Influence of CD4+ cell types on HIV-1 infection

Heeregrave, E.J.

Publication date
2010

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Submitted for publication

PREFERENTIAL INFECTION AND DEPLETION OF MYCOBACTERIUM TUBERCULOSIS (MTB)-SPECIFIC CD4+ T CELLS AFTER HIV-1 INFECTION

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ABSTRACT

HIV-1 infection results in the progressive loss of CD4+ T cells. Here we address how different pathogen-specific CD4+ T cells are affected by HIV-1 infection and the cellular parameters involved. We found striking differences in the depletion rates between CD4+ T cells to two common opportunistic pathogens, CMV and MTB. CMV-specific CD4+ T cells persisted after HIV-1 infection, whereas MTB-specific CD4+ T cells were depleted rapidly and contained more HIV-1 DNA than other memory CD4+ T cells. CMV-specific CD4+ T cells expressed a mature phenotype and produced very little IL-2, but large amounts of MIP-1β. In contrast, MTB-specific CD4+ T cells were less mature and most produced IL-2 but not MIP-1β. IL-2 production was associated with expression of CD25, and neutralization of IL-2 completely abrogated productive HIV-1 infection of antigen-specific CD4+ T cells *in vitro*. HIV-1 DNA was found to be most abundant in IL-2-producing, and least abundant in MIP-1β-producing MTB-specific CD4+ T cells from HIV-1 infected subjects with active TB. These data support the hypothesis that differences in function affect the susceptibility of pathogen-specific CD4+ T cells to HIV-1 infection and depletion *in vivo*, providing a potential mechanism to explain the rapid loss of MTB-specific CD4+ T cells after HIV-1 infection.

INTRODUCTION

HIV-1 infection is characterized by the progressive depletion of $CD4^+$ T helper cells eventually leading to the Acquired Immunodeficiency Syndrome (AIDS) and the onset of various opportunistic diseases. Highly active antiretroviral therapy (HAART) interrupts HIV-1 replication and leads to increases in $CD4^+$ T cell numbers and function, with associated clearance of many opportunistic infections. Data indicate that ongoing viral replication within $CD4^+$ T cells is among the most important parameters driving the massive, systemic depletion of memory $CD4^+$ T cells during acute Immunodeficiency Virus infection[1,2] and the subsequent slow depletion of $CD4^+$ T cells during chronic disease. Although $CD4^+$ T cell-mediated Delayed Type Hypersensitivity (DTH) reactions are lost during disease progression[3,4], it is largely unclear whether depletion of particular pathogen-specific $CD4^+$ T cells equates with susceptibility to given opportunistic infectious diseases. Some data suggest that this might be the case; e.g. cytomegalovirus (CMV) end organ disease is associated with and preceded by the loss of CMV-specific $CD4^+$ T cell responses[5,6]. Whether different pathogen-specific $CD4^+$ T cell populations are being differentially affected by HIV-1 and what pathogenic mechanisms contribute to the depletion of individual pathogen-specific $CD4^+$ T cells is unknown.

Pathogen-specific alpha/beta ($\alpha\beta$) $CD4^+$ T cells play a central role in the orchestration of adaptive immune responses and are important for protective immunity to many microbial pathogens, including Mycobacterium tuberculosis[7,8]. $CD4^+$ T cells are a crucial component of the response to acute MTB infection in the murine model[9]. Upon antigen-recognition MTB-specific $CD4^+$ T cells secrete interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) that activate infected macrophages, contribute to the containment of intra-endosomal bacilli[10-12] and to the formation of bactericidal granulomas[13]. Hence, MTB-specific $CD4^+$ T cells are thought to be of central importance in the efficient control of MTB infection and prevention of further dissemination to extrapulmonary sites.

Active pulmonary and extrapulmonary tuberculosis (TB) are among the most commonly observed opportunistic infectious diseases in HIV-1 infected subjects within MTB endemic areas and pulmonary TB is frequently the first manifestation of AIDS in such regions[14]. Within areas of high TB incidence, the risk of developing active TB is significantly increased even during the first year after HIV-1 infection when total $CD4^+$ T cell counts are still quite high[12,15] and TB was shown to be the cause of death in almost 50% of HIV-1 seropositive South African Gold miners[16]. Furthermore, evidence of active and disseminated TB was found in almost 50% of autopsies conducted on HIV-1 infected Kenyan subjects[17]. In the absence of HIV-1 infection or other immunodeficiencies, MTB is reasonably well controlled and only about 10% of MTB-exposed individuals develop active TB disease. Thus, the dramatic increase in active TB associated with HIV-1 infection suggests that MTB-specific immunity might be particularly vulnerable to HIV-associated immune damage.

In stark contrast to pulmonary TB, which frequently occurs in HIV-1 infected subjects with relatively high $CD4^+$ T cell counts, CMV-associated end organ disease

typically affects AIDS patients only after CD4 counts have fallen to very low levels. Although these pathogens differ substantially, MTB and CMV share a range of similarities. Both cause persistent or latent infections that are tightly controlled by the adaptive immune system in healthy individuals, but cause life threatening disease in immunocompromised states. Importantly, pathogen-specific CD4+ T cells play important roles in the control of both of these infections[5-8,18,19].

MTB-specific CD4+ T cell responses are depleted early after HIV-1 infection and are significantly decreased during chronic HIV-1 infection[20,21]. In contrast, CMV-specific CD4+ T cells are often detectable even late into chronic HIV-1 infection[22]. This difference provides an opportunity to study cellular parameters associated with the rapid depletion or persistence of pathogen-specific CD4+ T cells after HIV-1 infection and to further elucidate the mechanisms underlying the differential rates of depletion of various pathogen-specific CD4+ T cells. A better understanding of such mechanisms might help to elucidate general mechanisms of HIV-1 pathology and to define parameters that can protect vaccine-induced HIV-1 specific CD4+ T cells from preferential infection and depletion upon exposure to HIV-1.

MATERIALS AND METHODS

Study subjects

Subjects from three cohorts were included in this study. Most were part of a large well-characterized high-risk cohort of female bar workers (HISIS) enrolled in a prospective study of HIV-1 infection in the Mbeya region of Southwest Tanzania, and is described in detail elsewhere[23,24]. Subjects with evidence of active tuberculosis were enrolled at the NIMR-Mbeya Medical Research Programme (NIMR-MMRP) Tuberculosis Clinic in collaboration with the National Tuberculosis and Leprosy Program of Tanzania (NTLP). The purpose and the procedures of the study were explained thoroughly to potential participants. Only persons who gave voluntarily written informed consent in the presence of a witness were enrolled. Acid Fast Bacilli (AFB) smear positive tuberculosis patients were referred to the MMRP Tuberculosis clinic. Active TB was diagnosed by examination and culture (BACTEC™ MGIT, Becton Dickinson, Sparks, USA) of three separate sputum samples per patient. HIV status was determined using HIV 1/2 STAT-PAK (Chembio Diagnostic Systems, Medford, NY) and positive results were confirmed using Enzygnost Anti-HIV 1/2 Plus ELISA (DADE Behring). Patients eligible for TB treatment were referred to the NTLP for treatment according to national guidelines. Patients diagnosed with HIV infection were referred to the Southern Highland Care and Treatment Program. None of the subjects evaluated in the current study were on active antiretroviral therapy.

In addition, blood was obtained from HIV-positive antiretroviral therapy naïve Ghanaian patients with suspected TB attending the HIV clinic of Koforidua Hospital, Ghana, after informed consent. Ethical clearance was granted by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, NMIMR-IRB CPN 039/06-07. HIV infection was confirmed using the Determine HIV-1/2 test (Abbott) and the First response HIV 1-2.0 test (Premier Medical Corporation Ltd).

Peripheral blood mononuclear cells (PBMC) from these patient cohorts were isolated, cryopreserved, stored in liquid nitrogen, and used in subsequent immunological assays. Use of these patient materials was reviewed and approved by the ethics committees of all partners in compliance with national guidelines and institutional policies.

Conjugated antibodies

The following antibodies were used: IFN-γ–fluorescein isothiocyanate (FITC), Interleukin (IL)-2-allophycocyanin (APC), MIP-1β-phycoerythrin (PE), CD3-Cy7APC, CCR5-Cy7PE and CD25-Cy5PE (BD Biosciences), CD27-Cy5PE and CD45RO-Texas red-PE (Beckman Coulter), and CD4-PECy5.5 (Caltag). An anti-HIV-1 p24/p55 antibody (clone KC57, Beckman Coulter) was labelled with FITC or PE. CD8-quantum dot (QD) 655, CD57-QD565 and TNF-α-Alexa680 were conjugated in accordance with standard protocols (available at: http://drmr.com/abcon/index.html).

Antigens

For cell stimulations, Purified Protein Derivative (PPD, Statens Serum Institut) was used at a final concentration of 10 μg/ml. Two microliters of undiluted CMV grade 2 antigen (Microbix) was used to stimulate PBMC. Peptides fifteen amino acids in length and overlapping by eleven were designed for ESAT6 (AF420491.1) and CFP10 (AAC83445) by using the PeptGen peptide generator from the HIV Molecular Immunology Database (at: http://www.hiv.lanl.gov/content/immunology/) and were used at a final concentration of 2 μg/ml/peptide. For sorting experiments, MTB-specific $CD4^+$ T cells were stimulated overnight with a mix of PPD, ESAT6 and CFP10 peptides to maximize detection of the MTB-specific T cell population.

Stimulation and flow cytometric analysis

Previously cryopreserved PBMC were recovered by thawing and washing twice in Benzonase (4 μl of 25 kU Benzonase in 20 ml, Novagen) containing 37°C Complete Media (CM, 10% heat inactivated fetal calf serum, 100 U/ml penicillin G, 100 U/ml streptomycin sulfate, and 1.7 mm sodium glutamine). Cell stimulation was performed in CM in the presence of 1 μg/ml each of anti-CD28 and anti-CD49d (Pharmingen). Duration of stimulation varied in different assays. For the detection of MTB- and CMV-specific $CD4^+$ T-cell responses and analysis of their functionality and cell surface phenotype, PBMC were stimulated with specific antigens for 2 hours before adding Brefeldin A (BFA, Sigma) and further incubated overnight (14-18 hours, 6 hours for analysis of CD25 expression) at 37°C. For *in vitro* HIV-1 infection experiments, PBMC were stimulated for 48 hours and BFA was added 4 hours before flow cytometric analysis. Staining was performed using a modified version of a previously described method[25]. After stimulation, PBMC were washed once with PBS and stained with Vivid/Aqua (Molecular Probes)[26]. Anti-CCR5 antibody was then added for 10 minutes at RT in the dark. Subsequently, surface proteins were stained for 20 min. The cells were permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), after which they were stained for CD3, IFN-γ, MIP-1β, IL-2, and TNF-α. Cells were then washed and fixed with 1% paraformaldehyde. Cells were analyzed with a modified flow cytometer (LSR II; BD Immunocytometry Systems). Between 300,000 and 1,000,000 total events were collected from each sample. Electronic compensation was conducted with antibody capture beads (BD Biosciences) stained separately with the individual antibodies used in the test samples. Flow cytometry data were analyzed using FlowJo (version 8.8.3; TreeStar). For polychromatic analysis, initial gating for each sample set used a forward scatter area versus a forward scatter height plot to gate out cell aggregates. The cells were then gated through a forward scatter area versus a side scatter height plot to isolate small lymphocytes. CD3 versus Vivid/Aqua plot was then used to remove dead cells. The cut-off point for positive $CD4^+$ T cell responses was 2-fold the negative control and at least 0.1% cytokine positive after subtraction of the background.

Virus growth and characterization

CCR5-tropic HIV-1 BaL was grown on phytohaemagglutinin (PHA, Sigma)-stimulated PBMC in CM supplemented with IL-2 (Chiron). HIV-1 p24 antigen in cell culture supernatants was monitored by enzyme-linked immunosorbent assay (ELISA) (Coulter). Virus was harvested at

the peak of infection, on either day 4 or day 7. Viruses were concentrated by ultracentrifugation at 30,000 rpm for 70 min at 4°C, and virus pellets were resuspended in fresh RMPI at 10x to 50x to obtain clean, concentrated virus stocks. Control concentrated conditioned supernatant from uninfected PHA-stimulated T cells was collected as a control. The viral titers were determined by sensitive 14-day end-point titration assays using PHA- and IL-2-stimulated PBMC, and listed as 50% tissue culture infectious doses per ml ($TCID_{50}$/ml) as described previously[27]. The final titer of the stock of HIV-1 Bal was 2.6 x 10^6 $TCID_{50}$/ml.

In vitro HIV-1 infection of primary CD4+ T cells

Previously cryopreserved PBMC were thawed for coculture, washed thoroughly, and labeled with 0.25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescent dye (Invitrogen). CFSE-labeled cells were washed thoroughly and resuspended at 2 x 10^6 cells/tube in CM and stimulated for 24 hours with 1 μg/ml SEB at 37°C. Cells were then washed, resuspended in 200 μl of HIV-1 BaL stock (0.26 Multiplicity of Infection) and incubated at 37°C for 2 hours. The residual virus was then washed off and cells were resuspended in 1 ml of CM with 1 μg/ml SEB and incubated at 37°C for an additional 22 hours. BFA (10 μg/ml; Sigma-Aldrich) was added to all tubes during the last 4 hours before immediate analysis via flow cytometry. Stimulation was performed in the presence or absence of 1μM AZT or 10μg/ml anti-IL-2 antibody (Becton Dickinson).

Cell sorting

PBMC from 20 HIV-1+ subjects with active TB were thawed, washed twice in the presence of 50 U/ml Benzonase and resuspended at 3 x 10^6 cells/ml in CM. Costimulatory antibodies (αCD28 and αCD49d; 1 μg/ml final concentration) were added to cells before splitting them into different tubes for overnight stimulation at 37°C with either MTB antigens or no antigen. BFA (10 μg/ml) was added to all tubes 2 hours after stimulation. Following incubation, cells were washed and stained with pretitrated surface antibodies as described above. Cytokine-producing CD4+ T cells were sorted with a modified FACS Aria flow cytometer by gating tightly on live memory $CD3^+CD4^+$ T cells. MIP-$1\beta^+$TNF-α^+ memory CD4+ T cells were then sorted and all other memory CD4+ T cells were further delineated and sorted according to IFN-γ and IL-2 staining into IFN-γ^+IL-2^+MIP-$1\beta^-$, IFN-γ^+IL-2^-MIP-$1\beta^-$ and cytokine negative memory CD4+ T cells. Between 193 and 50,000 fixed, memory CD4+ T cells from each of the 4 different populations were collected depending on the number of PBMC available and the response level for each individual. Cells were then stored at -80°C prior to gag DNA analysis.

Quantification of HIV-1 gag viral DNA

HIV-1 DNA was quantified by qPCR with an ABI7700 (Perkin-Elmer) similar to a previously described protocol[28]. HIV-1 gag primers and probe were optimized for detection and quantification of East African subtype A and C strains. Gag primer position and sequences were 783gagF-GAGAGAGATGGGTGCGAGAGCGTC (Tm>60), 895gagR-CTKTCCAGCTCCCTGCTTGCCCA (Tm>60) and with a FAM labelled probe 844gagPr-ATTHG BTTAAGGCCAGGGGGAARGAAAMAAT. Sites where base sequences differed among HIV-1 isolates were made degenerate and are noted as follows: R (A or G), M (A or C), K (G or T), H (A or C or T), and B (C or G or T). To quantify the cell number in each reaction mix, the human albumin gene copy number was also assessed by qPCR. Albumin primer and probe sequences were as follows: hAlbFwd, TGCATGAGAAAACGCCAGTAA; hAlbRev, ATGGTCGCCTGTTCACCAA; and hAlbProbe, FAM-TGACAGAGTCACCAAATGCTGCACAGAA-BHQ1. Sorted T cells were lysed in 0.1 mg/ml proteinase K buffered with Tris-Cl for 1 h at 56°C and then for 10 min at 95°C to inactivate the enzyme. Five μl of lysate was used in a total reaction volume of 25 μl containing 12.5 μM primers, 5 μM probe, a 10 mM concentration of each deoxynucleoside triphosphate, 3.5 mM $MgCl_2$, 1.25 mM Blue 636 reference dye, and 0.625 U Platinum *Taq* in the supplied buffer.

Reaction conditions included 5 min activation at 95°C followed by 15 s at 95°C and 1 min at 60°C for 45 cycles. Quantification was generated using standard curves for HIV-1 gag and albumin.

Statistical analysis

Data analyses were carried out using GraphPad Prism version 4.0 software. Comparisons of two groups were performed using the Mann Whitney test. Comparisons of paired groups were performed using the Wilcoxon matched pairs test. Tests used for statistical analysis are described in the figure legends.

RESULTS

MTB-specific and CMV-specific CD4$^+$ T cell responses after HIV-1 infection

To determine the effect of HIV-1 on different pathogen-specific CD4$^+$ T cells, we first compared the frequencies of MTB- and CMV-specific CD4$^+$ T cell responses shortly before, early after, and during chronic HIV-1 infection in subjects without evidence of active TB or CMV disease. In 5 Tanzanian subjects who acquired HIV-1 infection during the study MTB- and CMV-specific responses were detectable at 3 months before the first HIV-1 seropositive study visit. During this baseline visit, the median frequencies of MTB- and CMV-specific memory CD4$^+$ T cells were 0.18% (range: 0.1-0.8) and 0.51% (range: 0.36-2.6), respectively. The median frequency of MTB-specific CD4$^+$ T cells decreased 89.4% (Fig. 1) within 6-12 months after HIV-1 seroconversion. MTB-specific CD4$^+$ T cells completely disappeared in 4 out of 5 subjects, and decreased more than 5-fold in the fifth subject. In contrast, CMV-specific CD4$^+$ T cells were still detectable after 6-12 months in all subjects with a median decrease of only 40.6% despite recent HIV-1 infection. In four subjects the frequencies of CMV-specific CD4$^+$ T cells were also determined during the first HIV-1 seropositive visit and/or at 3 or 6 months thereafter, but were always detectable (data not shown). These data demonstrate that the rate of *in vivo* CD4$^+$ T cell depletion early after HIV-1 infection can differ depending upon the specificity of the response.

The effect of HIV-1 infection on the frequencies of MTB- and CMV-specific CD4$^+$ T cells was also determined in a cross-sectional analysis of HIV-1 infected and uninfected Tanzanian subjects (n=17 HIV-1$^+$, 17 HIV-1$^-$, Fig. 1B and 1C). All subjects were clinically asymptomatic for MTB or CMV infection. HIV-1-infected subjects had a median CD4$^+$ T cell count of 640 cells/μl (range: 109-758). In line with previous reports[22], CMV-specific CD4$^+$ T cells were detectable in most samples and the median magnitude of the response was actually higher in HIV-1$^+$ than HIV-1$^-$ subjects (3.32% versus 1.72%, respectively). In contrast, HIV-1 infection was associated with significantly reduced magnitudes (p=0.009) and reduced prevalence of detectable MTB-specific responses. Six of 17 HIV-1$^+$ subjects had detectable MTB-specific CD4$^+$ T cell responses compared with 14 of 17 in HIV-1$^-$ subjects. Collectively these data demonstrate that early and chronic untreated HIV-1 infection is associated with severe depletion of MTB-specific CD4$^+$ T cell responses, whereas CMV-specific CD4$^+$ T cell responses tend to persist. These data suggest that MTB-specific CD4$^+$ T cells are more susceptible than CMV-specific CD4$^+$ T cells to *in vivo* HIV-1 infection and deletion.

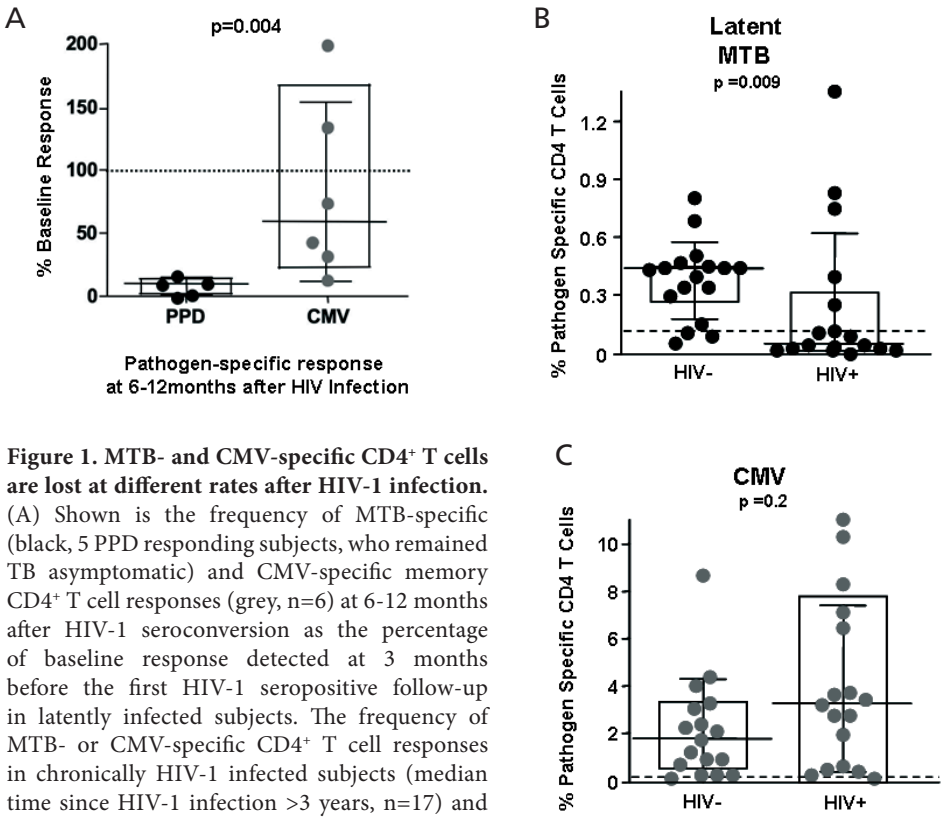


Figure 1. MTB- and CMV-specific CD4+ T cells are lost at different rates after HIV-1 infection. (A) Shown is the frequency of MTB-specific (black, 5 PPD responding subjects, who remained TB asymptomatic) and CMV-specific memory CD4+ T cell responses (grey, n=6) at 6-12 months after HIV-1 seroconversion as the percentage of baseline response detected at 3 months before the first HIV-1 seropositive follow-up in latently infected subjects. The frequency of MTB- or CMV-specific CD4+ T cell responses in chronically HIV-1 infected subjects (median time since HIV-1 infection >3 years, n=17) and a HIV-1- control group (n=17) is shown in (B) and (C), respectively. Memory CD4+ T cells were defined by expression of CD27 and CD45RO. IFN-γ+ memory CD4+ T cells were detected after *in vitro* stimulation of PBMC with Purified Protein Derivative or whole inactivated CMV virus and intracellular cytokine staining for IFN-γ. Statistical analysis was performed using the Mann-Whitney test.

Phenotype and function of MTB- and CMV-specific CD4+ T cells

To investigate factors that may be associated with the different rates of CD4+ T cell decline, we first assessed expression of the HIV-1 coreceptor, CCR5, on MTB- and CMV-specific CD4+ T cells. While CCR5 was present on CD4+ T cells of both specificities, there was no difference in the expression of CCR5 (percentage or mean fluorescent intensity) between CD4+ T cells of the two specificities (data not shown).

We next examined multiple other surface maturation markers and several functional characteristics within three different groups of pathogen-specific CD4+ T cells: MTB-specific CD4+ T cells in HIV-1- subjects in the absence of active TB disease; MTB-specific CD4+ T cells in association with active TB disease in HIV-1+ subjects; and CMV-specific CD4+ T cells (Fig. 2). Cellular maturation was studied using the differentiation markers CD27, CD45RO and CD57 that help to discriminate between

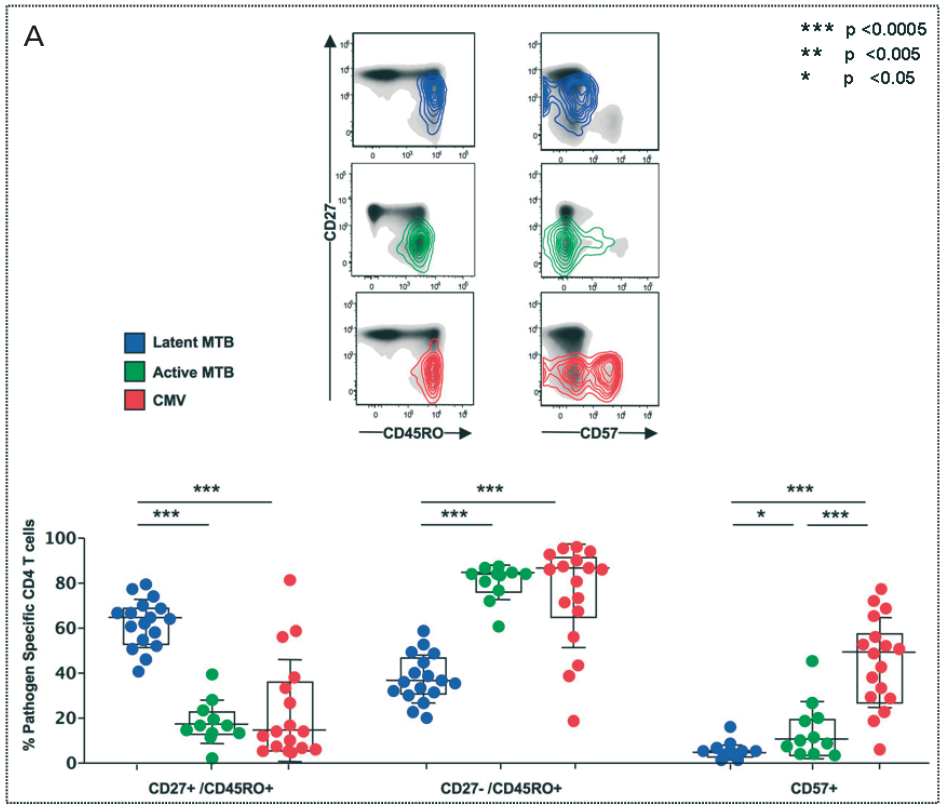
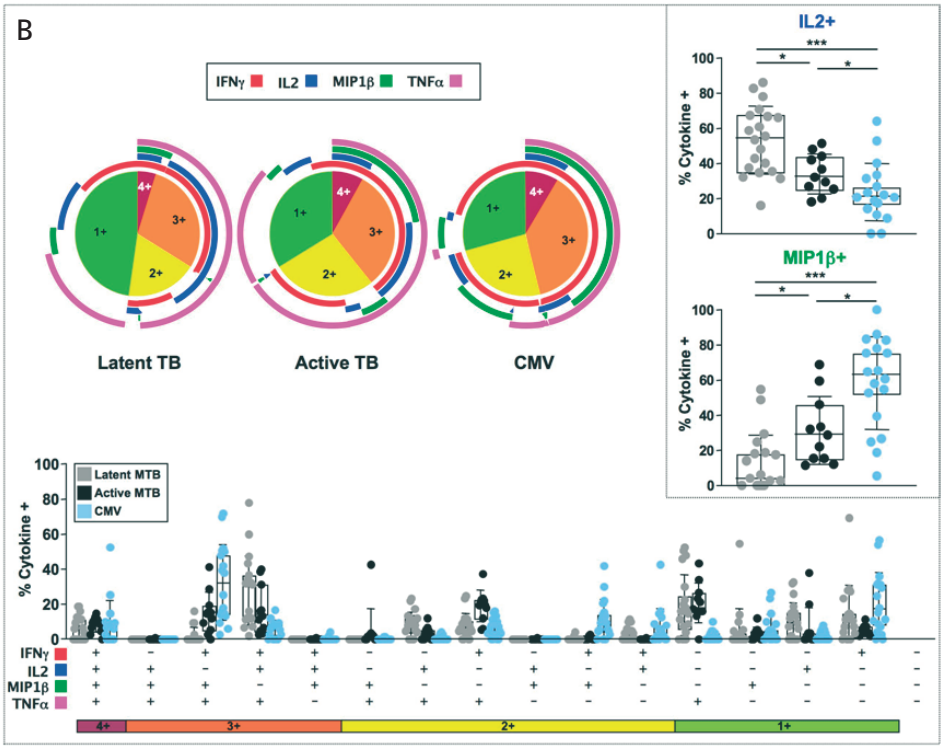


Figure 2. Differences in cellular maturation between MTB- and CMV-specific CD4⁺ T cells are diminished during active TB disease and are associated with diametrically opposed production of IL-2 and CCR5 ligand MIP-1β. The surface expression of maturation markers CD27, CD45RO and CD57 is shown in (A) for representative subjects as a density plot-overlay on total CD4⁺ T cells (black), for MTB-specific CD4⁺ T cells in the absence (blue, HIV-1⁻) or presence of active TB disease (green, HIV-1⁺), and for CMV-specific CD4⁺ T cells (red). The proportion of CD27⁺CD45RO⁺, CD27⁻CD45RO⁺ and CD57⁺ subsets within MTB- and CMV-specific CD4⁺ T cells from HIV-1⁻ subjects (n=17) and for HIV-1⁺ subjects with active TB (n=11, lower panel). (B) Flowcytometric analysis of IFN-γ, IL-2, MIP-1β and TNF-α production within pathogen-specific CD4⁺ T cells. PBMC from subjects with latent MTB infection (defined by positive response to RD1 antigens) or from HIV-1⁺ subjects with active TB were stimulated with MTB-antigens (PPD or a mix of PPD and RD1 peptide pools) or CMV whole antigen. The pie charts show the fraction of cells with 1, 2, 3 or 4 functions. The color coded circles indicate the proportion of the 4, 3, 2 and 1 functional responses response that is contributed by the single cytokines IFN-γ (red), IL-2 (blue), MIP-1β (green) and TNF-α (pink). The fraction of cells that produces MIP-1β (right upper panel) or IL-2 (right lower panel) is shown as percentage of total cytokine positive CD4⁺ T cells. The median is indicated. Further delineation of the 16 different possible cytokine combinations is shown in the lower panel. (C) Intracellular staining of IL-2 (y axis) and MIP-1β (x-axis) within CD27⁺CD45RO⁺, CD27⁻CD45RO⁺CD57⁻ and CD27⁻CD45RO⁺CD57⁺ CD4⁺ T cells after stimulation with PPD or CMV is shown for one representative subject (left panels) and for all studied responses further delineated by IL-2 and MIP-1β production as percent of total cytokine positive response (right panels).


B
IFNγ IL2 MIP1β TNFα
4+
3+
2+
1+
Latent TB
Active TB
CMV
IL2+
MIP1β+
% Cytokine +
Latent MTB
Active MTB
CMV
IFNγ
IL2
MIP1β
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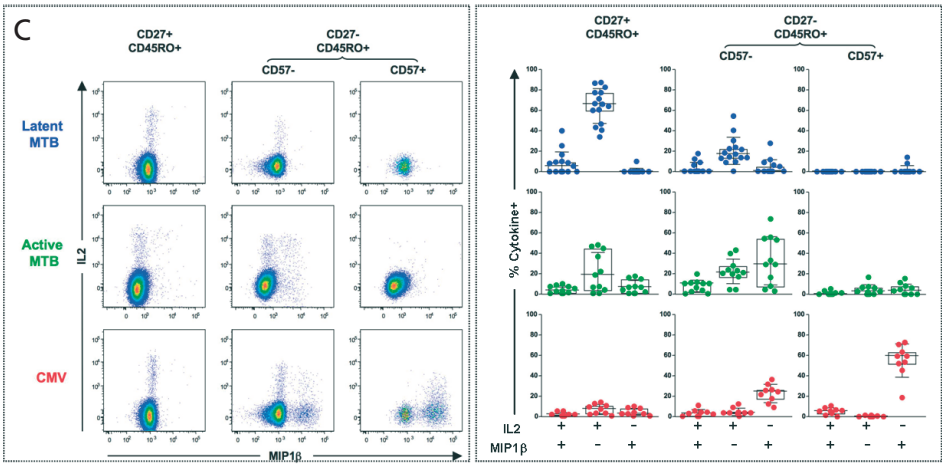
C
CD27+ CD45RO+
CD27- CD45RO+
CD57-
CD57+
Latent MTB
Active MTB
CMV
IL2
MIP1β
% Cytokine+

naïve ($CD27^+CD45RO^-$), central memory-like ($CD27^+CD45RO^+$), effector memory-like ($CD27^-CD45RO^+$) and terminally differentiated $CD4^+$ T cells ($CD57^+$)[29].

In HIV-1^- subjects with asymptomatic TB, the majority of MTB-specific $CD4^+$ T cells were $CD27^+CD45RO^+$ (median 64%) and thus had a central memory-like phenotype (Fig. 2A and B). A minority (median 36%) was $CD27^-CD45RO^+$; CD57 expression was not detectable or insignificant. In contrast, MTB-specific $CD4^+$ T cells in HIV-1^+ subjects with active TB were phenotypically distinct and were characterized by downregulation of CD27 (median 83% were $CD27^-$, $p<0.001$) and a significant increased expression of CD57 (median 9.5%, $p<0.05$). CMV-specific $CD4^+$ T cells also lacked CD27 expression (median 85.6% were $CD27^-$) and showed even more frequent expression of CD57 (median 48.4%).

To determine whether cell maturation was associated with functional changes within pathogen-specific $CD4^+$ T cells, we analyzed expression of IFN-γ, IL-2, TNF-α and MIP-1β within the three different groups of $CD4^+$ T cells and delineated the functions according to their maturation phenotype. As shown in figure 2C, diametrically opposed patterns of IL-2 and MIP-1β production were detected in MTB-specific and CMV-specific $CD4^+$ T cells among HIV-1^- TB asymptomatic subjects (right panel). Large fractions of CMV-specific $CD4^+$ T cells produced MIP-1β (median 62%), but few produced IL-2 (median 20%). In contrast most MTB-specific $CD4^+$ T cells in asymptomatic infection produced IL-2 ($p<0.001$, median 54%) but not MIP-1β ($p<0.001$, median 3%). Differences in MIP-1β and IL-2 production occurred throughout the 16 different possible cytokine combinations (lower panel). In contrast, no such differences were detected for TNF-α or IFN-γ production (data not shown). MTB-specific $CD4^+$ T cells in HIV-1^+ subjects with active TB were functionally distinct from those detected in HIV-1^- subjects during asymptomatic TB infection. The median fraction of MIP-$1\beta^+$ cells was higher (29%, $p<0.05$), whereas the median fraction of IL-2^+ cells was lower (32%, $p<0.05$) in HIV-1^+ subjects with active TB disease as compared with the response in HIV-1^- subjects with asymptomatic TB. Most notably, the subset of 3-functional cells that produced MIP-1β, IFN-γ and TNF-α but no IL-2, characteristic of the CMV-specific $CD4^+$ T cell response, was virtually absent from MTB-specific $CD4^+$ T cells during asymptomatic TB, but present during active TB.

Whether these distinct functional properties were associated with different cellular maturation was then analyzed by comparing pathogen-specific IL-2 and MIP-1β production by $CD27^+CD45RO^+$, $CD27^-CD45RO^+CD57^-$ and $CD27^-CD45RO^+CD57^+$ $CD4^+$ T cells. A representative dot plot for IL-2 and MIP-1β production for each group is shown in figure 2C (right panels). Independent of the pathogen-specificity, a $CD27^-$ phenotype was associated with more MIP-$1\beta^+$ and fewer IL-2^+ cells. Large fractions of $CD57^+$ $CD4^+$ T cells were only present within the CMV-specific response, and almost all expressed MIP-1β exclusively. Virtually none of the $CD57^+$ cells produced IL-2 irrespective of specificity. Together these data suggest that the capacity to produce IL-2 is reduced, whereas the capacity to produce MIP-1β is increased with maturation of pathogen-specific $CD4^+$ T cells.

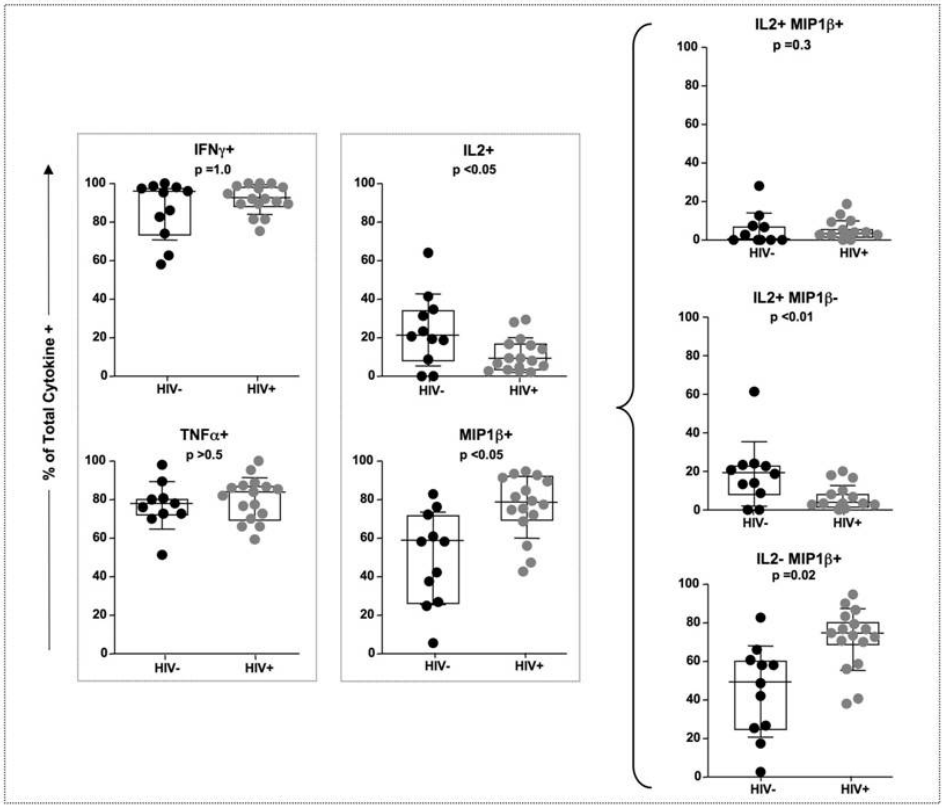

Figure 3. HIV-1 infection is characterized by increased fractions of MIP-1β+ and decreased fractions of IL-2+ CMV-specific CD4+ T cells. The fraction of IFN-γ^+, TNF-α^+, MIP-1β^+ or IL-2^+ among total cytokine positive CD4+ T cells (y-axis) is shown in Box & Whisker Plots (A) for HIV-1^- (n=11) and HIV-1^+ subjects (n=16) (left panels). The right panels show the fraction of IL-2^+MIP-$1\beta^+$, IL-2^+MIP-$1\beta^-$ and IL-2^-MIP-$1\beta^+$ among total cytokine positive CD4+ T cells. PBMC were stimulated overnight with whole inactivated CMV and the background was subtracted. Statistical analysis was performed using the Mann-Whitney test.

Effect of HIV-1 infection on CD4+ T cell function *in vivo*

Less IL-2 production and greater MIP-1β production among MTB-specific CD4+ T cells in HIV-1^+ versus HIV-1^- subjects, and between MTB and CMV-specific CD4+ T cells (Fig. 2) is consistent with the hypothesis that HIV-1 preferentially infects and depletes IL-2-producing CD4+ T cells and is partially inhibited from infecting and depleting MIP-1β-producing CD4+ T cells *in vivo*. We further addressed this possibility by comparing the proportion of CMV-specific cytokine-producing cells among HIV-1^+ and HIV-1^- subjects (Fig. 3). There was no difference in the proportion of IFN-γ- or TNF-α-producing CMV-specific CD4+ T cells in HIV-1^+ and HIV-1^- subjects. However there was a significantly smaller proportion of IL-2-producing cells ($p<0.05$, Fig. 3), and a significantly greater proportion of MIP-1β producing cells ($p<0.05$, Fig. 3) in HIV-1^+

versus HIV-1$^-$ subjects. This difference was reflected in cells that produced MIP-1β but not IL-2 and produced IL-2 but not MIP-1β, but not the cells that produced both effector molecules simultaneously (Fig. 3, right panel). These data are consistent with the hypothesis that with HIV-1 infection *in vivo*, IL-2-producing cells are more likely to get infected and depleted than MIP-1β-producing cells.

IL-2-dependence of HIV-1 infection *in vitro*

The *in vitro* inhibitory effects of CCR5 ligands on HIV-1 infection have been previously described[30-32]. To explore whether endogenous IL-2 production augments *in vitro* HIV-1 infection, we performed HIV-1 infection experiments in the presence or absence of a neutralizing anti-IL-2 antibody. SEB-stimulated PBMC were infected with the CCR5-tropic HIV-1 strain BAL and CD4$^+$ T cells were analyzed for HIV-1 p24 content in the context of cell division, CD25 and CCR5 surface expression (in the absence of exogenously added IL-2). The p24 staining in this system was the result of active viral replication, and not retained p24 from the virus inoculum, since it was inhibited by the addition of the reverse transcriptase inhibitor AZT (Fig. 4A). In the absence of the IL-2 neutralizing antibody, 48% of dividing cells (CFSE^{low}), but only 2.2% of the cells that had not undergone cell division were p24$^+$ (Fig. 4B, upper panel). Virtually all of these p24$^+$ cells expressed CD25 demonstrating that cellular HIV-1 infection occurred within cells that could respond to IL-2 signaling. Neutralization of IL-2 resulted in a 12.5-fold reduction of p24$^+$ cells and completely abrogated cell division (Fig. 4B, lower panel). The fraction of CD25$^+$ cells was reduced by 60% (Fig. 4B, lower panel), whereas

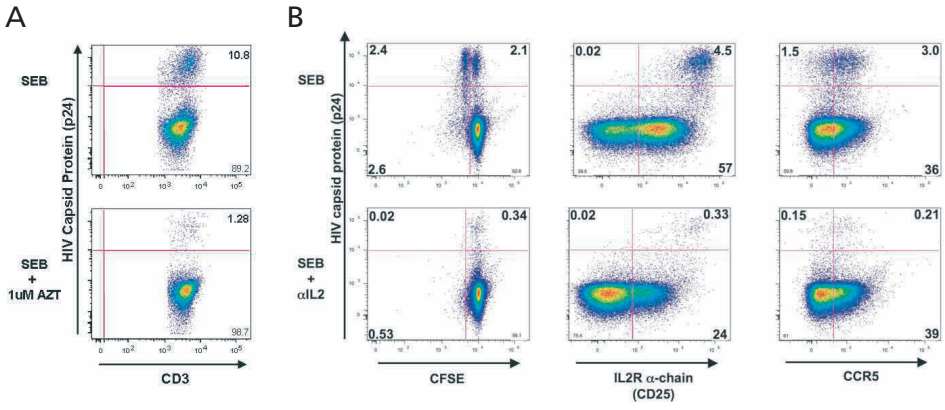

Figure 4. Productive HIV-1 infection of SEB-responding CD4$^+$ T cells is inhibited by *in vitro* neutralization of IL-2. Productive HIV-1 infection of CD4$^+$ T cells was analyzed after gating on CD4^{low} T cells and staining for HIV-1 CA-p24 (y-axis). Shown are dot plots from stimulated samples (A) in the presence or absence of 1μM AZT. (B) shows dot plots from stimulated samples in the presence (lower panel) or absence (upper panel) of a neutralizing anti-IL-2 antibody comparing CFSE-fluorescence intensity (x-axis, left panels), surface CD25 (x-axis, middle panels) and CCR5 (x-axis, right panels) with HIV-1 CA-p24 staining. PBMC samples were stimulated for 24h with SEB and then infected for another 24h with the CCR5-tropic HIV-1 strain BAL.

the fraction of CCR5+ cells was unaffected by the anti-IL-2 treatment, indicating that neutralization of IL-2 did not affect HIV-1 replication through modulation of the coreceptor, but by affecting post-entry events associated with cell division.

We next analyzed the expression of CD25 on IL-2+MIP-1β- and IL-2-MIP-1β+ CD4+ T cells after a 6 hr. SEB stimulation. Figure 5A shows a single experiment, and figure 5B the composite results from 7 experiments. These data demonstrate that a median of 39.5% of IL-2+MIP-1β-, but only 6.6% of MIP-1β+IL-2- CD4+ T cells express CD25 after stimulation. The expression of CD25 on the MIP-1β+IL-2- CD4+ T cells is the same as on all other non-responding memory CD4+ T cells, suggesting that IL-2 is specifically and intimately linked to expression of its high-affinity receptor, CD25.

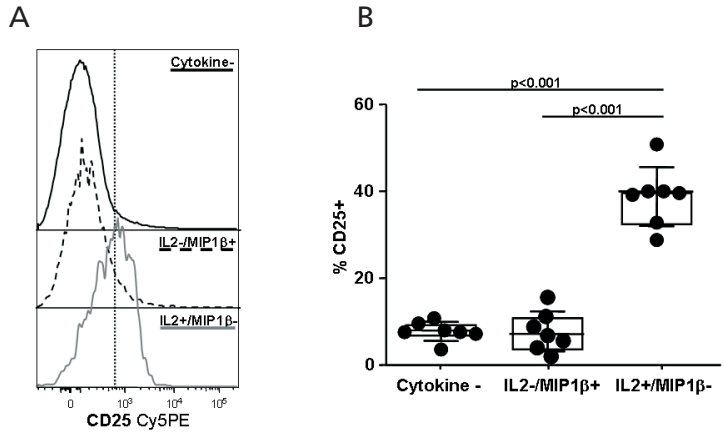

Figure 5. The capacity to secrete IL-2 by antigen-specific CD4+ T cells is associated with increased CD25 expression. Shown is (A) a representative histogram of the CD25 expression on cytokine negative, MIP-1β+IL-2- and MIP-1β-IL-2+ CD4+ T cells after 6h SEB stimulation of PBMC. The fraction of CD25+ CD4+ T cells among the same subsets is shown in a Box & Whisker Plot for 7 subjects in (B). Statistical analysis was performed using the Mann-Whitney test.

Cytokine expression delineates *in vivo* infection history of MTB-specific T cells

To determine *in vivo* cellular infection rates within MTB-specific and non-specific memory CD4+ T cells, we stimulated PBMC from HIV-1/MTB co-infected subjects with a mix of PPD, ESAT6 and CFP10, sorted the CD4+ T cells according to cytokine and chemokine expression (Fig. 6A) and quantified the HIV-1 gag DNA within the different subsets. Despite relatively low numbers of sorted cytokine/chemokine+ cells, we were able to quantify proviral DNA in 16 responding MTB-specific CD4+ T cell populations from 10 HIV-1 infected subjects with active TB. We first compared the number of HIV-1 gag DNA copies within MTB-specific, cytokine/chemokine+ CD4+ T cell samples with the non-responding CD4+ memory T cell compartment, identified by expression of CD27 and CD45RO. Twelve of 16 MTB-specific CD4+ T cell samples contained more

HIV-1 gag DNA copies than total memory CD4⁺ T cells. Next we determined whether MTB-specific CD4⁺ T cells expressing different patterns of IL-2 and MIP-1β contained different amounts of HIV-1 gag DNA. The median number of HIV-1 gag DNA copies per 10,000 cells was 1426.5 for IFN-γ⁺IL-2⁺MIP-1β⁻ (range 109-127500), 538 for IFN-γ⁺IL-2⁻MIP-1β⁻ (range 29-34167), 315 for MIP-1β⁺TNF-α⁺ (range 278 – 6136) and 268 for cytokine negative memory CD4⁺ T cells (range 8-19370). To reduce the inherent patient-to-patient variation in the HIV-1 gag DNA copy numbers and overcome the fact that many of the sorted samples contained too few cells to register a signal in the PCR assay, the results were expressed as a ratio of HIV-1 gag DNA copies in cytokine⁺/cytokine⁻ cells (Fig. 6B). Consistent with the raw data calculations described above, the IL-2-producing MTB-specific CD4⁺ T cells tended to harbor greater numbers of HIV-1

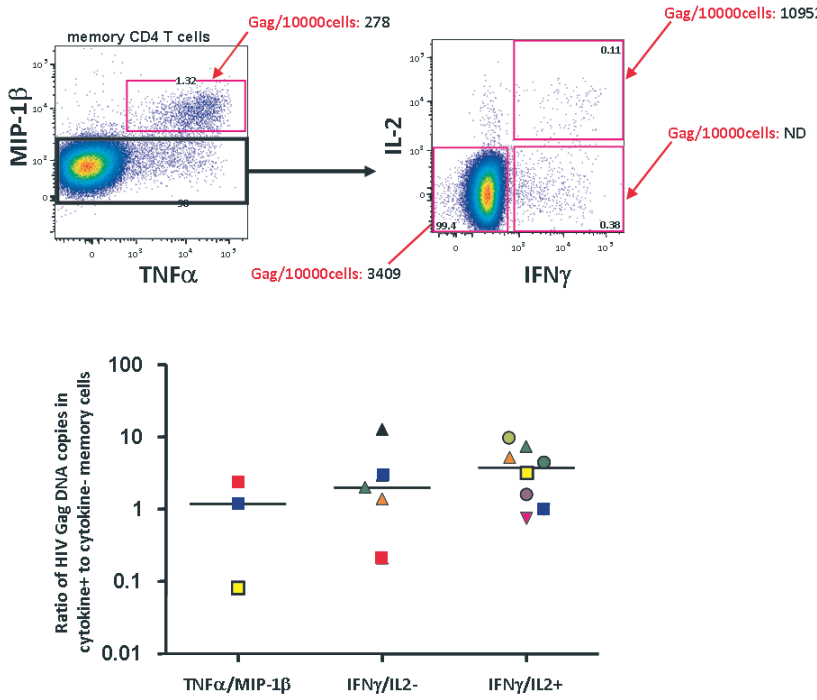

Figure 6. *In vivo* HIV-1 gag DNA in MTB-specific CD4⁺ T cells. The upper panel shows the gating/sorting strategy used during the sorting of different memory CD4⁺ T cell populations delineated by IFN-γ, IL-2 and MIP-1β production. MIP-1β negative memory CD4⁺ T cells were further delineated into IFN-γ⁺IL-2⁺, IFN-γ⁺IL-2⁻ and cytokine negative memory CD4⁺ T cells. The HIV-1 gag DNA/10,000 cells determined within these populations is indicated. The lower panel shows the ratio of HIV-1 gag copies/10,000 cells) detected within cytokine-positive CD4⁺ T cells divided to cytokine-negative memory T cells from 16 different cytokine-positive CD4⁺ T cell populations sorted from 10 HIV-1+ subjects (different symbols) with active TB. Memory CD4⁺ T cells were defined by expression of CD45RO and CD27. The PBMC were stimulated overnight with a mix of PPD and RD1 peptide pools O/N and further analyzed as described in the materials and methods section.

gag copies per cell as compared with the IL-2- and MIP-1β+ cells, though this trend was not statistically significant within the small number of subjects. Together these data support the hypothesis that the functional characteristics, and in particular the capacity to secrete IL-2 but not MIP-1β upon MTB-specific stimulation, are associated with increased susceptibility to HIV-1 infection.

DISCUSSION

We have recently demonstrated that MTB-specific CD4+ T cells are depleted early after HIV-1 infection and hypothesized that this loss might be caused by direct HIV-1 infection of this cell subset[20]. In contrast, CMV-specific CD4+ T cells are frequently detectable during chronic HIV-1 infection[22] and contain comparatively little proviral DNA[28]. We thus explored which functional and phenotypic characteristics might account for the rapid depletion of MTB-specific CD4+ T cells after HIV-1 infection and tested the hypothesis that differences in specific functions might contribute to differential HIV-1 infection of antigen-specific CD4+ T cells *in vivo*. Our results show that MTB-specific CD4+ T cells have higher infection rates compared with the total memory compartment and are comparable to those observed in HIV-1 specific CD4+ T cells[28] (median ratio 3.4).

We have previously shown that MTB-specific CD4+ T cells express comparatively high levels of the HIV-1 coreceptor CCR5. However, CCR5 expression was similar on MTB- and CMV-specific CD4+ T cells (data not shown) indicating that coreceptor expression alone could not account for any difference in rates of HIV-1 infection between MTB- and CMV-specific CD4+ T cells. Instead, we found significant differences in the capacity of MTB- and CMV-specific CD4+ T cells to produce IL-2 and CCR5 ligand MIP-1β. CMV-specific CD4+ T cells tended to produce MIP-1β but little IL-2, and displayed an effector memory phenotype, whereas MTB-specific CD4+ T cells produced IL-2 but less MIP-1β, and were less mature. Production of IL-2 was associated with a greater frequency of HIV-1 gag DNA within MTB-specific CD4+ T cells. These observations, together with the striking differences in the depletion dynamics between MTB- and CMV-specific CD4+ T cells early after HIV-1 infection, suggest that these differences in function might contribute to differential cellular susceptibility to HIV-1 infection and depletion of these two cell populations, and the associated differences in disease onset caused by these opportunistic pathogens.

The capacity of CCR5 ligands to block HIV-1 replication *in vitro* is well established[30,31]. Indeed CD4+ T cells with a relative resistance to CCR5-tropic HIV-1 strains are capable of producing comparatively high amounts of such CCR5 ligands, including MIP-1β[32]. We have recently shown that CMV-specific CD4+ T cells that produce MIP-1β and MIP-1α contain approximately 10-fold less HIV-1 gag DNA than CMV-specific CD4+ T cells that do not produce these chemokines[33]. These data indicate that autocrine production of MIP-1β protects CD4+ T cells from HIV-1 infection *in vivo*. Thus the lack of MIP-1β production by the less mature MTB-specific CD4+ T cells might contribute to their increased susceptibility to HIV-1 infection and subsequent depletion. In contrast, the production of MIP-1β by the more mature CMV-specific

$CD4^+$ T cells is likely to contribute to their relative resistance to HIV-1 infection[28,33] and thus to their persistence after HIV-1 infection. Indeed, as a common theme, the tendency of more differentiated $CD4^+$ T cells to produce MIP-1β could explain the previously described low HIV-1 infection rates within $CD57^+$ $CD4^+$ T cells *in vivo*[29].

IL-2 signaling pathway and HIV-1 infection

Whereas MTB-specific $CD4^+$ T cells lacked the capacity to produce MIP-1β in individuals with latent MTB infection, they had a remarkable capacity to produce IL-2. IL-2 and its high affinity IL-2 receptor α-chain CD25, whose expression rapidly increases upon T cell receptor engagement, are important in driving T cells into the cell cycle. In addition to being critical for T cell proliferation, IL-2 may also positively impact upon HIV-1 reverse transcription[34] by increasing the concentration of dNTPs in $CD25^+$ $CD4^+$ T cells. Indeed, CD25 expression and productive HIV-1 infection appear to be linked; *in vitro* depletion of $CD25^+$ $CD4^+$ T cells efficiently blocks viral replication[35-37]. CD25 expression on $CD4^+$ T cells correlates with PPD-induced HIV-1 replication[38] and it was recently demonstrated that productive HIV-1 infection within lymphoid tissue explants occurred predominantly in $CD4^+$ T cells expressing CD25[39]. *In vivo*, $CD25^+$ $CD4^+$ T cells contain up to 7-fold more immunodeficiency virus during the acute infection, a phenomenon that was particularly apparent within lymph nodes[40]. Our observations that *in vitro* neutralization of IL-2 reduced CD25 up-regulation, abrogated antigen-specific $CD4^+$ T cell proliferation and almost completely abrogated productive HIV-1 infection of $CD4^+$ T cells, supports a central role for the IL-2/CD25 signaling pathway in the infection of antigen-specific $CD4^+$ T cells. Our finding that peripheral MTB-specific $CD4^+$ T cells with the capacity to produce IL-2, but not MIP-1β, contained the highest amount of HIV-1 gag DNA *in vivo* supports the conclusion that IL-2 expression by MTB-specific $CD4^+$ T cells leads to their increased susceptibility to HIV-1 infection and depletion. The capacity to produce IL-2 in the absence of MIP-1β might create a microenvironment that very efficiently drives the replication of HIV-1 within MTB-specific $CD4^+$ T cells. Indeed the enormous potential of MTB-specific immune responses to contribute to an explosive replication of a single virus quasispecies *in vivo* has been demonstrated within SIV-infected Rhesus macaques; up to 35% of viral quasispecies in PBMC was closely related to the single major species that was isolated at the site of the Tuberculin skin test[41].

Impact of function and phenotype on HIV-1 infection

Activated, CD25 expressing $CD4^+$ T cells that are HIV-1 infected have been shown to preferentially undergo apoptosis in lymphoid tissue explants[39] and SIV infection of $CD4^+$ T cells at mucosal sites can reach extremely high frequencies during acute infection[1]. Our observations support the hypothesis that MTB-specific $IL\text{-}2^+MIP\text{-}1\beta^-$ $CD4^+$ T cells might represent a pool of $CD4^+$ T cells that are highly susceptible to HIV-1 infection, and may become rapidly depleted upon HIV-1 infection before they can further differentiate into effector cells, a mechanism that has been proposed previously[42,43]. In this scenario the few MTB-specific $CD4^+$ T cells that avoid HIV-1 infection could go on to mature further, and in the process lose IL-2 expression and

gain expression of MIP-1β, simultaneously making them less susceptible to HIV-1 infection. The resulting shift from IL-2 expression to MIP-1β expression concomitant with loss of CD27 and gain of CD57 expression that is predicted by this model is exactly what we observed in MTB- and CMV-specific CD4+ T cells in individuals who were infected with HIV-1 (Fig. 2C). Collectively our observations support the hypothesis that antigen-specific IL-2 production, particularly in the absence of CCR5 ligand MIP-1β, contributes to the productive HIV-1 infection and subsequent depletion of MTB-specific CD4+ T cells early after HIV-1 seroconversion.

During clinically latent TB the majority of MTB-specific CD4+ T cells expressed CD27 and can be considered as having a central memory-like phenotype (CCR7 was not measured), but also expressed higher levels of CCR5 compared with the total memory compartment[20]. In contrast, CMV-specific CD4+ T cells had a highly differentiated effector memory-like phenotype. HIV-1 infection was associated with a significantly increased fraction of senescent, CD57+ cells[44] and MIP-1β+ cells, and a reduced fraction of cells with the capacity to produce IL-2. Similarly in HIV-1 patients with active TB, MTB-specific CD4+ T cells were characterized by a more mature CD27low phenotype, a detectable fraction of CD57+ and MIP-1β+ CD4+ T cells, and a reduced capacity to produce IL-2. These differences might be driven by the strength and persistence of T cell receptor engagement and thus the frequency and duration of pathogen reactivation might contribute to the phenotype and function of pathogen-specific CD4+ T cell responses; CMV viral replication can be detected in almost ~20% of HIV-1 infected subjects[45,46] and MTB reactivation might account for the change in phenotype and function in subjects with active as compared with latent TB (Fig. 2). In addition, the phenotypic and functional changes associated with recurring TCR engagement might lead to reduced HIV-1 susceptibility of CD4+ T cells. The loss of IL-2 and gain in MIP-1β production upon antigen-specific stimulation might protect the more differentiated CD4+ T cells from productive HIV-1 infection[44], whereas increased HIV-1 infection among those cells still capable of producing IL-2 might preferentially deplete these less mature, central memory-like CD4+ T cells.

Implications for vaccines

Preferential infection of vaccine induced HIV-1 specific CD4+ T cells might reduce efficiency of vaccine induced immunity. Our results help to define cellular parameters associated with HIV-1 infection and either depletion or persistence of antigen-specific CD4+ T cells. These findings could help define the type of CD4 response one would hope to induce with a HIV-1 vaccine. Indeed, just recently it has been shown that vaccination with replication competent, recombinant CMV was able to induce differentiated, MIP-1β-producing HIV-1 specific T cells that were associated with protection against SIV challenge[47].

In conclusion, our data suggest that specific functional and maturational characteristics of MTB-specific CD4+ T cells might contribute to increased HIV-1 susceptibility and their subsequent depletion after HIV-1 infection. Strong antigenic stimulation during active TB might obscure this depleting effect despite high infection rates of MTB-specific memory CD4+ T cells and at the same time might drive

phenotypic and functional changes that confer partial cellular resistance to HIV-1 infection. These results suggest that MTB-specific adaptive immunity is particularly vulnerable to HIV-1 associated immune damage.

ACKNOWLEDGEMENTS

We thank Mr. Weston Assisya for the coordination of field studies and Jutta Jung for coordinating TB diagnosis in Mbeya, Tanzania, We would also like to thank Drs John Tetteh, Sampson F. Ofori and Mike Ofori for donor recruitment and sample collection in Ghana. We also thank all donors for their contribution to the study. This study has been supported by the European Commission through grants ICA_CT-1999-10007, ICA_CT-2002-10048, E.C. DGVIII – AIDCO - SANTE/2006/129-931.

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