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**Use of the regulatory protein Nef for  
vaccination against HIV-1**

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## Zusammenfassung

Das WHO/UNAIDS „Global summary of the AIDS epidemic“, erschienen im Dezember 2006, schätzt, dass 39,5 Millionen Menschen mit HIV leben, 4,3 Millionen in 2006 infiziert wurden und 2,9 Millionen Menschen im gleichen Jahr an AIDS gestorben sind. In den Industrieländern werden HIV-1 infizierte Personen mit einer Kombination von antiretroviralen Medikamenten behandelt (hochaktive antiretrovirale Therapie, HAART). Die Therapie verringert die Virenlast auf ein nicht nachweisbares Niveau, ist aber nicht in der Lage das Virus zu eliminieren. Folglich ist eine lebenslange HAART Therapie erforderlich. Einige Probleme sind mit HAART in den Industrieländern, in denen die meisten HIV-1 infizierten Menschen leben verbunden: Nebenwirkungen, vorschriftsmäßige Einnahme, Virusmutationen und hohe Kosten. Diese Beobachtungen heben die Notwendigkeit für die Entwicklung eines therapeutischen Impfstoffs gegen HIV-1 hervor.

Wir führten eine Sicherheits- und Immunogenitäts-Phase I Studie durch, in der wir einen Impfstoff, basierend auf einem modifizierten Vaccinia Virus Ankara (MVA) Vektor, der das HIV-1 regulierende Protein Nef exprimiert in zehn chronisch HIV-1 infizierten Patienten unter HAART verwendet haben. Die Studienteilnehmer wurden dreimal, in Woche 0, 2 und 16 subkutan geimpft. Danach wurden alle für mindestens ein Jahr beobachtet.

Der Impfstoff erwies sich als sicher und immunogen. Eine Zunahme der Nef-spezifischen CD4 T-Zell-Antworten, die zeitlich mit der Impfung in Zusammenhang steht, wurde in der Mehrheit der Patienten festgestellt und die beobachtete Immunantwort war der ähnlich der, die in einer Kohorte von Langzeit Nicht-Progressoren (LTNP) festgestellt wurde. Entsprechend dieser Daten verbessert sich der immunologische Status der Patienten und es erhöht sich die Wahrscheinlichkeit, dass geimpfte Personen die Virusproduktion kontrollieren, ähnlich wie es bei den LTNP der Fall ist. Interessanterweise waren die Nef-spezifischen CD4 T-Zellen ein Jahr nach der Impfung noch nachweisbar und demonstrierten die Eigenschaft des Impfstoffs, eine langfristige Gedächtnisantwort auszulösen.

MVA gehört zu dem vielversprechendsten attenuierten viralen Vektorsystem für die Präsentation von Antigenen und es ist einer der vielversprechendsten Impfstoffe gegen Pocken. HIV-1 infizierte Personen sind möglicherweise immunsupprimiert und werden als gefährdet für die Nebenwirkungen der klassischen Pockenschutzimpfung betrachtet. Die MVA-nef Impfstudie lieferte eine wertvolle Möglichkeit zur Analyse der Eigenschaften von MVA, Vaccinia-spezifische Immunantworten in chronisch HIV-1 infizierten Personen unter HAART zu induzieren. Nach der Impfung mit MVA-nef beobachteten wir eine starke

spezifische humorale und zelluläre Immunantwort gegen den viralen Vektor. Von Interesse ist hier, dass Vaccinia-spezifische Antikörper in der Lage waren, das Virus zu neutralisieren. Die Antikörper waren hauptsächlich von der IgG Klasse. Die Analyse der MVA-spezifischen und der Nef-spezifischen Immunantworten ergab eine mögliche Interferenz zwischen den CD4 T-Zellen, die den viralen Vektor erkennen und den CD4 T-Zellen, die spezifisch für Nef sind. Tatsächlich hatten die Patienten mit den niedrigsten Nef-spezifischen CD4 T-Zellantworten die höchsten CD4 T-Zellantworten gegen MVA und umgekehrt. Zusätzlich zeigte der Vaccinia naive Patient Nr. 10 die stärkste CD4 Antwort gegen Nef und eine niedrige CD4 Antwort gegen MVA. Diese Beobachtungen weisen auf einen möglichen negativen Effekt der vorhandenen Immunität gegen Vaccinia Virus hin, eine starke Immunantwort gegen das heterologe Antigen auszulösen.

Die Charakterisierung der gegen MVA gerichteten Immunantwort weist auf die mögliche Verwendung von MVA als alternativen Impfstoff gegen Pocken und die Eigenschaft des MVA-nef Vektors hin, eine doppelte Impfung zu ermöglichen. Diese Daten stellen das Grundprinzip für die weitere Entwicklung und Prüfung des MVA-nef Impfstoffs zur Verfügung.

## Summary

The WHO/UNAIDS “Global summary of the AIDS epidemic” released in December 2006, estimates that 39.5 million of people are living with HIV, 4.3 million were newly infected in 2006 and 2.9 million of people died of AIDS in the same year. In developed countries, HIV-1 infected individuals are treated with a combination of antiretroviral drugs (Highly Active Antiretroviral Treatment, HAART). This treatment normally reduces the viral load to undetectable levels but is not able to eradicate the virus. Therefore, life-long administration of HAART is required. Several problems are associated with HAART: side effects, compliance, virus escape and high cost for developing countries where most HIV-1 infected individuals reside. These considerations highlight the need for a therapeutic vaccine against HIV-1.

We performed a safety and immunogenicity phase I pilot study using a vaccine based on a modified vaccinia virus Ankara (MVA) vector expressing the HIV-1 regulatory protein Nef in ten chronically HIV-1 infected individuals undergoing HAART. Study subjects were vaccinated subcutaneously three times at week 0, 2 and 16. Afterwards, all the subjects were monitored for at least one year.

The vaccine resulted to be safe and immunogenic. An increase of the Nef-specific CD4 T-cell responses temporally associated with the administration of the vaccine was observed in the majority of the individuals and the observed magnitude was similar to that observed in a cohort of long-term non-progressors (LTNP). These data suggest an improvement of the immunological status and an increased chance of the vaccinated subject to control viral replication as it is the case for the LTNP. Interestingly, one year after the administration of the vaccine CD4 T-cells specific to Nef were still detectable demonstrating the capacity of the vaccine to elicit a long term memory response.

MVA is among the most promising live viral vector system for the delivery of pathogen-derived antigens and is one of the most promising safe vaccines against smallpox. HIV-1 infected subjects are potentially immunocompromised and are considered at risk for the side effects of the classical smallpox vaccination. The MVA-nef vaccination study provided a valuable chance to analyze the capacity of MVA to elicit vaccinia specific immune responses in chronically HIV-1 infected individuals under HAART. Following vaccination with MVA-nef, we observed a strong humoral and cellular immune response specific to the viral vector. Of note, vaccinia specific antibodies were able to neutralize the virus and were mainly of the IgG class, suggesting an effective immune response. The analysis of the MVA-specific and the Nef-specific immune responses highlighted a possible interference between CD4 T-cells

recognizing the viral vector and CD4 T-cells specific to Nef. In fact, subjects with the lowest Nef-specific CD4 T cell responses had the highest CD4 T cell responses to MVA and vice versa. In addition, the vaccinia naïve study subject number 10 was able to mount the strongest CD4 response to Nef and showed a low CD4 response to MVA. These important observations highlighted a possible negative effect of the pre-existing immunity to vaccinia virus on the capacity of the MVA vector to elicit a strong immune response to the genetic insert.

The characterization of the immune response directed to MVA, highlighted the potential use of MVA as alternative vaccine against smallpox and the capacity of the MVA-nef vector to provide a double vaccination. This data provide the rationale for further development and testing of the MVA-nef vaccine.



## **Background**

### ***AIDS and HIV an historical overview***

The first cases of Acquired Immunodeficiency Syndrome (AIDS) were described in 1981 in San Francisco and New York. Physicians noted that a group of young homosexual men was dying from infections and tumors that a fully competent immune system is able to control without problems. The first report (Gottlieb et al., 1981) described four homosexual men that contracted *Pneumocystis carinii* pneumonia, extensive mucosal candidiasis and multiple viral infections. All the patients were lymphopenic, they had no lymphocyte proliferative response to soluble antigens and their responses to phytohemagglutinin were reduced. Moreover, CD4+ T-helper cells were almost absent. Patients were clearly in an immunodeficient status.

In the same year, *Pneumocystis carinii* pneumonia associated with an immunodeficient status was observed in a group of injecting drug users (Masur et al., 1981), suggesting that the pathology was not restricted to the homosexual community. This observation was extended the next year when the pathology was observed in haemophiliacs (MMWR, 1982a) and in a 20-month old child that received multiple transfusion of blood (MMWR, 1982b). It was then clear that the disease was transmitted via the sexual route or via the blood.

In August 1982, the disease was being referred to as “Acquired Immune Deficiency Syndrome” (AIDS). This name was a summary of all the knowledge about this disease at that time. In fact, “Acquired” indicated that people acquired the condition rather than inherited it, the condition was the result of a deficiency within the immune system and it was a syndrome, with several manifestations, rather than a single disease.

In 1983, Montagnier and his colleagues at the Pasteur Institute in Paris reported the discovery of a T-lymphotropic retrovirus in a patient at risk of AIDS, this was the virus that we now call human immunodeficiency virus (HIV) (Barre-Sinoussi et al., 1983). Afterwards, Gallo and his colleagues showed that the virus discovered in Paris was the etiologic agent of AIDS (Gallo et al., 1984; Popovic et al., 1984; Sarngadharan et al., 1984; Schupbach et al., 1984) and succeeded to grow it in continuous T-cell cultures enabling the development of a blood test for detecting HIV. These two works opened the way to a period of intense discovery. The HIV genome was sequenced (Wain-Hobson et al., 1985), the HIV antigenic variation was discovered, the virus was found in the brain of AIDS patients (Gabuzda et al., 1986; Gartner et al., 1986; Stoler et al., 1986), genomic sequence variation was found in viral population from the same patient (von Briesen et al., 1987), macrophages were found to be target of HIV

(Wiley et al., 1986), various mode of transmission were elucidated, all HIV's genes and proteins were defined and the HIV receptor CD4 was identified (Dalglish et al., 1984; Klatzmann et al., 1984).

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 (Fischl et al., 1987). However, the same study showed severe adverse reaction including hematologic toxic effects, nausea, myalgia, insomnia, and severe headaches (Richman et al., 1987).

In 1989, a second drug, dideoxyinosine (ddI), was made available. Both AZT and ddI targeted the viral enzyme reverse transcriptase (RT), a key enzyme responsible for the retrotranscription of the viral RNA to DNA, a process that precedes the integration of the proviral DNA in the host cell genome. AZT and ddI are nucleoside analogues and they act as chain terminators in the RT reaction, blocking the virus immediately after its entry into the cell. The use of several nucleosides analogues was approved in the following years. However, the toxicity, the lack of activity in some cell types and the susceptibility to viral resistance were already important issues that limited the use of these drugs.

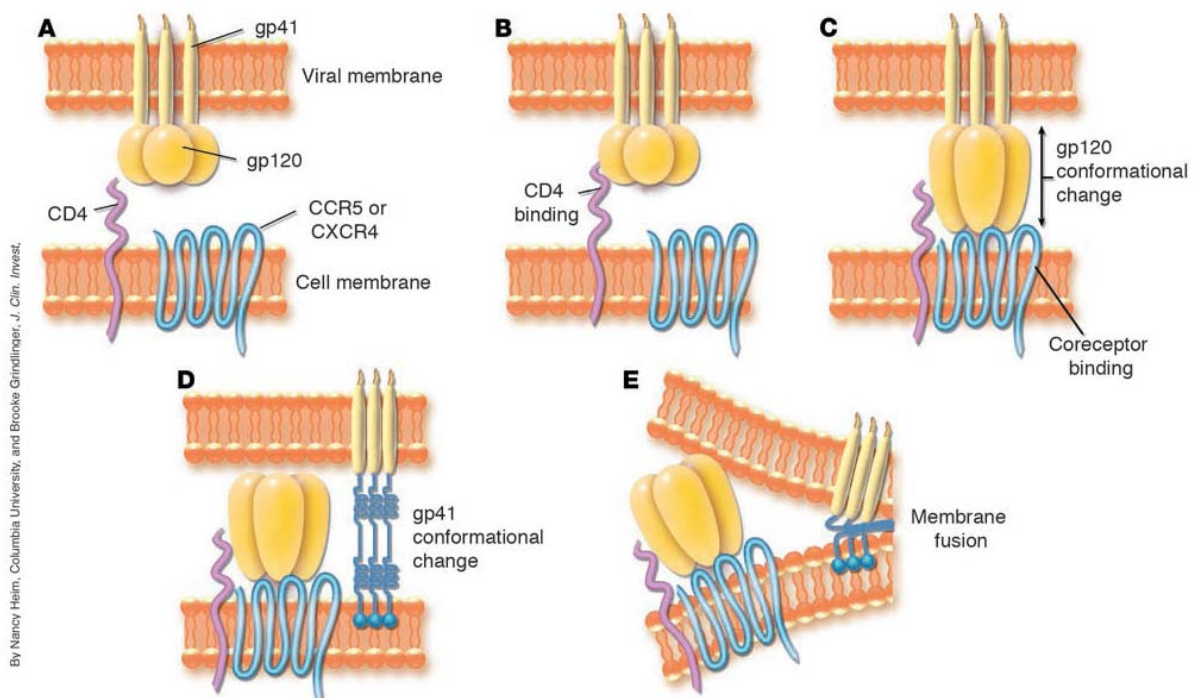
In 1995, two trials (ACT175 trial and Delta trial) demonstrated for the first time that a combination of two different nucleosides analogues was more effective than an AZT monotherapy in delaying disease progression.

Also in 1995, the FDA approved the drug saquinavir, the first member of a new class of anti-HIV drugs. Saquinavir is a HIV-specific protease inhibitor. This new class of antiretroviral drugs inhibits the viral protease that cleaves the viral precursor proteins needed for the construction of a mature virion. As consequence, infected cells release immature and non-infectious particles.

The next obvious step was the use of a combination of reverse transcriptase inhibitors together with protease inhibitors. The rationale behind this strategy was that the use of several antiviral drugs should suppress viral replication to such low level to avoid viral escape. This new strategy named highly active antiretroviral therapy (HAART) gave impressive results and in the 11<sup>th</sup> International Conference on AIDS in Vancouver (1996) numerous reports from clinical trials using HAART were presented. In 1997, the widespread use of HAART in developed countries drastically reduced AIDS related morbidity and mortality. However, in the following years it became also evident that the adverse events associated with the

administration of HAART and the capacity of the virus to escape would have been major obstacles to the complete eradication of HIV.

Between 1995 and 1996, the main HIV-1 co-receptors were identified. The discovery that the CC-chemokines RANTES, MIP-1 alpha and MIP-1 beta produced by CD8 T-cells are able to suppress HIV-1 (Cocchi et al., 1995), opened the way to the discovery of the main HIV-1 co-receptors CXCR4 and CCR5 (Lusso, 2006) and to the dissection of the entry mechanism of HIV-1. Virus entry in CD4 expressing cells is mediated by the envelope glycoprotein expressed on the surface of the virion (Berzofsky et al., 2004). The envelope protein is composed by an external subunit, the gp120, and a transmembrane subunit, the gp41 (Figure IA). These two subunits are arranged in trimers on the surface of the virus, with the three gp120 subunits forming a sort of shield protecting the three gp41 subunits. The gp120 binds first the main receptor CD4 (Figure IB) triggering a conformational change that exposes the coreceptor binding site (Figure IC). After engagement with the coreceptor (CCR5 or CXCR4), the fusion domain of the gp41 is exposed and can interact with the membrane of the target cell (Figure ID). Then, fusion between the viral and cellular membrane occurs (Figure IE).



**Figure I: Mechanism of HIV-1 entry in a target CD4 expressing cell. From Berzofsky et al., J. Clin. Invest. 2004.**

The distinctive use of CCR5 or CXCR4 coreceptors for entry into target cells, identify two phenotypic variant of HIV-1, R5 and X4 viruses, respectively. Usually, recently infected individuals harbor a R5 virus while X4 viruses predominate in the late stages of the disease.

The advent of HAART changed the public perception of HIV and AIDS. The public began to perceive HIV infection as a treatable chronic disease, and the attention dedicated to HIV and AIDS started to decrease drastically over the time. This change of perception lead also to a change of the affected population. In fact, in the United Kingdom in 1999, for the first time the number of newly diagnosed heterosexual infected with HIV was higher than the number of newly diagnosed homosexual.

In 1999, T20 an antiretroviral drug that targets the fusion process between the virus and the cell went into clinical trials and in 2002 at the Barcelona International Conference on AIDS, several groups reported on the efficacy of the new drug.

An important milestone in the fight against HIV was the conclusion of the first phase III efficacy trial of the VaxGen vaccine (McCarthy, 2003a; McCarthy, 2003b). The vaccine contained two recombinant form of the HIV-1 Envelope protein gp120 in a monomeric form. Two different trials were conducted in North America (AIDSVAX B/B) and in Thailand (AIDSVAX B/E), and in both the reduction of the HIV infection rate and progression to disease between vaccinated and placebo groups resulted to be not significant. Despite the disappointing result, this first trial finally demonstrates what had been already shown in vitro in several settings; gp120 specific antibodies plays a minor role in controlling HIV replication. However, Env variants that permit the exposure of conserved epitopes and oligomeric forms of the envelope protein that include the transmembrane protein gp41 are currently available and under study and may represent good candidate vaccine for future clinical trials (Pantophlet and Burton, 2006).

In 2006, the first “one a day” pill (Atripla) was approved for sale in the USA. It is aspected that this new pill will increase the compliance to HIV treatment and as direct consequence will decrease the emergence of escape mutants.

In the same year, a new threat emerged; several cases of extreme drug-resistant tuberculosis (XDR-TB) were recorded in South Africa. HIV infected people are more susceptible to *Mycobacterium tuberculosis* infection and to progression to active TB. As a consequence, HIV infected people with their compromised immune system represent a favourable host for the emergence of XDR-TB. High prevalence of HIV infection as been found also in individuals seeking treatment for malaria in Uganda (Kamya et al., 2006), underscoring the deadly interaction between HIV and other disease that commonly affect developing countries. On December 2006, two randomized controlled trial of male circumcision performed in Kenya and Uganda (Bailey et al., 2007; Gray et al., 2007) provided evidence of a strong association between circumcision intervention and a reduced risk of contracting HIV. In

regions with high HIV incidence and prevalence, circumcision may have a profound impact in saving life. However, large-scale implementation of circumcision will require improvement of the health care system and improvement of the communication with local authorities and community member.

### ***Natural history of HIV infection***

The detailed definition of the different stages of HIV infection in humans is an important field of research to highlight potential vulnerabilities of the virus. For obvious reasons the majority of the direct information regarding the different stages of the infection has been generated on the SIV model while a series of indirect observations in humans confirm the model of infection designed in non-human primates.

The dominant mode of transmission of HIV and SIV is through heterosexual intercourse and the first contact between the virus and the host occurs at the mucosal barrier (Haase, 2005). After crossing the mucosal barrier, the virus infects the resting memory CCR5+ CD4+ T-cells in the lamina propria. These are the first cells to be infected by HIV, probably because they are the more abundant cells that reside in the lamina propria. Although the memory CCR5+ CD4+ cells infected by HIV in the lamina propria have a typical resting phenotype, they are able to support viral replication and the virus is then able to infect activated CD4+ T-cell, macrophages and dendritic cells that reside as well in the lamina propria, but are less numerous than resting CD4+ T-cells. The observation of resting CD4+ T-cell in the lamina propria supporting SIV and HIV replication in vivo (Zhang et al., 1999; Zhang et al., 2004) contrasts with the data obtained in vitro, where lentiviral replication and T-cell activation are strictly connected. This contradiction might be related to the methods used to define the phenotype of the resting CD4 T-cells. That is probably these cells are not really resting.

After 4 to 6 days, virions disseminate from the small founding area in the lamina propria to the draining lymph node and afterwards establish infection in the peripheral lymph node, the spleen and the gut associated lymphoid tissue (GALT). All these organs contain highly packed target cells, a condition that favors viral replication, and between day 10 and 14 after exposure virus production reaches the highest level of any stage of the infection. Virus replication in the GALT plays a central role in HIV-1 and SIV pathogenesis. GALT contains nearly half the human body's total T-cells and the majority of the GALT CD4 T-cell expresses the CCR5 co-receptor. This distribution may account for the selection of CCR5 using viruses (R5 viruses) early in the course of infection. In the GALT, large numbers of memory CCR5+ CD4+ T-cells are killed by direct virus infection in few days after infection

(Li et al., 2005; Mattapallil et al., 2005). The degree of destruction of the CD4 memory compartment during acute infection predicts survival in monkey infected with SIV (Mattapallil et al., 2006). CCR5 using viruses are then maintained during all the chronic phase of infection and a shift towards CXCR4 using viruses is seen only during the progression to AIDS.

By two to four weeks after infection, the amount of virus in the host starts to drop down. Two contemporary acting mechanisms can explain the decline that follows the peak of viral load during the acute infection. First, the exhaustion of the resting CD4 T-cell killed by direct virus cytopathic effect and by virus induced apoptosis decreases the availability of target cells. Second, the immune system develops a strong HIV / SIV specific immune response able to counteract the viral production of the activated CD4 T-cells. Depletion studies performed in monkeys have nicely demonstrated that CD8 T-cells play a crucial role in controlling SHIV replication in primary infection (Matano et al., 1998).

After the acute phase of the infection, the viral load decreases to a lower level that is characteristic for each individual and is called “viral set point”. This is the typical chronic phase of the disease, characterized by the absence of symptom, detectable viral load (>50 copies RNA/ml) and a slow decline in the total CD4 counts. CD8 T-cells play an important role also in controlling the chronic phase of the infection as demonstrated by depletion studies in the SIV / *Macaca mulatta* model (Jin et al., 1999) and contribute to stabilize the viral load at the viral set point in the absence of antiretroviral therapy. During the acute infection and the chronic phase of the disease, we assist to the formation of the viral reservoirs that will be maintained throughout the life of the host. HIV and SIV reservoir include the follicular dendritic cell network, macrophages and resting memory CD4 T-cells (Noe et al., 2005).

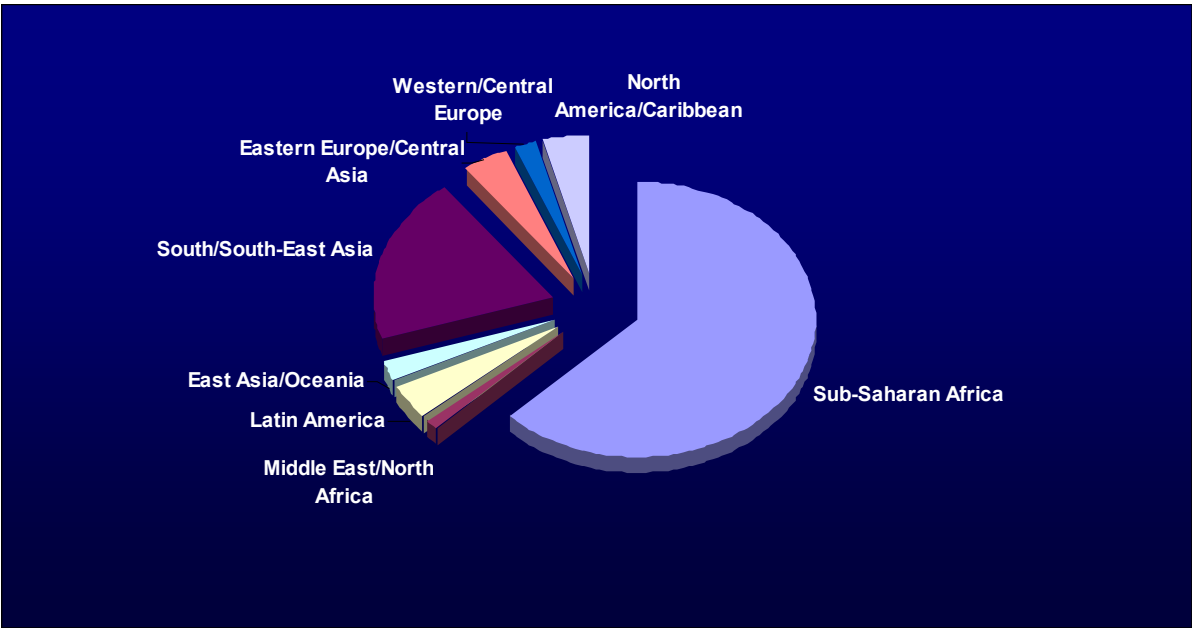
In the final stage of the disease, we assist to a rapid decline of the total CD4 T-cells and to the rise of the viral load. At this time, patients are in a clear immunodeficient status and opportunistic infections begin to occur when the CD4 count falls below 200 cells/ $\mu$ l. From a virologic point of view, the terminal phase of the HIV infection is characterized by a phenotypic shift from the R5 to the more pathogenic X4 virus. X4 viruses dominate the late stages of the HIV-1 disease and are responsible of dramatic damages to the immune system via their capacity to infect and destroy naïve CD4 T-cells (Moore et al., 2004).

In this scenario, the administration of antiretroviral drugs changes the natural course of HIV-1 infection and prolongs the chronic asymptomatic phase of the disease. After administration of HAART, the viral load falls below 50 RNA copies/ml, CD4 count remain stable and the probability to develop AIDS is strongly reduced. Despite the strong reduction in plasma viral

load, several studies theoretically demonstrated that eradication of HIV-1 might be achieved only after more than 60 years of continuous antiretroviral treatment (Pierson et al., 2000). The main obstacle to achieve eradication in a realistic time frame is the presence of viral reservoirs that form a sort of protected archive for the proviral DNA (Noe et al., 2005). Since proviral DNA may persist inside the cell without expression of viral protein, this reservoir cannot be targeted with the actual technology. Therefore, eradication of HIV remains an unrealistic scenario.

### **Vaccination**

The WHO/UNAIDS “Global summary of the AIDS epidemic” released in December 2006, estimates that 39.5 million of people are living with HIV, 4.3 million were newly infected in 2006 and 2.9 million of people died of AIDS in the same year. Twenty-five million HIV infected people live in Sub-Saharan Africa (Figure II) where only 1 million of people were receiving antiretroviral treatment by June 2006.



**Figure II: Global prevalence of people living with HIV in 2006.**

Despite the efforts made to increase the access to an effective treatment and to develop prevention programmes the number of people infected by HIV continues to grow. In many countries, HIV incidence increased in young adult between 15 and 24 year of age. In Sub-Saharan Africa, HIV epidemic affect more women than men. In South Africa 17% of young women (15-24 years) are infected by HIV, while only 4.4% of young men are infected (data from the year 2005).

These data highlights how important will be a vaccine against HIV, mainly in Sub-Saharan Africa. A prophylactic vaccine will certainly help in preventing the spread of the virus. On the other hand, a therapeutic vaccine is also urgently needed for the 40 million of people already infected by HIV. The aim of a therapeutic vaccine is to enhance pre-existing immune responses to such extent that a chronic pathogen can be kept under control or in the best scenario completely cleared (Autran et al., 2004). The main difference between a prophylactic and a therapeutic vaccine rely in the fact that a prophylactic vaccine has to face to a low amount of pathogen confined to a small area while a therapeutic vaccine has to face with large amount of pathogen and a generalized infection.

The final aim of the HIV vaccine research is to obtain a prophylactic vaccine able to prevent infection and a therapeutic vaccine able to eliminate the virus already present in the host. However, several line of evidence indicates that such vaccines will not be available in a short time frame and a more realistic goal will be to set up partially effective vaccines.

The aim of a partially effective prophylactic vaccine will be to limit the damage of the acute infection and delay the use of antiretroviral treatments. A partially effective prophylactic vaccine should reduce the amount of virus produced during the first weeks of infection; limiting the destruction of the memory CD4 T-cells in the gut associated lymphoid tissue. During the chronic phase of infection, the new vaccine should be able to lower the viral set point and delay the use of antiretroviral treatment.

The aim of a partially effective therapeutic vaccine will be to maximize the time without antiretroviral treatment and provide a valuable alternative for all the people that do not have access to antiretroviral treatments. Actually, a therapeutic vaccine has to face to two different scenarios:

- in a typical developed country where there is access to antiretroviral treatment for all the person that are in need and where there is the tendency to treat infected people as soon as possible, the vaccine will be administrated during treatment. Patients will have low or undetectable viral load and after vaccination they will have the option to interrupt treatment.
- in developing countries, as South Africa, the vaccine will be administrated mainly to untreated persons and probably the viral load and CD4 count of this people will be unknown. The vaccine will have to face a scenario of active viral replication.

For this reasons it will be necessary to develop two different therapeutic vaccines each one adapted to the situation in the field.



Several strategies of prophylactic and therapeutic vaccination that include the use of recombinant envelope proteins, synthetic peptides, virus like particles, whole inactivated viruses, plasmid DNA, recombinant orthopoxviruses and antigen pulsed dendritic cells have been already tested in humans (Egan, 2004; McMichael, 2006).

### **Recombinant envelope proteins**

Since, antibodies able to neutralize HIV-1 *in vitro* were exclusively directed against the viral Envelope, initial efforts to identify a vaccine were directed toward the development of a vaccine based on recombinant Envelope proteins. Successive findings that antibodies elicited by recombinant Envelope were able to neutralize laboratory strain of HIV but not primary isolates grown on PBMC, tempered the initial optimism. Nevertheless, studies on recombinant Envelope proteins underwent phase I, II, and III trials. The VaxGen vaccines AIDSVAX B/B and AIDSVAX B/E were the first vaccines against HIV-1 to be tested in two large phase III trials in USA and Thailand, respectively (McCarthy, 2003a; McCarthy, 2003b) (see also “AIDS and HIV an historical overview”). In the trial performed in North America, 5009 individuals were enrolled and in the three years study 5.8% of the placebo-receiving group and 5.7% of the vaccinated people became HIV-1 infected. The difference was not significant. The trial in Thailand confirmed the results obtained in North America. The results of these phase III trials were not unexpected and reflected the inability of the recombinant Envelope to elicit antibodies able to neutralize primary isolates *in vitro*.

Despite this debacle, several efforts to improve the formulation of Envelope based vaccines are under way. These include, trimeric forms, Envelopes with deletions in the variable regions (Erflé et al., 2005), and complexes between the Envelope protein and CD4, CCR5, and antibody. All this variants may express hidden determinants with a key role in virus neutralization. Hidden determinants may be expressed also in the context of the virion that carries several cell derived membrane proteins. This issue will be discussed later (see “Vaccines to cellular proteins”)

### **DNA vaccines**

DNA vaccines appeared ten years ago showing major advantages when compared to attenuated pathogens and live viral vectors. Attenuated pathogens induced primarily humoral immune responses and live viral vectors despite being able to induce a strong cytotoxic immune response, raised concerns about their safety in humans. In murine models, DNA vaccination was originally shown to induce strong and protective CD8 T-cell responses

(Ulmer et al., 1993). Unfortunately, when transferred to non-human primates and humans, DNA vaccination protocols failed in inducing optimal cellular and humoral immune responses and nowadays the main challenge is how to increase the potency of this vaccination strategy (Kutzler and Weiner, 2004). In a recent phase 1 safety and immunogenicity study (Graham et al., 2006), a DNA vaccine composed of 4 plasmids encoding Env glycoprotein from clades A, B, and C and an HIV-1 Gag-Pol-Nef fusion protein, induced humoral and cellular immune responses in the majority of the vaccinated volunteers (Table I). However, the detected responses were low in magnitudes and were directed only to the Env constructs. With the aim to improve the immunogenicity of the Gag, Pol, and Nef antigens, a new product was developed to express the genes on three different plasmids. The new vaccine formulation, composed by six plasmids encoding Env A, Env B, Env C, Gag B, Pol B, and Nef B, was tested in a small phase I evaluation trial and resulted in a better immunogenicity of the Gag, Pol, and Nef antigens (Catanzaro et al., 2007). Nevertheless, the magnitude of the immune response remained low. An overview of these clinical trials is shown in Table I. These studies also demonstrated how important could be the phenomenon of immune interference in the design of new vaccines. In fact, the insertion of several antigens in the same vaccine could be detrimental for the immunogenicity of the entire vaccine. As shown in Table I, immune interference is not an issue solely related to DNA vaccines. In fact, also a rAd5 vaccine developed by the Vaccine Research Center at the NIH to be combined to the DNA vaccine and expressing the fusion protein Gag-Pol resulted to be poorly immunogenic. Thus, single component vaccines needs to be evaluated more in deep before the construction of multi-antigen vaccines.

**Table I: DNA and Ad5 vaccination in humans (Vaccine Research Center, NIH)**

| Delivery   | Administration | Quantity<br>(n of subjects) | Antigens          | T-cells | Antibody | Endpoint            |
|--|----------------|-----------------------------|-------------------|---------|----------|---------------------|
| <b>Phase I dose-escalation study of a multiclade HIV-1 DNA vaccine (Graham, JID, 2006)</b>         |                |                             |                   |         |          |                     |
| needle-free<br>intramuscular   | w 0, 4, 8      | pl(10), 2mg(5)              | Env A, B, C       | Low     | Low      | Safe<br>Immunogenic |
|  |                | 4mg(20), 8mg(15)            | Gag-Pol-Nef (B)   | No      | No       |                     |
| <b>Phase I evaluation of a six plasmid multiclade HIV-1 DNA vaccine (Catanzaro, Vaccine, 2007)</b> |                |                             |                   |         |          |                     |
| needle-free<br>intramuscular   | w 0, 4, 8      | 4mg(14)                     | Env A, B, C       | Low     | Low      | Safe<br>Immunogenic |
|  |                |                             | Gag, Pol, Nef (B) | Low     | Low      |                     |

Phase I dose-escalation study of a multiclade HIV-1 rAd5 vaccine (Catanzaro, JID, 2006)

|               |     |  |             |     |     |                     |
|---------------|-----|--|-------------|-----|-----|---------------------|
| intramuscular | w 0 | pl(6), 10 <sup>9</sup> (10)                  | Env A, B, C | Low | Low | Safe<br>Immunogenic |
|               |     | 10 <sup>10</sup> (10), 10 <sup>11</sup> (10) | Gag-Pol (B) | No  | No  |                     |

To date, sparse data on therapeutic vaccination using DNA are available. The first human trial using DNA as therapeutic vaccine used DNA env and rev in therapy naïve subjects with CD4 count > 500. The vaccine resulted to be safe but poorly immunogenic (MacGregor et al., 1998). Successive studies in HIV-1 infected subjects undergoing HAART, demonstrated a better immunogenicity, however responses were never strong and generalized (Hejdeman et al., 2004). Recently, a DNA vaccine expressing HIV-1 gag p24/p17 and a string of CTL epitopes (pTHr.HIVA<sup>®</sup>) (Dorrell, 2005; Dorrell et al., 2006) tested on ten patients under HAART resulted to be poorly immunogenic. These studies demonstrated that currently used DNA vaccination strategies are unable to boost virus specific responses in HIV-1 infected individuals.

Several approaches are underway to enhance the immunogenicity of DNA vaccines. These include co-administration of cytokines genes, altering codon bias of the encoded gene, changing the cellular localization of the expressed antigen and using different adjuvants. In addition, one interesting option to increase the immunogenicity of DNA vaccines is to boost the responses with a subsequent vaccination performed with recombinant proteins or recombinant viruses, such as orthopoxviruses.

The co-administration of SIVmac239 Gag and HIV-1 89.6P Env together with the IL2 gene produced interesting results in rhesus monkeys challenged with a pathogenic SHIV-89.6P (Barouch et al., 2000). After challenge, control animals developed high plasma viral RNA levels, depletion of CD4 T lymphocytes and progression to disease. In contrast, seven out of eight vaccinated animals had no evidence of disease progression for more than two years after challenge. All the vaccinated animals showed a strong virus-specific CTL response and the lack of viral control in one animal correlated with escape from CTL recognition (Barouch et al., 2002). This DNA cytokine augmented set up constitute a valid example of improvement of a DNA based vaccine.

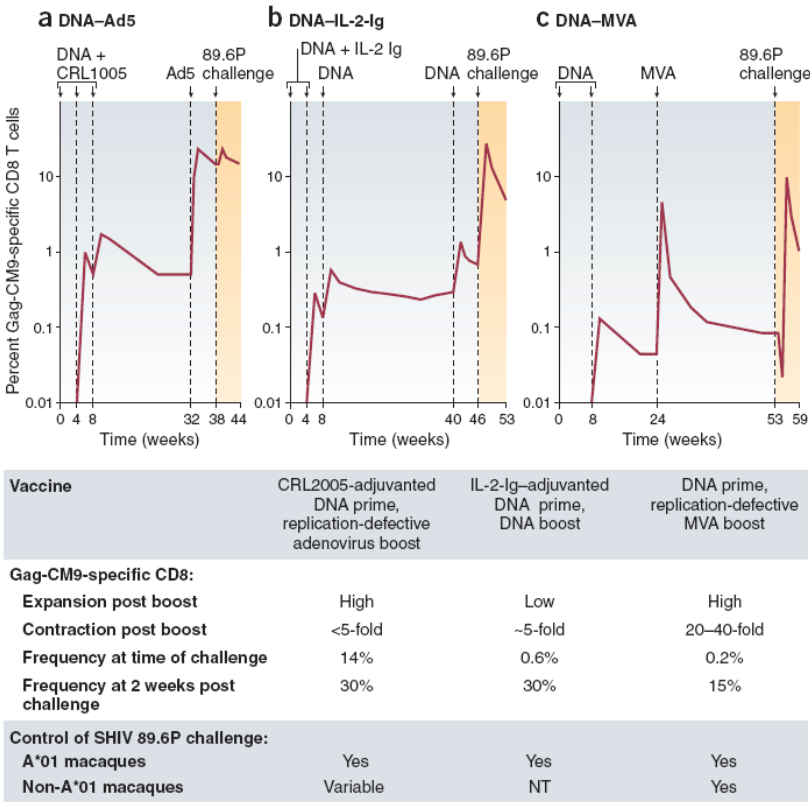
### Viral vectors

Several viral vectors have been used to develop vaccines against HIV. These include, mammalian pox-virus (MVA, NYVAC), Avian pox-virus (ALVAC, Fowlpox), Adenovirus (Ad5), Alphavirus, Rhabdovirus (vesicular stomatitis virus), Herpesvirus, and Picornavirus

(Robinson, 2002). The viral vectors that have had the most preclinical success and are now in a more advanced stage of clinical development are modified vaccinia Ankara (MVA) and adenovirus 5 (Ad5). Both viral vectors do not replicate in primate cells and represent ideal vectors to be used in immunocompromised populations. Thus, in developed countries, they can be used as therapeutic vaccines and in developing countries, they can be used to vaccinate people with an unknown HIV status. One potential problem in applying these vectors to humans is that their effectiveness can be limited by pre-existing host immunity. In fact, 90% of people in developing countries have pre-existing immunity to Ad5 and all people born before 1970 have been immunized against smallpox using vaccinia virus. Since variola virus the etiologic agent of smallpox is now eradicated and smallpox vaccination will not be necessary in the future, the problem of host pre-existing immunity to MVA will probably disappear along the time.

MVA and Ad5 vectors have been often used in a prime-boost strategy in order to boost immune responses previously elicited by DNA vaccines. Preclinical trials in monkey models performed with recombinant MVA and recombinant Ad5 had promising results. T-cell responses rose by prime-boost strategies using DNA and MVA or Ad5 vectors successfully controlled pathogenic challenges with the SHIV 89.6P chimera (see Table II). Strong virus specific CD8 responses were detected after the administration of the vaccine; with the DNA/Ad5 prime-boost combination being the more effective and the MVA/MVA combination being the least effective (see Table II and Figure III). Despite major differences in the immunogenicity, all the vaccination strategies shown in Table II and Figure III, were effective in reducing the viral set point after SHIV-89.6P challenge. The challenge resulted in a reduced viral set point in comparison to not vaccinated monkey even when live viral vectors were used alone and demonstrated to elicit low immune responses. Thus, measured immune responses did not predict the outcome of the challenge. In these trials, immune responses were monitored by following the expression of IFN- $\gamma$  after antigenic stimulation or by tracking the epitope specific T-cells with MHC multimer technology. Monkeys were usually typed for the *Mamu-A\*01* MHC allele and immune responses were tracked using Gag-CM9 MHC multimer. Despite the lack of correlation between measured immune responses and viral control, DNA/IL2 vaccinated monkey lost viral control after escape from specific CD8 responses (Barouch et al., 2002) and DNA/MVA vaccinated monkeys lost viral control after depletion of CD8 T-cells (Robinson and Amara, 2005). Thus, while control of viral replication after challenge correlates with the presence of vaccine elicited specific CD8 T-cells, the capacity to mount such protective response does not correlate with the magnitude of

vaccine-elicited CD8 T-cells. Trials including the experimental depletion of T-cell subpopulations after vaccine administration but before challenge should be undertaken to discover correlates for the efficacy of the vaccines.



**Figure III: Patterns of CD8 T-cells specific to the immunodominant Gag-CM9 epitope during vaccination and challenge for a DNA/Ad5 vaccine (a) (Shiver et al., 2002), a cytokine augmented DNA vaccine (b) (Barouch et al., 2000), and a DNA/MVA vaccine (c) (Amara et al., 2001). From H.L. Robinson and R.R. Amara, Nat Med 2005.**

**Table II: Prime-boost studies in the Rhesus macaque model challenged with SHIV-89.6P**

| Model  | Delivery | Administration       | Antigens                          | T-cells  | Antibody    | Challenge | Outcome              |                               |
|--|----------|----------------------|-----------------------------------|--|-------------|-----------|----------------------|-------------------------------|
| <b>Control of a mucosal challenge by a multiprotein DNA/MVA vaccine (Amara, Science 2001)</b>                                    |          |                      |                                   |  |             |           |                      |                               |
| Rhesus macaque   | Prime▶   | id, im (needle free) | w 0, 8 (DNA)                      | SIVmac239(Gag, Pol, Vif, Vpx Vpr) HIV89.6(Env, Tat, Rev) | Low         | Low       | m 7, intrarectal,    | Reduced viral set point       |
|  | Boost▶   | id, im (needle)      | w 24 (MVA 2x10 <sup>8</sup> )     | SIVmac239(Gag, Pol) HIV 89.6(Env)                        | Strong      | Low       | SHIV-89.6P           |                               |
| <b>Different patterns of immune responses but similar control of SHIV 89.6 by MVA and DNA/MVA vaccines (Amara, J Virol 2002)</b> |          |                      |                                   |  |             |           |                      |                               |
| Rhesus macaque   | Prime▶   | id                   | w 0, 8 (MVA 2x10 <sup>8</sup> )   | SIVmac239(Gag, Pol) HIV 89.6(Env)                        | Low         | High      | m 7, intrarectal,    | Reduced viral set point       |
|  | Boost▶   | id, im               | w 24 (MVA 2x10 <sup>8</sup> )     |  | Low         | High      | SHIV-89.6P           |                               |
| <b>Reduction of a SHIV 89.6P Viremia in Rhesus Monkey by rMVA vaccination (Barouch, J Virol 2001)</b>                            |          |                      |                                   |  |             |           |                      |                               |
| Rhesus monkey  |          | im                   | w 0, 4, 21 (MVA 10 <sup>8</sup> ) | SIVmac239(Gag, Pol) HIV 89.6(Env)                        | Low         | No        | w 27, iv, SHIV-89.6P | Reduced viral set point       |
| <b>Replication incompetent Ad5 elicit effective anti-immunodeficiency virus immunity (Shiver, Nature 2002)</b>                   |          |                      |                                   |  |             |           |                      |                               |
| Rhesus macaque monkey  | Prime▶   | im                   | w 0, 4, 8 (DNA)                   | SIVmac239(Gag)   | Low         | No        | w 12, iv, SHIV-89.6P | Reduced viral set point (3/3) |
|  | Boost▶   |                      | W 25 (DNA)                        |  | Medium      | No        |                      |                               |
|  | Prime▶   |                      | w 0, 6 (MVA)                      |  | Low         | No        |                      |                               |
|  | Boost▶   |                      | w 32 (MVA)                        |  | Low         | No        |                      | Reduced viral set point (2/3) |
|  | Prime▶   |                      | w 0, 6 (Ad5)                      |  | Medium      | No        |                      | Reduced viral set point (3/3) |
|  | Boost▶   |                      | w 32 (Ad5)                        |  | Strong      | No        |                      |                               |
|  | Prime▶   |                      | w 0, 4, 8 (DNA)                   |  | Medium      | No        |                      | Reduced viral set point (1/3) |
|  | Boost▶   |                      | w 32 (MVA)                        |  | Strong      | No        |                      |                               |
|  | Prime▶   |                      | w 0, 4, 8 (DNA)                   |  | Low         | No        |                      | Reduced viral set point (3/3) |
|  | Boost▶   |                      | w 32 (Ad5)                        |  | Very Strong | No        |                      |                               |

The relevance of the animal and challenge model used in these studies is not firmly established. The SHIV-89.6P chimera expresses a CXCR4 specific HIV envelope and after infection causes a dramatic decrease of total CD4 T-cell. AIDS develops in almost all the infected animals within six months from infection. This aggressive virus does not resemble HIV-1 that soon after infection establishes a long phase of latency that last for years before the first manifestations of AIDS related symptoms. Intriguingly, this aggressive virus seems easier to control by vaccination and studies conducted with the less aggressive CCR5 dependent viruses SIVmac239 and SIVmac251 resulted in a modest reduction of the viral set point (Aandahl et al., 2003; Horton et al., 2002; Vogel et al., 2003). In addition, recent studies addressing the influence of MHC class I haplotype on vaccine mediated protection, demonstrated that the expression of the MHC class I allele *Mamu-A\*01* was associated with a particularly efficient control of the SHIV-89.6P (Seaman et al., 2005). The same has been shown for vaccination and challenge studies using the less virulent SIVmac.

To overcome these problems Letvin et al. (Letvin et al., 2006) immunized monkeys that do not expressed *Mamu-A\*01* alleles and performed a challenge using the less virulent SIVmac strain. In addition, after challenge, monkeys were followed for more than three years to evaluate the long-term effect of the vaccine. DNA/Ad5 vaccinated monkeys showed only a limited reduction of the viral set point after challenge but the long-term follow up revealed significant difference in survival associated with the preservation of central memory CD4 T-cells. This study constitutes the base for further studies in monkeys and suggests a possible correlate of protection that can be analyzed in humans.

In summary, two main problems affect studies performed in monkeys: the relevance of the animal, and challenge model and the absence of a correlate of protection. In addition, it is not evident that these issues will be solved in the recent future. Therefore, it will be important to translate as soon as possible this vaccination protocols in clinical trials in order to establish their relevance for human beings.

MVA and Ad5 carrying different HIV-1 antigens have been already used in phase I and II clinical trials. Both vectors were safe and able to elicit immune responses to the inserted antigens. However, since efficacy studies in healthy subject require a large number of individuals and a long term follow up, at present time, no data exist on the efficacy of this vector as prophylactic vaccines. ALVAC vCP1521 vector is now in a more advanced stage of development. In October 2003, Sanofi Pasteur started a large phase III clinical trial to evaluate the efficacy of a prime/boost vaccine strategy employing an ALVAC vCP1521 (env

B, E; gag/pol) and the AIDSVAX B/E (protein env B, E). The placebo controlled double-blinded study will enroll 16,000 volunteers in Thailand and will end by June 2009.

MVA, Ad5 and ALVAC have been used in therapeutic vaccine settings. The therapeutic setting can provide information on efficacy even with a limited number of study subjects. However, despite safety and immunogenicity have been demonstrated, no indication of efficacy has been reported to date.

### **Vaccination using MVA**

The interesting data obtained in the monkey model fostered the use of MVA as vaccine vector in humans. MVA does not replicate in most mammalian cells and since the block in the replication cycle is at a late stage of morphogenesis, early and late gene expression is not impaired. This allows for a good expression of the inserted genes and for good safety characteristics.

The first report of a Phase I clinical trial using a recombinant MVA expressing an HIV-1 derived antigen is the main subject of this thesis (Cosma et al., 2003). Briefly, the vaccination of 10 chronically HIV-1 infected subjects with a MVA-HIV-1<sub>LAI</sub>-nef vector resulted to be safe and immunogenic. Interestingly, elicited immune responses were mainly mediated by CD4 T-cells. This study provided also valuable information upon the use of MVA as an alternative vaccine against smallpox in HIV-1 infected patients under HAART (Cosma et al., 2007).

Following this first report in HIV-1 infected individuals, the group of T. Hanke and A. J. McMichael demonstrated the capacity of MVA to induce HIV-1 specific responses in healthy subjects (Mwau et al., 2004). In this case, the MVA vector was expressing a consensus HIV-1 clade A Gag p24/p17 proteins fused to a string of clade A CTL epitopes (Hanke et al., 2002). These pioneering studies were followed by other clinical trial that showed the good immunogenicity of MVA as a vaccine against HIV-1 (Dorrell et al., 2006; Goonetilleke et al., 2006; Harrer et al., 2005).

### **Vaccines to cellular proteins**

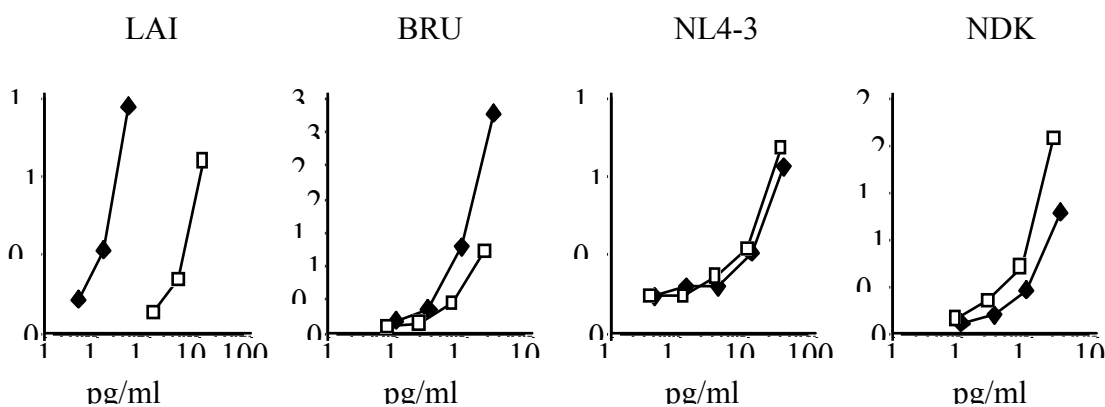
In 1995, Stott et al. (Stott and Almond, 1995) showed that monkeys vaccinated with inactivated SIV grown on human cells and challenged with the same virus were successful protected. When the challenge was performed with SIV grown on monkeys' cells no protection was observed. At that time, it was not clear if protection was mediated by allo-specific or xeno-specific humoral immune response. However, it was clear that cellular



molecules incorporated into virions played a role in the protection mechanism. Since, this vaccination strategy succeeded in achieving full protection, further investigations should be undertaken now that new technologies to dissect immune responses are available.

Host derived proteins are selectively incorporated into viral particles (Esser et al., 2001) and can confer new functions and new antigenic properties to the virus. For instance, ICAM-1, HLA class II and CD28 molecule accelerate the kinetic of viral entry by interacting with their corresponding counter-receptors displayed on the target cells (Giguere et al., 2005; Tremblay et al., 1998). The analysis of these molecules might be useful in the design of vaccines that can better mimic the envelope of the virus or can target important step in the virus life cycle.

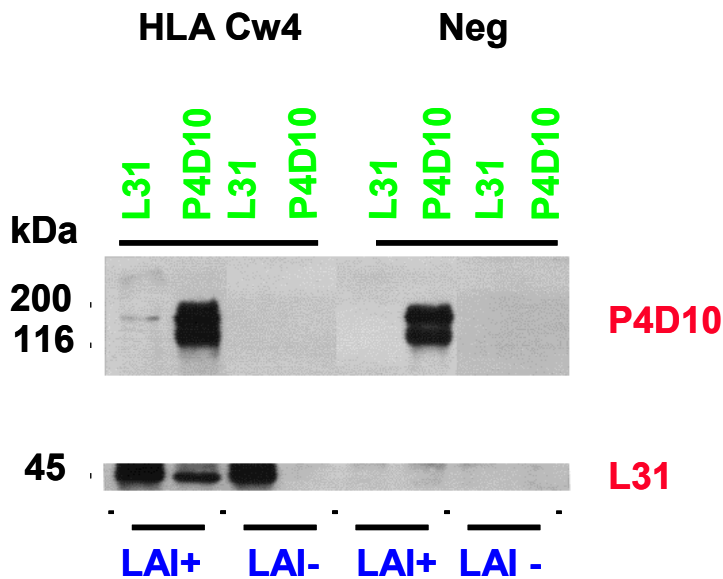
In 1999, we characterized the effect of the incorporation of HLA class I molecules into viral particles (Cosma et al., 1999). The study addressed in particular the role of the incorporation of the HLA Cw4 allele into CXCR4 dependent viruses. The incorporation of HLA-class I Cw4 alleles increased the infectivity of newly formed viral particle changing the conformation of the Envelope protein. As shown in Figure IV, the incorporation of HLA Cw4 into LAI and Bru virions increased the capacity of these viruses in entering an indicator CD4+ cell line. Similar results were observed upon incorporation of HLA Cw4 into primary isolates. The incorporation of HLA Cw4 had no effect on the NL4-3 and NDK viral strain.



**Figure IV: Effects of HLA Cw4 expression on infectivity.** Viruses generated from HLA Cw4 negative (open symbols) and positive (closed symbol) cells collected at the peak of infection were added to Hela-CD4-LTR-LacZ P4 indicator cells and efficiency of infection was measured by colorimetric determination of  $\beta$ -gal activity in cell extracts. pg/ml of virus added to assay are indicated on the x axis.  $\beta$ -gal activity in term of optical density is shown in the y axis. The different strain of HIV tested are shown on the top of each graph.

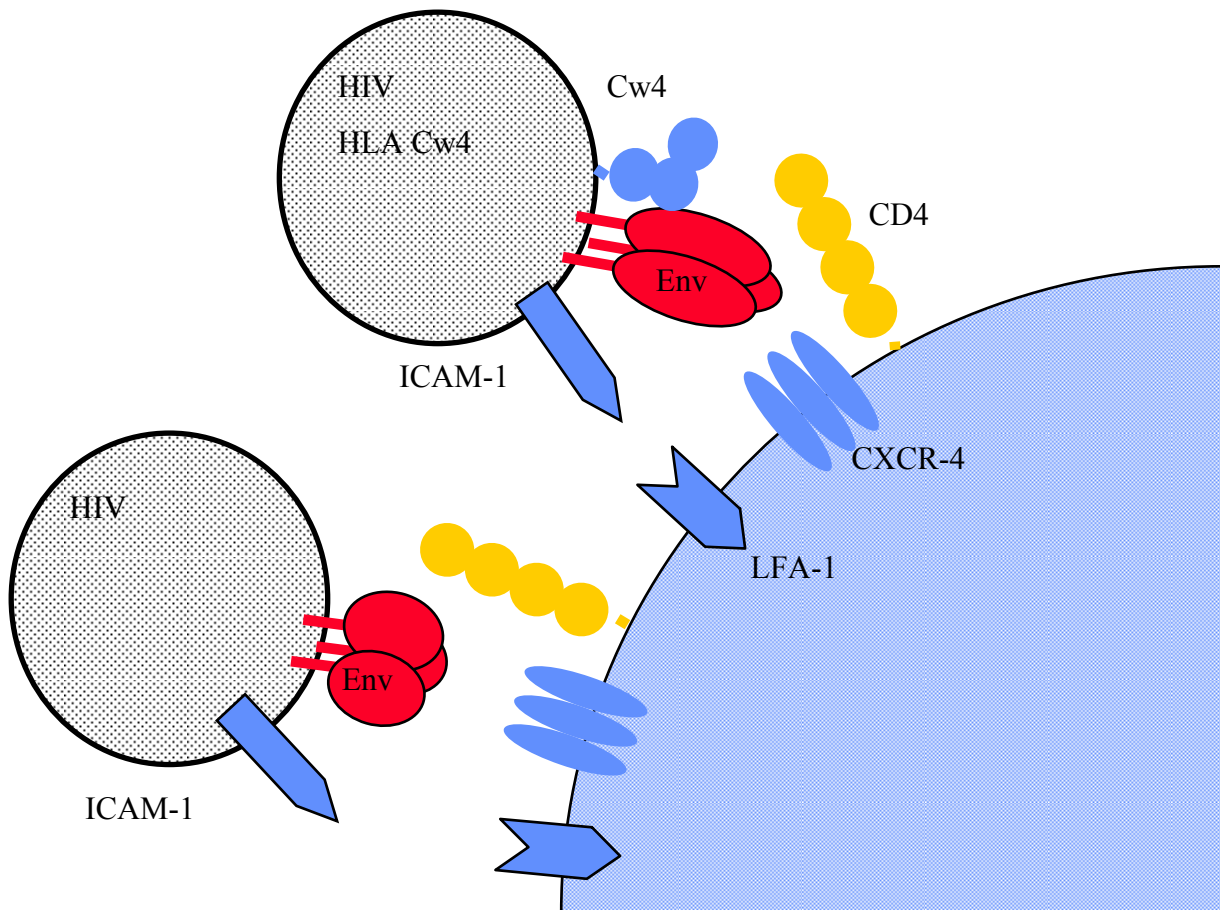
The observed increase in infectivity was associated with conformational changes in regions responsible for the viral tropism, such as the V3 loop and the epitopes normally induced by the interaction with the CD4 receptor. These changes were probably induced by direct

interaction of the Envelope protein and the HLA Cw4 molecule on the surface of the virions. In fact, we were able to co-precipitate the two molecules from the surface of actively HIV-1<sub>LAI</sub> producing cells (Figure V).



**Figure V:** Western blot analysis of immunoprecipitates from HIV-1<sub>LAI</sub> infected HLA-Cw4 expressing cells. Cell expressing HLA-Cw4 or not (as indicated at the top) were infected with HIV-1<sub>LAI</sub> (LAI+) or left untreated (LAI-). Then, lysates were immunoprecipitated with an antibody (P4D10) recognising the V3 loop of the envelope protein or an antibody (L31) recognising the HLA-C molecules (in green). Immunoprecipitated material was separated using SDS-PAGE and then transferred to a nitrocellulose membrane that was probed with the antibodies P4D10 and L31 (in red). Molecular weights are shown on the left.

These data indicate that a host derived molecule is able to modulate the conformation of the HIV-1 Envelope protein (Figure VI). These modifications include epitopes important for antibody specific virus neutralization and can unmask cryptic epitopes. Thus, complex of Envelope and HLA-Cw4 molecules can be used to build protein or cellular vaccines with different and may be better antigenic properties. It is also possible that the observation of Stott et al. may be better explained in terms of a different capacity of human and monkey host derived molecules to interact with the SIV Envelope.



**Figure VI: ICAM-1 enhance the infectivity of the HIV-1 viral particle via the interaction with its natural ligand expressed on the target cell. HLA-Cw4 acts in cis and changes the conformation of the Envelope protein on the viral particle. We hypothesized that these conformational changes allow a better interaction with the CD4 receptor and the CXCR4 co-receptor on the target cell. This conformational change can be used to formulate new antibody based vaccines.**

### ***Correlates of protection***

To evaluate AIDS vaccine formulations in humans is necessary to have indications about possible correlates of protection. Unfortunately, at present time, no clear correlates of protection or viral control exist and animal models did not provide this information.

One possibility to find correlates of protection is the study of humans that can naturally control infection or remain apparently uninfected despite repeatedly contacts with the virus.

### **Long Term Non-Progressor**

Long term non-progressor (LTNP) represent 1 to 5 % of the HIV-1 infected individuals and are characterized by a documented HIV-1 infection for more than seven years, stable and high total CD4 counts, usually more than 600 cell/mm<sup>3</sup>, low levels of viremia in peripheral blood, no history of anti-retroviral therapy and no symptoms of AIDS disease. The genetic

background of the host, the characteristic of the virus, a peculiar host immune response or a combination of all these factors may be responsible for the non-progressive status.

Polymorphisms in the genetic background of the host responsible of decreased susceptibility to infection and delayed progression to AIDS include the HIV co-receptors CCR5 and CCR2 (Dragic et al., 1996; Hogan and Hammer, 2001; Lee et al., 1998; Quillent et al., 1998), the chemokine SDF-1 (Meyer et al., 1999) and the HLA-B57 allele (Migueles et al., 2000).

In the virus genome, mutations and deletion in the regulatory gene *nef* have been clearly associated with the non-progressive status (Catucci et al., 2000).

While the genetic markers in the host and the virus have been clearly associated with a slow progression to AIDS, immunological markers are more difficult to track and up to now, we do not have clear evidences for immunological markers of protection or slow progression. However, the example of LTNP demonstrates that control of viral replication by the immune system in the context of the natural course of the disease is possible. The analysis of the 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. HIV specific CD4 T-cell plays a key role in this scenario. Indeed, proliferative capacity (Rosenberg et al., 1997), IL2 (Harari et al., 2004) and IFN- $\gamma$  (Pitcher et al., 1999) production were preferentially observed in HIV-specific CD4 T-cells derived from LTNP. Interestingly, better proliferative capacity and higher perforin expression was detected in HIV-specific CD8 T-cells derived from LTNP (Migueles et al., 2002). The capacity of HIV-specific CD8 T-cells to proliferate was associated with the presence of HIV-specific CD8 T-cells able to produce simultaneously IFN- $\gamma$  and IL-2 (Zimmerli et al., 2005). Despite the phenotype of proliferating IFN- $\gamma$ /IL-2 secreting CD8 T-cells represent an attractive marker of slow progression to AIDS, a similar population has been found in 30% - 40% of subjects successfully treated with HAART (Harari et al., 2006). Therefore, the preserved capacity to proliferate may merely represent a marker for a not yet compromised immune system. All this observations suggest that the immune system may be able to control viral replication. More recently, Betts et al. (Betts et al., 2006), observed that polyfunctional CD8 T-cell were preferentially observed in LTNP. Polyfunctionality was intended as simultaneous production/expression of CD107, IFN- $\gamma$ , MIP-1 $\beta$ , IL-2, and TNF- $\alpha$  and/or CD107, IFN- $\gamma$ , MIP-1 $\beta$ , and TNF- $\alpha$ . In contrast with previous studies expression of IL-2 was not associated with the LTNP status and significant differences between LTNP and progressor were mainly observed in polyfunctional CD8 T-cells lacking IL-2 expression.

In summary, from all these studies it is difficult to define clear protection markers, often each study is focalized only on one aspect of the immune response and the characteristics of the cohorts of patients differs from one study to another. In addition some studies are based on a limited number of subjects and conclusions could be biased by sampling problems.

### **Seronegative subjects exposed to HIV**

Exposure to HIV-1 does not always lead to infection as observed in person in high-risk group who are not infected despite frequent exposure to HIV-1. HIV-1 exposed seronegative (ESN) individuals are mostly found between long-term sexual partners of HIV-1 infected individuals (serodiscordant couples) and commercial sex workers suggesting that the frequency of the exposure is an important factor to be considered. This observation also supports the idea that acquired factors are likely involved in this resistance. As for the LTNP, several immunological mechanisms and markers have been studied in ESN. Since HIV is mainly sexually transmitted and the first contact with the host is at the level of the genital mucosa, the presence of HIV-specific mucosal IgA in HIV-1 negative partners of serodiscordant couples (Mazzoli et al., 1997) represents one important mechanism of protection involving the humoral immunity. Interestingly, when we purified IgA from ESN, we observed a clear neutralizing activity in 5 out of 15 subjects (Mazzoli et al., 1999). Of note, the neutralization assay was performed using HIV-1 primary isolates. These studies demonstrate that an HIV-specific immune response is present in HIV-1 negative and highly exposed subjects.

In our studies focused on ESN individuals, we also observed the presence of anti-HLA class I antibodies in seronegative injection drug users at risk for HIV exposure (Beretta et al., 1996b) and HIV-1 negative partners of serodiscordant couples (Beretta et al., 1996a; Lopalco et al., 2000). An immune response against cellular targets highlights the importance of cellular proteins incorporated into virions and the possible use of these molecules in vaccine formulations (see also “Vaccines to cellular proteins”).

On the side of the cellular immunity HIV-1 specific CD8 and CD4 T-cells have been found in several cohorts of female sex workers (Alimonti et al., 2006; Alimonti et al., 2005; Fowke et al., 2000; Jennes et al., 2004), suggesting a possible role of the cellular immune response in protection from infection. However, we cannot exclude an antibody mediated protective role at the mucosal site and a cellular immune response simply reflecting the contact with the virus.

## ***Summary by points of the main challenges for an AIDS vaccine***

Despite our knowledge of HIV-1 and its interaction with the host is increasing, the hope to have a vaccine against HIV-1 in a short term is not foreseen. Here are summarized a series of important challenges that we have to face to find an effective vaccine against HIV-1.

1. Neutralizing antibodies
  - a. Conserved epitopes are hidden by variable regions and glycosylation
2. Integrated proviral DNA
  - a. Has a long half-life
  - b. Forms an archive of all the virus infecting the host over the time
  - c. Can be reactivated to produce infective virions
  - d. Viral protein are not expressed in the latent phase
3. HIV genome is high variable
  - a. There are 12 known subtypes
  - b. Regional vaccines might be necessary
  - c. Escape mutants are constantly generated
4. Lack of a suitable animal model
5. Lack of a clear correlate of protection in humans

## Materials and Methods

### *Reagent Setup*

- **Culture medium:** prepare RPMI 1640 medium (Cambrex, cat.no. BE12-702F/U1) supplemented with 10% heat-inactivated FCS (Biochrom AG, cat.no. S0115) and 1% PenStrep (Cambrex, cat.no. DE17-602E) -> lab name is RPMI-10
- **Live/Dead staining solution:** Used for to count cells, Trypan Blue (Gibco, Invitrogen, Cat.no. 15250-061)
- **Costimulating antibodies (coAbs):** CD28 pure (BD, cat.no. 340975), CD49 pure (BD, cat.no. 340976)
- **Peptide pools:** use 2µg/ml peptide in the total volume of 200µl (after the addition of the BFA)
- **Negative & positive control:** negative control is only RPMI-10, positive control is with PMA + Ionomycin (PMA+I)
- **PMA:** Phorbol 12-myristate 13-acetate (Sigma, cat.no. P-8139), prepare a stock solution with a concentration of 0.1mg/ml in DMSO (Sigma, cat.no. D2650), store small single-use-aliquots at -20°C
- **Ionomycin:** (Sigma, cat.no. I-0634), prepare a stock solution with a concentration of 0.5 mg/ml in EtOH, store small single-use-aliquots at -20°C
- **BFA:** Brefeldin A (Sigma, cat.no. B-7651), prepare a stock solution with a concentration of 5mg/ml in DMSO, store small single-use-aliquots at -20°C
- **EMA:** Ethidium monoazide bromide (Invitrogen, Molecular Probe, cat.no. E-1374), live/dead discriminator, prepare a stock solution with a concentration of 2mg/ml in DMFA, store at -20°C for long time, once thawed keep at 4°C
- **FACS buffer:** (BD Pharmingen Stain Buffer 0,2% BSA, 0,09% Na Azide in DPBS, cat.no. 554657)
- **A3.01 cells:** Centre for AIDS Reagents (EU Programme EVA/AVIP), ARP098, Human CD4+ T Cell line
- **MVA-gfp:** single-use aliquot (115µl),  $5 \cdot 10^7$  IU/ml, stored at -80°C -> the amount of MVA-gfp has to be determined doing a titration for each viral preparation in order to obtain between 200 and 400 Gfp expressing cells
- **PBS:** phosphate buffered saline

- **Dianisidine solution:** Prepare a saturated solution of O-Dianisidine (D9143, Sigma) in EtOH, vortex at least 1', let stand at least 1h at room temperature protected by light, vortex, spin down. You can store this solution ten days at 4°C. Always vortex before use.

### ***Immune-staining of MVA infected cells***

The immune-staining is performed on cells forming a monolayer, such as CEF or HeLa. It is usually performed in 6 wells/plates on confluent cells

Wash the cells 1X PBS

- Fix with Acetone/Methanol (1:1) 5' room temperature

2X wash with PBS

- I Ab in PBS 3%FCS 1h room temperature (L31 1/500, P4D10 1/250)

2X wash in PBS 3%FCS

- II Ab goat anti mouse HRP (1/500) in PBS 3%FCS 45' room temperature

3X wash in PBS

- Add substrate (O-Dian.) solution

Prepare substrate solution just before adding to the cells:

add 200µl O-Dianisidine solution and 10µl H<sub>2</sub>O<sub>2</sub> to 10ml of PBS

Wait until plaques are visible and the background in the negative control remain clear. Then, simply wash away the O-Dianisidine solution with PBS.

Let dry the stained cells.

### ***Western Blot analysis of the expression of the Nef protein delivered by the recombinant vector MVA-nef***

#### **1) Infection of target cells with MVA-nef**

1.5 \* 10<sup>6</sup> B-LCL has to be infected with 10 pfu MVA/cell in a total volume of 225 µl of complete media. Perform the incubation in a 48 well plate.

Sample list:

B-LCL + mock infection

B-LCL + MVA wt

B-LCL + MVA nef (laboratory production)

B-LCL + MVA nef (GMP production)






2 hours, 37°C

1 wash

Transfer the cells in a 6 wells plate in a total 3 ml volume.



overnight at 37°

Take all the cells;  1500 rpm for 5'

Add 150µl of 1% NP40 in PBS (Cold)

Keep on ice for 20'

 14000 rpm, 2'

Take the supernatant and add 37.5µl of SB5x

Boil 5' at 95°C,



## **2) Analyze sample in SDS-PAGE and Western Blot**

### **3) Staining of the Western Blot**

The following buffer was used for the incubations: 5% BSA, 0.05% Tween in PBS (BSA-T-PBS). The washing steps were performed in the same buffer without BSA (T-PBS).

After the transfer, the nitrocellulose membrane is dried over-night.

The nitrocellulose membrane is carefully immersed in the T-PBS buffer.

3 wash with T-PBS

Overcoating: 1h in BSA-T-PBS

3 wash with PBS

I Ab 2hours 3D6 (1/200)

3x5' washes in T-PBS

II Ab Goat anti mouse HRP 1 hour (1/2000)

8x5' washes

Develop using Lumi Light

## ***Intracellular cytokine staining***

Peripheral blood was collected in heparin and processed using standard Ficoll (Biochrom, Berlin, German) density centrifugation. Lymphocytes were adjusted to  $6,6 \times 10^6$  cells/ml in

RPMI 1640 (Biochrom), 10% FCS, 1% antibiotics, 1,3 µg/ml anti CD28 and 1,3 µg/ml anti CD49d costimulatory antibodies (Becton Dickinson, Heidelberg, Germany). Then, 150 µl of cell suspension was plated in a 96 well plate together with peptides and left for 1 hour at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Brefeldin A (Sigma, Taufkirchen, Germany) was added to the cell suspension to a final concentration of 10 µg/ml and cells were subsequently incubated for 4 hours. Stimulated cells were incubated the photoreactive fluorescent label ethidium monoazide (EMA; Molecular Probe, Leiden, Netherlands) used as viability probe. Antibodies to surface antigens were added and incubation carried out on ice for 30 min. Then, cells were fixed and permeabilized, before adding antibody to the intracellular markers. Cells were analysed using a FACS Calibur (Becton Dickinson), a LSRII (Becton Dickinson) or a CYAN (Dako Cytomation) flow cytometer. The workflow for the intracellular cytokine staining procedure is shown in Figure VII. Four different sets of HIV peptides were used to stimulate lymphocytes: (i) 20-mer peptides overlapping by 10 amino acids corresponding to HIV strain LAI spanning the Nef, Tat and Rev proteins, (ii) 20-mer peptides overlapping by 10 amino acids corresponding to HIV strain SF2 spanning the p24 protein, (iii) 15-mer overlapping by 5 amino acids corresponding to HIV strain SF2 spanning the p17 protein, (iv) optimally defined epitopes from 8 to 11 amino acid in length derived from HIV-LAI Nef as described in the Los Alamos Molecular Immunology Database (Korber B., 2001), referred here as Nef CD8 Opt. The final concentration of each individual peptide was 0,4 µg/10<sup>6</sup> cells for all the experiments described. Alternatively, PBMC were infected with MVA in order to analyze vaccinia specific immune responses.

# IFN- $\gamma$ based Intracellular Cytokine Staining

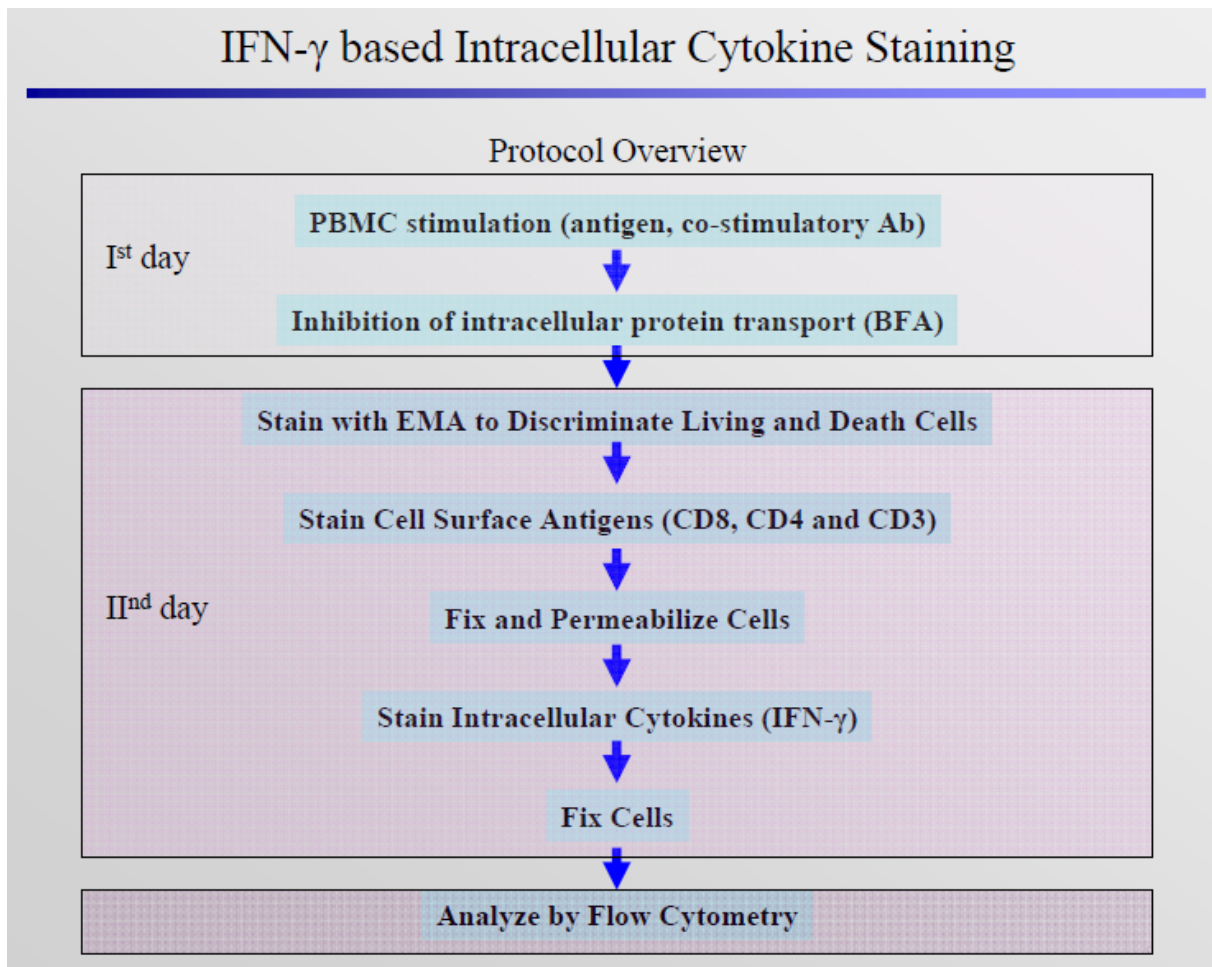


Figure VII: Example of workflow for the IFN- $\gamma$ -based intracellular cytokine staining

## Detailed protocol for the peptide stimulation of PBMC

Warm up the RPMI-10 to 37°C!

Use:  $1 \times 10^6$  cells/150 $\mu$ l RPMI-10 + coAbs for each experimental sample (Exp)

$0.5 \times 10^6$  cells/150 $\mu$ l RPMI-10 + coAbs for each compensation sample (Cmp)

### 1) RPMI-10 + coAbs:

Prepare always for 2-3 samples more than needed in a 50 ml Falcon tube

|            | RPMI-10 ( $\mu$ l) | CD28 ( $\mu$ l) | CD49d ( $\mu$ l) |
|------------|--------------------|-----------------|------------------|
| 1 sample   | 150                | 0.2             | 0.2              |
| 25 samples | 3750               | 5               | 5                |
| 40 samples | 6000               | 8               | 8                |

Resuspend the samples in RPMI-10 + coAbs and distribute 150 $\mu$ l in each well

### 2) Peptide pools (Antigens):

Use 2µg/ml peptide in the final 200µl (the total volume will be 200µl after the addition of the BFA)

Prepare PMA+I working solution: 20µl PMA stock solution + 1980µl PBS  
20µl I stock solution + 180µl PBS

Pipette PMA and I with 100µl-filtered tips

For 6 individuals:

|          | peptide (µl) | RPMI-10 (µl) |
|----------|--------------|--------------|
| Neg Ctrl | 0            | 60           |
| Nef      | 4.8          | 55.2         |
| Nef Opt  | 21.3         | 38.7         |
| Tat      | 2.6          | 57.4         |
| Rev      | 2.9          | 57.1         |
| p17      | 3.1          | 56.9         |
| p24      | 5.3          | 54.7         |
| PMA + I  | 22.5 + 18    | 19.5         |

Distribute 10µl of peptide pools in each experimental sample



1 hour at 37°C

### **3) BFA:**

Prepare working solution: 20µl BFA stock solution + 180µl PBS

Mix 192µl BFA working solution with 2200µl RPMI-10

Add 50µl/well (don't mix)



4 hours at 37°C

### **4) Stopping the stimulation:**

Cover the edges of the plate with parafilm and store over night at 4°C, protected by light.

On the next day start the staining following the appropriate protocol.

### **Detailed protocol for the MVA stimulation of PBMC**

4x10<sup>6</sup> frozen cells are used for each determination (-Ctrl and Sample)

Keep RPMI-10 at room temperature before starting the experiment

### **1) First day: MVA infection**

Thaw the cells

2 wash in RPMI-10 (use 50 ml Falcon tube) {MVA preparation}

Resuspend cells in 400 µl RPMI-10 (always in the same 50 ml Falcon tube)

Take out 50 µl as Neg Ctrl and 50 µl to be infected with MVA;

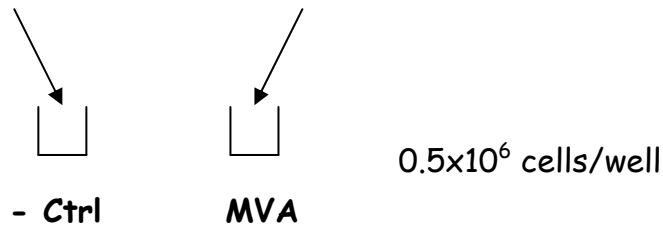


Plate them in a 96 well/plate flat bottom where the MVA ( $2.5 \times 10^6$  pfu; 5pfu/cell) was already plated. Mix well when adding the cells. Fill with PBS the wells around the samples.

Add 300 µl RPMI-10 to the 50 ml Falcon tube and leave the tubes in a slant position with the cap loosened in the incubator overnight

Leave RPMI-10 out of the fridge for the next day

### **2) MVA preparation**

Thaw MVA (Example: MVA\_F6 583 CEF  $2.1 \times 10^9$  pfu/ml)

Vortex 30'', put on ice, vortex, ice, vortex, ice

Use 1.2 µl MVA + 8.8 µl RPMI-10/sample

Plate in the 96 well/plate flat bottom before adding the cells

Example:

|        |    |    |    |    |    |
|--------|----|----|----|----|----|
| - Ctrl | #1 | #2 | #3 | #4 | #5 |
| MVA    | #1 | #2 | #3 | #4 | #5 |

### **3) Second day: stimulation**

Infected cells in 96 well/plate flat bottom:

Add 150 µl RPMI-10 (r.t.) and transfer cells to a 96 well/plate round bottom

2 wash with RPMI-10 (r.t.)

Leave the {pellet}

Cells in 50 ml Falcon:

Add 5 ml RPMI-10 (r.t.)

🌀 1500 rpm, 5', 21°C, discard media


Add 300 µl of RPMI-10 + 0.4 µl (0.4 µg) CD28 and CD49d Abs

Add the cells to the {pellet}; 150 µl for the –Ctrl well and 150 µl for the MVA infected well (1.5x10<sup>6</sup> cells/well)

Follow the standard ICS protocol


### Detailed protocol for the IFN-γ-based intracellular cytokine staining


Resuspend the cells with a multi-channel pipette.


 1500 rpm, 5', 4°C

Prepare EMA solution: 1µl in 1ml of FACS buffer (0,5% BSA, 0,02% NaAzide in PBS).

Add 50µl EMA sol. to each well

 20' on ice, in the dark

 10' on ice, to strong light


Add 150µl FACS buffer,  1500 rpm, 5', 4°C

2 wash with 200µl FACS buffer,  1500 rpm, 5', 4°C

Add 50µl FACS buffer and transfer the cell suspension to a 96 well plate were the antibodies to cell surface markers have been already plated.

|                |                 |             |                |
|----------------|-----------------|-------------|----------------|
| Anti CD8 PE    | 2µl/50µl test   | X42 samples | 84µl +         |
| Anti CD4 PerCP | 2µl/50µl test   |             | 84µl +         |
| Anti CD3 APC   | 0,5µl/50µl test |             | 21µl +         |
|                |                 | FACS buffer | <u>231µl =</u> |
|                |                 |             | 420µl          |

Distribute 10µl of antibodies dilution in each well.

 30' on ice, in the dark


Add 150µl FACS buffer,  1500 rpm, 5', 4°C

2 wash with 200µl FACS buffer,  1500 rpm, 5', 4°C

Add 200µl of 1X FACS Lysing sol (resuspend well!)



10' room temperature, in the dark


1 wash with 200µl FACS buffer,  1800 rpm, 5', room temperature

Add 100µl of 1X Permeabilization sol (resuspend well!)



10' room temperature, in the dark

Add 100µl FACS buffer,  1800 rpm, 5', room temperature

1 wash with 200µl FACS buffer,  1800 rpm, 5', room temperature

Add 50µl FACS buffer and transfer the cell suspension to a 96 well plate where the antibodies to intracellular markers have been already plated.


IgG2a FITC            0,25µl/50µl test            X6 samples            1,5µl + 58,5µl

Anti IFN $\gamma$  FITC            2µl/50µl test            X38 samples            76µl + 304µl




45' room temperature, in the dark

Add 150µl FACS buffer,  1800 rpm, 5', room temperature

1 wash with 200µl FACS buffer,  1800 rpm, 5', room temperature

Fix the cells with 1% Paraformaldehyde in PBS 400µl/sample

### **Detailed protocol for the IFN- $\gamma$ /IL-2/CD154 intracellular cytokine staining**

Resuspend the cells,  1600 rpm, 5', 4°C, discard the supernatant (SN)

#### **1) EMA staining:**

Prepare working solution: 1µl EMA stock solution in 1ml of FACS buffer

Resuspend the cells in 50µl/well



20' on ice, in the dark



10' on ice, to strong light

Add 150µl FACS buffer,  1600 rpm, 5', 4°C, discard the SN

2x wash with 200µl FACS buffer,  1600 rpm, 5', 4°C, discard the SN


## **2) Surface staining:**

Compensation antibodies:

|                | (µl) | FACS buffer (µl) | total (µl) | Position |
|----------------|------|------------------|------------|----------|
| Voltage Set Up | 0    | 20               | 20         | A 1      |
| No Abs Ctrl    | 0    | 20               | 20         | A 2      |
| CD8 FITC       | 4    | 16               | 20         | A 3      |
| CD8 PE         | 2    | 18               | 20         | A 4      |
| CD4 PerCP      | 5    | 15               | 20         | A 5      |
| CD8 PacB       | 2.5  | 17.5             | 20         | A 6      |
| CD8 APC        | 0.5  | 19.5             | 20         | A 7      |
| CD3 PE-Cy7     | 0    | 20               | 20         | A 8      |


Surface staining antibody mix:


| for 1 sample | (µl) |
|--------------|------|
| CD4 PerCP    | 5    |
| CD8 PacB     | 2.5  |
| FACS buffer  | 12.5 |
| total        | 20   |


 the Surface staining antibody mix at 12000 rpm, 3', 4°C -> transfer the SN to a new Eppendorf

Distribute 20µl of Surface staining antibody mix in the appropriate wells

Resuspend the cells in 50µl FACS buffer and transfer the cell suspension to the prepared plate where the antibodies to cell surface markers have been already plated.

 30' on ice, in the dark


Add 150µl FACS buffer,  1600 rpm, 5', 4°C, discard the SN


2x wash with 200µl FACS buffer,  1600 rpm, 5', 4°C, discard the SN


## **3) Fixation & Permeabilization:**

Resuspend well in 100µl Cytofix/Cytoperm reagent



 20' on ice, in the dark

Add 100µl Perm/Wash buffer,  1800 rpm, 5', 4°C, discard the SN

3x wash with 200µl Perm/Wash buffer,  1800 rpm, 5', 4°C, discard the SN


#### **4) Intracellular staining:**

Compensation antibodies:

|                | (µl) | FACS buffer (µl) | total (µl) | Position |
|----------------|------|------------------|------------|----------|
| Voltage Set Up | 0    | 30               | 30         | A 1      |
| No Abs Ctrl    | 0    | 30               | 30         | A 2      |
| CD8 FITC       | 0    | 30               | 30         | A 3      |
| CD8 PE         | 0    | 30               | 30         | A 4      |
| CD4 PerCP      | 0    | 30               | 30         | A 5      |
| CD8 PacB       | 0    | 30               | 30         | A 6      |
| CD8 APC        | 0    | 30               | 30         | A 7      |
| CD3 PE-Cy7     | 2    | 28               | 30         | A 8      |


Intracellular staining antibody mix Effector panel:


| for 1 sample      | (µl) |
|-------------------|------|
| IFN $\gamma$ FITC | 13   |
| CD154 PE          | 10   |
| IL2 APC           | 5    |
| CD3 PE-Cy7        | 2    |
| FACS buffer       | 0    |
| total             | 30   |


 the Intracellular staining antibody mix at 12000 rpm, 3', 4°C -> transfer the SN to a new Eppendorf

Distribute 30µl of Intracellular staining antibody mix in in the appropriate wells

Resuspend the cells in 50µl Perm/Wash buffer and transfer the cell suspension to the prepared plate where the antibodies to intracellular markers have been already plated.

 30' on ice, in the dark

Add 150µl Perm/Wash buffer,  1800 rpm, 5', 4°C, discard the SN

2x wash with 200µl Perm/Wash buffer,  1800 rpm, 5', 4°C, discard the SN

**5) Acquisiton:**

Acquisition with CYAN and LSRII:

Resuspend the samples in 350µl FACS buffer in 1ml titer-tubes

Vortex

Acquire all

Acquisition with HTS at LSRII:

Resuspend in 200µl FACS buffer in a 96well plate V-bottomed

After each individual fill 3 washing wells with FACS flow solution

loader settings:

|                           | Set Up | Samples | Washes |
|---------------------------|--------|---------|--------|
| Sample Flow Rate (µl/sec) | 1      | 2.5     | 3      |
| Sample Volume (µl)        | 150    | 150     | 200    |
| Mixing Volume (µl)        | 100    | 100     | 100    |
| Mixing Speed (µl/sec)     | 150    | 150     | 200    |
| Number of Mixes           | 5      | 5       | 0      |
| Wash Volume (µl)          | 800    | 800     | 800    |

Plate cheme:

experimental sample (Exp)

compensation sample (Cmp)

washing wells (wash)

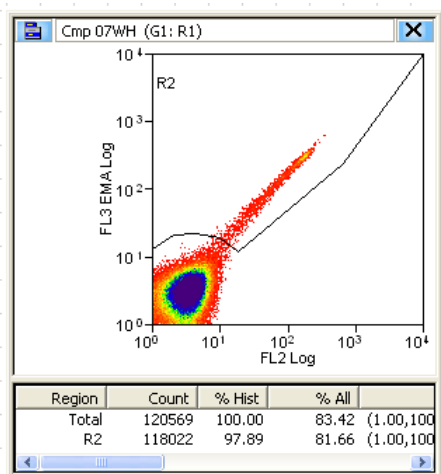
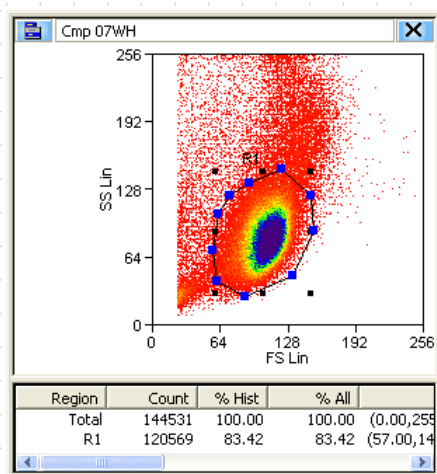
Cmp ----->

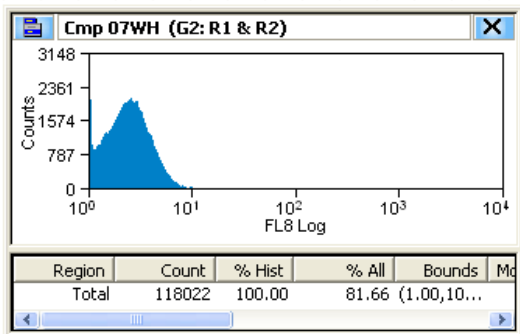
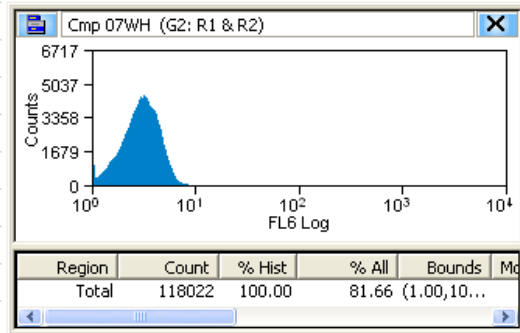
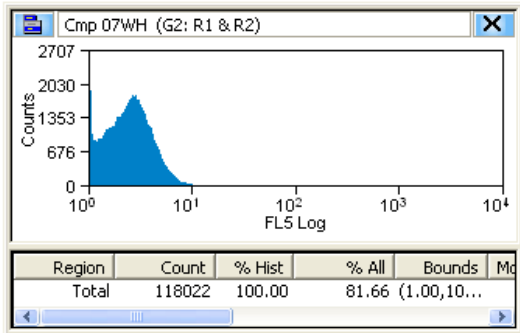
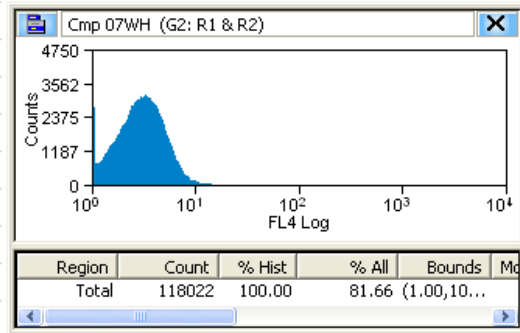
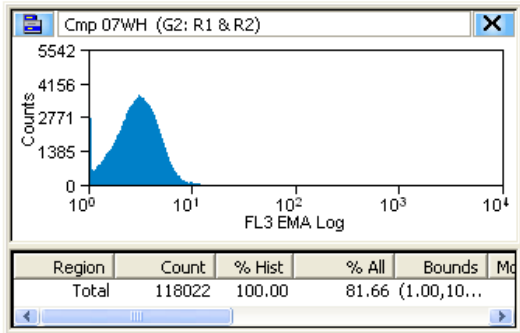
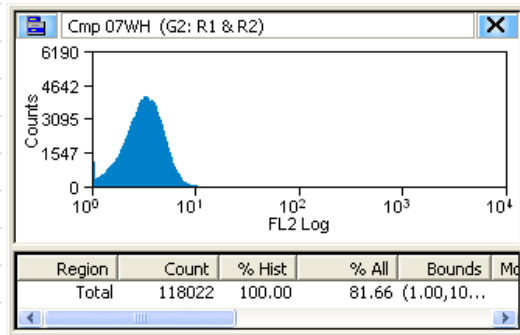
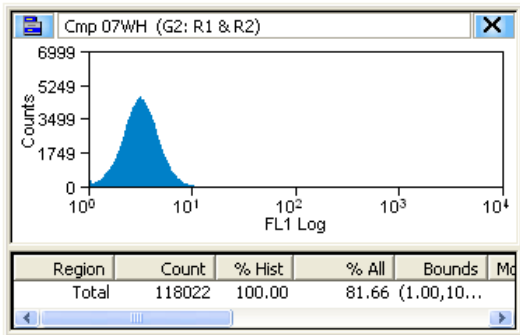
|                |             |          |         |           |          |         |           |  |      |      |      |
|----------------|-------------|----------|---------|-----------|----------|---------|-----------|--|------|------|------|
| Voltage Set Up | No Abs Ctrl | CD8 FITC | CD8 PE  | CD4 PerCP | CD8 PacB | CD8 APC | CD3 PECy7 |  | wash | wash | wash |
| Exp ID1        | Exp ID1     | Exp ID1  | Exp ID1 | Exp ID1   | Exp ID1  | Exp ID1 | Exp ID1   |  | wash | wash | wash |
| Exp ID2        | Exp ID2     | Exp ID2  | Exp ID2 | Exp ID2   | Exp ID2  | Exp ID2 | Exp ID2   |  | wash | wash | wash |

|         |         |         |         |         |         |         |         |  |      |      |      |
|---------|---------|---------|---------|---------|---------|---------|---------|--|------|------|------|
| Exp ID3 | Exp ID3 | Exp ID3 | Exp ID3 | Exp ID3 | Exp ID3 | Exp ID3 | Exp ID3 |  | wash | wash | wash |
| Exp ID4 | Exp ID4 | Exp ID4 | Exp ID4 | Exp ID4 | Exp ID4 | Exp ID4 | Exp ID4 |  | wash | wash | wash |

CYAN setting:

| Acquisition Parameters |               |         |      |
|------------------------|---------------|---------|------|
| Sample_1               |               |         |      |
| Threshold (%)          | 1.5           | Trigger | FS   |
| Name                   | Peak/Area/Log | Voltage | Gain |
| FS                     | Peak          | N/A     | 6.0  |
| SS                     | Peak          | 580     | 1.0  |
| FL1                    | Log           | 580     | 1.0  |
| FL2                    | Log           | 695     | 1.0  |
| FL3 EMA                | Log           | 695     | 1.0  |
| FL4                    | Log           | 680     | 1.0  |
| FL5                    | Log           | 750     | 1.0  |
| FL6                    | Log           | 620     | 1.0  |
| FL7                    | Log           | 620     | 1.0  |
| FL8                    | Log           | 920     | 1.0  |
| FL9                    | Log           | 950     | 1.0  |





LSRII setting:

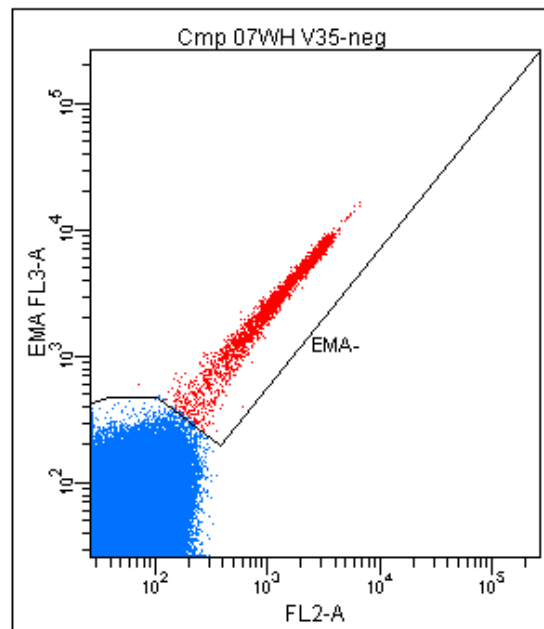
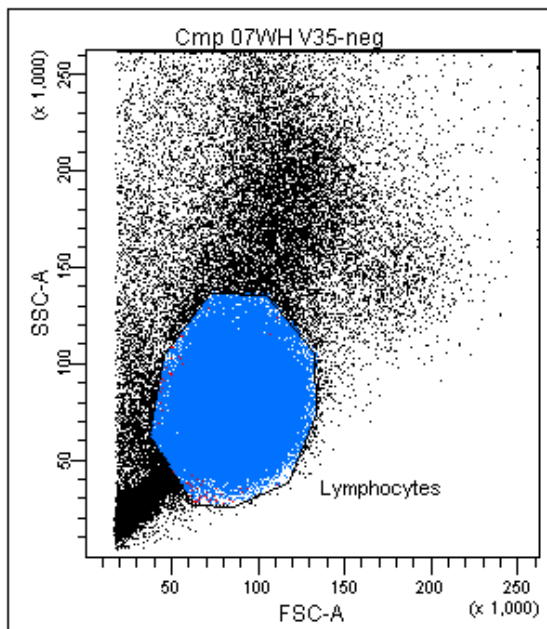
The left screenshot shows the 'Parameters' tab of the Instrument software. It contains a table with the following data:

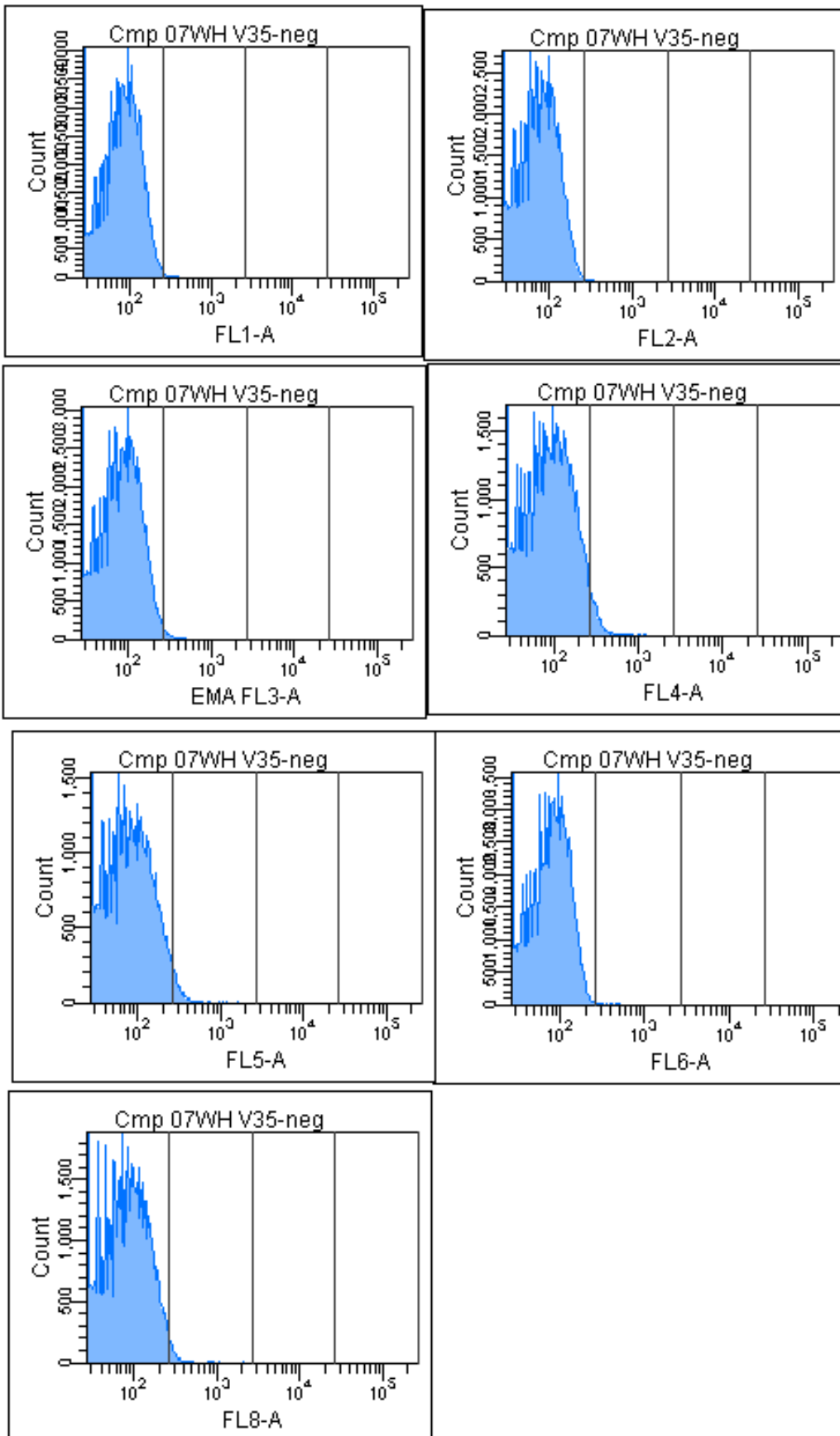
| Parameter | Voltage | Log                                 | A                                   | H                                   | W                        |
|-----------|---------|-------------------------------------|-------------------------------------|-------------------------------------|--------------------------|
| FSC       | 370     | <input type="checkbox"/>            | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | <input type="checkbox"/> |
| SSC       | 380     | <input type="checkbox"/>            | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input type="checkbox"/> |
| FL1       | 535     | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input type="checkbox"/> |
| FL2       | 510     | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input type="checkbox"/> |
| FL3       | 600     | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input type="checkbox"/> |
| FL4       | 660     | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input type="checkbox"/> |
| FL5       | 745     | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input type="checkbox"/> |
| FL6       | 495     | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input type="checkbox"/> |
| FL8       | 635     | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input type="checkbox"/> |

The right screenshot shows the 'Threshold' tab of the Instrument software. It contains a table with the following data:

| Parameter | Value  |
|-----------|--------|
| FSC       | 17,471 |

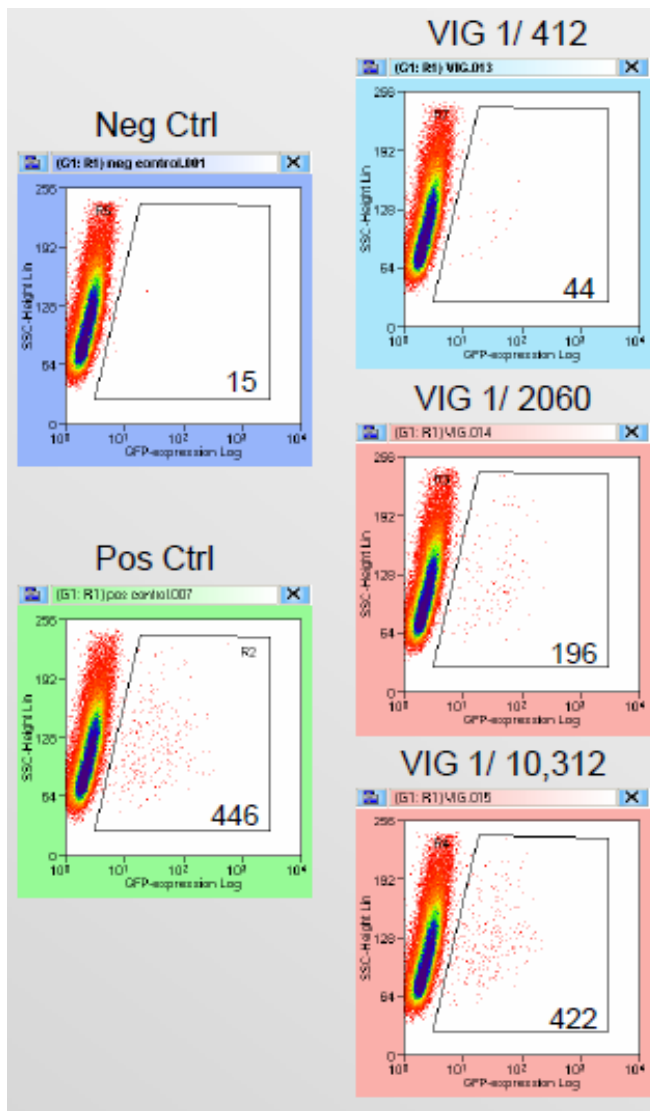
FSC setting may change after the regular BD service





### ***MVA-gfp neutralization assay***

The MVA-gfp neutralization assay was modified from its original description (Cosma et al., 2004). Briefly, heat inactivated sera were incubated with the MVA-gfp (multiplicity of infection of 0.1) for one hour at 37°C. Then,  $0.5 \times 10^6$  A3.01 were added and the incubation carried out for two more hours in the same conditions. Cells were washed in RPMI-10, transferred in 96 well flat-bottom tissue culture plate and kept overnight at 37°C. Then, cells were fixed in 1% paraformaldehyde. The percentage of MVA infected A3.01 was evaluated by measuring Gfp expression in a FACScalibur (Becton Dickinson) or CYAN ADP (Dako Cytomation). For each sample, 100,000 living cells were acquired. Representative pseudo-color dot plots are shown in Figure VIII. Five dilutions (1:16 to 1:10,000) were tested for each sample to obtain trend lines of percentages of neutralization and units of area under the curve (AUC) were calculated using GraphPad Prism version 4.03 (San Diego, California, USA).



**Figure VIII: MVA-gfp neutralization assay. Representative pseudo-color dot plots of a typical MVA-gfp neutralization assay. A negative control, a positive control and three dilutions of a preparation of neutralizing vaccinia virus immunoglobulin (VIG) are shown**

### **Detailed protocol for the MVA-nef neutralization assay**

1 day before starting seed A3.01 cells to a density of  $1 \times 10^6$ /ml in 20ml of RPMI-10

#### **1) Preparation of the human sera:**

Heat inactivation: 10 $\mu$ l sera in a Eppendorf tube

Heat inactivation 30' at 56°C

☺ max speed, 2', 21 °C

#### **2) Preparation of MVA-gfp:**

Thaw MVA-gfp

Vortex 3x for 1', keep on ice in between



For one plate: use 100 µl MVA-gfp + 2400 µl RPMI-10

Vortex

### **3) Preparation of the plate:**

Fill the wells with RPMI-10 according to the scheme, fill the wells around with 100µl PBS

|     |      |      |      |      |      |      |      |      |      |      |     |
|-----|------|------|------|------|------|------|------|------|------|------|-----|
| PBS | 40µl | 40µl | 40µl | 40µl | 40µl | PBS  | PBS  | PBS  | PBS  | PBS  | PBS |
| PBS | 40µl | 40µl | 40µl | 40µl | 40µl | 45µl | 40µl | 40µl | 40µl | 40µl | PBS |
| PBS | 45µl | 40µl | 40µl | 40µl | 40µl | 45µl | 40µl | 40µl | 40µl | 40µl | PBS |
| PBS | 45µl | 40µl | 40µl | 40µl | 40µl | 45µl | 40µl | 40µl | 40µl | 40µl | PBS |
| PBS | 45µl | 40µl | 40µl | 40µl | 40µl | 45µl | 40µl | 40µl | 40µl | 40µl | PBS |
| PBS | 45µl | 40µl | 40µl | 40µl | 40µl | 45µl | 40µl | 40µl | 40µl | 40µl | PBS |
| PBS | 45µl | 40µl | 40µl | 40µl | 40µl | 45µl | 40µl | 40µl | 40µl | 40µl | PBS |
| PBS | 45µl | 40µl | 40µl | 40µl | 40µl | 45µl | 40µl | 40µl | 40µl | 40µl | PBS |
| PBS | PBS  | PBS  | PBS  | PBS  | PBS  | PBS  | PBS  | PBS  | PBS  | PBS  | PBS |

### **4) Sera dilution:**

Add 5µl sera to the appropriate wells and perform 1/5 dilutions

10µl 10µl 10µl 10µl 10µl

|  |       |  |  |  |  |       |  |  |  |  |  |
|--|-------|--|--|--|--|-------|--|--|--|--|--|
|  |       |  |  |  |  |       |  |  |  |  |  |
|  |       |  |  |  |  | serum |  |  |  |  |  |
|  | serum |  |  |  |  | serum |  |  |  |  |  |
|  | serum |  |  |  |  | serum |  |  |  |  |  |
|  | serum |  |  |  |  | serum |  |  |  |  |  |
|  | serum |  |  |  |  | serum |  |  |  |  |  |
|  | serum |  |  |  |  | serum |  |  |  |  |  |
|  |       |  |  |  |  |       |  |  |  |  |  |

### **5) Plating the MVA-gfp & NegCtrl:**

Add 25µl of diluted MVA-gfp (vortex before adding) to the appropriate wells, for the NegCtrl wells add only 25µl RPMI-10

|  |         |         |         |         |         |     |     |     |     |     |  |
|--|---------|---------|---------|---------|---------|-----|-----|-----|-----|-----|--|
|  | NegCtrl | NegCtrl | NegCtrl | NegCtrl | NegCtrl |     |     |     |     |     |  |
|  | MVA     | MVA     | MVA     | MVA     | MVA     | MVA | MVA | MVA | MVA | MVA |  |
|  | MVA     | MVA     | MVA     | MVA     | MVA     | MVA | MVA | MVA | MVA | MVA |  |
|  | MVA     | MVA     | MVA     | MVA     | MVA     | MVA | MVA | MVA | MVA | MVA |  |
|  | MVA     | MVA     | MVA     | MVA     | MVA     | MVA | MVA | MVA | MVA | MVA |  |
|  | MVA     | MVA     | MVA     | MVA     | MVA     | MVA | MVA | MVA | MVA | MVA |  |
|  |         |         |         |         |         |     |     |     |     |     |  |



1 hour at 37°C

### **6) Preparation & adding of the A3.01 cells:**

We need  $0.5 \times 10^6$  cells in 50µl RPMI-10/well ->  $50 \times 10^6$  cells in 5ml/plate

☞ 1500 rpm, 5', 21°C, discard the SN

Resuspend the pellet in 10ml RPMI-10

Count the cells in a counting chamber (1/2 or 1/5 in Trypan Blue)

☞ 1500 rpm, 5', 21°C, discard the SN

Resuspend the pellet in 5ml RPMI-10

Add 50µl cells/well to all wells (except PBS-wells!)

Mix well before and after adding!



2 hours at 37°C

### **7) Over Night incubation:**

☞ 1500 rpm, 5', 21°C, discard the SN

2x wash with 200µl RPMI-10, ☞ 1500 rpm, 5', 21°C, discard the SN

Resuspend the cells in 110µl RPMI-10 and transfer to a in a 96well plate flat-bottomed, fill the wells around with 100µl PBS



over night at 37°C (approx. 16 hours)

### **8) Fixation:**

Resuspend the cells and transfer to a 96well plate U-bottomed

☞ 1500 rpm, 5', 21°C, discard the SN and resuspend the cells in 300µl of fixation solution

### ***Detection of vaccinia specific antibodies in ELISA***

Maxisorp plates (Nunc, Wiesbaden, Germany) were coated with sucrose-gradient purified MVA (at a protein concentration of 1µg/ml) for 3 h at 37 °C and overnight at 4 °C. The plates were blocked with PBS containing 0.05% Tween 20 and 10% FCS for 60 min at 37 °C. After

incubation of sera for 60 min at 37 °C, plates were washed five times with PBS. Then, a secondary anti-human immunoglobulin alkaline phosphatase conjugate was added for 30 min. According to the assay, affinity purified anti human IgG, IgM or IgG/IgM (Jackson ImmunoResearch, West Baltimore Pike, PA) were used. Following five washes, the plates were incubated with pNPP substrate (Sigma, Taufkirchen, Germany) at 37 °C, and the optical density was measured after 20 min at a wavelength of 405 nm. Eight dilutions (1:100 to 1:12,800) were tested for each sample and the obtained ODs were used to calculate units of AUC. Alternatively, plates were coated with 0.25 µg/ml of recombinant A27R or A33R protein overnight at room temperature and blocked with PBS containing 1% Tween (PBS-T) and 3% BSA for 2 hours at 37 °C. Sera were diluted in PBS-T and incubated for 60 min at 37 °C. After six washes with PBS-T, anti-human IgG/IgM peroxidase (Jackson ImmunoResearch) was added to the plate and incubation carried out for 1 hour 37 °C. Following 5 washes, the plate was incubated for 30 min with ABTS substrate (Sigma) before the measurement of the optical density at 405 nm. For each sample three dilutions (1:50 to 1:200) were tested and the obtained ODs were used to calculate units of AUC.

## **Results and Discussion**

### ***Rationale for the use of the MVA-HIV-1nef vector***

Vaccinia viruses engineered to produce recombinant proteins are promising vaccine candidates. However, in HIV infected individuals due to concerns about the side effects of the classical replication competent vaccinia virus, the delivery of HIV genes require the use of highly attenuated replication defective vaccinia virus strains. One such virus strain, MVA was chosen for our study. From a safety perspective, MVA was used as a smallpox vaccine in over 120,000 recipients without significant adverse reaction (Mayr et al., 1975). Moreover, in non-human primate models of AIDS, MVA vectors engineered with HIV derived genes resulted to be capable to confer protection against disease progression after viral challenge (Amara et al., 2002a; Amara et al., 2001; Amara et al., 2002b).

HIV-1 Nef is an early expressed regulatory protein, which plays an important immunomodulatory role. In fact, Nef is responsible for the downregulation of CD4, HLA class I and CCR5 on the surface of HIV infected cells. Moreover, Nef changes the activation state of the cells (Simmons et al., 2001). In vivo, Nef is essential for the maintenance of high levels of viral replication and progression to AIDS in SIV-infected monkeys. In humans, functional

deletions in the Nef gene have been shown to influence progression to AIDS (Learmont et al., 1999).

All these observations indicate that Nef is an essential protein for the pathogenesis of HIV. Thus, Nef represent an attractive component of HIV to be targeted in order to face the virus.

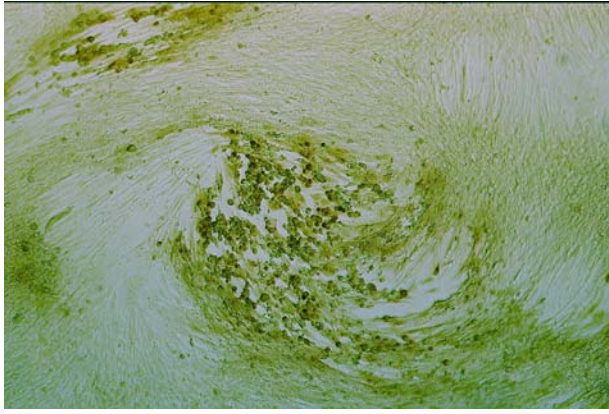
One important issue to be addressed is which HIV genes have to be included in a candidate vaccine against AIDS. The multi-protein approach, while partially eliminating the problem of the choice of the genes, does not give information on the immunogenicity at the single gene level. Thus, the information provided by the multi-protein approach does not permit to improve the vaccine in term of composition.

Our approach has as final target to evaluate the ability of the MVA-Nef vaccine to elicit an immune response and to improve the immune control of HIV in chronically HIV-1 infected individuals. We want also to collect information about the immunogenicity of Nef alone in order to build a collection of data to be used for the development of new vaccines against HIV.

### ***Characterization of the MVA-nef vector***

#### **Expression in chicken embryo fibroblast**

The MVA vector expressing the HIV-1<sub>LAI</sub> Nef gene was first characterized on chicken embryo fibroblast (CEF). CEF are usually used to amplify preparations of recombinant MVA (Sutter and Staib, 2003). The use of CEF monolayer and serial dilutions of the MVA vector ensure the visualization of single plaques generated by single infectious units. Nef expression was detected in all the plaques (Figure IX), demonstrating the purity and stability of the MVA-nef vector.



**Figure IX: Immunostaining of a typical MVA plaque on CEF. CEF monolayer infected with different dilutions of MVA-nef were fixed and stained with a polyclonal rabbit serum specific to Nef. Anti-rabbit HRP conjugated secondary antibody and dianisidine substrate were used to develop the assay. A representative plaque generated by infection with MVA-nef is shown. The darker cells express the Nef protein.**

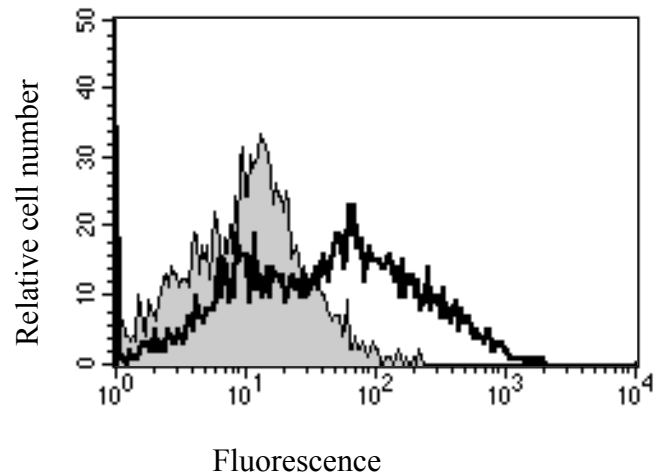
This staining confirmed the stability of the MVA-nef vector. However, further experiments in human cells are required to assess the expression of Nef in a system relevant for the successive use of the vector as a vaccine in human beings.

### **Expression in human B-LCL**

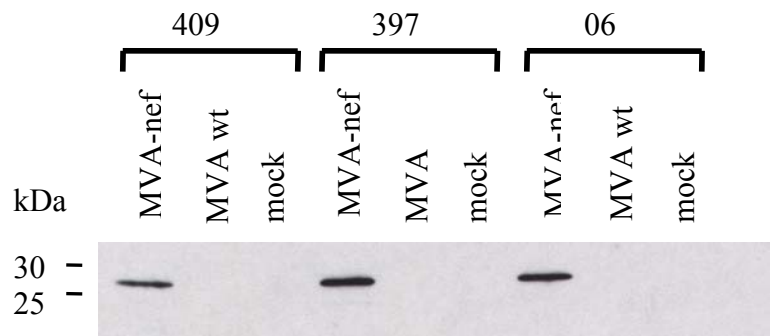
Expression of the Nef protein in MVA-nef infected human B-LCL was measured by intracellular staining and Western blot. Intracellular staining showed that in more than 50% of the cells the Nef protein was detectable at 16 hours post-infection. A representative histogram plot obtained with MVA-nef infected B-LCL from individual 3975 is shown in Figure X. Similar results were obtained with individuals 4097 and 063 B-LCL (data not shown). The same samples were analyzed for the expression of Nef in Western blot. A band with a molecular weight of 27 kDa corresponding to Nef was detected in all three samples (Figure X).

Intracellular staining

█ MVA-wt  
█ MVA-nef



Western blot analysis

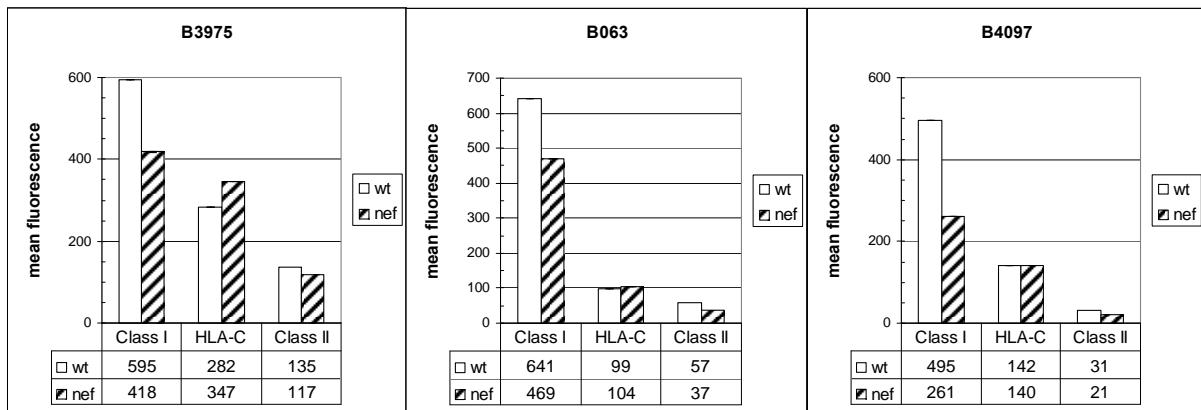


**Figure X: Nef protein expression in MVA-nef infected B-LCL analyzed by intracellular staining and Western blot.** B-LCL were infected with 8 pfu/cell of MVA-nef or MVA-wt and incubated overnight at 37°C in 5% CO<sub>2</sub>. As additional control, B-LCLs were mock infected. The Nef-specific mAb 3E6 followed by a FITC-conjugated goat anti mouse antibody was used for the intracellular staining. The same cells were analyzed in Western blot using the Nef-specific mAb 3D6.

### Functional characterization

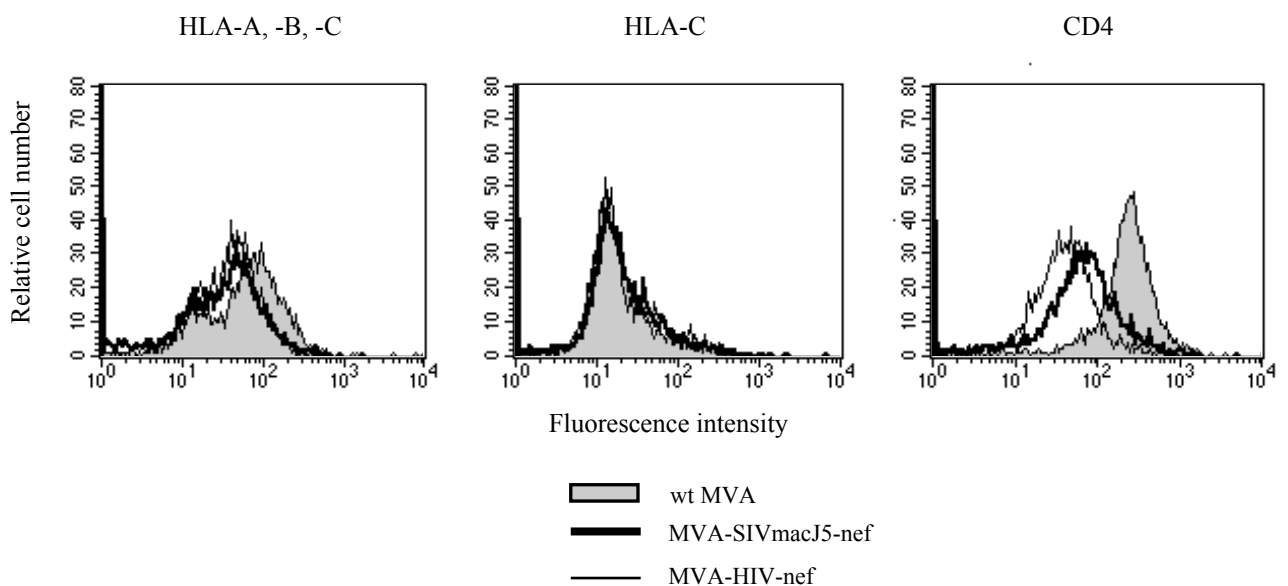
The capacity of Nef to downregulate the surface expression of HLA class I molecules was measured in the three B-cell lines used in Figure X. B-LCL were infected as described above with MVA-nef or MVA-wt and surface expression of HLA-A,-B,-C, HLA-C and HLA class II molecules was measured in flow cytometry (Figure XI). The mAb W6/32 (Serotec) was used to measure the expression of HLA-A,-B and -C molecules while the mAb L31 (Setini et al., 1996) was used to specifically measure the expression of HLA-C molecules. The mAb WR18 (Serotec) was used to detect HLA-class II molecules. The total surface expression of HLA class I decreased upon infection with MVA-nef in all the B-LCL tested while no differences were observed in the expression of HLA-C between MVA-nef and MVA-wt

infected B-LCL. The surface expression of HLA class II was only slightly affected by the expression of the Nef protein.



**Figure XI: Functional characterization of the Nef protein expressed by the MVA-nef vector in different B-cell lines. B-LCL were infected with 8 pfu/cell of MVA-nef or MVA-wt and incubated overnight at 37°C in 5% CO<sub>2</sub>. Each panel represents a B-LCL derived from a different individual.**

To characterize further the functionality of the Nef protein encoded by MVA-nef, a human CD4<sup>+</sup> T-cell line (A3.01) was used. The intracellular expression of the Nef protein together with the surface expression of CD4 and HLA class I were evaluated after infection with MVA-HIV-nef, MVA-SIVmacJ5-nef or wtMVA. The Nef protein was expressed in 80% of the cells with mean fluorescence intensity (MFI) of 109 (data not shown). As shown in Figure XII, the CD4 expression was downregulated from an MFI of 265 to 54 in more than 90% of the cells. As expected we observed a selective downregulation of HLA-A and -B (from an MFI of 83 to 48) but not HLA-C molecules (Figure XII).



**Figure XII: Functional characterization of the Nef protein expressed by the MVA-nef vector in the A3.01 T-cell line. Cells were infected with 8 pfu/cell of MVA-HIV-nef, MVA-SIVmacJ5-nef or wtMVA for 3**

hours and after 16 hour at 37°C in 5% CO<sub>2</sub> the expression of HLA class I, HLA-C and CD4 molecules was evaluated in flow cytometry.

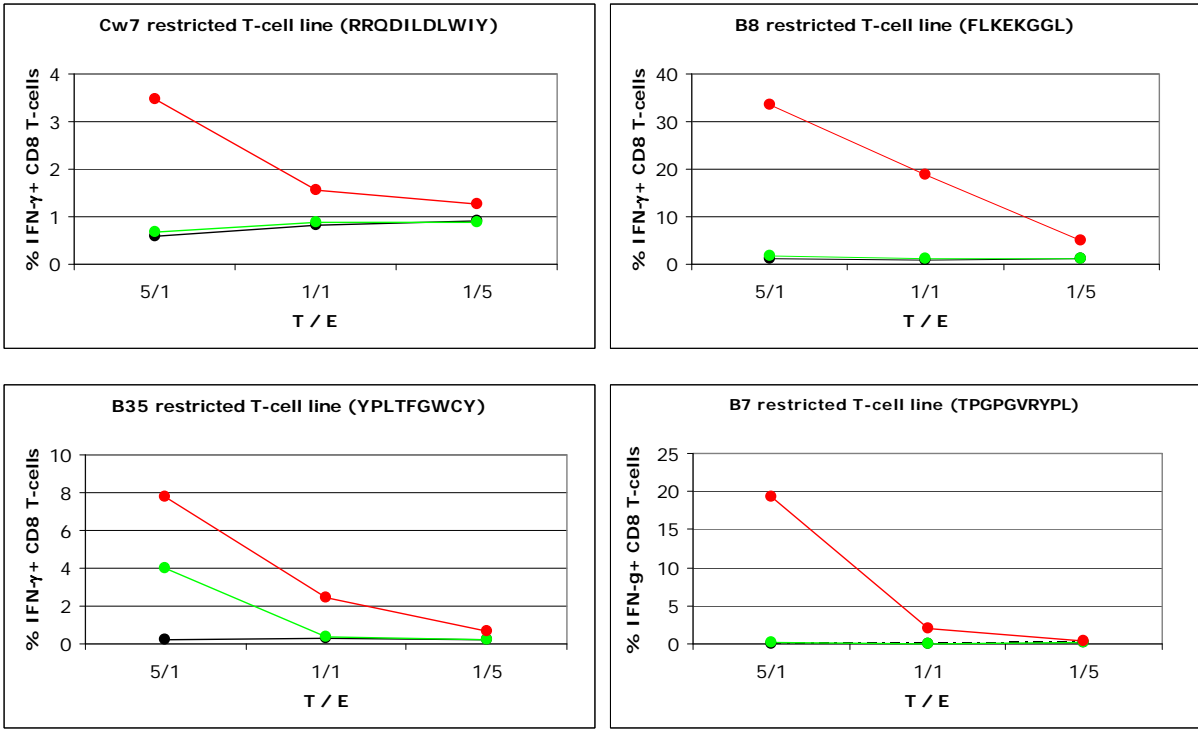
These results showed that the MVA-nef vector can be used to express the Nef protein in human cells and that this protein was functional concerning its capacity to downregulate CD4 and HLA class I molecules.

### **Characterization of the ability of the MVA-nef vector to present Nef derived epitopes**

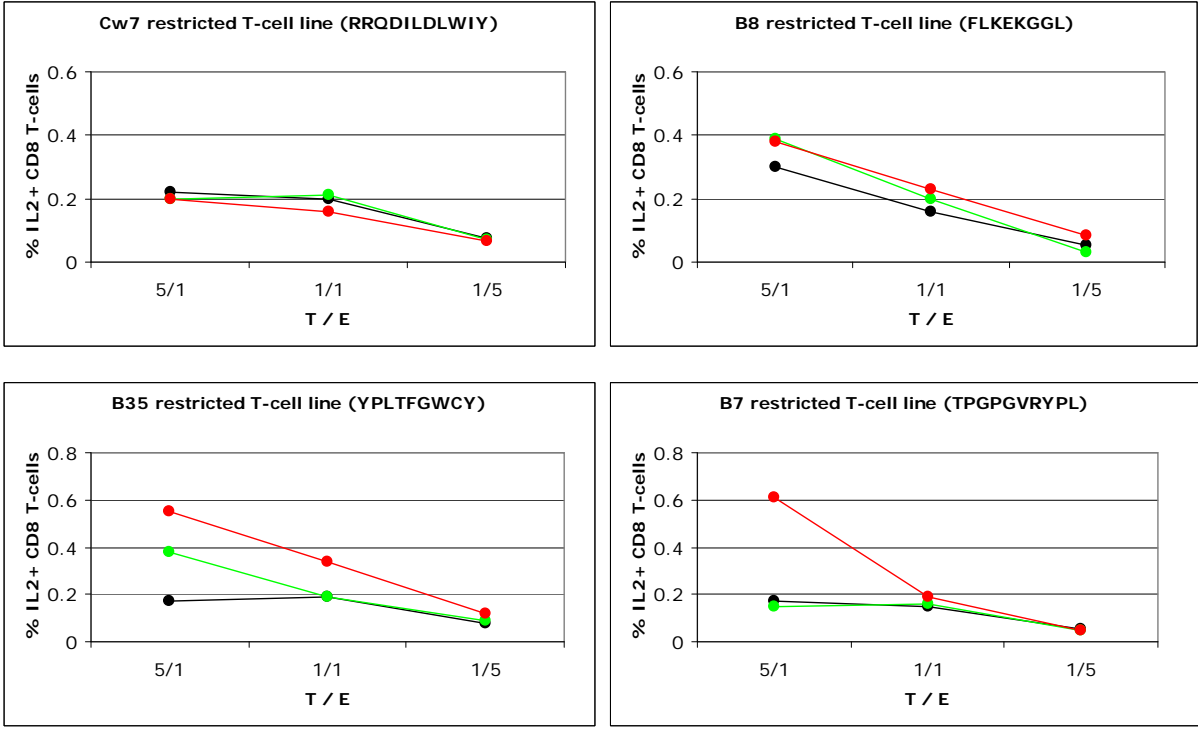
Viral interference with MHC-class I molecules is considered an important mechanism of immune escape adopted by several viruses that infect humans (Alcami and Koszinowski, 2000). HIV Nef protein downregulates HLA-A and B but not HLA-C and -E alleles (Le Gall et al., 1998; Schwartz et al., 1996). This selective Nef-mediated downregulation of HLA class I molecules was shown to correlate with protection of infected primary T lymphocytes from killing by CTL (Cohen et al., 1999) and from lysis by NK cell (Collins et al., 1998). The downregulation of HLA-A and -B molecules in MVA-nef infected T and B-cells might impede the correct presentation of Nef epitopes to specific CD8 T-cells, thus decreasing the capacity of the vaccine in inducing Nef-specific CD8 responses.

To address the effect of the MVA-nef induced HLA class I downregulation on antigen presentation, we used B-LCL infected with MVA-nef and T-cell lines specific for defined Nef epitopes. Nef specific T-cell lines derived from HIV-1 infected patients were mixed with different amounts of autologous B-LCLs infected with MVA-nef or MVA-wt. As an additional control mock-infected B-LCL were used. After five hours of co-incubation, IFN- $\gamma$  and IL2 production in CD3<sup>+</sup> CD8<sup>+</sup> cells were determined using intracellular cytokine staining. MVA-nef infected B-LCLs were able to stimulate the production of IFN- $\gamma$  in all the four T-cell line tested (Figure XIII). The percentage of IFN- $\gamma$  producing cells at the maximal T/E ratio varied from 3.5% to 35% of the total CD3<sup>+</sup> CD8<sup>+</sup> cells. Only the T-cell line restricted to B35 and B7 were able to respond to the antigenic stimulation producing IL2 (Figure XIV). Of note, an MVA-wt specific response with production of IFN- $\gamma$  and IL2 was observed with the B35 restricted T-cell line. Since this T-cell line was derived from an MVA-nef vaccinated subject, we cannot exclude a contamination by MVA-specific CD8 T-cells during the expansion of the Nef specific T-cells.

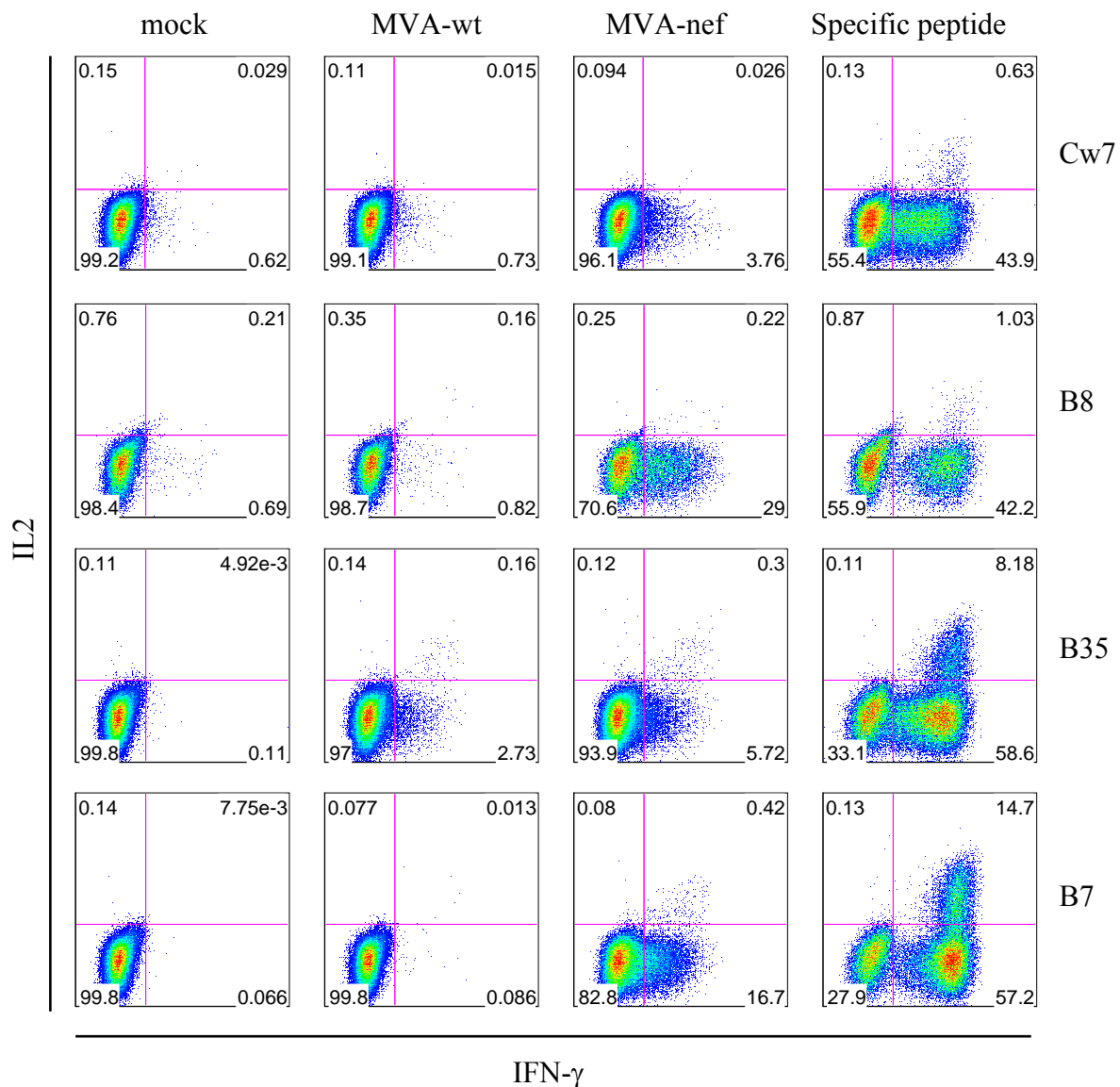




**Figure XIII: Expression of IFN- $\gamma$  in T-cell lines stimulated with MVA-nef infected B-LCL. Nef specific CD8 T-cell lines were stimulated with B-LCL infected with MVA-nef (red), MVA-wt (green) or mock infected (black) and stained for the expression of intracellular cytokines. Each graph represents a different CD8 T-cell line stimulated with autologous B-LCL. Different amounts of B-LCL were used and the Target / Effector ratio is shown on the x axis. Epitope specificity and HLA restriction are indicated in each graph.**



**Figure XIV: Expression of IL2 in T-cell lines stimulated with MVA-nef infected B-LCL.** Nef specific CD8 T-cell lines were stimulated with B-LCL infected with MVA-nef (red), MVA-wt (green) or mock infected (black) and stained for the expression of intracellular cytokines. Each graph represents a different CD8 T-cell line stimulated with autologous B-LCL. Different amounts of B-LCL were used and the Target / Effector ration is shown on the x axis. Epitope specificity and HLA restriction are indicated in each graph. As positive control, B-LCLs were loaded with the peptides recognized by the respective T-cell line. As expected, peptide loaded B-LCLs were able to stimulate strongly the specific T-cells (Figure XV). IFN- $\gamma$  production varied between 43% and 72% of the total CD3+ CD8+ cells. Of note, the B8 and Cw7 restricted T-cell lines that did not produced IL2 after stimulation with MVA-nef infected B-LCL, produced low amount of IL2 after the strong stimulation provided by the peptide loaded B-LCL. Thus, the absence of IL2 production in these T-cell lines was not a consequence of the Nef expression in the antigen presenting cells but it is an intrinsic characteristic of the specific T-cell lines.



**Figure XV: Expression of IFN- $\gamma$  and IL2 in T-cell lines stimulated with MVA-nef infected B-LCL. Nef specific CD8 T-cell lines were stimulated with B-LCL loaded with peptides corresponding to the epitopes recognized by the autologous B-LCL or they were infected with MVA-nef (red), MVA-wt (green) or mock infected (black). After 5 hours of stimulation, cells were stained for the expression of IFN- $\gamma$  and IL2. The quadrant gate in the pseudo color dot plots define the percentages of CD8+ T-cell that produce only IFN- $\gamma$ , only IL2 or both. The HLA restriction of the different T-cell lines is indicated on the right of the panel. Stimulation is indicated on the top of the panel. Only the graphs representing a Target / Effector ratio of 5 / 1 are shown.**

Similar results were obtained using a classical chromium release assay. The four epitopes specific T-cell lines were able to lyse efficiently B-LCL infected with the MVA-nef vector (data not shown). Altogether, these results indicate that Nef epitopes are generally presented by antigen presenting cells as B-LCL after infection with MVA-nef.

In summary, we demonstrated that the MVA vector expressing the HIV-1<sub>LAI</sub> Nef gene is able to express Nef in chicken embryo fibroblast, immortalized human B-cells (B-LCL) and human CD4 T-cell lines. The expressed Nef protein is functional in regard to its capacity to selectively downregulate HLA-A and -B molecules and CD4. Finally, Nef epitopes are correctly presented to the immune system by professional APC infected with MVA-nef.

### ***Therapeutic vaccination with MVA-HIV-1 nef in chronically HIV-1 infected individuals.***

#### **Description of the phase I vaccination trial**

Ten chronically HIV-1 infected patients were included in the study according to the following inclusion criteria:

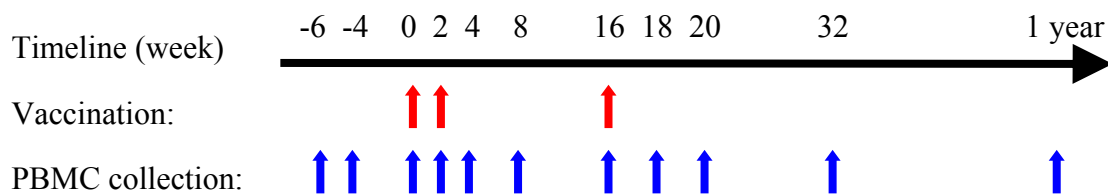
- Male or female subjects, aged >18 years
- Asymptomatic HIV-infection with two documented positive HIV-1 antibody tests
- Stable on anti-retroviral therapy for at least 6 months
- Karnofsky performance status >80%
- CD4 cell counts above 400/microliter (mean of two determination)
- Written informed consent signed prior to study entry
- Negative pregnancy test

Exclusion criteria were the following:

- Uncontrolled infection i.e. not responding to antimicrobial therapy
- Recent (less than 6 months) myocardial infarction

- Creatine > 2mg/dl
- Hemoglobin (Hb) <9g/dl
- Leukocytes <3000/microliter
- Platelets<50000/microliter
- Liver function Tests (LFT) > 5x upper limit of normal
- Any continuous therapy that may influence CD4 counts other than anti-retroviral therapy
- Any immune modifying therapy within 4 weeks prior to entry
- Participation in any other investigational drug trial

Ten subjects matching the entry criteria received three immunizations given by subcutaneous route at week 0, 2 and 16. Each vaccine dose consisted of  $5 \times 10^8$  infectious units of MVA-HIV-1<sub>LAI</sub>-nef in 1ml of phosphate buffer. Blood was collected for three times before vaccine administration, after each vaccine administration and one year after the third vaccination as shown in Figure XVI.



**Figure XVI: Timing of vaccination and blood cells collection**

### **Safety of the MVA-nef vaccination**

The vaccine was safe and we did not observe any adverse reaction for the entire follow up. In particular, total CD4 counts and viral load were maintained for all the study period and no changes associated with the vaccine administration were observed. For a detailed description, see Cosma et al. (Cosma et al., 2003).

### **Characterization of the Nef-specific cellular immune response**

CD8 and CD4 T-cell immune responses were assessed using an IFN- $\gamma$  based intracellular cytokine staining (ICS). Pools of overlapping peptides were used to stimulate freshly isolated PBMC before staining for extracellular markers (CD3, CD8 and CD4) and intracellular IFN- $\gamma$ . The pools used to stimulate the PBMC are shown in Table III. The immune response to Nef

was characterized using both 20mer overlapping by 10 and a selection of optimal CD8 epitopes (Nef Opt). As controls, we used pools of overlapping peptides derived from the HIV regulatory proteins Tat and Rev, and from the HIV structural proteins p17 and p24.

**Table III**

| Protein       | Peptide length | Overlap         | HIV-1 strain |
|---------------|----------------|-----------------|--------------|
| Nef           | 20mer          | 10              | LAI          |
| Nef (Nef Opt) | 8 to 11        | Not overlapping | LAI and SF2  |
| Tat           | 20mer          | 10              | LAI          |
| Rev           | 20mer          | 10              | LAI          |
| p17           | 15mer          | 5               | SF2          |
| p24           | 20mer          | 10              | SF2          |

Before the administration of the MVA-nef vaccine, all the 10 subjects showed a CD8 Nef-specific immune response at least against one of the two Nef pools tested (Figure XVII A and C, and Figure XVIII). During the longitudinal follow up of the CD8 T-cell response, we observed a clear increase of the Nef-specific immune response temporally associated with the vaccine administration in subject 8 and 10. While in the other eight subjects an association between vaccination and increase of the Nef-specific CD8 immune response was not evident (Figure XVIII). CD4 immune response to Nef was present only in two subjects before the administration of the vaccine (subject 3 and 10). Interestingly, after the first vaccine administration at week 2, eight out of ten subjects showed an increased CD4 immune response to Nef (Figure XVII C and Figure XIX) and this response was boosted after the second and third vaccination in subject 4 and 10. Of note in subject 10, we were able to detect 1% of CD4 T-cells specific to Nef after the third vaccination at week 18. For a detailed description see also Cosma et al. (Cosma et al., 2003). The follow up of the CD8 and CD4 immune response to Tat, Rev, p17 and p24 was carried out in parallel to the characterization of the Nef-specific immune response to monitor changes in the anti-HIV immune response and verify the specificity of the Nef-specific responses elicited by the vaccine. CD8 immune responses to Tat, Rev, p17 and p24 remained mostly unchanged during the follow up (Figure XX). Five out of ten subjects showed already a p24-specific immune response to Nef before the vaccine administration and in two of them (subject 3 and 8) an increase of the p24-specific CD8 immune response was detected after the first and the second vaccination, respectively. Subject number 1 had a strong and variable immune response to Tat for all the follow up

period. Similarly, CD4 immune responses remain unchanged and almost undetectable for all the time of the study (Figure XXI). However, in subjects 1, 3 and 8, we observed an increase of the p24-specific CD4 immune response temporally associated with the vaccine administration. In summary, except for the p24-specific immune response, temporally associated variations of the immune response directed to the other HIV-1 proteins tested were not detected.

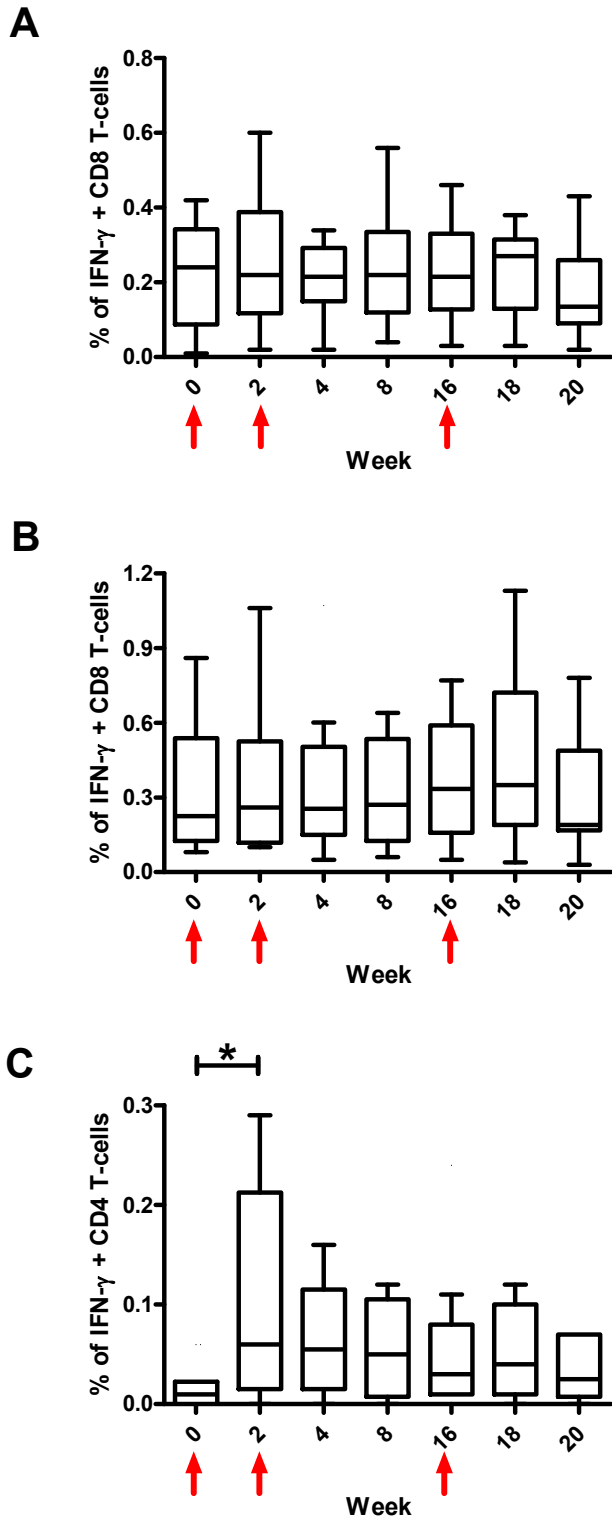


Figure XVII: CD8 and CD4 T-cell responses to Nef. Tukey Whiskers plots of the CD8 (A, B) and CD4 (C) responses to Nef measured using overlapping peptide (A, C) or a pool of optimal CD8 epitopes (B) are shown for each time point. The red arrows indicate the time of vaccination. The asterisk indicates a p value < 0.05 using a Wilcoxon matched pair test.

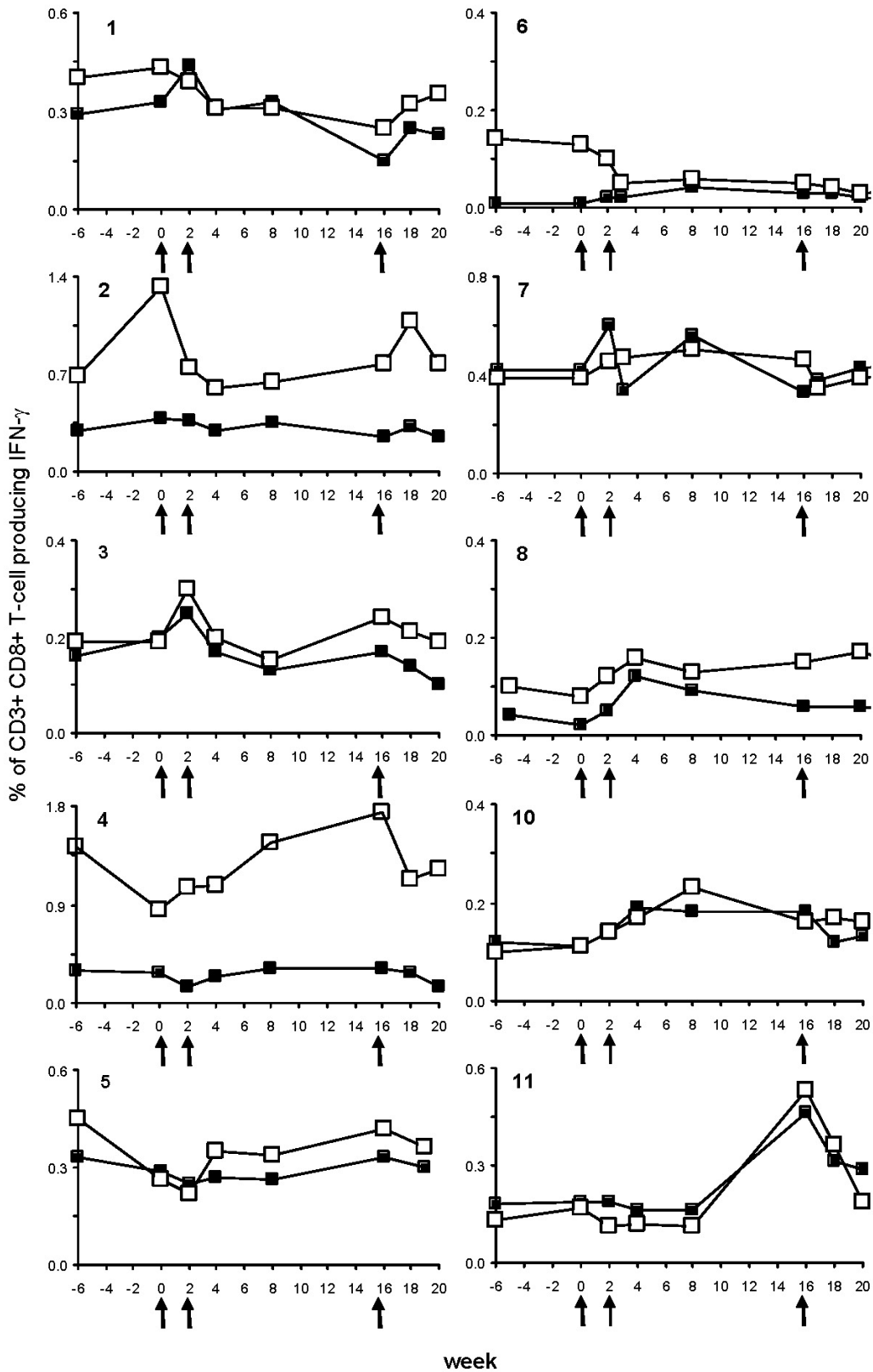
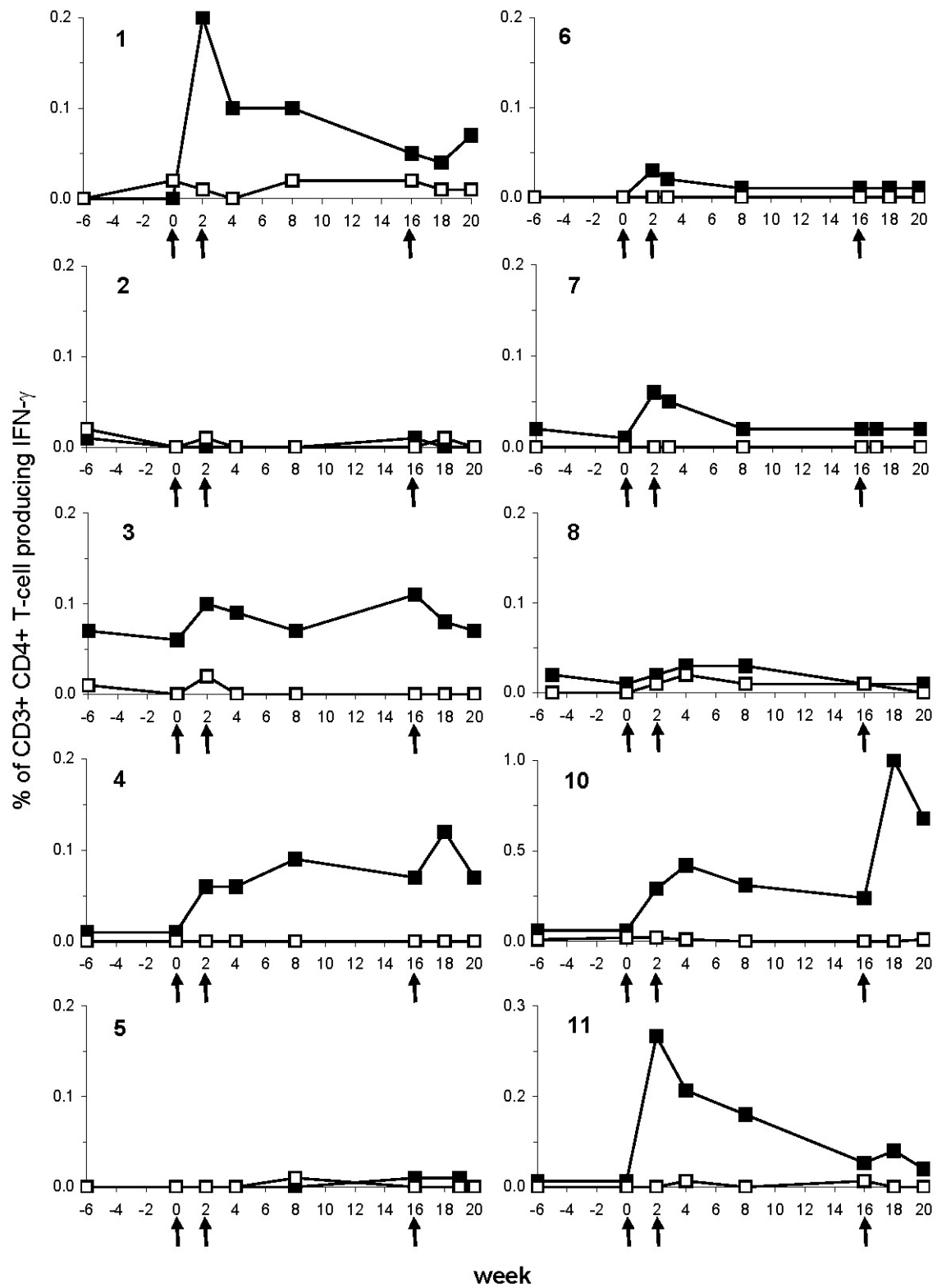
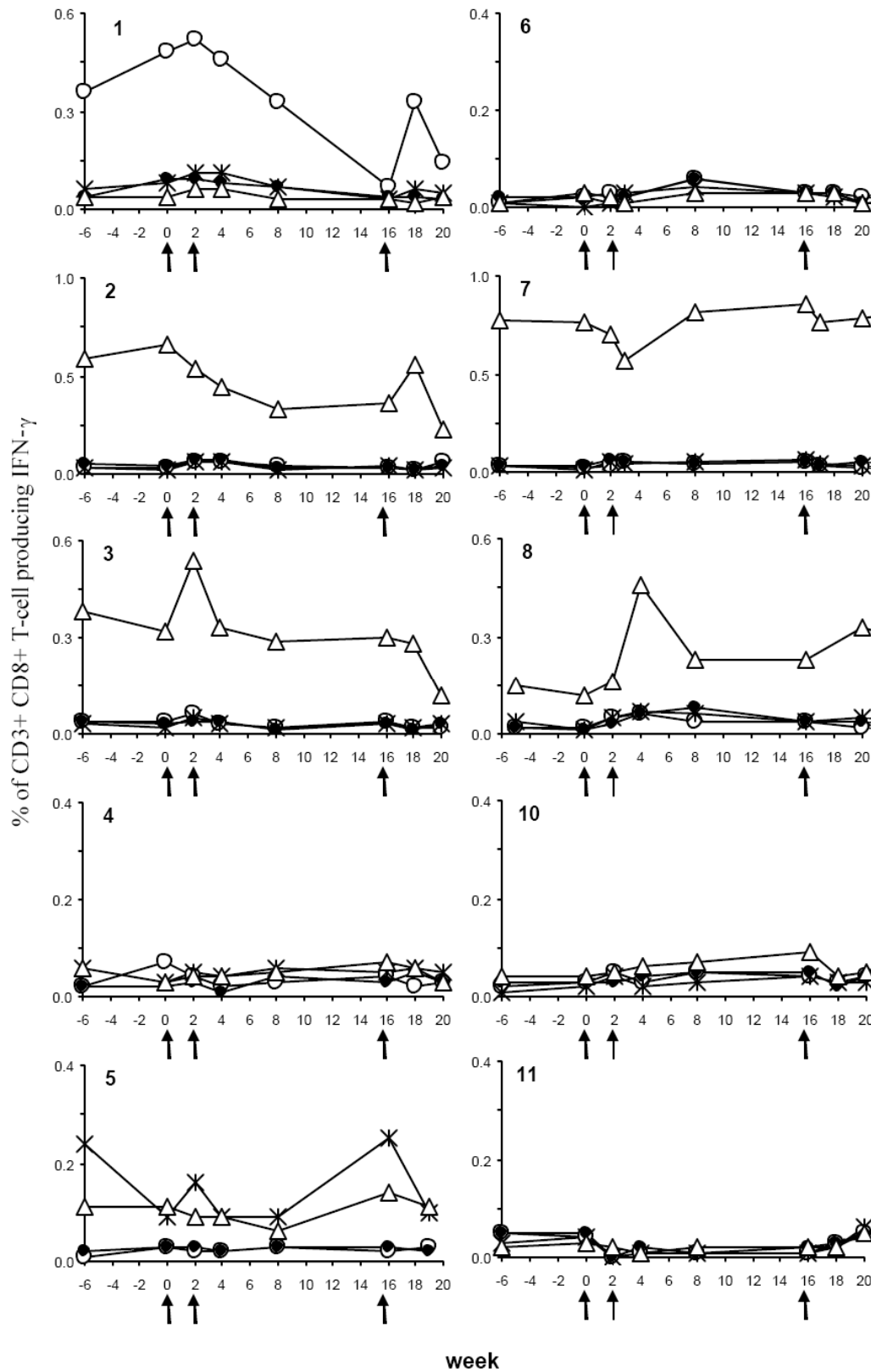


Figure XVIII: Follow up of the CD8 T-cell responses to Nef. PBMC were stimulated with 20mer peptide overlapping by 10 (closed square) or a pool of optimal CD8 epitopes (open circles). Each graph depicts the immune response observed in one individual. The arrows indicate the time of vaccination.

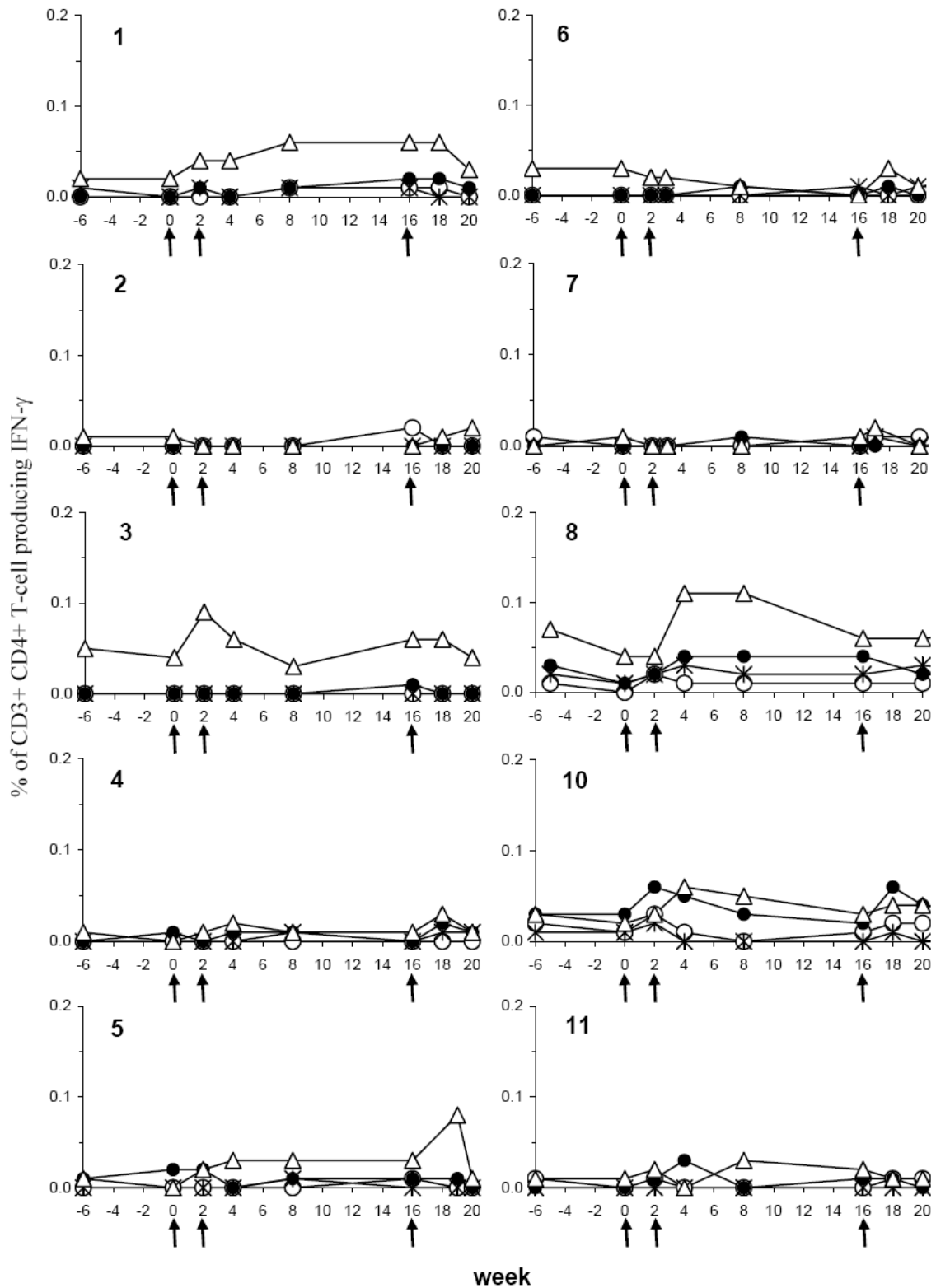




**Figure XIX: Follow up of the CD4 T-cell responses to Nef. PBMC were stimulated with 20mer peptide overlapping by 10 (closed square) or a pool of optimal CD8 epitopes (open circles). In this case, the pool of optimal CD8 epitopes serve as negative control. Each graph depicts the immune response observed in one individual. The arrows indicate the time of vaccination**



**Figure XX: Follow up of the CD8 T-cell responses to Tat (open circle), Rev (asterisk), p17 (closed circle) and p24 (open triangle). Each graph depict the immune response observed in one individual. The arrows indicate the time of vaccination.**



**Figure XXI: Follow up of the CD4 T-cell responses to Tat (open circle), Rev (asterix), p17 (closed circle) and p24 (open triangle). Each graph depict the immune response observed in one individual. The arrows indicate the time of vaccine administration.**

### Characterization of the long-lasting memory immune response

One year after the last vaccine administration, we assessed if the CD4 immune response elicited by the vaccination was still detectable in the eight responder individuals. It was not possible to include subject 3 in our study, since he interrupted the antiretroviral therapy immediately after the end of our study without the advice of the physicians. All the other 7 patients continued to assume regularly the antiretroviral therapy and blood samples were collected to measure the residual CD4 response specific to Nef. Results are shown in Figure XXII.

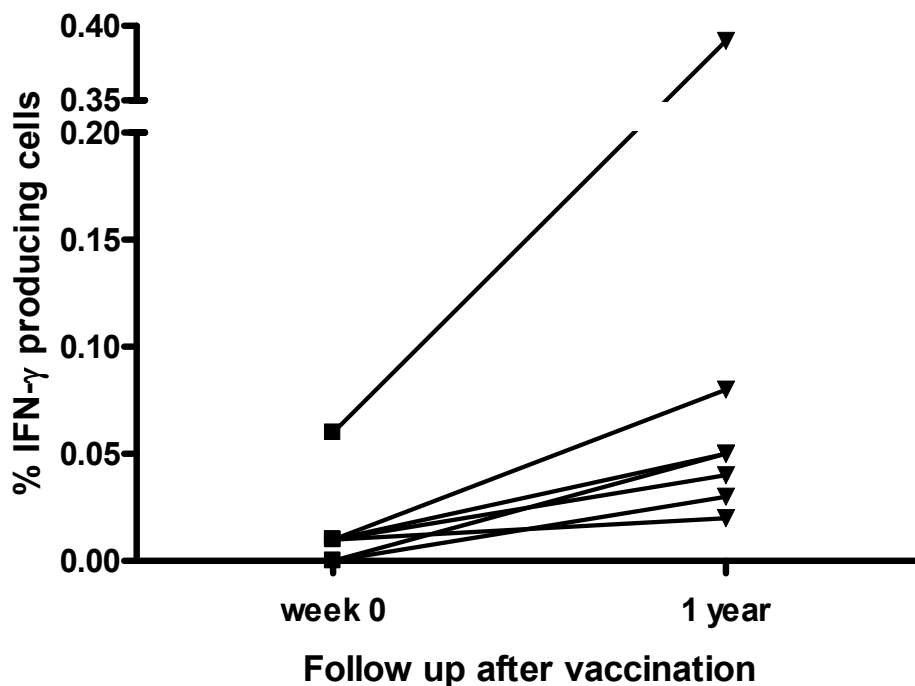


Figure XXII: Characterization of the Nef-specific CD4 immune response one year after the last vaccine administration. The following subjects are shown: 1, 4, 6, 7, 8, 10 and 11.

CD4 T-cells producing IFN- $\gamma$  after stimulation with the pool of Nef derived peptides were detected in all the seven subjects and frequencies were significantly higher than frequencies detected before the administration of the vaccine in the same subjects ( $p=0,0156$ , Wilcoxon signed rank test). Thus, the MVA-nef vaccine was able to elicit a long lasting CD4 immune response specific to Nef.

### ***Comparison between the immune response elicited by MVA-nef and the immune response observed in LTNP***

To understand better the quality of the immune response elicited by the MVA-nef vaccine, we analyzed the Nef-specific CD4 and CD8 immune response in a cohort of chronically HIV infected individual and in a cohort of long term non-progressor. As controls, CD4 and CD8 immune responses specific to Tat, Rev, p17 and p24 were also screened. Specific immune responses were defined by IFN- $\gamma$  production in CD8 and CD4 T-cells after stimulation with pools of overlapping peptides (see Table III).

The cohort of chronically HIV infected individuals was composed of 18 individuals diagnosed as HIV-1 infected for a median of 71 months (range 26 to 183 months) and treated for a median of 62 months (range 23 to 171 months). The median CD4 count was 513 cells/mm<sup>3</sup>, with a range between 285 and 1110 cells/mm<sup>3</sup>. Seven individuals had detectable but low viral loads ranging from 105 to 20417 copies RNA/ml. In all the other individuals, the viral load was less than 50 copies RNA/ml. The clinical characteristic of the single patients at the time of sample evaluation are shown in Table IV.

**Table IV Clinical characteristic of the 18 chronically HIV infected subjects**

| Patient ID | Date of birth | HIV+<br>diagnosis<br>(months) | Antiretroviral<br>treatment<br>(months) | Plasma viral<br>load (RNA<br>copies/ml) | CD4<br>counts<br>(cells/mm <sup>3</sup> ) |
|------------|---------------|-------------------------------|---|---|---|
| 01-0910    | 25/4/1957     | 99                            | 96                                      | 15849                                   | 334                                       |
| 01-1610    | 6/1/1942      | 70                            | 56                                      | 1122                                    | 285                                       |
| 01-2310    | 27/3/1956     | 109                           | 57                                      | 50                                      | 737                                       |
| 02-0910    | 5/8/1977      | 26                            | 23                                      | 1047                                    | 502                                       |
| 03-0910    | 30/5/1948     | 116                           | 110                                     | 2455                                    | 444                                       |
| 03-1610    | 17/9/1940     | 183                           | 171                                     | 118                                     | 780                                       |
| 04-2310    | 3/8/1960      | 62                            | 58                                      | 50                                      | 524                                       |
| 04-1610    | 25/1/1965     | 60                            | 59                                      | 50                                      | 588                                       |
| 05-2310    | 10/1/1959     | 63                            | 61                                      | 50                                      | 317                                       |
| 05-1610    | 24/1/1948     | 72                            | 70                                      | 105                                     | 459                                       |
| 06-1610    | 8/5/1945      | 65                            | 63                                      | 20417                                   | 634                                       |
| 09-2210    | 29/3/1954     | 115                           | 59                                      | 50                                      | 1110                                      |
| 01-1003    | 1/2/1962      | 76                            | 75                                      | 50                                      | 969                                       |
| 02-1003    | 20/6/1970     | 116                           | 74                                      | 50                                      | 609                                       |
| 03-1003    | 30/5/1966     | 72                            | 69                                      | 50                                      | 347                                       |
| 04-0604    | 9/11/1969     | 45                            | 44                                      | 50                                      | 334                                       |
| 05-1904    | 22/9/1943     | 55                            | 53                                      | 50                                      | 688                                       |
| 06-1904    | 2/8/1967      | 66                            | 64                                      | 50                                      | 455                                       |

The characteristics of the ten chronically HIV infected individuals enrolled in the MVA-nef vaccination trial, sampled six weeks before the administration of the vaccine, are shown in Table V. They were diagnosed as HIV-1 infected for a median of 124 months (range 25 to 209 months) and treated for a median of 61.5 months (range 24 to 146 months). The median CD4 count was 565.5 cells/mm<sup>3</sup> (range 407 to 1421 cells/mm<sup>3</sup>). Eight individuals had undetectable viral load while in two had detectable but stable viral load.

**Table V Clinical characteristic of the 10 chronically HIV infected subjects enrolled in the MVA-nef vaccination trial**

| Patients | Date of birth | HIV+ diagnosis (months) | Antiretroviral treatment (months) | Plasma viral load (RNA copies/ml) | CD4 counts (cells/mm <sup>3</sup> ) |
|----------|---------------|-------------------------|-----------------------------------|-----------------------------------|-------------------------------------|
| 1        | 6/10/1959     | 185                     | 54                                | 50                                | 407                                 |
| 2        | 13/8/1944     | 149                     | 64                                | 6077                              | 803                                 |
| 3        | 3/7/1955      | 209                     | 66                                | 8710                              | 1116                                |
| 4        | 23/6/1955     | 48                      | 34                                | 50                                | 1421                                |
| 5        | 11/3/1962     | 187                     | 66                                | 50                                | 584                                 |
| 6        | 22/5/1937     | 99                      | 41                                | 50                                | 782                                 |
| 7        | 1/7/1949      | 66                      | 60                                | 50                                | 473                                 |
| 8        | 6/7/1960      | 83                      | 63                                | 50                                | 549                                 |
| 10       | 16/7/1963     | 197                     | 146                               | 50                                | 488                                 |
| 11       | 6/1/1962      | 25                      | 24                                | 50                                | 488                                 |

Statistical analysis did not reveal any significant difference between the ten chronically HIV infected individuals enrolled in the MVA-nef vaccination trial and the group of 18 chronically HIV-1 infected subjects (Figure XXIII). Therefore, the 18 chronically HIV-1 infected individuals were taken as reference group to study changes in the status of the immune response following MVA-nef vaccination.

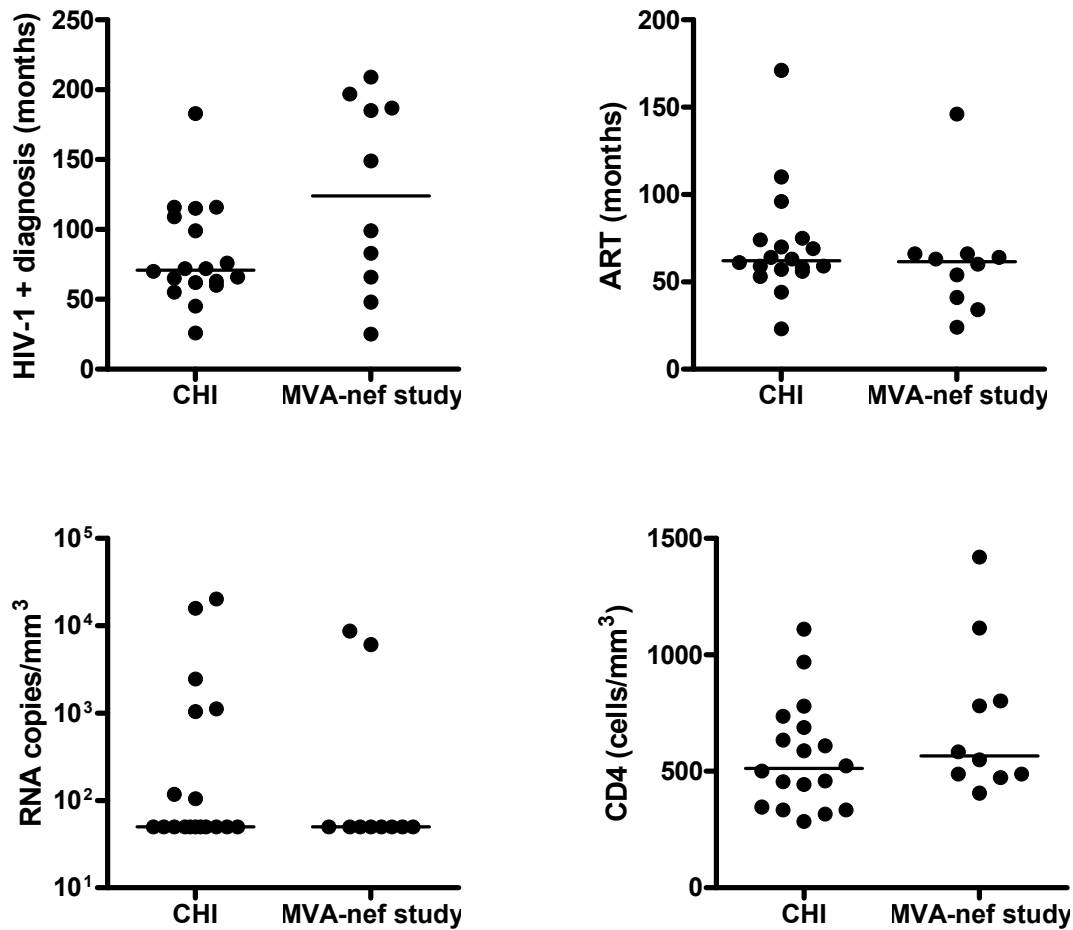


Figure XXIII: Comparison between the clinical characteristics observed in 18 chronically HIV-1 infected individuals (CHI) and in the 10 chronically HIV-1 infected individuals enrolled in the MVA-nef study (MVA-nef study).

The cohort of LTNP was composed of 12 individuals. All the subjects were antiretroviral naïve at the time of sample evaluation and were diagnosed as HIV-1 infected between 58 and 228 months (median 200 months). The viral load ranged from 214 to 91201 copies RNA/ml (median 1600 copies RNA/ml) and total CD4 counts ranged from 134 to 626 cells/mm<sup>3</sup> (median 338 cells/mm<sup>3</sup>). The characteristic of the single patients at the time of sample evaluation are shown in Table VI.

Table VI: Clinical characteristics of the 12 LTNP

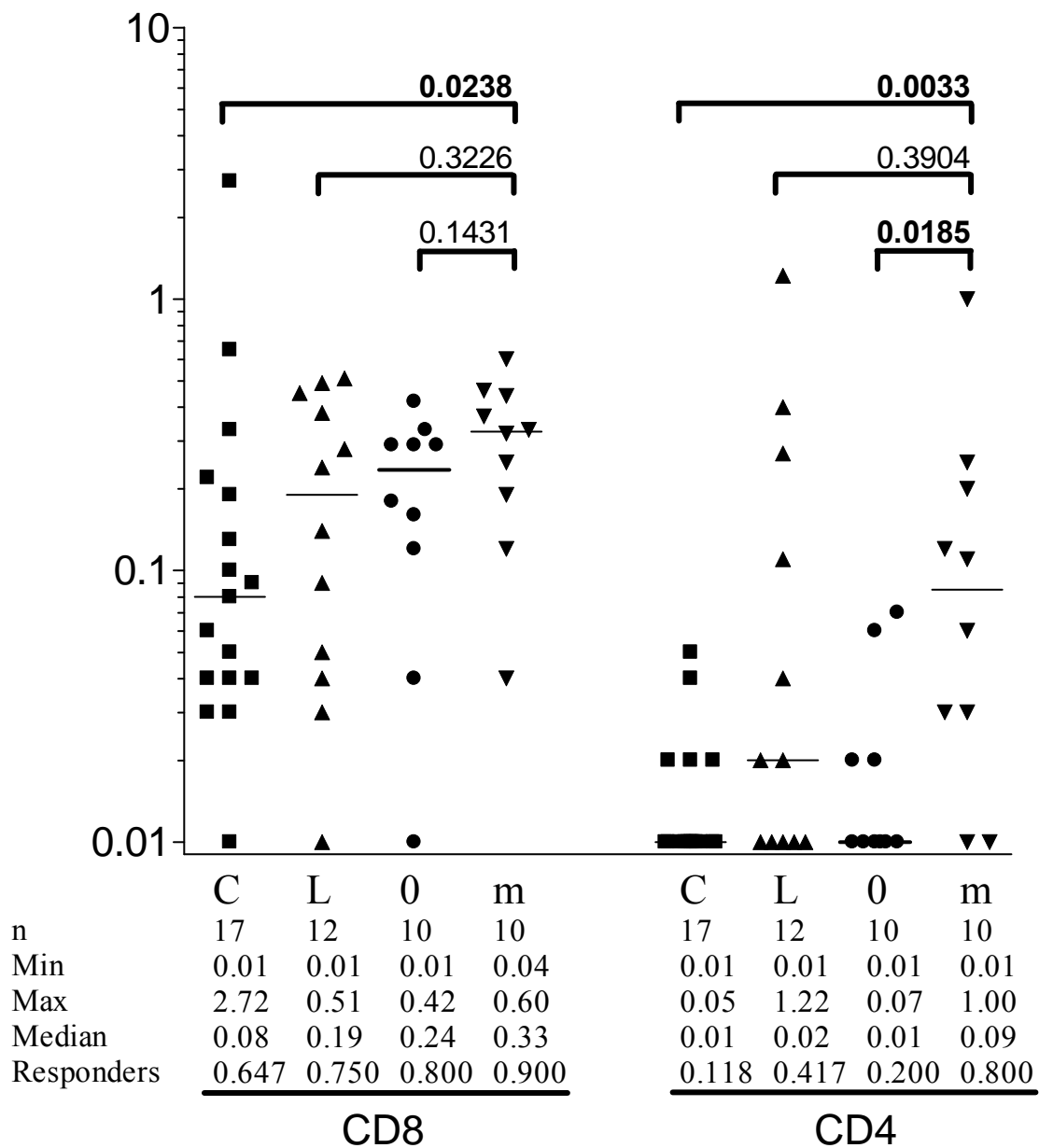
| Patients | Date of birth | HIV+ diagnosis (months) | Antiretroviral treatment (months) | Plasma viral load (RNA copies/ml) | CD4 counts (cells/mm <sup>3</sup> ) |
|----------|---------------|-------------------------|-----------------------------------|-----------------------------------|-------------------------------------|
| L1       | 4/2/1948      | 210                     | -                                 | 3631                              | 354                                 |
| L2       | 9/5/1965      | 217                     | -                                 | 31623                             | 377                                 |



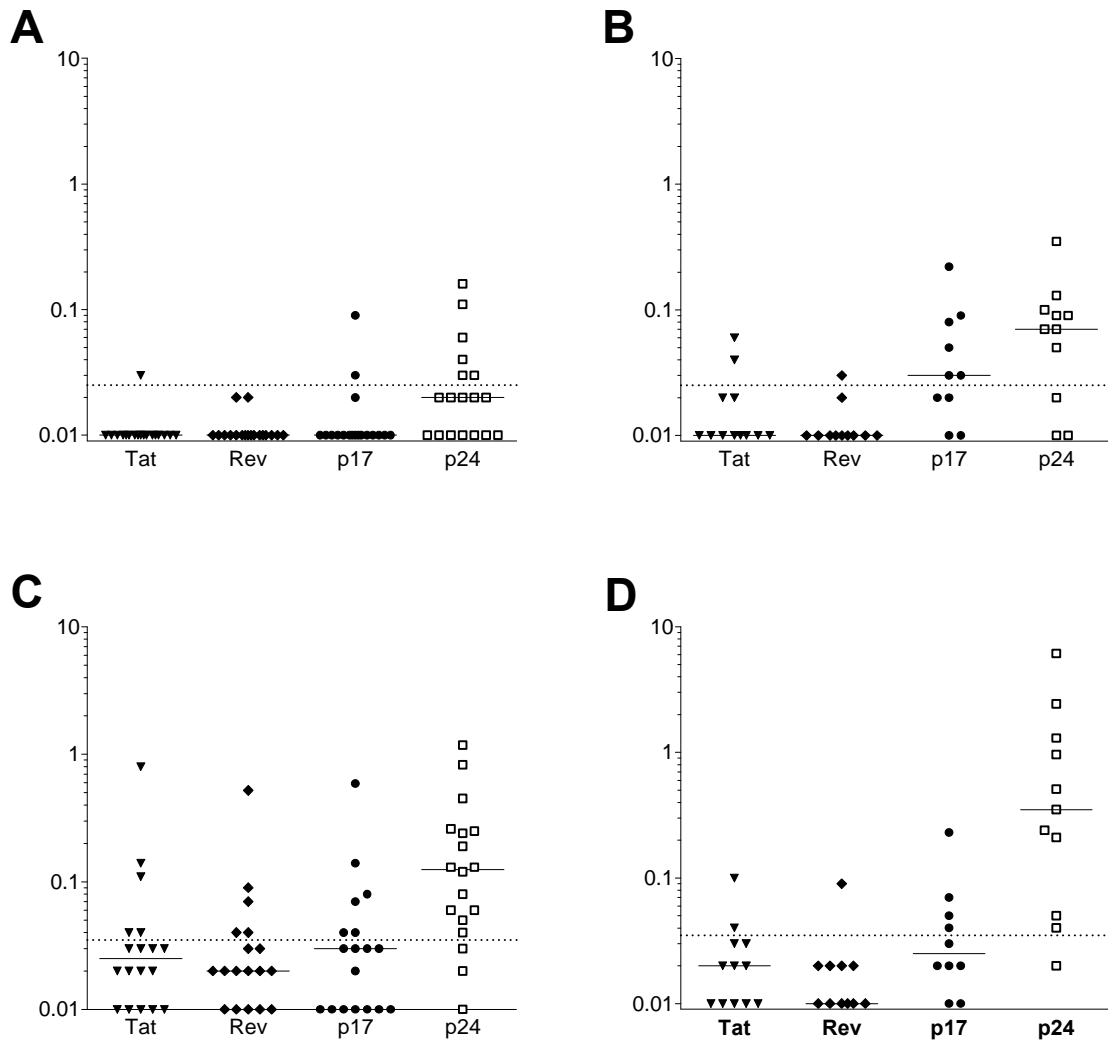
|     |            |     |   |       |     |
|-----|------------|-----|---|-------|-----|
| L3  | 25/4/1961  | 190 | - | 1023  | 276 |
| L4  | 15/10/1970 | 115 | - | 8511  | 322 |
| L5a | 1/2/1962   | 213 | - | 91201 | 281 |
| L6  | 22/9/1953  | 225 | - | 214   | 626 |
| L7  | 24/5/1958  | 195 | - | 1300  | 134 |
| L8  | 19/1/1952  | 160 | - | 720   | 466 |
| L9  | 31/10/1961 | 205 | - | 1100  | 421 |
| L10 | 5/9/1964   | 58  | - | 1400  | 204 |
| L11 | 22/6/1961  | 228 | - | 1800  | 274 |
| L12 | 14/7/1953  | 101 | - | 2400  | 525 |

To compare the immune response elicited by the MVA-nef vaccine with the immune responses usually observed in LTNP and chronically HIV infected subjects, we decided to consider the maximal immune response observed after vaccine administration during the 32 weeks study period in the 10 vaccinated subjects. In keeping with the results shown previously, the median Nef-specific CD4 response after vaccine administration was significantly higher than the median observed before the administration of the vaccine (Figure XXIV). Interestingly, the median Nef-specific CD4 response was also significantly higher than the median observed in 18 chronically HIV-1 infected individuals, thus demonstrating a significant change not only in comparison to the previous responses but also in comparison to the general responses observed in chronically HIV-1 infected individuals. When we analyzed the Nef-specific CD4 T-cell responses in a cohort of LTNP (Table VI), we observed a heterogeneous response. Seven LTNP did not show Nef-specific CD4 responses, while five LTNP showed responses similar to the MVA-nef vaccinated subjects. These data suggest that the MVA-nef vaccine is able to stimulate Nef-specific CD4 responses with a magnitude similar to that observed in LTNP. The analysis of the Nef-specific CD8 responses did not reveal major differences between study subjects sampled before and after vaccination. However, CD8 Nef responses in the chronically HIV-1 infected individuals enrolled in the MVA-nef study were in general higher than CD8 Nef responses in the reference cohort of HIV-1 infected individuals (Figure XXIV). As control immune response to Tat, Rev, p17 and, p24 were analyzed in chronically HIV-1 infected subjects and LTNP (Figure XXV). CD4 responses to p17 and p24 were detected more frequently in LTNP than in chronically HIV-1 infected individuals. Eight out eleven (72%) LTNP showed a significant CD4 T-cell response to p24 while only six out of eighteen (33%) showed the same response in chronically HIV-1 infected individuals. A similar pattern was observed for the p17 specific CD4 responses. CD8 responses to p24 were equally found in LTNP and chronically HIV-1 infected individuals. However, the magnitudes of the responses were higher in LTNP. No differences between

LTNP and chronically HIV-1 infected individuals were observed for immune responses specific to Tat and Rev.



**Figure XXIV: The Nef-specific CD4 responses elicited by MVA-nef are similar to CD4 responses observed in LTNP. Comparison of Nef-specific CD8 and CD4 responses measured in chronically HIV-1 infected subjects (C), LTNP (L), study subjects before (0) and after MVA-nef vaccination (m). Immune response were determined measuring the production of IFN- $\gamma$  following stimulation with Nef derived peptides as described previously. The median is shown for each group.**



**Figure XXV: Analysis of the CD4 and CD8 immune responses in LTNP and chronically HIV-1 infected individuals. CD4 (A and B) and CD8 (C and D) responses were measured in LTNP (B and D) and chronically HIV-1 infected individuals (A and C). Each point represents one individuals. Median is shown for each cohort and antigen tested. Dotted lines represent the cut off for a positive CD8 and CD4 response. As previously discussed, a clear correlate of protection does not exist. However, we can deduce that our MVA-nef vaccine was able to change the immunological status of the vaccinated chronic HIV-1 infected individuals, rendering them more similar to LTNP. Obviously, this change is focused only on the Nef-specific immune response.**

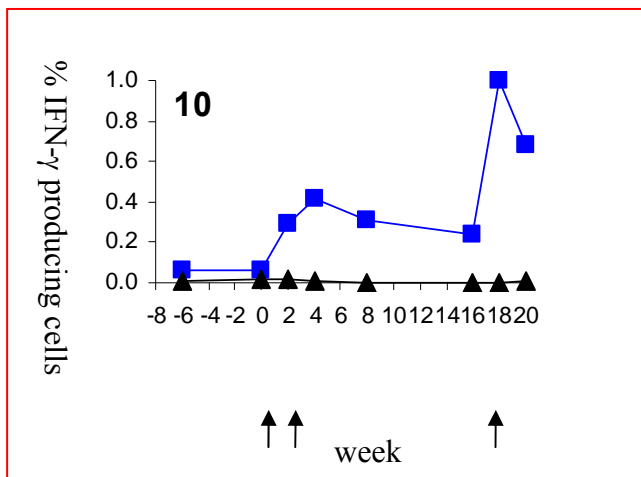
### ***Evaluation of MVA as alternative vaccine against smallpox***

Although increasing evidences in animal models suggest that MVA might be an alternative vaccine against smallpox for individuals at risk of the side effects induced by the classical smallpox vaccine (Belyakov et al., 2003; Earl et al., 2004; Wyatt et al., 2004), the immune responses elicited by MVA in these high risk individuals have not yet been characterized. In

addition, in our study we notice that the only subject not receiving smallpox vaccination in his childhood demonstrated the strongest immune response to Nef (Figure XXVI). Therefore, in our study to evaluate safety and immunogenicity of the MVA-nef vector, we included a complete characterization of the vaccinia specific immune response elicited by the vaccine.

**Table I Patient characteristics**

| Subject         | Date of birth | Smallpox vaccination |
|-----------------|---------------|----------------------|
| 1 <sup>a</sup>  | 1959          | yes                  |
| 2 <sup>a</sup>  | 1944          | yes                  |
| 3 <sup>a</sup>  | 1955          | yes                  |
| 4 <sup>a</sup>  | 1955          | yes                  |
| 5 <sup>a</sup>  | 1962          | yes                  |
| 6 <sup>a</sup>  | 1937          | yes                  |
| 7 <sup>a</sup>  | 1949          | yes                  |
| 8 <sup>a</sup>  | 1960          | yes                  |
| 10 <sup>a</sup> | 1963          | no                   |
| 11 <sup>a</sup> | 1962          | yes                  |



**Figure XXVI: In subject 10, following the MVA-nef vaccination we observed the higher frequency of CD4+ lymphocytes producing IFN- $\gamma$ , a boost of the immune response at each vaccine administration and an expansion of the targeted Nef epitopes.**

### **Vaccinia specific immune responses were readily detected after MVA-nef vaccination**

Humoral immune responses were measured using an innovative neutralization assay developed in our laboratory (Cosma et al., 2004), together with a classical ELISA that used highly purified viral particles or recombinant vaccinia virus proteins. Cellular immune responses were measured using the same intracellular cytokine staining used to measure the Nef-specific immune response. In this case, however, PBMC were stimulated with autologous PBMC previously infected with MVA. MVA was able to elicit vaccinia specific humoral and cellular immune in all the ten vaccinated subjects (Figure XXVII) (Cosma et al., 2007). These responses were maintained over one year demonstrating the capacity of MVA to elicit long lasting immune responses.

To assess whether humoral immune responses and CD8 T-cell responses were equally regulated we performed a Spearman's rank correlation between neutralizing titers and % of

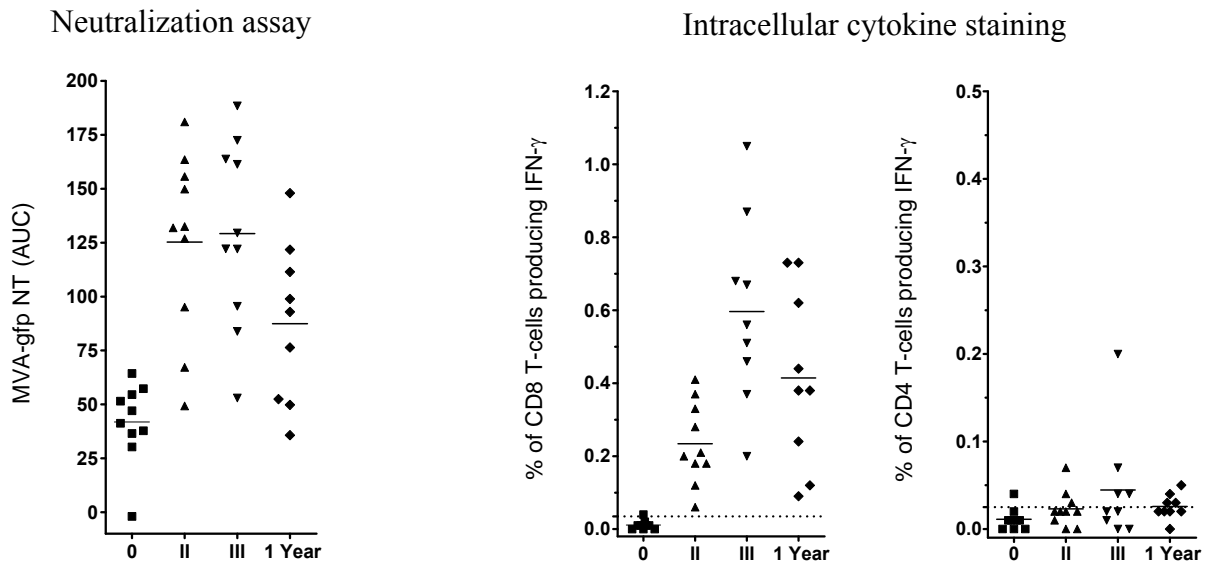
IFN- $\gamma$  producing CD8 T-cells for all the time points tested. As shown in Figure XXVIII no correlations were found. For instance, subject number 2 who showed the lowest neutralizing activity demonstrated a strong CD8 T-cell response after the second MVA administration (0.37%) and maintained a strong response for one year (0.38%). Similarly, subject 10, the only one that was not vaccinated against smallpox as a child and in which we observed low titers of antibodies, demonstrated a strong CD8 T-cell response with 0.20% of IFN- $\gamma$  producing cell after two MVA administrations, 0.46% after three MVA administrations and 0.24% one year after the last immunization.

### **Antibodies detected after vaccination with MVA were mainly of the IgG class**

A possible association between protection from monkeypox and presence of vaccinia specific IgG was recently described in SIV infected monkeys (Edghill-Smith et al., 2005a). SIV infected monkeys vaccinated with Dryvax or MVA followed by Dryvax were fully protected after a lethal monkeypox challenge only when their CD4 T-cell levels were above 300 cells/mm<sup>3</sup>. Monkeys with CD4 T-cells levels above 300 cells/mm<sup>3</sup> had high IgG titers and low IgM titers while highly immunocompromised monkeys with CD4 T-cells levels < 300 cells/mm<sup>3</sup> had low IgM and IgG titers. These data suggest that the presence of high titers of vaccinia specific IgG is a protection marker for monkeypox in monkeys and could be similar for smallpox in humans.

Since CD4 T-cell counts in our cohort of chronically HIV-1 infected individuals were > 400 cells/mm<sup>3</sup> (Table VII), MVA should be able to induce high titers of vaccinia specific IgG. To verify this hypothesis, we performed an ELISA to measure vaccinia specific IgG and vaccinia specific IgM on the sera collected after the third MVA administration.

As shown in Table VII, IgG titers ranged between > 25,600 and 1600 while IgM titers were significantly lower ranging between 200 and < 100 (Mann-Whitney U test,  $p < 0.0001$ ). The observed IgG and IgM titers were consistent with titers observed in monkeypox protected monkeys (Edghill-Smith et al., 2005a).



**Figure XXVII: MVA-specific humoral and cellular immune responses in MVA-nef vaccinated subjects.** Samples were tested before MVA administration (0), after two immunizations (II), after three immunizations (III), and 1 year after the third immunization (1 Year). Humoral immune responses were measured using a neutralization assay and the neutralization capacity is expressed in terms of area under the curve (AUC). CD8 and CD4 T-cells specific to MVA were measured using an IFN- $\gamma$ -based intracellular cytokine staining. A cut off of 0.04% was applied to CD8 responses while a cut off of 0.03% was applied to CD4 responses. The cut off line is shown in each graph.

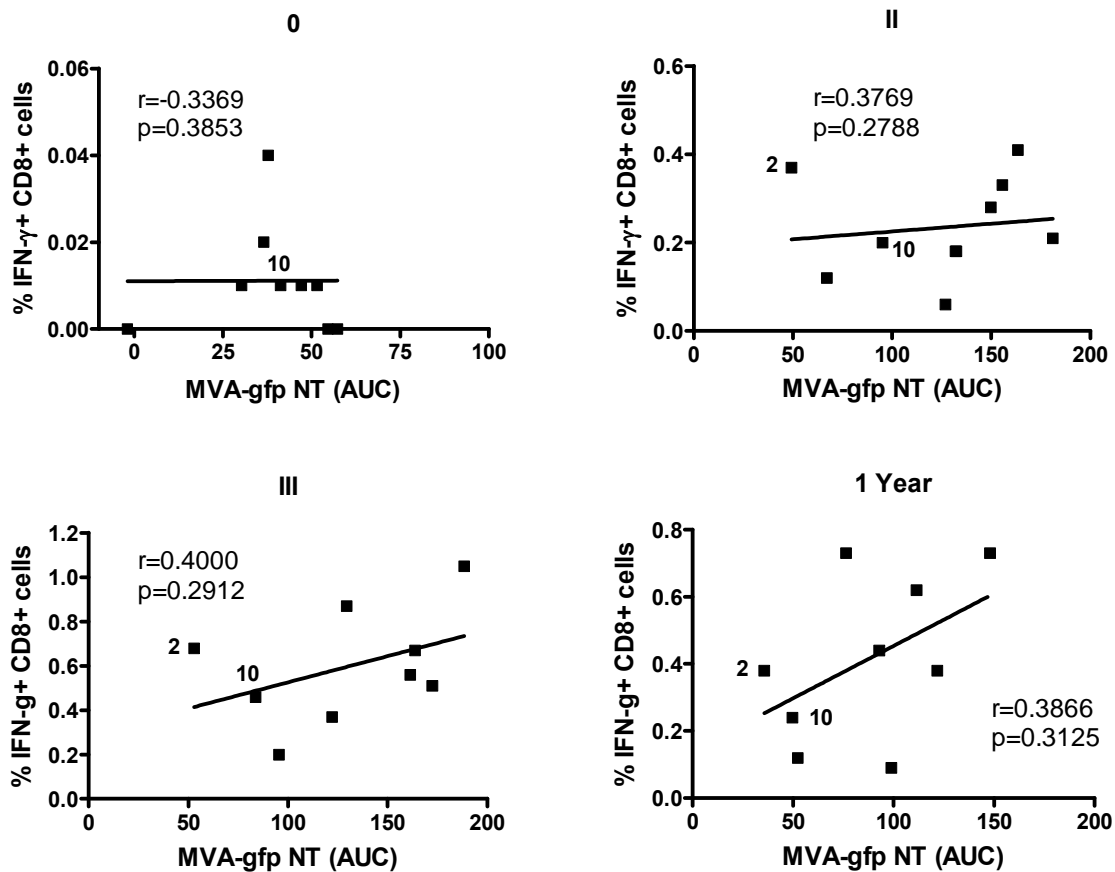


Figure XXVIII: Correlation analysis between humoral and cellular immune response to vaccinia virus. The correlation between neutralizing activity (MVA-gfp NT) and frequency of vaccinia specific CD8 T-cells (% of IFN- $\gamma$ + CD8+ cells) was determined by Spearman's rank correlation. R and P value are shown in each figure. The solid line represents a regression line. Data points relative to the individuals discussed in the text are indicated. Subject 10 was vaccinia naïve while subject 2 received smallpox vaccination as children. Since PBMC were not available, the cellular immune response for subject number 2 at (0) and subject number 4 at (III) were not assessed.

**Table VII: ELISA titer and total CD4 counts. (0) before vaccination, (III) after the third vaccination**

| Subject | CD4 counts | MVA ELISA |         |         |      |
|---------|------------|-----------|---------|---------|------|
|         | Baseline   | IgG/IgM   |         | IgG     | IgM  |
|         |            | 0         | III     | III     | III  |
| 1       | 407        | 800       | 3,200   | 6,400   | 200  |
| 2       | 803        | 400       | 1,600   | 1,600   | 100  |
| 3       | 1116       | 800       | 3,200   | 12,800  | 100  |
| 4       | 1421       | 400       | 3,200   | 12,800  | 100  |
| 5       | 584        | 400       | 12,800  | 12,800  | 100  |
| 6       | 782        | 800       | 12,800  | 12,800  | <100 |
| 7       | 473        | 400       | 3,200   | 6,400   | <100 |
| 8       | 549        | 1600      | 6,400   | 12,800  | <100 |
| 10      | 488        | 400       | 3,200   | 3,200   | <100 |
| 11      | 488        | 1600      | >12,800 | >25,600 | 100  |

Eradication of smallpox demonstrated that the immune response elicited by the classical vaccination was remarkably effective. However, since variola virus is now eradicated, it is not possible to establish if the residual immunity still detectable in individuals vaccinated more than 30 years ago is protective. The same applies to immune responses elicited by alternative vaccines against smallpox such as MVA. Therefore, to determine the efficacy of the immune response elicited by MVA in chronically HIV infected subjects we characterized the vaccinia specific immune response in four healthy subjects that were recently vaccinated with the classical smallpox vaccine and/or MVA. Theoretically, these subjects should have a vaccinia specific immune response similar or higher to that that was present during the smallpox vaccination campaign. Vaccination status and results are summarized in Table VIII. Subject 32 demonstrated the highest titer of neutralizing antibodies. The neutralizing titer was similar to the mean observed in chronically HIV-1 infected individuals one year after the last vaccination with MVA (Figure XXVII). In the same subject, cellular immune responses were under the limit of detection. The opposite pattern was observed in subject 33, 0.15% of CD8 T cells specific to vaccinia virus were detected while the neutralization titer was low. Subject 31 and 34 had a weak but detectable CD8 T cell response and intermediate neutralizing titers.



Overall, the immune responses detected were similar or lower than the average responses detected in chronically HIV-1 infected individuals vaccinated with MVA.

**Table VIII: Vaccinia specific immune response in subjects repeatedly vaccinated with the classical smallpox vaccination and MVA. Blood samples were collected in 2004. Cellular immune responses are expressed in terms of % of CD4 or CD8 T-cells expressing IFN- $\gamma$  after stimulation with autologous MVA infected PBMC.**

| Subject | Year of vaccination    |                  | Humoral response (MVA-gfp NT) | Cellular response |      |
|---------|------------------------|------------------|-------------------------------|-------------------|------|
|         | Vaccinia               | MVA              | AUC                           | CD8               | CD4  |
| 31      | 1954, 1965, 1975       | 2001             | 46.2                          | 0.06              | 0.02 |
| 32      | 1970                   | 2000, 2001       | 88.8                          | 0.04              | 0.01 |
| 33      | childhood              | 2001             | 23.7                          | 0.15              | 0.00 |
| 34      | 1958, 1969, 1995, 2000 | 1990, 2000, 2001 | 57.7                          | 0.05              | 0.03 |

### **Analysis of the relationship between MVA-specific and Nef-specific immune responses.**

Following MVA-nef vaccination, we observed new immune responses directed to the encoded protein (Cosma et al., 2003) and the viral vector. The relationship between immune responses directed to the encoded Nef protein and to the MVA vector was assessed by means of correlation analysis. CD4 and CD8 T-cell responses to Nef were compared to CD4 and CD8 T-cell responses to MVA as well as to humoral immune responses to MVA measured by mean of ELISA and neutralization assay. Correlation coefficients and P values were calculated after two and three administrations of the vaccine, as well as for immune responses measured 1 year after the vaccination. Interestingly, we observed a significant inverse correlation between MVA-specific and Nef-specific CD4 T-cell responses after the third vaccination (Figure XXIX). Subjects with the lowest Nef-specific CD4 T-cell responses had the highest CD4 T-cell responses to MVA and vice versa. The correlation coefficient and the P values indicated a significant inverse correlation also when the subject with the highest CD4 T-cell response to Nef (subject 10;  $r=-0.9507$ ,  $p=0.0011$ ) or the subject with the highest response to MVA (subject 2;  $r=-0.7454$ ,  $p=0.0368$ ) were excluded from the analysis. This correlation was not evident after two MVA-nef immunizations or one year after the last immunization, but only after the third immunization when we observed the peak of the MVA-specific cellular response. This relationship highlights interference between MVA-specific

and Nef specific CD4 responses. CD8 T-cell responses to Nef correlated neither with cellular nor humoral MVA-specific immune responses at all the time points tested (data not shown). Nevertheless, the presence of a strong Nef-specific CD8 response before the administration of the MVA-nef vaccine might have masked a possible relationship.

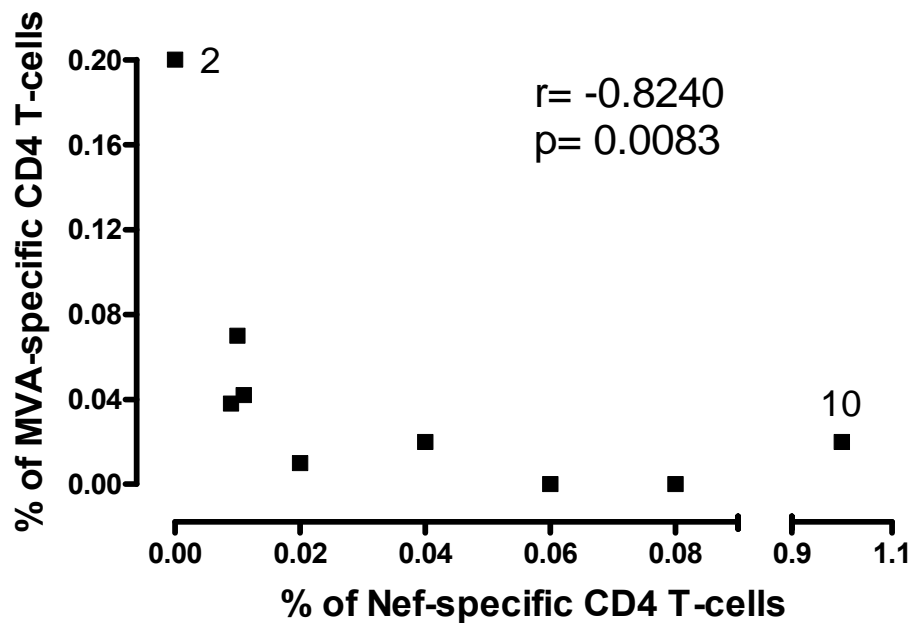


Figure XXIX: Relationship between MVA-specific and Nef-specific CD4 responses after three administrations of the MVA-nef vaccine. The correlation between MVA-specific and Nef-specific CD4 responses determined by IFN- $\gamma$  based intracellular cytokine staining was determined by Spearman's rank correlation analysis. R and P values are shown. Data points relative to the subjects discussed in the text are shown. The analysis was carried out on nine subjects since PBMCs from subject number 4 after the third vaccination were not available.

In summary, MVA was able to elicit humoral and cellular immune responses to vaccinia virus in a cohort of chronically HIV-1 infected individuals undergoing HAART. Of note, the elicited immune responses were similar to responses that are considered protective in healthy subjects recently vaccinated with vaccinia and/or MVA. Unfortunately, immune interference was detected between Nef-specific CD4 responses and MVA-specific CD4 responses.

## Discussion

CD8 T-cell response is crucial in controlling the acute phase of HIV infection (Borrow et al., 1994; Koup et al., 1994). However, in the chronic phase of the disease CD4 T-cell response seems to be important in controlling viral load as seen in LTNP (Pitcher et al., 1999; Rosenberg et al., 1997) and patients treated with HAART early during primary infection (Rosenberg et al., 2000). The MVA-nef vector is able to elicit and expand Nef-specific CD4 immune responses in the majority of the vaccinated subjects, increasing both the magnitude and breadth of the immune response. These results suggest that CD4 T-cell responses could be stimulated in chronically HIV infected individuals by means of therapeutic vaccination. Moreover, MVA-nef vaccination is able to amplify the frequencies of CD4 lymphocytes specific to Nef to reach levels comparable or even higher to the ones observed in LTNP, indicating a modulation of the virus-specific immune status in these chronically HIV-1 infected individuals.

Whereas a CD4 T-cell response was clearly observed, the CD8 immune response specific to Nef elicited by MVA-nef was faint or absent. There are several potential explanations for the lack of a strong Nef specific CD8 immune response after MVA-nef vaccination: 1) the expression of the functional product of the nef gene might induce HLA class I downregulation in MVA-nef-infected antigen-presenting cells inhibiting the antigen presentation to CTL (Collins et al., 1998); 2) since all the subjects enrolled in the study showed a Nef specific CD8 immune response before vaccination (Nef pool: median 0,24%, min 0,01%; max 0,42%; Nef Opt pool: median 0,23%, min 0,08%, max 1,33%) the T-cell precursors specific to Nef might be exhausted; 3) because of the strong pre-existing Nef-specific CD8 response, weak responses elicited by the vaccine might be masked by high variability in the determination of the responses 4) the MVA vector might be more effective in inducing a CD4 T-cell response in this clinical setting. Nevertheless, the increase observed in subjects 8 and 10 together with the expansion of the breadth of the CD8 T-cell response in subject 1, indicates that the MVA-nef vector is able to stimulate also CD8 T-cell responses.

Of note, an increase of the CD8 and CD4 T-cell responses to p24 has been observed in some subjects immediately after vaccination (Figure XX and Figure XXI; subjects 3 and 8). Cytokine-driven bystander activation or stimulation of HIV-infected antigen-presenting cells by vaccine-elicited T-cells might explain the clonal expansion of T-cells specific for the p24 antigen; indicating that undetectable precursors specific to p24 might be present in chronically HIV infected individuals. Moreover, these increases in the p24 specific immune response

suggest that the MVA-nef vaccine could promote epitope spreading towards other HIV proteins.

Whether a clinical benefit can be achieved by MVA-nef vaccination, is an important issue that has yet to be addressed. However, several lines of evidence highlight the importance of virus-specific T-helper cells in chronic viral infections. First, T-helper cells play a critical role in maintaining effective immunity in murine models of chronic viral infections (Battegay et al., 1994; Matloubian et al., 1994). Second, successful treatment of acute HIV-1 infection in humans leads to augmentation of T-helper-cell immune responses and enhances the immune control of the HIV-1 infection (Rosenberg et al., 2000). Third, we found a relatively robust Nef-specific CD4 immune response in LTNP. Thus, the established importance of T-helper cells in controlling chronic viral infections together with the clear demonstration that a T-helper immune response can be augmented in chronically HIV infected individuals using an MVA-nef vector provide a rationale to further explore immunotherapeutic intervention in chronic HIV-infection. More interestingly, the Nef-specific T-helper response detected after MVA-nef vaccination might suggest that the immunological status of these HIV chronic infected individuals was, at least for a limited period of time, similar to that described in LTNP.

Finally, this clinical study shows for the first time that the highly attenuated vaccinia-virus vector MVA can be used as safe vector in a cohort of immuno-compromised individuals. We believe this pilot study establishes the scientific rationale for future use of the MVA vector in individuals for which the use of a not attenuated vaccinia vector is not advisable, such as patients who undergo cancer therapy, long-term corticosteroid therapy, organ transplant recipients and patients with congenital immunodeficiency disorders (Bartlett, 2003). We also demonstrate that recombinant MVA vaccines can elicit immune responses to a target antigen also in individuals that have been previously vaccinated against smallpox. Nevertheless, the most prominent Nef-specific immune response was found in subject 10, the one that was not previously immunised with vaccinia virus (Figure XXVI).

In summary, the MVA-nef vector is able to safely elicit a CD4 T-cell immune response specific to Nef in a cohort of chronically HIV infected individuals, thus changing the chronic unbalanced immunological status of these subjects. These results suggest that a therapy interruption after MVA-nef vaccination might be valuable to further amplify HIV-specific immune responses. Of note, as shown in Figure XXII, Nef-specific CD4 T-cells elicited by the MVA-nef vaccine were still present one year after the third vaccine administration indicating that the MVA-nef vaccine is able to elicit long lasting T-helper memory cells.

Although increasing evidence from animal models suggests that MVA might be an alternative vaccine against smallpox for individuals at risk for the side effects induced by the classical smallpox vaccine (Belyakov et al., 2003; Earl et al., 2004; Slifka, 2005; Wyatt et al., 2004), the immune responses elicited by MVA in these high risk individuals have not yet been fully characterized. We characterized the vaccinia-specific immune responses elicited in ten chronically HIV-1 infected individuals undergoing HAART after three administrations of an MVA vaccine. Our results demonstrated that MVA was able to evoke strong and durable humoral and cellular immune responses. MVA was able to elicit vaccinia-specific immune responses in subjects that were vaccinated against smallpox as children and in one subject that was completely naïve to vaccinia. Thus, MVA was able to reestablish vaccinia-specific immune response in individuals vaccinated long ago and at the same time, it was able to elicit *de novo* vaccinia-specific immune responses.

The analysis of the humoral immune response demonstrated that MVA was able to elicit both neutralizing and binding antibodies. Notably, the magnitude of the humoral immune response elicited by MVA was similar to that observed in healthy subjects repeatedly vaccinated with vaccinia and/or MVA.

Of note, no correlation was found between humoral immune response and CD8 T-cell response. This observation is consistent with the analysis performed by Hammarlund et al. (Hammarlund et al., 2003) on a cohort of 306 individuals vaccinated against smallpox. Overall, the data demonstrated that specific antibodies and IFN- $\gamma$  producing CD8 T-cells are two independent biomarkers.

While neutralizing antibodies and CD8 T-cells were readily elicited in all subjects, vaccinia specific CD4 T-cells were observed in only 4 subjects. Several hypotheses may explain the lack of generalized CD4 T-cell responses to vaccinia. First, MVA may have a reduced capacity in inducing CD4 T-cell responses in this clinical setting. However, in the same subjects a strong CD4 immune response to the recombinant nef gene was observed (Cosma et al., 2003) confirming the capacity of MVA to elicit CD4 immune responses. Second, it may be possible that another cytokine is the basis for the vaccinia-specific CD4 immune response induced by MVA. Third, since this is a retrospective study, frozen PBMCs were used in all experiments and CD4 T-cells are more sensitive to freezing and thawing than CD8 T cells.

Safety, capacity to accommodate different and not related genes and ability to stimulate the innate immune system make MVA an optimal vector to develop novel vaccines. However, the encoded protein has to compete against the numerous vector-derived antigens for T-cell

recognition. Therefore, antigen dominance and immune interference play an important role to determine the immunogenicity of the encoded product and the viral vector (Kedl et al., 2003). Our results demonstrated a significant inverse correlation between MVA-specific and Nef – specific CD4 responses. A possible explanation for this relationship is the existence of a competition at the level of the CD4 responses. Our data indicate a general advantage for the CD4 T-cells specific to Nef and this advantage seems to be more pronounced in the vaccinia naïve subject number 10. If this is the case, the lack of a generalized CD4 T-cell response to vaccinia might be the consequence of antigen competition.

The respective role of neutralizing antibodies, CD8 and CD4 T-cells in protection from smallpox is still poorly understood. Moreover, since eradication of smallpox preceded the advent of modern techniques to measure T-cell responses, such as intracellular cytokine staining, ELISPOT and tetramer staining, historical data on the protective vaccinia-specific immune response rely entirely on the analysis of the presence of vaccinia-specific antibodies and no comparisons with cellular immune responses exist. Recently, several studies have addressed this issue in animal models. Antibodies were found to be sufficient in protecting mice from a lethal challenge with vaccinia virus in the absence of specific CD8 immune response, while in the absence of antibodies, CD8 T-cells were able to prevent mortality and disease progression (Belyakov et al., 2003; Xu et al., 2004). In mice undergoing a secondary infection with ectromelia virus, antibodies were sufficient and necessary for protection while CD8 response failed in protecting mice from lethal ectromelia infection (Fang and Sigal, 2005; Panchanathan et al., 2006). In immunocompromised monkeys, the presence of high titers of vaccinia specific IgGs have been shown to correlate with protection against a lethal monkeypox challenge (Edghill-Smith et al., 2005a). Finally, a recent report analyzing the immunological mechanism of protection in Rhesus macaques vaccinated with Dryvax and then challenged intravenously with monkeypox virus, demonstrated that specific antibodies are necessary and sufficient for protection (Edghill-Smith et al., 2005b). In the same report, depletion of CD8 and CD4 T-cells performed after vaccination did not affect the outcome of the subsequent challenge. Overall, studies performed in the Rhesus macaque model indicate a pivotal role of the humoral response, while some of the studies performed in the mouse model suggest an additional contribution of the cellular immune response.

In our cohort of chronically HIV-1 infected individuals, the MVA vaccine fulfills the requirements of stimulating both neutralizing antibodies and specific CD8 T-cells. Moreover, we detected high titers of neutralizing IgGs. Thus, the data support our proposal to use MVA as an alternative smallpox vaccine in potentially immunocompromised individuals.

In a parallel study, a similar cohort of HIV-1 infected individuals was vaccinated with an MVA HIV-1 nef vector vaccine (Harrer et al., 2005). In this study, the characterization of the vaccinia specific immune response was limited to the measurement of specific IgG using an ELISA and specific CD8 T-cells using an ELISPOT assay. Consistent with our results, vaccinia specific IgGs were readily detected after the administration of the MVA vaccine.

HIV-1 infected individuals are considered potentially immunocompromised and CD4 counts have been shown to predict the capacity of these individuals in mounting a correct immune response to a vaccine (Rodriguez-Barradas et al., 1992; Rousseau et al., 1999). Our data showed that in a cohort of chronically HIV infected subjects with CD4 T-cell counts between 407 and 1421 cells/mm<sup>3</sup>, MVA was able to elicit a durable immune response.

CD4 counts also predict the risk of adverse effects following classical vaccination against smallpox, likely because the immune system is better able to control the spreading of the replication-competent vaccinia virus classically used to vaccinate against smallpox. Some studies involving small numbers of patients indicated that the classical smallpox vaccination might be safe in subjects with CD4 counts >200 cells/mm<sup>3</sup> (Tasker et al., 2004), while below this threshold adverse effects were observed. However, in the absence of large scale clinical trials the Centers for Disease Control and Prevention (Atlanta, GA) recommended against the use of the replication competent vaccinia virus to vaccinate individuals with HIV infection, regardless of their CD4 cell counts (Bartlett, 2003). Thus, replication-deficient MVA because of its immunogenicity and safety may represent a better alternative. Nevertheless, a series of limitations of the present study needs to be emphasized. The study was conducted on a cohort of chronically HIV-1 infected individuals undergoing HAART with CD4 count >400 cells/mm<sup>3</sup> and undetectable viral load. The ability of this specific cohort in mounting a MVA specific response is certainly better than what we can expect in a cohort of HIV-1 infected individuals with CD4 counts <400 cells/mm<sup>3</sup> and detectable viral load. Therefore, further studies in cohorts of HIV infected individuals with CD4 counts <400 cells/mm<sup>3</sup> and with higher viral loads are required. In addition, since 9 of the 10 HIV infected individuals received smallpox vaccination as children, the role of MVA in priming vaccinia specific immune responses rely only on the characterization of the immune responses observed in subject number 10 who was not immunized as a child. Further studies in cohorts of vaccinia naïve HIV infected individuals should corroborate whether the immune responses observed in this subject are characteristic of the priming capacity of MVA. Finally, the vaccine used in our study encoded the HIV-1 regulatory protein Nef. The Nef protein promotes HIV-1 immune escape via the downregulation of CD28, CD4 and MHC class I and class II



molecules. Therefore, we cannot exclude such an effect in the context of the MVA vector. If this is the case, the wild type MVA might be more effective in eliciting vaccinia specific immune responses.

This study provides a complete characterization of the vaccinia specific immune response following the administration of MVA to chronically HIV-1 infected individuals with a long history of antiretroviral treatment and with CD4 T-cells counts  $>400$  cells/mm<sup>3</sup>. The observed immune response suggests that MVA can be used as alternative vaccine against smallpox in this specific cohort of individuals.

## Conclusions

With this research, we demonstrate that the MVA HIV-1<sub>LAI</sub> nef vaccine is safe and immunogenic in chronically HIV-1 infected individuals under HAART. The observed Nef-specific immune responses were similar in magnitude to responses usually detected in LTNP. In addition, the elicited responses were detectable one year after the administration of the third vaccination, thus demonstrating the capacity of the vaccine to elicit long lasting responses.

The natural follow up of this study will be an efficacy trial in which therapy will be interrupted after the administration of the vaccine.

In addition, since this study had a therapeutic setting, it will be useful to test the MVA HIV-1<sub>LAI</sub> nef vaccine in a prophylactic study.

Another important aspect of this study is the consideration that MVA can be used as alternative vaccine against smallpox in chronically HIV-1 infected individuals under HAART. This study highlighted also a potential problem of immune interference between Nef-specific immune responses and MVA-specific immune responses. Therefore, in the next trial we will also evaluate immune responses in vaccinia naïve individuals.

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## Curriculum Vitae

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Languages: Italian, French, English.



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**Overview** More than 10 years of experience in HIV research in different institutions in Italy, France and Germany. Main area of expertise: Immunology and Virology.

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**Main Research Interests** Characterization of cellular and humoral immune responses in humans  
Multicolour flow cytometry  
Development of new high-throughput immunoassays

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### Research Activity

Since 2000 Research Scientist c/o Prof. Frank D. Goebel  
Medizinische Poliklinik Innenstadt Klinikum der  
Ludwig-Maximilians-Universität München

- Immunogenicity of the MVA-nef vaccine in HIV infected individuals
- Evaluation of MVA as alternative vaccine against smallpox
- Development of new immunoassay to evaluate humoral and cellular responses against HIV and vaccinia
- Design of databases to store, organize and analyze data

1997-1999 Research Scientist c/o Dr Alberto Beretta and Prof. Luc Montagnier

CIRBS-World Foundation for AIDS Research and Prevention,  
Paris, France

- Role of HLA class I molecules in HIV-1 replication and infectivity
- Development of non-radioactive methods for specific selection and labeling of cellular protein

1994-1996

Fellowship c/o Pr. Antonio Siccardi and Dr. Alberto Beretta  
DIBIT-San Raffaele Hospital, Milan, Italy

- HIV-1-specific immunity in seronegative individuals at high risk for HIV infection

1994

Visiting fellowship c/o Dr. Patrizio Giacomini  
Centre of Experimental Research “Regina Elena”, Roma, Italy

- Characterization of HLA class I alleles and isoforms using 1D-isoelectrofocusing

1991-1992

Student in Biological Sciences c/o Prof. Bruno Curti  
“Università degli Studi”, Milan, Italy

- Purification and characterization of the proteolysis products derived from the D-amino acid oxidase

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### Teaching activity

Since 2002

Supervisor for biology and medicine PhD students

1997

Immunology course for medical student, University of Milan, Italy

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### Education

1992

Degree (*Laurea*) in Biological Sciences with full marks (110/110) and honours (*lode*).

Thesis Title: « Structural and functional study of the limited proteolysis products from D-amino acid oxidase »

Subject: Biochemistry

Supervisor: Prof. B. Curti, Biological Chemistry, “Università degli Studi di Milano”, Milan, Italy.

1987 High school degree “Maturità Scientifica”  
Liceo Scientifico Bertrand Russel, Milan, Italy

## Publications

2007

- 16) M. Mamani-Matsuda, **A. Cosma**, S. Weller, A. Faili, C. Staib, L. Garçon, O. Hermine, D. Adoue, C. Fieschi, J.O. Pers, N. Arakelyan, B. Varet, A. Sauvanet, A. Berger, F. Paye, J.M. Andrieu, M. Michel, B. Godeau, P. Buffet, C.A. Reynaud J.C. Weill  
*The human spleen is the main reservoir for life long memory B-cells*  
(Submitted)
- 15) D. Hoffmann, J. Seebach, **A. Cosma**, F.D. Goebel, K. Strimmer, H.M. Schätzl, and V. Erfle  
*Therapeutic vaccination reduces HIV protein sequence variability*  
FASEB Journal (in press)
- 14) A. Bråve, L. Gudmundsdotter; G. Gasteiger, K. Hallermalm, W. Kastenmuller, E. Rollman, A. Boberg, G. Engström, S. Reiland, **A. Cosma**, I. Drexler, J. Hinkula, B. Wahren, V. Erfle  
*Immunization of mice with the nef gene from Human Immunodeficiency Virus type 1: Study of immunological memory and long-term toxicology*  
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Affinity Biotinylation

United States Patent No. 6,150,123