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Immunomonitoring technologies for the evaluation of Modified Vaccinia Virus Ankara expressing HIV-1 *nef* as a vaccine against AIDS

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Erklärung

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I TABLE OF CONTENS

II ABBREVIATION LIST

AIDS	Acquired Immune Deficiency Syndrome
Al700	Alexa 700
APC (fluorescent dye)	Allophycocyanin
APC	Antigen presenting cell
BFA	Brefeldin A
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
Cy 7	Cyanin 7
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylendiamintetraacetate
ELISPOT	Enzyme Linked Immuno Spot Technique
EMA	Ethidium monoazide bromide
ER	Endoplasmatic reticulum
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FSC	Forward scatter channel
ICS	Intracellular cytokine stain
IE	immediate early
IFN	Interferon
IL	Interleukin
IU	Infectious units
HAART	Highly active anti-retroviral therapy
LMP	Latent membrane protein
LTNP	Long term non-progressor
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein

MVA	Modified Vaccinia virus Ankara
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PacB	Pacific blue
PBMC	Peripheral blood monocyte
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridininchlorophyll protein
pp65	Phosphoprotein 65
RNA	Ribonucleic Acid
s.c.	Subcutaneous
SCC	Sideward scatter channel
wt	Wildtype

1 Zusammenfassung

Der WHO/UNAIDS Bericht „Global summary of the AIDS epidemic", veröffentlicht im Dezember 2009 schätzt, dass 33 Millionen Menschen mit HIV leben, dass sich im vergangenem Jahr 2,7 Millionen Menschen mit HIV infizierten und 2 Millionen an AIDS starben. Trotz über zwei jahrzehntelanger Forschung existiert bis heute weder eine präventive noch eine therapeutische Vakzine. Und auch definitive Korrelate einer immunologischen Protektion gegen die HIV-Infektion sind nicht bekannt.

T-Zell vermittelte Immunität wird für die Kontrolle der HIV Infektion und der Progression zu AIDS als essentiell erachtet. Mehrere potentielle Impfstoff-Kandidaten haben die Stimulation von zellulären Immunantworten zum Ziel und werden derzeit in klinischen Studien der Phase I bis Phase III geprüft. Daher ist es erforderlich Assays, die eine verlässliche, informative und sensitive Messung von CD4- und CD8 T-Zell Antworten ermöglichen, zu etablieren. Unter den vielversprechendsten Impfstoffkandidaten befinden sich rekombinante modifizierte Vaccinia Viren vom Typ Ankara (MVA), lebend-virale Vekorsysteme für den Transport von HIV Antigenen. Solche rekombinanten MVA Vektoren werden und wurden in mehreren klinischen Impfstudien getestet.

Derzeit wird der auf der Messung von IFN-γ vermittelten T-Zell Immunantworten basierende ELISPOT Assay als Gold Standard erachtet und stellt den primären und bevorzugten in Impfstudien verwendeten Assay dar. Trotz der hohen Sensitivität des Tests, kann durch die reine Messung von IFN-γ Produktion durch T-Zellen jedoch nur beschränkt eine Aussage über die Qualität einer Immunantwort gemacht werden. Auf polychromatischer Durchflusszytometrie beruhende Methoden, wie intrazelluläre Zytokinfärbung (ICS) eröffnen die Möglichkeit mehrere Parameter auf einer einzelnen Zelle zu detektieren. Ergebnisse aus Quer- und Längsschnitt- Studien, die sich mit der Untersuchung verschiedener Grade von HIV Kontrolle befassten, heben die Wichtigkeit von Assays, die die simultane Messung multipler Parameter auf Einzel-Zell Niveau ermöglichen, hervor. Dadurch empfielt sich die Anwendung von polychromatischer Durchflusszytometrie zum Monitoring von zellulären Immunantworten.

In dieser Arbeit wurden auf polychromatischer Durchflusszytometrie basierende Methoden zum Zwecke des T-Zell Immunmonitorings entwickelt, optimiert und standardisiert. Zusätzlich wurden diese intrazellulären Zytokinfärbungs-Methoden mit ELISPOT Assays verglichen, die jeweils in spezialisierten Laboratorien durchgeführt wurden. Die komparative Analyse zeigte, dass durch die Anwendung einer speziellen Analysemethode die Sensitivität

des ICS bis zu einem Niveau erhöht werden konnte, das vergleichbar zu demjenigen eines ELISPOT Assays war. Diese Erkenntnisse sind bedeutend für die Auswahl geeigneter Immunassays, die sowohl Phänotyp als auch Funktionen spezifischer T-Zellen auf akkurate und sensitive Weise charakterisieren.

Der etablierte ICS kam in Kombination mit einem polychromatischen CFSE-basierten Proliferations-Assay in der Re-Evaluation einer klinischen Studie zur Anwendung, in der ein rekombinantes HIV-1 Nef exprimierendes MVA (MVA-*nef*) in HIV-1 infizierten, antiretroviral behandelten Individuen getestet wurde. In dieser Studie wurde der Einfluss der immunologischen Intervention mit MVA-*nef* auf die Nef spezifische zelluläre Immunantwort im Hinblick auf Zytokin- und Chemokinproduktion (IFN-γ, IL-2, MIP-1β), Aktivierungs- und Differenzierungsmarkerexpression (CD154, CD45RA) und proliferatives Potential untersucht. Vakzine-induzierte Polyfunktionalität und proliferative Kapazität, die in einigen Studien mit nicht progressiver HIV-Infektion assoziiert wurden, konnten durch die Kombination beider oben beschriebener Immunassays beobachtet werden. Anhand des kürzeren ICS konnte ein der Impfung folgender signifikanter Anstieg von polyfunktionalen CD4 T-Zellen beobachtet werden, die gleichzeitig IFN-γ, IL-2 and CD154 exprimierten. Mit der MVA-*nef* Immunisierung assoziierte Änderungen der Qualität der CD8 T-Zell Immunantwort konnten nicht festgestellt werden. Nur die zusätzliche Anwendung des polychromatischen CFSE-basierten Proliferationsassay, der eine längere *ex vivo* Stimulationsdauer einschließt, zeigte auch vakzine-induzierte, Nef spezifische CD8 T-Zellen mit proliferativem Potential. Die signifikante Korrelation zwischen dem MVA-*nef* induzierten Anstieg der IL-2 Produktion in CD4 T-Zellen und dem Anstieg der proliferierenden, Nef spezifischen CD8 T-Zellen lässt einen möglichen kausalen Zusammenhang zwischen beiden Funktionen vermuten.

Das Verständnis, das in dieser Arbeit gewonnen werden konnte übertrifft bei Weitem die Informationen, die aus der primären Analyse der klinischen MVA-*nef* Studie durch Anwendung einfacher IFN-γ basierter Tests resultierten. Die Ergebnisse heben die Wichtigkeit der Kombination hoch entwickelter Immunmonitoring Methoden hervor um versteckte Effekte immnologischer Interventionen aufzuzeigen. Die Daten unterstützen des Weiteren die Anwendung des von Pockenviren abstammenden MVA Vektors zur Stimulation effektiver HIV-spezifischer T-Zell Immunantworten. Aus technischer Sicht sind die Ergebnisse dieser Arbeit bedeutend um geeignete Assays zur Messung antigen spezifischer zellulärer Immunantworten in klinischen Studien zur Verfügung zu stellen, die eine sensitive Untersuchung von Funktion und Phänotyp ermöglichen.

2 Summary

The WHO/UNAIDS "Global summary of the AIDS epidemic" released in December 2009, estimates that 33 million of people are living with HIV, 2.7 million were newly infected and 2.0 million of people died of AIDS in the last year. After more than two decades of research an effective preventive or therapeutic vaccine against HIV remains elusive and immunological correlates of protection remain unknown.

T-cell mediated immunity is considered to play an important role in controlling HIV infection and progression to AIDS. Several candidate vaccines against HIV aiming to stimulate cellular immune responses are investigated in phase I to phase III clinical trials and assays enabling for a reliable, informative and sensitive measurement of CD4 and CD8 T-cell need to be implemented. Among the most promising vaccine candidates is recombinant modified vaccinia virus Ankara (MVA), a live viral vector system for the delivery of HIV-derived antigens. Several vaccination trials have made use of the modified vaccinia virus Ankara (MVA) as delivery vector.

At present, the IFN-γ-based ELISPOT assay is considered as a gold standard and preferred primary assay in vaccine trials. However, despite its high sensitivity the measurement of the sole IFN-γ production provides limited information on the quality of the immune response. Polychromatic flow-cytometry-based assays as intracellular cytokine staining (ICS) offer the possibility to detect several markers on the same cell. Several findings from cross-sectional and longitudinal studies investigating different grades of HIV control highlight the importance of developing assays able to simultaneously measure several parameters on a single-cell level and strongly suggest the use of flow cytometry to monitor immune responses. In this work polychromatic flow-cytometry based assays were developed, optimized, and standardized for T-cell immunomonitoring purposes. In addition, these ICS based methods were compared with ELISPOT assays performed in two different experienced laboratories. The comparative study provided evidence that by the use of a special analysis system, the sensitivity of the ICS could be increased up to levels comparable to the sensitivity of the ELISPOT assay.

The established polychromatic ICS together with a polychromatic CFSE-based proliferation assay were applied to a re-evaluation study of a vaccination trial using recombinant MVA expressing HIV-1-Nef (MVA-*nef*) in HIV-1 infected HAART treated individuals. In this study, the impact of the immunologic intervention with MVA-*nef* on the specific anti-Nef T-cell immune response was investigated in regard to cytokine production (IFN-γ and IL-2), chemokine production (MIP-1β), activation and differentiation marker expression (CD154 and CD45RA, respectively) and proliferative potential. Vaccine-induced polyfunctionality

and proliferative capacity, which were associated with nonprogressive HIV-1 infection in several studies, were detectable by combining the two immune assays. By means of short-term ICS, a significant increase of polyfunctional Nef-specific CD4 T cells expressing IFN-γ, IL-2 and CD154 was observed following vaccination, whereas changes in the quality of the CD8 T-cell response could not be observed. Only the additional use of a long-term polychromatic CFSE-based proliferation assay revealed vaccine-induced Nef-specific CD8 as well as CD4 T cells with proliferative capacity. The correlation between the vaccine-induced IL-2 production by CD4 T cells and the increase of proliferating Nef-specific CD8 T cells suggests a causal link between these two functions. The insight gathered in this reevaluation study exceeded by far the information obtained in the original work using a simple IFN-γ-based immune assay. These results highlight the importance of combining sophisticated immunomonitoring tools to unravel concealed effects of immunologic interventions and support the use of the poxvirus-derived MVA vector to stimulate effective HIV-specific T-cell responses. From a technical point of view, these findings are important to guide the choice for suitable immune assays able to characterize the phenotype and function of specific T-cells in a highly sensitive way.

3 Introduction

3.1 Human Immunodeficiency Virus (HIV)

3.1.1 Epidemiological profile

The WHO resport dating from the end of 2008 indicates that to date around 33 million people are living with HIV, the virus that causes AIDS. Each year around 2.7 million more people become infected with HIV. Young people, 15–24 years of age account for about 45% of all new HIV infections in adults. Every year, approximately 2 million people die of AIDS related causes (http://www.unaids.org; "Report on the global AIDS epidemic"). Although HIV and AIDS are found in all parts of the world, the worst affected region is sub-Saharan Africa, where in some countries more than one in five adults is infected with HIV. Worldwide prevalence is shown in Figure 1. The epidemic is spreading most rapidly in Eastern Europe and Central Asia, where the number of people living with HIV increased of 66% between 2001 and 2008 (http://www.unaids.org; "AIDS epidemic update 2009") [1].

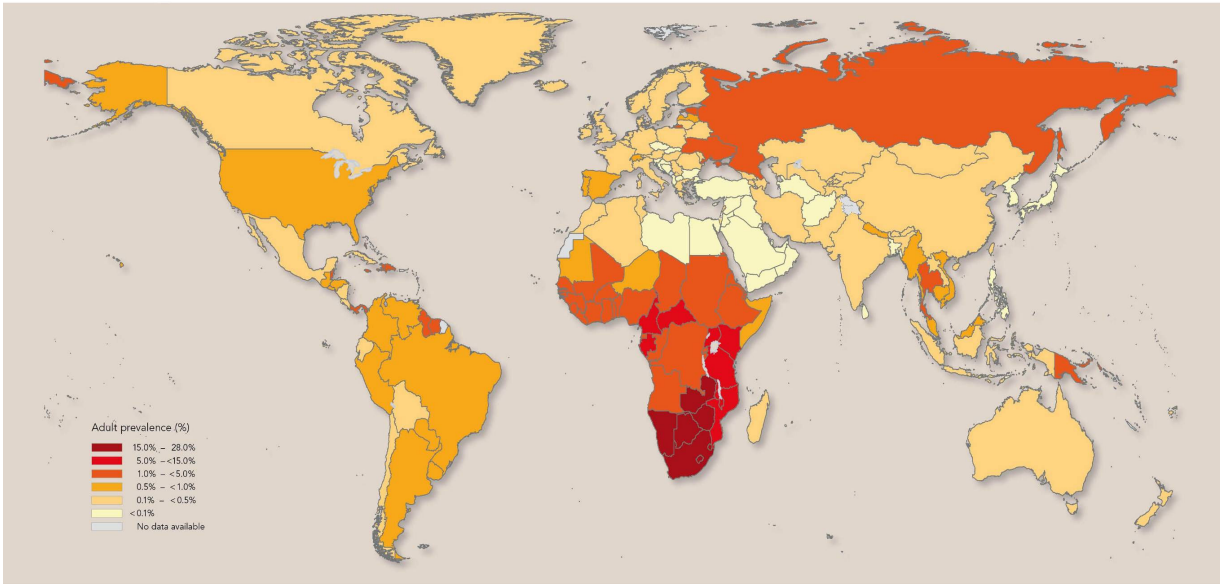

Figure 1. **A global view of HIV infection, 2007.** 33.4 million [31.1-35.8 million] of people are living with HIV infection. The global distribution of adult prevalence is shown as indicated by the color coding in the picture. Although HIV and AIDS are found in all parts of the world, the most affected region is sub-Saharan Africa, where in a few countries more than one in five adults is infected with HIV. Taken fromUNAIDS: "Report on the global AIDS epidemic 2008"

Epidemiologic data from Germany where since the beginning of the epidemic about 28,000 people died of AIDS related causes, are shown in Figure 2. Each year there are still 3000 people newly infected with HIV and about 1100 start suffering from AIDS.

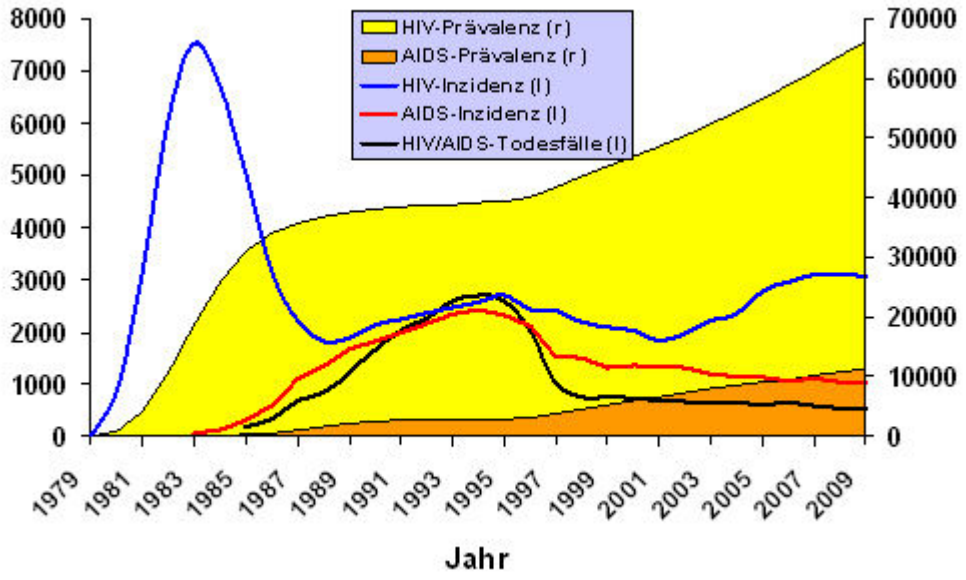

Figure 2. **Estimated HIV and AIDS incidence, prevalence and deaths in Germany 2009.** HIV incidence (blue line), AIDS incidence (red line), HIV/AIDS related deaths (black line) relate to left y-axes scaling (incidence); HIV prevalence (yellow area), AIDS prevalence (orange area) relate to right y-axes scaling (prevalence). Epidemiologisches Bulletin 48/2009; Robert Koch Institut.

3.1.2 Virological profile

Between the two types HIV-1 and HIV-2 [2-4] the latter is less easily transmitted, and the period between initial infection and illness is longer in the case of HIV-2 [5, 6]. Worldwide, the predominant virus is HIV-1. HIV-2 is concentrated in West Africa and is rarely found elsewhere [1]. On the basis of differences in the env gene, three groups of HIV-1 have been identified: M, N, and O [7]. Group M is the most prevalent and is subdivided into eight subtypes (clades) A through I, based on the whole genome, which are geographically distinct. The most prevalent is subtype B (mainly in North America and Europe), A and D (mainly in Africa), and C (mainly in Africa and Asia) [8, 9]. The closely HIV related simian immunodeficiency virus (SIV) exhibits a somewhat different behavior: in its natural hosts, African green monkeys and sooty mangabeys, the retrovirus is present in high levels in the blood, but evokes only a mild immune response [10], does not cause the development of simian AIDS [11], and does not undergo the extensive mutation and recombination typical of HIV [12]. By contrast, infection of heterologous hosts (rhesus or cynomolgus macaques) with SIV results in the generation of genetic diversity that is on the same order as HIV in infected humans; these heterologous hosts also develop simian AIDS [13, 14].

In 1983, Barre-Sinoussi et al. reported the discovery of a T-lymphotropic retrovirus in a patient at risk of AIDS. This was the virus that we now call HIV [15]. Afterwards, Gallo et al. showed that the discovered virus was the etiologic agent of AIDS [16-19] and succeeded to grow it in continuous T-cell cultures enabling the development of a blood test for detection of HIV. These two works opened the way to a period of intense research and discovery. The HIV genome was sequenced [20, 21], the HIV antigenic variation was discovered [22], macrophages were found to be target of HIV [23], various modes of transmission were elucidated [24-26], all HIV genes and proteins were defined and the HIV receptor CD4 was identified [27, 28].

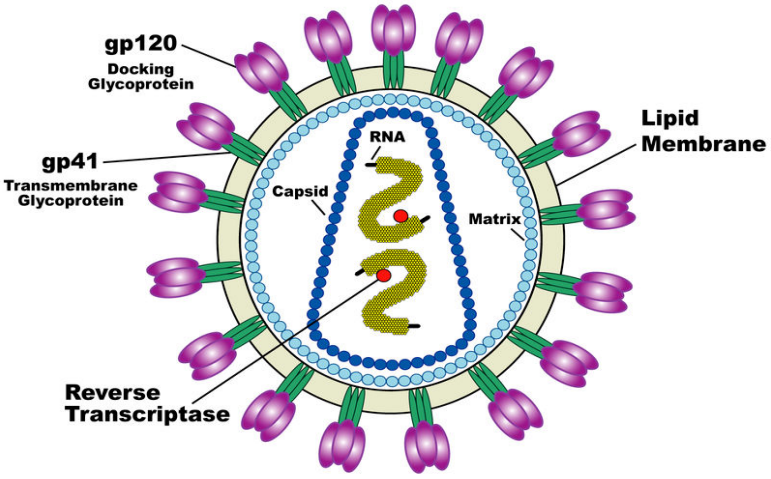

Figure 3. Structure of HIV-1. HIV-1 is composed of two copies of single-stranded RNA enclosed by a conical capsid comprising the viral protein p24 (in dark blue), typical of lentiviruses. An association of the matrix protein p17 (in light blue) is surrounded by a plasma membrane of host-cell origin. The envelope includes the glycoproteins gp120 and gp41. Source: http://en.wikipedia.org/wiki/HIV

HIV belongs to the lentivirus subgroup of retroviruses and is one of two important human T-cell lymphotropic retroviruses. HIV is around 120 nm in diameter and roughly spherical. It is composed of two copies of positive single-stranded RNA enclosed by a conical capsid composed of subunits of the viral protein p24. A matrix composed of subunits of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle, In turn, this is surrounded by the viral envelope, a plasma membrane of host-cell origin. Proteins from the host cell are embedded in the viral envelope with the complex HIV protein Env that protrudes through the surface of the virus particle. Env consists of a cap made of three molecules glycoprotein (gp) 120, and a stem consisting of three molecules gp41 that anchors the structure into the viral envelope [29].

The 10kB RNA genome codes for the virus's nine genes; group specific antigen (*gag)*, polymerase (*pol)*, envelope (*env)*, transcription transactivator (*tat)*, regulator of virion gene expression (*rev)*, negative factor (*nef)*, viral infectivity factor (*vif)*, viral protein r (*vpr) and viral protein u* (*vpu*). Two of these genes, *gag* and *env*, contain information needed to make the structural proteins for new virus particles:

- The *env* gene codes for a protein called gp160 that is cleaved in the host ER to form the two envelop proteins gp120 and gp41. This glycoprotein complex enables the virus to attach to and fuse with target cells [29].
- The *gag* gene encodes the two internal core proteins p17 (matrix protein) and p24 (capsid), with p24 used as antigen for serological tests. Furthermore, it encodes the nucleocapsid proteins p6 and p7 which are tightly bound to the viral RNA.

Three of the genes, *pol, tat* and *rev*, are regulatory genes that are required for viral replication:

- The *pol* gene encodes several proteins, including 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 codes for two Tat proteins (p16 and p14), which are transcriptional transactivators for the long terminal repeat (LTR) promoter and activate transcription of viral genes [30].
- The *rev* gene codes for the Rev protein, which is involved in shuttling RNAs from the nucleus and the cytoplasm [31].

The four remaining genes *nef*, *vif*, *vpr*, and *vpu* (or *vpx* in the case of HIV-2) encode for proteins that control the ability of HIV to infect cells, or to cause disease.

- The *nef* gene codes for the Nef protein, which down-regulates CD4 [32, 33], as well as the MHC class I and class II molecules [34-36]. Nef furthermore induces apoptosis in uninfected CD4 and CD8 T cells [37, 38]. Further description of Nef can be found in the section.3.3.2 Nef
- The *vif* gene encodes the Vif protein. This protein enhances infectivity by preventing the action of APOBEC3G (a cellular protein that causes hypermutation by deaminating cytosins in both mRNA and retroviral DNA. Thereby these molecules are inactivated and infectivity is reduced) [211].

- The *vpr* gene encodes the Vpr protein, which transports viral core from cytoplasm into nucleus in non-dividing cells and arrests cell division at G2/M [211].
- The *vpu* gene encodes the Vpu protein, which enhances the release of new virus particles from infected cells [211].

The ends of each strand of HIV RNA contain the RNA sequence called LTR. Regions in the LTR (e.g. the Psi element or the SLIP element) act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell.

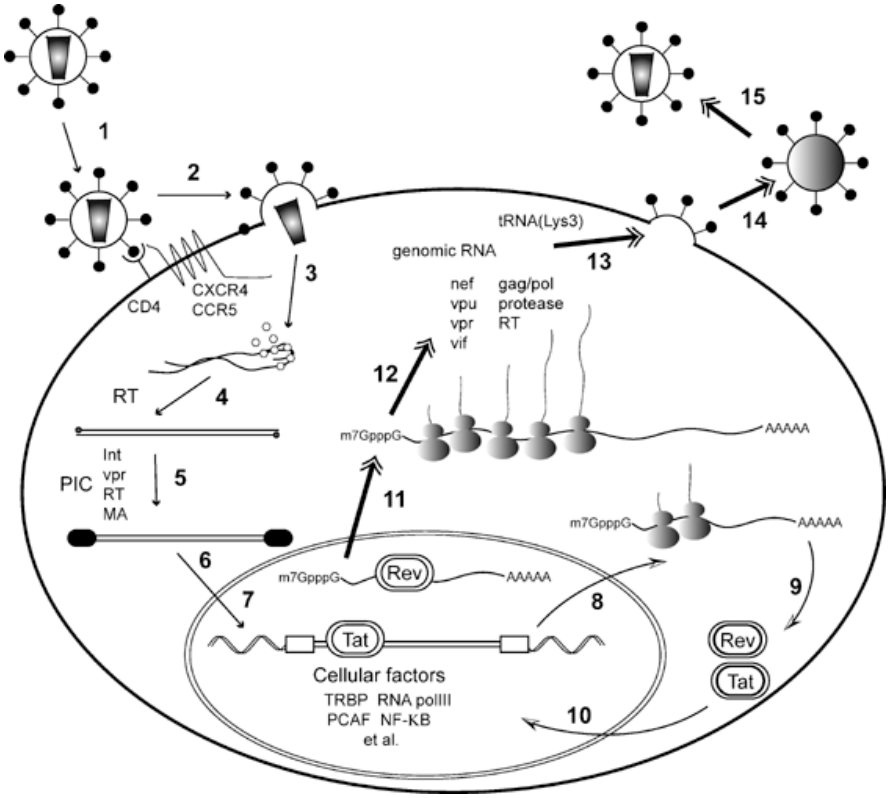

Figure 4. HIV life cycle. Small arrowheads, viral entry to integration. Curved arrows, early replication. Double-headed arrows, late replication. (1) Adsorption to CD4 receptor and either CXCR4 or CCR5 co-receptor. (2) Fusion. (3) Uncoating of viral genomic RNA dimer. (4) Reverse transcription (RT, reverse transcriptase). (5) Formation of pre-integration complex (PIC). (6) Nuclear import of PIC. (7) Integration of proviral DNA into host genome. (8) Transcription of early multiply spliced mRNAs. (9) Translation of early regulatory proteins, Tat and Rev. (10) Nuclear import of Tat and Rev. Tat increases transcription of viral mRNAs. (11) Rev mediates export of singly spliced and unspliced viral mRNAs. (12) Translation of viral structural proteins. (13) Assembly at the plasma membrane of viral genomic RNA, proteins, and cellular factors including tRNA(Lys3), the obligate primer for reverse transcription. (14) Viral budding. (15) Viral maturation. Cellular factors involved in viral transcription: RNA polIII, TRBP, NF-κB and PCAF [39]. Source: [39]

The replication cycle of HIV is shown and described in Figure 4. In general, it follows the typical retroviral cycle. The initial step in the entry of HIV into the cell is the binding of gp120 to the main receptor CD4 triggering a conformational change that exposes the coreceptor binding site. It then interacts with the coreceptor (CCR5 or CXCR4) (1); the fusion

domain of the gp41 is exposed and can interact with the membrane of the target cell, leading to fusion (2) and entry of the virion into the cell [40]. The coreceptors CCR5 and CXCR4 identify two phenotypic variants of HIV-1, R5 (M tropic) and X4 (T tropic) viruses, respectively. Generally, recently infected individuals harbor a R5 virus while X4 viruses, which are more pathogenic, predominate in the late stages of the disease. Mutations in the CCR5 encoding gene, such as the well described "delta32" mutation endow the individual with protection from HIV infection or disease progression [41]. After uncoating (3), the reverse transcriptase (RNA dependant DNA polymerase) transcribes the viral RNA into double stranded DNA. One reason for the high mutation rate of HIV is the lack of an editing function in this reverse transcription process. Following the formation of a pre-integration complex (PIC) (5) and transportation to the nucleus (6), the viral genome integrates into the host cell, mediated by the viral integrase (endonuclease) (7). Early multiply spliced mRNAs are transcribed (8) and the early regulatory proteins Tat and Rev are translated (9). Tat and Rev are imported into the nuceleus (10) and Tat increases transcription of viral mRNAs. Rev mediates export of singly spliced and unspliced viral mRNAs (11). Viral mRNAs are translated into several large polyproteins (12). In the following there is assembly at the plasma membrane of viral genomic RNA, proteins, and cellular factors including tRNA(Lys3), the obligate primer for reverse transcription (13). Cleavage of the Gag and Pol polyproteins by the viral protease (the Env polyprotein is cleaved by a cellular protease) occurs as the immature virion buds from the cell membrane (14). This cleavage process results in a mature, infectious virion (15). [39].

3.1.3 Clinical profile

The clinical course of HIV infection can be divided into three stages: an early, acute stage; an asymptomatic, latent stage; and a late, immunodeficiency stage [42] [211]. As indicated in Figure 5, the acute stage usually begins 2-4 weeks after infection. Mononucleosis-like symptoms with fever, lethargy, sore throat and generalized lymphadenopathy occurs. The acute stage, typically with high level viremia, usually resolves spontaneously in about 2 weeks. Resolution of the acute stage is accompanied by a decreasing viremia and increase of CD8 T cells directed against HIV. Antibodies to HIV typically appear 10-14 days after infection and most patients will seroconvert by 3-4 weeks after infection. After initial viremia, a viral set point is reached, which can be different from in individual cases and usually remains stable over the years of the latent infection period. In the latent stage, lasting usually 7-11 years, patients are asymptomatic. A syndrome called AIDS Related Complex (ARC) can occur during the latent period with manifestations of persistent fevers, fatigue, weight loss and

lymphadenopathy. Although plasma viremia usually is low to undetectable in this period, the virus itself does not enter a latent phase as a large amount of HIV is produced and sequestered within the lymph node. Nevertheless, plasma viral RNA, together with CD4 T-cell counts serve to guide treatment decisions and prognosis [43]. The late stage of HIV infection is AIDS, manifested by a decline of CD4 T-cell counts to below 400cells/µl and an increase in the frequency of and severity of opportunistic infections. The two most characteristic manifestations of AIDS are *Pneumocystis* pneumoniae and Kaposi's sarcoma, but also other opportunistic infections occur. These include viral infections such as disseminated Herpes simplex, Herpes zoster, Cytomegalovirus infections; fungal infections such as thrush (caused by *Candida albicans*), cryptococcal meningitits and disseminated histoplasmosis; protozoal infections such as toxoplasmosis; and disseminated bacterial infections such as those caused by Mycobacterium tuberculosis. Many AIDS patients have severe neurological problems, e.g. dementia and neuropathy either caused by HIV infection of the brain or opportunistic organisms.

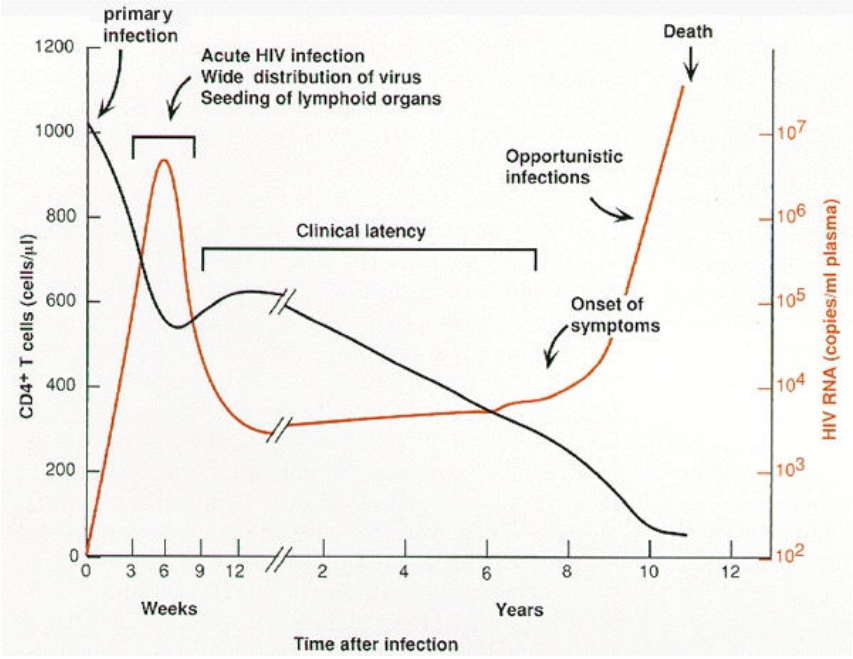

Figure 5. Schematic diagram of the course of HIV-1 infection. The diagram illustrates the relationship between HIV-1 virus load (brown line) and CD4+ T-cell count (black line) over time in a typical case of untreated HIV-1 infection. The different stages of infections are indicated in the graph. Taken from: [John M. Coffin, Stephen H Hughes, Harold E. Varmus: Course of HIV and SIV infection. In: Retroviruses. Cold Spring Harbour Press, 1997, ISBN 0-87969-571-4]

3.1.4 Therapy options

To date, with no vaccine available, the only effective remedy against HIV-1 infection remains highly active antiretroviral therapy (HAART). By inhibiting viral replication HAART leads

to reversion of symptoms caused by the infection, to a halt of disease progression and to achieve clinically relevant immune reconstitution [44].

In 1987, the first anti-HIV drug entered in clinical use. One year before, a study published in the New England Journal of Medicine demonstrated that a drug called azidothymidine (AZT) decreased mortality and the frequency of opportunistic infections in subjects with AIDS [45]. AZT belongs to the class of nucleoside and nucleotide reverse transcriptase inhibitors (NRTI). NRTI target the viral enzyme reverse transcriptase (RT), a key enzyme responsible for the retrotranscription of viral RNA to DNA, a process that precedes the integration of the proviral DNA in the host cell genome. NRTI are incorporated into the newly synthesized viral DNA and prevent its further elongation. In 1995, the FDA approved the drug saquinavir, the first member of a new class of anti-HIV drugs. Saquinavir is an HIV-specific protease inhibitor (PI) [46]. The drugs belonging to this new class of antiretroviral drugs inhibit the viral protease. This enzyme cleaves viral precursor proteins needed for the assembly of a mature virion. As consequence, infected cells release immature and non-infectious particles. In 1996 the FDA approved the first non-nucleoside reverse transcriptase inhibitor (NNRTI) Nevirapin [47]. NNRTIs inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function. A number of more recently developed drug substances target further viral or cell associated structures leading to an interference with viral replication [48-51]. The fusion inhibitor Enfuvirtide is a 36-mer synthetic peptide derived from the HIV-1 gp41 transmembrane protein and was approved in 2003 for treatment of advanced HIV infection. Maraviroc is another entry inhibitor which blocks the chemokine receptor and HIV co-receptor CCR5. It was approved by the FDA in 2007 for use in drug experienced patients. To predict efficacy, HIV tropism has to be determined prior to treatment. The first integrase inhibitor approved by the FDA in 2007 was Raltegravir which can be administered in combination with optimized background therapy. Standard antiretroviral therapy regimen consist of the use of at least three antiretroviral drugs to maximally suppress the HIV virus and stop the progression of HIV disease [52][210]. Viral load reduction of 0.6 to 2 log10 decreases morbidity and mortality [53]. A decrease of viral load to 20-50 RNA copies/ml is able to impede development of resistance and consecutive therapy failure. Preferred combinations are shown in the scheme below.

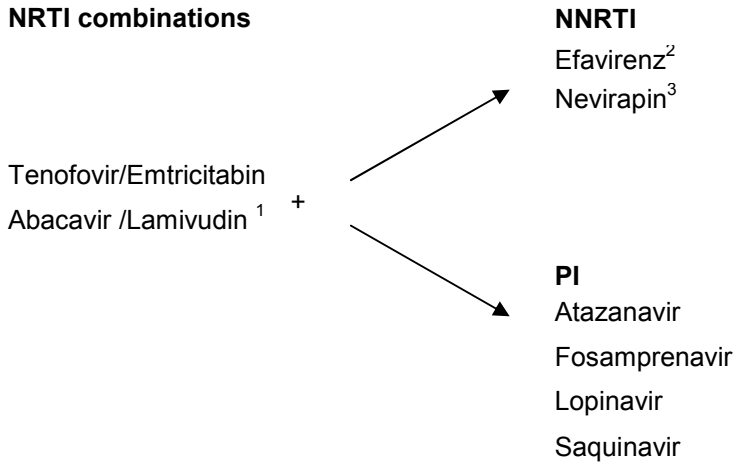

Figure 6. Preferred combinations of antiretroviral drugs
[1] After negative screening for HLAB5701, with reservation if plasma viral load $>10^5$ copies/mL and high cardiovascular risk (Framingham score >20%). [2] No administration during pregnancy or wish/risk of pregnancy [3]With reservation in case of liver disease, male patients with >400 CD4 T-cells/µl, female patients with >250 CD4 T-cells/µl. [Leitlinie zur antiretroviralen Therapie der HIV Infektion; Deutsche AIDS Gesellschaft; September 2008]

HAART contributed substantially to reducing AIDS-related opportunistic infections and death [44]. However, HAART is not able to clear HIV-1 infection and virus still persists in resting CD4 T-cells [54]. As a result, cessation of HAART is accompanied by a rebound in viremia in the quasi totality of patients. Moreover, HAART has several short term side effects that have yet to be entirely discovered. The most common side effects include gastrointestinal problems such as bloating, nausea and diarrhea, lipodystrophy, hepatotoxicity, lactic acidosis, osteoporosis and skin rash [55]. Nevertheless, its use is strictly necessary to control HIV replication. In addition, in developing countries the high cost and the required lifelong adherence are major limitations for the diffusion of HAART. Although from 2001 to 2007, the number of people receiving antiretroviral medicines in low- and middle-income countries has increased ten-fold, reaching almost 3 million people by the end of 2007 there are still millions without access to any treatment, an unacceptable situation (http://www.unaids.org; "Report on the global AIDS epidemic").

In this scenario, a preventive vaccine is desperately needed and therapeutic vaccination may represent an alternative strategy aiming to re-induce immunological competence and to impede or delay progression to AIDS [56, 57].

3.2 Vaccination

Vaccine development has become more and more complex in the last decades, pursuing new strategies for stimulating immune responses against infectious agents of viral, bacterial or parasitic origin and against cancer. There is no doubt, that a prophylactic, as well as a therapeutic vaccine against HIV is desperately needed as they might be the only hope to stop the worldwide AIDS pandemic. Numerous vaccination strategies that include the use of recombinant envelope proteins, synthetic peptides, virus like particles, whole inactivated viruses, plasmid DNA, recombinant orthopoxviruses or adenoviruses and antigen pulsed dendritic cells have been already tested in humans [58, 59].

3.2.1 Prophylactic HIV vaccination

A number of unique characteristics of the biology of HIV infections make the creation of an HIV vaccine particularly difficult. High levels of viral replication persist in infected individuals despite ongoing humoral and cell-mediated immune responses towards HIV. This persistence of viral replication could indicate that it is problematical to generate immune protection against viral infection. Thus, the way to a successful HIV vaccine represents an unprecedented scientific challenge. The traditional approaches for creating effective antiviral vaccines have proved inadequate for making one against HIV-1 [60, 61]. The challenges involved in the development have accumulated from the time of the first clinical trials to the highly publicized Vaxgen trial [60, 62, 63] and the recent “STEP” [64, 65] and “THAI” trial [66]. A challenging aspect of HIV biology regarding vaccine development is the extraordinary genetic diversity of the virus [67]. This diversity is apparent both in a single infected individual and at a global level in geographically disparate infected people. Because of the inaccuracy of the replication machinery of the virus, new mutations are introduced into virtually every virion generated in an infected individual. As many as a billion new and unique viral particles can be created each day in an infected person, so the virus population in an individual must be considered a swarm or quasi-species. At a global level, the genetic diversity of the virus is manifest in distinct HIV clades. Several HIV envelope–based vaccines were aimed at inducing neutralizing antibody responses, as several groups had shown that passive transfer of large amounts of neutralizing antibodies could protect primates against infection [68-71]. Unfortunately, these trials failed. The main reasons for the failures are likely to include the genetic variability of the viral envelope proteins, which allows the virus to escape neutralizing antibodies and the difficulty in identifying immunogens and immunization platforms that consistently induce antibodies able to neutralize several HIV clades as explained above [72]. In the same way, vaccine candidates aiming to induce cellular

immune responses have to overcome immune escape caused by continuous viral mutation. HIV is transmitted both as cell-free and as cell-associated virus. Because cell-free virus can only be eliminated through binding to neutralizing antibody and cell-associated virus by cell-mediated immune responses, a vaccine may have to elicit both types of immune responses to protect against infection with HIV. When antibodies fail to prevent infection an effective T cell response will probably be required to control infection. Because the set-point level of viremia after infection predicts both disease progression and the likelihood of subsequent transmission, a vaccine that does not protect against infection could still be effective if it induced sufficient immune responses to dramatically lower the steady-state viral load in case of infection [73]. The rationale for a T-cell-based AIDS vaccine stems from data in monkey models and in humans indicating that T-cells play a role in control of HIV. CD8 T-cell depletion during acute [74] or chronic [75] SIV infection increases viral load. Expression of particular HLA class I alleles correlates with delayed disease progression in HIV-1 infected humans [76, 77]. CD8 T-cells drive a strong selective pressure in SIV [78] and HIV-1 [79] infection. However, the mechanism by which T-cells contain SIV- and HIV-replication remains unclear.

Viral vector vaccines expressing one or several HIV-1 genes generally aim to stimulate mainly cellular immune responses and have been evaluated in preclinical and clinical studies ranging from Phase I to Phase III [58, 80-83]. One of these candidate vaccines, adenovirus serotype 5 vectors expressing either HIV-1 gag, pol or nef (MRKAd5 HIV-1 gag/pol/nef) was tested in the so called STEP trial, a double-blind, placebo controlled phase II, test-of-concept study with 3000 HIV-1-seronegative participants at 34 different sites in North America, the Caribbean, South America, and Australia [64]. The vaccine did not reduce plasma viraemia after infection, and HIV-1 incidence was higher in vaccine-treated than in placebo-treated men with pre-existing adenovirus serotype 5 (Ad5) immunity. Nevertheless the vaccine was highly immunogenic for inducing HIV-1-specific CD8 T cells [84] and thus the question has to be asked why the induced CD8 T cells failed to confer any protection. The most recent large scale clinical vaccination trial ("THAI trial") was a community-based, randomized, multicenter, double-blind, placebo-controlled efficacy trial with 16,402 healthy participants. Efficacy of four priming injections of a recombinant canarypox vector vaccine (ALVAC-HIV [vCP1521]) plus two booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) was evaluated. Several previous trials of diverse canarypox–HIV vector primes and boosters containing subunit gp120 or gp160 had

established the prime–boost concept as a candidate for advanced testing [85], although the phase 3 trial of bivalent gp120 AIDSVAX B/E vaccine alone had shown no effect on HIV-1 acquisition [86]. In the "THAI trial" the vaccine efficacy was 31.2% (95% CI, 1.1 to 51.2; P=0.04), but vaccination did not affect the degree of viremia or the CD4+ T-cell count in subjects in whom HIV-1 infection was subsequently diagnosed. The partial success of a combination of the AIDSVAX B/E vaccine, mainly aiming at the induction of neutralizing antibodies, with the canaypox-based ALVAC vaccine, mainly aiming at inducing T-cell immune responses, support further evaluation of vaccine regimens aiming at eliciting both, humoral and cellular immune responses. In spite of this modest success, the results brought hope to the scientific community as for the first time an indication for vaccine-mediated protection against HIV infection was observed. Furthermore, this trial supports the use of pox virus derived vectors in future HIV vaccine development.

A major problem of HIV vaccine research is that despite of the extensive research, various studies and increasing knowledge reliable immunological correlates of protection against HIV infection do not yet exist. Therefore predicting the efficacy T-cell responses elicited by a vaccine remains difficult and complex. An appropriate assessment of T-cell responses includes various distinct properties and requires sophisticated tools and methods [87].

3.2.2 Therapeutic HIV vaccination

Despite substantial benefits, HAART is not able to clear HIV-1 infection and the virus still persists in resting CD4 T cells [88]. Since interruption of the antiviral treatment inevitably leads to disease progression, lifelong HAART administration is required. Unfortunately, HAART administration is associated with serious side effects, long-term toxicity and selection of multidrug-resistant viral strains. In addition, in developing countries the high cost and the required lifelong adherence are major limitations for the diffusion of HAART. In this scenario, therapeutic vaccination may represent an alternative strategy aiming to induce immunological competence and to impede or delay progression to AIDS [56, 57].

Requirements for a therapeutic vaccine are substantially different from those for a prophylactic vaccine. A prophylactic vaccine aims at preventing infection and has to face a limited amount of pathogen confined to a small area. A therapeutic vaccine has to face either a generalized infection with large amounts of pathogen in untreated HIV infection or pharmacologically suppressed viral replication in ART treated patients. The former scenario

is found mainly in developing countries, where viral load and CD4 T-cell counts are usually unknown; the latter scenario is found mainly in developed countries with access to antiretroviral treatment for HIV infected patients, where a therapeutic vaccine would be administrated during HAART treatment. Treated patients have low or undetectable viral load and following vaccination, they will have the option to interrupt treatment. For these reasons it might be necessary to develop two different therapeutic HIV vaccines, each one adapted to the specific situation of application. Whatever the case may be, the aim of a therapeutic vaccine would be to enhance pre-existing immune responses to such extent that the chronic pathogen can be controlled or in the best scenario completely cleared [89].

3.3 Modified Vaccinia Virus Ankara (MVA) and MVA-nef

3.3.1 MVA

Vaccinia virus (VV) is considered to be the best known member of the poxvirus family and to be the prototype live viral vaccine. It is closely related to the virus that causes cowpox. VV is a large, complex, enveloped virus with a linear, double-stranded DNA genome approximately 190 kbp in length, which encodes for approximately 250 genes [90, 91]. VV replicates in the cytoplasm of the host cell, its DNA does not integrate into the host cell genome and it is non-oncogenic [92]. The genome of VV can accommodate large amounts of heterologuos DNA and viral gene expression is highly efficient. VV is therefore an attractive candidate for the development of viral vaccine vectors [91].

Modified Vaccinia Virus Ankara (MVA) was attenuated from the parental strain chorioallantois vaccinia virus Ankara by 516 serial passages in primary chicken embryo fibroblast cells (CEF) [93]. During passaging, MVA has suffered a multitude of mutations within its genome and six major deletions resulting in the loss of 15% (30kbp) of original genetic information. The deletions affect a number of virulence and host range genes as well as the gene for the Type A inclusion bodies [94]. 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 [95, 96]. Therefore non-recombinant MVA was used in more than 120,000 human subjects in a smallpox vaccination campaign in Germany [97]. Recombinant MVA viruses, encoding tumor antigens or other antigens of immunological interest, were shown to generate efficient immune responses in vivo protecting animals from challenges with both tumor cells and infectious agents like influenza or parainfluenza viruses, immunodeficiency viruses, or malaria parasites

[96, 98-100]. Considering the characteristics of MVA, which are the restricted host range, the efficient expression of heterologous genes, the immunogenicity, the avirulence in animal models and the excellent safety record as a smallpox vaccine, the recombinant MVA is a promising human vaccine candidate against HIV and other infectious agents.

3.3.2 Nef

The misnamed negative factor Nef is an early-expressed, highly conserved, accessory protein of primate lentiviruses. It is a small cytoplasmic protein of 27 kDa in HIV-1 and 34 kDa in HIV-2 and SIV [101] that is expressed in abundance in the early phase of HIV infection from episomal as well as integrated proviruses [102] and is a virulence factor critical for attaining high virus loads and the development of AIDS [103]. Being mainly a conglomerate of protein–protein interaction domains, Nef does not have any enzymatic activity. It carries out its functions by establishing connections between its targets and effectors, which are usually part of trafficking or signalling pathways, to assist in viral escape from host immune attack [104].

Nef mainly interferes with three important pathways in the cell: downregulation of CD4 as well as major histocompatibility complex I downregulation (MHC I) expression on the cell surface and perturbation of the cellular signal transduction pathways (Pak2 activation, and enhancement of virion infectivity) [105]. Furthermore it has been suggested that simian immunodeficiency viruses are non-pathogenic in their hosts because they have Nef being able to downregulate CD3 which is not the case for HIV-1 Nef [106]. The expression of Nef in T cells unleashes a series of signaling events that are similar to those that occur following T-cell activation through the TCR and co-stimulatory pathways [107]. Nef influences apoptosis in both infected and uninfected immune effector cells [108]. It induces apoptosis in HIV specific CD8 T-cells [109] and blocks death signalling pathways within an infected cell to aid resistance to apoptosis [110, 111].

The replication of HIV-1 in vitro is restricted to dividing (activated) cells. In macrophages, the first cell type to be infected by HIV, Nef intersects the CD40 signaling pathway leading ultimately to T-lymphocyte proliferation through the activation of B-cells [112]. In addition, the ability of Nef to induce the release of two CC chemokines in HIV-1-infected macrophages leads to chemotaxis and activation of resting T lymphocytes. This generates an environment that is promoting viral replication in the host by increasing the pool of substrate lymphocytes without additional stimuli [113]. In resting T cells, Nef can be expressed from unintegrated

viral DNA and increases T cell activation and viral replication [114]. The interference of Nef in T-cell receptor (TCR) or nuclear factor of activated T cells (NFAT) signaling pathways also promotes the susceptibility of T cells to HIV replication [115, 116]. In primary cells, in which the effects of Nef are most profound, Nef increases viral replication in the infected cell and improves infectivity of progeny virions after their release. This effect is dependent on the association of this protein with the plasma membrane and is determined at the stage of virus particle formation [117]. Due to these features, the Nef gene has been employed in several approaches to generate a HIV vaccine.

3.3.3 MVA-HIV-1LAI-nef (MVA-nef)

Recombinant MVA expressing one or several different HIV genes has been extensively tested in humans [82, 118, 119]. One of the candidate vaccines is MVA-HIV-1_{LAI}-nef (MVA-nef). The MVA-nef construct was generated using the MVA-F6 clone originating from the 574th passage of MVA in primary chicken embryo fibroblast cells. The gene encoding the HIV-1 Nef gene under the control of the vaccinia early/late promotor P7.5 has been inserted into the site of deletion II by homologous recombination using flanking MVA sequences. MVA-nef has been already tested in several preclinical [120] and independent clinical trials summarized in Table 1. In all studies vaccination resulted to be safe with minor side effects. All studies demonstrated that the vaccine is immunogenic. However, approved correlates of HIV-1 protection do not yet exist. Yet, recent advances in immune monitoring technologies offer the possibility to assess immunological benefits following MVA-nef vaccination and can possibly predict the efficacy of MVA-nef administration. Since the vaccine is safe and immunogenic in both, healthy and HIV-1 positive individuals, it has the potential to be used in settings where the HIV-1 status of the individuals is unknown (i.e. large vaccination campaign in developing countries).

Table 1. Summary of clinical trials using MVA-nef

Reference	VACCINE	HIV status, nr subjects	Administration	Phase	Notes/ Results	Safety	Reference
Cosma et al. [119]	$5x10^8$ MVA-Nef	HIV+, 10	Sc; 0, 2, 16 weeks	I	CD4 increase or new: 8/10 CD8 increase 2/10	confirmed	Cosma et al. [119]
Harrer et al. [118]	$5x10^8$ MVA-Nef	HIV+, 14	Sc; 0, 4, 16 weeks	I	New CD8 9/14 New CD4 2/14	confirmed	Harrer et al. [118]
Harrer et al. CROI conference, 2006	$5x10^8$ MVA-Nef	HIV-, 14	Sc; 0, 4, 16 weeks	I	CD8 or CD4 9/14	confirmed	Harrer et al. CROI conference, 2006
Harrer et al. AIDS Vaccine Conference, Seattle 2007 and Cape Town 2008	$1x10^8$ MVA-wt $1x10^8$ MVA-Nef $5x10^8$ MVA-Nef	HIV+, 26, 25, 26	0, 8, 16 weeks	II	T-cell responses: Dose $1x10^8$ 44% Dose $5x10^8$ 62%	confirmed	Harrer et al. AIDS Vaccine Conference, Seattle 2007 and Cape Town 2008

3.4 Immunonmonitoring technologies

3.4.1 Flow cytometry

Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5µm to 40µm diameter. Cells are hydro-dynamically focused in a sheath of PBS before intercepting an optimally focused light source (laser). As cells or particles of interest intercept the light source, they scatter light and fluorochromes are excited to a higher energy state. This energy is released as a photon of light with specific spectral properties unique to different fluorochromes. Light is sent to different detectors by using optical filters. The most common type of detector used in flow cytometry is the photomultiplier tube (PMT). The electrical pulses originating from light detected by the PMTs are processed by a series of linear and log amplifiers. The data generated by flow-cytometers can be plotted in a single dimension, to produce a histogram, in two-dimensional dot plots or even in three dimensions. The regions on these plots can be sequentially separated, based on forward scatter, side scatter and fluorescence intensity, by creating a series of subset extractions, termed "gates". Because different fluorescent dyes' emission spectra overlap, signals at the detectors have to be compensated electronically as well as computationally. Data accumulated using a flow cytometer can be analyzed using specialized software.

Immunfluorescence staining and flow cytometric analysis are used for the simultaneous analysis of surface molecules and intracellular cytokines at a single-cell level. For the detection of cellular immune responses cells usually are re-stimulated ex vivo, stained for surface antigens and then fixed and permeabilized to allow for anti-cytokine antibodies to stain intracellularly. Ex vivo re-stimulation of cells is usually required for detection of cytokines by flow cytometry since otherwise cytokine levels are typically too low for detection. Stimulation of cells with the appropriate antigenic reagent depends on the cell type and the experimental conditions. The establishment of a flow-cytometry based intracellular cytokine staining assay should include several controls:

- The Isotype control address nonspecific binding of an antibody of a particular isotype, e.g. IgG1 conjugated to a particular fluorochrome, e.g. FITC. Unspecific binding should be excluded.
- Fluorescence minus one (FMO) controls check for the residual spillover of different antibody-fluorochrome-conjugates in a detection channel after compensation. For each antibody-fluorochrome-conjugate there is a sample including all ABs except the one of interest. The combination of several antibody-fluorochrome-conjugates can result in a loss of resolution sensitivity, as there can be residual spillover as background. The FMO control is a possible gating control for markers which don‘t have a very distinct division between positive and negative populations.
- Biological comparison controls (standard negative and positive control) are unstimulated or irrelevantly stimulated samples. They have to be included in each experiment. During the simulation, these wells do not contain any antigenic peptide or an irrelevant peptide to determine the biological background of functional markers, produced in response to antigens. This control generally serves to set negative and positive gates. Usually the biological background is subtracted from the response observed for re-stimulated cells.

3.4.2 T-cell immunomonitoring

Along with vaccine development, immunomonitoring technologies have become more complex in the last decades. An effective prophylactic or therapeutic HIV vaccine, such as vaccines against several other intracellular pathogens like plasmodia or mycobacteria as well as against cancer will need to elicit effective T-cell immune responses. Therefore, methods that qualify and quantify antigen-specific, functional T cells in a precise, sensitive, and robust way are essential.

Function-dependent assays usually require a brief in vitro restimulation to induce specific effector functions, such as the enzyme-linked immune spot assay (ELISPOT) and intracellular cytokine staining (ICS) or secretion assays. T cells responding specifically to antigen are detected through their ability to rapidly produce effector cytokines such as IFN-γ, TNF-α, IL-2, IL-4, IL-5 or MIP-1β. Other methods determine proliferation or degranulation of T cells in response to antigen-recognition, which can be quantified by flow cytometry-compatible labelling or staining techniques (e.g. CFSE and CD107 staining). All these assays detect antigen-specific T cells based on effector functions, like rapid production of cytokines or induction of cell proliferation in response to in vitro restimulation with antigen; only T cells capable of responding with the readout effector function under the chosen in vitro restimulation conditions can be detected. It is unlikely that function-dependent T-cell detection assays will be able to detect the entire population as some antigen specific T-cells possibly do not produce any effector cytokine. In this case, T-cell detection methods that are independent of effector functions, like MHC class I and class II tetramer staining are more suitable. Tetramer staining methods that do not require *ex vivo* re-stimulation have proven to be most useful for extensive phenotypic characterizations of antigen-specific T cell populations. Several surface and/or intracellular markers have been identified, which indicate whether a single T cell belongs to a certain subtype of effector or memory T cells. Combination of staining for several different T cell markers with MHC multimers by using multicolor flow cytometry has recently opened a new level of T cell phenotyping. Based on these different technologies, a substantial number of parameters for antigen-specific T cells can be monitored: absolute frequencies, phenotypical subpopulations, functional capacities (cytokine secretion, degranulation, proliferation, cytotoxicity), functional and structural avidity. It becomes more and more clear, that it is necessary to determine most of these parameters to obtain a meaningful immune monitoring analysis.

At present, the standard assays that are commonly used for this purpose are IFN-γ based ELISPOT, HLA class I and class II multimer staining and ICS. The ELISPOT assay is currently considered the gold standard in vaccine trials due to its sensitivity and extensive standardization and validation [121-124]. In fact, several reports demonstrated that the ELISPOT assay is more sensitive in detecting weak responses when compared to the ICS assay [125-128], a feature that represents an important advantage for the detection and measurement of the immune response in vaccine trials [129]. The most commonly used

ELISPOT assay measures IFN-γ secretion by total PBMC stimulated by specific antigens. Albeit ELISPOT assays being able to measure the secretion of two different cytokines have been recently established [130], it is unlikely that future development will increase the simultaneous measurement of cytokines for this kind of assays. On the other hand, the introduction of new reagents, instruments and software, strongly improved the capacity of flow cytometry based assays such ICS and multimer staining to simultaneously measure several parameters in the same sample and on the same cell [131-133]. However, between ICS and multimer staining, the former seems to be more suited to be employed in vaccine trials since it does not require previous HLA typing and *a priori* knowledge of specific epitopes [134, 135]. Hence, it is generally accepted that ICS provides more information regarding the quality of the immune response whereas ELISPOT grants a high capacity of detecting low magnitude responses, while multimer staining is the method of choice for a detailed analysis of the immune response in a selected and limited number of samples.

Clear immunological correlates of protection from HIV infection and disease progression do not yet exist although there is strong evidence that CD4 and CD8 T-cells play a role in the control of viral replication [73]. However, neither the magnitude of the immune response (measured as production of IFN-γ) nor the breadth of recognized epitopes constitute *per se* valid correlates of protection [136-138]. Recently, studies have shown that polyfunctional CD8 T-cell responses are preferentially observed in long term non-progressors (LTNP) when compared to persons with progressive disease [139]. Furthermore, antigen-specific terminally differentiated CD8 T-cells, defined by the lineage markers CCR7 and CD45RA, have been preferentially found in long-term non-progressors [140] and early infections with future control of HIV-1 viremia [141]. These findings highlight the importance of developing assays able to simultaneously measure several parameters in the same sample and strongly suggest the use of flow cytometry to monitor immune responses.

4 Results

4.1 Development of flow-cytometry based assays for the characterization of T-cell immune responses

Several candidate vaccines against HIV-1, among them MVA expressing HIV-1 Nef, aim at stimulating cellular immune responses, either alone or together with the induction of neutralizing antibodies, and assays able to measure CD8 and CD4 T-cell responses need to be implemented. At present, the IFN-γ-based ELISPOT assay is considered the gold standard and it is broadly preferred as primary assay for detection of antigen-specific T-cell responses in vaccine trials. However, in spite of its high sensitivity, the measurement of the sole IFN-γ production provides limited information on the quality of the immune response. The introduction of polychromatic flow-cytometry-based assays such as the intracellular cytokine staining (ICS) and the continuous technical advancements in flow cytometry strongly improved the capacity to detect several markers on a single cell level.

The first task of this work was the development, optimization and standardization of flow-cytometry based assays able to determine the function and the memory phenotype of antigen specific CD4 and CD8 T-cells, suitable to be applied in clinical HIV vaccination trials and also other settings. Based on literature research, markers that should be included in the flow-cytometric staining panel were defined. As for the application in clinical trials, a certain grade of standardization was necessary.

4.1.1 Establishment of 9 – color Intracellular cytokine staining (ICS)

A 9-color ICS that allowed the simultaneous determination of the function and the memory phenotype of antigen specific CD4 and CD8 T-cells was developed. The established assay had the capacity to detect the expression of IFN-γ, representing the reference marker to detect and quantify specific antiviral T-cell immune responses [142]; the cytokine IL-2, which is associated with helper function and essential for proliferation of antigen-specific T cells and memory development [133, 143-145]; the chemokine MIP-1β, which has been shown to dominate HIV-1-specific immune responses [139] and the CD4 T-cell-specific activation marker CD154 (CD40L), which allows the interaction with CD40 expressing antigen-presenting cells (APCs) and therefore represents a link between innate and adaptive immunity [146-148]. For the characterization of the memory phenotype, we used CD45RA, an isoform of a membrane phosphatase that is expressed by both naïve and terminally differentiated T-

cells [149]. By the expression of CD45RA discrimination between effector and memory T cells [149] was possible.

The experimental procedure is shown in Figure 7. It generally started with the isolation of peripheral blood mononuclear cells (PBMC) from heparinized blood. Isolated PBMC subsequently were either cryopreserved or directly processed. After isolation, or in case of crypreserved PBMC, after thawing, $1x10^6$ PBMC were resuspended in medium supplemented with 10% FCS and 1% PenStrep. The ex vivo re-stimulation with peptides was performed in the presence of costimulatory antibodies. Following 60 minutes incubation, Brefeldin A was added to the cell suspension and the incubation was carried out for additional four hours. Brefeldin A interferes with protein transport from the Golgi apparatus to the endoplasmic reticulum. This leads to proteins accumulating inside the ER and allows their intracellular detection. After an optional overnight storage at 4°C, stimulated cells were resuspended in staining buffer and incubated with the photoreactive fluorescent label ethidiummonoazide to asses their viability. Usually cell surface molecules are now stained with fluorochrome-conjugated antibodies. This step was omitted to reduce duration of the assay for routine application. During the establishment of the assay, all staining methods have been carefully compared (data not shown). The next step was fixation and permeabilization to render the cells permeable for fluorochrome-conjugated staining antibodies. A particular two-step protocol, which was established additionally, allows freezing at -80°C after the fixation and permeabilization step. This protocol is suitable for clinical trials at different sites, where fresh cells can be stimulated, frozen and sent to a centralized analysis facility. It holds the advantage that in this way stimulation with complete proteins is possible and the immune responses observed in fresh cells usually are higher compared to cryopreserved cells. The suitability of the two-step protocol has been tested in collaboration with an Italian and Egyptian laboratory for clinical investigations of HIV and HCV/schistosoma specific immune responses, respectively (Figure 8). It is furthermore planned to use the protocol for future clinical trials in sub-Saharan Africa. After fixation and permeabilization or after thawing in case of the two-step protocol, cells were incubated with the following fluorochrome-conjugated antibodies: CD8-PacB, CD3-AmCyan, CD4-PerCP, CD45RA-PECy7, CD154-FITC, IFN-γ-Al700, IL-2-APC and MIP1β-PE. After washing, cells were acquired using an LSRII flow cytometer equipped with a high throughput system. Sample analysis was performed using FlowJo software. FMO control and Isotype control experiments were

performed during the establishment of the panel and a biological negative control was included in each experiment to enable for background subtraction.

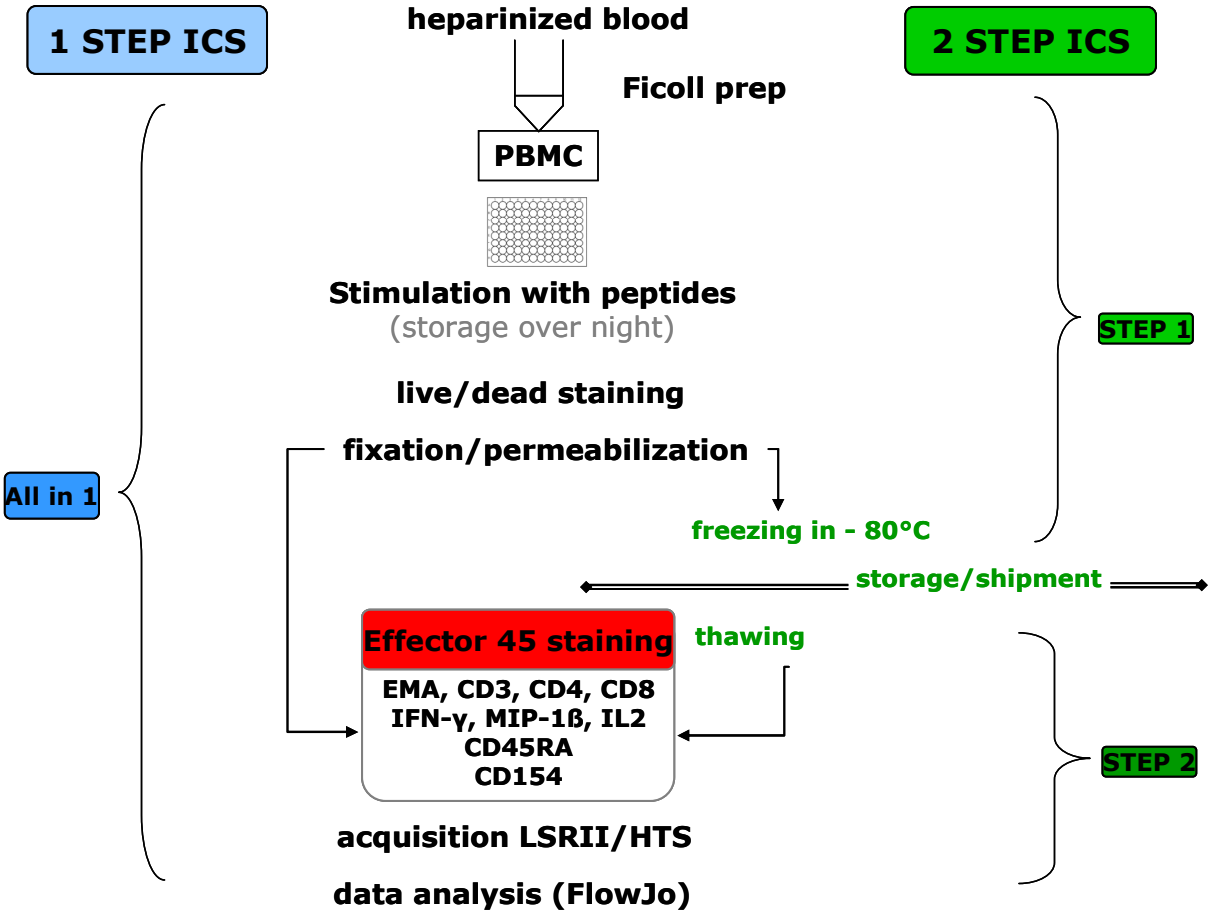

Figure 7. Experimental procedure. PBMC are isolated and restimulated with antigenic peptide formulations for five hours. After an optional overnight storage at 4°C stimulated cells are resuspended in staining buffer and incubated with the photoreactive fluorescent label ethidiummonoazide to asses their viability. Following fixation and permeabilization an optional freezing step can be included and allows storage or transportaion. After fixation and permeabilization or after thawing in case of the two-step protocol, cells are incubated with the following fluorochrome-conjugated antibodies: CD8-PacB, CD3-AmCyan, CD4-PerCP, CD45RA-PECy7, CD154-FITC, IFN-γ-Al700, IL-2-APC and MIP1β-PE. Samples are acquired using an LSRII flow cytometer (Becton Dickinson) equipped with a high throughput system. Sample analysis is performed using FlowJo software.

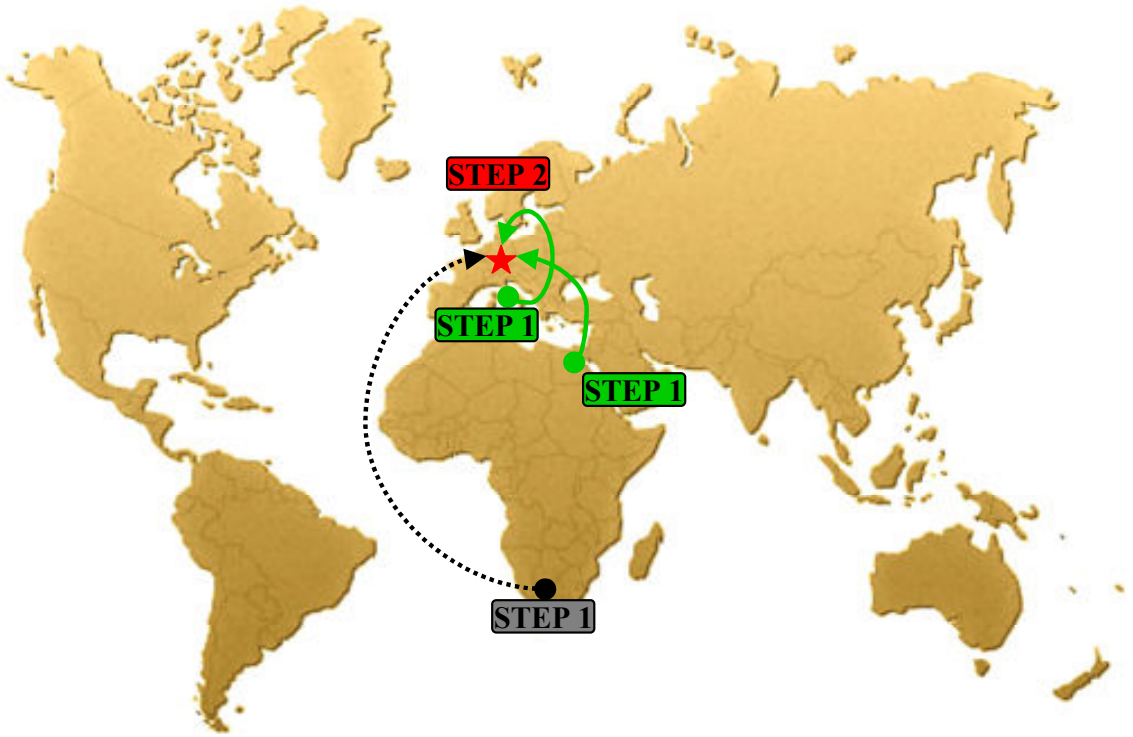

Figure 8. Two-step ICS effector 45. A particular two-step protocol if the effector 45 ICS was established to allow freezing at -80°C after the fixation and permeabilization step. This protocol is suitable for clinical trials at different sites, where fresh cells can be stimulated, EMA stained, fixed and permeabilized (STEP 1), frozen and then sent to a centralized analysis facility for staining acquisition and data anlalysis (STEP 2). It holds the advantage that in this way stimulation with complete proteins is possible and the immune responses observed in fresh cells usually are higher compared to cryopreserved cells. The suitability of the two-step protocol has been tested in collaboration with an Italian and Egyptian laboratory for clinical investigations of HIV and HCV/schistosoma specific immune responses, respectively. Further application is planned in future clinical trials in Africa.

A gating strategy has been developed and is shown in Figure 9. Lymphocytes were gated on a forward scatter area versus side scatter area pseudo-color dot plot and dead cells were removed according to EMA staining. CD3+ events were gated versus IFN-γ, IL-2, MIP-1β and CD154 to account for activation induced down-regulation. CD3+ events were then combined together using the Boolean operator "Or". The same procedure was used to subsequently gate CD8+ events. CD4+ events were excluded before creating a gate for each function or phenotype. CD4+ events were defined vice versa. By calculating every possible combination of the five resulting populations, Boolean gating analysis identified 32 response patterns. Response patterns with at least one positive functional marker were taken in consideration for analysis, thus resulting in a total of 30 immune response patterns.

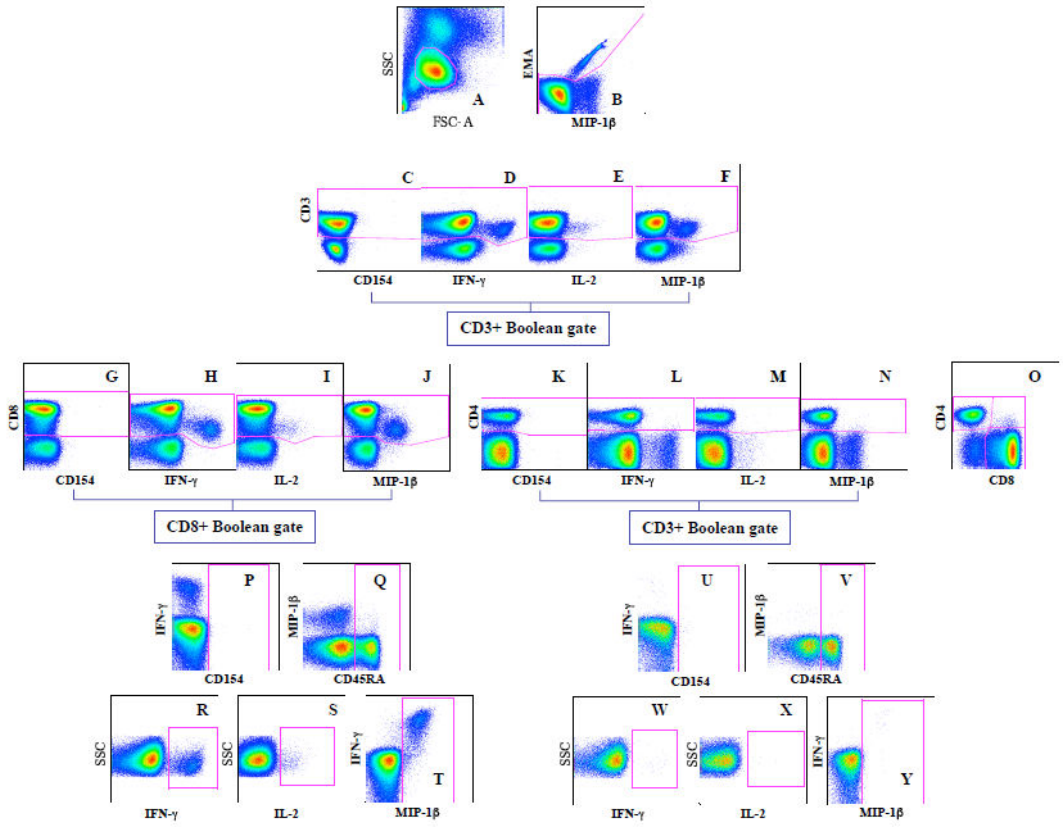

Figure 9. Gating strategy. Representative example showing the gating strategy of the 9 color ICS applied on a PBMC sample stimulated with peptide pool 6. Lymphocytes are gated on a forward scatter area (FSC-A) versus side scatter area (SSC-A) pseudo-color dot plot (A) and dead cells are removed according to EMA staining (B). CD3+ events are gated versus CD154 (C), IFN-γ (D), IL-2 (E) and MIP-1β (F) to account for down-regulation. CD3+ events are then combined together using the Boolean operator "Or". The same procedure is used to subsequently gate CD8+ (G, H, I and J) and CD4+ (K, L, M and N) events. CD4+ events are excluded from the CD8+ population using the exclusion gate in O before creating a gate for each function or phenotype (P, Q, R, S and T). CD8+ events are excluded from the CD4+ population using the exclusion gate in O before creating a gate for each function or phenotype (U, V, W, X and Y).

4.1.2 Establishment of a Carboxyfluorescein succinimidyl ester (CFSE) - based proliferation assay

Cell division can be measured accurately within CFSE-labelled populations because of the reproducibility, efficiency and stability of CFSE-labeling of cytoplasmic proteins, resulting in an exceptionally bright and uniform signal that is divided between daughter cells with high conformity. A polychromatic CFSE-based proliferation assay was developed in order to allow for simultaneous evaluation of the proliferative capacity in combination with the expression pattern of the functional markers IFN-γ, IL-2 and MIP-1β in CD4 and CD8 T cells. CD45RA was also included in the staining combination, but as this differentiation marker was downregulated after the prolonged *ex-vivo* re-stimulation of 5 days, it was excluded from further analysis.

After thawing, PBMC were resuspended in 0.1 µM CFSE. Cells were incubated for 10' at 37° C in the dark for staining and plated at 10^6 cells/200µl per well. In the presence of antigenic peptides and costimulatory antibodies cells were cultured for 5 days in complete medium without addition of any further exogenous stimulus. During cell division in these 5 days CFSE staining is diluted with each division of a cell resulting in a low staining level in proliferating cells and bright staining for non-proliferating cells. At day 5, media was exchanged and cells were restimulated applying the same procedure and conditions as for the ICS. Following EMA staining and fixation/permeabilization, cells were stained with the fluorochrome-conjugated antibodies used in the ICS protocol. The CD154-FITC antibody was omitted not to interfere with the detection of the CFSE. Acquisition and sample analysis were performed in concordance with the ICS protocol. Functional markers and ex vivo proliferative activity, defined by the low CFSE staining level, were gated according to a gating strategy depicted Figure 10. According to the differential expression of CD45RA, IFN-γ, IL-2, MIP1β and the CFSE staining level, 30 responding CD4 and CD8 T-cell subpopulations were identified.

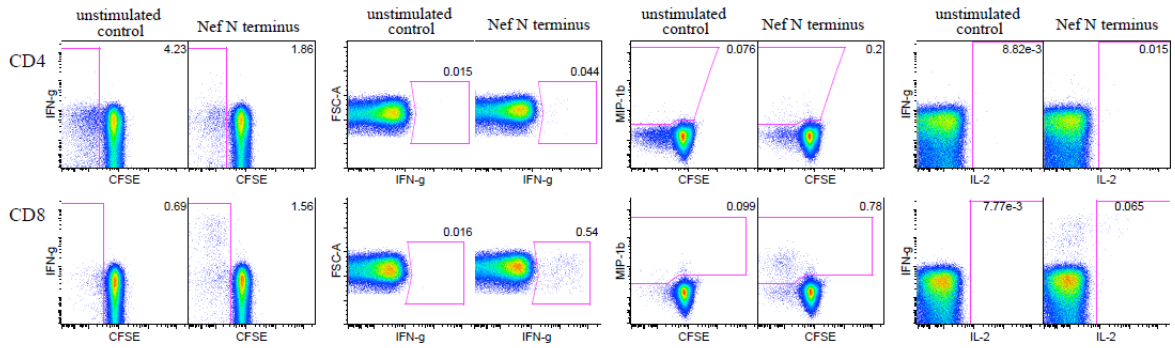

Figure 10. Gating strategy polychromatic CFSE-based proliferation assay. Representative example (subject V11) of the gating strategy for the polychromatic intracellular cytokine staining assay (ICS) in A and polychromatic CFSE-based proliferation assay in B. From left to right the gates for CD154, IFN-γ, MIP-1β and IL-2 are shown in A. From left to right the gates for CFSE staining level, IFN-γ, MIP-1β and IL-2 are shown in B. Upper panels show CD4 T-cell gating. Dot plots represent total CD4 T cells and numbers indicate the positive events as defined by the gate in percentage of total CD4 T cells. Lower panels show CD8 T-cell gating.

4.1.3 Data processing and storage

Following the application of the defined gating strategy for each assay, Boolean combination of all positive populations identified 32 response patterns. Response patterns with at least one positive functional marker were taken in consideration for analysis, thus resulting in a total of 30 immune response patterns in ICS, as well as in the proliferation assay. Via classical excel tables data were transferred to Pestle to subtract background. Since background levels varied between subpopulations, i.e. CD154 staining showed a higher background than IFN-γ and the

combination of 3 or more functions had extremely low background, an individual threshold level for each subpopulation was calculated. The threshold level was defined for each functional combination as the 90th percentile of the distribution of negative values from a total of 147 samples stimulated with mainly Nef and Tat derived antigenic formulations from 37 HIV-1 infected individuals, including the study participants. Values lower than the respective individual threshold level were set to 0. Furthermore, a general threshold of 0.005% was applied for all CD8+ and CD4+ T-cell subsets to exclude minor responses. As a result the minimal threshold was ranging from 0.005% of the parent population to 0.11% and 0.06% for CD8 and CD4 T-cell response subpopulations, respectively.

For the CFSE-based proliferation assay the threshold level was defined for each functional combination as the 95th percentile of the distribution of negative values from a total of 201 samples stimulated with mainly Nef and Tat derived antigenic formulations from 16 HIV-1 infected individuals, including the study participants. The 95th percentile was chosen for the proliferation assay as in long-term assays T-cell responses as well as background levels tend to have a higher variability than in short-term assays. A general threshold of 0.005% for all CD8+ and CD4+ T-cell subsets was applied to exclude minor responses. All data derived from FlowJo were furthermore inserted into a database, where results could be connected to clinical data. Data processing workflow is described in Figure 11.

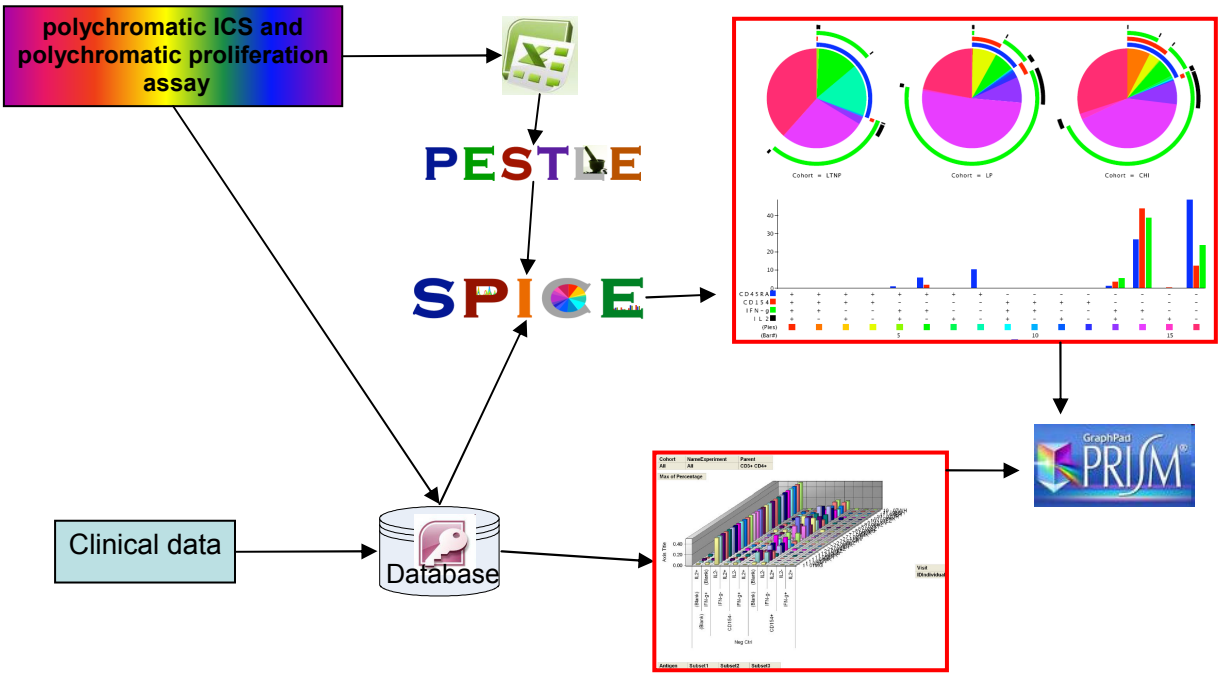

Figure 11. Data processing workflow.

4.1.4 Characterization of the phenotype in ICS and MHC class I tetramer staining

The memory phenotype of CD8 T-cells from 4 different HIV infected individuals were compared as detected by ICS and MHC class I tetramer staining regarding CD45RA expression. Cells from the same blood collection and freezing procedure were used both assays. In ICS PBMC were stimulated with the same antigenic peptide (optimal CD8 epitope) that was loaded on the tetramer.

Responding functional CD8 T cells as detected by ICS were compared to antigen specific CD8 T cells as detected by tetramer staining. Figure 12 displays the expression of IFN-γ compared to MIP-1β in subject 4. In this case, the measurement of MIP-1β detects approximately the double amount of responding cells (7.64% versus 4.28%) but does not reach the number of antigen specific cells detected using tetramer staining (9.18%). Although varying in different subjects, as shown in Table 2, generally more Nef-specific CD8 T cells were producing MIP-1β than IFN-γ. Thus, the extent to which antigen specific cells are detected by functional assays was depending on the respective functional marker as well as on patients and antigens.

Another question to be answered was, if using the ICS including a 5 hours in vitro re-stimulation and an overnight rest at 4°C, the observed CD45RA expression phenotype of antigen specific cells was comparable to the results of the tetramer staining without *ex vivo* re-stimulation. Figure 12 shows a representative example displaying the CD45RA phenotype in both assays. A ratio of the frequency of antigen specific CD8+CD45RA+ T-cells divided by the frequency of CD45RA- antigen specific CD8 T cells (CD45RA+/CD45RA-) as detected by ICS and tetramer staining was built for several functional subsets. The detection of CD45RA expression was found to be very similar in both assays, thus indicating that the memory phenotype does not change after 5 h *ex vivo* re-stimulation and storage over night. Tetramer staining and ICS were therefore equivalent regarding the investigation of CD45RA expression.

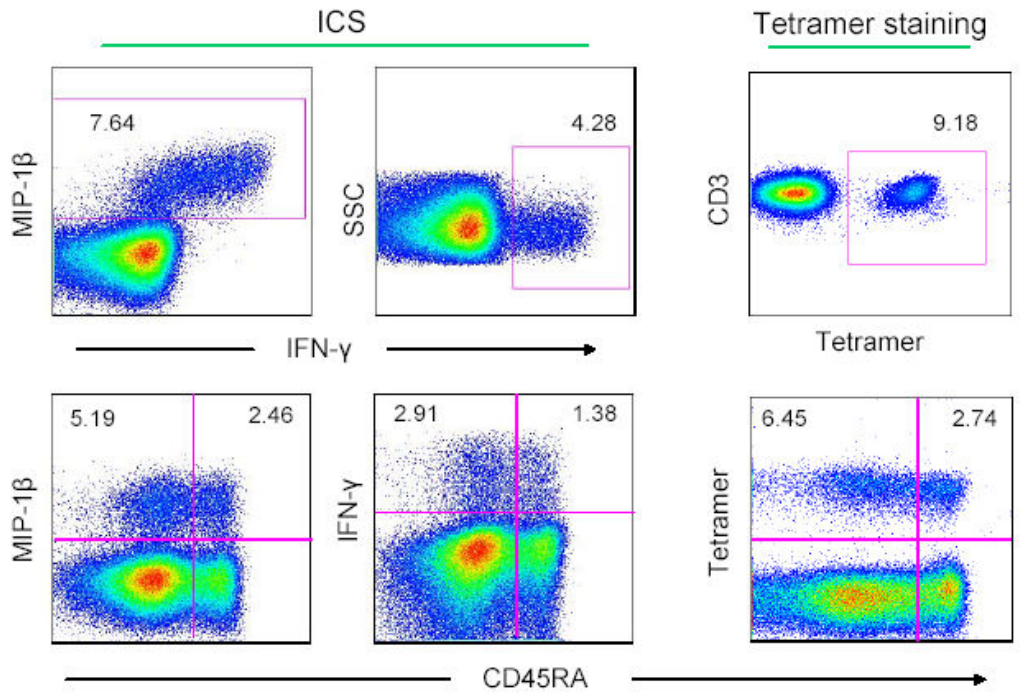

Figure 12. Analysis of the CD45RA phenotype in ICS and MHC class I tetramer staining. Expression of IFN-γ and MIP-1β upon stimulation with the HIV-1 Nef peptide FL8 in CD8+ T cells of subject V4. The results in ICS are compared with a MCH class I tetramer staining using the specific tetramer loaded with the same peptide. The CD45RA expression is compared in IFN-γ+, MIP1-ß+ and tetramer+ cells. The percentage numbers after background subtraction are indicated on the graph.

Tetramer/ epitope	**MIP1b+** [% of CD8 T cells]	**ratio CD45RA+/-**	**IFNg+** [% of CD8 T cells]	**ratio CD45RA+/-**	**tetramer+** [% of CD8 T cells]	**ratio CD45RA+/-**
V04 FL8 B8	7.64	0.47	4.28	0.48	9.18	0.44
V05 TL10 B7	0.56	all CD45RA-	0.69	all CD45RA-	0.88	all CD45RA-
V06 TL10 B7	1.02	all CD45RA-	0.89	all CD45RA-	1.42	0.01
V11 YY9 B35	0.61	0.08	0.49	0.06	0.37	0.02

Table 2. Expression of IFN-γ and MIP-1β in CD8+ T cells of 4 subjects (V04,V05,V06,V11) stimulated with different peptides (ICS) compared to the detection of antigen specific cells via MHC class I tetramer staining. The column besides the marker expression lists the ratio of the percentage of CD45RA+ marker expressing to CD45RA- marker expressing CD8 T cells for the respective antigen and patient.

4.1.5 Establishment of an analysis system based on the evaluation of of IFN-γ+ MIP-1β+ T-cells

The cumulative analysis of 275 samples obtained from 31 HIV-1 positive individuals stimulated with peptides derived from 5 different HIV-1 proteins using the polychromatic ICS, revealed an interesting feature of HIV-1 specific IFN-γ-based responses. Upon antigenic stimulation the majority of the IFN-γ producing CD8 T-cells were also producing MIP-1β (IFN-γ+ MIP-1β+ CD8 T-cells in %: mean±SD, 0.245±0.6341), whereas CD8 T-cells characterized by the sole production of IFN-γ were rarely detected (IFN-γ+ MIP-1β- CD8 T-cells in %: mean±SD, 0.016±0.0652) (Figure 13 A). This trend was observed for all the CD8 T-cell responses whereas the few detected CD4 T-cell responses were more heterogeneous, since antigen-specific cells producing IFN-γ but not MIP-1β were detectable (IFN-γ+ MIP-1β+ CD4 T-cells in %: mean±SD, 0.±0.; IFN-γ+ MIP-1β- CD4 T-cells in %: mean±SD, 0. ±0.; Figure 13 B).

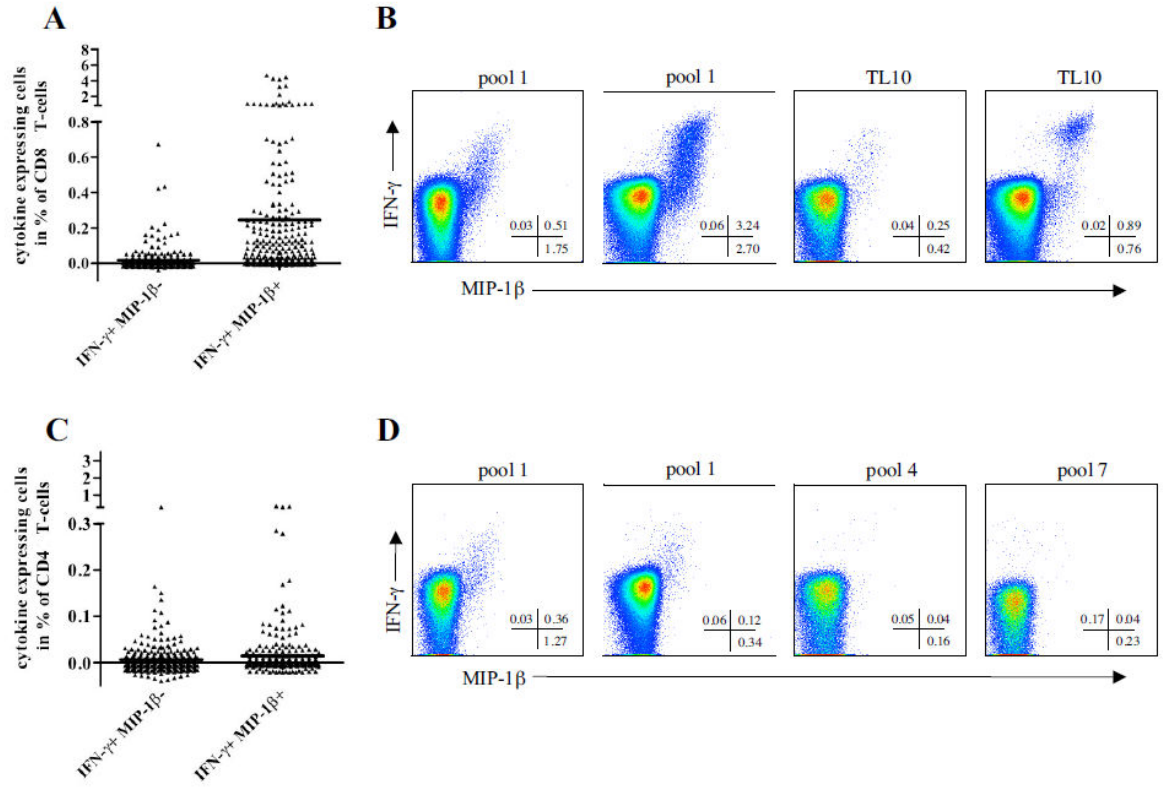

Figure 13. IFN-γ and MIP1-β expression in CD8 and CD4 T-cells stimulated with HIV-1-derived antigens. IFN-γ and MIP1-β expression in CD8 and CD4 T-cells stimulated with HIV-1-derived antigens. Percentages of IFN-γ+ MIP-1β- and IFN-γ+ MIP-1β+ CD8 (A) or CD4 (C) T-cells are shown for a total of 275 samples. The mean is depicted for each T-cell population. Representative pseudo-color dot plots of data gated on living CD8+ CD3+ lymphocytes (B) or living CD4+ CD3+ lymphocytes (D) from 4 different patients are shown. In each plot the percentage of IFN-γ+ MIP-1β-, IFN-γ+ MIP-1β+ and IFN-γ- MIP-1β+ is indicated in the bottom right corner. The pools used for PBMC stimulation are described in Table 1. TL10, TPGPGVRYPL.

The analysis of T cells positive for both markers was of particular interest, since the simultaneous evaluation of two or more functions is supposed to decrease the non-specific background [139]. In order to investigate this observation in our experimental setting, 52 mock stimulated samples from 31 HIV-1 positive subjects were analyzed. Mock stimulated samples were run for each analyzed patient to measure spontaneous cytokine production and unspecific antibody staining. They were processed just as the other samples but in the absence of antigenic peptides. The measured background in the mock stimulated samples was significantly reduced (around 4-fold lower; $p<0.0001$, Wilcoxon matched pairs test) in the IFN-γ+ MIP-1β+ CD8 T-cells when compared to the total IFN-γ+ CD8 T-cells (Figure 14 A). Similarly, a 7-fold decrease ($p<0.0001$, Wilcoxon matched pairs test) of the non-specific background in IFN-γ+ MIP-1β+ CD4 T-cells was observed when compared to total IFN-γ+ CD4 T-cells (Figure 14 B). Representative plots of responding CD8 T-cells and their negative control are shown in Figure 14 C.

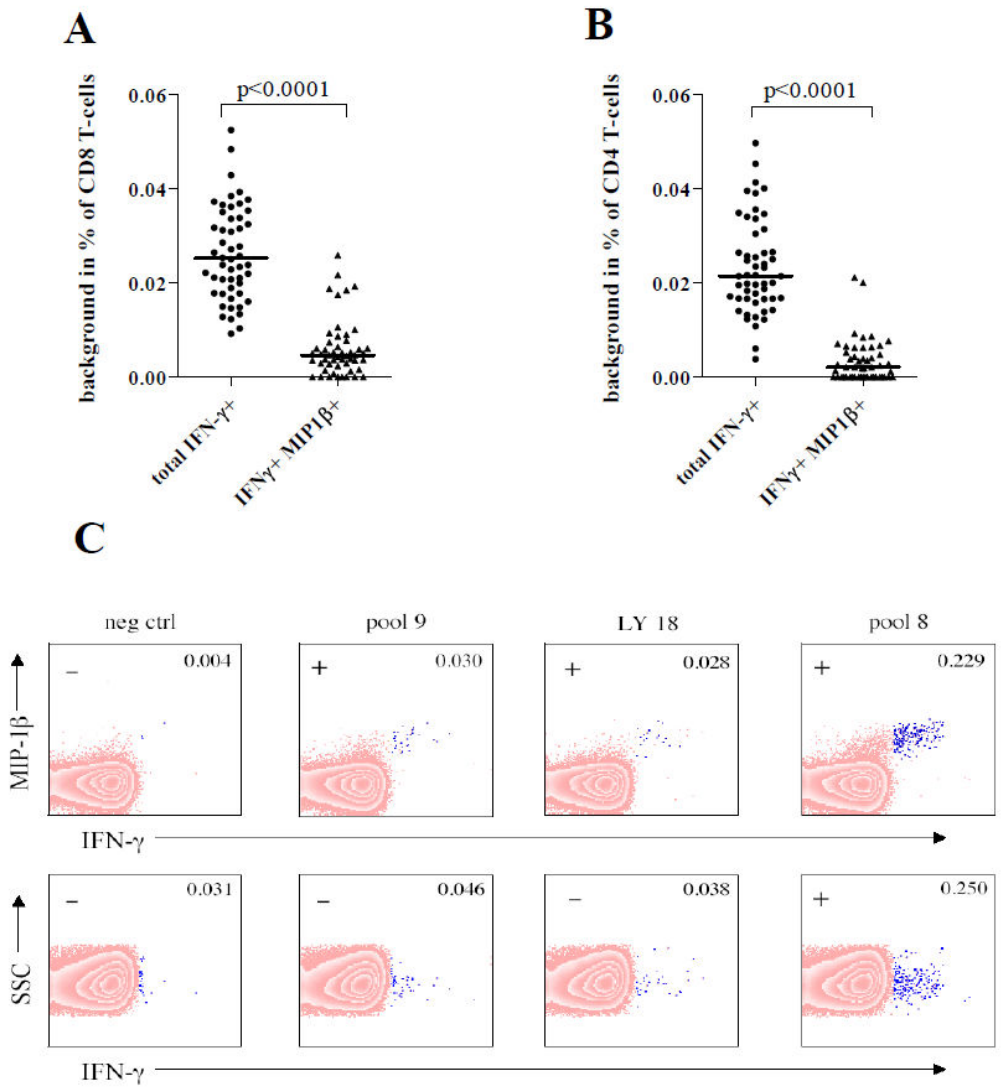

Figure 14. Magnitude of IFN-γ+ MIP-1β+ T-cells and IFN-γ+ T-cells in mock stimulated samples. Percentages of total IFN-γ+ and IFN-γ+ MIP-1β+ CD8 (A) or CD4 (B) T-cells are shown. The lines indicate the median percentage of the observed background. P values were determined by Wilcoxon matched pairs test. In (C) representative data from one study subject are shown. PBMC are gated on CD8+ CD3+ lymphocytes and were stimulated as indicated at the top of the figure. The peptide LDLWIYHTQGYFPDWQNY (LY18), included in pool 8, was here used alone. Data were analyzed with the IFN-γ+ MIP-1β+ (zebra plot in the upper row) or the total IFN-γ+ (pseudo-color dot plot in the bottom row) data analysis system. IFN-γ+ MIP-1β+ CD8 T-cells are depicted in blue whereas a red gate select for total IFN-γ+ CD8 T-cells. The percentage of IFN-γ+ MIP-1β+ and total IFN-γ+ CD8 T-cells is indicated in the upper-right corner of each plot. According to the rule described in the Methods section, samples were scored as positive or negative (upper-left corner).

A linear regression analysis was performed to examine the correlation between percentages of total IFN-γ+ and percentages of IFN-γ+ MIP-1β+ CD8 and CD4 T cells in samples stimulated with HIV-1-derived peptides. Percentages of total IFN-γ+ and IFN-γ+ MIP-1β+ CD8 T cells showed a goodness of fit of r^2=0.9929 and a slope of 1.052 demonstrating an almost perfect linearity of the two measurements (Figure 15 A). The goodness of fit was slightly lower for CD4 T cells; although it was still characterized by an r^2 value of 0.7817 (Figure 15 B). The

slope was 1.190, confirming the presence of HIV-1 specific CD4 T-cells producing IFN-γ but not MIP-1β, as previously shown (Figure 13 B).

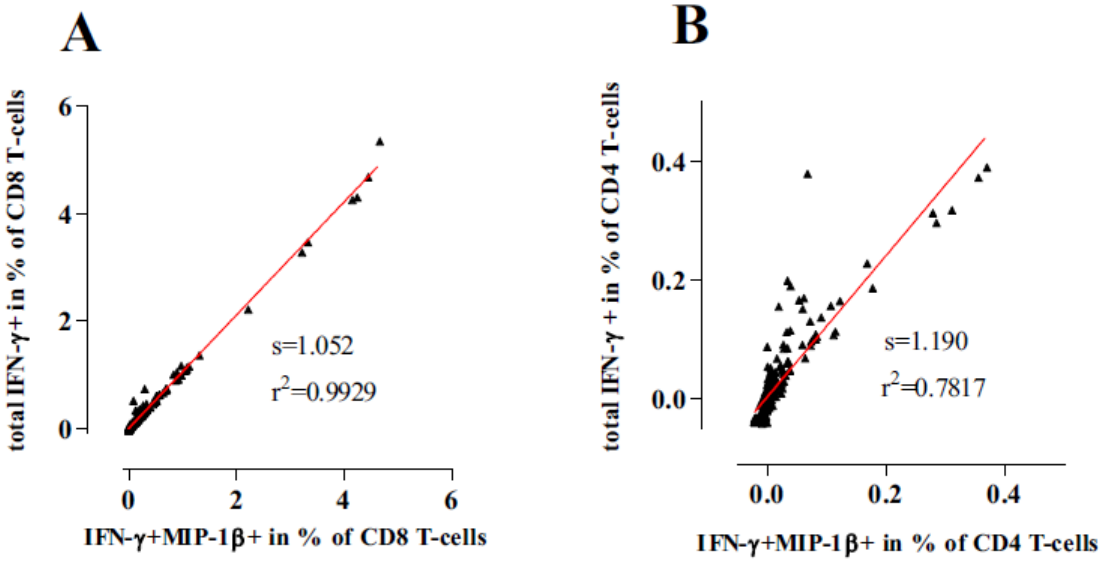

Figure 15. Linear regression analysis. Linear regression analysis between frequencies of IFN-γ+ MIP-1β+ T-cells and total IFN-γ+ T-cells is shown for CD8 (A) and CD4 (B) T-cells. The slope (s) and the goodness of fit (r^2) are indicated in each graph. The regression line is depicted in each graph.

Since the numbers of IFN-γ+ MIP-1β+ T-cells were essentially equivalent to those of total IFN-γ+ T-cells whereas the background was strongly decreased in the former, the assumption was made, that the evaluation of double positive IFN-γ+ MIP-1β+ T-cells could represent an interesting option to increase the sensitivity of the ICS assay in the detection of IFN-γ mediated HIV-1-specific responses.

In order to compare the sensitivity of the two modalities to evaluate the IFN-γ T-cell response, the previously described 275 independent samples (Figure 16 A) were analyzed. The 90th percentile of the negative values after background subtraction was calculated for total IFN-γ+ and IFN-γ+ MIP-1β+ T cells. This value was considered as a threshold. Samples were considered positive when higher than the threshold and at least 2-fold higher than their respective negative control. In the CD8 T-cell population, 187 positive responses were detected using the IFN-γ+ MIP-1β+ data evaluation, while only 146 positive responses were detected using the total IFN-γ+ data evaluation. The difference was significant performing a Fisher's exact test (p=0.0005). The difference between positive CD4 T-cell responses calculated using the two modalities was not significant. When CD8 and CD4 T-cell responses were considered together, the difference achieved significance with a p value of 0.0058 (Fisher's exact test). The contingency tables in Figure 16 B show that the IFN-γ+ MIP-1β+

data evaluation allowed the detection of 41 CD8 responses that were otherwise missed by evaluation of the total IFN-γ+ T-cells. As expected, the simultaneous detection of IFN-γ+ and MIP-1β+ did not increase the capacity to detect antigen-specific CD4 T-cell responses. In fact, 11 CD4 T-cell responses were exclusively detected by the total IFN-γ+ data evaluation whereas 5 were exclusively observed with the simultaneous detection of IFN-γ+ and MIP-1β+.

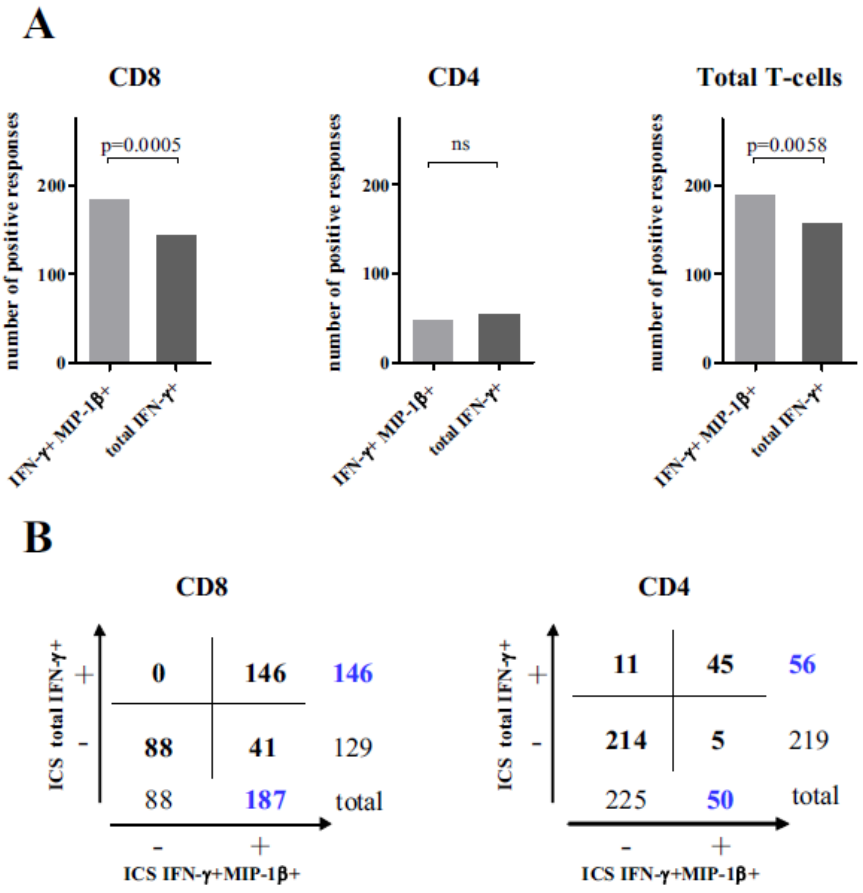

Figure 16. Number of detected positive responses. (A) The histogram plots show the number of positive CD8, CD4 or total T-cell responses detected with the IFN-γ+ MIP-1β+ and the total IFN-γ+ data evaluation systems. The p values (Fisher's exact test) are shown for each graph. Not significant difference (ns). (B) 2x2 contingency tables comparing the two data evaluation systems are shown for CD8 and CD4 T-cell responses.

4.1.6 Comparison of the IFNγ+ MIP1β+ data analysis system in comparison to the ELISPOT assay

ICS is generally considered less sensitive than ELISPOT in detecting low magnitude responses [125-127]. Therefore, it was tested whether the simultaneous detection of MIP-1β and IFN-γ might increase its sensitivity in comparison to two ELISPOT assays performed in independent laboratories. Each laboratory used its own ELISPOT method, including a different ELISPOT reader and a different procedure to determine positive responses (see

Methods). To facilitate the comparison with the ELISPOT, ICS results were expressed as the sum of the CD8 and CD4 T-cell responses and a response in ICS was considered positive when a CD8 or a CD4 T-cell response was scored as positive.

Laboratory 1 analyzed 67 samples from 17 HIV-1 infected subjects stimulated with 14 different peptide formulations derived from two different HIV-1 proteins. Correlation analysis of the responses measured by ELISPOT and by ICS expressed in terms of IFN-γ+ MIP-1β+ CD8 T-cells or total IFN-γ+ CD8 T-cells demonstrated in both cases a significant correlation (Figure 17A). The ELISPOT detected 50 positive responses in 67 samples, while in the ICS positive responses expressed as IFN-γ+ MIP-1β+ CD8 or CD4 T-cells were 55 and in the ICS positive responses expressed as total IFN-γ+ CD8 or CD4 T-cells were 45. By measuring IFN-γ+ MIP-1β+ T-cells 6 positive responses were detected that were otherwise missed by ELISPOT whereas, in contrast, only 1 response detected by ELISPOT was missed in the ICS determination. Determination of the total IFN-γ+ cells, enabled to detect 4 positive responses that were missed by the ELIPOT, but the ELISPOT was able to detect 9 responses missed by the ICS.

Laboratory 2 analyzed 29 samples obtained from 3 HIV-1 infected subjects stimulated with 18 different peptide formulations derived from 5 HIV-1 proteins using an ELISPOT assay approved by the Cancer Vaccine Consortium [150]. As observed with the results generated from the first laboratory, the correlation with the ELISPOT results was significant for both ICS methodologies (Figure 17 B). A total of 29 positive responses were detected by ELISPOT, while 27 and 20 positive responses were detected by ICS using the IFN-γ+ MIP-1β+ and the total IFN-γ+ methods, respectively. Only 2 positive responses were lost by the IFN-γ+ MIP-1β+ data evaluation system, whereas 9 responses were lost by the total IFN-γ+ data evaluation system in comparison to the ELISPOT performed in laboratory 2. These combined results of the 2 laboratories demonstrated that the new evaluation method based on the simultaneous detection of IFN-γ and MIP-1β increased the capacity of the ICS to detect low-magnitude responses that would be otherwise missed using the ELISPOT or the detection of the total IFN-γ+ T-cells.

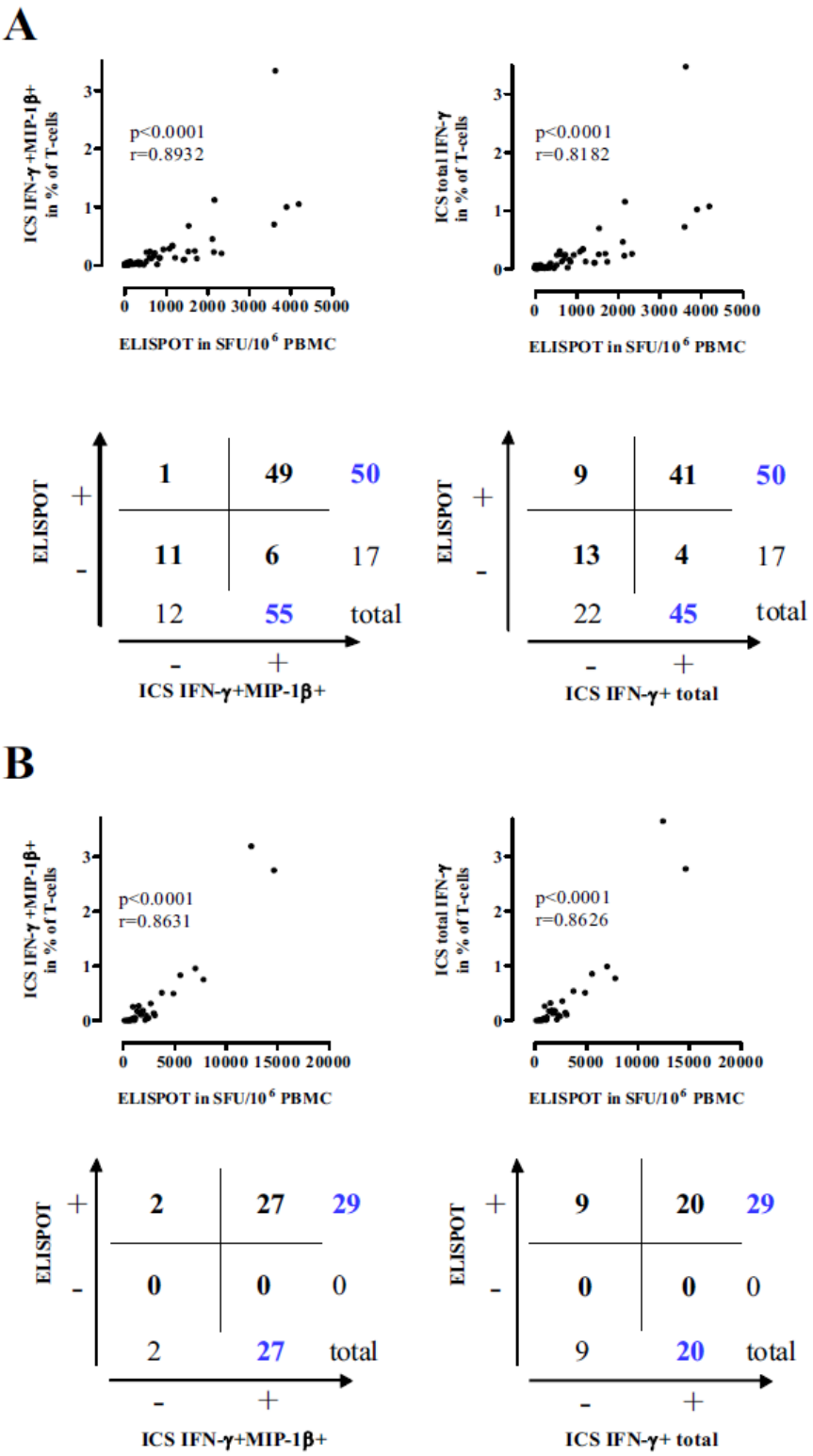

Figure 17. Comparison between the ICS and two independently performed ELISPOT assays. The two ICS data evaluation systems are compared with ELISPOT assays performed by laboratory 1 (A) and laboratory 2 (B). Correlations between frequencies of responding T-cells detected by ELISPOT and by ICS using the IFN-γ+ MIP-1β+ or the total IFN-γ+ data evaluation system are determined by Spearman's rank correlation. r and p values are shown in each graph. 2x2 contingency tables comparing the positive T-cell responses detected by ELISPOT and by ICS with the two data evaluation systems are also shown.

4.1.7 Influence of the variation in cell number input in the ICS assay

Cell counting is a basic technique in use in all cell culture laboratories. Nevertheless, it constitutes an important source of experimental error [150]. The number of cells per sample is a critical parameter in the ELISPOT assay, since results are directly calculated from the total amount of cells seeded in each well. In contrast, in the ICS assay responding cells are calculated as a percentage of total CD4 or CD8 T cells and therefore the results are independent from the total number of cells used in each experimental sample. However, variation in the cell number might still affect the experimental outcome because of changes in the proportion between the amount of cells, growth factors and stimulants. Therefore, the impact of varying the amount of PBMC per experimental sample in our 9-color ICS assay we tested. Stimulation with 2 different peptides representing optimal CD8 T-cell epitopes was performed using 0.45, 0.91, 1.82 and 3.66 million of cells/well, while the amount of peptides was kept constant at 2 μg/ml. There was neither a trend nor a high variation between the results for either the total IFN-γ+ response as well as for the combined IFN-γ and MIP-1β positive cells (Figure 18). Of note, the background levels were not affected by the number of cells seeded per well.

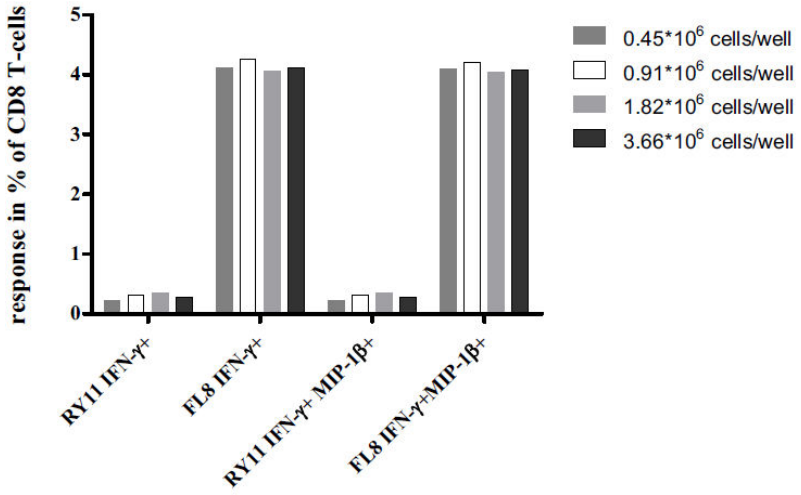

Figure 18. Variation in the number of cells/well in ICS assay. Different amounts of PBMC were stimulated with 2 different Nef derived optimal CD8 epitopes (FLKEKGGL, FL8 and RRQDILDLWIY, RY11). Analyzed responses are shown on the x axis.

4.2 Re-evaluation of a therapeutic vaccination trial using MVA-nef in HIV-1 infected individuals

In a previous Phase I study, it was shown that a modified vaccinia virus Ankara vector expressing HIV-1_{LAI}-*nef* (MVA-*nef*) was safe and immunogenic in 10 HIV-1-infected individuals under HAART. Participants received MVA-*nef* vaccination twice (week 0 and 2) with a third vaccination 12 weeks after the second administration (week 16). MVA-*nef* was able to elicit or increase Nef-specific CD4 T-cell responses in 8 out of 10 participants, as measured by IFN-γ-based ICS assay [119].

As the sole evaluation of IFN-γ provides limited information on the quality of antigen-specific T-cell responses [139, 151-153], a re-evaluation of the study was performed using the methods established as described in 4.1. The study mainly focused on the evaluation of the quality of the immune response in regard to several markers and the *ex-vivo* proliferative potential. Therefore data were analyzed using a more complex system than the IFN-γ+ MIP-1β+ data evaluation system. The data analysis system applied in the re-evaluation of the clinical trial is described in section 4.1.1 Establishment of 9 – color Intracellular cytokine staining (ICS), 4.1.2 Establishment of a Carboxyfluorescein succinimidyl ester (CFSE) -based proliferation assay and 4.1.3 Data processing and storage.

4.2.1 MVA-nef vaccination increases the magnitude of the total Nef-specific CD4 T-cell response

The above described polychromatic ICS was used to examine Nef-specific CD4 and CD8 T cells in cryopreserved PBMC samples obtained from 9 vaccine recipients. The analysis included PBMC collected before the first and after the second administration of the vaccine. The ICS assay included the measurement of four functional markers (IFN-γ, IL-2, MIP-1β and CD154) and one differentiation marker (CD45RA) in CD4 and CD8 T-cell subsets. By summing the frequencies of CD4 or CD8 T cells of each unique T-cell population expressing at least one functional marker, the magnitude of the total specific response (Figure 19 A and C) was analyzed. The total frequency of Nef-specific CD4 T cells was significantly increased by two immunizations ($p=0.0078$; Wilcoxon signed rank test) and was further boosted after the third administration in study subjects V01, V03, V04 and V08. The magnitude of the CD4 T-cell response ranged from 0 to 0.204% (median 0.022%) before the administration of the vaccine, from 0 to 1.028% (median 0.074%) and 0 to 0.967% (median 0.066%) after the second and the third immunization, respectively. The total frequency of Nef-specific CD8 T cells did not show any significant variation temporally associated with the administration of

the vaccine. The magnitude of the CD8 T-cell response ranged from 0.012 to 0.461% (median 0.27%) before the administration of the vaccine, from 0.01 to 0.566 (median 0.206%) and from 0.028 to 0.582% (median 0.249%) after the second and the third immunization, respectively. As a control, CD4 and CD8 T-cell responses to the regulatory HIV protein Tat were equally monitored, and significant variations temporally associated with the administration of the vaccine were not observed (data not shown). These data confirm the previous first-line characterization of the T-cell response induced by the MVA-*nef* vaccine as measured by IFN-γ-based ICS [119], in that Nef-specific CD4 but no CD8 T-cell responses were induced by the administration of the vaccine.

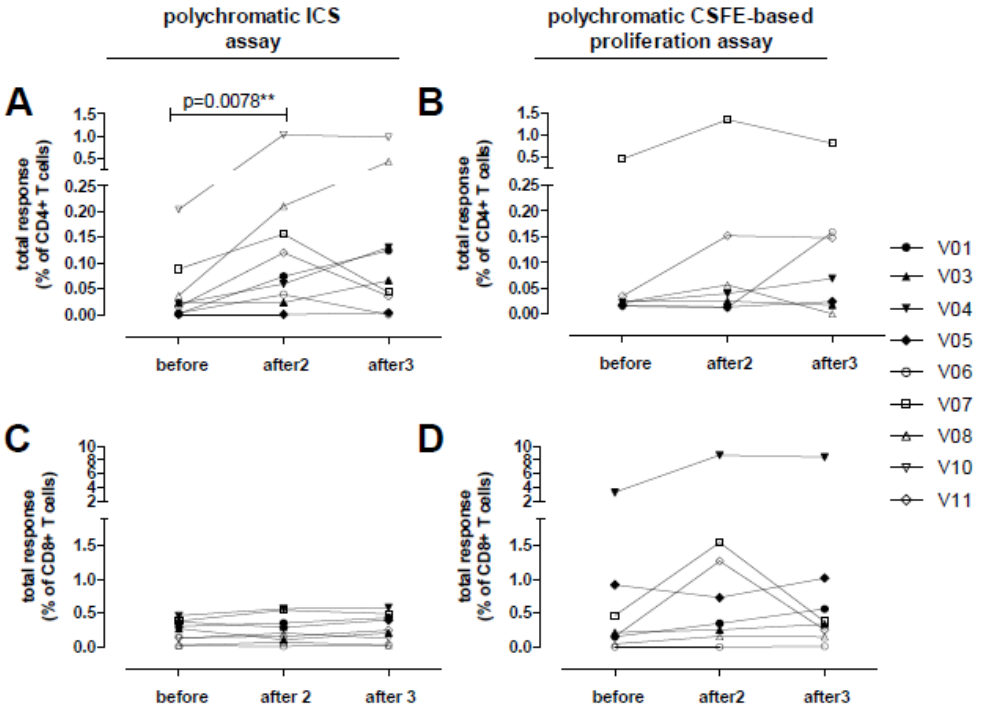

Figure 19. Total CD4 and CD8 T-cell responses throughout the course of the MVA-*nef* vaccination trial detected by polychromatic ICS and polychromatic CFSE-based proliferation assay. Total Nef-specific CD4 (A and B) and CD8 (C and D) T cells are shown before, after 2 and after 3 MVA-*nef* administrations as detected by ICS (A and C) and CFSE-based proliferation assay (B and D). Nine subjects were analyzed by ICS; the total response is calculated by summing all responding subsets defined by IFN-γ, IL-2, MIP-1β secretion and CD154 expression. Eight subjects are analyzed by CFSE-based proliferation assay; the total response is calculated by summing all responding subsets defined by IFN-γ, IL-2, MIP-1β secretion and a low CFSE staining level representing proliferative activity. All the data are background subtracted. Minor responses are excluded by the use of a predefined threshold system. To account for multiple comparison differences between the three time-points we applied nonparametric Friedman's test for three-way comparison followed by a two-way Wilcoxon signed rank test.

4.2.2 MVA-nef vaccination induces Nef-specific CD4 and CD8 T cells able to proliferate

Since proliferative potential of antigen-specific T cells is considered a key factor in maintaining or restoring effective antiviral immunity [154-156], it was of special interest to simultaneously evaluate the functional profile and the proliferative potential in combination with functional markers of Nef-specific CD4 and CD8 T cells during the course of the study. Since there was not enough cell material available from study subject V10, the analysis of the proliferative potential included only 8 study subjects. By summing the frequencies of CD4 or CD8 T cells of each unique T-cell population positive for at least one functional marker or able to proliferate, the magnitude of the total specific response was analyzed (Figure 19 B and D). The magnitude of the total Nef-specific CD4 T cells ranged from 0.015 to 0.467% (median 0.023) before the administration of the vaccine. Following the second and the third immunization the specific CD4 T-cell response ranged from 0.012 to 1.357% (median 0.04%) and from 0 to 0.825% (median 0.069%). Thus, the magnitude of the CD4 T-cell response detected by proliferation assay was in the same quantitative range as the response detected by standard ICS (Figure 19 A and, B). Interestingly, the CFSE-based proliferation assay revealed a clear increase of functional and proliferating Nef-specific CD8 T cells following MVA-*nef* vaccination (Figure 19 D). The magnitude of the total Nef-specific CD8 T cell responses ranged from 0 to 3.093% (median 0.152%) before the administration of the vaccine. Following the second and the third administration, the CD8 T cells responding to Nef ranged from 0 to 8.698% (median 0.537%) and from 0 to 8.042% (median 0.355%), respectively.

Figure 20 shows representative staining examples of ICS and proliferation assay performed before and after two vaccine administrations for study subject V11. CD4 T-cell responses to Nef were similarly detected using the two immune assays (Figure 20 A and B), whereas the proliferation assay turned out to be dramatically more effective in detecting the vaccine induced CD8 T-cell responses in comparison to the ICS (Figure 20 C and D). These results highlight a differential capacity of the ICS and proliferation assay to reveal specific immune responses. In particular, the proliferation assay allowed the detection of CD8 T-cell responses elicited by the vaccine, not observed using the ICS assay.

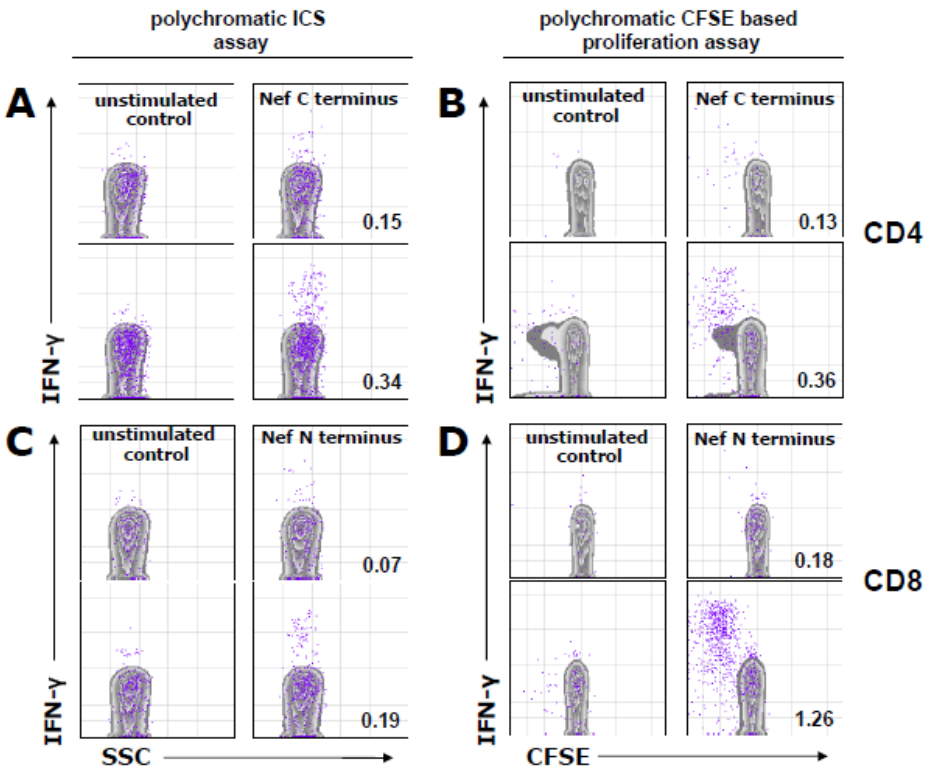

Figure 20. Representative CD4 and CD8 T-cell responses before and after 2 administrations of MVA-*nef* as measured by polychromatic ICS and polychromatic CFSE-based proliferation assay. Density plots show total CD4 (A and B) or CD8 (C and D) T cells in gray, overlaid by total responding, marker-expressing cells of study subject V11 in violet. Results obtained from polychromatic ICS are depicted in A and C, whereas results obtained from the polychromatic CFSE-based proliferation assay are depicted in B and D. In each panel, left plots show the unstimulated negative controls and right plots samples stimulated with the Nef N terminus or Nef C terminus peptide pool. The upper plots show the time point before the administration of MVA-*nef*, whereas the lower plots show the time point after two MVA- *nef* administrations. Numbers indicated on the graphs are the % of total responding cells relative to the parent population (CD4 or CD8) after background subtraction.

4.2.3 MVA-nef vaccination increases the grade of functionality of CD4 T cells

The breakdown of the total Nef-specific response into specific functional categories as detected by polychromatic ICS assay is shown in Figure 21. First the CD4 T-cell responses were analyzed (Figure 21 A). CD4 T cells responding to Nef were mainly CD45RA negative. Only a single subject showed a clear CD45RA positive Nef-specific subset before the administration of the vaccine, which declined after the 2nd vaccination and disappeared following the 3rd administration of the vaccine. This suggests a shift towards the CD45RA negative memory population in this specific individual. As it is not possible to draw conclusions from a single subject, CD45RA was excluded from further CD4 T cell analysis. The analysis of the degree of functionality showed that before the administration of the vaccine mono-, bi-, tri- and tetra-functional Nef-specific CD4 T cells accounted for 52.5, 29.7, 16.6 and 1.3% of the total response, respectively. After two vaccine administrations tri-

and tetra-functional specific T cells increased to 21.2 and 6.6% of the total CD4 response, respectively. Following the third administration of the vaccine, a further increase to 30.6 and 7.6% of the total responding CD4 T cells was observed. The breakdown into specific functional categories demonstrated a significant increase of the Nef-specific CD4 T cells expressing CD154, IFN-γ and IL-2 following the second ($p=0.031$) and the third ($p=0.023$) administration of the vaccine. A significant increase of the Nef-specific CD4 T cells expressing CD154 and IFN-γ ($p=0.031$) was additionally observed following the second administration of the vaccine. CD4 T cells expressing CD154, IFN-γ, IL-2 and MIP-1β were detected only in one study subject (V10) before the administration of the vaccine. Following the administration of the vaccine these tetra-functional T cells became detectable in three additional study subjects (V01, V04 and V11) and they were strongly boosted in study subject V10. Remarkably, CD154 expression on Nef-specific CD4 T cells was increased after the administration of MVA-*nef*, indicating an improved capacity of these CD4 T cells to interact with antigen-presenting cells.

The degree of functionality of Nef-specific CD8 T cells did not change following vaccination, and the breakdown of the response into specific functional categories did not show any significant variation associated with the administration of the vaccine (Figure 21 B). Expression of CD45RA on CD8 T cells showed a memory phenotype that differed among individuals but was not influenced by the administration of MVA-*nef*.

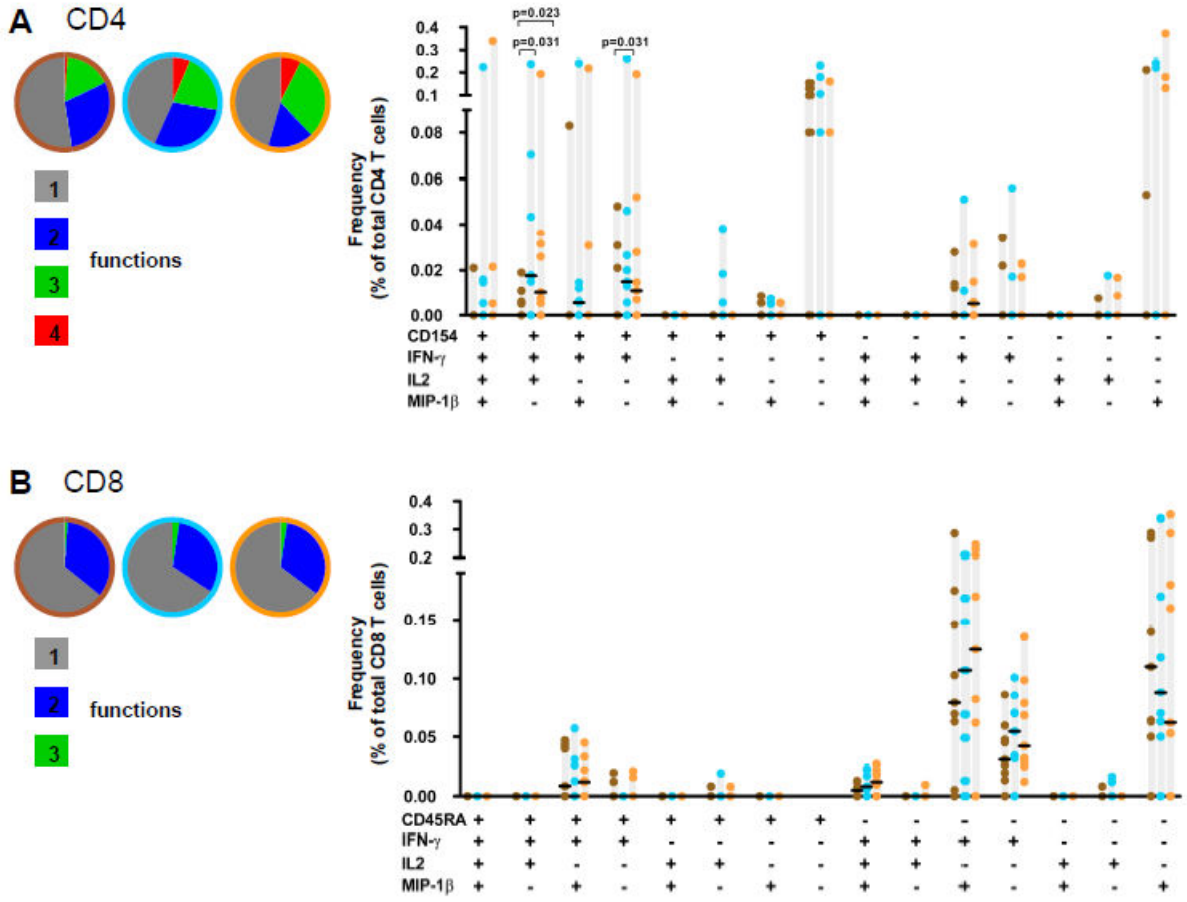

Figure 21. Functional breakdown of total CD4 and CD8 T-cell responses as measured by polychromatic ICS. Pie charts and aligned dot plots are shown for CD4 (A) and CD8 (B) T-cell responses of all study participants. Pie charts show the functional subsets grouped according to their grade of functionality. Fractions of tetra- (red), tri- (green), bi- (blue), and monofunctional (grey) responding T cells are shown for samples collected before (brown border), after 2 (turquoise border) and after 3 (orange border) vaccine administrations. CD4 T cell functions taken into account were CD154 expression and IFN-γ, IL-2 and MIP-1β secretion. CD8 T-cell functions taken into account were IFN-γ, IL-2 and MIP-1β secretion. Aligned dot plots show all possible combinations of 1 to 4 markers in absolute % of the parent populations before (brown) and after 2 (turquoise) or 3 (orange) vaccine administrations. Markers taken into account are specified in the respective graph. To account for multiple comparisons between the 3 time-points, we applied a nonparametric Friedman's test for three-way comparison followed by a two-way Wilcoxon signed rank test.

4.2.4 Detailed analysis of the CD4 T-cell immune response throughout the trial in a single subject V04

To obtain a more detailed picture of the impact of MVA-*nef* on the T-cell immune response, an in depth analysis of subject V04 was conducted, from which sufficient cell material was available. The immune response of V04 was of particular interest as this subject underwent a therapy interruption one year after the last administration of MVA-*nef*. Proximately, this participant maintained viral control and stable CD4 counts for the following 6 years.

The CD4 T-cell response against Nef was separately analysed for the N-terminal and the C-terminal part of the protein using two separate pools of peptides. Samples were selected from the timepoints indicated in the scheme below.

Before MVA-*nef* immunization there was no detectable CD4 T-cell response against the N-terminal Nef and a response with a maximum of two functions against the C-terminal Nef. After the second administration of MVA-*nef*, a CD4 T-cell response against the N-terminal Nef, which included tetra-functional subsets, was elicited *de novo* and the maximal grade of functionality in the response against the C-terminal part of Nef increased from bi- to tetra-functional. Specific tetra-functional CD4 T-cells against the N-terminal part of Nef were still present after one year. Functionality of CD4 T-cell responses directed against the C-terminal part of Nef was reduced to two functions after one year and returned to pre-vaccination level. Of note, neither the quantity nor the quality of the CD8 T-cell response against none of the different regions of Nef was changed significantly by the administration of MVA-*nef* (Figure 22).

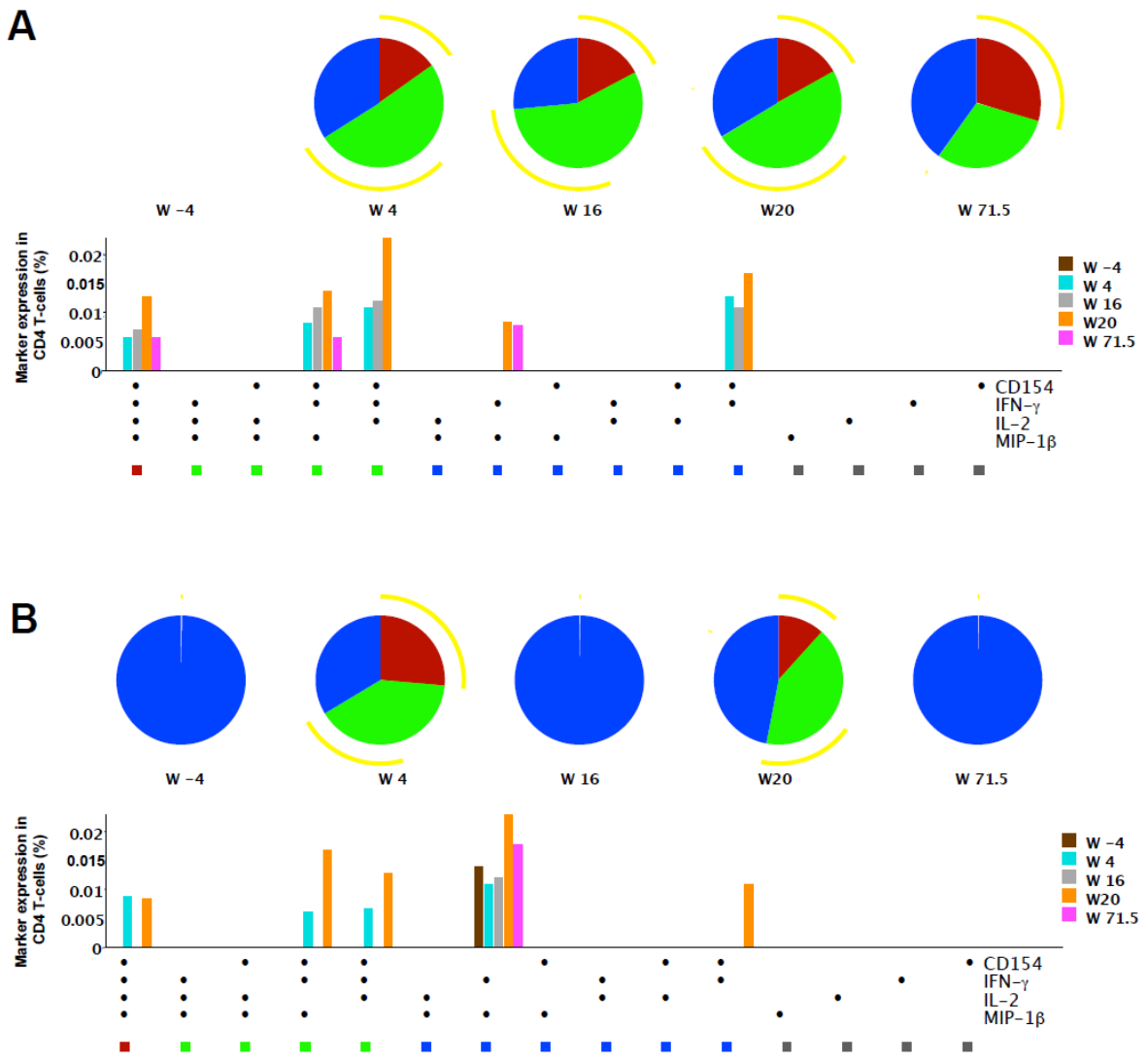

Figure 22. Detailed analysis of Nef specific CD4 T-cell responses of a single subject. Pie charts and bar charts show the CD4 T-cell response against the N-terminal (A) and C-terminal (B) Nef of subject V04. Pie charts show the functional subsets grouped according to their grade of functionality. Fractions of tetra- (red), tri- (green), bi- (blue), and monofunctional (grey) responding T cells are shown for samples collected at week -4 , week 4, week 16, week 20 and week 71.5 respective to the first MVA-*nef* administration. IL-2 production is indicated as yellow circle around the pie charts. CD4 T cell functions taken into account are CD154 expression and IFN-γ, IL-2 and MIP-1β secretion. Bar charts show all possible combinations of 1 to 4 markers in absolute % of the parent populations at week -4 (brown), week 4 (turquoise), week 16 (grey), week 20 (orange) and week 71.5 (pink).

4.2.5 Functional characteristics of the proliferating Nef-specific CD4 and CD8 T cells

Nef-specific CD4 (Figure 23 A) or CD8 (Figure 23 B) T cells detected by proliferation assay were dissected into functional categories according to their differential expression of IFN-γ, IL-2 MIP-1β and the capacity to proliferate. The total amount of CFSE low Nef-specific CD4 T cells increased after the second administration of the vaccine from a median of 0% (range: 0 to 0.022%) to 0.04% (range: 0 to 0.232%). The functional breakdown showed that these cells

were mainly producing IFN-γ and MIP-1β, but the increase was limited to few study subjects and did not reach statistical significance. On the contrary, a strong effect of the vaccination on the capacity of Nef-specific CD8 T cells to proliferate was observed. The total amount of CFSE low Nef-specific CD8 T cells ranged from 0 to 3.093% (median 0.152%) before the administration of the vaccine, from 0 to 8.395 (median 0.482%) and from 0 to 8.042% (median 0.2%) after the second and the third immunization, respectively. In addition, significant differences were observed in specific CD8 T cells holding a proliferative potential and producing MIP-1β alone or MIP-1β and IFN-γ following the second (p=0.016; Wilcoxon signed rank test) and the third administration (p=0.023; Wilcoxon signed rank test), respectively.

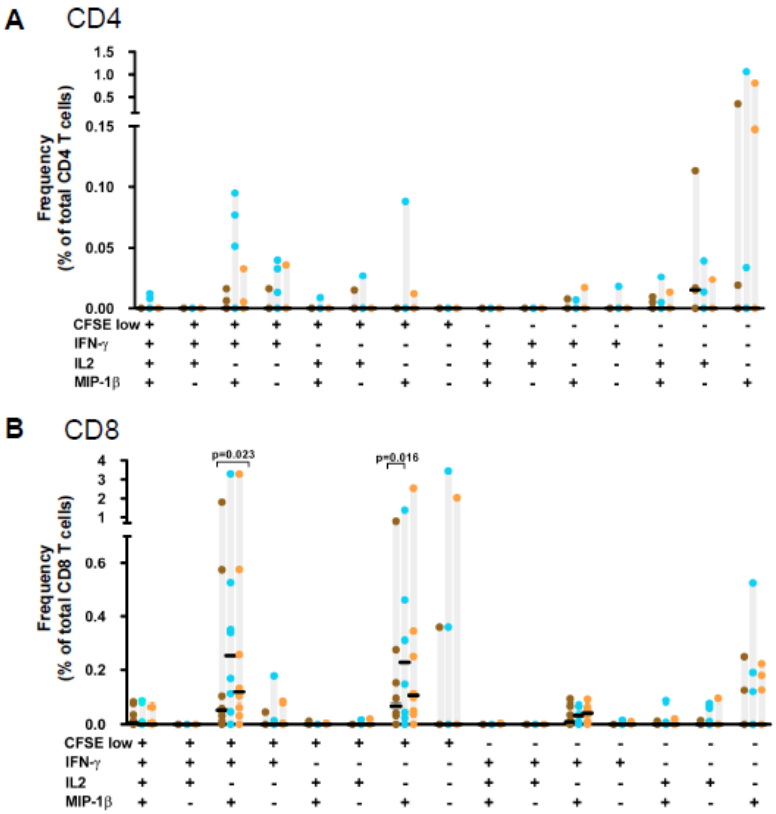

Figure 23. Functional breakdown of total CD4 and CD8 T-cell responses as measured by polychromatic CFSE-based proliferation assay. Aligned dot plots are shown for CD4 (A) and CD8 (B) T-cell responses. All possible combinations of 1 to 4 markers in absolute % of the parent populations (CD4 or CD8 T cells) before (brown) and after 2 (turquoise) or 3 (orange) vaccine administrations are shown. To account for multiple comparisons between the 3 time points, we applied a Friedman's test followed by a Wilcoxon signed rank test.

4.2.6 Correlation between CD4 T-cell responses and CD8 T-cell proliferative activity

In HIV-infected subjects, a causal link between IL-2 production by CD4 T cells and T-cell proliferative capacity has been proposed [143, 157]. Therefore, it was determined whether

there was a correlation between the increase of the Nef-specific CD4 T-cell response detected by ICS assay and the increase in the proliferation activity observed in the Nef-specific CD8 T cells. The analysis included the 8 study subjects that had been analyzed in both assays and compared the increment of the responses observed after the second and the third administration of the vaccine. Following the second administration of MVA-*nef*, a significant correlation between the increase of Nef-specific CD4 T cells co-expressing CD154, IFN-γ and IL-2 as detected by ICS assay and the increase of total Nef-specific CD8 T cells as detected by proliferation assay was shown [p=0.022; Spearman's correlation test (data not shown)]. Following the third vaccination, a significant correlation between the increase of Nef-specific CD4 T cells co-expressing CD154, IFN-γ and IL-2 as detected by ICS assay and the increase of total Nef-specific CD8 T cells as well as the proliferating MIP-1β+ IFN-γ+ subset as detected by proliferation assay was found (p=0.028 and p=0.022, respectively; Spearman's correlation test). In addition, correlation analysis showed a significant association between the increase of total IL-2 production by CD4 T cells detected by ICS assay with the increase of total proliferating CD8 T cells following two and three administrations of MVA-*nef* (p=0.022 and p=0.007, respectively; Spearman's correlation test).

4.2.7 Vaccine induced polyfunctional CD4 T cells produce high quantities of cytokines

Polyfunctional T cells produce more cytokines on a single-cell level than mono- or bi-functional T cells [158-160]. A high level of cytokine expression might account for an effective control of viral replication by polyfunctional T cells [152]. To assess the MFI of the polyfunctional Nef-specific CD4 T cells elicited by the vaccine, the responding subsets were grouped according to their grade of functionality and then the relative MFI (rMFI) for the expression of IFN-γ, IL-2, CD154 and MIP-1β was calculated (Figure 24). rMFI values were calculated by dividing the MFI of the functional subsets by the MFI of nonfunctional CD4 T cells. Tetra-functional Nef-specific CD4 T cells expressed significantly higher levels of IFN-γ than tri-, bi- and mono-functional cells. A similar pattern was observed for the expression of CD154 with the exception that tetra- and tri-functional T cells had similar relative MFI. The distribution of the MFI for IL-2 expression was identical to that observed for CD154, but since a small number of bi- and monofunctional CD4 T cells expressed IL-2, differences were not statistically significant. Finally, MIP-1β expression was significantly lower in monofunctional than in tetra-, tri- and bi-functional responding CD4 T cells, but among poly-, tri- and bi-functional CD4 T cells no differences were observed. These data suggest that the Nef-specific polyfunctional CD4 T cells observed in this study present the typical phenotype

of highly functional effector cells. The overlaid dot plots in Figure 24 B show the florescence intensity of the mono-, bi-, tri- and tetra-functional cells in the representative study subject V10 during the course of the study. Following the administration of the vaccine, a clear increase of the Nef-specific CD4 T cells with a polyfunctional phenotype clearly expressing highest amounts of IFN-γ, IL2 and CD154 on a per-cell basis was observed.

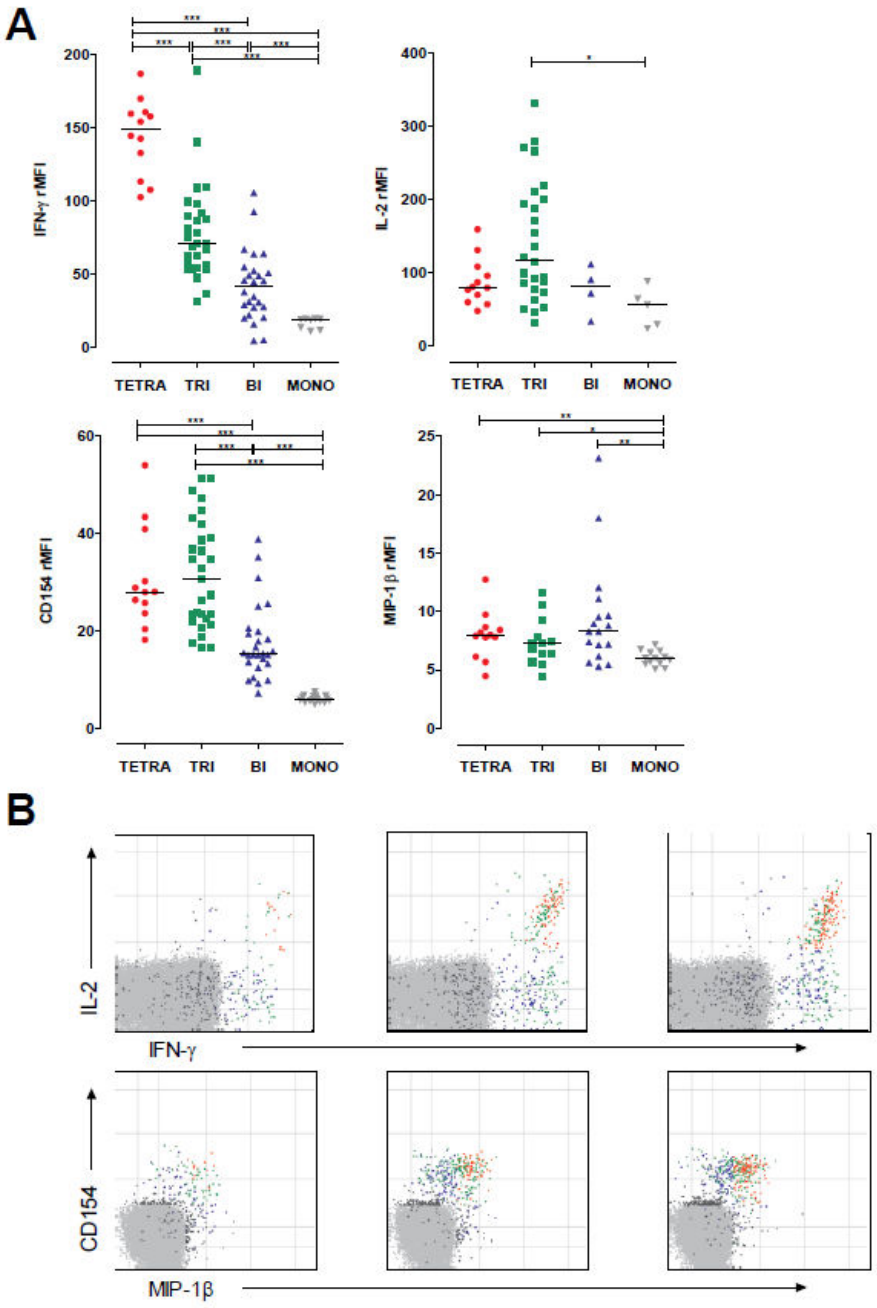

Figure 24. Characterization of polyfunctional CD4 T cells. (A) CD4 T cells responding to stimulation with Nef derived peptide pools are grouped according to their grade of functionality and expression of IFN-γ, IL-2, MIP-1β or CD154 into tetra- (red), tri- (green), bi- (blue), and mono-functional (grey). Relative MFI (rMFI) values were calculated by dividing the MFI of the functional subsets by the MFI of nonfunctional CD4 T cells. Each point represents a positive CD4 T-cell response detected with the Nef peptide pools covering either the N terminus or the C terminus of the Nef protein. To account for multiple comparisons, differences between the functional groups were assessed by nonparametric Kruskal-Wallis test for four-way comparison followed by a pairwise Mann-Whitney test. (B) Representative flow cytometric data from subject V10 are depicted in overlaid dot plots. Mono-, bi-, tri- and tetra-functional Nef-specific CD4 T cells are indicated using the same color coding as for the graphs in (A). Asterisks indicate the level of significance with *** indicating $p \leq 0.0001$; ** indicating $p \leq 0.001$; * indicating $p \leq 0.05$.

4.2.8 Comparison of the CD4 T-cell immune response in MVA-nef vaccinated subjects to CD4 T-cell responses in persistent but controlled viral infections regarding functionality

To investigate the role of Nef-specific CD4 T-cell subsets with different grades of functionality, they were compared to CD4 T-cell responses in controlled chronic viral infections. CMV and EBV specific CD4 T-cell responses in 23 healthy individuals were investigated. The expression profile of CD154, IFN-γ, IL-2 and MIP-1β was evaluated in order to be compared to the responses seen in vaccinated HIV-1 infected study participants. CD4 T-cells responding upon stimulation with the immunodominant phospho-protein pp65 and the immediate-early protein IE-1 of CMV, and the latent membrane proteins LMP-1 and LMP-1 of EBV were assessed. A CD4 T-cell response towards pp65 was observed in 14 out of 23 individuals [median: 0.172% (0.006%-2.557%)]; towards IE-1 in 8 out of 23 individuals [median: 0.028% (0.005%-0.089%)]; towards LMP-1 in 6 out of 23 individuals [median: 0.038% (0.006%-0.208%)]; and towards LMP-2 in 5 out of 23 individuals [median: 0.013% (0.006%-0.185%)]. The functional breakdown of the responses revealed that 9 out of 14 responses towards CMV pp65 contained a tetra-functional fraction with a median of 0.022% (0.009%-0.983%). Tri-functional cells expressing CD154, IFN-γ and MIP-1β were present in 8 of the 14 responses towards CMV pp65 [median: 0.022% (0.000%-1.246%)] and tri-functional cells expressing CD154, IFN-γ and IL-2 were present in 11 out of 14 responses towards CMV pp65 [median: 0.028% (0.000%-0.170%)] (Figure 25 A). No tetra-functional cells were detected within the response towards CMV IE-1. Tri-functional cells expressing CD154, IFN-γ and IL-2 were detected in 7 out of 8 responses towards IE-1 with a median of 0.011% (0.005%-0.055%) (Figure 25 B). 1 out of 6 responses towards EBV LMP-1 and 1 out of 5 responses towards EBV LMP-1 contained a tetra-functional fraction with a frequency of 0.012% and 0.023% of CD4 T-cells, respectively (Figure 25 C and D). Taken together it can be stated that the CD4 T-cell responses against antigens of controlled chronic viral infections were highly functional.

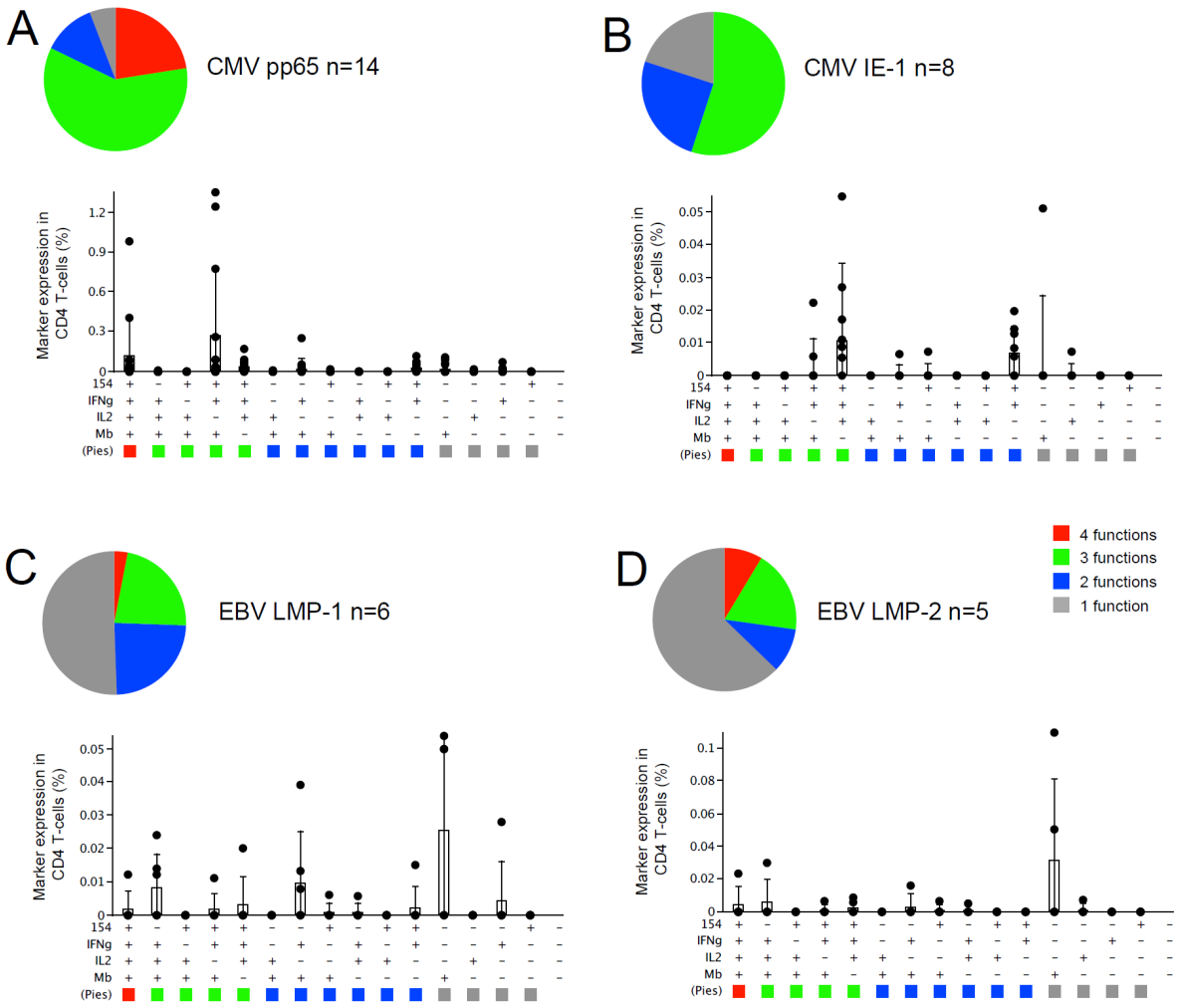

Figure 25. Analysis of CD4 T-cell responses against CMV and EBV derived antigens as examples for controlled chronic viral infections. The analysis summarizes a study including 23 healthy volunteers. Pie charts and aligned dot plots show the CD4 T-cell response against the CMV pp65 (A), CMV IE-1 (B), EBV LMP-1 (C) and EBV LMP-2 (D). The number of responding individuals is indicated in the graph as n. Pie charts show the functional subsets grouped according to their grade of functionality. Tetra- (red), tri- (green), bi- (blue), and monofunctional (grey) responding T cells are shown for all responders. CD4 T cell functions taken into account are CD154 expression and IFN-γ, IL-2 and MIP-1β secretion. Aligned dot plots show all possible combinations of 1 to 4 markers in absolute % of the CD4 T-cell population.

5 Discussion

To date, the only effective remedy against HIV and AIDS remains antiretroviral therapy. Despite of substantial benefits, HAART is not able to clear HIV infection and has several short-term and long-term side effects, yet, its use is strictly necessary to control HIV replication. Thus, alternative therapeutic and prophylactic solutions to cure progression to AIDS and prevent HIV infection have to be found.

Vaccination is one of the possible alternative strategies to contain HIV, both in a therapeutic or in prophylactic setting. The rationale behind a therapeutic vaccine is to improve the magnitude and the quality of the anti-HIV immune responses to an extent that will be able to control viral replication without the help of anti-retroviral therapy while a prophylactic vaccine should aim at eliciting an immune response able to face the first encounter with the virus. The example of long term non-progressors (LTNP) demonstrates that control of viral replication by the immune system is possible in the context of the natural course of the disease. The comparative analysis of anti-HIV immune response in LTNP, chronically HIV infected individuals under HAART and individuals with progressive disease highlighted some possible correlates of protection and some possible immunological mechanisms of viral control [140, 141, 154, 161-164]. Thus, it can be suggested that the immune system might be able to control viral replication. However, from all these studies it is difficult to define clear protection markers, since often studies are focalized on one specific aspect of the immune response. The available methods for analysis of antigen-specific T cells are still technically challenging, and further improvements, as well as the establishment of generally accepted standard operation procedures are currently being developed [165]. Therefore, the first aim of this work was the establishment of assays holding the potential to evaluate simultaneously several markers with a particular role in HIV infection to gain further insights in the functional profile, differentiation phenotype, proliferative potential and activation state of HIV specific T cells. The developed assays were then applied to a setting of a therapeutic vaccination using MVA-nef, including a small comparative study with immune responses towards CMV and EBV derived antigens as an example for controlled viral infections in regard to qualitative aspects.

5.1 Establishment of flow-cytometry based assays for the characterization of T-cell immune responses

The polychromatic ICS and CFSE-based proliferation assay established in this work are function-dependent assays such as the ELISPOT. They detect antigen-specific T cells based on effector functions, like production of cytokines and chemokines or induction of cell proliferation in response to antigenic *ex vivo* re-stimulation; only T cells capable of responding with the readout effector function under the chosen *ex-vivo* re-stimulation conditions can be detected. T cell detection assays will be able to detect the entire population. For this reason, T cell detection methods that are independent of effector functions and do not require *in vitro* incubation, especially MHC class I tetramers have proven to be useful for extensive phenotypic characterization of antigen-specific T cell populations. Several surface and/or intracellular markers (CD127, CD69, CD38, CD45RA, CCR7, CD62L), which indicate whether a single T cell belongs to a certain subtype of effector or memory T cells have been identified [166]. When evaluating the T-cell differentiation in diverse viral infections such as CMV, EBV and HIV it has been shown, that the maturation phenotype is skewed mainly regarding the CD45RA expression [167]. Detection of this marker is usually combined with the measurement of the homing receptor CCR7 to distinguish between T_{CM} (central memory, CD45RA-CCR7+) T_{EM} (effector memory CD45RA-CCR7-), T_{EMRA} (terminally differentiated, CD45RA+CCR7-) and naïve cells (CD45RA+CCR7+) [149]. When combined with at least one functional marker CD45RA alone in theory is able to discriminate between T_{CM}/T_{EM} and T_{EMRA} cells as the large majority of functional, responding cells is not naïve. Two studies recently could demonstrate the potential protective role of an HIV specific, IFN-γ functional, CD8 T_{EMRA} cell subset in different HIV infected cohorts. They revealed that a CD45RA positive phenotype of IFN-γ producing functional cells in the early state of infection correlates with a lower viral load in later phases [141]. Furthermore there could be indicated a relationship between the CD45RA+IFN-γ+ cell subset and a long term nonprogressor status [140]. Therefore, this marker had been included in the staining combination of the polychromatic assays. It has been reported that CD45RA expression is varying depending on the time elapsed since antigen stimulation. After a stimulation of two days the loss of CD45RA expression was observed to be approximately 14-fold [168]. The direct comparison performed in this work provides a confirmation that the memory phenotype regarding the CD45RA expression observed in the ICS assay after 5 hours of in vitro restimulation and an overnight rest at 4°C is comparable to the results of the MHC class II tetramer staining without *ex vivo* re-stimulation. The results prove that the developed ICS

assay allows detection of the memory phenotype regarding the CD45RA expression and is equivalent to a specific tetramer staining from this point of view. On the contrary, when using the CFSE based proliferation assay incorporating the prolonged *ex-vivo* re-stimulation of 5 days, CD45RA was downregulated on antigen-specific cells and was therefore excluded from further analysis in this assay.

It is generally established that the number of detected functional cells by ICS after stimulation with single peptides in general does not reach the range of cells detected by specific tetramer staining using the same epitope, because not all antigen specific cells are supposed to be functional in cytokine production and cytotoxic activity [135, 169]. The experiments display that the extent to which antigen specific T cells are detected by a functional assay is depending on the respective functional markers being measured and on patient specific characteristics. It could be shown that the 9-color ICS investigating MIP-1β can reach almost the range of cells detected by a tetramer staining using the same antigenic epitope.

The ELISPOT assay is currently considered the gold standard in vaccine trials due to its sensitivity and extensive standardization and validation [121-124, 150]. In fact, several reports demonstrated that the ELISPOT assay is more sensitive in detecting weak responses when compared to the ICS assay [125-128], a supporting issue in favour of the use of the IFN-γ ELISPOT as primary assay in vaccine trials [129]. In this work, experimental evidence is provided in support of the combined IFN-γ and MIP-1β ICS method that, unlike commonly used methods based on the flow-cytometric detection of IFN-γ, achieved sensitivity comparable to that typical of ELISPOT assays. In this regard, the key observation was that the majority of the IFN-γ producing T-cells were simultaneously producing MIP-1β rendering this new modality of evaluation equivalent to the measurement of the total IFN-γ producing T-cells. With the relevant advantage of a consistent decrease of background the sensitivity of the assay can be increased. The results provide support for an expanded use of polychromatic flow cytometry as primary assay in vaccine trials. The 9-color ICS method optimized during this work allows the simultaneous measurement of several fluorescence markers without losing sensitivity in comparison to the gold standard IFN-γ ELISPOT. However, instead of a 9-color assay the same method could be applied to any staining combination including IFN-γ and MIP-1β in combination with the appropriate lineage markers. Thus, for investigators with no access to sophisticated flow cytometers, a simplified panel could be used for immune-monitoring purpose as alternative to the ELISPOT not losing sensitivity and with the

advantage to discriminate CD4 and CD8 T-cell mediated responses. Alternatively, more complex staining combinations could be designed for laboratory facilities where complex instrumentation is available, provided the inclusion of the simultaneous measurement of IFN-γ and MIP-1β. The present study comparing the combined IFN-γ and MIP-1β detection method to the classical IFN-γ based ICS was limited to the analysis of the HIV-1-specific T-cell responses. Nevertheless, this method can be extended to other antigen specific immune responses if T-cells expressing IFN-γ and MIP-1β represent the majority of the total IFN-γ producing T-cells. In this regard, a possible extension of the methodology is the coupling of an activation marker (i.e. CD69, CD154, etc.) to the measurement of cytokines or chemokines (i.e. IFN-γ, IL-2 and MIP-1β). Generally, targeting 2 or more molecules on the same cell population should increase the sensitivity of the assay for the selected cell population. Since flow cytometry is continuously advanced by the development of new instrumentation and reagents, the inclusion of more markers in a single sample should aim not only to increase the amount of information per cell but also to increase the sensitivity for populations of special interest. The cell number study demonstrated that the amount of cells used in each experimental sample does not affect the readout of the 9-color ICS. Since the procedure of manual cell counting is a usual source of experimental error and the number of cells directly affects the ELISPOT readout, the data support the concept of a reduced experimental error associated with the use of ICS assays and strengthens the idea to apply ICS as primary assay in vaccine trials.

The polychromatic CFSE-based proliferation assay established in the present work can be regarded as a combination of a classical CFSE staining method [170] and the classical ICS [171]. It allowed for simultaneous evaluation of the proliferative capacity in combination with the expression pattern of the functional markers IFN-γ, IL-2 and MIP-1β in CD4 and CD8 T cells. The two methods have been previously combined for mouse T-cell evaluation [172, 173] and recently a similar method has been established for human PBMC [174]. Nevertheless, the assay established in this work is unique in that no exogenous proliferative or anti-apoptotic cytokines are added and observed proliferation, survival, and cytokine production depend solely on endogenous cytokines produced in response to the antigen. The combination of the standard ICS and the CFSE-based proliferation assay proved extremely useful to examine qualitative and quantitative aspects of the MVA-*nef*-induced, anti-Nef-specific immunity.

5.2 Re-evaluation of a therapeutic vaccination trial using MVA-nef in HIV-1 infected individuals

Therapeutic vaccination may represent a valuable tool to achieve viral control in HIV-1-infected individuals by boosting the virus-specific immune response. Various HIV-1 immunogens have been tested in HIV-1-infected individuals and were able to stimulate specific CD4 and CD8 T-cell responses [118, 119, 175-180]. Vaccine-specific immune responses were evaluated with immune assays such as ELISPOT [118, 179, 181], proliferation assay [175, 176, 179, 181] and ICS [177, 179, 181]. However, most of these studies investigated single parameters like the secretion of IFN-γ [177, 178] or proliferative responses [175, 176]. Recently, Ondondo et al. observed an increase of the production of IFN-γ and IL-2 in HIV-1 infected individuals immunized with an MVA vector expressing HIV-1 gag p24/p17 as investigated by ICS [181].

The first clinical trial demonstrating safety and immunogenicity of an MVA vectored vaccine in chronically HIV-1 infected individuals was performed in the Institute of Molecular Virology of the Helmholtz Zentrum München (formerly GSF) in 2001-2002. Ten chronically HIV infected individuals undergoing HAART were vaccinated with a modified vaccinia virus Ankara (MVA)-HIV-1_{LAI}-*nef* vector [119]. The MVA-*nef* vaccine was administered three times. No significant adverse events were observed during the course of vaccination and during one year of follow up indicating for the first time that the highly attenuated vaccinia-virus vector MVA is safe in HIV-1 infected individuals. The first line analysis demonstrated that recombinant MVA-*nef* was immunogenic in regard to CD4 T-cell, but not CD8 T-cell responses as detected by a simple IFN-γ-based ICS. CD4 T-cell responses directed to previously unidentified Nef epitopes were detected in two subjects. As already described in this work, the sole evaluation of IFN-γ provides limited information about the quality of antigen-specific T-cell responses and other important parameters.

Applying the polychromatic flow cytometry based methods, established in this work, a re-evaluation on the samples collected during the MVA-*nef* vaccination trial was performed to further investigate the T-cell responses elicited by the vaccine. As observed in this re-evaluation, the MVA-*nef* vaccine was able to increase significantly the frequencies of Nef-specific CD4 T cells expressing CD154 and IFN-γ, as well as CD4 T cells expressing CD154, IFN-γ and IL-2. In addition, Nef-specific CD4 T cells expressing CD154, IFN-γ, IL-2 and MIP-1β were induced in four out of nine subjects. As demonstrated by the MFI analysis, the

vaccine-elicited polyfunctional CD4 T cells showed increased production of IFN-γ, CD154 and IL-2 on a per-cell level whereas the quantity of produced MIP-1β was independent from the grade of functionality. These data indicate that the polyfunctional CD4 T cells secreting highest amounts of cytokines observed in the present study are similar to polyfunctional T cells that have been associated with successful immune responses reviewed by Seder et al. [152]. A correlation between the presence of antigen-specific CD4 T cells co-producing multiple cytokines and nonprogressive chronic HIV-1 infection has been shown in several cross-sectional studies [162, 182-184]. Multifunctional CD4 or CD8 T cells are furthermore proposed to represent a correlate of vaccine-mediated protection against various infectious diseases [159, 160, 185]. While initial studies characterized the expression profile of IFN-γ and IL-2 [162, 182, 183], more recent studies have expanded the investigation and analyzed more complex functional patterns [139, 184].

To compare the CD4 T-cell immune responses found in HIV infected individuals during the clinical trial to the responses in controlled human viral infections like the human cytomegalus virus (CMV) and Epstein-Barr virus (EBV) using the established methods, the polychromatic ICS was applied to a study including 23 healthy volunteers. CMV and EBV establish persistent viral infections and provide well-established models to study the role of effective host T-cell responses [186, 187]. In these models CD4 T-cell help could be shown to be important for the induction and maintenance of an effective immune response in animal models and in humans [188-191]. Especially CMV pp65 specific CD4 T-cells have been found to exhibit direct antiviral effector functions (e.g. IFN-γ, IL-2, MIP-1β, CD154, Perforin), suggesting a role in viral control beyond cognate help in this setting [191, 192].

Nef specific CD4 T-cell responses in the MVA-*nef* vaccinated study participants were compared to CD4 T-cell responses towards the CMV proteins pp65 and IE-1, and the EBV proteins LMP-1 and LMP-2 in healthy individuals using them as an example of effective T-cell mediated control of persistent viral infections. Concordant with other data, CMV pp65 specific CD4 T-cells were detected in more individuals and in higher frequencies than CMV IE-1 specific CD4 T-cells [193, 194]. Frequencies of EBV specific CD4 T-cell responses were low, but generally CMV and EBV specific immune responses in healthy individuals were more functional than Nef specific responses before MVA-*nef* vaccination in HIV infected individuals. If a polyfunctional CD4 T-cell phenotype is sufficient to explain control of CMV and EBV in healthy individuals remains to be clarified, but production of highest

amounts of several functional markers can be clearly seen as a sign of an actual good functional state.

IL-2 secretion by CD4 T cells is associated with control of HIV-1 replication in infected individuals and is related to the proliferative potential of CD4 and CD8 T cells [87, 143, 157]. HIV-1 viremia, in particular, impedes the establishment of IL-2-producing HIV-1-specific memory CD4 T cells endowed with proliferative capacity [195]. Several lines of evidence indicate that the proliferative capacity is maintained in primary and nonprogressive HIV-1 infection and impaired in chronic progressive infection [143, 154, 196]. Whether the proliferative impairment is reversible by endogenous or exogenous IL-2 [143, 155, 157] is still a matter of discussion. Whatever the case may be, the MVA-*nef* vaccine was able to increase the number of Nef-specific IL-2 producing CD4 T cells and Nef-specific CD4 and CD8 T cells capable to proliferate *ex vivo*. This indicates an improved capacity of effector CD4 and particularly CD8 T cells to expand in response to HIV-1 antigens. Along with the functional profile, the proliferative capacity of antigen-specific T cells is believed to be a key factor in maintaining or restoring effective antiviral immunity [154, 155]. In the present study, it could be verified that MVA-*nef* was able to induce specific CD4 and CD8 T cells endowed with proliferative potential. In addition, following vaccination, a significant correlation between the increase in IL-2 production by specific CD4 T cells and the increase in proliferative activity of CD8 T-cells has been observed, suggesting a causal link between the two functions.

Mono-functional CD4 T-cells secreting only IFN-γ are associated with progressive HIV-1 infection [195, 197] and indicate ongoing exhaustion also in other chronic infectious diseases like tuberculosis and HCV infection [198, 199]. A clearly decreased mono-functional fraction of the Nef specific CD4 T-cell response was observed following MVA-nef vaccination. CD4 T-cells producing anti-viral chemokines like MIP-1β offer the potential to protect virus-specific CD4 T-cells from HIV infection [200]. Higher amounts of beta-chemokines have been found in successfully treated, chronically infected patients [201]. In this study, increased numbers of MIP-1β producing CD4 T-cells were observed along with the increased total Nef specific CD4 T-cell response upon MVA-nef vaccination. The induction of CD154+ CD4 T cells is typically impaired in HIV-1 infection [202] and in progressive infection CD154-expressing CD4 T cells are selectively depleted [147]. Thus, the vaccine-induced increase of

Nef-specific CD4 T cells expressing CD154 suggests a reversion of the impaired CD154 expression and likely, an improved interaction potential between CD4 T cells and APC.

Taken together, it can be stated, that the MVA-*nef* vaccine was able to modify the Nef-specific response towards a T-cell phenotype associated with a good prognosis of HIV-1 disease progression and observed in effective immune responses towards controlled human viral infections. Nevertheless, it has not been assured if these polyfunctional and proliferative immune responses are able to provide protection against HIV-1 infection or to improve viral control in already HIV-1-infected individuals. Polyfunctional CD8 and CD4 T-cells which are usually associated with successful immune responses have been detected in healthy subjects vaccinated with a defective Adenovirus vaccine vector [203]. However, the same vaccine was ineffective in protecting against infection in a proof of concept phase IIb trial [204]. Therefore, though the MVA-*nef* vaccine was able to elicit responses normally associated with slow HIV-1 disease progression in infected individuals and with naturally controlled infectious diseases, efficacy studies will be warranted to assess eventual clinical benefits provided by the vaccine. In this regard, future immunization studies should incorporate a period of HAART discontinuation to assess whether vaccine-elicited responses are associated with improved control of viral replication.

From a methodological point of view, the re-evaluation of the therapeutic MVA-*nef* vaccination trial highlighted how the use of the polychromatic CFSE-based proliferation assay augmented the perceived immunogenicity of the MVA-*nef* vaccine. In fact, both the *ex vivo* IFN-γ-based ICS performed in a previous study [119] and the polychromatic ICS performed in the present study underestimated the vaccine-induced Nef-specific CD8 T-cell responses clearly detected by the proliferation assay. Recently, Winstone and colleagues [205] used a cultured IFN-γ ELISPOT assay to re-examine vaccine-induced T-cell responses in trial IAVI-006. The cultured IFN-γ ELISPOT assay detected 5 times more vaccine-induced responses in study participants as compared to the classical ELISPOT [82, 206]. The proliferation assay used in the re-evaluation study and the cultured IFN-γ ELISPOT assay used to evaluate the IAVI-006 trial have in common several days of incubation in the presence of the antigen, suggesting that an expansion step may be required to reveal specific responses otherwise undetectable. The polychromatic CFSE-based proliferation assay simultaneously assessing functional markers and proliferative capacity represents therefore an innovative and sensitive immunoassay, well-suited to reveal concealed effects of immunologic interventions. The

combination of the polychromatic ICS and the CFSE-based proliferation assay was particularly important to assess qualitative and quantitative aspects of the MVA-*nef*-induced, anti-Nef-specific immunity.

The re-evaluation data, alongside previous reports in which an HIV-1 gag/multiepitope immunogen was delivered by the MVA vector [181], demonstrate the capacity of MVA to improve the quality of the HIV-1-specific immune response in infected individuals. In the re-evaluation, as well as in the first-line analysis, both using short stimulation periods, more CD4 than CD8 T-cell specific to Nef were detected. Recently, it has been reported, that compared to New York vaccinia virus (NYVAC), MVA is able to stimulate in an equilibrated way both CD4 and CD8 T-cell responses in rhesus macaques primed with a DNA vaccine [207]. As a possible explanation for the fact that in this study more CD4 than CD8 T-cell specific to Nef were detected it could be suggested that the expansion of CD4 T-cells requires sustained antigen presentation in contrast to CD8 T-cells [208]. It can be hypothesized that CD4 T-cell expansion is favored in chronic infection where there is continuous presence of the antigen. In addition, induction of apoptosis in cells infected by the MVA vector followed by MHC class II restricted antigen crosspresentation might be a possible reason for the preferential elicitation of specific CD4 T cells. Nevertheless, the mechanism for the differential stimulation of CD4 and CD8 T-cell responses remains to be clarified by mechanistic studies.
The Nef specific CD4 T-cell response in a single subject V04 before the vaccination consisted solely of CD4 T-cells expressing IFN-γ and MIP-1β directed against the C-terminal part of Nef. This response was altered by MVA-*nef* and shortly after vaccine administration tetrafunctional subsets were detectable. MVA-*nef* was furthermore able to induce *de novo* tetrafunctional CD4 T cells expressing CD154, IFN-γ, IL-2 and MIP-1β directed against the N-terminal Nef. The distinct characteristics of the response to the different regions of the protein suggested that MVA-*nef* is able to elicit an immune response targeting also new epitopes but holds also the potential to elicit fully functional CD4 T-cell responses against epitopes already targeted by the host. This finding goes along with the results of a study conducted by Ondondo et al. [181]. Taken together, a general capacity of the MVA vector to induce highly functional responses against heterologous genetic inserts can be suggested. Specifically, this study demonstrates the elicitation of T-cell responses against the nonstructural, highly conserved and early expressed Nef protein encoded by the MVA vector.

6 Conclusions

After more than two decades of research, an effective preventive or therapeutic vaccine against HIV-1 remains elusive. Despite some indications, definite immunological correlates of protection against HIV infection as well as disease progression remain unknown.

T-cell mediated immunity is considered to play an important role in controlling HIV infection and progression to AIDS. Reliable and informative assays able to measure CD8 and CD4 T-cell responses need to be implemented. Polychromatic flow cytometry based immune assays established in this work, namely an ICS including a short-term ex vivo re-stimulation and a CFSE based proliferation assay including a long-term ex vivo re-stimulation offer the possibility to investigate several functional, differentiation and activation markers simultaneously. The methods were optimized, standardized and compared to other widely used immune assays. At present, the IFN-γ-based ELISPOT assay is considered as a gold standard and preferred primary assay in vaccine trials. In this work, it could be demonstrated that the use of the combined detection of IFN-γ and MIP-1β could scale-up the sensitivity of ICS assays to levels comparable to those of IFN-γ-based ELISPOT for detection of HIV specific T-cell immune responses. The application of the IFN-γ+ MIP-1β+ method in other diseases and immunological fields remains to be assessed.

Several vaccination trials are making use of MVA as delivery vector for HIV-1 derived and other antigens. The established highly sophisticated polychromatic flow cytometry based methods were applied to a re-evaluation of a therapeutic vaccination trial using MVA-*nef*. The impact of the immunologic intervention with MVA-*nef* on the specific anti-Nef T-cell immune response in regard to cytokine production (IFN-γ and IL-2), chemokine production, activation marker expression (CD154) and proliferative potential in HAART treated, HIV-1 infected individuals was assessed. By means of short-term polychromatic ICS, we observed a significant increase of polyfunctional Nef-specific CD4 T cells expressing IFN-γ, IL-2 and CD154 following vaccination, whereas changes in the quality of the CD8 T-cell response could not be observed. Only the additional use of the long-term polychromatic CFSE-based proliferation assay revealed vaccine-induced Nef-specific CD8 as well as CD4 T cells with proliferative capacity. The correlation between the vaccine-induced IL-2 production by CD4 T cells and the increase of proliferating Nef-specific CD8 T cells suggests a causal link between these two functions. In conclusion, the MVA-*nef* vaccine was able to change the quality and quantity of the Nef-specific immune response in HIV-1-infected subjects under HAART. Whether highly functional HIV-1-specific CD4 and CD8 T cells endowed with

proliferative capacity will be clinically relevant in HIV-1 infection remains to be determined. Further clinical trials designed to test the immunogenicity in combination with the therapeutic efficacy of MVA-*nef* vaccination will help to define the clinical relevance of the observed immune responses.

The insight gathered in this re-evaluation study exceeds by far the information obtained in the original study using simple IFN-γ-based immune assays. These data are important to guide the choice for suitable immune assays and to build reagent panels able to characterize accurately the phenotype and function of responding T-cells in an extensive and highly sensitive way.

The results concerning recombinant MVA expressing a single gene of HIV-1 are novel and important, and may encourage the use of pox virus derived, viral vector vaccines in the HIV-1 field as well as for other infectious diseases. This work highlights the importance of combining sophisticated immunomonitoring tools to unravel concealed effects of immunologic interventions and shall encourage further clinical trials designed to test the immunogenicity in combination with the therapeutic efficacy of MVA-nef and similar recombinant viral vector vaccines.

7 Materials and Methods

7.1 Patients and samples

7.1.1 Mva-nef vaccination trial

Inclusion criteria required participants to have CD4 counts above 400/µl and stable viral loads for at least 6 months. Patient's clinical characteristics are shown in Table 3. Ten subjects have been enrolled in the vaccination trial and they were all under therapy for 71.7 months on average. Participants were 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. Eight out of 9 participants had undetectable viral load. In subject V3, the viral load was 8710 RNA copies/ml. CD4 T-cell counts ranged from 407 to 1421 cells/mm^3 with a median of 549 [119]. Samples from prevaccination timepoints as well as samples from timepoints after the second and third vaccine administration were investigated in the reevaluation study. PBMC obtained from 9 individuals could be re-examined in the present study according to available cryopreserved banked samples. Pre-vaccination samples were not available from study subject V02 and this participant was excluded from the present evaluation. Subject V10 was analyzed only by polychromatic ICS, since not enough material was available to perform the proliferation assay.

Patients characteristics

Subject	Date of birth	Year of diagnosis	Duration of therapy (months)	Baseline findings		Smallpox vaccination
				Plasma viral load (RNA copies/ml)[a]	CD4-cell counts (cells/mm^3)[a]	
1	1959	July 1986	65	<50	407	Yes
2	1944	July 1989	75	6077	803	Yes
3	1955	July 1984	77	8710	1116	Yes
4	1955	January 1998	42	<50	1421	Yes
5	1962	June 1986	76	<50	584	Yes
6	1937	October 1993	51	<50	782	Yes
7	1949	July 1996	70	<50	473	Yes
8	1960	February 1995	73	<50	549	Yes
10	1963	August 1985	156	<50	488	No
11	1962	February 2000	32	<50	488	Yes

[a] Measured at the time of HIV-1-specific T-cell analysis at week −6.

Table 3. Patients characteristics. [119]

Patient ID	Vaccination timepoints (week)	Sample timepoints ICS (week)	Sample timepoints CFSE (week)
V01	W0 / W2 / W16	W-4 / W4 / W18	W-4 / W8 / W18
V02	W0 / W2 / W16	-	-
V03	W0 / W2 / W16	W-4 / W4 / W18	W-4 / W4 / W18
V04	W0 / W2 / W16	W-4 / W4 / W20	W-4 / W4 / W20
V05	W0 / W2 / W16	W0 / W4 / W19	W0 / W4 / W19
V06	W0 / W2 / W16	W-4 / W3 / W18	W-4 / W3 / W18
V07	W0 / W2 / W16	W-4 / W3 / W17	W-4 / W3 / W32
V08	W0 / W2 / W16	W-4 / W4 / W20	W-4 / W4 / W20
V10	W0 / W2 / W16	W-6 / W4 / W20	-
V11	W0 / W2 / W16	W-4 / W4 / W18	W-4 / W8 / W20

Table 4. Specification of exact timepoints of sampling for the re-evaluation study relative to the first administarion of MVA-*nef*.

7.1.2 IFN-γ+ MIP-1β+ data evaluation and ELISPOT comparison

275 PBMC samples obtained from 31 HIV-1 infected individuals were analyzed for the establishment of the IFN-γ+ MIP-1β+ data evaluation and its comparison to the ELISPOT. The median CD4 T-cell count was 502 cells/µl (range 229 to 1,042). Twenty-one study subjects were under antiretroviral therapy and 16 of them had undetectable viral load. When detectable the median viral load was 2,581 RNA copies/ml (range 151 to 50,577). Six of the study subjects under antiretroviral therapy with undetectable (<50 copies of RNA/ml) viral load underwent treatment interruption and their range of viremia was then from 600 to 49,600 RNA copies/ml at the time of sampling.

7.1.3 Evaluation of CMV and EBV specific immune responses in healthy volunteers

23 healthy volunteers were included in the study. Median age was 29 years (range: 25-67). The study included 10 male and 13 female participants. CMV and EBV status was unknown prior to the investigation.

7.2 Materials

7.2.1 Reagents and solutions

Reagents:

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 H_2O (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		
CFSE stock solution 25mg powder diluted in 4,48ml DMSO to get the 10mM stock solution	Carboxyfluorescein succinimidyl ester	Invitrogen	C1157
Cytofix/Cytoperm reagent kit	Cytofix/Cytoperm Perm/Wash	Becton Dickinson	554714

7.2.2 Peptides

Peptide pools:
2µg/ml peptide in a total sample volume of 200µl

HIV derived peptides:

Pool	Antigen	HIV-1 subtype	Length (aa)	Overlap (aa)	# of peptides	amount/ sample (µl)	Source
1	Nef	LAI	20	10	20	0.80	NIBSC, London, UK
2	Tat	LAI	20	10	8	0.43	NIBSC
3	Rev	LAI	20	10	11	0.48	NIBSC
4	p24	LAI	20	10	22	0.88	NIBSC
5	p17	SF2	15	5	13	0.52	NIBSC
6	Nef opt	LAI	8-11	NA	16	3.55	NIBSC
7	Nef (1-96)	Bru	variable	variable	15	6	NIBSC
8	Nef (96-205)	Bru	variable	variable	15	6	NIBSC
9	Tat	BH10	variable	variable	11	4.4	NIBSC

aa=Amino acids; NA=not applicable

(1) 20-mer peptides overlapping by 10 amino acids spanning the HIV-1 LAI Nef protein;

(2) 20-mer peptides overlapping by 10 amino acids spanning the HIV-1 LAI Tat protein;

(3) 20-mer peptides overlapping by 10 amino acids spanning the HIV-1 LAI Rev protein;

(4) 20-mer peptides overlapping by 10 amino acids spanning the HIV-1 LAI p24 protein;

(5) 15-mer peptides overlapping by 5 amino acids spanning the HIV-1 SF2 p17 protein;
(6) pool of 16 Nef derived peptides corresponding to previously described optimal CD8 epitopes [9];
(7) variable length overlapping peptides spanning the 1 to 96 region of HIV-1 Bru Nef;
(8) variable length overlapping peptides spanning the 96 to 205 region of HIV-1 Bru Nef and
(9) variable length overlapping peptides spanning HIV-1 BH10 Tat.
Pool 1 to 6 and 7 to 9 were previously described by Cosma et al [119] and Vardas et al. [209], respectively. The peptide sets were validated for use in cytokine secretion T-cell ELISPOT as well as in intracellular-cytokine staining assay within the European Commission-sponsored AIDS Vaccine Integrated project (AVIP) consortium. Peptides were obtained through the Centre for AIDS Reagents, National Institute for Biological Standards and Control, Hertfordshire, UK. Several peptides contained in the pools 7, 8 and 9 were used alone in some experiments. The following peptides corresponding to previously described optimal CD8 epitopes [9] were also used in some stimulation experiments: FLKEKGGL (FL8), TPGPGVRYPL (TL10), YPLTFGWCY and RRQDILDLWIY (RY11). All the peptide pools were tested for specificity in healthy subjects in previous studies [119, 209]

CMV and EBV derived peptides:

Pool	Origin	Antigen	Length (aa)	Overlap (aa)	# of peptides	amount/ sample (µl)	Source
1	CMV	pp65	15	11	138	4	JPT, Berlin, Germany
2	CMV	IE-1	15	11	120	4	JPT
3	EBV	LMP-1	15	11	94	4	JPT
4	EBV	LMP-2	15	11	122	4	JPT

(1) 15-mer peptides overlapping by 11 amino acids spanning the CMV pp65 protein;
(2) 15-mer peptides overlapping by 11 amino acids spanning the CMV IE-1 protein;
(3) 15-mer peptides overlapping by 11 amino acids spanning the EBV LMP-1 protein;
(4) 20-mer peptides overlapping by 10 amino acids spanning the EBV LMP-2 protein;
Peptide pools (25µg each) were diluted in 40µl DMSO and 210µl PBS.

7.2.3 Antibodies

Antibody		Company	Cat.no.
Costimulating antibodies	CD28 pure	Becton Dickinson,	340975
	CD49 pure	Becton Dickinson	340976

Compensation antibodies:

Antibody	Company	Cat.no.	(µl)
CD8-FITC	Becton Dickinson	345772	4
CD8-PE	Becton Dickinson	345773	2
CD4-PerCP	Becton Dickinson	345770	5
CD8-PacB	Biozol	DAK-PB984	2.5
CD8-APC	Becton Dickinson	345775	0.5
CD45RA-PECy7	Becton Dickinson	341111	0.7
CD3-AmCyan	Becton Dickinson	339186	1
CD3-Al700	Becton Dickinson	557943	0,5

Intracellular staining antibody mix:

for 1 sample	Company	Cat.no.	(µl)
CD3-AmCyan	Becton Dickinson	339186	1
CD8-PacB	Biozol	DAK-PB984	2.5
CD4-PerCP	Becton Dickinson	345770	5
CD154-FITC	Becton Dickinson Pharmingen	555699	5
IL2-APC	Becton Dickinson	341116	5
IFNγ-Al700	Becton Dickinson Pharmingen	557995	0,4
MIP1ß-PE	Becton Dickinson Pharmingen	550078	0,2
CD45RA-PECy7	Becton Dickinson	337186	0,7

7.2.4 Consumables

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

7.2.5 Laboratory equipment

Laboratory Equipment:

Equipment	Model/ type	manufacturer
CO2 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
Laminar flow	Hera safe ClassII Type A7B3	Haereus NuAire, Plymouth, USA
Nitrogen container	Arpege 170	Air Liquide, Düsseldorf, Germany
	Cryo 200	Thermo Scientific
Eppendorf pipettes	Pipetman P10-1000	Gilson, Middleton,USA
Multi Channel pipettes	Pipetman Ultra 20-300µl	Gilson
	SL-Pette 5-50µl	Südlabor, Gauting, Germany
„Mr. Frosty"		Nunc
Pipettus	accu-jet pro	Brand, Wertheim, Germany

7.2.6 Software

Software:

Product	**Manufacturer**
FacsDIVA	Becton Dickinson
FlowJo version 8.5.3	Treestar, Ashland, USA
GraphpadPrism 4	Graph Pad Software, San Diego, USA
MS Office	Microsoft, Redmond, USA
Spice version 4.1.5	kindly provided by Mario Roederer, NIH, USA
Pestle version 5.0.1	kindly provided by Mario Roederer

7.3 Methods

7.3.1 Purification of Peripheral Blood Mononuclear Cells (PBMC) using Ficoll density gradient

Heparinated blood was centrifuged in 50ml Falcon tubes for 10 min at 1800 rpm at 21°C. Plasma was removed from the blood and separately stored in small aliquots at -80°C. Prewarmed RPMI (room temperature) was added to refill the volume to 35ml, and mixed gently. The diluted blood was slowly layered above Ficoll (15ml, previously prepared in 50ml Falkon tubes) and centrifuged for 17 min at 2100 rpm at 21°C without brake. The lymphocyte ring was aspirated together with media and Ficoll using a 10ml shorty-pipette and transferred to a new 50ml Falcon tube. Lymphocytes were washed in 50ml, volume refilled with RPMI, and centrifuged for 13 min at 1600 rpm at 21°C. Supernatant was discarded, PBMC pellet dissolved and two additional wash steps in 25ml RPMI were performed (1600 rpm, 13', 21°C and 1300 rpm, 5', 21°C).

7.3.2 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 containing 10% DMSO in FCS. Cells were slowly cooled down (-1°C/min) in "Mr. Frosty" to -80° in a -80° freezer. The following day they were transferred to liquid N_2 for long-term storage.

7.3.3 Thawing PBMCs

PBMC stored in cryotubes in liquid N_2, were thawed at 37°C in a waterbath and subsequently washed three times with RPMI-10 prewarmed at room temperature to remove DMSO. Centrifuging steps during the washes were carried out for 5 min at1500 rpm in 21°C.

7.3.4 Intracellular cytokine staining (ICS)

After thawing in media at room temperature cryopreserved PBMC were washed twice. 10^6 PBMC were resuspended in 150 µl culture medium RPMI 1640 supplemented with 10% FCS and 1% PenStrep. Peptide-stimulated and mock-stimulated samples were run in parallel to define the background. 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 (0.2 µl/well). Following 60 min of incubation, 10 µg/ml of Brefeldin A was added to the cell suspension and the incubation carried out for additional 4 h. Stimulated cells were labelled with the photoreactive fluorescent viability marker ethidium monoazide, fixed and permeabilized using the BD Cytofix/CytopermTM Kit during 20 minutes of incubation. Cells

were stained with the following fluorochrome-conjugated antibodies: CD3-AmCyan, CD8-PacB, CD4-PerCP, CD45RA-PECy7, CD154-FITC, IFN-γ-Al700, IL-2-APC and MIP1β-PE. Samples were acquired using an LSRII flow cytometer equipped with a high throughput system. Analysis was performed using FlowJo version 8.5.3. Gating strategy is shown in Figure 9. According to the differential expression of CD45RA, CD154, IFN-γ, IL-2 and MIP1β, 30 responding CD4 and CD8 T-cell subpopulations were identified. A threshold level for each subpopulation was calculated following background subtraction, and responses lower than the respective threshold level, were set to 0. Furthermore, a general threshold of 0.005% was applied for all CD8+ and CD4+ T-cell subsets to exclude minor responses.

7.3.5 CFSE proliferation assay

After thawing, PBMC were resuspended in 0.1 μM CFSE. Cells were incubated for 10' at 37° C in the dark, rinsed in complete medium and plated at 200μl/well (10^6 cells/200μl). Peptide antigens and costimulatory antibodies were added as for the ICS, and cells were cultured for 5 days in complete medium. At day 5, cells were resuspended in 200μl culture media and restimulated applying the same procedure and conditions as for the ICS. Following EMA staining and fixation/permeabilization, cells were stained with the fluorochrome-conjugated antibodies used in the ICS protocol. The CD154-FITC antibody was omitted not to interfere with the detection of the CFSE. Acquisition and sample analysis were performed in concordance with the ICS protocol. Functional markers and ex vivo proliferative activity, defined by low CFSE staining level, were gated according to a gating strategy depicted in Figure 10. According to the differential expression of CD45RA, IFN-γ, IL-2, MIP1β and the CFSE staining level, 30 responding CD4 and CD8 T-cell subpopulations were identified. An individual threshold was calculated as described for the ICS procedure and general threshold of 0.005% for all CD8+ and CD4+ T-cell subsets was applied to exclude minor responses.

7.3.6 Tetramer staining

Thawed PBMC were adjusted to 5×10^6 cells/ml in RPMI 1640 (Cambrex), 10% FCS, 1% PenStrep (Cambrex) and incubated EMA (Molecular Probes, Leiden, The Netherlands) used as viability dye. After washing cells were stained with the Tetramer and CCR7-FITC and fixed. Then they were stained with the following antibodies: CD3-PacB (DAKO cytomation, Hamburg, Germany), CD28-PerCP-Cy5.5, CD45RA-Pe-Cy7, CD27-APC (Becton Dickinson, Heidelberg, Germany), CD8-APC-Cy7 (Caltag, Hamburg, Germany). Cells were acquired after washing with an LSRII flow cytometer (Becton Dickinson) and data were analyzed

using FlowJo software (Tree Star, San Carlos, CA). Tetramer staining experiments were kindly conducted by Rashmi Nagaraj. Tetram

7.3.7 ELISPOT assay (laboratory 1)

Laboratory 1 used the TriSpot™ Human IFN-γ/IL-2 ELISPOT Kit (Endogen, Rockford, IL/USA) according to the manufacturer instructions. Briefly, PBMC from ACD whole blood were separated on Lymphoprep™ (Axis-Shield PoC, Oslo, Norway), washed in RPMI medium (RPMI 1640, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, all from BioWhittaker Europe, Verviers, Belgium) and counted by Trypan Blue exclusion for assessing viability. After resuspension in complete medium (RPMI medium supplemented with 10% heat inactivated fetal bovine serum, BioWhittaker), PBMC were transferred to the ELISPOT plate with a concentration of 0.8 to $2x10^5$cells/well in duplicate. Peptides were added at a final concentration of 3 µg/ml each. PBMC in medium alone or stimulated with phytohemagglutinin (PHA-P, Sigma) at 5 µg/ml were used as negative and positive controls, respectively. Incubation was carried out at 37°C in a 5% CO_2 incubator for 18 hours. The resulting spots were counted using the Automated ELISA-Spot Assay Video Analysis System Eli-Scan with the software Eli.Analyse V4.2 (A.EL.VIS, Hannover, Germany). PBMC from each study subject were mock stimulated in duplicate and the mean background value subtracted from the mean of the duplicate samples. Responses were empirically scored as positive when the stimulated sample minus background value was >50 SFU per 10^6 PBMC and higher than the mean value of the negative controls plus two standard deviations. This ELISPOT assay was kindly conducted by Silvia Heltai.

7.3.8 ELISPOT assay (laboratory 2)

Frozen PBMC were thawed, washed with CTL Wash™ Supplement culture medium (Cellular Technology Ltd., Cleveland, Ohio) plus benzonase nuclease (50 U/ml; Novagen, Madison, WI), rested for 3h at 37 °C, counted and seeded at 1 to $2x10^5$ cells in triplicates on antibody precoated PVDF plates (Mabtech AB, Nacka, Sweden). The capture antibody (Mabtech) was the IFN-γ-specific clone 1-D1K. Beforehand, the plates were incubated at 37°C in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin/streptomycin (100 U/ml) and 10% human AB serum (BioWhittaker, Verviers, Belgium) to block unspecific binding. The PBMC were stimulated directly with different peptides and peptide pools (2 µg/ml), and assessed in the ELISPOT assay after 24 h of culture

in CTL Test™ medium. The development of the spots was performed as described previously (Brill2006, Frankenberger2005, Pohla2000) with the following exceptions: the plates were extensively washed first with PBS/0.05% Tween20, then with only PBS, incubated with a directly streptavidin-alkaline phosphatase (ALP) conjugated biotinylated detection antibody clone 7-B6-1 (Mabtech), washed again and a ready-to-use BCIP/NBT-plus substrate solution was used (Mabtech). Spots were counted using the AID reader system ELR03 with the software version 4.0 (AID Autoimmun Diagnostika GmbH, Strassberg, Germany). Responses were scored as positive if the test wells contained a mean number of spot-forming units (SFU) higher than the mean value plus two standard deviations in negative control wells. The ELISPOT standard operation procedure was approved in the international panel analysis of the Cancer Vaccine Consortium [150]. This ELISPOT assay was kindly conducted by Birgit Stadlbauer.

7.3.9 Data processing and statistical analysis

Spice version 4.1.5 (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health) and Prism version 5.01 (GraphPad Software Inc., San Diego, CA) were used for graphical representation and statistical analysis.
Nonparametric statistical tests were applied in all cases.
Background comparison was performed using Wilcoxon matched pairs tests. A linear regression analysis was performed to examine the correlation between assays. Fisher's exact test was used for sensitivity comparison of different ICS modalities and between ICS and ELISPOT.
For MVA-*nef* trial re-evaluation Friedman tests accounting for multiple comparisons, followed by pair-wise post-hoc Wilcoxon signed rank tests were used to assess significance of change in values between study timepoints. Spearman's correlation test was used for correlation analysis. A confidence interval of 95% was used for all statistical considerations.

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9 Curriculum Vitae

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EDUCATION

04/2007-03/2008	**Ludwig-Maximilians-Universität München,** Germany PhD studies
04/2001-11/2006	**Bayerische Julius-Maximilians-Universität Würzburg,** Germany Studies of Pharmacy; final result: 1.0; overall result: 1.85 (top 5%)
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RESEARCH & PROFESSIONAL EXPERIENCE

Research since 02/2007	**Institute of Virology, Helmholtz Zentrum München,** Germany PhD studies: **Immunmonitoring:** polychromatic flow-cytometry, T-cell analysis, intracellular cytokine staining (ICS), ELISPOT, standard methods in molecular biology and cell culture **Clinical trials:** re-evaluation of MVA-*nef* therapeutic HIV vaccination trial, design and preparation of Phase II clinical trial with MVA-*nef*

Internship	**GlaxoSmithKline & Co. KG,** Munich, Germany
11/2005-04/2006	Clinical research (Vaccine development, GCP, management and coordination of clinical trials, international investigator satisfaction survey)
Pharmaceutical practise	
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MEMBERSHIP

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AWARDS

Award for the best achievment in the final universitary exam of the Department of Chemistry and Pharmacy "Fakulätspreis", 2005, Bayerische Julius-Maximilians-Universität Würzburg

Award for the best achievement in Biology and Music "Abiturpreis", 2000, Hölderlin Gymnasium

10 List of publications

Journal publications

Kutscher S, Dembek CJ, Allgayer S, Heltai S, Stadlbauer B, Biswas P, Nozza S, Tambussi G, Bogner JR, Stellbrink HJ, et al: *The intracellular detection of MIP-1beta enhances the capacity to detect IFN-gamma mediated HIV-1-specific CD8 T-cell responses in a flow cytometric setting providing a sensitive alternative to the ELISPOT.* AIDS Res Ther 2008, 5:22.

Kutscher S, Allgayer S, Dembek CJ, Bogner JR, Protzer U, Goebel FD, Erfle V and Cosma A: *MVA-nef induces HIV-1 specific polyfunctional and proliferative T-cell responses revealed by combination of short- and long-term immune assays.*
Revision for publication in Gene Therapy

Dembek CJ, **Kutscher S**, Heltai S, Allgayer S, Biswas P, Ghezzi S, Vicenzi E, Hoffmann 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, Erfle V, Malnati M and Cosma A
HIV-1-Specific CD45RA+ CD8+ T Cells Secreting MIP-1β but Not IFN-γ Are Associated with Nonprogressive Infection.
Manuscript in preparation

Congress contributions

Kutscher, S., et al., *MVA-nef vaccination induces polyfunctional CD4 T-cells in HIV-1 infected individuals unter HAART.*
Abstracts from AIDS Vaccine 2008. Cape Town, South Africa. October 13-16, 2008. AIDS Res Hum Retroviruses, 2008. 24 Suppl 1: p. 3-162. P17-01

Kutscher, S., et al., *Development and use of a 9 color intracellular cytokine staining as alternative to the ELISPOT. Abstracts from AIDS Vaccine 2008. Cape Town, South Africa. October 13-16, 2008.* AIDS Res Hum Retroviruses, 2008. 24 Suppl 1: p. 3-162. P15-40.

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Abstracts of AIDS Vaccine 2009. Paris, France. October 19-22, 2009. Retrovirology, 2009. 6 Suppl 3: p. O1-52, P1-425. P18-04

Kutscher, S., et al., *MVA-nef vaccination induces polyfunctional CD4 T-cells and increases the proliferative capacity of CD8 T-cells in HIV-1 infected individuals under HAART*.
Abstracts of the 2nd European Congress of Immunology. September 13-16, 2009. Berlin, Germany. Eur J Immunol, 2009. 39 Suppl 1: p. S3-760.PD01/2

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