IL-12 is necessary for the priming of CD4+ T cells required during the elicitation of gp120 specific CTL function
4.1 Introduction

The immune system comprises of a composite array of cells that preserve the integrity of the organism by eliminating the pathogens. In the immune system, innate and adaptive arms play a vital role in clearance of pathogens. The key players of adaptive cellular immune response are T lymphocytes, which also includes cells having a cytotoxic effector function called cytotoxic T lymphocytes (CTL) (Andersen et al, 2006). CTL kills the target cells by one of at least three distinct pathways. The first two involves direct cell-cell contact between effector and targets. The first one is binding of FasL (expressed on effector CTLs) to Fas receptor present on target cells, which leads to apoptosis of target cells (Nagata, 1996). Second pathway involves release of perforin and granzyme from CTLs. Perforin is a soluble pore forming cytolytic protein which is sequestered into cytotoxic granules. Upon the formation of immunological synapse between target and CTL, cytotoxic granules fuse with the plasma membrane of the CTLs and release their content (which also includes granzyme) into the synapse. In the synapse, perforin synergies with granzyme into the target cells, which leads to target cell death (Heuse et al, 1994; Trapani et al, 2002; Trapani et al, 2003). The third one is mediated through IFNγ and TNFα, which are produced by effector cells after TCR stimulation and act on the target cell, which are at the distal end.

Several studies suggest that CTLs play a critical role in antiviral immunity. Direct evidence for the protective role of CD8⁺ T cell was provided in SIV virus model in rhesus macaques, in which elimination of CD8⁺ T cell resulted in dramatic increase in viral load (Schmitz et al, 1999). However, in spite of the presence of HIV specific CTLs in infected individual viral production continues at a high level. It is unclear why CD8⁺ T cells provide only partial protection and are ultimately unable to prevent progression of AIDS. There are several reasons for this dysfunction, first targeting of essential helper CD4⁺ T cells by HIV may be one important cause for the progressive loss of CTL function. Second HIV infection is also characterized by the dysregulation of cytokine expression. HIV-1 infection often result in the over expression of immunosuppressive agents and concomitant impairment of type1 cytokine production from accessory and effector cells (Lee et al, 1996; Valdez et al, 1997; Shearer et al, 1998).
Generation of effector CTLs appear to require three chronological signals: TCR ligation, co-stimulatory signal (B7 with CD28 and CTLA4) (Greenwald et al, 2005) and interaction of CD40L present on T cell with CD40 expressed on APC. Studies have demonstrated that CD40 and CD40L (CD154) interaction induces IL-12 from APCs (Schoenberg et al, 1998) and also priming of CTL response depends upon CD40 signaling (O'Sullivan et al, 2003). IL-12 is hetero-dimer produced by phagocyte and antigen presenting cells in both innate and adaptive immunity and exerts immunoregulatory effects on T cells and NK cells (Trinchieri G, 1995). IL-12 is believed to play an important role in differentiation of naïve T cells into IFNγ producing effector cell. Studies have been performed to identify the role of IL-12 by stimulating T cells with plastic beads coated with MHC ligand plus peptide in the presence of exogenously added IL-12 (Curtsinger et al, 1999; Curtsinger et al, 2003). Studies have also demonstrated the significance of endogenous IL-12 by using IL-12 deficient mice; however, there are contradictory reports in experiments using IL-12 deficient mice. Some reports suggest that IL-12 plays an important role in induction of IFNγ, therefore IL-12 deficient mice is defective in IFNγ secretion (Magran et al, 1996). On the other hand there are findings, which suggest that IL-12 deficient mice are able to clear infection (Oxenius et al, 1999). There are reports showing that immune response can be induced against different HIV-1 proteins by immunization with genes encoding viral proteins (Wang et al, 1997; Calarota et al, 1998). Such immunization leads to expression of delivered foreign gene, resulting in the induction of specific immune response against the in vivo produced antigen. In the present work, we have examined the role of IL-12 in induction of CTL generation using HIV-1 Env protein gp120 as a model antigen using DNA immunization in mice. Our results show for the first time that IL-12 is required in priming of gp120 specific CD8+ T cell response and production of IFNγ. Impairment of CD4+ T cells in absence of IL-12 could be the possible cause of dysfunction of CD8+ T cells, which is crucial for CTL effector function.
4.2 Material and Methods

4.2.1 Plasmid preparation
The gp120 sequence was amplified from a subtype C Indian isolates p93IN301904 (NIH AIDS research and reagent program, USA) (Lole et al, 1999) by polymerase chain reaction and was cloned into pCDNA3.1 vector (Clontech, USA). The cloning of gp120 in pCDNA 3.1 was confirmed by restriction digestion and sequence analysis. Expression of gp120 by pCDNAGp120 was confirmed in vitro and in vivo as described in chapter 2. pMG-Z1-mIL-12 vector expressing both p35 and p40 subunit of IL-12 was obtained from Invivogen, USA. Endotoxin free plasmids were prepared by using QIAGEN columns according to manufacturer's protocol.

4.2.2 Mice and immunization
BALB/c wild type, IFNγ, CD40 and IL-12 deficient (6-8 week old) mice on BALB/c background were obtained from Jackson Lab (Bar Harbor, ME, USA) and were maintained in Experimental Animal Facility of National Center of Cell Science, Pune. Mice were injected with 0.025 % of bupivacaine intramuscularly (i.m.) in the quadriceps muscle 48 h prior to DNA immunization. Mice were later immunized intramuscularly using 26-gauge needle with 3 doses of 100 µg pCDNAGp120 either alone or along with pMG-Z1-mIL-12 on day 0, 15 and 30. The spleen was taken out 10 days after the last immunization and the cells were used for assays described below. The experiments accorded with the institutional committee for the purpose of control and supervision of experiments on animal approved protocols.
T cell purification and CTL assay by JAM Test have been described in section 3.2 of previous chapter.

4.2.3 RT-PCR
Total RNA was isolated from APC-T cell co-culture using Trizol (Invitrogen, USA) according to manufacturers instructions (Invitrogen, USA). 5 µg RNA was used for first strand cDNA synthesis using MMLV reverse transcriptase. The cDNA was then used as
template for PCR amplification of mouse IFNγ, perforin and granzyme B. The primers used for PCR are

IFNγ
Forward 5’-AAC GCT ACA CAC TGC ATC TTG G-3’
Reverse 5’-CTCATGAATGCATCCTTTTTCG-3’;
Perforin:
Forward CTCGCATGTACAGTTTTCGCCTGG
Reverse: TGTGAGCCCATTCAAGGTTCAGCTG
GranzymeB
Forward CTCGACCCTACATGGCCTTAC
Reverse: CCAGCCACATAGCACAACATC
β-actin
forward 5’-GTG GGC CGC TCT AGG CAC CA-3’
Reverse 5’-TGG CC TTA GGG TTC AGG GGG-3’.
Each sample was amplified for mouse β-actin to ensure equal input.

4.3 Results

4.3.1 gp120 mediated immune response in wild type and IL-12 deficient mice
In order to study the immune response generated against gp120 using DNA immunization with the vectors indicated in the methods, gp120 specific CTL assays were performed with splenocyte isolated from immunized mice. gp120 specific CTL lysis was well observed in pCgp120 immunized mice as compared to pCDNA immunized mice (Fig 4.1A). In order to investigate the role of IL-12 in gp120 specific CTL response, we have immunized BALB/c and IL-12 deficient mice with pCDNA and pCgp120 and analyzed gp120 specific CTL response. CTL lysis was impaired in IL-12 deficient mice immunized with pCgp120, which clearly indicates the role of IL-12 in generation of gp120 specific CTL response (Fig 4.1B). Our finding suggests that IL-12 plays a role in elicitation of CTL response (Komastu et al, 1998).
Fig 4.1. Immune response against gp120 in mice using DNA immunization

A. gp120 specific CTL response in pCDNA and pCgp120 immunized WT mice. BALB/c was immunized with pCDNA and pCgp120. Splenocytes from pCDNA and pCgp120 injected mice were plated 2x10⁶ per well in 24 well plate with gp120 peptide pulsed 1x10⁶ irradiated naïve splenocytes. After 5 days of culture viable CD8⁺ T cells were harvested and plated against ³H thymidine-incorporated gp120 peptide pulsed p815 cells and tested for their cytolytic activity in standard 3½ h JAM test. The E:T ratio used are shown in the figure. Each data point is the mean of triplicate samples. The results represent three individual experiments and error bar represent the mean±SD of a given group. B. gp120 specific CTL response in pCDNA and pCgp120 immunized IL-12 deficient mice. WT and IL-12 mice were immunized with pCDNA and pCgp120 and gp120 specific CTL assay was performed as described above.

4.3.2 IL-12 is required during priming of CTL and APC derived IL-12 during restimulation is not useful for elicitation of gp120 specific CTL

There are several reports, which have demonstrated that IL-12 induces IFNγ, which is required for effective CTL lysis. However the phase of requirement for IL-12 in IFNγ production is ambiguous in literature so we have studied the role of IL-12 and IL-12 dependent IFNγ in priming/effector phase of CTL elicitation. We have immunized mice with pCgp120 alone or pCgp120 along with plasmid encoding IL-12 in wild type, IL-12 and IFNγ deficient mice. gp120 specific CTL lysis was impaired in IL-12 and IFNγ deficient mice (Fig 4.2A) as compared to WT mice (p<0.05). We have used WT and IL-12 deficient APC for restimulation but interestingly there is no significant difference in CTL response in WT and IL-12 deficient APC. These observations have suggested that IL-12 might play role in priming and is not required during in vitro restimulation of
Fig 4.2 APC derived IL-12 is not required for restimulation but IFNγ is necessary for CTL response. BALB/c, IL-12 and IFNγ deficient mice was immunized with pCgp120 and Cgp120+IL-12 vector. Splenocytes from pCgp120 and pCgp120+IL-12 injected WT, IL-12, IFNγ mice were plated 2x10^6 per well in 24 well plate with gp120 peptide pulse 1x10^6 irradiated naïve WT or IL-12/-/- splenocytes. After 5 days of culture cells were harvested and plated against ^3^H thymidine-incorporated gp120 pulsed p815 cells and tested for their cytolytic activity in standard 3½ h JAM test. The E:T ratio used are shown in the figure. Each data point is the mean of triplicate samples. The results represent three individual experiments and error bar represent the mean±SD of a given group. A. gp120 specific CTL response in pCgp120 immunized WT, IL-12 and IFNγ deficient mice stimulated with WT or IL-12/-/- APCs. B. gp120 specific CTL response in pCgp120 or pCgp120+IL-12 immunized WT, IL-12 and IFNγ deficient mice stimulated with WT or IL-12/-/- mice. C. RT-PCR for IFNγ was performed with RNA isolated from the stimulated cells using gene specific primers. D. RT-PCR for perforin was performed with RNA isolated from the stimulated cells using gene specific primers.
gp120 specific CTLs. In contrast co-priming with IL-12 vector rescued the CTL activity in IL-12 deficient mice (Fig 4.2B) (p<0.05) but not in IFNγ deficient mice indicating the significance of IL-12 in the priming of CTLs but probably not in effector function of CTLs. Results suggest that IFNγ, induced by IL-12 in priming might help in CTL maturation perhaps associated with the acquisition of cytotoxic property. This observation is consistent with other finding (Paganin et al, 1995). We have done RT-PCR for IFNγ gene and observed that expression was enhanced when IL-12 vector was immunized along with pCgp120 in WT and IL-12 mice (Fig 4.2C). The impaired functional activity of cytotoxic T lymphocytes during HIV-1 infection has recently been attributed to decreased intracellular levels of perforin (Ang et al, 2005). Therefore we have also analyzed the expression of perforin gene. Analysis of perforin demonstrates that co-priming with IL-12 vector induced perforin in WT and IL-12 deficient mice (Fig 4.2D).

4.3.3 gp120 specific primed CD4+ T cells are required for CTL effector function

The generation of functional CTL is critically dependent on CD4+ T cell help (Wodarz et al, 2001; Castellino et al, 2006). Studies have demonstrated that IL-12 can directly act on CD4+ T cells to induce IFNγ (Seder et al, 1993). So we have investigated the role of gp120 specific CD4+ T cells in priming of CD8+ T cell to become effector CTLs. We have immunized WT, IL-12 and CD40 deficient mice with pCDNA and pCgp120 and isolated CD4+ and CD8+ T cells from these mice and co-cultured them for 5 days in presence of gp120 peptide pulsed irradiated naïve macrophages, which was used as APC. CD8+ T cells isolated from pCDNA immunized WT mice and co-cultured with six different group of CD4+ T cells from WT, IL-12 and CD40 deficient mice did not show CTL lysis as expected (Fig 4.3A), however, in case of CD8+ T cells isolated from pCgp120 immunized mice and co-cultured with CD4+ T cells from WT, IL-12 and CD40 deficient mice, CTL activity was only observed in co-culture containing CD4+ T cells from wild type pCgp120 immunized mice (Fig 4.3B). This result suggests that antigen specific primed CD4+ T cell was a crucial requirement for an effective CTL lysis and in absence of IL-12, insufficient priming of CD4+ T cell happens. These unprimed CD4+ T cells fails to provide help to CD8+ T cell as a result there was no lysis observed in co-
culture of CD8⁺ T cells isolated from pCgp120 immunized WT mice and CD4⁺ T cells isolated from pCgp120 immunized IL-12⁻/⁻ and CD40⁻/⁻ mice.

Fig 4.3. CD4⁺ T cells primed in presence of IL-12 can only provide help to CD8⁺ T cells for CTL function. BALB/c WT, IL-12 and CD40 deficient mice were immunized with pCDNA and pCgp120 and CD4⁺ T cells were isolated. CD8⁺ T cells were isolated from pCgp120 immunized WT mice. Both CD8⁺ and CD4⁺ cells from different mice were co-cultured with WT gp120 peptide pulsed macrophage as APC. After 5 days of culture viable CD8⁺ T cells were harvested and plated against ³H thymidine-incorporated gp120 pulsed p815 cells and tested for their cytolytic activity in standard 3½ h JAM test. The E:T ratio used are shown in the figure. Each data point is the mean of triplicate samples. The results represent three individual experiments and error bar represent the mean±SD of a given group. A. gp120 specific CTL activity in CD8⁺ T cells isolated from pCDNA immunized mice. B. gp120 specific CTL activity in CD8⁺ T cells isolated from pCgp120 immunized mice.

4.3.4 IL-12 vector restores the CD4⁺ T cell priming and help of CD8⁺ T cells
We then immunized WT, IL-12⁻/⁻ and CD40⁻/⁻ mice with pCgp120 and pCgp120 along with IL-12 vector on day 0, 15 and 30. The mice were sacrificed 10 days after the last immunization. CD4⁺ T cells were isolated from these immunized mice. For the same experiment we have isolated CD8⁺ T cells from only WT mice immunized with pCgp120. These CD8⁺ T cells were co-cultured with 6 group of CD4⁺ T cells as mentioned above and CTL assay was performed as described earlier. The results show that IL-12 co-immunized CD4⁺ T cells not only induce CTL activity of WT gp120 specific T cells but it also rescues the CTL activity in the co-cultures of CD4⁺ T cells.
from IL-12−/− and CD40−/− mice and CD8+ T cells from WT mice (Fig 4.4A). This finding suggests that CD4+ T cells primed with antigen (gp120) in presence of IL-12 are capable of providing help to CD8+ T cells for effective lysis. We have also performed RT-PCR from IFNγ, perforin and granzyme B from the co-culture experiment described above. The results obtained from RT-PCR shows the up regulation of IFNγ, Perforin and granzyme in IL-12 vector immunized mice (Fig 4.4B), which are known to be involved in effective CTL lysis. The collective results obtained from CTL lysis and RT-PCR experiments above clearly shows that IL-12 plays a role in priming of antigen specific CD4+ T cells, which provide help to CD8+ T cells for CTL effector function.

**Fig 4.4. IL-12 co-immunization restores the CD4+ T cell priming and help of CD8+ T cells in IL-12 and CD40 deficient mice.** BALB/c, IL-12 and CD40 deficient was immunized with pCgp120 or pCgp120 + IL-12 vector and CD4+ T cells were isolated. CD8+ T cells were isolated from pCgp120 immunized WT mice. Both CD8+ and CD4+ cells from different mice were co-cultured with WT gp120 peptide pulsed macrophage as APC. After 5 days of culture viable CD8+ T cells were harvested and plated against 3H thymidine-incorporated gp120 pulsed p815 cells and tested for their cytolytic activity in standard 3½ h JAM test. The E: T ratio used are shown in the figure. Each data point is the mean of triplicate samples. The results represent three individual experiments and error bar represent the mean±SD of a given group. RNA was isolated and RT-PCR was done for IFNγ, perforin and granzyme B using gene specific primers. **A.** gp120 specific CTL activity in CD8+ T cells isolated from pCgp120 immunized mice in presence of CD4+ cells from IL-12 co-immunized mice. **B.** Perforin, Granzyme B and IFNγ expression by RT-PCR in the co-culture described in A.
4.4 Discussion

During HIV infection various cytokines are over produced in early stages whereas in advanced disease cytokines secreted from T cells are selectively deficient. Peripheral Blood Mononuclear Cells (PBMCs) from HIV infected patients show impaired IL-12 induction as compared to uninfected controls (Chehimi et al, 1994; Chougnet et al, 1996; Yoo et al, 1996). This suggests the significance of IL-12, as it results in inability of the patient to mount a strong cellular immune response against the virus.

We have immunized WT and IL-12 deficient mice with pCgp120 and observed impaired CTL lysis in IL-12^-^- mice. Our results indicate the role of IL-12 in CTL response. We have also immunized WT, IL-12 and IFNγ deficient mice with pCgp120 or pCgp120 along with IL-12 vector. CTL response was augmented when IL-12 vector is immunized along with pCgp120 as compared to pCgp120 alone (Boyer et al, 2000). We have also observed that IL-12 vector rescues the impaired CTL response in IL-12 deficient mice; however, immunization of IL-12 vector in IFNγ deficient mice was unable to restore the CTL response. These observations have suggested that IL-12 mediated IFNγ is vital for effective CTL lysis. To analyze the role of APC derived IL-12 for restimulation we have co-cultured the primed T cells from immunized mice with WT and IL-12 deficient mice APCs for 6 days and then observed CTL response. We could not observe any significant difference in WT and IL-12 deficient APCs. This implies the IL-12 plays a role in initial priming of T cells and not at the stage of restimulation.

The production of IL-12 by phagocytic cell is induced by a variety of mechanisms that can be T cell dependent or T cell independent. Induction of IL-12 in T cell independent mechanism is due to interaction of adhesion molecules, for example CD44 with low molecular weight fragments of the extracellular matrix glycosamine hyaluronan (LMW-HA) that accumulate during inflammation by intracellular pathogens, fungi, virus or by their by product such as LPS and bacterial DNA (Kennedy et al, 1996; Hodge-Dufour et al, 1998). The T cell dependent mechanism of IL-12 production is dependent on the ability of CD40 ligand expressed on activated T cells to interact with CD40 receptor on the surface of monocytes or macrophages and dendritic cells (Kelsall et al, 1997). The T cell dependent mechanism plays an important role in immunoregulatory role of IL-12 in maintenance of Th1 response (Stuber et al, 1996; Vanham et al, 1999). We have isolated
CD4+ T cells from pCgp120 immunized WT, IL-12 and CD40 deficient mice and co-cultured them with CD8+ T cells isolated from pCDNA and pCgp120 immunized mice and have observed impaired CTL lysis in all groups except WT CD4+ T cells. This observation leads to suggest that there is T cell dependent induction of IL-12 in DNA immunization as CTL lysis was impaired in IL-12 and CD40 deficient mice and it also suggests the requirement of antigen specific CD4+ T cell help for effector function of CTL.

Recent findings suggest that CD40 engagement enhances antigen presentation in Langerhans cells and primes T cell to induce IFNγ independent of IL-12 (Gorbachev et al, 2004). In order to investigate the role of CD40 induced IL-12 in elicitation of gp120 specific CTL activity, we have isolated CD8+ T cells from pCgp120 immunized WT mice and co-cultured them with CD4+ T cells from pCgp120 or pCgp120+IL-12 vector immunized WT, IL-12−/− and CD40−/− mice. The results clearly indicate that immunization with IL-12 vector restores the CTL lysis in IL-12 and CD40 deficient mice and also augments CTL lysis by increased expression of perforin, granzyme B and IFNγ. In conclusion present data suggest that impaired IL-12 induced priming of antigen specific CD4+ T cells can lead to improper function of CD8+ T cells or CTL dysfunction. It could be beneficial to increase antigen induced IL-12 in AIDS patients in order to enhance Th-1 response.
4.5 References


