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Potential immune correlates of protection in HIV-1 infected individuals

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Claudia Dembek

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Abbreviation List

Abbreviation	Full name
´	Minutes
AIDS	Acquired immune deficiency syndrome
AI700	Alexa 700
APC	Allophycocyanin
ART	Antiretroviral therapy
AZT	Azidothymidine
BFA	Brefeldin A
CD	Cluster of differentiation
CMV	Cytomegalovirus
Cy 7	Cyanin 7
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISPOT	Enzyme Linked Immuno Spot Technique
EMA	Ethidium monoazide bromide
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDA	Food and Drug Administration
FSC	Forward scatter channel
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
ICS	Intracellular cytokine staining
IFN	Interferon
IL	Interleukin
LTNP	Long term non-progressor
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MIRA	CD45RA+ IFN-γ- IL-2- MIP-1β+ / CD45RA+ IFN-γ- MIP-1β+
ml	Milliliter
µl	Microliter
MVA	Modified Vaccinia virus Ankara
NP	Non-progressor
NRTI	Nucleoside reverse transcriptase inhibitor
PacB	Pacific blue
PBMC	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridininchlorophyll protein
PR	Progressor
RNA	Ribonucleic Acid
rpm	Rounds per minute
SCC	Sideward scatter channel
SHIV	Simian/human immunodeficiency virus
SIV	Simian immunodeficiency virus

1 Summary

The WHO/UNAIDS “Global summary of the AIDS epidemic” released in December 2008, estimates that 33.4 million people are currently living with HIV, some 2.7 million were newly infected in 2008 and 2.0 million people died of AIDS in the same year. In developed countries HIV-1 infected individuals are generally treated with highly active antiretroviral therapy (HAART, ART). This treatment reduces the viral load to undetectable levels but is not able to completely eradicate the virus, therefore, life-long administration of ART is required. Several problems are associated with ART: side effects, compliance, virus escape and high costs for developing countries where the majority of HIV-1 infected individuals reside. These considerations emphasize the need for an effective preventive or therapeutic vaccine against HIV-1, which remains elusive after more than two decades of research. A major problem in the development of a vaccine against HIV-1 is that immune correlates of protection from disease progression remain unknown.

The aim of the present study was to investigate the adaptive immune response directed to HIV-1 in order to get insights in possible correlates of immune control. Since T-cell mediated immunity is considered to play an important role in controlling HIV-1 infection and progression to AIDS, HIV-specific CD4+ and CD8+ T-cell responses were characterized using multiparameter flow cytometry.

In a cross-sectional set up, HIV-1 infected individuals with different levels of control of viral replication were analyzed: HIV-1 infected non-progressors (NP), progressors (PR) and ART-treated individuals. A distinctive feature of NP was the presence of Nef-specific CD45RA+ CD8+ T cells secreting MIP-1β but not IFN-γ. Since these cells were detected in response to Nef stimulation in 7 out of 11 NP, in only 1 out of 10 PR and were completely absent in 22 ART-treated patients they represent a possible immune correlate against disease progression. CD45RA+ IFN-γ- MIP-1β+ CD8+ T cells were termed MIRA (**MI**P-1β+ CD45**RA**+) CD8+ T cells. Characterization of MIRA CD8+ T cells revealed that they have limited capacity to degranulate and do not express the fas ligand. They provide

moderate anti-viral activity by MIP-1β but may prevent hyperactivation of the immune system in the absence of IFN-γ production.

In a longitudinal set up, the immune response of an HIV-1 infected partial controller was analyzed, who had been vaccinated with MVA-Nef and had MIRA CD8+ T cells in response to Nef-stimulation.

In this patient, following ART interruption, viral replication remained stable for three years but increased consistently in the subsequent two years until ART had to be re-initiated. The ART-free period was therefore divided into a phase of stable viremia and a loss of control phase. High frequencies of HIV-1-specific CD4+ and CD8+ T cells were not sufficient to prevent loss of viral control. Detailed analysis of these responses revealed a gradual loss of functionality in dominant HIV-1-specific CD4+ and CD8+ T-cell responses that was characterized by viral load-dependent decrease of IFN-γ and increase in MIP-1β production. Furthermore, loss of terminally differentiated HIV-1-specific CD8+ T cells occurred independently of viral load and possibly preceded the uncontrolled phase of infection.

This study highlights the possible correlates of protection from HIV-1 disease progression and contributes towards understanding the mechanisms involved in the failure of the host immune system.

2 Introduction

2.1 HIV

2.1.1 History of HIV and AIDS

The first cases of acquired immune deficiency syndrome (AIDS) were described in 1981 in San Francisco and New York. Today it is known that cases fitting to the description of AIDS existed at least four years before. Because the first reports [1], [2] described only homosexual patients suffering from pneumocystis carnii pneumonia, extensive mucosal candidis and multiple viral infections, the disease was associated to homosexuality and primarily named GRID (gay related immuno deficiency). Already in 1981, pneumocystis carinii pneumonia associated with an immuno deficient status was observed in a group of injecting drug users suggesting that the pathology was not restricted to the homosexual community [3]. In 1982 the pathology was observed in haemophiliacs [4] and in a 20-month old child that received multiple transfusion of blood [5]. This clarified the routes of disease transmission: sexual intercourse or blood interexchange with an infected person. In August 1982, the name AIDS (acquired immune deficiency syndrome) established, describing all known features of this new disease. In 1983, Montagnier and colleagues reported the discovery of a T-lymphotropic retrovirus in a patient at risk of AIDS; this was the virus that we now call human immuno deficiency virus (HIV) [6]. One year later, Gallo and colleagues proved this virus to be the etiologic agent of AIDS [7], [8], [9], [10] and succeeded to grow it in T-cell cultures. In March 1987, the U.S. Food and Drug Administration (FDA) approved azidothymidine (AZT), a nucleoside analog reverse transcriptase inhibitor (NRTI) as the first antiretroviral drug to be used as a treatment for AIDS. AZT decreased mortality and the frequency of opportunistic infections in patients with AIDS [11], nevertheless severe adverse reaction including hematologic toxic effects, nausea, myalgia, insomnia, and severe headaches were reported [12]. The protease inhibitor Saquinavir was approved for use by the FDA in 1995 [13]. This drug inhibits the viral protease, which

cleaves the viral precursor protein into mature viral particles. The administration of Saquinavir consequently causes the release of immature and noninfectious particles. The use of a combination of reverse transcriptase inhibitors together with protease inhibitors was the next obvious step, because the use of combined antiviral drugs should suppress viral replication to minimal levels to avoid viral escape. This new strategy named highly active antiretroviral therapy (HAART) gave impressive results and numerous reports from clinical trials using HAART were presented in the 11th International Conference on AIDS in Vancouver (1996). In 1997, the widespread use of HAART in developed countries drastically reduced AIDS related morbidity and mortality. In 2003, Enfuvirtide, a drug inhibiting the fusion of HIV and the host-cell was approved by the FDA [14]. Despite the tremendous advances in the development of life-extending Anti-HIV-pharmaceutics, HIV pandemic is still a major global burden. The numbers of HIV infected persons increased continuously until these days.

By the end of 2008, 33.4 millions of people are living with HIV. 2.7 million new infections were estimated worldwide in the year 2008. An estimated 1.9 million people became newly infected only in sub-Saharan Africa, where AIDS is still the largest cause of mortality (UNAIDS/WHO/2008). A vaccine against HIV is urgently needed.

2.1.2 Course of untreated HIV infection

The clinical course of HIV infection can be divided into three stages: an early, acute stage; an asymptomatic, latent stage; and a late, immuno deficiency stage [15] (Figure 1). The typical course of untreated HIV infection begins with an acute influenza-like viral illness in the first few weeks after infection. This phase is also called seroconversion and features high titers of virus in the peripheral blood. An adaptive HIV-specific CD8+ T-cell immune response develops which follows the peak of the initial burst of viral load. Viremia is usually controlled few weeks after infection and levels of CD4+ T-cell counts are largely restored, but the immune response fails to eradicate the virus and HIV-1 infection enters a phase of clinical

latency. With the progressive decline of CD4+ T-cell counts after the asymptomatic phase, symptoms describing AIDS such as the susceptibility for opportunistic infections, B-cell lymphoma and the caposi-sarcoma become more frequent and finally cause death.

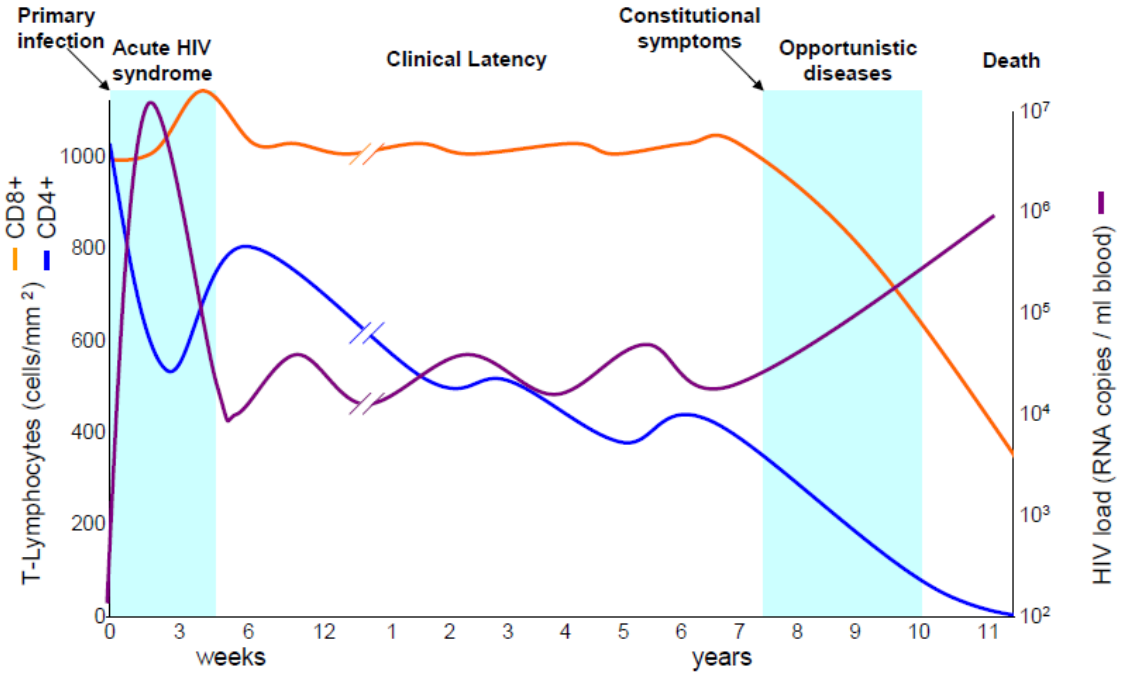


Figure 1: Clinical course of untreated HIV-1 infection. During the period following primary infection, HIV spreads widely in the body and an abrupt decrease in CD4+ T cells in the peripheral circulation is usual. An immune response to HIV rises ensuring a decrease in detectable viremia. During the following period of clinical latency follows, CD4+ T-cell counts continue to decrease, until they fall to a critical level below which there is a substantial hazard of opportunistic infections.

2.1.3 Human immuno deficiency virus

2.1.3.1 Subtypes

HIV is divided into two distinct subtypes: HIV-1 and HIV-2. HIV-1 infection is predominant and spread worldwide. It is the major cause of AIDS in humans. On the contrary HIV-2 infection is usually characterized by a long clinical latent period of 10 years or more, resulting in a mortality rate two-thirds lower compared to that for HIV-1 [16], [17], [18]. Indeed HIV-2 infection often does not progress to AIDS [19]. In HIV-2 infected patients the observed viral load is typically lower when compared to HIV-1 infected patients. HIV-2 has been

isolated primarily in West Africa countries such as Senegal, Guinea Bissau and Ivory Coast, with some cases also identified in the USA and Western Europe.
Because HIV-1 displays the far greater global burden compared to HIV-2, the present study, as the majority of studies on HIV worldwide, concerns only HIV-1. HIV-1 is divided into three subgroups: the major subgroup (M) and two minor subgroups (N and O). HIV-1 subgroup M has been further divided into more than 10 clades with distinct global distribution. In northern Europe and the United States the predominant HIV-1 clade is B. In Africa most of the HIV-1 clades are found, with the predominance of clade C. In Asia HIV-1 infections are predominated by clade C and E. Due to the predominance of HIV-1 clade B in Western Europe and the United States, the majority of studies on HIV are currently performed on this HIV subtype, so is the present study.

2.1.3.2 Genome

2.1.3.2.1 HIV genome

The genomeof HIV is approximately 10Kb of size and consists of nine viral genes (Figure 2). *env and gag* genes encode **structural proteins** and viral enzymes which are essential components of the retroviral particle.

- The *env* gene codes for a glycoprotein called gp160. gp160 is cleaved in the host ER to form the two envelop proteins gp120 and gp41. The gp120/gp41 complex enables the virus to attach to and fuse with target cells [20].
- The *gag* gene codes for the two internal core proteins p17 (matrix protein) and p24 (capsid). The capsid protein p24 is used as antigen for serological tests. Furthermore, *gag* encodes the nucleocapsid proteins p6 and p7 which are tightly bound to the viral RNA.

The **regulatory proteins**, encoded in *pol*, *tat* and *rev* genes modulate transcriptional and posttranscriptional steps of virus gene expression and are essential for virus propagation.

- *pol* is one of the main retroviral genes. It encodes four proteins: the RNAse H - which breaks down the retroviral genome following infection of a cell, the reverse transcriptase, which synthesizes DNA by using the viral RNA as a template, the integrase that integrates viral into cellular DNA, and the protease that cleaves the various viral precursor proteins.
- The *tat* gene encodes the two Tat proteins p16 and p14, which are transcriptional transactivators for the long terminal repeat (LTR) promoter and activate transcription of viral genes [21].
- The Rev protein encoded in the *rev* gene is involved in shuttling RNAs from the nucleus and the cytoplasm [22].

Accessory proteins encoded in *vif*, *vpr*, *vpu* and *nef* genes are conserved in the different isolates, suggesting an important role *in vivo* although these proteins are not necessary for viral propagation in tissue culture.

- The Nef protein encoded in the *nef* gene down-regulates CD4 [23], [24] as well as the MHC class I and class II molecules [25], [26], [27]. Furthermore Nef induces apoptosis in uninfected CD4 and CD8 T cells [28], [29]. Detailed description of Nef can be found in the next section 1.1.3.2.2 HIV-Nef.
- The *vif* gene codes for the Vif protein. Vif enhances infectivity by preventing the action of APOBEC3G in infected cells. APOBEC3G is a cellular protein that causes hypermutation by deaminating cytosins in both mRNA and retroviral DNA. Thereby these molecules are inactivated and infectivity is reduced [150].
- The Vpr protein is incoded in the *vpr* gene. Vpr transports viral core from cytoplasm into nucleus in non-dividing cells and arrests cell division at G2/M [150].
- The *vpu* gene encodes the Vpu protein, which enhances the release of new virus particles from infected cells [150].

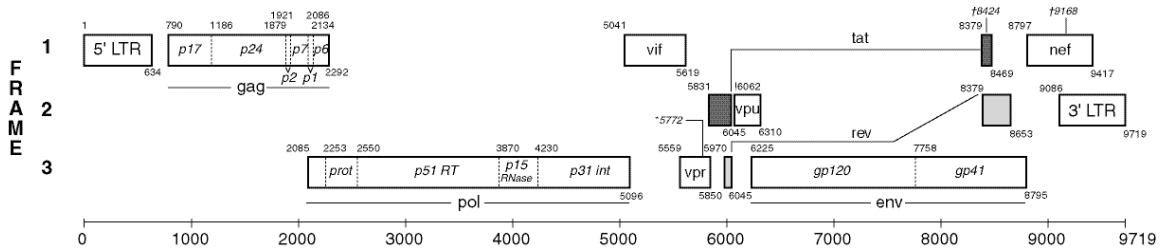


Figure 2: Illustration of the HIV-1 genome showing the organization of genes and their transcriptional splicing (dashed lines).
Source: http://www.hiv.lanl.gov/content/sequence/HIV/IMAGES/hxb2genome.gif

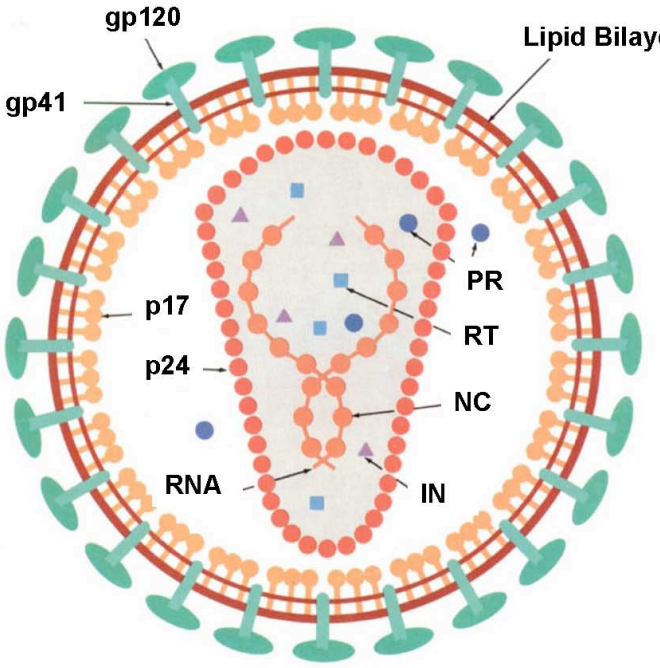
2.1.3.2.2 HIV-Nef

The *nef* gene encodes for the accessory protein Nef, originally named “negative factor”. Nef is a virulence factor, critical for viral replication and pathogenesis. The pathogenicity of Nef was mainly shown by the lack of disease progression in patients infected with Nef-deficient or -altered HIV [30], [31]. Data from SIV Nef vaccinated macaques provided evidence that Nef-specific CD8+ T cells might contribute to the control of SIV infection [32]. Nef is myristoylated and primarily localized in the paranuclear region and the plasma membrane. It basically serves as adaptor protein to divert host cell proteins to perverted functions leading to viral replication [33], [34]. By now there are four in vitro activities of HIV-1 Nef that have been extensively documented: 1) Nef downregulates cell surface levels of CD4, the primary viral receptor [35], [36], [37], [38], [39]. 2) Nef downregulates the expression of major histocompatibility complex class I (MHC-I) molecules on the cell surface [40], [26], [41]. 3) Nef activates the infected cell and mediates cellular signaling [42], [43], [44], [45] and 4) Nef enhances viral particle infectivity [46], [47], [48]. Nef is one of the first HIV proteins to be produced in infected cells and represents together with gag the most immunogenic target for the host immune response [49]. Due to these features, the *Nef* gene has been employed in several approaches to generate a HIV vaccine. Nef represents further a candidate HIV vaccine within the AIDS Vaccine Integrated Project (AVIP, http://www.avip-eu.org), by which this work was funded. Therefore and due to the

discribed antigenic characteristics of Nef the present study focus on Nef-specific T-cell responses.

2.1.3.3 Structure

HIV is a lentivirus belonging to the family of retroviruses. Its structure is in detail described Figure 3. HIV is roughly spherical and approximately 120 nm in diameter. The virus is composed of two copies of positive single-stranded RNA enclosed by a conical capsid, which is composed of subunits of the viral protein p24. A matrix composed of viral protein p17-subunits surrounds the capsid ensuring the integrity of the virion particle. In turn, the capsid is surrounded by the viral envelope, a host-cell originated plasma membrane. Proteins from the host cell are embedded in the viral envelope together with the complex HIV protein Env that protrudes through the surface of the virus particle. Env consists of a cap consisting of three molecules glycoprotein (gp) 120, and a stem made of three molecules gp41 that anchors the structure into the viral envelope [20].



ORF	protein	ORF	protein	ORF	protein
pol	PR (protease) RT (reverse transcriptase) IN (integrase)	*gag*	p17 p24 NC (nucleo capsid)	*env*	gp120 gp41

Figure 3: Structure of an HIV particle. HIV is composed of two copies of positive single-stranded RNA enclosed by a capsid comprising the viral protein p24. The RNA is tightly bound to the nucleocapsid proteins (NC), p6, p7. Enzymes that are crucial for the development of the virion, such as reverse transcriptase (RT) and integrase (IN) are enclosed in the capsid. The viral enzyme protease (PR) is present in the capsid as well as in the compartment between capsid and a matrix composed of an association of the viral protein p17 which surrounds the capsid, ensuring the integrity of the virion particle. The envelope is generated when the capsid buds from the host cell, capturing some of the host-cell membrane with it (lipid bilayer). The envelope contains the glycoproteins gp120 and gp41. ORF: open reading frame.
Source http://www.chm.tcu.edu/HIV.jpg

2.1.3.4 Life cycle

HIV infects macrophages, CD4+ T cells and asterocytes. Also dendritic cells play an important role in capturing and transporting the virus to lymphoid organs. The infection of HIV begins with the binding of the trimeric envelope complex gp120 to the CD4 surface molecule on macrophages or T-lymphocytes [50], triggering a conformational change that exposes the coreceptor binding site of gp120. Env subsequently interacts with the chemokine receptor CCR5 or CXCR4 [51], [52]. According to the coreceptors CCR5 and CXCR4 two phenotypic variants of

HIV-1, are distinguished: R5 (M tropic) and X4 (T tropic) viruses, respectively. Generally, recently infected individuals harbor a R5 virus while the more pathogenic X4 viruses predominate in the late stages of the disease. Mutations in the CCR5 encoding gene, such as the well described “delta32” mutation provide the individual with protection from HIV infection or disease progression [53]. The interactions with these receptors in turn cause conformational changes in envelope complex gp41, which mediates fusion between the virus and the host cell [54] and allows the virion the entry into the cell. Once internalized, HIV is uncoated. The viral single stranded RNA genome is transcribed into double stranded DNA by the enzyme reverse transcriptase. Following the formation of a pre-integration complex and transportation to the nucleus, the viral genome integrates into the host genome, mediated by the viral integrase. Early multiply spliced mRNAs are transcribed inducing a weak transcription of the provirus, which allows the translation of the regulatory proteins Tat and Rev, T-cell activation promotes this process. The proteins Tat and Rev are imported into the nuceleus. Tat increases transcription of viral mRNAs and Rev mediates export of unspliced and singly spliced viral mRNAs. Viral mRNAs are translated into several large polyproteins. Viral genomic RNA, proteins, and cellular factors including tRNA (the obligate primer for reverse transcription) assemble in the following at the plasma membrane. Cleavage of the Gag and Pol polyproteins, mediated by the viral protease occurs as the immature virion buds from the cell membrane. This cleavage process results in a mature, infectious virion [55].

2.2 Vaccines against HIV

2.2.1 Examples of HIV vaccines

Neutralizing antibodies were the first choice for vaccine induced immunity against HIV. Antibodies able to neutralize HIV are exclusively directed against the viral envelope. Therefore, initial efforts to identify a vaccine were directed toward the development of a vaccine based on recombinant envelope proteins. The first

vaccines against HIV-1 tested in two large phase III trials in USA and Thailand were the VaxGen vaccines AIDSVAX B/B and AIDSVAX B/E [56], [57]. These vaccines contained recombinant forms of the HIV surface protein gp120, which resemble the gp120 proteins found on HIV strain subtypes B and E, common in Asia. Unfortunately, both phase III trials failed to reduce the infection rates in individuals receiving the vaccine in comparison to the placebo groups. The vaccines were unable to elicit competent antibodies to neutralize primary isolates in vitro.

DNA vaccination was originally shown to induce strong and protective CD8+ T-cell responses in murine models [58]. When transferred to non-human primates and humans, unfortunately DNA vaccination protocols failed to induce optimal cellular and humoral immune responses and nowadays the main challenge is to increase the potency of this vaccination strategy [59].

Vaccines produced in viral vectors derived from recombinant Adenovirus (rAd5), mammalian pox-virus (MVA, NYVAC), Avian pox-virus (ALVAC, Fowlpox), etc have been purposely chosen for the development of vaccines against HIV, because these viruses are replication-defective in human cells. MVA as example has suffered during passaging a multitude of mutations within its genome. Six major deletions result in the loss of 15% (30kbp) of the original genetic information. The deletions affect a number of host range and virulence genes. As a consequence, MVA exhibits a severely restricted host range, and replicates only very poorly, if at all, in most mammalian cell types, including primary human cells and most transformed human cell lines [60], [61]. The idea in using viral vector vaccines is to stimulate the immune system's killer CD8+ T cells to hunt for the virus more aggressively. Since the vaccine virus cannot replicate in the host cell, the antigen dose is orders of magnitudes less than can be achieved by live attenuated viral vaccines. MVA and Ad5 vectors have been often used in a prime-boost strategy in order to boost immune responses previously elicited by DNA vaccines. Preclinical trials in monkey models performed with recombinant MVA and recombinant Ad5 had promising results. MVA and Ad5 carrying different HIV-1 antigens have been used in phase I and II clinical trials. Both

vectors were considered as safe and able to elicit immune responses to the inserted antigens. Nevertheless the STEP trial [62], [63], [64] is a bad example of a HIV vaccine study failing in a phase III clinical trial. Sponsored by pharmaceutical giant Merck & Co. and the federally funded HIV Vaccine Trials Network (HVTN), the STEP trial was a large phase III clinical trial, using recombinant adenovirus rAd5 expressing the HIV-1 *gag*, *pol* and *Nef* genes. The study enrolled 3000 healthy volunteers. In September 2007 the trial was stopped, because the data and safety monitoring board concluded after its first interim analysis, that the vaccine did not prevent HIV-1 infection. The results further revealed that a preexisting immunity against adenovirus resulted in a three times higher risk of HIV-1 infection in the vaccine group compared to the placebo group. This example once again shows that the way towards an effective vaccine is without question very difficult. However encouraging results in the AIDS vaccine field were recently presented by Rerks-Ngarm and colleagues [65]. In a large scale placebo controlled efficacy trial the group combined a recombinant canarypox vector vaccine (ALVAC-HIV) with a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) in a prime boost vaccination setting and reports a trend toward the prevention of HIV-1 infection among the vaccine recipients, with a vaccine efficacy of 26.4% (intention-to-treat analysis involving 16,402 patients), 26.2% (perprotocol analysis involving 12,542 patients) or 31.2% (modified intention-to-treat analysis involving 16,395 patients). Nevertheless, besides these supporting results there is still no vaccine available reducing the risk of HIV infection.

2.2.2 Challenges to an AIDS vaccine

Compared to other viruses like polio, measles, Hepatitis B, influenza and mumps, against which effective immunizations succeed to elicit protective immune and memory immune responses, HIV features several characteristics handicapping the development of an effective vaccine.

2.2.2.1 Sequence diversity

First, the error-prone reverse transcriptase (RT) activity causes an enormous sequence diversity. The mutation rate is approximately $3x10^{-5}$ per nucleotide base per cycle of replication [66]. The capacity of HIV for mutation is further enhanced by the high propensity for recombination and an extremely rapid turnover in vivo. Viruses within the same HIV-1 subtype can vary up to 20% and especially in places where different HIV-1 clades exist, the highly variable envelope protein can differ by up to 38%.

2.2.2.2 Infection of critical immune cells

HIV specifically targets CD4+ T-lymphocytes. These cells exhibit critical T-helper functions important for the initiation of cytotoxic T-Lymphocyte and thymus dependant responses. Already within the first days of HIV exposure, massive infection and loss of memory CD4+ T cells occur, resulting in a considerable immune impairment in early HIV infection [67].

2.2.2.3 Immune avoidance

HIV evades the recognition of the host immune system by several ways. The HIV accessory protein Nef for example mediates down regulation of MHC class I molecules [40], which are crucial for T-cell recognition of infected cells.

The surface envelope protein of HIV, target for neutralizing antibodies, is highly glycosylated and large carbohydrate molecules serve to mask potential protein epitopes. Antibody recognition of the HIV envelope is further hindered by variable regions in Env and enormous sequence variation among different HIV clades. An effective vaccine induced antibody response must therefore be able to bind and neutralize millions of different viruses present in the global pandemic [68].

2.2.2.4 Latency

One of the greatest challenges of the immune system in coping with HIV represents the latent reservoir of infected lymphocytes by the integration of the viral genetic material into the host chromosome. This process is irreversible. It

occurs immediately after infection and ends with the cell death. Life-long infection of the host is therewith maintained, even in the case of antiretroviral medication [69]. During the latent phase, viral proteins are not expressed, but infectious viral particles can be produced as soon as the T-cell becomes activated. The integrated proviral DNA forms an archive of all viruses infected the host during lifetime.

2.2.2.5 Lack of suitable animal models

The most convenient animal models consist of infection of rhesus macaques with a simian immuno deficiency virus (SIV). Although these models are useful for particular applications, the fact that SIV is distinct from HIV-1 represents a significant limitation to their use.

In order to create an animal model mimicking human infection Reiman et al. generated a chimeric simian/human immuno deficiency virus (SHIV) [70]. The new virus SHIV89.6P is composed of SIVmac239 expressing HIV-1 *env*, *tat*, *vpu* and *rev*. The main difference between infection of macaques by SHIV89.6P and HIV infection in humans is the development of AIDS. Since AIDS develops within months in SHIV infected macaques, the SHIV induced disease is an acute infection. HIV infection in humans results in AIDS after years; the cause why HIV induced disease is chronic. By now, an animal model that can be progressively infected by HIV remains elusive.

2.3 Correlates of immune control

Prior to evaluate HIV/AIDS vaccine formulations in humans it is necessary to identify immune correlates of protection against HIV. At present time, no clear correlates of protection or viral control exist.

One possibility to find correlates of protection is to study the HIV-specific immune response of individuals that show different degrees of control of HIV-1 infection.

2.3.1 Patient cohorts with different degrees of HIV control

2.3.1.1 Long term non-progressors

Long term non-progressor (LTNP, NP) represent a small proportion of the HIV-1 infected individuals (1-5%). The status is usually characterized by a documented HIV-1 infection for more than seven years, low levels of plasma viral load, high and stable CD4+ T-cell counts (above 600 cells/µl), naivety to antiretroviral therapy and no symptoms of AIDS disease. Responsible for the non-progressive status can be the host genetic predisposition, the nature of the virus, a particular host immune response or a combination of the listed factors. While some genetic markers in the host and the virus have been clearly associated with a slow progression to AIDS, immunological markers are more difficult to track and up to now, immunological markers of protection or slow disease progression remain unknown. However, the example of LTNP demonstrates that control of viral replication by the immune system in the context of the natural course of the disease is possible.

2.3.1.2 Elite controllers

Like LTNP, "elite" or "natural controllers" are infected with HIV-1 for 15 to 20 years, maintain high CD4+ and CD8+ T-cell counts and remain therapy naive. These individuals show plasma HIV-RNA values persistently below 50 copies/ml throughout the course of infection. Elite controllers represent much less than 1% of the total HIV-1 infected population [71].

2.3.1.3 Viremic controllers

These HIV-1 infected individuals are characterized by maintaining moderate plasma viral load (ca 10.000 - 50.000 copies/ml) and stable CD4+ T-cell counts (above 500 cells/µl) for several years. In the presence of viral replication, the HIV-1-specific immune response might be effective in these patients.

2.3.1.4 Progressors

Progressors (PR) are untreated HIV-1 infected patients, which do not control HIV-1 replication. They are characterized by high plasma viral load (over 100.000 copies/ml) and low CD4+ counts (around 200 cells/µl). Usually PR are infected with HIV-1 for several years, although often the exact timepoint of infection is unknown in these patients. This study cohort incorporates patients with untreated progressing HIV-1 infection and provides an appropriate counterpart to compare the immune responses against HIV-1 with the one of patients with non-progressive HIV-1 infection.

2.3.1.5 ART-treated individuals

Plasma viral load is artificially suppressed by ART to undetectable or low levels in this cohort, since these HIV-1 infected patients are unable to control viral replication naturally. ART-treated individuals therefore represent the interesting case of low plasma viral load and impaired immune system and allow to study amongst others the impact of antigen load to host immune responses.

2.3.2 Correlates of immune control: state of the art of science

Although up to now there are no immune correlates known to fully control HIV-1 infection or replication, modern technologies allow scientists to discover distinct features of the immune system associated with non-progressive HIV-1 infection or delayed disease progression.

Humoral immune response clearly contributes to prevent HIV-1 infection, but its role in controlling established HIV-1-infection is still unclear. The administration of neutralizing antibodies helps to control viral replication [72]. Using primary macrophages as target cells in neutralization assays, scientists found an inverse correlation between neutralizing titers in patient sera and viral load, suggesting a potential role of neutralizing antibodies in early virus control [73]. Studies on patients who remain persistently uninfected despite repeated exposures to HIV-1, demonstrate that exposure to HIV-1 without seroconversion or delayed

HIV-induced disease is, in some individuals associated with HIV-1-neutralizing antibodies [74], [75].

Although progressed HIV-1 infection is characterized by gradual loss of helper CD4+ T cells, HIV-1-specific CD4+ T cells secreting IFN-γ usually remain abundant long time during infection. On the contrary, proliferative capacity [76] and IL2 production were preferentially observed in HIV-1-specific CD4+ T cells derived from LTNP [77]. Therefore, the problem is not the lack of HIV-1-specific CD4+ T cells, but rather a skewing towards a functional CD4+ T-cell population.

Studies in humans and in nonhuman primate models of HIV-1 infection indicate that CD8+ T cells play a direct role in controlling or limiting HIV-1 replication. CD8+ T-cell depletion during acute [78] or chronic [79] SIV infection is associated with a significant increase in viral load. Furthermore CD8+ T cells exert a strong selective pressure on SIV [80] and HIV-1 [81]. The introduction of polychromatic flow cytometry technology uncovered a high level of complexity in terms of CD8+ T-cell functional and differentiation markers, and it is now well accepted that the sole evaluation of IFN-γ, commonly in ELISPOT assays, provides only limited information on the quality of antigen-specific CD8+ T-cell responses [82], [83]. Better proliferative capacity and higher perforin expression was detected in HIV-1-specific CD8+ T cells derived from LTNP [84] or elite controllers [85]. Furthermore HIV-1-specific CD8+ T cells, which express the activation marker HLA-DR and expand rapidly upon antigenic stimulation were observed in HIV-1 infected controllers [86]. Recent studies demonstrated the association of polyfunctional HIV-1-specific CD8+ T cells and non-progressive HIV-1 infection [87]. In addition, measurement of IFN-γ secretion in combination with the differentiation markers CCR7 and CD45RA revealed an enrichment of HIV-1-specific, fully differentiated effector cells in HIV-1 infected LTNP [88]. Also in early infected individuals, a low viral set point was associated with the presence of fully differentiated effector cells [89]. In these studies, ART naive individuals with detectable viremia were chosen as controls and compared to HIV-1 infected LTNPs with low or undetectable viremia. Thus, it was not clear whether these HIV-1-specific T-cell populations were the cause or the

consequence of low viremia and the non-progressive status. Interestingly, a successive longitudinal study on a cohort of individuals starting ART and followed for more than two years showed the emergence of polyfunctional CD8+ T cells after prolonged suppression of viremia, suggesting that polyfunctional CD8+ T cells are lost under the condition of high antigen exposure and recovered or maintained when the antigen level is low [90].

In summary, despite all these studies it is difficult to define clear markers of immune control. Often studies are focused only on one aspect of the immune response and the characteristics of the patients cohorts differ from one study to another.

2.4 Cross-sectional vs longitudinal studies

There are two basic study designs in medical research, both may be essential in finding immune correlates of protection against HIV-1.

2.4.1 Cross-sectional study

This study design is very common in HIV research. Usually several cohorts of HIV-1 infected patients with different degrees of HIV-1 control compared at the same time, this makes the study quick, comparatively cheap and easy to manage, as no follow up is required. Cross-sectional studies are the best way to determine prevalences and are useful to identify associations and correlations. The main difficuliy of this study design is to differentiate cause and consequence from simple association, as cross-sectional studies provide no explanation for their findings.

2.4.2 Longitudinal study

Thisresearch study design involves repeated observations of the same item(s) over long periods of time. Due to repeated observation at the individual level,

longitudinal studies have more power than cross-sectional observational studies, by virtue of observing the temporal order of events. They allow assumptions about cause and consequence relations.

3 Materials and Methods

3.1 Material

3.1.1 Reagents and solutions

Reagent	Identification	Manufacturer	Catalogue number
Culture medium	RPMI 1640 medium	Cambrex, Taufkirchen, Germany	BE12-702F/U1
FCS Supplement (10%)	heat-inactivated FCS	Biochrom AG, Berlin, Germany	S0115
Penstrep Supplement (1%)	PenStrep	Cambrex	DE17-602E
Live dead discriminator for counting	Trypan Blue	Gibco, Invitrogen	15250-061
freezing media	10% DMSO in FCS (see above)	Sigma	D2650
Ficoll separating solution density 1.077g/ml	Bicoll	Biochrom AG	L6115
BFA (Golgi stop) stock solution with a concentration of 5mg/ml in DMSO, store small single-use-aliquots at -20°C	Brefeldin A	Sigma-Aldrich, Taufkirchen, Germany	B-7651
EMA (live/dead discriminator) stock solution with a concentration of 2mg/ml in DMFA, store at -20°C for long time, once thawed to keep at 4°C	Ethidium monoazide bromide	Molecular Probes/Invitroge, Karlsruhe, Germany	E-1374
Buffer solutions	FACS staining buffer, (0,2% BSA, 0,09% Na Azide in DPBS)	Becton Dickinson, Heidelberg, Germany	554657
	Perm/Wash solution 10x to dilute with H2O (from Kit)	Becton Dickinson	554714
PBS buffer pH 7.4	0.14 M NaCl 2.7 mM KCl 3.2 mM Na_2HPO_4 1.5 mM KH_2PO_4		
Cytofix/Cytoperm reagent kit	Cytofix/Cytoperm, Perm/Wash	Becton Dickinson	554714

3.1.2 Antibodies

3.1.2.1 Costimulatory antibodies (coAbs)

CD28 pure (BD, cat.no. 340975)

CD49 pure (BD, cat.no. 340976)

3.1.2.2 Fluorescencent labeled Antibodies

Antigen	conjugate	Company	Cat.no.	µl (sample)
CD107a	PE-Cy5	BD	555802	2.5
CD137	APC	BD	550890	5
CD14	ECD	Beckman Coulter	IM2707U	1
CD154	Fitc	BD	555699	5
CD19	ECD	Beckman Coulter	A07770	3
CD27	APC-Al750	eBioscience	27-0279-82	0.3
CD3	Al700	BD	557943	0.5
CD3	AmCyan	BD	339186	1
CD3	PE-Cy5	BD	555334	20
CD4	PerCP	BD	345770	5
CD45RA	PECy7	BD	337186	0.7
CD57	APC	Biozol	BLD-322313	5
CD8	APC	BD	345775	0.5
CD8	Fitc	BD	345772	4
CD8	PacB	Biozol	DAK-PB984	2.5
CD8	PE	BD	345773	2
CD95L	Fitc	Biozol	LS-C35869	1, 2.5, 5
HLA-DR	PerCP	BD	347402	20
IFN-γ	Al700	BD	557995	0.4
IL-2	APC	BD	341116	5
MIP-1ß	PE	BD	550078	0.2
Perforin	Fitc	BD	556577	2.5

3.1.3 Peptides

2µg/ml peptide were used in a total sample volume of 200µl.

3.1.3.1 Peptide pools

3.1.3.1.1 20-mer peptides overlapping by 10 amino acids

Kindly provided by the “Centralised Facility for AIDS Reagents”, NIBSC, London, UK

Name	Antigen	HIV-1 subtype	# of peptides
nef	Nef	LAI	20
tat	Tat	LAI	8
rev	Rev	LAI	11
p24	p24	LAI	22
p17	p17	SF2	13

3.1.3.1.2 Pools of variable length overlapping peptides

Kindly provided by the “Centralised Facility for AIDS Reagents”, NIBSC, London, UK

Name	Antigen	HIV-1 subtype	# of peptides
Nef 4	Nef (1–96)	Bru	15
Nef 5	Nef (95–205)	Bru	15
Tat 2	Tat	BH10	11

3.1.3.1.3 Pool of optimal CD8+ T-cell epitopes

Kindly provided by the “Centralised Facility for AIDS Reagents”, NIBSC, London, UK

Name	Antigen	HIV-1 subtype	# of peptides
Nef Opt	Nef	LAI	16

3.1.3.2 Single Peptides

3.1.3.2.1 Single peptides derived from peptide pools Nef 4 and Nef 5

peptide pool	name	sequence	position in Nef
Nef 4	p2	SVVGWPTVRERMRR|AEPAA	9-27
	p3	VRERMRRAEPAADGVGAA	16-33
	p4	AEPAADGVGAASRDLEK	23-39
	p5	GVGAASRDLEKHGAIT	29-44
	p11	VGFPVTPQVPL|RPMTYK	66-82
	p13	RPMTYKAAVDLSHFLK	77-92
	p14	AVDLSHFLKEKGGLEGL	84-97
	p15	FLKEKGGLEGLI	90-101
Nef 5	p16	LEGLIHSQRRQDILDLWIY	97-155
	p17	QRRQDILDLWIYHTQGY(F)	104-121
	p18	LDLWIYHTQGYFPDWQNY	110-127
	p19	YHTQGYFPDWQNYT	115-128
	p20	YFPDWQNYTPGPGVRY (PL)	120-137
	p29	VLEWRFDSRL|AFHHVAREL (H)	180-198
	p30	DSRLAFHHVARELHPEYF	186-203

3.1.3.2.2 Optimal Epitopes

Name	Sequence	Antigen	HLA-restriction	HIV-1 subtype	source
FL8	FLKEKGGL	Nef	B8	LAI	NIBSC
RH10	RQDILDLWVH	Nef	Cw7	autologue	proimmune, Oxford, UK
EI8	EIYKRWII	Gag p24	B8	autologue	peptides and elephants, Potsdam, Germany
NL8	NPDCKTIL	Gag p24	B8	autologue	peptides and elephants

3.1.4 Consumables

Product	Manufacturer
Cell culture plates, 96-well (3799&3598)	Corning, New York, USA
Cryotubes	Nunc, Wiesbaden, Germany
Eppendorf tubes 0.5µl-2,0µl	Eppendorf, Hamburg, Germany
FACS plates, 96-well, v-bottom	Falcon/BD Pharmingen, Hamburg, Germany
FACS tubes	Bio-Rad, Munich, Germany
Falcon tubes (15 ml, 50 ml; PS, PP)	BD Pharmingen, Hamburg, Germany
Gloves	Kimberly-Clark, Mainz, Germany
Pipettes, 5ml, 10 ml, 25ml, 10ml shorty Cellstar	Greiner, Nürtingen, Germany

3.1.5 Laboratory equipment

Equipment	Model/ type	manufacturer
CO_2 Incubator	CB 150	Binder, Tuttlingen, Germany
Fridge (4°C)	Profi Line	Liebherr, Biberach, Germany
Freezer (-20°C)	Premium	Liebherr
Freezer (-80°C)	VIP-Series	Sanyo, Pfaffenhofen, Germany
Microscope	Telaval 31	Carl Zeiss, Carl Zeiss, Oberkochen, Germany
Centrifuge	Rotanta 400R, Micro 200R	Heraeus, Hanau, Germany
Flow Cytometer	LSRII with HTS	Beckton Dickinson
Haematocytometer	Neubauer counting chamber	Karl Hecht KG, Sondheim, Germany
Vortexer Scientific Industries	Lab dancer	IKA, Staufen, Germany

3.1.6 Software

Product	Manufacturer
FacsDIVA 5.0.3	Becton Dickinson
FlowJo version 8.8.6	Treestar, Ashland, USA
GraphpadPrism 5	Graph Pad Software, San Diego, USA
MS Office 2001	Microsoft, Redmond, USA
Spice version 4.3	kindly provided by Mario Roederer, NIH, USA
Pestle version 1.6.2	kindly provided by Mario Roederer

3.2 Methods

3.2.1 Purification of peripheral blood mononuclear cells (PBMC) using ficoll density gradient

3.2.1.1 Background

Ficoll is a hydrophilic sucrose polymer with a molecular weight of 400.000 Dalton, commonly used for the production of density [91]. Density gradients allow for the separation of cells and subcellular components which sediment during centrifugation due to gravity. The reactivity and stability of Ficoll is based on the glycoside bonds within the sucrose residues and on its hydroxyl groups.

3.2.1.2 Procedure

Plasma was separated from heparinated blood (10', 1800 rpm, 21°C) and removed. Blood was refilled with prewarmed RPMI mediumto 35ml and cell pellet was dissolved by gently mixing. The diluted blood was carefully layered above 15 ml Ficoll separating solution. During centrifugation (17', 2100 rpm, 21°C, off brake) PBMC were separated from residual blood components according to their density and accumulated as visible lymphocyte ring within the Ficoll separating solution. The lymphocyte ring including media and Ficoll was aspirated, centrifuged (13', 1600 rpm, 21°C) and washed two additional times in 25 ml RPMI (13', 1600 rpm, 21°C and 5', 1300 rpm, 21°C).

3.2.2 Counting & freezing PBMC

PBMC were live/dead stained with Trypan Blue and counted under the Microscope using a Neubauer counting chamber. They were frozen in the desired concentration in 1 ml freshly prepared freezing media. Before storing in liquid N_2, they were allowed to slowly cool down in "Mr. Frosty" to -80°C.

3.2.3 Thawing PBMC

PBMC stored in cryotubes in liquid N_2, were thawed at 37°C in the waterbath and subsequently washed three times with RPMI supplemented with 10% FCS (RPMI-10) (5', 1500 rpm, 21°C) to remove DMSO.

3.2.4 Intracellular Cytokine Staining (ICS)

ICS was performed in 96 well plates. Single color stained "compensation samples" run along with every multi color staining experiment. RPMI-10 was used for mock-stimulation (neg ctrl). The workflow of ICS is illustrated in Figure 4, the experimental scheme in Figure 5.

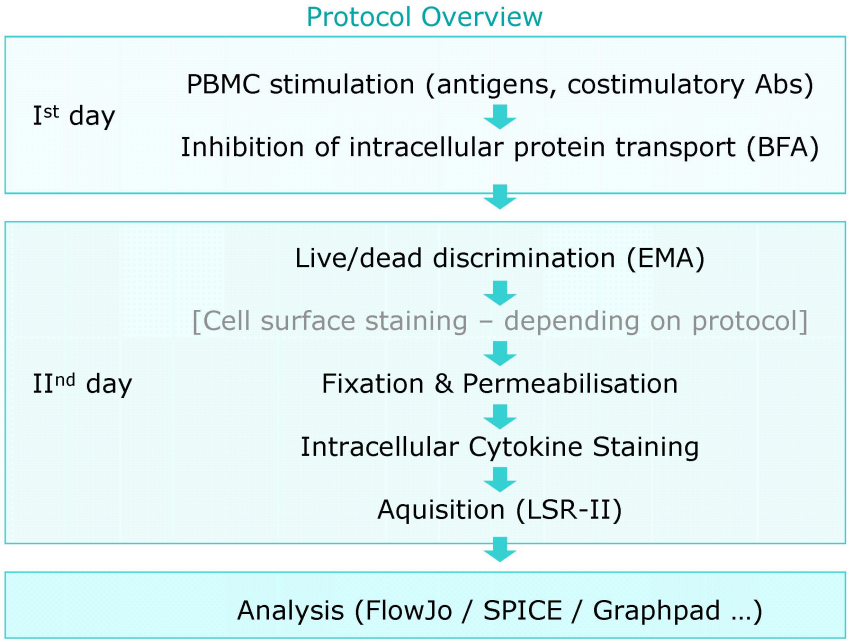


Figure 4: Workflow of Intracellular Cytokine Staining

3.2.4.1 Experimental Scheme

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Cmp	Cmp	Cmp	Cmp	Cmp	Cmp	Cmp	Cmp	Cmp	Cmp			
B	Exp	Exp	Exp	Exp	Exp	Exp	Exp						ID 1
C	Exp	Exp	Exp	Exp	Exp	Exp	Exp						ID 2
D	Exp	Exp	Exp	Exp	Exp	Exp	Exp						ID 3
E	Exp	Exp	Exp	Exp	Exp	Exp	Exp						ID 4
F													
G													
H	Neg Ctrl	Pep 1	Pep 2	Pep 3	Pep 4	Pep 5	Pep 6						

Figure 5: Experimental ICS scheme in 96 well plate. A1–A10: unstimulated PBMC, $0.5x10^6$. A1: Unstained set up control for instrument calibration. A2: Unstained neg ctrl. A3-A10: single color stained compensation samples (cmp). ID1-4: Individual 1-4, colors represent the stimulation different peptide stimulations.

3.2.4.2 Standard ICS Protocol (45RA-panel)

Developed by Sarah Kutscher and Dr. Antonio Cosma

3.2.4.2.1 Stimulation

After purification or thawing 10^6 PBMC were resuspended in 150 µl RPMI-10. The stimulation was performed with 0.4 µg peptide/10^6 cells in the presence of 1.3 µg/ml anti CD28 and 1.3 µg/ml anti CD49d costimulatory antibodies. After incubation for one hour at 37°C in 5% CO_2, 10 µg/ml BFA in a total volume of 50 µl RPMI-10 were added to the cell suspension and incubation was carried out for additional 4 hours. The 96 well plate was closed by parafilm and stored at 4°C over night.

3.2.4.2.2 Live/dead discrimination by EMA staining

a) Background

The photoreactive fluorescent label ethidium monoazide (EMA) diffuses into dead cells and intercalates in DNA. Upon exposure to strong light, EMA binds covalently to DNA. Therefore EMA is commonly used to stain dead cells by flow cytometry. In comparison to propidium iodide (PI) which would leak after

permeabilization of cells, EMA suits excellent for intracellular staining. The excitation peak of EMA is 462 nm (LSRII: blue laser excitation) with an emission peak at 625 nm (LSRII: FL3).

b) Procedure

Stimulated PBMC were pelleted (5', 1600 rpm, 4°C) and resuspended in FACS staining buffer. To assess their viability they were incubated with EMA for 20 min on ice in the dark and for additional 10 min on ice in strong light. Cells were subsequently washed three times in 200 µl FACS staining buffer (5', 1600 rpm, 4°C).

3.2.4.2.3 Fixation and Permeabilisation

Live/dead stained PBMC were resuspended in 100 µl Cytofix/Cytoperm reagent solution, incubated for 20 min on ice in the dark and subsequently washed four times in a total volume of 200 µl Perm/Wash solution (5', 1800 rpm, 4°C).

3.2.4.2.4 Intracellular staining

The fixed and permeabilized cells were incubated with the following fluorochrome conjugated antibodies for 30 min at 4°C: CD154 Fitc, MIP-1ß PE, CD4 PerCP, CD45RA PECy7, CD8 PacB, CD3 AmCyan, IL-2 APC, IFN-γ Al700. Single colour staining was performed on unstimulated compensation samples using CD8 Fitc, CD8 PE, CD4 PerCP, CD45RA PECy7, CD8 PacB, CD3 AmCyan, CD8 APC, and CD3 Al700. Stained PBMC were washed four times in a total volume of 200 µl Perm/Wash solution (5', 1800 rpm, 4°C), to remove unbound antibodies.

3.2.4.2.5 Acquisition

Cells were resuspended in 350 µl FACS staining buffer in 1ml titer-tubes FACS tubes for manual acquisition or in 200 µl FACS staining buffer in a 96 well plate v-bottomed for automated acquisition using high throughput system. Acquisition was performed on LSRII flowcytometer (BD) using BD FACSDiva 5.0.3.

3.2.4.3 Phenotyping ICS Protocol (57-panel)

Besides the unstimulated neg ctrl sample a second unstimulated sample was required to be stained without CD27 APC-Al750 (CD27- neg ctrl) for subsequent estimation of the CD27 staining.

ICS was performed according to the Standard ICS Protocol (45RA-panel) with the following aberrations after Live/dead staining:

3.2.4.3.1 Surface staining

The live/dead stained PBMC were incubated with CD57 APC and CD27 APC-Al750 for 30 min at 4°C for surface staining. CD27- neg ctrl was stained only with CD57 APC. Likewise, for compensation single color staining was performed on unstimulated PBMC using CD57 APC and CD27 APC-Al750.

Surface stained PBMC were washed three times in a total volume of 200µl FACS staining solution (5', 1600 rpm, 4°C) to remove unbound antibodies. Fixation and Permeabilisation was performed as described in Standard ICS Protocol (45RA-panel).

3.2.4.3.2 Intracellular staining

The fixed and permeabilized cells were incubated with the following fluorochrome conjugated antibodies for 30 min at 4°C: Perforin Fitc, MIP-1ß PE, HLA-DR PerCP, CD45RA PECy7, CD8 PacB, CD3 AmCyan and IFN-γ Al700. Intracellular single colour staining was performed on unstimulated compensation samples using Perforin Fitc, CD8 PE, HLA-DR PerCP, CD45RA PECy7, CD8 PacB, CD3 AmCyan and CD3 Al700.

Stained PBMC were washed four times in a total volume of 200µl Perm/Wash solution (5', 1800 rpm, 4°C) to remove unbound antibodies. Acquisition was performed as described in Standard ICS Protocol (45RA-panel).

3.2.4.4 Degranulation ICS Protocol (107a-panel)

ICS was performed according to the Standard ICS Protocol (45RA-panel) with the following aberrations in stimulation:

3.2.4.4.1 Stimulation

After purification or thawing 10^6 PBMC were resuspended in 150 µl RPMI-10. Cells were surface stained with CD107a PE-Cy5 prior to stimulation. The stimulation was performed with 0.4 µg peptide/10^6 cells in the presence of 1.3 µg/ml anti CD28 and 1.3 µg/ml anti CD49d costimulatory antibodies. After incubation for one hour at 37°C in 5% CO_2, 10 µg/ml BFA in a total volume of 50 µl RPMI-10 was added to the cell suspension and the incubation carried out for additional 4 hours. The 96 well plate was closed by parafilm and stored at 4°C overnight.

Live/dead staining and Fixation and Permeabilisation were performed as described in Standard ICS Protocol (45RA-panel).

3.2.4.4.2 Intracellular staining

To reduce the CD107a background, monocytes and B-cells were excluded from analysis by staining with anti CD14 and anti CD19 respectively. The fixed and permeabilised cells were incubated with the following fluorochrome conjugated antibodies for 30 min at 4°C: Perf Fitc, MIP1ß PE, CD14 ECD, CD19 ECD, CD45RA PECy7, CD8 PacB, CD3 AmCyan and IFN-γ Al700. Single colour staining was performed on unstimulated compensation samples using Perforin Fitc, CD8 PE, CD19 ECD, CD3 PE-Cy5, CD45RA PE-Cy7, CD8 PacB, CD3 AmCyan, CD3 Al700.

Stained PBMC were washed four times in a total volume of 200µl Perm/Wash solution (5', 1800 rpm, 4°C) to remove unbound antibodies. Acquisition was performed as described in Standard ICS Protocol (45RA-panel).

3.2.4.5 Data Analysis

3.2.4.5.1 Colour compensation

a) Background

A fluorochrome absorbs incident photons and emits photons of lower energy with consequently longer wavelength. Fluorescein (Fitc) for example absorbs blue light and emits green. Hence, every fluorophore has its characteristic emission spectrum. One consequence of the physics of fluorescence is that most emission spectra exhibit a tail extending toward longer wavelengths. The LSR-II (Becton Dickinson) used in this study like most flow cytometers uses a number of bandpass and dichroic filters to separate fluorescence emission from the excitation light source and to resolve different colors. Bandpass filters pass the emission maximum of the respective fluorochrome to the detector. Each detection channel has one bandpass filter. For Fitc (FL-1) the 530/30 filter was used, this filter can pass light from 515 nm to 545 nm (Figure 6). When added the phycoerythrin (PE) bandpass filter (FL-2, 575/26), this filter is able to pass light from 562-588 nm, the emission maximum of PE and a spectrum, where also Fitc emits weak light (called spillover of Fitc).

To analyse properly multicolor flow cytometry data it is necessary to correct for spillover. The percentage of spillover for a given fluorochrome was calculated using samples stained only with that fluorochrome. The calculated percentages of spillover were then used to correct the signal in samples containing all fluorochomes. This process is called colour compensation.

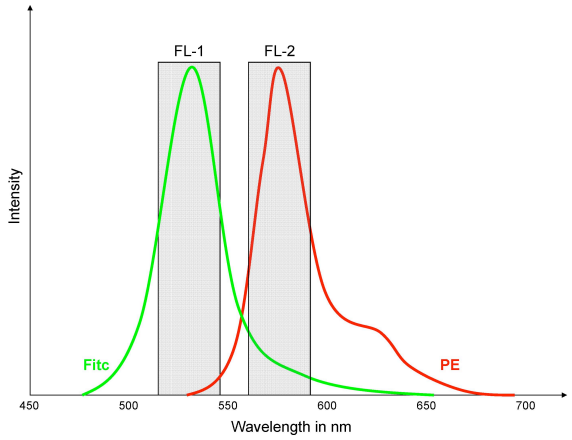


Figure 6: Emission spectrum of Fitc and PE with the corresponding channels FL-1 and FL-2.

b) Single color compensation

Prior to compensation, single color stained PBMC were gated for lymphocytes according to their characteristic shape in forward versus side scatter plot (FSC vs SSC) see Figure 8. The lymphocytes were further gated for live cells according to the EMA-staining in FL2 vs FL3. On the live (EMA-) cells, dim and bride stained cell populations were defined as positive or negative for the respective fluorescence. By means of these defined values for positive fluorescence labelled populations a so called compensation matrix was constructed (using wizard operation) calculating the proportion of external spillover fluorescences to be subtracted in the single channels. Compensation was regarded as correct once positive and negative populations regarding the single color staining in the appropriate channel had same median fluorescence intensities regarding all residual fluorescent channels (Figure 7).

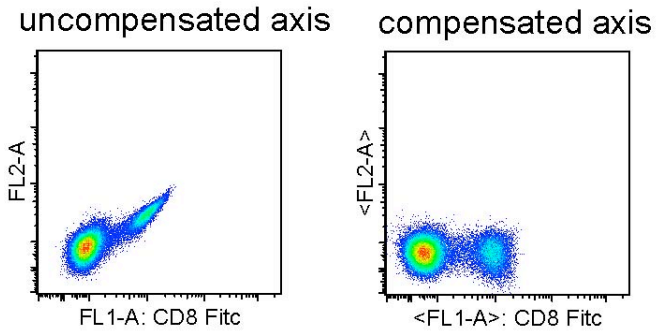


Figure 7: CD8 Fitc single color stained PBMC. Left plot shows uncompensated und right plot compensated axis. In compensated plot the median FL-2 fluorescence intensities are identical for FL-1 dim and FL-1 bright cell populations.

3.2.4.5.2 Data Analysis

The data analysis is in detail described for the Standard ICS Protocol (45RA-panel).

a) Gating strategy

Data analysis was performed using FlowJo version 8.8.6 (Tree Star, Ashland, OR). PBMC were gated for lymphocytes according to their characteristic shape in forward versus side scatter plot (FSC vs SSC) (Figure 8). The lymphocytes were

further gated for living cells (EMA-) according to the EMA-staining in FL2 vs FL3. On EMA- cells CD3+ events were gated versus IFN-γ, IL-2, MIP-1β and CD154 to account for down-regulation. CD3+ events were then combined together using the Boolean operator "Or" (gate A). On the CD3+ population the same procedure was used to define CD4+ (gate B) and CD8+ T cells (gate C). On the CD4+ T-cell population gates were performed to define responding (IFN-γ, IL-2, MIP-1β and CD154) or CD45RA+ cells, according to the following gating rules developed in the laboratory, this study was performed in:

The gates were set on the neg ctrl sample, taking in consideration the down regulation and fluorescence intensity of responding cells in the stimulated samples.

IFN-γ gate: [IFN-γ vs SSC, pseudocolor dot plot] gate was performed with backround values below 0.04%.

IL-2 gate: [IL-2 vs SSC, pseudocolor dot plot] gate was performed with backround values below 0.02%.

MIP-1β gate: [MIP-1β vs IFN-γ, zebra plot] gate was performed on the border of the zebra plot, with attention to not pass the MIP-1β- population in [CD45RA vs MIP-1β].

CD154 gate: [CD154 vs IFN-γ, pseudocolor dot plot] gate was performed on the border of the zebra plot.

CD45RA gate: [CD45RA vs MIP-1β, zebra plot] gate was performed at the narrowest point between CD45RA- and CD45RA+ population → this gate was copied to the CD8+ T-cell population.

On the CD8+ T-cell population gates were performed to define responding (IFN-γ, IL-2, MIP-1β and CD154) or CD45RA+ cells as described for the CD4+ population. Populations of all possible combinations of responding or CD45RA+ cells were created by Boolean operation “create combination of gates” for CD4+ and CD8+ T cells, resulting in 32 subpopulations of responding cells.

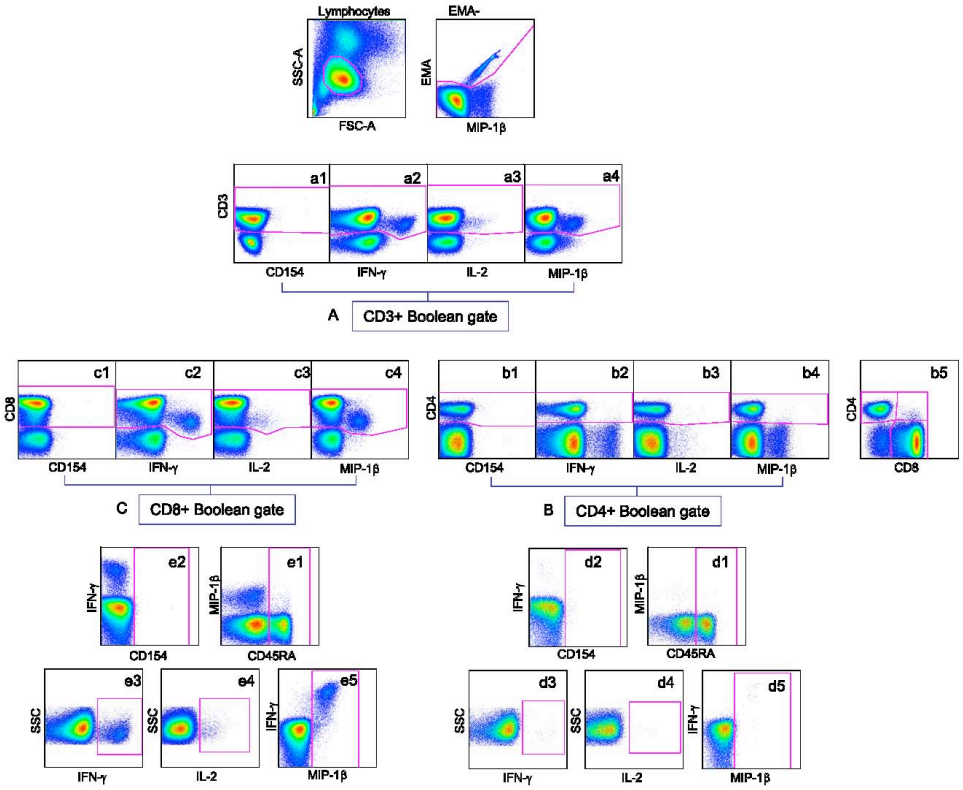


Figure 8: Gating strategy. Representative example showing the gating strategy of the 9 colour ICS (45RA-panel) applied on a PBMC sample stimulated with peptide pool Nef Opt. Lymphocytes are gated on a forward scatter area (FSC-A) versus side scatter area (SSC-A) pseudo-colour dot plot and dead cells are removed according to EMA staining. CD3+ events are gated versus CD154 (a1), IFN-γ (a2), IL-2 (a3) and MIP-1β (a4) to account for down-regulation. CD3+ events are then combined together using the Boolean operator "Or" (A). The same procedure was used to subsequently gate CD4+ (b1-4) and CD8+ (c1-4) events. CD4+ events are excluded from the CD8+ population using the exclusion gate in b5 before creating a gate for each function or phenotype (d1-5). CD8+ events are excluded from the CD4+ population using the exclusion gate in b5 before creating a gate for each function or phenotype (e1-5) [92].

b) Individual threshold system

After background subtraction, performed for each of the 32 subpopulation of responding CD8+ T cells, the 90th percentile of the negative values was calculated for each subpopulation of responding cells. These values were considered as individual threshold. Samples were regarded as positive the respective subpopulation when higher than the threshold value. Data were analysed in Spice version 4.3 (SPICE Software Mario Roederer, Vaccine Research Center, NIAID/NIH, Bethesda, MD) and Prism® version 5 (GraphPad Software Inc., San Diego, CA).

3.3 Patients

3.3.1 Participants of the cross-sectional study

Eleven NP, 10 PR and 23 ART-treated patients were included in the present cross-sectional study. Of the 11 NP patients, 10 matched the definition of long term non-progressors (LTNP), i.e. naive to ART with a documented HIV-1 infection of >9 years (median: 19.5 years), CD4+ T-cell counts ranging between 421 and 1042 (median: 522 cells/µl). In comparison to the other NP, study patient NP11 had lower levels of CD4+ T-cell counts (274 cells/µl). Nevertheless he was included in the NP cohort because of 19 years of documented history of HIV-1 infection with stable CD4+ T-cell counts over a one year follow-up post-sampling (range: 256-310 cells/µl). The median plasma viral load in the 11 NP was 900 HIV-1 RNA copies/ml (range: <50-10756). PR had poor restriction of viral replication (HIV-1 RNA copies/ml >99000) and declining CD4+ T-cell counts (median: 303 cells/µl; range: 132-474). All PR were ART naive. PR05 was the only rapid progressor in the PR cohort. Already five month after putative HIV-1 infection (the timepoint of blood sampling) CD4+ T-cell counts dropped in this patient to 208 counts/ml without recovery and persistently high level viremia. The 23 ART-treated patients were on treatment for 2 or more years. Twenty ART-treated patients had undetectable viral load, whereas patients ART12, ART11 and ART03V had 36600, 3362 and 13965 HIV-1 RNA copies/ml, respectively. The median CD4+ T-cell count in the 23 ART-treated patients was 488 cells/µl (range: 229-969 cells/µl). Study patients ART12, ART14, ART15 and ART16 were previously classified as NP but initiated ART more than 4 years before blood sampling for the present study and therefore are here included in the ART-treated cohort. Six of the ART-treated individuals (ART01, ART02, ART03, ART04, ART05 and ART06) underwent a single cycle of therapy interruption (TI). From these patients PBMC were obtained before TI (Table 1) and at the peak of the immune response after TI. Viremia was monitored weekly and treatment restarted at the first viral load determination >100000 HIV-1 RNA copies/ml. Patients remained off treatment for a median of 31 days (range:

26-182). The study was approved by the local Institutional Review Boards (Comitato Etico – Ospedale San Raffaele; Ethic Commitee of the Azienda Ospedaliera della Provincia di Lodi; Ethikkommission der Medizinischen Fakultät der Ludwig-Maximilians-Universität München; Ethikkommission der Ärztekammer Hamburg). Written informed consent was obtained for all study participants.

Table 1 Patient characteristics:

Patient	Years of known seropositivity	Years of ART	CD4+ counts (cells/µl)	CD8+ counts (cells/µl)	HIV-1 RNA Copies/ml of Plasma
NP06	19	-	626	3341	214
NP08	14	-	466	957	720
NP09	17	-	421	864	1100
NP11	19	-	274	747	1800
NP13	13	-	502	967	10756
NP14	20	-	461	464	488
NP15	20	-	532	991	8954
NP16	21	-	1042	1091	50
NP17	23	-	924	1030	1083
NP18	25	-	511	500	900
NP19	9	-	842	914	196
PR03	6	-	466	2322	316212
PR05	0	-	208	237	610000
PR11	6	-	214	808	>500000
PR12	2	-	457	1162	489978
PR25	1	-	474	1180	268919
PR34	10	-	326	2628	113164
PR36	19	-	132	834	>500000
PR77	9	-	226	1265	105488
PR86	1	-	406	2448	99402
PR95	0	-	280	1186	123818
ART12	23	4	413	740	36600
ART14	23	8	470	1926	<40
ART15	22	9	345	478	<40
ART16	23	9	609	888	<40
ART01*	7	7	969	789	<50
ART02*	10	6	609	1033	<50
ART03*	6	6	347	1542	<50
ART04*	4	4	334	455	<50
ART05*	5	5	688	643	<50
ART06*	6	6	455	1378	<50
ART07	23	15	532	840	<50
ART08	23	16	229	833	<50
ART09	13	13	351	281	<50
ART10	22	11	777	850	<40
ART11	24	15	271	1350	3362
ART01V	15	4	401	1893	<50
ART03V	17	5	954	2403	13965
ART05V	16	6	593	1671	<50
ART06V	9	4	715	846	<50
ART07V	6	5	488	851	<50
ART08V	7	6	708	NA	<50
ART10V	17	13	488	460	<50
ART11V	2	2	698	NA	<50

*These patients were later enrolled in a single cycle therapy interruption (TI).

3.3.2 Participant of the longitudinal study and characterization of MIRA CD8+ T cells

HIV-1 infected patient V4 has been enrolled in the vaccination trial and was under therapy for 4.3 years. V4 was vaccinated with MVA-*Nef* and received three subcutaneous immunizations of $5x10^8$ pfu of MVA-*Nef* at week 0, 2 and 16 to assess safety and immunogenicity of the vaccine. ART was interrupted 1.4 years after vaccination and reinitiated 6.3 years later when HIV-1 RNA were 180000 copies/ml and CD4+ counts 357 cells/µl.

4 Results

4.1 Correlates of immune control in HIV-1 infected non-progressors

4.1.1 CD45RA+ IFN-γ- IL-2- MIP-1β+ (MIRA) CD8+ T cells are associated with non-progressive HIV-1 infection

4.1.1.1 Background and Study design

Long term survival of HIV-1 infected individuals is usually achieved by continuous administration of ART. An exception to this scenario is represented by HIV-1 infected non-progressors (NP), which maintain relatively high numbers of circulating CD4+ T cells without clinical symptoms for several years in the absence of ART. In order to improve the understanding of the relationship between cellular immune response and non-progressive HIV-1 infection, the CD8+ T-cell response in the peripheral blood compartment of HIV-1 infected individuals with different histories of infection was analyzed. Eleven NP were compared to 10 progressors (PR) with unrestricted control of viral replication. NP and PR had not received ART before. In addition, 23 ART-treated patients were analyzed in whom HIV-1 replication was pharmacologically controlled and the role of the immune system is less relevant. Finally, the immune response of 6 ART-treated patients who interrupted ART was analyzed investigating the effect of rebounding virus replication on the HIV-1-specific CD8+ T-cell responses. The study was focused on the role of specific CD8+ T cells with respect to the non-structural HIV-1 proteins Nef and Tat. Indeed, these two nonstructural proteins are known to strongly influence HIV-1 replication, pathogenicity and the host immune response [93], [94]. Since previous studies associated the presence of polyfunctional [87] terminally differentiated [88], [89], [95] CD8+ T cells with the capacity to control viral replication, the simultaneous detection of 4 functional markers, i.e. IFN-γ, IL-2, CD154 and MIP-1β was coupled with the detection of CD45RA expression by intracellular staining. The use of CD45RA allowed the

discrimination between antigen-specific terminally-differentiated effector CD8+ T cells (CD45RA+), also termed T_{EMRA}, and the precursor CD45RA- memory CD8+ T cells, subdivided into central memory, T_{CM} and effector memory, T_{EM}.

4.1.1.2 HIV-1-specific CD45RA+ IFN-γ- IL-2- MIP-1β+ (MIRA) CD8+ T cells are a specific signature of NP.

Nef- and Tat-specific CD8+ T-cell responses were analyzed by multicolor flow cytometry in a cohort of NP and compared to responses observed in PR and ART-treated patients (Table 1). Following stimulation with a pool of 30 overlapping peptides (Nef 4 and Nef 5, Materials and Methods) covering the HIV-1 clade B Nef protein, the expression of CD45RA and the production of IFN-γ, IL-2, CD154 and MIP-1β were measured simultaneously. Nef-specific CD8+ T-cell responses were detected in most individuals.

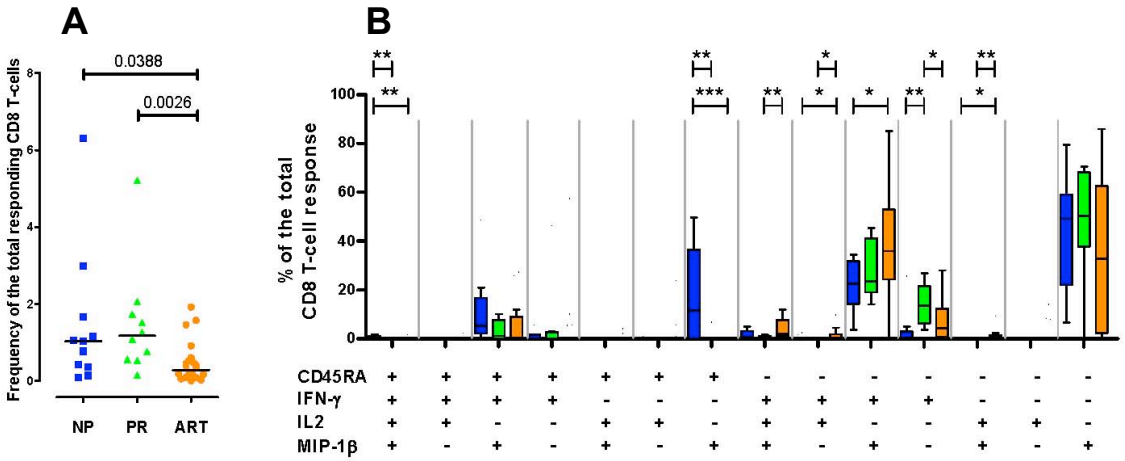


Figure 9: HIV-1-Nef-specific CD8+ T-cell responses in NP, PR and ART-treated-individuals. (**A**) Frequency of the total Nef-specific CD8+ T cells in 11 NP, 3 LP and 22 ART-treated individuals. Nef-specific responses were not analyzed in patient ART07. Medians are represented by horizontal bars. (**B**) Quality of the Nef-specific response. All the possible combinations of the responses are shown on the X axis for NP (blue box), PR (green box) and ART-treated individuals (orange box). Tukey boxes and whisker plots are shown. Significant differences are noted above the graph: (*) p <0.05, (**) p <0.01 and (***) p < 0.001.

NP and PR showed higher frequencies of total Nef-specific CD8+ T cells when compared to ART-treated patients (Figure 9A). To assess the quality of the specific responses, all possible combinations of expression of CD45RA, IFN-γ, IL-2 and MIP-1β in the responding CD8+ T cells (Figure 9B) were calculated. In

agreement with previous reports [96], CD8+ T cells expressing CD154 were not detectable: this marker was excluded from the analysis. Nef-specific responses were mainly composed of single positive MIP-1β+ or double positive MIP-1β+ and IFN-γ+ cells. Highly statistically significant differences ($p<0.01$) among the proportion of responding CD8+ T cells in NP, PR and ART-treated patients were found in CD45RA+ IFN-γ+ IL-2+ MIP-1β+, CD45RA+ IFN-γ- IL-2- MIP-1β+ (MIRA), CD45RA- IFN-γ+ IL-2+ MIP-1β+, CD45RA- IFN-γ+ IL-2- MIP-1β- and CD45RA- IFN-γ- IL-2+ MIP-1β+ CD8+ T-cell populations.

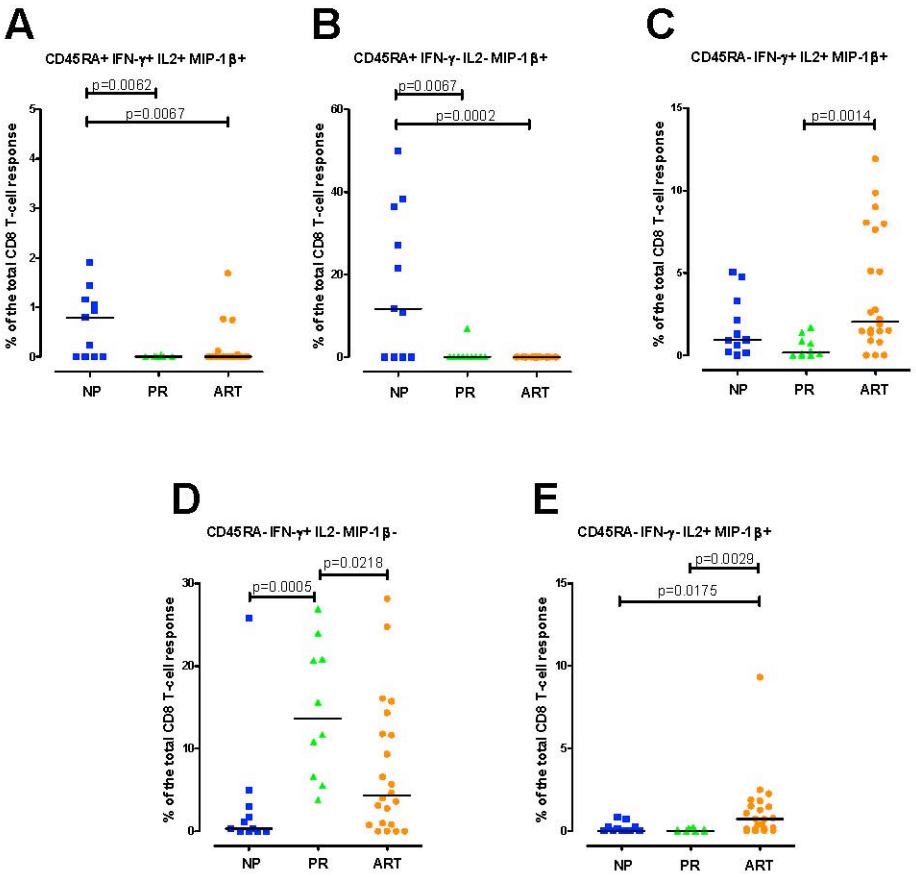


Figure 10: Individual data point representation of the HIV-1-Nef-specific CD8+ T-cell populations showing highly significant differences among NP, PR and ART-treated patients. Percentages of the total responses are shown for Nef-specific CD45RA+ IFN-γ+ IL-2+ MIP-1β+ (**A**), CD45RA+ IFN-γ- IL-2- MIP-1β+ (MIRA) (**B**), CD45RA- IFN-γ+ IL-2+ MIP-1β+ (**C**), CD45RA- IFN-γ+ IL-2- MIP-1β- and CD45RA- IFN-γ- IL-2+ MIP-1β+ CD8+ T cells.

In Figure 10, individual data points are shown for the populations with highly significant differences among the three cohorts. The proportion of polyfunctional (IFN-γ+ IL-2+ MIP-1β+) Nef-specific CD45RA+ CD8+ T cells was significantly

higher in NP than in PR or ART-treated individuals (Figure 2A), whereas the proportion of polyfunctional CD45RA- CD8+ T cells was significantly higher in ART-treated patients than in PR (Figure 10C). ART-treated individuals showed higher proportion of responding CD45RA- IFN-γ- IL-2+ MIP-1β+ CD8+ T cells in comparison to PR and NP (Figure 10E). On the other hand, monofunctional Nef-specific CD45RA- IFN-γ+ IL-2- MIP-1β- CD8+ T cells were detected in significantly higher proportion in PR than in NP and ART-treated individuals (Figure 10D). Surprisingly, the proportion of responding CD45RA+ IFN-γ- IL-2- MIP-1β+ (MIRA) CD8+ T cells in NP was significantly higher than in PR and ART-treated patients with extremely low p values ($p=0.0067$ and $p=0.0002$, respectively; Figure 10B). Indeed, CD45RA+ IFN-γ- IL-2- MIP-1β+ (MIRA) responding CD8+ T cells were detected in 7 out of 11 NP (64%) and 1 out of 10 PR (10%), whereas they were completely undetectable in the 22 ART-treated patients analyzed (Nef-specific responses were not analyzed in patient ART07). Interestingly, Nef-specific CD45RA+ IFN-γ- IL-2- MIP-1β+ (MIRA) CD8+ T cells in NP, when detectable, represented a high proportion of the total response (range: 10.7 to 49.9%). The same population detected in one PR (PR05) represented only 6.8% of the total response. Due to the exceptional association of CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8+ T cells with non-progressive HIV-1 infection this population was termed MIRA reflecting its characteristics MIP-1β production and the expression of CD45RA.

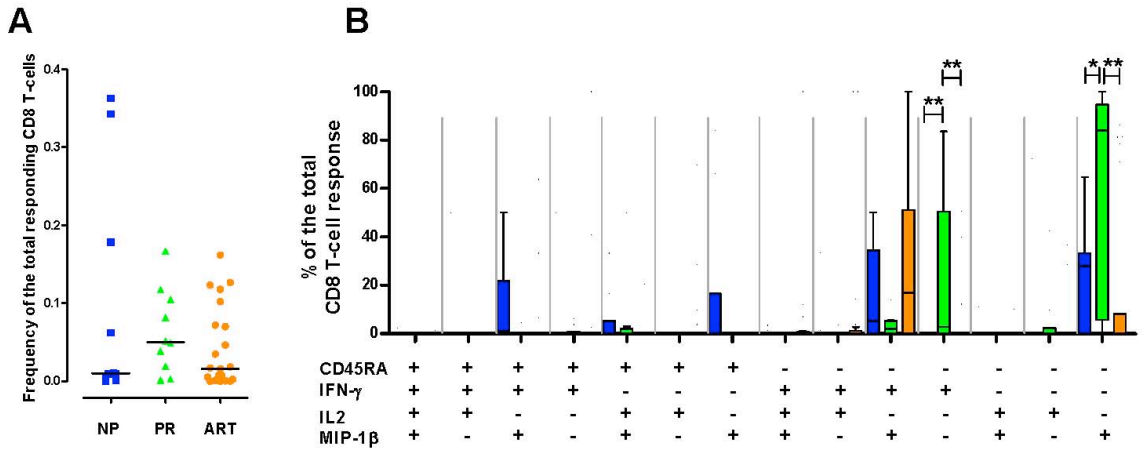


Figure 11: HIV-1-tat-specific CD8+ T-cell responses in NP, PR and ART-treated individuals. (A) Frequency of the total Tat-specific CD8+ T cells in 10 NP, 3 LP and 23 ART-treated patients. Tat-specific responses were not analyzed in NP11. (B) Quality of the Tat-specific response. All the possible combinations of the responses are shown on the X axis for NP (blue box), PR (green box) and ART-treated individuals (orange box). Tukey boxes and whisker plots are shown.

In comparison to Nef-specific responses, Tat-specific CD8+ T cells were characterized by lower magnitude and, worthy of note, no significant differences were observed in the total CD8+ T-cell responses among the cohorts herein analyzed (Figure 11A). Significantly higher proportions of Tat-specific CD45RA- IFN-γ+ IL-2- MIP-1β- and CD45RA- IFN-γ- IL-2- MIP-1β+ CD8+ T cells were observed in NP than in PR and ART-treated individuals (Figure 11B and 12B and 12C). Interestingly, MIRA responding CD8+ T cells were found in 2 NP that showed Tat-specific CD8+ T-cell responses, while none of the remaining Tat responders in the other groups showed this cell population (Figure 12A).

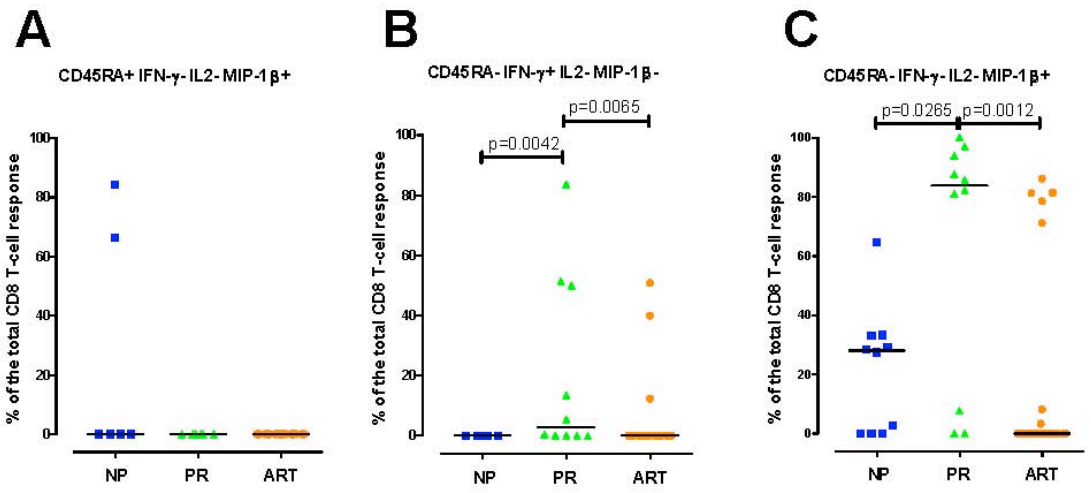


Figure 12: Individual data point representation of the HIV-1-tat-specific CD8+ T-cell populations. Percentages of the total responses are shown for Nef-specific MIRA (**A**), CD45RA- IFN-γ+ IL-2- MIP-1β+ (**B**) and CD45RA- IFN-γ- IL-2- MIP-1β+ (**C**) CD8+ T cells.

Overall was observed that monofunctional CD8+ T cells were prevalent in PR whereas polyfunctional CD8+ T cells were prevalent in individuals in whom the viral load was kept under control either naturally or with the help of antiretroviral treatment. Of particular interest, a novel HIV-1-specific MIRA CD8+ T-cell population was identified specifically associated with prolonged spontaneous control of HIV-1 disease progression in the absence of ART.

4.1.1.3 HIV-1-specific MIRA CD8+ T cells are not driven by in vivo viral replication.

The potential effect of differences in the level of viremia on the presence of MIRA responding CD8+ T cells in NP was explored next. The NP cohort was characterized by a prolonged exposure to HIV-1 antigens since their seropositivity was diagnosed with a median of 19 years (range: 9-25). In addition, NP showed detectable plasma viremia, albeit at low levels (range: 50-10,756 RNA copies/ml). Therefore, antigen exposure could have played a direct role in generating MIRA CD8+ T cells. However, the analysis of the relationship between plasma viremia and Nef-specific MIRA CD8+ T cells expressed as percentage of the total Nef-specific response or as percentage of the total CD8+ T cells revealed no significant correlation (Figure 13). Furthermore, only one PR

(10%) showed detectable levels of Nef-specific CD45RA+ MIP-1β+ CD8+ T cells, corroborating the idea that this novel CD8+ T-cell population is not directly influenced by antigen levels.

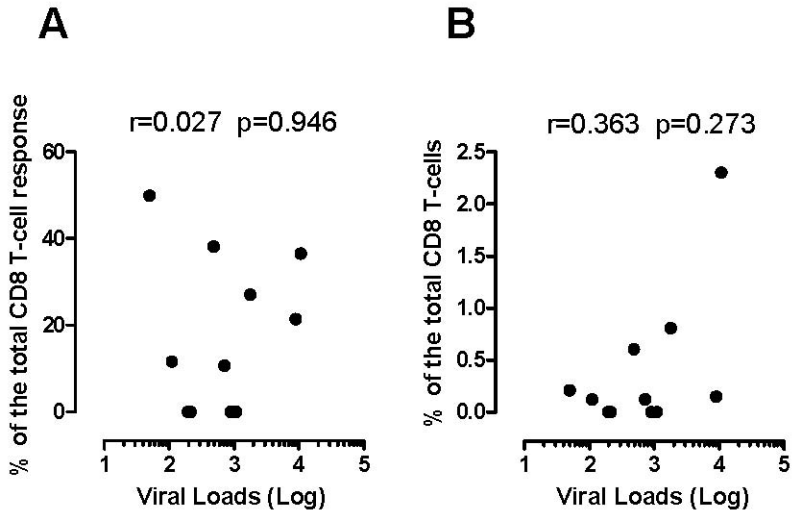


Figure 13: Relationship between HIV-1-Nef-specific MIRA CD8+ T cells and viral load. The relationship between viral loads and frequencies of MIRA cells expressed as total responding CD8+ T cells (**A**) or total CD8+ T cells (**B**) was determined by Spearman's correlation analysis. R and p values are shown.

To investigate further the role of in vivo HIV-1 replication in inducing MIRA CD8+ T cells, Nef- and Tat-specific CD8+ T-cell responses were analyzed in a longitudinal cohort. Six ART-treated patients with highly suppressed viremia (ART01, ART02, ART03, ART04, ART05 and ART06) underwent a single cycle of therapy interruption (TI). Viremia became detectable in all patients between day 5 and 21 after TI, and ART was resumed between day 27 and 185 when viremia levels reached >100,000 HIV-1 RNA copies/ml. A significant expansion of the Nef-specific CD8+ T-cell responses was observed in all the patients analyzed (Figure 14A). However, the quality of the CD8+ T-cell response remained unchanged in that MIRA CD8+ T cells remained undetectable even during the boost of the total Nef-specific CD8+ T-cell response that followed the peak of virus replication post-TI (Figure 14B). Of note, a decrease of Nef-specific CD8+ T cells expressing multiple effector functions was observed and an increase of Nef-specific CD8+ T cells expressing the sole IFN-γ, but these differences were not significant, probably due to the low number of patients

included in the longitudinal analysis. The Tat-specific CD8+ T-cell response was substantially undetectable both before and after TI (data not shown).

Thus, in the present experimental setting MIRA CD8+ T cells were not driven by in vivo viral replication.

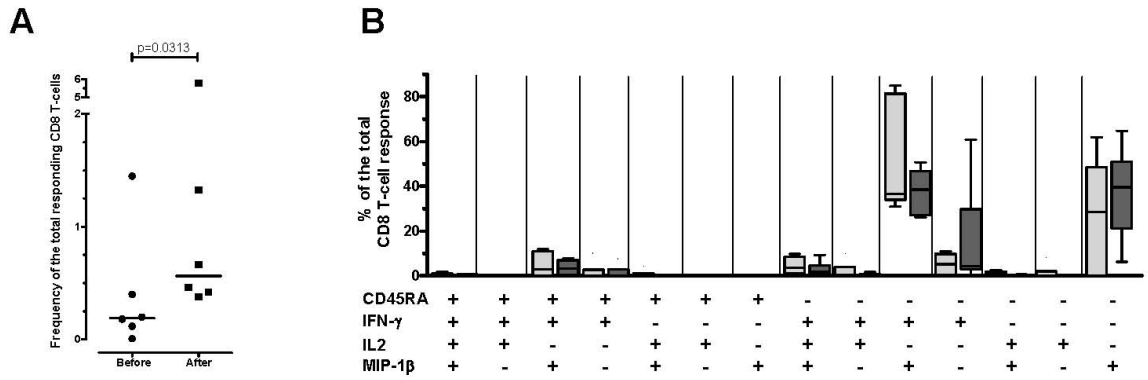


Figure 14: Nef-specific CD8+ T-cell responses during TI. (A) Frequency of the total Nef-specific response before and after TI. The median is shown for each group. (**B**) Quality of the Nef-specific CD8+ T-cell responses before (light gray boxes) and after (dark gray boxes) TI. All possible combinations of responses are shown on the X axis. Tukey boxes and whisker plots are shown.

4.1.1.4 IL-2 is not an essential marker to define the exclusive detection of MIRA CD8+ T cells in NP.

Since in all cohorts of HIV-1 infected patients IL-2-producing cells were rarely detected, data shown in Figure 9B were reanalyzed considering only the combined expression of CD45RA, IFN-γ and MIP-1β. The proportion of responding CD45RA+ IFN-γ- MIP-1β+ CD8+ T cells was significantly higher in NP than in PR and ART-treated patients (p=0.0069 and p=0.0012, respectively). This observation indicates that IL-2 expression represents neither an essential marker of non-progressive HIV-1 infection nor a distinctive feature of CD45RA+ IFN-γ- MIP-1β+ CD8+ T cells in NP.

4.1.2 Functional and phenotypic characterization of MIRA CD8+ T cells

4.1.2.1 Background and study participants

In the previous chapter MIRA CD8+ T cells were described as a potential immune correlate of protection, since their appearance was strongly associated with non-progressive HIV-1 infection. To analyze in detail the function and phenotype of MIRA CD8+ T cells, 4 out of 11 NP from the cross-sectional study were selected with a viral load ranging from >50 to $1x10^4$ copies/ml (NP13, NP14, NP15 and NP16). The time points of blood sampling was three months after the blood sampling of the cross-sectional study.

In addition to the 4 NP, a viremic controller (V4) was included in the study of the functional and phenotypic characterization of MIRA CD8+ T cells. Patient V4 was included in the study because of his exceptional status as viremic controller. V4 has been vaccinated with MVA-Nef and had strong Nef-specific T-cell responses. At the timepoint of blood sampling, V4 was ART free for 4.1 years (VL: $1.2x10^5$ copies/ml, CD4 counts: 508 cells/µl).

4.1.2.2 MIRA CD8+ T cells are epitope specific

Nef-specific MIRA CD8+ T cells were identified using pooled peptides. Therefore, it was not clear whether this novel T-cell population is restricted to certain HIV-1 Nef epitopes or represent a general characteristic of the Nef-specific response. CD8+ T-cell responses in 4 NP (NP13, NP14, NP15 and NP16) and patient V4 were analyzed using a pool of 30 overlapping peptides (Nef 4 and Nef 5, Materials and Methods) covering the HIV-1 clade B Nef protein. MIRA CD8+ T cells were detected in 3 NP (NP13, NP15 and NP16) and patient V4. Since the Nef pool was separated into an N-terminal (Nef 4) and a C-terminal (Nef 5) part, evidently MIRA CD8+ T cells appeared in some patients only in response to the N-terminal part of Nef, whereas in other patients MIRA CD8+ T cells emerged only in response to the C-terminal part of Nef, suggesting that MIRA CD8+ T cells are epitope specific (Figure 15A). Pre-screening by IFN-γ ELISPOT performed by Dr. Mauro Malnati provided information about which peptides within

the pools Nef 4 and Nef 5 pools elicited T-cell responses in the 4 NP. Patient V4 was pre-screened for positive peptides by flowcytometry (data not shown). PBMC from study participants were stimulated with the respective positive peptides derived from Nef 4 and Nef 5 pools (Materials and Methods), in case of two peptides covered the same HLA matched epitope, these two peptides were used together in the stimulation mixture. The production of IFN-γ and MIP-1β as well as the expression of CD45RA was subsequently determined on CD8+ T cells.

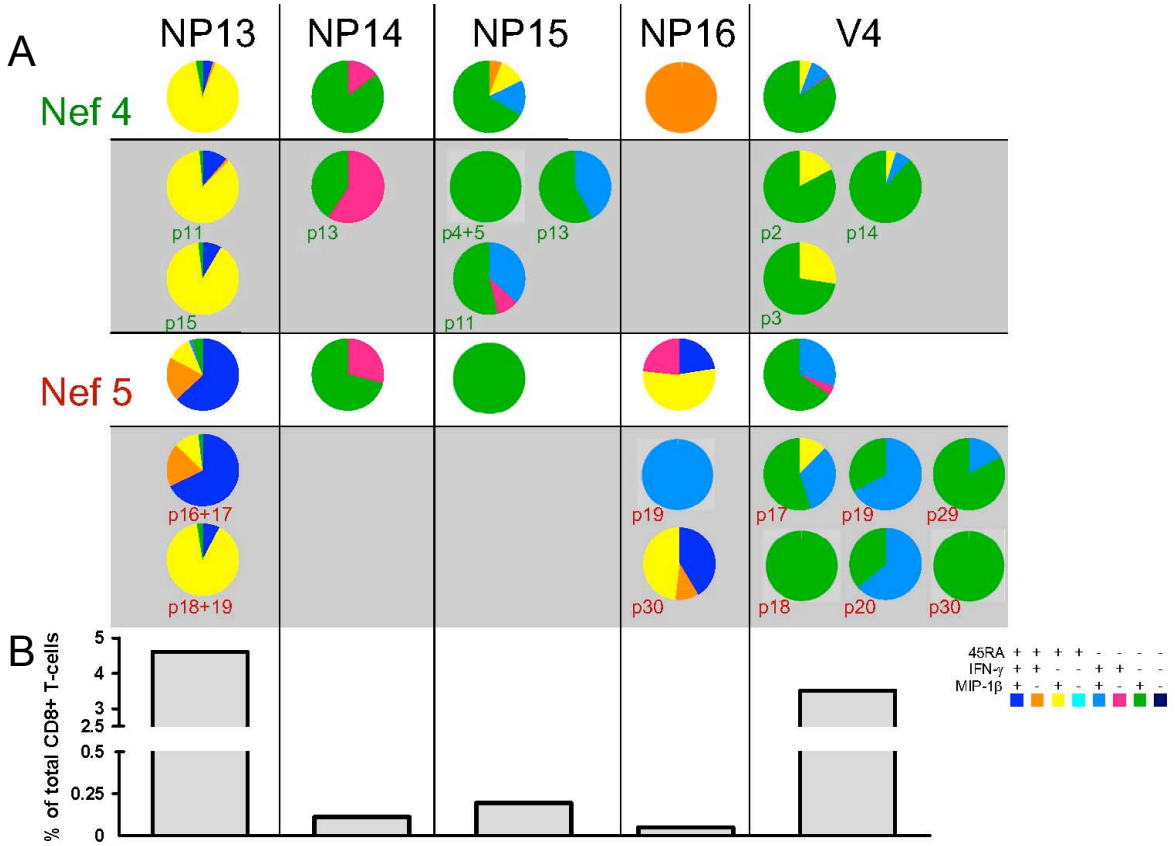


Figure 15: Quality and magnitude of Nef-specific CD8+ T-cell response. A) The quality of Nef-specific immune response is peptide specific. PBMC from non-progressors or partial controller V4 were stimulated with the N-terminal (Nef 4) or the C-terminal (Nef 5) peptide-pool on white background or peptides derived from these pools on grey background (green: peptides derived from Nef 4; red: peptides derived from Nef 5). Pi charts represent the quality of response regarding the marker CD45RA, IFN-γ and MIP-1β, the color code is explained in the legend. B) The magnitude of response varies between the patients. Bars show the total responding CD8+ T cells to Nef (Nef 4 and Nef 5 summed) in % of total CD8+ T cells.

The magnitude of response differed strongly between the patients (Figure 15B). To guarantee veritable positive responses a general threshold of 0.008 was

implemented in addition to the individual threshold. The quality of response within the same patient varied strongly from peptide to peptide, confirming the hypothesis that MIRA CD8+ T cells were specific to certain epitopes. The CD8+ T-cell response of patient NP13 against Nef 4 as well as the response against the single peptides p11 and p15 originated from Nef 4 was dominated by MIRA CD8+ T cells. CD8+ T cells producing IFN-γ, MIP-1β and expressing CD45RA on the contrary dominated the response of the same patient against Nef 5. The stimulation with single peptides from Nef 5 allowed the identification of two epitopes with different qualities of CD8+ T-cell responses. CD8+ T cells specific for the epitope in p16 and p17 had a comparable quality to CD8+ T cells responding to the pool Nef 5. Whereas MIRA CD8+ T cells dominated the quality of response against the epitope in p18 and p19. Following stimulation of PBMC from patient 16LR with single peptides from Nef 5 also two epitopes with different qualities of CD8+ T-cell responses were identified. 48 % of CD8+ T cells specific to p30 were MIRA CD8+ T cells, similar to the response against Nef 5 peptide pool. However, CD8+ T cells responding to p19 were CD45RA- IFN-γ+ MIP-1β+ and therefore qualitatively different to the response against Nef 5. In patient V4, the response against p14 was similar to the response against Nef 4, whereas the CD8+ T-cell responses to p2 and p3 differed from the response towards Nef 4. In patient V4, MIRA CD8+ T cells were undetectable in response to the peptide pool Nef 5, but appeared in response to the single peptide p17, derived from Nef 5 pool. Altogether, these data showed that the quality of CD8+ T-cell response was epitope specific and further, that MIRA CD8+ T cells were detectable only in response to certain epitopes.

4.1.2.3 MIRA CD8+ T cells are CD27- and Perforin^{high}

To characterize further the phenotype of MIRA CD8+ T cells the following functional and phenotypic markers were monitored: the differentiation marker CD27, the marker for activated CD8+ T cells HLA-DR, the marker for terminally differentiation CD57 and perforin. Figure 16 shows representative staining of PBMC from NP13 stimulated with the peptides 11 and 15 (Nef 4 derived) or

peptides 16+17 and 18+19 (Nef 5 derived). All responding CD8+ T cells from patient NP13 expressed CD45RA on their surface and hence belonged to the CD8+ effector T-cell population. HLA-DR was homogenously expressed on CD8+ T cells in this patient and no striking difference was noticed between MIRA CD8+ T cells and the other responding populations or total CD8+ T cells.

CD57+ CD8+ T cells were described as terminally differentiated effector cells, being replicative senescent and undergoing antigen-induced apoptotic cell death [97]. MIRA CD8+ T cells were equally distributed in both, the CD57+ and CD57- CD8+ T-cell populations demonstrating that they are not prone to undergo apoptosis.

The MIRA CD8+ T-cell population was clearly CD27-, indicating the affiliation to the effector (CD45RA+/CD27-) CD8+ T-cell population.

The expression of perforin allowed no bimodal separation between positive and perforin populations. For this reason the mean fluorescence intensity (MFI) of perforin was evaluated for MIRA CD8+ T cells. Data from patient NP13 indicate that MIRA CD8+ T cells appeared to have a higher mean fluorescence intensity of perforin compared to the other responding cell populations.

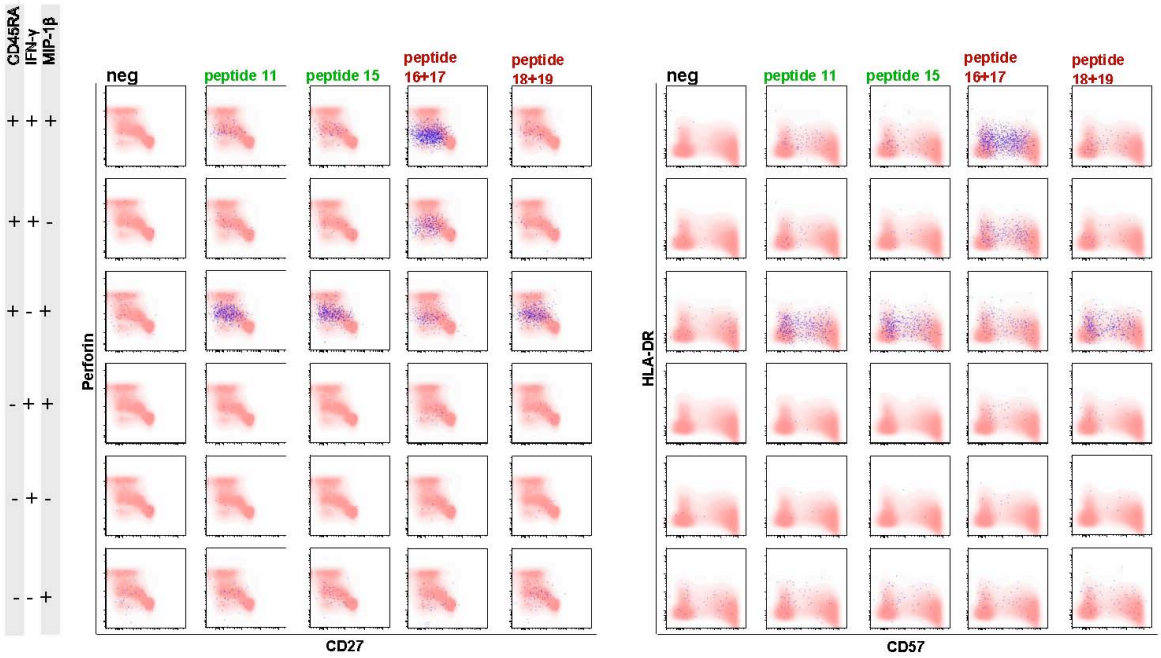


Figure 16: Phenotypic characterization of CD8+ T-cell responses in patient 13NP. Eleven-color flow cytometry was performed on PBMC after stimulation with peptides derived from the N-terminal (green) or C-terminal (red) Nef pools to characterize the phenotype of responding CD8+ T cells. Blue dots depict responding CD8+ T cells, with their functional profile described on the left, overlaid onto the red density plot of the total CD8+ T cells, as determined by CD27 and Perforin, CD57 and HLA-DR respectively.

All responses described in Figure 15 were analyzed regarding the marker Perforin, CD27 or HLA-DR. Relative Fluorescence Intensities (rMFI) were calculated by dividing the MFI of the responding CD8+ T-cell population by the MFI of the marker-negative population in total CD8+ T-cells. Thus, rMFIs were calculated for all six possible combinations, considering the marker CD45RA, IFN-γ and MIP-1β, of responding CD8+ T cells (Figure 17A-C). All combinations of responding CD8+ T cells had similar rMFI (perforin) when stimulated with both pools Nef 4 or Nef 5. A slightly increased rMFI (perforin) was recognized in MIRA CD8+ T cells (CD45RA+ IFN-γ - MIP-1β+). When considering the immune responses against single peptides, MIRA CD8+ T cells expressed a significant higher level of perforin compared to all other responding CD8+ T-cell populations ($p=0.0003$). Already the CD8+ T-cell response against the pools Nef 4 and Nef 5 showed clearly that the CD45RA+ populations expressed fewer surface CD27 than the CD45RA- populations. These differences amplified in the CD8+ T-cell

responses against the single peptides. Interestingly, the responding CD45RA- CD8+ T-cell populations exhibited higher rMFI (HLA-DR) compared to the CD45RA+ CD8+ T-cell populations. This difference was particularly noticed in CD45RA- IFN-γ- MIP-1β+ cells after stimulation with the pools Nef 4 and Nef 5, as well as in all CD45RA- CD8+ T-cell populations after stimulation with the single peptides. MIRA CD8+ T cells as the other responding CD45RA+ CD8+ T-cell populations hence expressed a relatively low level of the activation marker HLA-DR.

Figure 16 illustrates the bimodal distribution of CD8+ T cells regarding the differentiation marker CD57. Therefore rather than analyzing the rMFI (CD57), the percentage of CD57+ cells was compared in all populations of responding CD8+ T cells (Figure 17D). No significant differences were detected between MIRA CD8+ T cells and the other responding cell populations regarding the expression of CD57, indicating that MIRA CD8+ T cells, as the other responding CD8+ T cells, were not obligatory terminally differentiated and therefore not necessarily forced to undergo apoptosis.

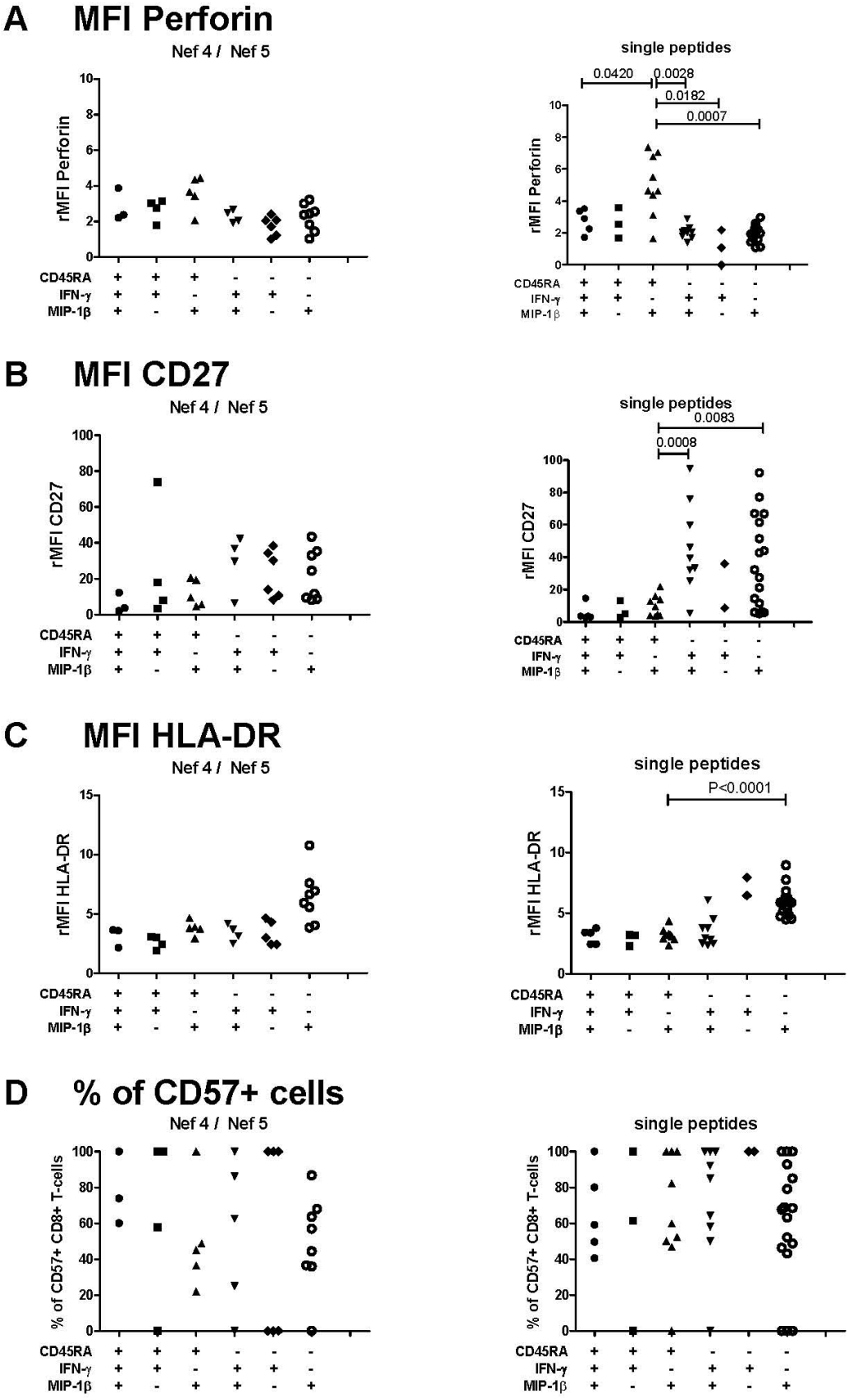

A MFI Perforin
Nef 4 / Nef 5
rMFI Perforin
CD45RA
IFN-γ
MIP-1β
single peptides
0.0420
0.0028
0.0182
0.0007
B MFI CD27
rMFI CD27
0.0083
0.0008
C MFI HLA-DR
rMFI HLA-DR
P<0.0001
D % of CD57+ cells
% of CD57+ CD8+ T-cells

Figure 17: Phenotypic characterization of CD8+ T-cell responses in 4 NPs and the viremic controller V4. Responses against the two Nef pools Nef 4 and Nef 5 (left panels) or the single peptides (right panels) were separated according to their expression of CD45RA and their IFN-γ and MIP-1β production (x-axis). The relative mean fluorescent intensity (rMFI) regarding the marker Perforin (a), CD27 (b) and HLA-DR (c) were calculated the following: mean fluorescent intensity (MFI) of the responding CD8+ T cells divided through the MFI of the negative population regarding the respective marker in total CD8+ T cells. d) The percentage of CD57+ cells within the responding CD8+ T-cell populations.

4.1.2.4 MIRA CD8+ T cells keep high levels of perforin because they do not degranulate

The expression of cell surface CD107a was shown to be associated with the loss of intracellular perforin [98]. Since MIRA CD8+ T cells feature high perforin levels, they are not suggested to exhibit cytotoxic killing function, because they do not release the intracellular perforin by degranulation. In order to address this assumption, PBMC from patient V4 were stimulated with the two pools Nef 4 and Nef 5 or the optimal HLA-matched CD8+ T-cell epitopes HLA-Cw7-restricted RQDILDLWVH (RH10) and HLA-B8-restricted FLKEKGGGL (FL8). PBMC were costained for CD3, CD8, CD45RA, IFN-γ, MIP-1β, perforin and CD107a. The bulk of MIRA CD8+ T-cell population, carrying high levels of intracellular perforin, did not express the degranulation marker CD107a on their cell surfaces (Figure 18). Since it was shown, that the expression of surface CD107a directly correlates with cytotoxic activity [98] it can further be concluded that MIRA CD8+ T cells exhibit due to their low level of surface CD107a and their high level of intracellular perforin no cytotoxic function by perforin release. However, it was recently shown, that antibody clone δG9, used in the present, detects perforin granules but does not detect de novo perforin synthesis, while clone D48 recognizes more conformations of perforin than clone δG9 [85]. The perforin production might therefore be underestimated in the present study.

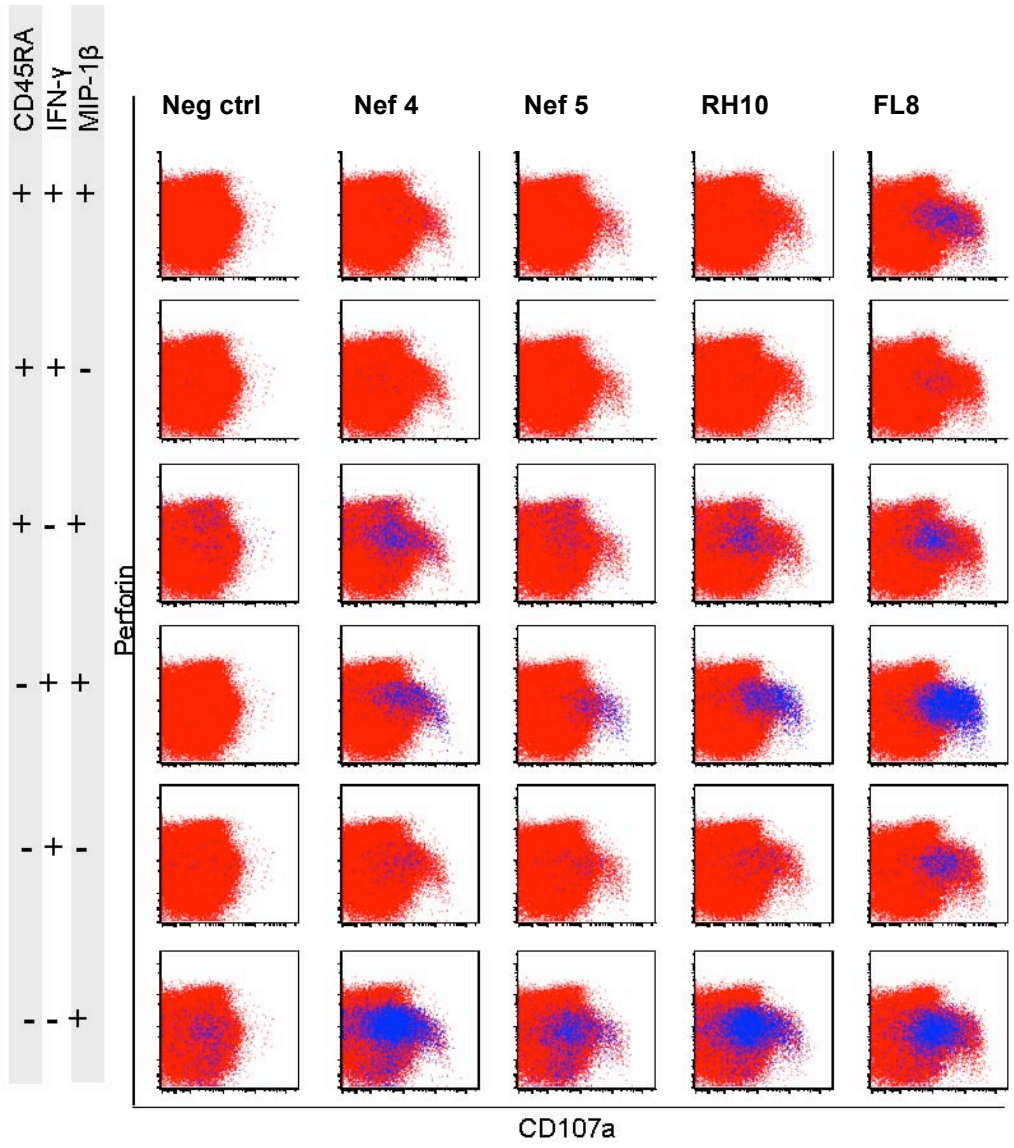


Figure 18: Assessment of the killing capacity via perforin degranulation of CD8+ T-cell responses in patient V4. Ten-color flow cytometry was performed on PBMC after stimulation with the N-terminal or C-terminal Nef pool or the optimal CD8+ T-cell epitopes RH10, FL8 respectively to characterize the perforin intensity and the expression of the degranulation marker CD107a of responding CD8+ T cells. Blue dots depict responding CD8+ T cells, with their functional profile described on the left onto the red dot plot of the total CD8+ T cells.

4.1.2.5 MIRA CD8+ T cells do not express the fasL

Cytotoxic CD8+ T lymphocytes mediate killing of target T cells through two major pathways, a granule dependant (perforin, granzyme) and independent (ligand-ligand induced cell death, for example fas-fasL) mechanism [99]. Facing the granule dependant pathway it was shown above that MIRA CD8+ T cells do not kill by releasing perforin. The next question to address was whether MIRA

CD8+ T cells express the fasL and are therefore able to kill their targets via ligand-ligand induced cell death. PBMC from patient V4 were stimulated with the Nef 4 and Nef 5 or the optimal CD8+ T-cell epitopes RH10 and FL8 and stained for cell surface CD95L (fasL) followed by intracellular staining of CD45RA, CD3, CD8, CD4, IFN-γ and MIP-1β. Results show that T cells which produce the cytokines IFN-γ or MIP-1β were negative for the expression of fasL on their surface (Figure 19). Thus also MIRA CD8+ T cells, producing MIP-1β, were negative for the expression of fasL. In conclusion MIRA CD8+ T cells that do not kill via the perforin pathway were shown not to induce apoptosis on target T cells via the fas/fasL pathway.

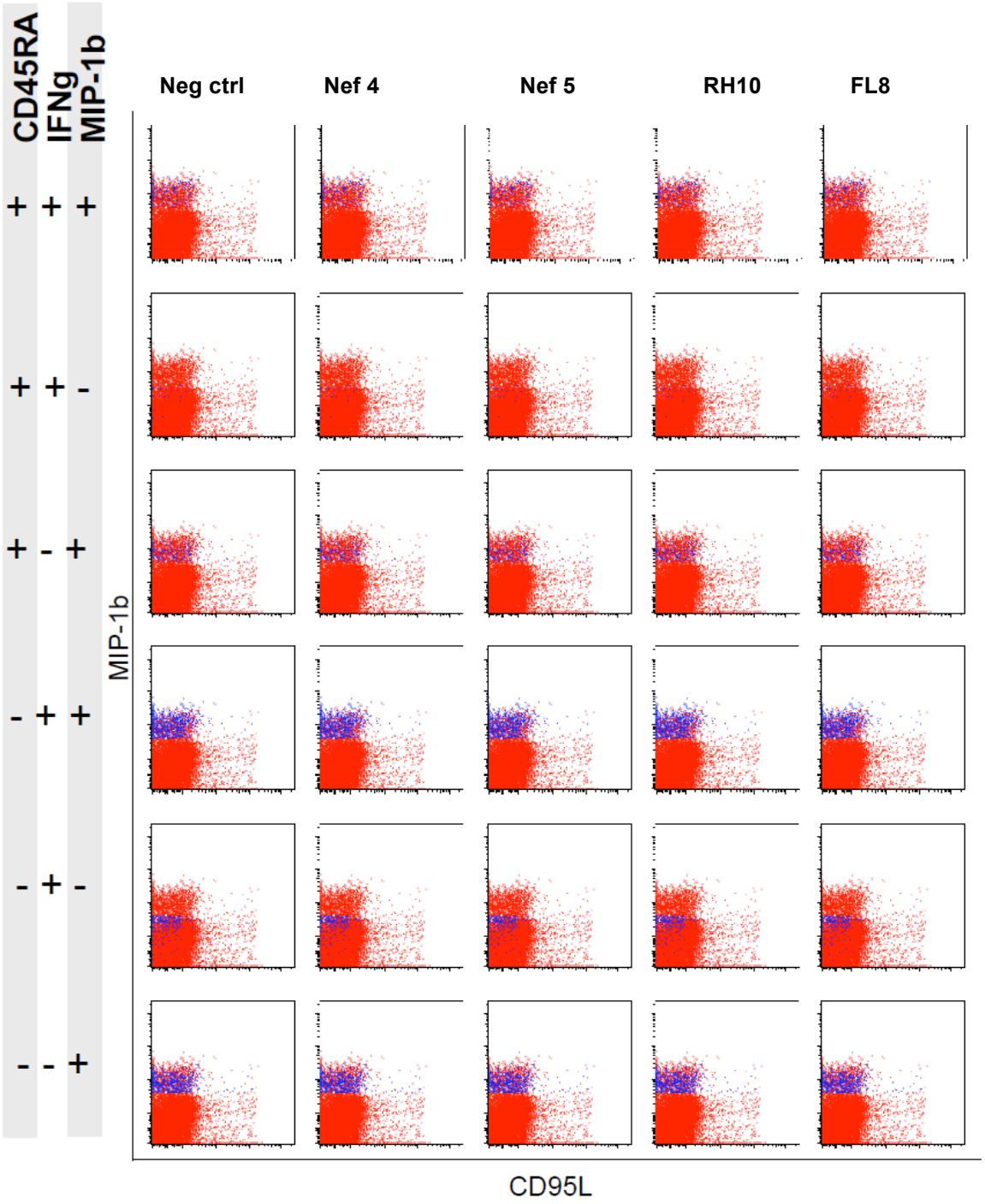


Figure 19: Assessment of the killing capacity by fasL (CD95L) expression of CD8+ T-cell responses in patient V4. Ten-color flow cytometry was performed on PBMC after stimulation with the N-terminal or C-terminal Nef pool or the optimal CD8+ T-cell epitopes RH10, FL8 respectively to characterize the expression surface fasL of responding CD8+ T cells. Blue dots depict responding CD8+ T cells, with their functional profile described on the left onto the red dot plot of the total CD8+ T cells.

4.1.2.6 MIRA CD8+ T cells produce high amounts of intracellular CD137 five hours post stimulation

CD137 is described to be expressed 24h after stimulation on the cell surface of antigen specific activated effector memory cells [100], [101]. Since the interest of the present study was to monitor intracellular cytokine production, 24h stimulation without BFA in order to detect surface CD137 would have been inconclusive. To estimate the activation potential of MIRA CD8+ T cells, PBMC from patient V4 were stimulated with the optimal CD8+ T cell epitope FL8 following the standard stimulation protocol for 1 hour and 4 additional hours in the presence of BFA. The production of IFN-γ and MIP-1β as well as the expression of CD45RA, CD154 and CD137 was evaluated subsequently. MIRA CD8+ T cells appeared to produce high amounts of CD137 which accumulated intracellularly due to BFA treatment (Figure 20). The activation potential of MIRA CD8+ T cells was even higher compared to other responding CD8+ T-cell populations.

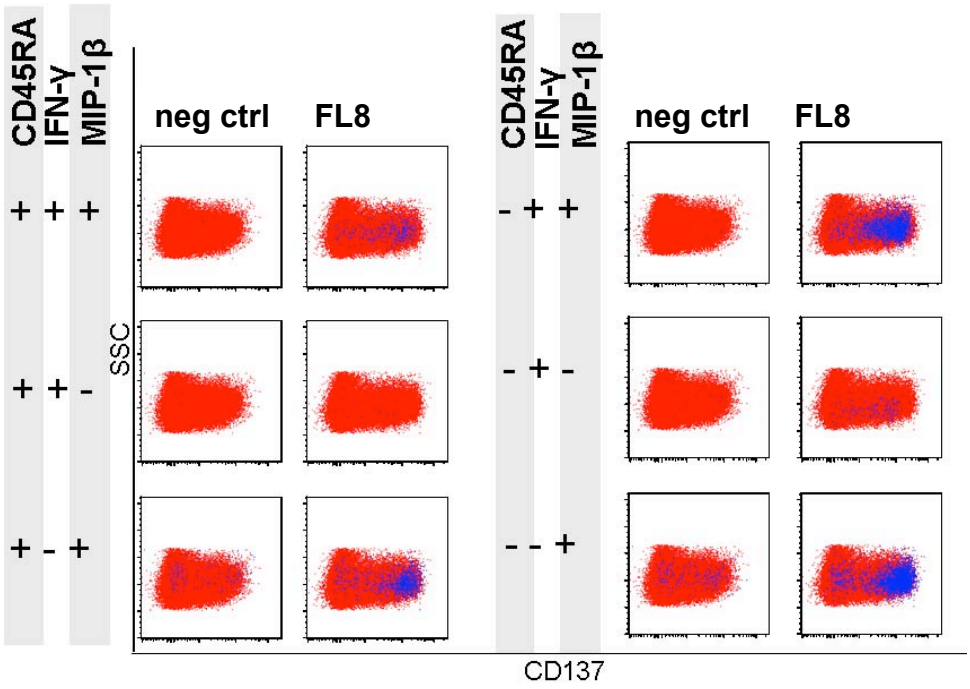


Figure 20: Assessment of the activation potential of CD8+ T-cell responses from patient V4. Ten-color flow cytometry was performed on PBMC after stimulation with the optimal CD8+ T-cell epitope FL8 to characterize the levels of intracellular CD137 in responding CD8+ T cells. Blue dots depict responding CD8+ T cells, with their functional profile described on the left onto the red dot plot of the total CD8+ T cells.

4.2 Longitudinal follow up of an HIV-1 infected patient with partial control of viral replication

4.2.1 Background

Lots of cross-sectional studies including the present one have been performed in order to find immune correlates of protection against disease progression in HIV-1 infection. Besides the important role of such studies in identifying such correlations they depict only snapshots in the histories of HIV-1 infected study participants. To monitor cause and consequence assumptions of host immune response and viral replication longitudinal studies are the adequate study designs.

Since the main interest of the cross-sectional study was due to the association of Nef-specific MIRA CD8+ T cells to non-progressive HIV-1 infection, addressed to Nef-specific T-cells responses, also the longitudinal follow up was performed in order to shed light on Nef-specific T-cell responses. Therefor an HIV-1 infected patient with strong Nef-specific T-cell responses was selected. This part of the study is dedicated to the patient V4. In intervals from 4 to 8 weeks the parameters of HIV-1 infection (CD4 counts and plasma viral load) and the HIV-1-specific immune response was monitored over a period of 11 years including initiation, interruption and restart of ART.

4.2.2 Clinical course of HIV-1 infection in patient V4

Patient V4 was diagnosed as HIV-1 infected in January 1998 (year 0). ART was initiated when plasma viral load increased (over $6.9x10^4$ copies/ml) and CD4 counts declined (below 600 cells/µl blood) at year 0.9 after diagnosis (Figure 21). After ART initiation viral replication was rapidly controlled (<50 copies/ml) and CD4 counts recovered to 800-2100 cells/µl blood. Patient V4 participated in a phase I clinical trial to assess safety of an HIV-1 Nef-based vaccine delivered by a modified vaccinia virus Ankara (MVA-Nef) administered 3 times (year 3.8) [102]. One year after MVA-Nef vaccination (year 5.2) ART was interrupted. After

ART interruption viremia rebounded to $1.7x10^4$ copies/ml (year 5.4). During the ART-free period (duration 5.7 years) a slow but constant disease progression was observed with decreasing CD4 counts and increasing levels of plasma viral load. However, a sudden shift of both clinical parameters towards disease progression was noticed at year 8.6 after diagnosis. Plasma viral load increased for the first time above $1x10^5$ copies/ml, while CD4 T cells counts dropped below 500 cells/µl (year 8.8). Both parameters did not regenerate until ART was restarted, 5 years after the interruption, when viremia increased to $1.8x10^5$ copies/ml and CD4 counts declined to 360 cells/µl blood. Based on the abrupt impairment of both clinical parameters CD4 counts and plasma viral load, the ART-free period was divided into two phases: stable viremia (year 5.5 - 8.6 after diagnosis) and loss of control (year 8.6 - 10.2 after diagnosis). The HIV-1-specific immune response of patient V4 was monitored intensively over a follow up period of 7 years (year 4.1 – 11.2 after diagnosis).

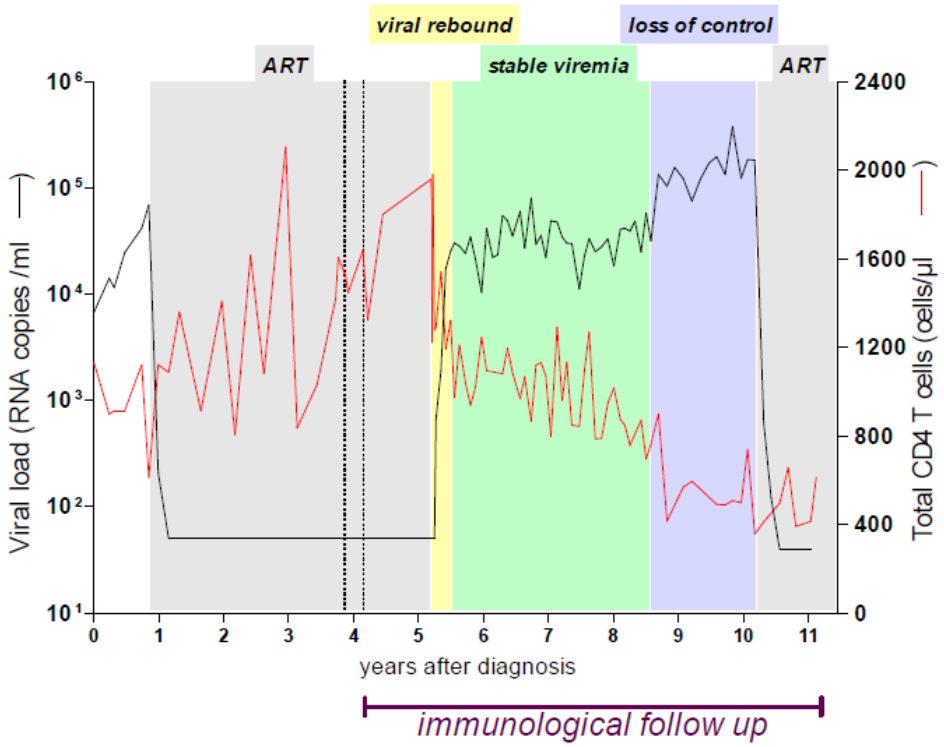


Figure 21: Clinical characteristics of HIV-1-infection of patient V4. Plasma viral load and CD4 counts are shown over a period of 11.2 years. Shadings depict distinct clinical phases: ART, viral rebound, stable viremia and loss of viral control. Dashed line indicate timepoints of MVA-Nef immunizations. The period of the immunological follow up is indicated.

4.2.3 HIV-1-specific T-cell response during the follow up period

HIV-1-specific CD4+ and CD8+ T-cell responses were monitored during the follow up period of 7 years by measuring IFN-γ production upon stimulation with peptide pools covering HIV-1 proteins Nef, Gag p24 (p24), Gag p17 (p17), Rev and Tat using intracellular cytokine staining (ICS). Highest CD4+ and CD8+ IFN-γ responses were elicited upon stimulation with Nef and to a lower extend with p24 peptide pools (Table 1). Only marginal numbers of IFN-γ-secreting T cells were detected in response to p17, Rev and Tat stimulation. Therefore, immune response against Nef and p24 was analyzed in more detail. Production of IFN-γ, IL-2 and MIP-1β, as well as the expression of CD45RA and CD154 were determined simultaneously by polychromatic flow cytometry.

Table 2: HIV-1-specific IFN-γ secreting cells

	CD4+ T-cell responses (%)		CD8+ T-cell responses (%)	
HIV-protein	median	range	median	range
Nef	0.3	0-1.14	0.78	0-1.81
p24	0.15	0-0.47	0.23	0-1.62
p17	0.05	0-0.21	0.09	0-0.41
Rev	0.01	0-0.7	0.03	0-0.23
Tat	0.01	0-0.06	0.03	0-0.15

* sixtyseven timepoints were analyzed (year 3.7 - 11.2 after diagnosis).

4.2.4 Frequency of Nef- and p24-specific CD4+ T cells during the follow up period

Total Nef- and p24-specific CD4+ T-cell response was determined by summing each unique CD4+ T-cell subpopulation, positive for at least one of the monitored functional markers IFN-γ, IL-2, MIP-1β or CD154 (Figure 22).

As plasma viral load increased after ART interruption Nef- and p24-specific CD4+ T-cell response raised immediately. During the phase of stable viremia Nef-specific CD4+ T-cell response progressively increased reaching a maximum of 2 % of total CD4 T cells during the loss of control phase. Thereafter, a rapid decline was observed and following ART initiation Nef-specific CD4 T cells were only 0.35% of total CD4 T cells.

In contrast to Nef-, p24-specific CD4+ T-cell response remained nearly constant (ranging from 0.34 to 0.48 %) for 2 years during the phase of stable viremia and decreased suddenly to 0.04 % at the end of this phase. Within the loss of control phase p24-specific CD4+ T cells were present at low levels (median 0.15 %, range 0.08 – 0.19%). ART re-initiation was accompanied by a short term increase of p24-specific CD4+ T cells which declined thereafter.

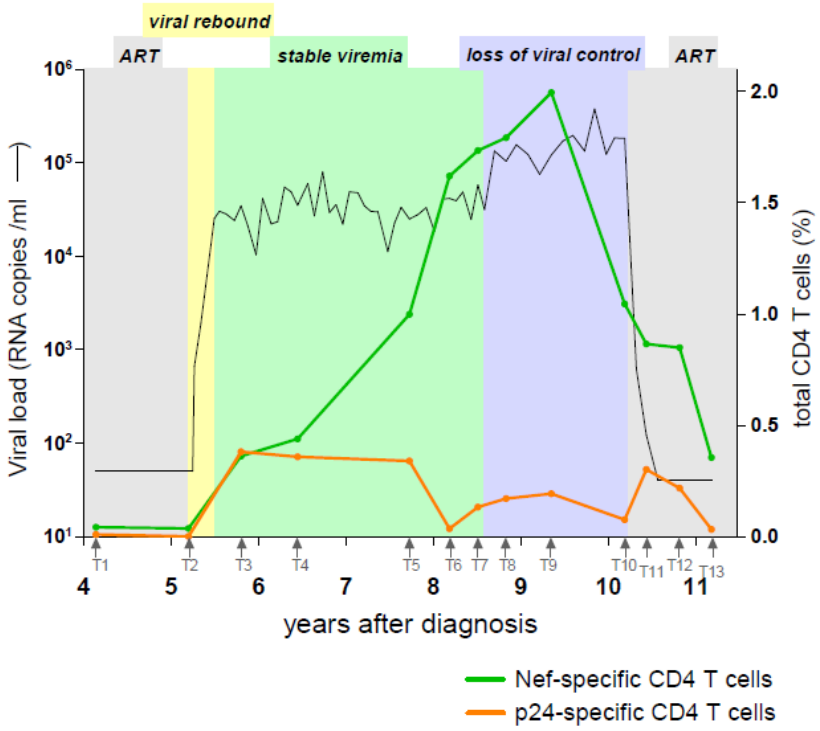


Figure 22: Magnitude of HIV-1-specific CD4+ T-cell response during a 7-year follow up period. Total response considering all CD4+ T cells positive for at least one of the following marker: IL-2, IFN-γ, MIP-1β or CD154Stimulation was performed using peptide pools covering Nef or p24 protein. Thirteen timepoints were analyzed (T1-T13). Shadings depict distinct clinical phases: ART, viral rebound, stable viremia and loss of viral control. Plasma viral load is shown.

4.2.5 Exhaustion of Nef- but not p24-specific CD4+ T-cell response

The functionality of Nef- and p24-specific CD4+ T cells was analyzed regarding IFN-γ, IL-2 and MIP-1β production as well as the expression of CD154 (Figure 23).

An exhaustion of Nef-specific CD4+ T cells was observed with progressive loss of IL-2, CD154 and IFN-γ already after ART interruption and during the phase of stable viremia. In contrast the proportion of MIP-1β secreting cells increased

steadily with nearly all Nef-specific CD4+ T cells producing MIP-1β in the loss of control phase. The exhausted, widely monofunctional MIP-1β+ IFN-γ+/– IL-2– CD154– phenotype of Nef-specific CD4+ T cells persisted during the loss of control phase.

To evaluate the contribution of plasma viral load regarding the qualitative changes in the Nef-specific CD4+ T-cell response, these responses were analyzed statistically during the ART-free period. The proportion of IFN-γ producing and CD154 expressing CD4+ T cells within total Nef-specific CD4+ T-cell response correlated inversely with plasma viral load, while the proportion of Nef-specific MIP-1β producing CD4+ T cells correlated positively with levels of plasma viral load (Table 3). On the other hand, the proportion of MIP-1β and IFN-γ secreting cells increased strongly throughout the phase of stable viremia in the p24-specific CD4+ T-cell population. During this phase, nearly all cells maintained CD154 expression, while the number of IL-2 producing cells was consistently low. No correlations between the function of p24-specific CD4+ T cells and plasma viral load were detected.

Table 3: Spearman correlation of plasma viral load and the expression of CD154, IFN-γ, IL-2 and MIP-1β in total Nef- and p24-specific CD4+ or CD8+ T cells.

T cell subset	Protein	marker	Spearman r	P value (two-tailed)	P value summary
CD4	Nef	CD154	-0.83	0.02	*
		IFN-γ	-0.83	0.02	*
		IL-2	-0.67	0.08	ns
		MIP-1β	0.86	0.01	*
CD8	Nef	IFN-γ	-0.93	0	**
		IL-2	-0.62	0.11	ns
		MIP-1β	0.67	0.08	ns
	p24	IFN-γ	-0.69	0.07	ns
		IL-2	0.02	0.98	ns
		MIP-1β	0.76	0.04	*

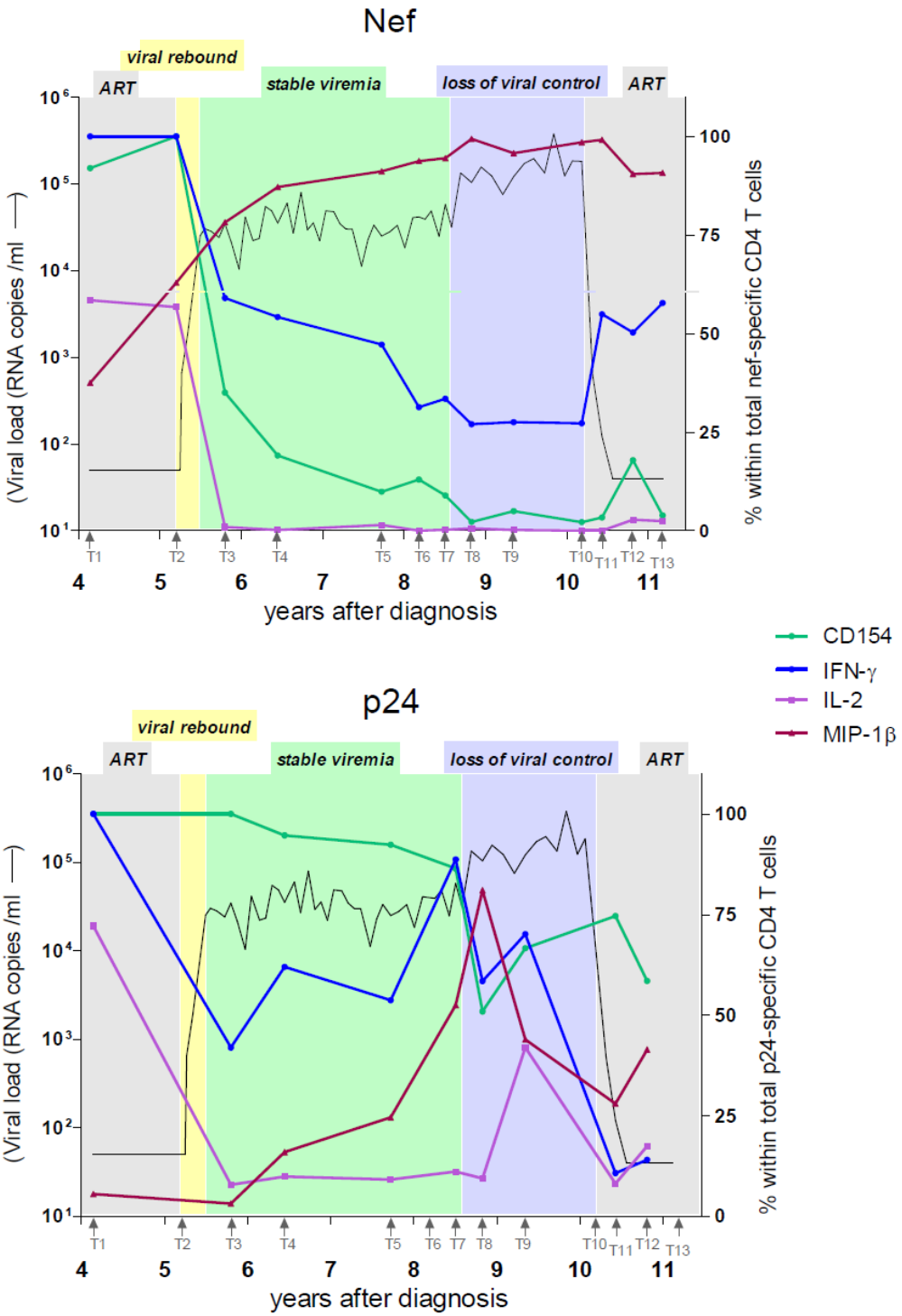


Figure 23: Quality of HIV-1-specific CD4+ T-cell response during a 7-year follow up period. Stimulation was performed using peptide pools covering Nef or p24 protein. Thirteen timepoints were analyzed for Nef-response (T1-T13). T2, T6, T10 and T13 were not analysed for p24 response. Shadings depict distinct clinical phases: ART, viral rebound, stable viremia and loss of viral control. Plasma viral load is shown. Percentage of IFN-γ, IL-2 and MIP-1β producing and CD154 expressing cells are shown on total Nef- and p24-specific CD4+ T-ell response.

4.2.6 Frequency of Nef- and p24-specific CD8+ T cells during the follow up period

Frequency of total CD8+ T-cell response was determined by summing each unique CD8+ T cell subpopulation positive for at least one of the monitored functional markers IFN-γ, IL-2 or MIP-1β (Figure 24). As CD154 is not expressed on CD8+ T cells, this marker was excluded from CD8+ T cell analysis.

With the increase of plasma viral load after ART interruption higher numbers of both Nef- and p24-specific CD8+ T cells were detectable. Frequencies of Nef-specific CD8+ T cells reached a maximum of 3.5 % of total CD8+ T cells during the phase of stable viremia followed by a rapid decrease to 2.1 %. The response fluctuated (median 2.5 %; range 2.0 - 2.9 %) until the end of the loss of control phase and decreased to 0.7 % after ART re-initiation. The frequencies of p24-specific (maximum of 2.5 %) were generally lower compared to Nef-specific CD8+ T cells but increased also immediately after ART interruption and remained at stable levels. During and after the loss of control phase the numbers of p24-specific CD8+ T cells were similar (median 1.5 % / range 1.3-1.7 %) and decreased one year after ART re-initiation.

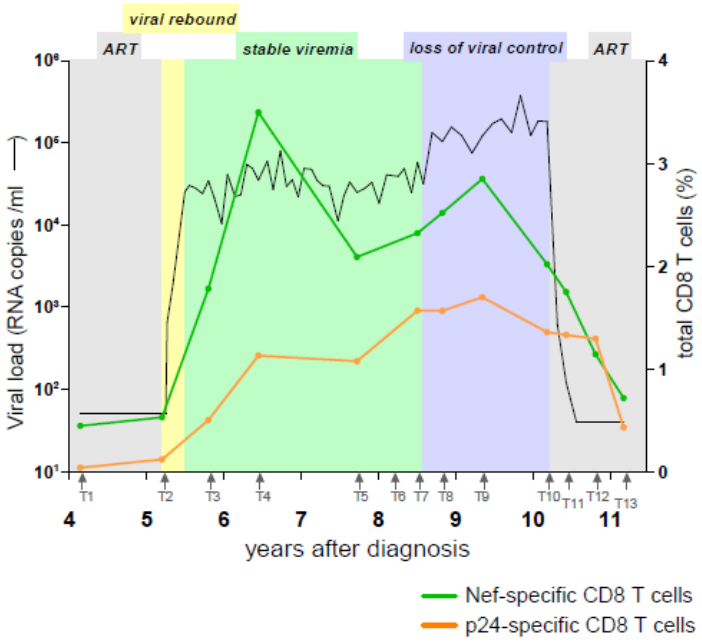


Figure 24: Magnitude and quality of HIV-1-specific CD8T-cell response during a 7-year follow up period. Stimulation was performed using peptide pools covering Nef or p24 protein. Total response considering all CD8+ T cells positive for at least one of the following marker: IL-2, IFN-γ or MIP-1β Thirteen timepoints were analyzed (T1-T13). Shadings depict distinct clinical phases: ART, viral rebound, stable viremia and loss of viral control. Plasma viral load is shown.

4.2.7 Progressive exhaustion of Nef- and p24-specific CD8+ T-cell responses

The functionality of Nef- and p24-specific CD8+ T-cell responses was determined regarding the production of IFN-γ, IL-2 and MIP-1β (Figure 25).

In contrast to the CD4+ T-cell response Nef- and p24-specific CD8+ T-cell responses were similar, not only in quantity but also in quality. IL-2 production became nearly undetectable and shortly after ART interruption, almost all antigen-specific CD8+ T cells produced MIP-1β. Progressive exhaustion was observed during follow up period by decreasing numbers of IFN-γ producing cells. After ART re-initiation, the numbers of Nef- and p24-specific bi-functional MIP-1β+ IFN-γ+ IL-2– and tri-functional MIP-1β+ IFN-γ+ IL-2+ CD8+ T cells recovered (data not shown).

Statistical analysis during the ART-free period revealed the contribution of plasma viral load to the functional changes in Nef- and p24-specific CD8+ T-cell response. The proportion of IFN-γ production in total Nef-specific CD8+ T cells correlated inversely with plasma viral load and the proportion of IFN-γ production in total p24-specific CD8+ T-cell response showed similar tendencies (table 3). While the proportions of MIP-1β producing cells in total p24-specific CD8+ T cells correlated with plasma viral load and the proportions of MIP-1β producing cells in total Nef-specific CD8+ T-cell response showed a similar tendency.

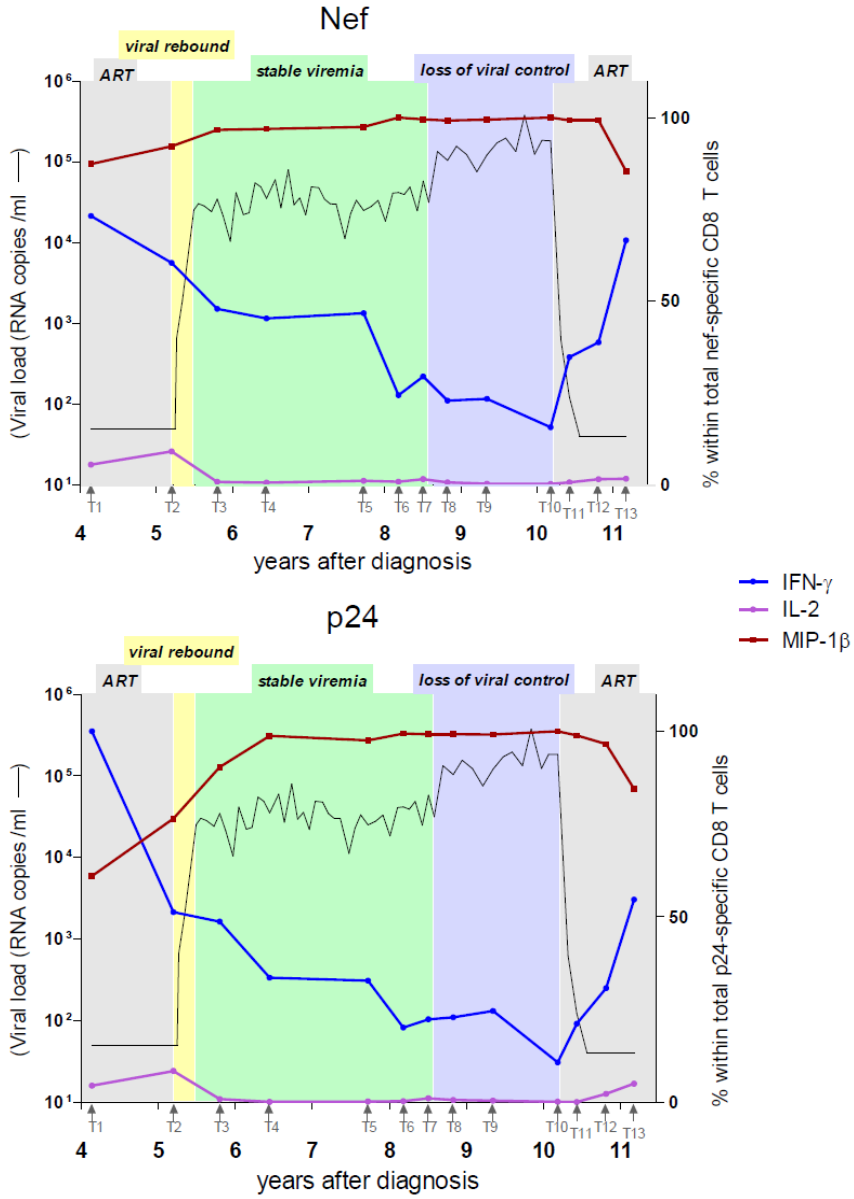


Figure 25: Quality of HIV-1-specific CD8 T-cell response during a 7-year follow up period. Stimulation was performed using peptide pools covering Nef or p24 protein. Thirteen timepoints were analyzed (T1-T13). Percentage of IFN-γ, IL-2 and MIP-1β producing cells are shown on total Nef- and p24-specific CD8+ T-ell response. Shadings depict distinct clinical phases: ART, viral rebound, stable viremia and loss of viral control. Plasma viral load is shown.

4.2.8 Kinetic of MIRA CD8+ T cells in patient V4

In order to determine the presence of MIRA CD8+ T cells in the follow up of patient V4, PBMC were stimulated with Nef 4 and Nef 5. The frequency of Nef-specific MIRA CD8+ T cells increased continuously after ART interruption with a maximum of 25.8 % at T5 during the phase of stable viremia (Figure 26). Interestingly after this peak the proportion of Nef-specific MIRA CD8+ T cell decreased strongly at the end of the phase of stable viremia and fluctuated between 5.7 % and 13.6 % during the loss of control phase.

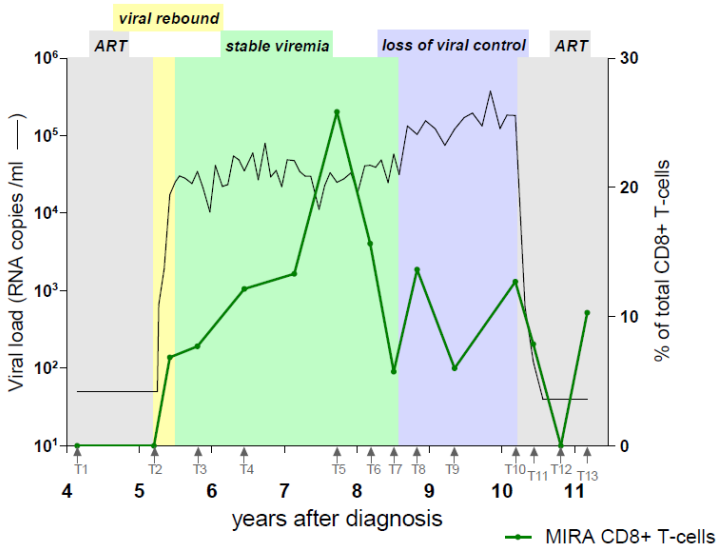


Figure 26: Kinetik of MIRA CD8+ T cell in patient V4 during the follow up period of 7 years. Relative amount of MIRA CD8+ T cells within total Nef-specific CD8+ T-cell response. Stimulation has been performed with Nef 4 and Nef 5 peptide pools. 13 timepoints were analysed (T1-T13). Shadings depict distinct clinical phases: ART (grey), viral rebound phase (yellow), phase of stable viremia (green), loss of viral control phase (blue). Plasma viral load is shown by a grey line.

4.2.9 Epitope specificity of Nef- and p24-specific CD8+ T cells

To identify the immunodominant CD8+ T-cell epitopes, responsible for Nef- and p24-specific CD8+ T-cell response, the single peptides derived from the peptide

pools Nef and p24 were first screened one by one. Within the responding single peptides, optimal HLA-matched CD8+ T-cell epitopes were screened subsequently. The two most immunogenic CD8+ T-cell epitopes within the Nef protein were B8-restricted FL8 and Cw7-restricted RH10. The most immunogenic CD8+ T-cell epitopes within the p24 protein were B8-restricted EI8 and NL8.

The strongest CD8+ T-cell response was elicited by FL8 (Figure 27). Total FL8-specific CD8+ T cells rose strongly with the increase of plasma viral load after ART interruption to consistently high levels during the phase of stable viremia (~7 % of total CD8+ T cells) and a maximum of 10.2 % of total CD8+ T cells during the loss of control phase. Plasma viral load suppression by ART re-initiation was accompanied by a decrease of FL8-specific CD8+ T cells (4.7 %).

Interestingly the kinetic of total RH10-, EI8- and NL8-specific CD8+ T-cell responses were very similar throughout the course of infection, but lower compared to the FL8-specific CD8+ T-cell response. These subdominant CD8+ responses also increased during the phase of stable viremia (maximum: 2.2 % - 3.3 %), dropped subsequently (1.4 %- 1.6 %) before peaking again (2.6 % - 4.6 %) during or the loss of control phase.

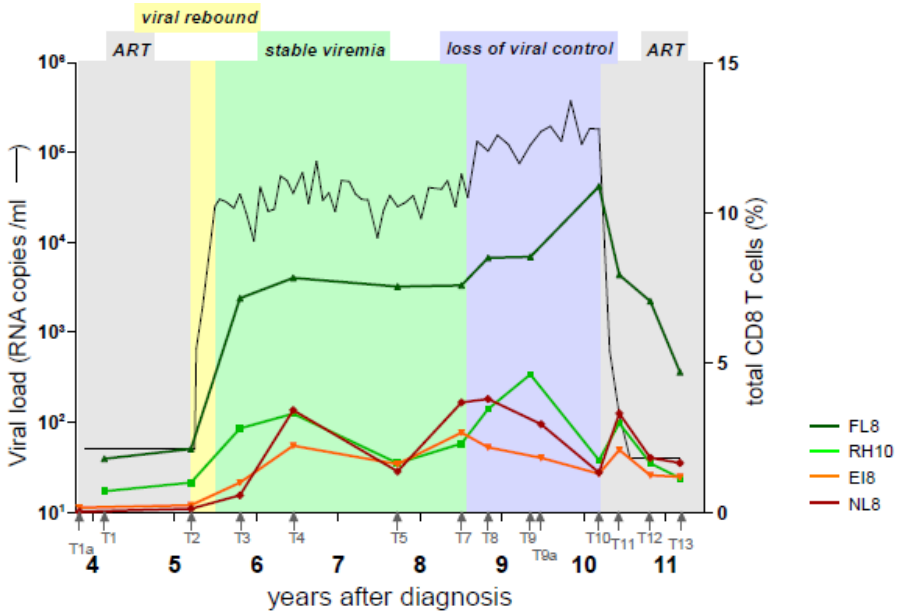


Figure 27: Magnitude of CD8+ T-cell response specific to optimal CD8+ T-cell epitopes. Stimulation was performed using the following peptides: Nef-derived HLA-Cw7-restricted RH10 and HLA-B8-restricted FL8 as well as p24-derived HLA-B8-restricted EI8 and NL8. Total response considering all CD8+ T cells positive for at least one of the following marker: IL-2, IFN-γ or MIP-1β. Twelve timepoints were analyzed, T6 was excluded and T1a and T9a replace T1 and T9 in EI8 and NL8 stimulation.

4.2.10 Terminally differentiated HIV-1-specific CD8+ T cells were frequently detectable during the phase of stable viremia

Terminally differentiated CCR7- CD45RA+ effector CD8+ T cells were recently associated with non-progressive status of HIV-1 infection [88], [89].

The use of the sole CD45RA marker cannot discriminate between experienced and naive T cells, when not associated with other cellular markers such as CCR7 or CD27. However, in the present study, cells were analyzed that were able to produce cytokines or chemokines following a short (5 hours) antigenic peptide stimulation. Thus, only experienced (memory or effector T cells) can be detected by this assay, since the number of circulating naive T cells carrying a T-cell receptor specific for a given peptide is too low to be detected by short term assays.

For detailed validation of the differentiation status of HIV-1-specific CD8+ T-cell response, epitope-specific CD8+ T-cell analysis was shown to provide adequate information [88].

Therefore the differentiation state of Nef- (FL8 and RH10) and p24- (EI8 and NL8) epitope-specific CD8+ T-cell responses (Figure 28) were characterized. After ART interruption, the proportion of fully differentiated CD45RA expressing cells within total epitope-specific CD8+ T cells showed a 10 % increase. However, already within the phase of stable viremia the proportion of fully differentiated CD45RA+ cells progressively declined in all epitope-specific CD8+ T-cell populations about 10 %. This phenotype persisted during the loss of control phase. CD45RA expression on epitope-specific CD8+ T cells recovering after ART re-initiation.

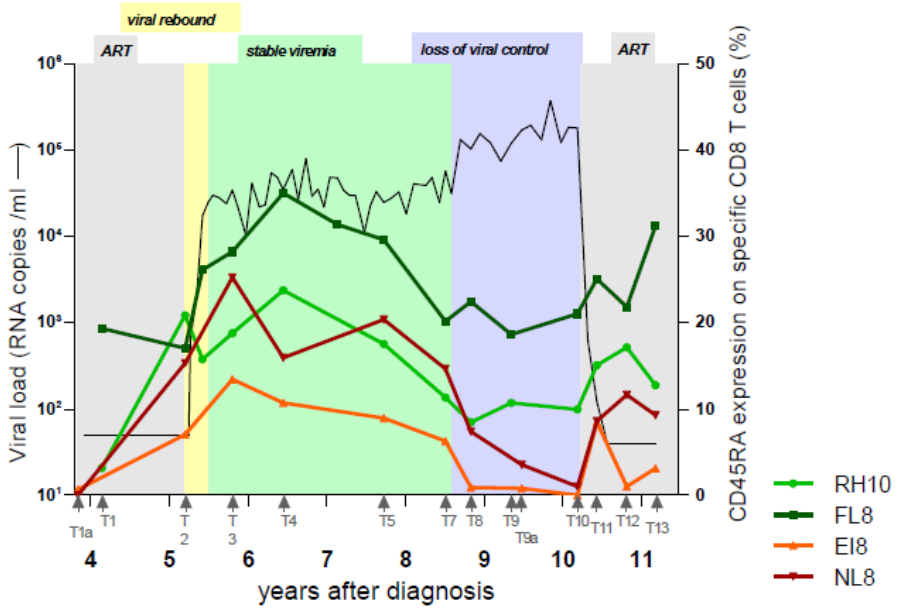


Figure 28: Quality of CD8+ T-cell response specific to optimal CD8+ T-cell epitopes. Stimulation was performed using the following peptides: Nef-derived HLA-Cw7-restricted RH10 and HLA-B8-restricted FL8 as well as p24-derived HLA-B8-restricted EI8 and NL8. Percentage of CD45RA expressing cells are shown on total specific CD8+ T-cell response. Twelve timepoints were analyzed, T6 was excluded and T1a and T9a replace T1 and T9 in EI8 and NL8 stimulation.

5 Discussion

5.1 MIRA CD8+ T cells

5.1.1 MIRA CD8+ T cells are associated with non-progressive HIV-1 infection

A fundamental prerequisite for the development of both immune-based therapies and an effective vaccine against HIV-1 is the identification of solid immune correlates of disease progression. In order to identify such correlates, in this study Nef- and Tat-specific CD8+ T-cell immune responses were compared in three cohorts of HIV-1 infected individuals with different degree of HIV-1 control: NP, PR and ART-treated patients. With this setting, a novel population of CD8+ T cells associated with non-progressive HIV-1 infection was identified. CD45RA+ IFN-γ- MIP-1β+ CD8+ T cells that henceforth were entitled MIRA (MIP-1β+ CD45RA+) CD8+ T cells potentially represent a valuable immune correlate of disease progression, since they were detected in response to Nef stimulation in 7 out of 11 NP, in only 1 out of 10 PR and were completely absent in 22 ART-treated patients. On 6 ART-treated patients undergoing single cycle TI was further demonstrated that the presence of MIRA CD8+ T cells is independent of plasma viral load. These observations render this novel population particularly interesting as a potential surrogate clinical marker of immunological reconstitution or maintenance after immune-based interventions.

Since MIRA CD8+ T cells express CD45RA but not CD27 they belong to the effector T cell population [103]. Several studies have suggested a possible role of the fully differentiated HIV-1-specific T effector memory CD45RA+ (T_{EMRA}) CD8+ T cells in the effective control of HIV-1 replication: IFN-γ producing T_{EMRA} CD8+ T cells have been associated with the control of virus replication in NP [88] and in individuals with early infection and low viral set point thereafter [89]. Furthermore, antigen-specific T_{EMRA} CD8+ T cells were preferentially detected in acutely infected individuals who achieved control of viremia either spontaneously or after

structured TI [95]. A pre-terminally differentiation status or skewed maturation phenotype mainly composed by T effector memory (T_{EM}) cells has been reported for HIV-1-specific CD8+ T cells in therapy-naive viremic patients [104], [105]. The skewed maturation of HIV-1-specific CD8+ T cells in comparison to other better controlled persistent infections has been considered as a defective immune response. Altogether, these studies indicate an important role of terminally differentiated CD8+ T cells in the control of HIV-1 replication *in vivo*. The present study supports and extends the link between HIV-1-specific T_{EMRA} CD8+ T cells and slow disease progression by identification of a novel HIV-1-specific population of effector cells specific for non-progressive HIV-1-infection.

Polyfunctional CD8+ T cells have been previously described in NP [87]. This observation is consistent with the present study, in which the proportion of responding CD45RA+ IFN-γ+ IL-2+ MIP-1β+ CD8+ T cells was significantly higher in NP than in ART-treated individuals (p=0.0067). In addition, higher proportions of polyfunctional CD45RA- CD8+ T cells were observed in ART-treated individuals in comparison to PR and higher proportions of monofunctional (IFN-γ+ IL-2- MIP-1β-) CD45RA- CD8+ T cells were observed in PR in comparison to NP and ART-treated individuals. These data support the idea that polyfunctional CD8+ T cells are lost during progressive HIV-1 replication and are maintained or recovered during non-progressive infection or treatment with ART. A recent longitudinal study demonstrated that polyfunctional CD8+ T cells re-emerge following prolonged ART-mediated viral suppression [90]. Furthermore, Streeck et al. [106] demonstrated that monofunctional HIV-1-specific CD8+ T cells decrease upon removal of antigenic stimulation. Together with these previous studies, the data presented inhere suggest that persistent stimulation by antigen can cause functional CD8+ T-cell impairment and may lead to enrichment of monofunctional IFN-γ producing HIV-1-specific CD8+ T cells.

In chronic viral infections, the main obstacle to the definition of a correlate of disease progression is the ability to discriminate between phenotypes responsible for the control of viral replication and phenotypes that are the

consequence of a different infection history [90]. MIRA CD8+ T cells were undetectable in a group of 4 ART-treated patients with a previous history as NP, suggesting that this cell population is absent when patients lose the capacity to control virus replication. The absence of MIRA CD8+ T cells in 9 out of 10 PR and 3 ART-treated patients with detectable viremia together with the analysis of a group of 6 ART-treated patients undergoing a single cycle of TI demonstrated that MIRA CD8+ T cells are not induced or regulated by the *in vivo* levels of HIV-1 replication. In this regard, no correlation was found between the proportion of MIRA CD8+ T cells and the levels of viremia in the 11 NP analyzed. These observations suggest that MIRA CD8+ T cells are not the direct consequence of ongoing *in vivo* antigen exposure, but possibly represent a true correlate of HIV-1 disease progression. However, more detailed longitudinal studies will be necessary to definitively demonstrate the role of MIRA CD8+ T cells in HIV-1 infection.

It was demonstrated that the sole measurement of the Nef-specific response may be sufficient to define MIRA CD8+ T cells as a correlate of nonprogression in HIV-1 disease. In addition, the presence of MIRA CD8+ T cells in 2 Tat-responding NP and the absence of the same population in the Tat-responding PR and ART-treated patients suggest that MIRA CD8+ T cells may represent a correlate of nonprogression independently of the targeted viral protein.

HIV-1 infection causes hyperactivation of the immune system leading to immune exhaustion and disease progression [107], [108], [109]. Since MIRA CD8+ T cells produce neither IFN-γ nor IL-2, it can be suggested that due to this limited effector function they do not contribute to hyperactivation in chronic HIV-1 infection. In contrast, in early infection, CD8+ T cell effector function is essential in controlling the initial viral replication [110]. Thus, the limited effector function of MIRA CD8+ T cells may contribute to rapid progression in early stage of HIV-1 infection. This could explain why MIRA CD8+ T cells were detected in progressor PR05, the only patient within the PR cohort who presented with the clinical phenotype of a rapid progressor.

Since the present study was observational, it was not the objective to clarify whether MIRA CD8+ T cells exert a direct protective function. However, it has been shown, that MIP-1β dominates HIV-1-specific CD8+ T-cell responses [87], [92] and that high levels of MIP-1β are associated with decreased risk of progression to AIDS [111]. Furthermore, MIP-1β is a potent natural inhibitor of CCR5-mediated HIV-1 entry [112]. IFN-γ was shown to be capable to upregulate HIV-1 replication [113], [114] and to induce the expression of HIV-1 in persistently infected cells in [115]. It can therefore be speculate that in the absence of IFN-γ, MIP-1β secreted by MIRA CD8+ T cells provide HIV-1 inhibitory functions.

Taken together, the present cross-sectional study presents a novel population of HIV-1-specific effector CD8+ T cells associated with non-progressive HIV-1 infection. This population, named MIRA, expresses CD45RA and produces MIP-1β but not IFN-γ and was shown to be independent of *in vivo* viral replication.

5.1.2 Functional and phenotypic characterization of MIRA CD8+ T cells

MIRA CD8+ T cells were identified as potential correlates of protection against HIV-1 disease progression. In order to clarify their role in non-progressive HIV-1 infection, qualitative analysis of these cells was performed to provide information about their specificity, activation state, cytotoxic capacity and differentiation state.

An important issue derived from the epitopic study was, that the quality of CD8+ T-cell responses against a single epitope does not represent the quality of CD8+ T-cell responses against the entire protein when stimulated with a pool of overlapping peptides. Each epitope elicits it´s characteristic CD8+ T-cell response in terms of quality and only a battery of optimal epitopes spanning one protein could provide a broad picture of the quality of CD8+ T-cell response against a requested protein. This reasoning explains the epitope specific appearance of MIRA CD8+ T cells. To establish a vaccine against HIV-1 it is

therefore of particular importance to select the epitopes according to the immune response they elicit. Epitopes eliciting MIRA CD8+ T-cell response might be favorable, as MIRA CD8+ T cells are associated with non-progressive HIV-1 infection.

Since MIRA CD8+ T cells did not express CD27 they were classified as effector CD8+ T cells. It is known that HIV-1-specific CD45RA+ effector memory T cells are associated with control of viral replication [89]. HIV-1-specific CD8+ T cells in long term non-progressors have been described to be enriched of the CD27- CD45RA+ effector subset [116]. In a study on HIV-1 chronically infected, untreated patients was reported that HIV-1-specific CD8+ T cells do not maturate to the effector subset CD27- $CD57^{high}$, like CD8+ T cells against other controlled long persisting viral infections like CMV or EBV, but accumulate in an atypical $CD27^{high}$ $CD57^{low}$ subset of undifferentiated memory state [117]. This accumulation was correlated with HIV-1 plasma viremia.

The subset of MIRA CD8+ T cells, which has been described to be exclusively found in non-progressors or patients with partial control of viral replication, has this protective CD45RA+ CD27- effector memory phenotype. However, MIRA CD8+ T cells were not necessarily CD57 positive; their relative amount of CD57 positive cells was rather comparable to that of other responding CD8+ T-cell subpopulations. In contrast to $CD57^{high}$ effector memory cells, MIRA CD8+ T cells are therefore not prone to undergo apoptosis [97].

To evaluate the killing capacity of MIRA CD8+ T cells the expression of intracellular perforin was regarded as suitable indicator, since the granzymes Grzm A or Grzm B, are responsible for the DNA fragmentation observed in the target cell, but require the presence of perforin for their activity [118], [119]. The ability of MIRA CD8+ T cells to upregulate perforin expression represents a precise picture of their antiviral potential, since the correlation between perforin expression and cytotoxic function was recently described [83].

Interestingly significant differences between responding CD8+ T-cell populations were more frequently detectable after stimulation with single peptides containing HLA-matched epitopes than after stimulation with the peptide pools Nef 4 and

Nef 5. This observation shows that stimulation with peptide pools are suitable to indicate functional and phenotypic tendencies without considering the HLA-type of the single patients. A more detailed picture of function and phenotype of responding cells can be certainly obtained by stimulation with single peptides containing HLA matched epitopes.

HIV-1-specific CD8+ T cells were analyzed in regard of perforin production and the expression of the degranulation marker CD107a. Interestingly a tendency of reverse correlation between these two markers was observed; the perforin^{high} CD8+ T cells degranulated less than the perforin^{low} CD8+ T cells, meaning that perforin^{high} CD8+ T cells keep higher levels of perforin because they do not release perforin by degranulation. MIRA CD8+ T cells, expressing very high levels of perforin, did not express the degranulation marker CD107a and therefore exhibit no cytotoxic function via perforin.

Apart from the granule dependant pathway, requiring perforin, granzyme and CD107a, cytotoxic CD8+ T lymphocytes have been shown to mediate killing of target cells through a ligand-ligand induced mechanism for example by fas-fasL binding [99]. In patient V4 all responding CD8+ T cells, including MIRA CD8+ T cells, were negative for the expression of the apoptosis inducer fasL. It was shown in the murine model that perforin- and fas-based mechanisms account for all T cell mediated cytotoxicity [120]. Having shown that MIRA CD8+ T cells do neither release perforin by degranulation nor express fasL on their cell surface consequently leads to the conclusion that MIRA CD8+ T cells are not cytotoxic killer cells.

CD137 (4-1BB), a member of the TNFR superfamily is a costimulatory molecule that is transiently up-regulated following TCR engagement accompanied by CD28 costimulation [121], [122], [123], [124]. CD137 was shown to stimulate CD8+ T-cell proliferation [125], [126], survival [127] and IFN-γ production [128]. Furthermore, CD137 costimulation has been shown to play a role in antiviral CD8+ T-cell responses [129], [130], [131], [132]. Recent studies on mice show, that CD137 deficiency has minor defects in controlling acute viral infections [133]. However CD137 deficient mice are unable to control chronic viral infections,

suggesting an essential role of CD137 in memory CD8+T-cell response [132], [134], which was confirmed in human studies [101]. Technically, it was not possible until now to isolate MIRA CD8+ T cells and explore surface CD137 expression after 24h stimulation as common protocols suggest. However, in this study was shown that all antigen specific CD8+ T cells, independently of the CD45RA isoform, produce high levels of intracellular CD137 already 5 hour post stimulation. MIRA CD8+ T cells and CD45RA- IFN-γ- MIP-1β+ CD8+ T cells produced the highest levels of intracellular CD137. MIRA CD8+ T cells respond therefore to antigenic stimulation by production of high amounts of CD137, which after expression on the cell surface and binding to CD137L is suggested to prevent MIRA CD8+ T cells from dying and promote proliferation and cytokine production. MIRA CD8+ T cells therefore gain dramatically in importance, because compared to terminally differentiated effector CD8+ T cells, MIRA CD8+T cells potentially pass their effector functions to following generations.

5.1.3 Longitudinal follow up of an HIV-1 infected patient with partial control of viral replication

In the first part of the present study, HIV-1-specific T-cell responses of infected patients with different degrees of viral control were analyzed in order to identify associations with non-progressive HIV-1 infection. To assume cause and consequence relations between host T-cell responses and viral replication, HIV-1-specific T-cell responses were analyzed longitudinally in a patient who underwent both, controlled and uncontrolled HIV-1 infection. Since Nef-specific MIRA CD8+ T cells were identified as potential immune correlate of protection from disease progression, a patient with strong Nef-specific T-cell response was chosen for the longitudinal follow up. Patient V4 was vaccinated with MVA-Nef during ART treatment [102].

The clinical parameters of HIV-1 infection were monitored longitudinally in patient V4. After ART treatment interruption viremia was stable for three years, before control of viral replication was suddenly lost. Using multiparameter flow

cytometry, two major modifications of HIV-1-specific T-cell responses, which coincided with the loss of viral control, were determined. First, progressive loss of IFN-γ as well as increase of MIP-1β production dependant on plasma viral load in dominant HIV-1-specific CD4+ and CD8+ but not in subdominant CD4+ T-cell responses was detected. Second, at the epitopic level was observed that terminally differentiated HIV-1-specific CD8+ T cells were more frequently within the phase of stable viremia than during loss of viral control, independently of plasma viral load levels.

Patient V4 has been vaccinated with MVA-Nef, which was described to elicit and enhance highly functional Nef-specific effector CD4+ T-cell responses in ART-treated HIV-1 infected individuals [102], [135]. In patient V4 the MVA-Nef-primed CD4+ T-cell response was boosted by ART interruption induced HIV-1 rebound resulting in a strong Nef-specific CD4+ T-cell response after ART interruption. This strong vaccine primed Nef-specific CD4+ T-cell response was insufficient to prevent loss of control of viral replication.

Polyfunctional HIV-1-specific CD4+ T cells have been linked to successful immune response [136], [137]. Indeed, a high degree of functionality was observed during the phase of stable viremia with a progressive loss of IL-2, CD154 and IFN-γ in Nef-specific CD4+ T cells. Once functionally exhausted Nef-specific MIP-1β+ IFN-γ+/- IL-2- CD154- CD4+ T cells might be incapable to contribute in controlling viral replication during the loss of control phase.

CD154 expression on CD4+ T cells, necessary for interaction between CD4+ T cells and antigen presenting cells, is typically impaired in HIV-1-infection [138]. Yet the activation of CD4+ T cells by CD154 expression also enhances HIV-1 replication in these cells [139], [140].Therefore, the early downregulation of CD154 on Nef-specific CD4+ T cells might prevent uncontrolled viral replication during the phase of stable viremia. However almost complete loss of CD154 expression on these cells (only 2.1-5% of total Nef-specific CD4+ T cells) coincided with loss of viral control probably due to missing interaction between Nef-specific CD4+ T cells and antigen presenting cells. Since both, CD154 expression and IFN-γ production correlated inversely with plasma viral load

during the ART-free period, downregulation of these two functions seemed to be influenced by levels of HIV-1 antigen.

HIV-1 is known to preferentially infect HIV-1-specific CD4+ T cells [141], therefore high amounts of Nef-specific CD4+ T cells would obviously be an excellent target for HIV-1 replication. Because autokrine MIP-1β production was shown to prevent HIV-1-infection [142], Nef-specific MIP-1β producing CD4+ T cells might contribute to limited HIV-1 replication during the phase of stable viremia. Nevertheless, MIP-1β production positively correlated with levels of plasma viral load during the ART free period. In this regard, plasma viral load induced functional exhaustion in Nef-specific CD4+ T cells in patient V4 was described, characterised by reduction of IFN-γ production and CD154 expression as well as enhanced MIP-1β production.

In contrast to the strong vaccine primed Nef-specific CD4+ T-cell response, p24-specific CD4+ T cells did not loose but rather increased functionality during stable viremia. The proportions of IFN-γ, IL-2 and MIP-1β production, as well as the proportion of CD154 expression did not correlate with plasma viral load. Nevertheless, loss of viral control could not be prevented by polyfunctional p24-specific CD4+ T cells. Possibly the numbers of p24-specific CD4+ T cells have been too low, or a stable functional phenotype is necessary to control viral replication that could not be established during the loss of control phase.

Therapeutic MVA-Nef vaccination did, in contrast to CD4, not enhance Nef-specific effector CD8+ T-cell response [102], [135] in vaccinees during ART. Indeed Nef- and p24-specific CD8+ T-cell response of patient V4 were comparable in quality and quantity after ART interruption.

In the model of CD8+ T-cell exhaustion in chronic viral infections, hierarchically reduction of IL-2 production occurs already before loss of IFN-γ production [143]. In this regard, the majority of Nef- and p24-specific CD8+ T cells were already partially exhausted during the phase of stable viremia since they have lost IL-2 production earlier. Both, Nef-and p24-specific CD8+ T cells further exhausted during stable viremia and loss of control phase by progressively reduced IFN-γ production. Throughout the ART free period, the proportions of IFN-γ in

Nef-specific CD8+ T-cell response strongly inversely correlated with plasma viral load, the same tendency was detected in p24-specific CD8+ T cells. Beyond doubt HIV-1 takes advantage of reduced IFN-γ production, since besides its antiviral activity [144], it is known to inhibit HIV-1 replication in latently infected cells [145].

MIP-1β, which blocks viral entry, belongs to the major HIV-1-suppressive factors produced by CD8+ T cells [112], [146]. However, it has been shown that high quantities of MIP-1β producing CD8+ T cells are maintained in both non-progressive and progressive HIV-1 infection. Another recent study described no differences in serum MIP-1β levels between progressors and slow progressors and therefore contributes no role in control of viremia to this chemokine [147]. This indicates that MIP-1β production alone does not decide on control of viral replication. Interestingly in the present study the proportions of MIP-1β producing p24-specific CD8+ T cells even correlated with plasma viral load during the ART-free period and similar tendencies were observed in Nef-specific CD8+ T cells. These observations suggest a plasma viral load dependant change in HIV-1-specific CD8+ T cells towards reduced IFN-γ and increased MIP-1β production.

In agreement with previous studies a reduced grade of functionality in HIV-1-specific T cells was associate with loss of viral control, [148], [77], [149]. The present data of patient V4 support the hypothesis that loss of functionality is a consequence of exposure to viral antigen [90], [106]. A negative correlation between plasma viral load and IFN-γ production as well as a positive correlation between plasma viral load and MIP-1β production was observed. It can be concluded that plasma viral load influences HIV-1-specific T-cell functions and not vice versa since both the abrupt increase of plasma viral load due to ART interruption as well as the prompt decrease of plasma viral load due to ART re-initiation causes alterations in the functional profile of major CD4+ and CD8+ T-cell responses.

Stimulation with pools of 20mer peptides overlapping by 10 amino acids covering the complete HIV-1 proteins Nef and p24 provided an overview of protein specific

CD4+ and CD8+ T-cell response. However it has been recently described that CD45RA expression, indicating the differentiation status of antigen-specific T cells, needs to be determined on epitope-specific CD8+ T-cell responses as CD45RA expression is not reflected by peptide pool stimulation corresponding to the same HIV-1 protein [88]. Therefore stimulation with optimal HLA-matched Nef derived CD8+ T cell epitopes has been performed.

The fully differentiated effector memory CD45RA+ phenotype of HIV-1-specific CD8+ T cells is associated with controlled HIV-1 infection [88] and if detectable in early infection, associated with future control of HIV-1 [89]. Impaired effector CD8+ T cell maturation may contribute to chronic progressive disease.

In V4 was observed, that CD8+ T cells specific for the optimal Nef and p24 epitopes FL8, RH10, EI8 and NL8 expressed high proportions of CD45RA at the beginning of the phase of stable viremia. This phenotype exhausted slowly during the phase of stable viremia by reduction of CD45RA expression. During this time CD8+ T cells specific to optimal CTL epitopes might be still able to control viremia but below a certain level of CD45RA expression, (FL8-specific CD8+ T cells: 20.2% of total CD8+ T cells, RH10-specific CD8+ T cells: 11.3% of total CD8+ T cells, NL8-specific CD8+ T cells: 14.7% of total CD8+ T cells and EI8-specific CD8+ T cells: 6.3% of total CD8+ T cells) these dominant CD8+ T cell populations might became incapable in controlling viremia, resulting the loss of control of viral replication. Especially the frequencies of exhausted, impaired, widely CD45RA- FL8-specific CD8+ T increased to severely high levels (maximum of total responding FL8-specific CD8+ T cells: 10.2% of total CD8+ T cells, thereof 79% CD45RA-) during loss of control phase and might drove the HIV-1-specific CD8+ T cell immune response towards a non protective one way road.

The associations between progressive HIV-1-infection and both, loss of T-cell function and loss of terminally differentiated HIV-1-specific CD8+ T cells are in agreement with previous findings obtained from cross-sectional studies that compare patient cohorts with different degrees of HIV-1 control [87], [88], [89]. While cross-sectional studies analyse single “snap-shots” the present longitudinal

study provides additional information about the course of modifications in HIV-1-specific T-cell response between the phase of stable viremia and loss of control. Moreover, in the present case study the immunological parameters, which changed between phase of stable viremia and the loss of control phase were linked to plasma viral load. In this regard the reduced functionality of major antigen specific T cells in patient V4 within the loss of control phase is suggested to be a consequence of plasma viral load, while the loss of terminally differentiated HIV-1-specific CD8+ T cells seemed to be an immunological modification which precedes loss of viral control and occurs independently of plasma viral load.

Finally, the proportion of MIRA CD8+ T cells, that have been associated with non-progressive HIV-1 infection in the cross-sectional part of the present study, decreased with the loss of control inpatient V4. This confirms the association of MIRA CD8+ T cells and non-progressive infection.

Taken together two main qualitative modifications were detected in the present longitudinal case study in HIV-1-specific T cells between controlled and uncontrolled phase of infection: First, progressive loss of function in major HIV-1-specific CD4+ and CD8+ T-cell responses was determined by plasma viral load dependant loss of IFN-γ and increase of MIP-1β production. Second, decreasing frequencies of terminally differentiated HIV-1-specific CD8+ T cells precedes loss of viral control and occurred independently of plasma viral load. Loss of terminally differentiated HIV-1-specific CD8+ T cells might therefore predict or even cause loss of viral control. In this regard, the study sheds light on potential correlates of protection and their loss in the course of a progressive HIV-1 infection and contributes to understand the failure of the host immune system against HIV-1.

6 Conclusions

Understanding the mechanisms by which the host immune response controls HIV-1 replication is important for the development of effective vaccines and immune based therapies. It is therefore essential to compare the immune response in HIV-1 infected patients with different degrees of HIV-1 control.

The cross-sectional study represents a novel population of HIV-1-specific effector CD8+ T cells associated with non-progressive HIV-1 infection. This population, which was named MIRA CD8+ T cells, expresses CD45RA and produces MIP-1β but not IFN-γ.

MIRA CD8+ T cells did not degranulate or express the fasL and, therefore, probably do not contribute to limiting viremia. Due to their CD137 and moderate CD57 expression they are long-living effector T cells with limited anti-viral activity that might prevent hyperactivation of the immune system.

MIRA CD8+ T cells were also present in an HIV-1 infected partial controller, who underwent both controlled and uncontrolled phases of HIV-1 infection. Longitudinal follow up of this patient revealed that high frequencies of HIV-1-specific CD4+ and CD8+ T-cell responses were unable to prevent loss of viral control. Progressive loss of function in major HIV-1-specific CD4+ and CD8+ T-cell responses were characterized by plasma viral load dependant decrease of IFN-γ and increase of MIP-1β production. Furthermore, progressive loss of terminally differentiated HIV-1-specific CD8+ T cells occurred independently of plasma viral load and possibly precedes the uncontrolled phase of infection.

The present study highlights the contribution of T-cell function and the differentiation in protection from HIV-1 disease progression and contributes towards understanding the mechanisms involved in the failure of the host immune system in patients with HIV-1 infection.

7 References

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8 Curriculum Vitae

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Education

February 2007 – August 2010	**PhD thesis** *Helmholtz-Zentrum München, Institute of Virology, Munich, Germany* „Immune correlates of protection in HIV-1 infection“ Techniques used: Advanced polychromatic flow cytometry
July 2006	**Diploma in Biology** (1.4) *Ludwig-Maximilians Universität München, Munich Germany* Main Subjects: Cell Biology, Immunology, Neurobiology, Psychology
October 2005-July 2006	**Diploma Thesis** (1.3) *University of Leicester, Leicester, Great Britain, UK* “Murine properdin protein: Its function and expression” Techniques used: general cell culture techniques, complement activation assay on Streptococcus *pneumoniae*, transformation, chemical transfection, electroporation, Ca^{2+}-phosphate precipitation, flow

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June 2000	**Abitur** (2.6) *Gymnasium Unterhaching, Munich, Germany* Main Courses English, Biology

Skills and experience

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Applications	MS Word, Excel, PowerPoint and Access; Graphpad Prism; Mac OS

9 List of Publications

Journal publications

Dembek CJ, Kutscher S, Allgayer S, Bauer T, Hoffmann D, Goebel FD, Bogner JR, Erfle V, and Cosma A: **Loss of viral control accompanied by exhaustion of dominant HIV-1-specific T-cell responses: a case report of HIV infection**. Manuscript in preparation.

Dembek CJ, Kutscher S, Heltai S, Allgayer S, Biswas P, Ghezzi S, Vicenzi E, Hoffman D, Reitmeir P, Tambussi G, Bogner JR, Lusso P, Stellbrink HJ, Santagostino E, Vollbrecht T, Goebel FD, Protzer U, Draenert R, Tinelli M, Poli G, Erfe V, Malnati M and Cosma A: **Nef-Specific CD45RA+ CD8+ T cells Secreting MIP-1β but Not IFN-γ Are Associated with Nonprogressive HIV-1 Infection**. *AIDS Res Ther* 2010, 7:20

Kutscher S, Allgayer S, **Dembek CJ**, Bogner JR, Protzer U, Goebel FD, Erfle V and Cosma A: **Modified Vaccinia Virus Ankara expressing HIV-1 Nef induces specific polyfunctional and proliferative T-cell responses revealed by combination of short- and long-term immune assays**. *Gene Therapy* 2010.

Di Bonito P, Grasso F, Mochi S, Petrone L, Fanales-Belasio E, Mei A, Cesolini A, Laconi G, Conrad H, Bernhard H, **Dembek CJ**, Cosma A, Santini SM, Lapenta C, Donati S, Muratori C, Giorgi C, Federico M. **Anti-tumor CD8+ T cell immunity elicited by HIV-1-based virus-like particles incorporating HPV-16 E7 protein**. *Virology* 2009, 395:45-55.

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Congress contributions

Dembek CJ, Kutscher S, Allgayer S, Goebel FD, Bogner RJ, Erfle V and Cosma A: **Loss of viral control accompanied by fatigue of dominant HIV-1-specific T-cell responses: a case report of HIV infection.** *4th MASIR Conference* 2010, Mykonos, Greece

Dembek CJ, Kutscher S, Allgayer S and Cosma A: **Nef- and Tat-Specific CD45RA+ CD8+ T cells Secreting MIP-1β but Not IFN-γ Are Associated with Nonprogressive HIV-1 Infection.** *4th MASIR Conference* 2010, Mykonos, Greece

Dembek CJ, Kutscher S, Allgayer S, Goebel FD, Bogner RJ, Erfle V and Cosma C: **Longitudinal follow up of an HIV-1 infected subject with partial control of viral replication.** *2nd European Congress of Immunology* 2009, Berlin, Germany

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