (BLCL-4). (*, $P<.05$; t test)

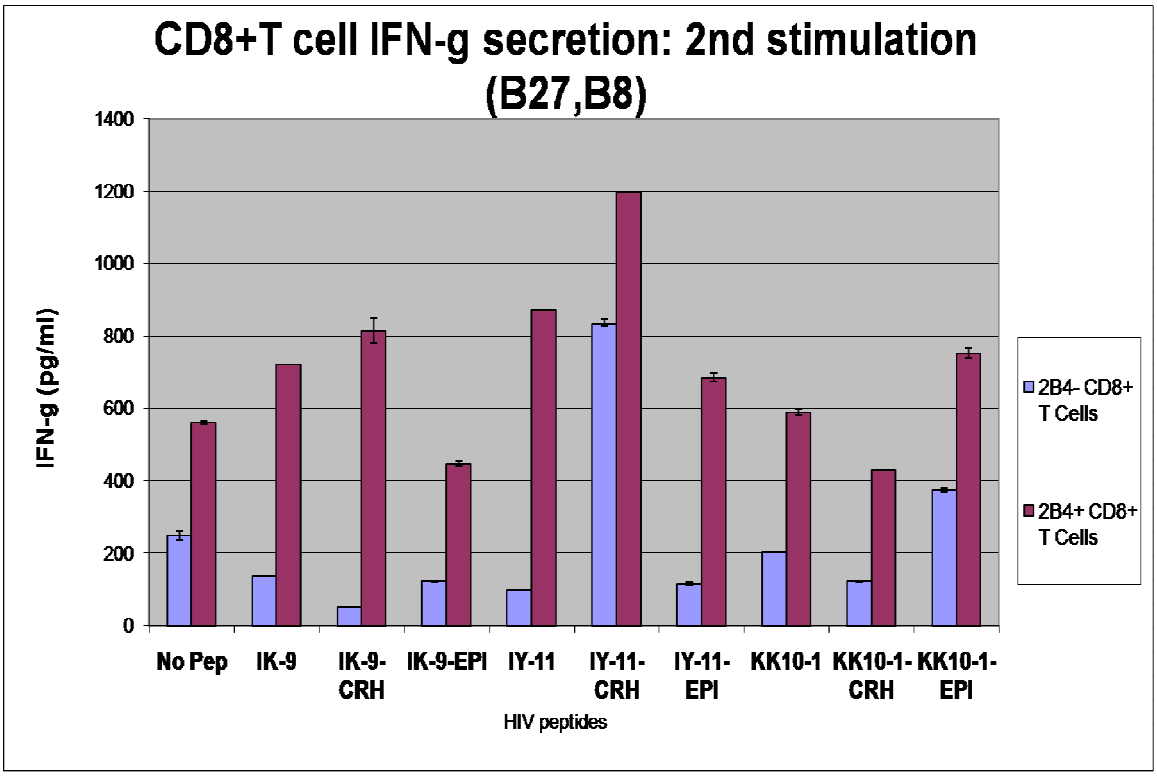

Figure 4.6b In heterogeneous HLA B27, B8 donor, $2B4^+$ $CD8^+$ T cells secrete more IFN-γ than $2B4^-$ $CD8^+$ T cells following the second APC stimulation.

B. $2B4^+$ and $2B4^-$ $CD8^+$ T cells primed with APC's that present HIV epitopes. Data shown is IFN-γ ELISA after second stimulation with APC's, approximately 16 days after initial stimulation with APC's. Cells from this experiment are from an HLA B27 donor (BLCL-4). (*, $P<.05$; t test)

Mean Fluorescence Intensity (MFI) for 2B4 Expression HLA (B27, B8)				
	2B4- CD8+ T Cells		2B4+ CD8+ T Cells	
Baseline Day 0	5.6		8.9	
Peptide	Day 8	Day 16	Day 8	Day 16
CD8 Only	6.24	7.8	12.6	17.3
No Peptide	13.1	27.8	15.5	19.5
FL-8	13.6	16.9	16.4	31.3
IK-9	11.9	17.5	15.9	25.2
IY-11	10.8	18.7	15.8	37.4
KK10-1	11.9	16.9	16.7	25.5
KK10-2	12.7	30.2	19.3	52.5

Figure 4.7a In heterogeneous HLA B27, B8 donors, $2B4^+$ $CD8^+$ T cells upregulate more 2B4 expression following first and second APC-HIV epitope stimulation.

A. $2B4^+$ and $2B4^-$ $CD8^+$ T cells primed with APC's that present HIV epitopes. Data shown is flow cytometry mean fluorescence intensity after first and second stimulation with APC's, approximately 8 days and 16 days after initial stimulation with APC's. CRH and EPI were added to the culture during stimulation. Cells for this experiment are from an HLA B27 donor.

Mean Fluorescence Intensity (MFI) for 2B4 Expression HLA (B27, B8)				
	2B4- CD8+ T Cells		2B4+ CD8+ T Cells	
Baseline Day 0	5.6		8.9	
Peptide	Day 8	Day 16	Day 8	Day 16
CD8 Only	6.24	7.8	12.6	17.3
No Peptide	13.1	27.8	15.5	19.5
No Peptide CRH	11.5	28.7	17.2	31.7
No Peptide EPI	12.4	18.5	16.3	37.7
IK-9	11.9	17.5	15.9	25.2
IK-9 CRH	11.3	15.6	15.6	20.4
IK-9 EPI	13.5	18.7	17.4	20.8
IY-11	10.8	18.7	15.8	37.4
IY-11 CRH	11.4	16.6	15.5	19.2
IY-11 EPI	12.7	15.8	16	24.2
KK10-1	11.9	16.9	16.7	25.5
KK10-1 CRH	13.2	36.6	17	28.8
KK10-1 EPI	13.2	26.1	19.1	43.6

Fig4.7b In heterogeneous HLA B27, B8 donors, $2B4^+$ $CD8^+$ T cells upregulate more 2B4 expression following first and second APC-HIV epitope stimulation, however, activation in the presence of CRH or EPI does not cause significant increases in 2B4 expression.

B. $2B4^+$ and $2B4^-$ $CD8^+$ T cells primed with APC's that present HIV epitopes. Data shown is flow cytometry mean fluorescence intensity after first and second stimulation with APC's, approximately 8 days and 16 days after initial stimulation with APC's. CRH and EPI were added to the culture during stimulation. Cells for this experiment are from an HLA B27 donor.

CHAPTER V

ASSESSING THE HIV ANTIGEN SPECIFIC CYTOTOXICITY DIFFERENCES IN $2B4^-$ AND $2B4^+$ $CD8^+$ T CELLS IN THE PRESENCE AND ABSENCE OF NEUROENDOCRINE HORMONES

Lastly, by uniting the aims in the last two chapters we will gain more perspective into the function of these $2B4^+$ and $2B4^-$ $CD8^+$ T cells. Cytotoxicity assays using effector $CD8^+$ T cells and target B27 or B14 BLCLs can show the level of specificity of these cytotoxic T cells toward the HIV epitopes used to prime them as well as variations in killing ability between $2B4^+$ and $2B4^-$ $CD8^+$ T cells. Although IFN-γ secretion will provide a measure of immunogenicity of the HIV epitopes, we will not know if the $CD8^+$ T cells are activated nonspecifically towards the epitope we used to stimulate them until we compare the killing activity of these cells against a target that mimics infection by presenting the peptide on MHC class I.

After 16 days of coculture and two rounds of activation with the APCs we harvested the $2B4^-$ and $2B4^+$ $CD8^+$ T cells and used them as our effector cells in a sodium chromate (^{51}Cr) release cytotoxicity assay.

RESULTS

Figures 5.1 and 5.2 represent the same assay performed on cells from the donor that was used to create BLCL-4, heterogeneously expressing HLA B27 and B8. Figure

5.1 shows the killing activity of both $2B4^-$ and $2B4^+$ $CD8^+$ T cells previously activated with the peptides listed alone for 16 days. The graph shows that after activation with the HIV epitopes, inhibition of killing occurred when compared to $CD8^+$ T cells that were not activated with peptide but did receive peptide pulsed dendritic cells in the culture and to FL-8 peptide activated T cells. Because the FL-8 activated $CD8^+$ T cell killing activity was higher than the group that was not activated with peptide or activated with HIV peptide, it is apparent that the peptide itself was not the cause of this inhibition; it was the HIV epitope that was the cause of lowered killing activity. Figure 5.2 seems to show the opposite in $2B4^+$ T cells in most cases, the presence of CRH or EPI during activation with or without peptide caused higher levels of killing activity, except in the case of IY-11 EPI and all KK10-1 activated $CD8^+$ T cells, nevertheless killing activity is lowered in HIV epitope activated T cells as compared to non peptide culturing.

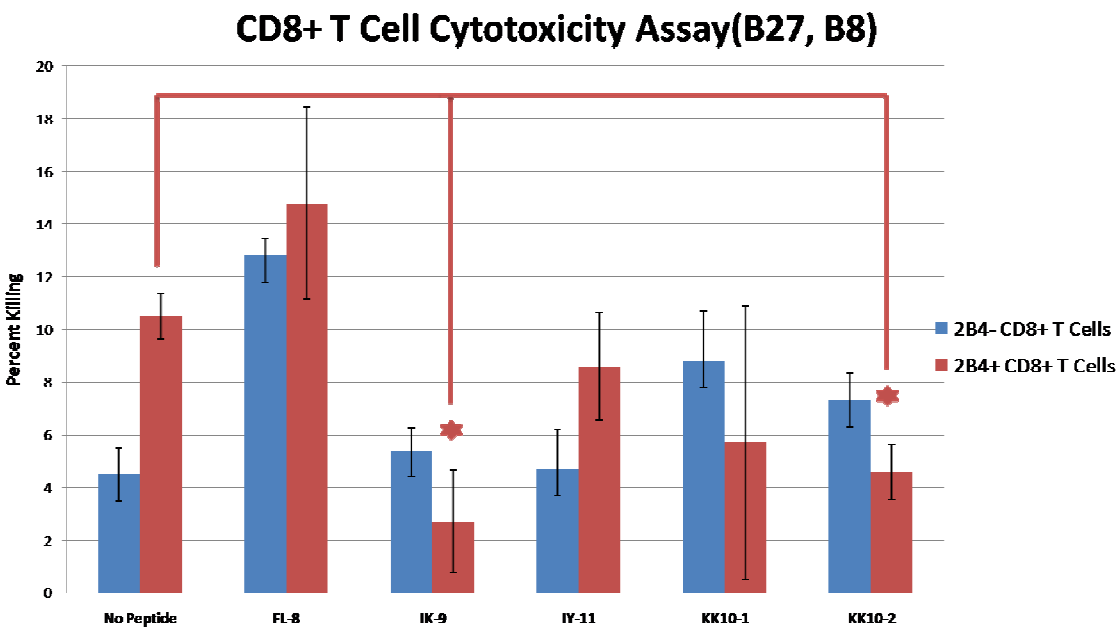

Figure 5.1 HIV peptide activation of $2B4^+$ $CD8^+$ T cells inhibits cytotoxic activity.

$2B4^+$ and $2B4^-$ $CD8^+$ T cells primed with APC's that present HIV epitopes after 16 days of coculture. Data shown is sodium chromate (^{51}Cr) release cytotoxicity assay percent killing after the second stimulation with APC's, approximately 16 days after initial stimulation with APC's. Cells from this experiment are from an HLA B27 donor (BLCL-4). (*, $P<.05$; t test)

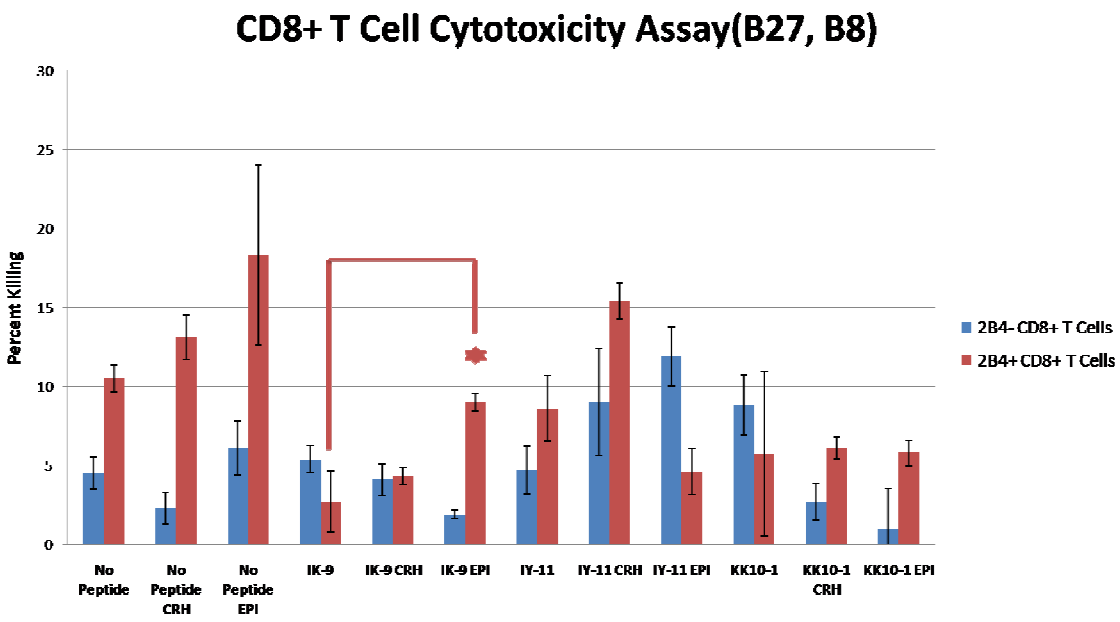

Figure 5.2 HIV peptide activation of $2B4^{+}$ $CD8^{+}$ T cells cultured in the presence of CRH or EPI increases cytotoxic activity in non peptide cultured and IK-9 activated cells.

$2B4^{+}$ and $2B4^{-}$ $CD8^{+}$ T cells primed with APC's that present HIV epitopes after 16 days of coculture. Data shown is sodium chromate (^{51}Cr) release cytotoxicity assay percent killing after the second stimulation with APC's, approximately 16 days after initial stimulation with APC's. Cells from this experiment are from an HLA B27 donor (BLCL-4). (*, $P<.05$; t test)

CHAPTER VI

DISCUSSION AND SUMMARY

While Perrit et al. (1999) has reported that $2B4^+$ T cells have higher cytolytic activity and that the blockage of the 2B4 receptor on T cells can decrease killing activity, they did not find any statistically significant difference. Surprisingly though they also reported that people that have progressively worse AIDS disease have lower $CD4^+$ T cells correlated with a higher population of $2B4^+$ $CD8^+$ T cells. Therefore the question remains, is 2B4 beneficial or inhibitory with respect to killing in $CD8^+$ T cells involved in HIV disease. If 2B4 is upregulated during the later stages of HIV disease, is it causing further disease progression by inhibiting the killing of the virus or is it the "last ditch" effort by the immune system to upregulate the molecules necessary to fight the virus at all costs and becomes overwhelmed by the sheer amount of pathogen spread throughout the body.

Together, the data presented in this study suggests that human $2B4^+$ $CD8^+$ T cell activation with APC's is inhibited during *in vitro* HIV peptide stimulation, leading to decreased killing activity. As shown in chapter 4, after 8 days of incubation with APC's, the HIV stimulated $2B4^+$ $CD8^+$ T cells secreted less IFN-γ when compared to non stimulated and influenza peptide stimulated $2B4^+$ T cells. This finding is surprising because 2B4 expression on $CD8^+$ T cells highlights a population of activated memory T cells (Altvater et al. 2009). This inhibition suggests that 2B4 expression on $CD8^+$ T cells

plays a role in antigen recognition and activation via MHC class I-HIV epitope complex. When comparing this finding to other reports on natural HIV disease (Peritt et al. 1999), it explains why upregulation of 2B4 in $CD8^+$ T cells correlates with worsening HIV disease progression. As this molecule is being over expressed, likely due to repeated activation of $CD8^+$ T cells with HIV epitopes (shown to be a factor over the course of two stimulations, figures 4.7a and b), cytokine secretion is downregulated. Usually, cytokines are needed in order to dictate the function of immune cells, and without sufficient amounts of IFN-γ, $CD8^+$ T cells will not function as efficiently in terms of their cytotoxic activity. Other factors that allow 2B4 to function can also play a role. As discussed in chapter 1, the ligand for 2B4, CD48, is downregulated in HIV infected cells (primarily monocytes, macrophages and $CD4^+$ T cells). In this study we have shown an upregulation of the 2B4 receptor after two stimulations with HIV peptide pulsed dendritic cells, nevertheless inhibition of IFN-γ secretion and killing activity was seen in one B27 donor. This finding could be explained by CD48 downregulation in BLCL targets after HIV peptide-pulsing or downregulation of SAP molecules leaving 2B4-CD48 interactions with an inhibitory effect.

Throughout this study, we have also shown that CRH and EPI added during $2B4^+$ $CD8^+$ T cell stimulation with APC's subsequently increases IFN-γ secretion and killing activity in most cases. Presence of CRH and EPI during experiments with HLA (B27, B44) expressing cells showed that the inhibition of IFN-γ seen in IK-9 stimulated cells was overcome due to the addition of these hormones during the first round of stimulation.

Experiments with HLA (B27, B8) expressing cells showed CRH and EPI culturing upregulated the secretion of IFN-γ in $2B4^-$ $CD8^+$ T cells to similar levels seen in the $2B4^+$ $CD8^+$ T cells, correlating with what was seen in the previous donor, however there was no significant increase in the $2B4^+$ $CD8^+$ T cell cytokine secretion when compared to culturing without CRH or EPI. Cytotoxicity assays on HLA (B27, B8) with CRH or EPI added in the culture during stimulation did not correlate with IFN-γ secretion seen in this same donor. CRH normally elicits a proinflammatory response, however reports of the opposite effect have been observed (Baigent et al. 2000). EPI is an anti-inflammatory cytokine, therefore we expected that CRH and EPI would have opposite effects on cytokine secretion and killing activity. The upregulation of 2B4 molecules between HIV epitope stimulated $CD8^+$ T cells with and without CRH and EPI present was not significant, removing this molecule as being a factor in increased IFN-γ secretion seen after CRH and EPI treatment. Other dynamic molecules must be involved, but because we found such conflicting data between two people that both express the HLA B27 type, it's difficult to make a conclusion without more experimentation on additional B27 donors.

As with all studies there were a few potential drawbacks due to the methods used and data gathered. One of the main limitations of this lengthy experimental design is that each experiment must work and be accurate in order for the outcome to be correct. The non availability or unwillingness of donors hampers the experiment. In chapter 3,

although the experimental protocol was established by many others, when performed on these particular BLCLs we found that it was very difficult to standardize. The data represented shows only a few experiments that were successful. In order to successfully perform the experiment many factors had to be implemented. First, the pH of the stripping solution was unique to each cell line, even though the cells were of the same HLA allele, the variance of pH had to be tested and consistent each time we did the experiment. Second, the concentration of the citric acid solution had to be strong enough to remove the self peptide, but weak enough to hinder cell lysis. Third, the stripping time was distinctive to each cell line, as too much exposure to the citric acid solution would likely cause the cells to lyse also. It took many months to test this technique with a variety of different concentrations, pH and stripping times.

Chapter 4 was one of the most difficult experiments performed because it involved donor blood draws. Knowing the scheme of what we wanted to accomplish, several protocols were joined together to create the 28 day timeline used for the whole experiment. Within these 28 days there were two donor blood draws, two monocyte differentiation experiments to dendritic cells which were then induced to present the HIV epitopes, two IFN-γ ELISAs, 2B4 expression measurements using flow cytometry and a cytotoxicity assay. The coculture incubation of APC's and $CD8^+$ T cells in itself was 16 days. Due to the low populations of $2B4^+$ $CD8^+$ T cells found in a normal individual, the second blood draw and subsequent cell sorting caused many difficulties. First, we were

limited by the amount of blood we could take without causing major inconvenience to the donors. Every blood draw with similar amounts of blood could give different percentages of these cells therefore we needed to take a sufficient amount that would allow us to isolate enough T cells of each type to culture in the many wells. Secondly, after 16 days of incubations and stimulations with a variety of different peptides, the proliferation of these T cells was unpredictable. Some wells, such as the non peptide stimulated and those containing only $CD8^+$ T cells had very little proliferation. This then became a problem when we reached the end of the experiments and had to perform the cytotoxicity assay (chapter 5). Another problem with the vastly different amounts of cells in the coculture stimulations after this period of time was the standardization of IFN-γ secretion after 8 and 16 days. We overcame this obstacle by counting the cells in each peptide stimulated coculture every time an ELISA was performed and normalized the data for IFN-γ secretion.

Another problem with the data gathered in this study was the lack of consistency between different individuals. When repeated the data was not as consistent between individuals and even between experiments done on the same individual. Depending on the state of the donor's monocytes and $CD8^+$ T cells at the time that blood was drawn, we could see that there were vastly different numbers of these populations of cells. Also IFN-γ secretion and cytotoxicity could not be compared in the same individual from one experiment to the other, even though trends of this data could be compared in some cases.

In Conclusion, the phenomenon of 2B4 upregulation in $CD8^+$ T cells after activation with HIV peptides correlates with studies of natural HIV infection. This study highlights the possible reason for the observation by Perrit et al. (2009), that worsening AIDS disease occurs even though more 2B4 is being expressed, by showing that activation with HIV epitopes cause $2B4^+$ $CD8^+$ T cells have inhibited cytokine secretion and cytotoxicity as compared to $2B4^-$ $CD8^+$ T cells.

CHAPTER VII

FUTURE DIRECTIONS

Due to the *in vitro* nature of this study, we cannot conclude that similar reactions will occur during the course of HIV infection. However, the knowledge gained has the potential for use as the stepping stone to future *in vivo* studies of the role of $2B4^+$ $CD8^+$ T cells in natural HIV infection. In addition, *in vivo* humanized mouse models can be used to gather the function of CRH and EPI hormones during HIV disease progression. Mechanistic studies on the role of 2B4-CD48 interactions, as well as downstream signaling pathways should also be explored in order to understand the dynamic nature of the 2B4 receptor during HIV.

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