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Immune Correlates of SIV-Protection and HIV-Control: Study of an Effective ALVAC/SIV – gp120/Alum Vaccine and of HIV-infected Long Term Non Progressors

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ABSTRACT

Background

The ALVAC/HIV+ gp120/Alum RV144 vaccine trial conducted in Thailand resulted in 31% vaccine efficacy in a low-risk cohort of 16.000 volunteers. We aimed to mirror these results in an SIV_{mac251} model, to explore the effect of the adjuvant MF59 in a similar vaccine regimen and to investigate the immune correlates of protection. Post-hoc analysis of RV144 demonstrated that antibodies targeting the V2 region of gp120 correlated with decreased risk of infection. The same response has been detected in SIV-infected rhesus macaques that achived substained SIV control after treatment with monoclonal antibody anti-α4β7 and an intermittent period of antiretroviral therapy. To date, V2 antibody response data in HIV infected individual that naturally control HIV progression are lacking. We compared the V2 humoral response in a group of chronic HIV infected patients that never received ART and compare that to the response in HIV infected patients Long Term Non Progressors (LTNPs).

Methods

We immunized 54 rhesus macaques with an RV144 like vaccine regimen adjuvanted either in alum or MF59. We compared the efficacy of the vaccine to 24 simultaneous controls, as well as 23 historical controls. We challenged all the animals with repeated intra-rectal low doses of SIV_{mac251}. We evaluated vaccine efficacy, measured humoral systemic and mucosal humoral responses by multiple antibody detection assays (with particular regard to the V2 response), we measured frequency and quality of plasma-cell precursors, Plasmablasts and Natural Killer cells by flow-citometry. We enrolled 14 HIV italian patients LTNP and 12 pre-ART chronic HIV-infected (CHI) patients and studied their systemic response to V2, with both linear and cyclic peptides.

Results

Our study mirrored the RV144 human trial: the ALVAC-simian immunodeficiency virus (SIV) and gp120 alum (ALVAC-SIV + gp120) reduced the risk of SIV mucosal infection. Interestingly the ALVAC-SIV + gp120 MF59 vaccine did not, despite the general higher systemic and mucosal immunogenicity. Vaccine efficacy was associated with alum-induced envelope Env-dependent mucosal NK-cells that produce interleukin 17 (IL-17), as well as with mucosal IgG to the gp120 variable region 2 (V2). Notably, this latter humoral mucosal response was the only response higher in the alum group. MF59 skewed the traffic of PBs from the mucosal to the inflammatory sites. We observed similar differences, consistent with the macaque results, in α4β7 levels on circulating PBs in humans immunized in the RV135 and RV132 trials, which used the RV144 immunogens adjuvanted either with alum or MF59, respectively. The systemic antibody V2 response in LTNP was higher compared to the V2 response in pre-ART CHI patients.

Conclusions

Our data confirm the protection achieved in human by the immunization with a prime/boost approach with an ALVAC-HIV + gp120-Alum vaccine in a SIV_{mac251} model. We found novel immune features (NK-cells and PBs) that might represent immune correlated of protection. Furthermore, we confirmed in our model similar responses showed in humans vaccinated with the RV144 regimen. As already described in vaccinated humans, we now demonstrate the importance of the antibodies to V2 in prevention from SIV mucosal acquisition. The importance of antibody to V2 region of SIV/HIV has been recently corroborated by a rhesus macaque study in which substained virologic control was achived after ART suspension. We remarkably found in a group of italian LTNPs an higher antibody response to the linear and cyclic form of the V2 region of gp120 compared to a group of pre-ART CHI patients. Understanding the mechanism in which these antibody might contribute to mediate protection from HIV/SIV acqusition and HIV/SIV control will require further studies

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Historical remarks

Acquired immune deficiency syndrome was recognized for the first time in the U.S. in 1981 when the Centers for Disease Control and Prevention (C.D.C.) reported an unexplained increased number of cases of Pneumocystis jiroveci (known as P. carinii) pneumonia and of Kaposi's sarcoma in previously healthy homosexual men in Los Angeles and in New York [1-4]. Initially, as it was certified that in all these patients a severe impairment of their immune system occurred, the disease was called Gay- Related Immunodeficiency (GRID). However, within a few months the same disorder was found in haemophilics [5] and in recipients of blood transfusion [6] , and later in both male and female injection drug users [7]. As the epidemiologic pattern unfolded and the number of cases increasingly rose according to a contagious disease fashion, it was evident that it was a previously unknown infectious disease, which was not affecting young homosexual men only, but could be transmitted to anyone both through sexual activity and blood contact. In 1982 it was proposed by C.D.C. to name the disease according to the profound alteration of the immune response it was strictly associated with: the Acquired Immune Deficiency Syndrome (AIDS). AIDS, in fact, included all kinds of opportunistic infections and all forms of rare tumors that were frequently found in those patients.

In 1983, two separate research groups led by Luc Montagnier and Robert Gallo independently declared that a novel retrovirus may have been infecting AIDS patients. At the Pasteur Institute, in Paris, Montagnier's group observed by electromicroscopy a virus from a patient presenting with swelling of the lymph nodes of the neck and physical weakness, two typical symptoms of AIDS, and accordingly named the virus Lymphadenopathy-Associated Virus (LAV) [8]. On the other hand, Gallo, who was leading a virology laboratory at the National Cancer Institute, in Bethesda, claimed that his group

had isolated a virus from an AIDS patient. This virus was strikingly similar to other retroviruses, namely Human T Lymphotropic Viruses (HTLVs), and called the newly isolated virus HTLV-III [9]. However, by March 1985 it became clear that LAV and HTLVIII were the same retrovirus and that it was, in turn, the etiologic agent of AIDS. One year later, the International Committee on Taxonomy on Viruses ruled that Human Immunodeficiency Virus (HIV) was to be the new name from then on.

HIV structure

Two major types of HIV have been characterized. HIV type 1 (HIV-1) is the virus that was first isolated as LAV in 1983, and the most common cause of HIV disease throughout the world. It is closely related to SIV_{cpz}, a specific strain of *Simian Immunodeficiency Virus* virus that infects *Pan troglodytes troglodytes* species of chimpanzees, which have been thought to be the wild source of human infection [10-13]. On the other hand, HIV type 2 (HIV-2) is confined to some West African regions, probably due to the fact that it is less virulent and less infective than HIV-1, and is more strictly related to the African sooty mangabeys strains of SIV (known as SIV_{smm}) [14, 15] .

The *Human Immunodeficiency Virus* is roughly spherical, with a diameter of 120 nm. Its envelope partially derives from the host cell plasma membrane following the budding process, and is studded with 10-100 spikes that protrude through the surface of the virion [16]. Each of these spikes comprises a trimer of gp120 (i.e., glycoprotein 120), a heavily glycosylated protein which enables the virus to attach the target cell, and gp41, a transmembrane protein which plays a critical role in the fusion of the virion with the target cell. Both of them are coded by HIV *env* gene and represent important targets for drug treatment and vaccines against HIV. The inner layer of the envelope presents p17 MA (i.e., matrix antigen), a viral *gag* gene product. The core of the virion is covered with p24 CA (i.e., capsid antigen), another *gag* gene product that gives it its cone-like shape. Within the core, there are two copies of single-stranded viral genomic RNA and viral proteins that either are

required for the assembly of the virion (p7 nucleocapsid protein, NC) or are necessary to its replication process (such as reverse transcriptase and integrase) [17]. Similarly to any other retrovirus, the HIV genome comprises structural genes, encoding for major proteins and essential enzymes, and non-structural genes, encoding for proteins involved in regulation of gene expression or modulations of the host cell interactions in order to augment viral replication.

The structural genes are *gag*, *pol*, and *env*, sometimes referred to as 5' *gag-pol-env* 3', according to their position within the viral genome. *Gag* (i.e., group-specific-antigen) gene encodes for the precursor polyprotein, processed by viral protease into a number of structural proteins:

- MA p17 (matrix antigen), which forms the inner layer of the lipid-containing envelope;
- CA p24 (capsid antigen), which constitutes the skeleton of the viral core;
- NC p7 (nucleocapsid), which binds to the RNA genome within the viral core;
- p6, which interacts the with *vpr* protein.

Pol (i.e., polymerase) gene encodes for a variety of viral enzymes, including:

- PR p10 (protease), which is essential for the cleavage of *gag* precursor polyprotein and for the processing of all viral enzymes;
- RT (reverse transcriptase) and RNAse H, a RNA-dependent DNA polymerase and a ribonuclease, respectively, which are required to transcribe double-stranded DNA from the RNA genome;
- IN p32 (integrase), which catalyzes the integration of viral DNA into the host genome.

Env (i.e., envelope) encodes for gp160, a glycoprotein that is cleaved in the endoplasmic reticulum into two smaller glycoproteins [18]:

- gp120, which mediates the attachment of the virus to the target cell and elicits a specific humoral immune response [19, 20];
- gp41, which catalyzes the fusion of the virion to the host cell.

Furthermore, in HIV genome two essential regulatory elements can be identified. *Tat* (transactivator), a transcriptional activator that binds to the TAR (i.e., *tat*-responsive element) RNA region to initiate transcription and elongation from the LTR promoter *(*Long Terminal Repeats (LTR) are terminally present on both ends of RNA genome and contain sequences involved in several regulatory functions, including the initiation of transcription, prevention of premature termination, polyadenylation and integration). *Rev* (regulator of expression of viral proteins), a regulatory factor that binds to the RRE (i.e., *rev*-responsive element) RNA region and inhibits viral RNA splicing, promoting nuclear export of incompletely spliced viral mRNAs. Finally, other proteins are encoded by HIV non-structural genes. *Nef* (i.e., negative effector) gene encodes for a phosphoprotein that promotes the down-regulation of surface CD4 receptors and MHC-I molecules, blocks cell apoptosis, increases viral infectivity and somehow alters the state of cellular activation. *Vif* (i.e., viral infectivity factor) encodes for a phosphoprotein in the absence of which the produced viral particles are defective. *Vpr* (i.e., viral protein R) product is involved in the nuclear import of pre-integration complexes, cell growth arrest during G2-phase of the cell cycle and transactivation of cellular genes. *Vpu* (i.e., viral protein U) is a gene expressed by HIV-1 only, and encodes for a protein involved in the degradation of CD4 in the endoplasmic reticulum and in the enhancement of virion release from the plasma membrane. *Vpx* (i.e., viral protein X) product is a protein expressed by HIV-2 genome only, known to be a homolog of HIV-1 *vpr*, but further research is needed to fully understand its role in viral replication.

HIV pathogenesis. Long-Term Non-Progressors and Elite Controllers

Before the introduction of antiretroviral therapy as a means to reduce AIDS-related morbidity and mortality, the course of HIV infection resulted in a typical pattern in most of the untreated patients. After HIV transmission and the establishment of infection, referred to as *primary infection*, the patient presents high levels of HIV RNA copies (millions per milliliter) in the peripheral blood and an almost simultaneous and abrupt drop of $CD4^+$ T cells (Fig. 1).

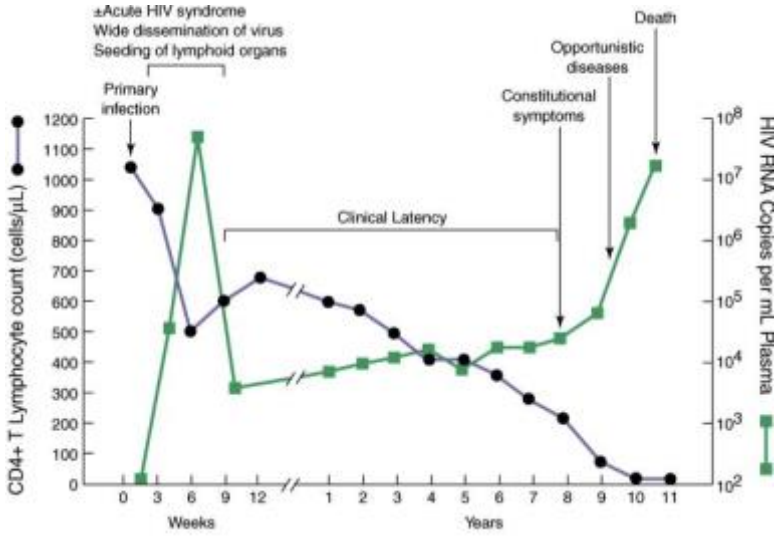

Fig. 1 | Typical course of an untreated HIV-infected individual (From Fauci AS, Braunwald E, Kasper DL, Hauser SL, Longo DL, Jameson JL, Loscalzo J. *Harrison's Principles of Internal Medicine*. 18th ed. New York, NY: McGraw-Hill Medical Publishing Division. 2011).

About 50% of recently infected patients develop the acute HIV syndrome, a cluster of acute mononucleosis-like symptoms, reflecting the wide dissemination of virus and the seeding of lymphoid organs. Following a partial rebound of $CD4^+$ T-cell count, a long period of *clinical latency* takes place, during which the patient is relatively asymptomatic. However, the term is not to be confused with *microbiologic latency*, since even in patients where HIV RNA levels cannot be detected there is always some degree of ongoing viral replication, resulting in fact in a progressive and relentless decrease of $CD4^+$ T lymphocytes. It is estimated that an average of ten years are necessary for the $CD4^+$ T count to decrease significantly, so

that the *Acquired Immune Deficiency Syndrome* (also referred to as AIDS) becomes clinically evident, and is primarily characterized by severe opportunistic infections and neoplasms. In the absence of therapy, most patients are bound to eventually die mainly as a result of the profound impairment of the immune system (Fig. 1).

Individuals with HIV infection show variable rates of disease progression and viral control. Whereas some subgroups of individuals control infection very well and remain asymptomatic for several years, others show rapid immunological and clinical progression. A number of terms have been used to describe individuals at these extremes of the clinical spectrum, including Long-Term Non-Progressors (LTNPs) and Elite Controllers (ECs). Although these groups have been the focus of intense study, there is no consistency in how they have been defined. A recent work conducted a systematic review of the literature [21]. It described the heterogeneity in the definitions used, and identified common definitions that may provide a framework for developing consensus definitions LTNPs are defined as subject followed up from 1 to 25 years with 10 years being the most common duration of follow-up required. The $CD4^+$ cell count for this patients range between 300 and 1000 cells/μl. Only 36% of 159 unique definitions for LTNPs in the literature include a HIV-RNA threshold. The absence of clinical symptoms is a prominent criterion.

Fifthy unique definitions of ECs are present in the literature. As expected from the terminology, HIV-RNA thresholds appeared in all definitions listed, with thresholds ranging from 40 to 500 copies/ml. The most frequent HIV-RNA threshold used was 50 copies/ml. The duration of follow-up varies from 6 months to 16 years, from definition to definition.

The possible explanations that lie underneath this phenomenon have been extensively reviewed [22]. Research in the field has undergone considerable development in recent years and LTNPs offer a piece of the puzzle in understanding the ways that infected individuals can naturally control HIV-1 infection. Their method of control is based on viral, genetic and immunological components. With respect to virological features, genomic sequencing has shown that some LTNPs are infected with attenuated strains of HIV-1 and harbour mutant nef, vpr, vif or rev genes that contain single nuclear polymorphisms, or less

frequently, large deletions, in conserved domains. Studies have also shown that some LTNPs have unique genetic advantages, including heterozygosity for the CCR5-Δ32 polymorphism, and have been found excitatory mutations that upregulate the production of the chemokines that competitively inhibit HIV-1 binding to CCR5 or CXCR4. Lastly, immunological factors are crucial for providing LTNPs with a natural form of control, the most important being robust HIV-specific $CD4^+$ and $CD8^+$ T-cell responses that correlate with lower viral loads. Many LTNPs carry the HLA class I B57 allele that enhances presentation of antigenic peptides on the surface of infected $CD4^+$ cells to cytotoxic $CD8^+$ T cells [23]. Notwithstanding the extensive literature produced on the viral, genetic and immunological feautures of these patients, the exact mechanisms for LTNP are still under investigation. For all these reasons, LTNPs and ECs serve as an ideal model for HIV-1 vaccine development due to their natural control of HIV-1 infection.

HIV VACCINES

Vaccines and adjuvants overview

A vaccine is a biological preparation that creates immunity against a particular pathogen, in the effort of providing effective protection to the host. The term vaccine derives from Edward Jenner's 1796 use of cowpox (in Latin variola vaccinia), to inoculate humans, thereby establishing protection against smallpox. Current vaccines fall in four major categories: attenuated, inactivated, subunit, and toxoid [24] . Live attenuated vaccines are the major class of vaccines, especially for intracellular pathogens. The agents that are inoculated replicate throughout the body and result in the development of active infections. Suitably attenuated strains require low doses of vaccine and lead to a long-lasting form of immunity, which is both antibody-mediated and cytotoxic. Furthermore, they are usually administered orally and, thus, tend to induce effective mucosal immunity. On the other hand, the stability of the vaccine may be poor and reversion to the virulent phenotype may occur.

Since its discovery by Louis Pasteur, attenuation became the main route for the development of new vaccines. In its easiest form, attenuation simply required the repeated passage of the agent in culture until a strain was obtained that exhibited reduced pathogenicity but elicited, nevertheless, good immunity. A historical example of this approach is the development of the BCG (i.e., Bacillus Calmette-Guerin) vaccine for tuberculosis. Although some of them remain the only choice for prevention of certain infectious diseases, inactivated vaccines tend to be less attractive than attenuated ones. Indeed, the costs are normally higher, since the agent is not able to replicate throughout the body and needs to be administered parentally and in greater amounts. The immune response elicited is antibody-mediated, and both cytotoxic and mucosal immunity are not significantly induced. Usually, also an adjuvant is needed to prime a better immune response, in contrast to attenuated vaccines, for which no adjuvant is required. Nevertheless, killed vaccines cannot revert to the virulent phenotype and can rely on a good stability of the agent. Inactivation is generally achieved with formaldehyde, ethylenemines or β-propionolactone. Subunit vaccines are vaccines developed against individual viral or bacterial components that play a crucial role in eliciting protective immunity. Although the quest for such vaccines initiated before recombinant DNA technology, current developments are underpinned by the ability to clone, sequence and express large quantities of the relevant antigens in adequate hosts (typically bacteria or yeasts). Subunit vaccines therefore are a product of recombinant DNA technology. The most important subunit vaccine is represented by HBV vaccine, which largely contributed to decrease the prevalence of hepatitis B infection throughout the Western World in the last thirty years. Finally, vaccines based on toxoids can provide protective immunity in diseases that result from the activity of specific bacterial exotoxins. They are made from inactivated toxic compounds that maintain their immunogenicity. This concept has been thoroughly demonstrated with the successful

development of vaccines for diphtheria and tetanus early this century. The early procedure of toxin inactivation with formaldehyde is still adequate; alternatively, chemical treatment with formalin can be used. Furthermore, there is a broad spectrum of newly optimized vaccines that may be useful in the establishment of protective immune responses towards pathogens for which no current vaccine is available, such as HIV. Conjugate vaccines are created by covalently attaching a poorly immunogenic (usually polysaccharide) antigen to a carrier protein (preferably from the same microorganism) or a toxoid, thereby enhancing its ability to induce an effective and long-lasting immune response. This technique is used, for instance, for the development of Haemophilus influenzae type B vaccine. Virus-like particle-based vaccines can be obtained through the separate introduction of one or more genes that encode for structural proteins and one or more genes that encode for immunogen proteins into a vector, which allows their constant expression within infected cells. Viral proteins, then, self-assembly spontaneously and bud from the infected cells, giving rise to particles that share the backbone structure with wild-type virus, but are not able to actively replicate. A considerable number of viruses that are not able to replicate or are not pathogenic, namely viral vectors, are currently available to provide the backbone for the cloning of genes belonging to micro-organisms that cannot be attenuated properly. Main vectors for this purpose are Poxviruses, Alphaviruses, Adenoviruses and Vescicular Stomatitis Viruses. In recent years, also DNA vaccination has been established as a novel technique to deliver bacterial or viral antigens and prime a protective immune response. The gene of interest is inserted into a plasmidic vector, which allows protein expression within eukaryotic cells. Naked DNA is then injected intramuscularly or intradermically and engulfed by cells, thereby paving the way to the synthesis of an antigen protein and the subsequent induction of cellular-mediated immune responses [25].

An adjuvant is an immunological agent that enhances the immune response to an antigen.

Adjuvants are inorganic or organic chemicals, macromolecules or entire cells obtained from certain killed bacteria, which may be included in a vaccine to strengthen the recipient's immune response. Adjuvants have been used in human vaccines for almost a century, yet very few adjuvants are licensed for human use. This has been due, in part, to a lack of understanding of their mechanism of action. However, recent insights into the innate immune system and its importance in initiating the adaptive immune response have sparked the rational design and development of the next generation of adjuvants. Several studies have validated one class of pattern recognition receptors (PRRs), namely Toll-Like Receptors (TLRs), as vaccine adjuvant targets. Various TLR agonists have been tested in humans and the TLR4 agonist monophosphoryl-lipid A (MPL) has been recently licensed in Europe and the USA for a vaccine that prevents human papilloma virus (HPV) infection[26]. The two important subsets of adjuvants are: inorganic adjuvants (such as aluminum salts) and organic adjuvants (such as oil-based adjuvants).

Aluminum salts (*alum*) have been in wide use with human vaccines for almost a century. This class of adjuvants, which includes aluminum phosphate, aluminum hydroxide, and aluminum hydroxyphosphate, is a component of various viral and bacterial vaccines such as diphtheria, tetanus, pertussis, hepatitis A and B, rabies, anthrax, and has also been used in the RV-144 trial that first verified the partial efficacy of a vaccine regimen against HIV infection in Thailand. Alum formulations are particulate in nature, to which the vaccine antigens are adsorbed, albeit with distinct characteristics among the different forms of alum salts. The initial assumption that alum creates a depot *in situ*, thereby allowing slow release of antigen over time and prolonged exposure to the immune system[27]. Particulate vaccine formulations generally are more readily internalized by antigen-presenting cells (APCs) than

are soluble antigens and the same is true for alum-adsorbed antigens. The mechanism by which antigen uptake is facilitated is not yet clear. Crystalline alum, however, was shown to bind lipids on the surface of APCs and trigger a cellular activation cascade leading to initiation of an immune response, but without internalized itself by the cells[28], suggesting an indirect role in delivering antigen into the antigen-processing pathway. These results are in contrast with a previous study using confocal microscopy showing that alum was internalized by APCs.[159] However, injection of vaccines containing alum elicits profound broad local effects on the immune system. Within a few hours after injection, pro-inflammatory cytokines are released and there is an influx of inflammatory monocytes followed by dendritic cells (DCs), natural killer (NK) cells, neutrophils, and eosinophils by 24 h [29, 30]. During this time, a constellation of cytokines and chemokines are secreted and they may facilitate the recruitment and activation of APCs at the site of injection. These APCs may then internalize vaccine antigens and migrate to the draining lymph node to prime lymphocytes [31]. The immunostimulatory effects of alum are broad, rapid, and seem to involve multiple pathways, both direct and indirect. It provokes a strong TH2 response, but is rather ineffective against pathogens that require TH1-cell-mediated immunity and determines APC recruitment. However, more investigation will be required to fully elucidate these mechanisms.

Oil-in-water emulsions are licensed for use only in human influenza vaccines. These include MF59, which was originally licensed for influenza vaccines for the elderly and is now being tested in the RV-144 follow-up trial in South Africa instead of alum (HVTN 100 and HVTN 702), and AS03, which was recently approved for pandemic influenza vaccines. *MF59* consists of uniform particles (~160 nm in size) generated by microfluidics

technology and its main constituents are the naturally occurring oil squalene and two non-ionic surfactants (Tween 80 and Span 85) [26]. Differently from alum, recent evidence have shown that MF59 is quickly drained from the injection site [32]. In addition, unlike alum, the adjuvant effects of MF59 can be maintained even when the antigen alone is administered up to 24 h after injection of MF59 at the same site [32], suggesting that MF59 creates a sort of immuno-competent environment within the muscle that could facilitate the development of antigen-specific immune responses. Studies conducted on cells *in vitro* demonstrated that MF59 increased phagocytosis and pinocytosis, and promoted antigen uptake by APCs [33]. In that study, MF59 stimulated monocytes, macrophages, and granulocytes to produce the chemokines CCL2, CXCL8, CCL3, and CCL4. Stimulated monocytes underwent phenotypic changes in accordance with their differentiation toward DCs. These data suggested that MF59 does not directly target DCs to internalize antigen, but may act upstream by inducing recruitment of DC precursors and their subsequent differentiation [33]. Moreover, MF59 significantly increased the number of antigen-loaded APCs in draining lymph nodes compared to alum or non-adjuvanted vaccine [34], suggesting a potential role in immune-cell presentation. Finally, unlike alum, it elicits both TH1 and TH2 potent immune responses, and seems to upregulate interferon-related genes and to play an active role in prompting local inflammatory responses [35]. Nevertheless, as is the case for alum, further studies are required to better understand the mode of action of MF59.

Brief HIV vaccines history: from Zagury to the RV144 trial

In April 1984, U.S. Health and Human Services Secretary Margaret Heckler stated that an HIV-vaccine would have been ready for testing in humans in about two years. From that moment more than thirty years have passed, 35 million people are living with HIV and

25 million individual died of it. In 2013 more than 2 million new HIV-1 infections have been counted globally [36]. The introduction of the highly active antiretroviral therapy, the most recent advances in the prophylactic approaches with the introduction of tenofovir/emtricitabine administrated to reduce the risk of HIV sexual transmission [37] decreased but did not eliminated the HIV-1 pandemic. 90% of the infected population live in underdeveloped area of the words where these tools are not available [36]. The development of a safe and effective prophylactic HIV-1 vaccine would represent the best solution to control the AIDS pandemic. 3 years after the discovery of HIV, the French researcher Daniel Zagury tested the first vaccine candidate without any regulatory approval and without adequate preclinical testing. He inoculated himself with a vaccine candidate containing a genetically engineered version of an HIV protein inside a viral vector based on the vaccinia virus (the same virus used in the smallpox vaccine). Zagury also vaccinates nine HIV-uninfected children from Zaire (now the Democratic Republic of the Congo), making this the first unofficial preventive AIDS vaccine trial [38]. More than 250 clinical trials had been conducted, most of them ended in a phase I or II [39]. For the majority of licensed vaccines, neutralizing antibodies have provided the best correlate of vaccine efficacy. Therefore, in the first 10 years of research on HIV-1 vaccine, scientists tried to use the monomeric env gp120 protein to induce env specific humoral immune response. In 1998 the first efficacy trial of an AIDS vaccine candidate started. VaxGen launched a Phase III efficacy trial: AIDSVAX. The trial enrolled 5,400 volunteers, mostly men who have sex with men (MSM), in United States, Canada, Netherlands, and Puerto Rico. A year later, another arm of the trial began in Thailand, involving nearly 2,500 injection drug users. In both trials was administered the HIV gp120 protein and both trials failed to protect from HIV-1 infection regardless the induction of HIV-1 env gp120 specific response. In previous phase I and II clinical trials the same immunogens failed to induce broadly neutralizing antibodies (bNabs).

All these observations made clear the fact that type-specific antibody response alone was not providing protection against HIV-1 infection. In 1992 researchers reported that rhesus macaques vaccinated with a live, attenuated simian immunodeficiency virus (SIV) were protected against infection, raising hopes that this might be a feasible approach to HIV vaccine development. In 1994 early studies in monkeys opened the hypothesis that the protection could come from cellular immune response [40]. In this analysis animals with a lower viral set point had a specific T lymphocyte response after homologous SIV challenges. The negative results coming from the specific env antibody responses in humans together with the observations made in rhesus macaques vaccine models lead the way to start in 2004 the most important phase IIb vaccine trial focused on t cell immunity: the HIV Vaccine Trials Network (HVTN) 502 also known as STEP trial. This vaccine was formulated as a trivalent mixture of rAd5 vector expressing HIV-1 clade B Gag, Pol and Nef. The vaccine was tested in North and South America, the Caribbean islands, and Australia in 3,000 volunteers with the aim to either prevent HIV infection or in reduce viral load among vaccinated that would have acquired the virus [41]. In 2007 Vaccinations in the STEP trial were discontinued after a data safety monitoring board determines that the vaccine was not effective. Subsequent data shows that Ad5 may have increased the risk of HIV acquisition among a subset of vaccinated [41]. In 2009 a second Ad5 phase IIb exploratory clinical trial started. Based on what was known about the Step study findings, there were no signs of increased susceptibility to HIV infection among male vaccine recipients who were circumcised and lacked antibody responses to Ad5 at enrollment. That is why the HVTN 505 study was designed to include only those male volunteers with these characteristics. HVTN 505 evaluated two different vaccines in a prime-boost strategy. The HVTN 505 vaccine regimen consisted of a recombinant DNA-based vaccine (the prime vaccine). The DNA prime vaccine (called VRC-HIVDNA016-00-VP) contained genetic material expressing

antigens representing Gag, Pol, Nef, Env (clade B). Following completion of the series of three immunizations with the prime, vaccine recipients received a single immunization in week 24 with a recombinant vaccine (the boosting vaccine) based on a weakened adenovirus type 5 (Ad5) to carry the genetic material expressing a matching set of HIV antigens to stimulate the immune system. The HVTN 505 study team enrolled 2,504 HIV-negative MSMs and transgenders. The volunteers were from 18 to 50 years old and lived in the United States. During its scheduled interim review, the HVTN 505 DSMB found that the investigational vaccine regimen neither prevented HIV infection nor reduced viral load among vaccine recipients who later became infected with the virus. In April 2013, the primary analysis looked at volunteers who were diagnosed with HIV infection after having been in the study a minimum of 28 weeks. In this analysis, 27 HIV infections occurred among the vaccine recipients, and 21 HIV infections occurred among the placebo vaccine recipients. Based on these findings, the researcheres recommended that to suspend the injections of the investigational vaccine. The National Insitute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health, partner of the study, instructed all HVTN 505 study sites to immediately cease administering injections.

The surprising results of the RV-144 trial, an efficacy trial that was started in Thailand in 2003 with funding from NIAID and the US Army were published at the end of 2009. The trial, conducted by the Thailand Ministry of Public Health, tested a combination of two vaccine candidates (Sanofi Pasteur's recombinant canarypox vector vaccine candidate ALVAC-HIV vCP1521 and VaxGen's AIDSVAX B/E) in 16,402 healthy men and women between the ages of 18 and 30, primarily at heterosexual risk for HIV infection. Four priming injections of a ALVAC-HIV [vCP1521]) plus two booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E). In the modified intention-to-treat analysis the vaccine efficacy was 31.2%. Vaccination did not affect the degree of viremia or the CD4+ T-cell

count in subjects in whom HIV-1 infection was subsequently diagnosed [42]. The immune correlates of protection of this trial indicated that specific antibodies responses against V1V2 region of Env may have contributed to the protection against HIV-1 infection, whereas high levels of Env-specific IgA antibodies may have increased the risk of HIV-1 acquisition. This analysis did not find neutralization antibody as correlate but remarkably it seemed that the humoral response was effective through mediating other functions as antibody-dependent cell-mediated cytotoxicity (ADCC) [43]. Rolland et al. confirmed in an association between high V1/V2-binding antibodies and a reduced risk of HIV-1 acquisition providing evidences that vaccine-induced V2 responses plausibly had a role in the partial protection conferred by the RV144 regimen [44]. The V2 region is a highly variable region of HIV [45] and an immunization directed toward that specific region of the gp120 could not be effective when used with clades that do not share the same V2 region structure.

The development of an AIDS vaccine is affected by the range of virus subtypes as well as by the wide variety of human populations that differ in their genetic make-up and their routes of exposure to HIV. In particular, the occurrence of superinfection indicates that an immune response triggered by a vaccine to prevent infection by one clade of HIV may not protect against all other strains. The increasing variety of sub-types found within countries suggests that the effectiveness of a vaccine is likely to vary between populations, unless an innovative method is developed which guards against many virus strains [46]. Inevitably, different types of candidate vaccines will have to be tested against various viral strains in multiple vaccine trials, conducted in both developed and developing countries. However, in January 2015 a phase I/II Vaccine Immunogenicity Trial, HVTN 100 has started in South Africa. The vaccine regimen used in this study it is similar to the one used in the RV144. Nevertheless two major changes that could affect the outcome have been applied: the products that have been used are matching the circulating HIV-1 subtypes C and the adjuvant used to conjugate the gp120

clade C protein it is an oil in water emulsion, MF59. HVTN has been completed in October 2016. In order to start an Efficacy phase III trial on human based on the immunogens of the HVTN 100 the following four “go” criteria were evaluated:

1. Presence of IgG binding antibodies to at least 2 of the 3 gp120 vaccine antigens administrated;

2. a non-inferior IgG binding antibody magnitute to gp120 vaccine antigens compared to the RV144 trial;

3. a non-inferior response rate of Env specific CD4+ T cells expressing IL-2 or IFN-gamma or CD40L compared to RV144;

4. Prevalence of IgG binding antibodies to at least 1 clade C V1V2 Env region in more then 56% of the vaccinated. All the criteria were met and the beginning of the Efficacy Phase III study (HVTN 702) was approved.

The RV-144 Trial

There have only ever been four large phase III efficacy trials of HIV vaccines in humans. The first two were different versions of a vaccine called AIDSVAX; we learnt in 2003 that neither version prevented infection. The third was the STEP trial, which closed in September 2007. This vaccine, which had looked promising in animal studies, proved not only to have no effect in humans but actually to increase the likelihood of infection in a subset of participants. However, in October 2009, the RV144 trial proved to be modestly, and unexpectedly, efficacious, reducing HIV infections in recipients by 31%. The study was the result of a collaboration between U.S. and Thai governments [42]. It was a community-based, randomized, multicenter, double blind, placebo-controlled trial which involved 16,402 men and women between the ages of 18 and 30 in Rayong and Chon Buri provinces. It is currently referred to as *RV-144 trial*, or *Thai trial*. The vaccine strategy consisted of a prime-

boost approach. Four doses of ALVAC-HIV from Sanofi Pasteur (Swiftwater, PA, USA) were administered. The last two administration of ALVAC were coupled with AIDSVAX from VaxGene (San Francisco, CA, USA) [47]. ALVAC-HIV is a recombinant canarypox vector (vCP1521) where clade B-derived *gag* and protease and clade E-derived *env* genes were inserted inside the DNA genome, thus determining the formation of virus-like particles that bud from the cell membrane and elicit a T cell-mediated immune response. AIDSVAX B/E is a bivalent subunit vaccine, composed of a mixture of two highly purified glycoproteins produced by recombinant DNA in Chinese hamster ovary cells from subtype B (MN) and E (A244). The gp120 proteins were covalently bound to a 27-amino acid sequence found in the gD protein of herpes simplex virus type 1 (HSV-1), which was then cleaved during the purification procedure. The prime (ALVAC-HIV) was administered at baseline (day 0), 4 weeks, 12 weeks, and 24 weeks. Boosting with AIDSVAX B/E occurred at weeks 12 and 24. Testing for HIV was performed at day 0, at 24 and 26 weeks, and every six-months during the 3-year follow-up period. Similarly, assessment of behavior associated with increased risk of infection occurred at baseline, at week 26 and every six months thenceforth. Two primary endpoints were established: prevention of HIV infection and effect of vaccination on early viral load after HIV acquisition. The former was assessed on the basis of repeated positive results on enzyme immunoassay and Western blotting. The latter was defined as the mean among three measurements of HIV-1 RNA levels within 6 weeks after serodiagnosis. Despite the enrollment of 16,402 volunteers, 7 of them were found to be HIV-positive before the beginning of the study: although they underwent randomization and were included in the intention-to-treat (ITT) group, they were not considered in the modified ITT group, which was made of 16,395 individuals, 8197 receiving vaccine and 9198 receiving placebo. Finally, 6176 and 6366 volunteers from each group, respectively, were included in the per-protocol analysis group, as the remainders were ruled out due to

inappropriate or incomplete administration of vaccine or placebo. Participants were equally allocated in each group (vaccine and placebo) according to several baseline characteristics, including sex, age, province of provenience, marital status, number of sex partners, risk behavior (e.g., needle sharing, no condom use, symptoms of sexually transmitted disease, drug injection, commercial sex work). The study demonstrated for the first time the induction of protection from HIV-1 infection following vaccine administration: in the modified intention-to-treat analysis a significant difference was established between the two groups (vaccinees and controls) in regard of rate of HIV-1 infection, showing a vaccine efficacy of 31.2% (p = 0.04) (Fig. 2).

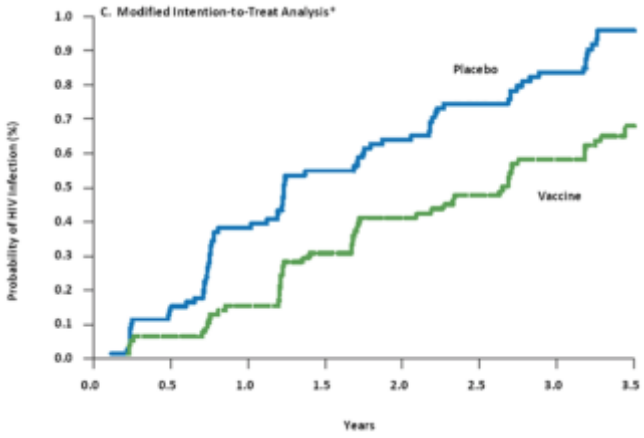

Fig. 2 | Kaplan-Meier cumulative rate of infection, according to Modified Intention-to-treat Analysis, showing a vaccine efficacy of 31.2% (From Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med*. 2009;361:2209-2220).

In addition, a consistent, although not significant, trend was confirmed both in the ITT analysis and in the per-protocol analysis, where vaccine efficacy was 26.4% (p = 0.08) and 26.2% (p = 0.16), respectively. However, there was no significant difference in the mean viral load among subjects who were found to be HIV-positive in the vaccine group, as compared to those in the placebo group. Some questions, of course, remained unaddressed, such as the possibility that the vaccine might be more effective in persons at lower risk of infection, or that the efficacy in protected individuals might have decreased over

time. Furthermore, a broad constellation of immune responses was induced by the prime-boost approach strategy, including T-cell line adapted neutralizing antibodies, antibody-dependent cell-mediated cytotoxicity, $CD4^+$ T-cell proliferation and $CD8^+$ T-cell activation. However, at the time of the trial it was not known yet whether these responses were related to protection. Haynes and colleagues conducted an in-depth study of protection correlates with a consortium having broad-based skills in immunologic investigation and performing statistical analyses [43]. The consortium conducted a case-control study that focused on the relationship of six primary immune-response variables to the risk of HIV-1 infection. The findings indicated that binding IgG antibodies to variable regions 1 and 2 (V1-V2) of the HIV-1 envelope (Env) glycoprotein 120 correlated inversely with the rate of HIV-1 infection. In addition, a complex relationship to risk was observed with levels of monomeric plasma IgA antibodies, which were not associated with an overall increased risk of infection as compared with controls, but which appeared to mitigate the protective effect of IgG antibodies to V1-V2. These IgA responses also appeared to modulate, in a similar fashion, a variety of possible protective mechanisms associated with other immune responses, such as the avidity of IgG antibodies, antibody-dependent cellular cytotoxicity, neutralizing antibodies, and $CD4^+$ T-cell responses. The mechanism for the apparent effect of IgAs in inhibition of the protective effect observed with anti-V1V2 IgG antibodies against HIV-1 infection is yet to be determined. Nevertheless, the only samples available for IgA measurements were from peripheral blood, so that immune responses in key compartments, such as mucosal sites, remain unknown.

ANIMAL MODELS

Limitations and streghts

HIV group M is genetically close to the SIV_{cpz}, a particular strain of SIV which infects chimpanzees and causes an AIDS-like illness in the wild [10, 13, 48] . Nevertheless, SIV infection in chimpanzees cannot be used as a model since chimpanzees are a protected

species. An adequate animal model requires the production of all proteins (including receptors and enzymes) that the virus would use within the host in order to replicate. Therefore, it is evident that CD4 receptors and CCR5 and CXCR4 co-receptors as well as some transcription factors and nuclear export factors all need to be expressed by target cells [49]. However, several restriction factors (proteins that are upregulated following production of type I interferons and innate immunity activation), which inhibit the establishment of HIV infection, have been recently identified, thus accounting for the inability of HIV to infect a variety of species other than humans [50]. The major restriction factors are tripartite-motif-containing protein 5 (TRIM5), apolipoprotein B-editing catalytic subunit-like 3 (APOBEC3), Tetherin and SAM-domain- and HD-domain-containing protein 1 (SAMDH1). These restrictions factor act a diferent leves but they all result in an aborted infection [51-54].

Non-human primate models

SIV is a lentivirus that comprises a number of species-specific strains. For instance, SIV_{cpz} typically infects African chimpanzees (*Pan troglodytes troglodytes*) and is phylogenetically related to HIV-1, at least to M group [10, 13]. On the other hand, SIV_{smm} is specific for the African sooty mangabey (*Cercocebus atys*) and gave rise to HIV-2 in humans and SIV_{mac} in macaques, upon cross-species transmission[55]. Indeed, SIV_{smm} and SIV_{mac} sequences show great similarity with HIV genome. SIV does not cause any disease in its natural hosts (i.e., Old World monkeys), even if it preserves its infectivity [56]. The only exception is represented by SIV_{cpz}, which is associated with an increase in mortality, although not to the same levels as HIV in humans or SIV in non-natural hosts [57]. By contrast, SIV strains that are able to infect Asian monkeys do cause a disease, named *SAIDS*, which closely resembles AIDS [58]. Some similarities in natural and non-natural hosts have been demonstrated, including high viremia (high viral load), short lifespan of productively infected cells, significant depletion of mucosal $CD4^+$ T cells during acute infection, significant activation of both innate and adaptive immune response, ineffective immune control of infection as showing protection from AIDS does not result from an

adaptive immune response that allows the suppression of viral replication. However, even more differences have been identified, since in natural hosts the following features have been shown: high levels of peripheral $CD4^+$ T cells, preservation of mucosal immunity and absence of microbial translocation, acute immune activation resolution and absence of chronic immune activation, preferential tropism for effector memory T cells (T_{em}) instead of central memory T cells (T_{cm}), normal lymph node structure and function, rare pattern of vertical transmission. Given that Old World monkeys cannot be used as a model for HIV infection, non-natural hosts, such as Asian macaques, where SIV infection closely resembles HIV-1 in humans, have become the most common animal model for HIV research. Indeed, Asian macaques are being extensively used in many studies for the development of effective vaccine strategies and microbicides against HIV-1. Three species of macaques are currently utilized: the rhesus macaque (*Macaca mulatta*), the pig-tailed macaques (*Macaca nemestrina*), and the cynomologus macaque (*Macaca fascicularis*). Rhesus macaques are by far the most widely used and the best characterized animal model for HIV infection.

Rhesus macaques

SIV infection, as previously described, is very similar to HIV-1 as for the impairment of the adaptive immunity and depletion of $CD4^+$ T cells, but it differs from HIV insofar as the velocity of disease progression [49]. In humans from 8 to 10 years are necessary to the infection to progress to AIDS, whereas in rhesus macaques SAIDS manifestations occur within 2 years, resulting in an even more convenient model in terms of both practicality and costs. In humans, at the time of infection only few variants of the virus are transmitted to the host (genetic bottleneck theory), and genetic variability of infective virions in a single individual is due to a variety of mechanisms that occur after the establishment of infection. In order to recapitulate these findings in rhesus macaques, it has been recently demonstrated that intrarectal challenge with low and repeated doses of SIV_{mac251} similarly results in the transmission of few virus variants, as seen in humans, whereas high-dose

challenge does not [59]. Before the beginning of every study, it needs to be considered that there are at least two restriction factors that might play an important role after the challenge with SIV_{mac}. First, some MHC-I molecules are strongly associated with better containment of replication: Mamu A*01, Mamu B*08 and Mamu B*17 all correlate with low levels of viral load[60]. Secondly, TRIM5-α actually seems not to alter the susceptibility of macaques to SIV_{mac251} infection [61]. Finally, the limitations of this model need to be addressed. SIV strains are less sensitive to drugs that are currently being used as standard in antiretroviral therapy SIV strains do not tend to bind CXCR4, as opposed to HIV-1, and use some other co-receptors, instead, so that testing of entry inhibitors-based therapy might be further complicated [62]. Finally, the scientific community is still making a lot of efforts to assess a strategy to create chimeric recombinant genomes, namely SHIV (i.e., Simian Human Immunodeficiency Virus), which can infect macaques and express *env* or *pol* HIV-1 proteins. Rhesus macaque model is still the most reliable for HIV research: recently, it was possible to mimic the results of Thai trial in Indian rhesus macaques by using similar vaccine regimens and challenging with low and repeated doses of SIV_{mac251} [63]. However, the refinement of pre-existing models and perhaps the development of new ones will pave the way to more relevant models.

HIV/SIV CONTROL and HIV/SIV PREVENTION.

Many laboratories in the world have faced the issue of trying to suppress HIV viral load in the absence of antiretroviral therapy (ART). The scientific community has reached the extraordinarily success in inducing long term HIV-1 remission under ART. Nevertheless, replication competent virus is still present in individuals even several years after the initiation of ART and effective suppression of viral load. Discontinuation of therapy results in viral rebound even in people that well control after years of it [64]. There are two scientifical strategies that address persistence: eradicate the reservoir (classical “cure”) or control viral

rebound (sustained virologic remission). Many groups have tried different ways to find a cure, without success. They tried to use latency-reversing agents to deplete HIV reservoirs, administer immunotoxic therapy directed at reservoir, perform stem cell transplantation and gene editing. On the other hand researchers who aimed to a sustained virologic remission have as a goal to discontinue ART and reach control of viremia by either inducing a natural HIV-specific immunity, administering therapeutic vaccinations or infusing HIV-specific antibodies [65]. HIV envelope induces aberrant signal that increase viral replication, increases the susceptibility of the cells to be infected by HIV and induces dysfunction in NK cells [66-69]. NK cells lack of CD4 or CCR5 receptors. Considered that, the most probable way in which HIV-1 envelope can bind those cells is through α4β7 integrine. The primary function of α4β7 is to mediate migration and retention of leuckocytes in the gut. Indeed HIV-1 evelope protein is able to bind to α4β7 and to induce intracellular signals through α4β7 expressed on CD4 [70]. The three natural ligands of α4β7 are MadCAM, VCAM-1 and Fibronectin. These molecules bind to α4β7 through a tripeptide loop with a common central aspartic core (respectively LDT, IDS, LDV). Of note, this tripeptide is very conserved amongst HIV and SIV sequences. The central role of α4β7 during HIV acute infection has been recently demonstrated by Ansari *et al.* [71]. They demonstrated that blocking of α4β7 during acute infection leads to decreased levels of viral loads in plasma and gastrointestinal tissues in SIV-infected rhesus macaques. Based on this observations researcher started to investigate the hypothesis that a monoclonal antibody directed α4β7 could protect against mucosal transmission of SIV. The results of this study were stunning [72]. Targeting α4β7 integrin reduced mucosal transmission of SIV and protected gut-associated lymphoid tissue from infection. In summary, this study showed that the infusion of anti α4β7 antibody in SIV_{mac251}-mucosally exposed-monkey could protect from SIV infection and could induce

sustained decrease in proviral load in GALT and other peripheral lymphoid tissues following termination of anti-α4β7 treatment in infected animals. These results led the researchers to perform another study, in an SIV model, in which they tested the effect of the infusion of anti-α4β7 antibody in SIV-infected rhesus under ART [73]. Infusing anti α4β7 antibody to NHPs together with a 90-day course of ART initiated 5 weeks post-infection with SIV_{mac239} induced long term viral load suppression following discontinuation of antiretrovirals. The treated animals showed restoration of CD4+ T-cells in gut and blood. Several potential correlates were observed in this study including the increase of the frequency of cytokine-synthesizing NKp44+ cells and the induction of an antibody response toward the gp120 V2 domain. It is of interest that the SIV_{mac239} V2 peptide target of the response in this study overlaps the site of antibody immune pressure in HIV-1 V2 that correlated with decreased transmission risk of infection in the RV144 vaccine efficacy trial. The significance of this findings in human are unclear and further studies are necessary.

AIM OF THE STUDY

Today, more people living with HIV than ever before have access to life- antiretroviral therapy, which delay the progression of the disease and reduces the likelihood to transmit the virus to others. In addition, others who are at high risk for HIV infection have access to the Pre-exposure Prophylaxis, being used to prevent HIV. Yet, unfortunately, approximately 50,000 Americans and 2 million people worldwide became newly infected with HIV in 2014. To control and ultimately end HIV globally, a powerful array of HIV prevention tools widely accessible to all who would benefit from them are needed Vaccines historically have been the most effective means to prevent and even eradicate infectious diseases. They safely and cost-effectively prevent illness, disability, and death. Developing safe, effective, and affordable vaccines that can prevent HIV infection in uninfected people is the best hope for controlling and ultimately ending the HIV/AIDS pandemic.

As previously described, among human clinical studies, only one, the RV-144 trial, completed in Thailand, showed marginal efficacy (31.2%) [42]. A preliminary pilot study demonstrated that it is possible to recapitulate the protection seen in the Thai trial in an animal model for HIV infection [63]. In this model Indian rhesus macaques were vaccinated with ALVAC canarypox vector delivering SIV genes and boosted with SIV gp120 protein. Similarly to Thai trial, the adjuvant used was alum. Four weeks after the last vaccination animals were challenged with repeated low doses of SIV_{mac251} intrarectally, resulting in 27.3% rate of protection from SIV acquisition.

This study seeks to expand upon those data, in order to define vaccine efficacy and immune correlates of protection induced by vaccination in the animal model. As the preliminary study, a group of 27 Indian rhesus macaques will be vaccinated with the same prime-boost approach (ALVAC/gp120). Furthermore, a second group of animals will be recruited. In this group, MF59 will be used instead of alum, aiming at assessing vaccine

efficacy in a regimen that differed only in the type of adjuvant. Recent studies demonstrated that immune correlates of HIV prevention (induction of antibodies against the V2 of gp120) are induced in rhesus macaques that control viral replication in absence of ART [72, 73]. Therefore, we will explore and investigate the role of some immune correlates (V2 response) of protection in a group of LTNP HIV patients.

MATERIALS AND METHODS

ANIMAL INFORMATION

The animals used in this study were colony bread Indian rhesus macaques (*Macaca mulatta*) obtained from Covance Research Products (Alice, TX). They were from 3 to 10 years old, and their weight before the study was greater than 3 kilograms. The animals were housed and maintained in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Advanced BioScience Laboratories, Inc., Institutional Animal Care and Use Committee approved the protocol. All efforts were made to minimize suffering. All macaques were negative for simian retrovirus, simian T-cell leukemia virus type 1 and herpes B.

STUDY DESIGN AND VACCINE IMMUNOGENS

All animals used in this study were colony-bred Rhesus macaques (*Macaca mulatta*), obtained from Covance Research Products (Alice, TX). The animals were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International. Both care and use of the animals were in compliance with all relevant institutional (US National Institutes of Health) guidelines. The protocol (AUP 491) was approved by the Advanced BioScience Laboratories's Institutional Animal Care and Use Committee.

A total of 54 juvenile macaques were randomized into two groups of 27 macaques, according to their major histocompatibility status, gender and weight (average, 3 kg). Statistical analysis suggested an 80% probability of detecting significant vaccine efficacy in a range of 30–40% (likelihood ratio test) [74]. Although the investigators were unblinded, the animal handlers were blinded to the vaccine groups. All 54 macaques were immunized at

weeks 0, 4, 12 and 24 with intramuscular inoculations of 10^8 plaque-forming units (PFU) of ALVAC (vCP2432) expressing SIV genes *gag-pro* and *gp120TM* (Sanofi Pasteur). The sequence of the SIV genes was obtained from a mucosally transmitted founder variant of SIV_{mac251} designated M766r [75]. The *gag-pro* gene was codon-optimized and constructed using human-codon bias, leaving the stem loop and slippery sequences intact to allow for the one frame-shift required for *pol* translation. The *gag-pro* encodes the entire *gag*, followed by *pol*, through the end of the protease. The *gp120TM* gene was codon-optimized and constructed using human-codon bias. The native signal peptide was replaced with a synthetic signal based on the human tissue plasminogen activator (tPa) signal peptide (MDAMKRGLCCVLLLCGAVFVTTTEA). The SIVM766 gp120 from I20 through R527, and the 22-residue gp41 transmembrane (TM) domain from Y695 to L716 follows (numbering is based on SIV_{mac251}). Six residues of the HIV-1LAI cytoplasmic tail (N706 to G711) follow the TM domain (numbering based on HIV-1HXB2 numbering convention).

At weeks 12 and 24, 27 animals consisting of 12 females and 15 males, 5 MamuA*01$^+$ and 4 MamuB*17$^+$ (average weight, 5.4 g), were given a bivalent monomeric-gp120 protein boost in the opposite thigh of the vector immunization, containing 200 µg each of the mucosally transmitted founder variants, SIV_{mac251}-M766 [75] gp120-gD (referred to in the text as gp120) and SIV_{smE660} gp120-gD CG7V [76] (referred to in the text as gp120), both formulated in alum. The diluted proteins were mixed 1:1 with Alhydrogel 2% (Invivogen, Cat# vac-alu-50) by rocking at room temperature for 10 min. An additional group of 27 animals (13 females and 14 males, six MamuA*01$^+$ and four MamuB*17$^+$; average weight, 6.7 g) were given only 100 µg (as per Novartis's recommendation) of both gp120-gD proteins in MF59.

This study was planned with the provision of using 24 simultaneous controls that received alum (*n* = 6), MF59 (*n* = 12) or nothing (*n* = 6), as well as 23 historical controls that received

alum (*n* = 11)[6] or gp96-Ig (*n* = 12)[77]. All controls were housed in the same facility and were challenged by the same operators with the same challenge-virus stock. The control group had ten MamuA*01^{+} and six MamuB*17^{+} animals and an equivalent number of females and males. Animals were not screened for TRIM-5α because we previously demonstrated a lack of sensitivity to TRIM-5α restriction of this SIV_{mac251} stock used at the same dose (120 TCID50) [61].

VIRAL CHALLENGE

Four weeks after the final immunization, all animals were challenged with 120 TCID50 SIV_{mac251} via the rectal route. Animals testing negative for SIV RNA in plasma were re-exposed, and a maximum of ten weekly challenges were used. SIV_{mac251} acquisition did not differ in the simultaneous and historical controls.

CELLS, PLASMA AND MUCOSAL SECRETION ISOLATION

Plasma has been collected as described: blood was collected in EDTA and centrifuged after bleeding at 2000 rpm for 10 minutes. Plasma was aliquoted of 0.5-1 ml vials and stored immediately at -80C for long term storage. Peripheral blood, plasma and rectal swabs were collected from all animals before vaccination (4 weeks prior to immunization) and after the fourth immunization (week 25). For immunized human samples (studies RV135 and RV132) blood was collected before the immunization and 2 weeks after last vaccination. Peripheral blood mononuclear cells (PBMCs) were collected by using Isopaque-Ficoll technique relying on differential density gradients to extract PBMCs from whole blood. Rectal swabs were placed in 1 ml of cold elution buffer (1 x protease inhibitor cocktail [Calbiochem], 0.25% BSA) after collection, in order to prevent antibody degradation. Antibodies were eluted by spinning 2 times at 16,000 x g for 15 to 20 minutes at 4C.

MEASUREMENT OF VIRAL RNA, DNA AND CD4$^+$ T CELLS

SIV_{mac251} RNA in plasma was quantified by nucleic acid sequence–based amplification, as previously described [78]. SIV DNA was quantified in mucosal tissues 3 weeks after infection by a real-time qPCR assay with sensitivity up to ten copies × 10^6 cells, as previously described [79]. CD4$^+$ T cell counts were periodically determined from whole blood by flow cytometry, as previously described [80].

IgG- and IgA- BINDING ASSAY

The SIV Env-specific IgG and IgA antibodies in serum and rectal mucosa were determined by a custom SIV bAb multiplex assay (SIV-BAMA), as previously described [44, 81]. For mucosal samples, specific activity was calculated by the ratio of MFI (linear range of standard curve)/μg/ml total macaque IgG, which was measured by macaque IgG or IgA ELISA to normalize the data to recovery of antibody. Rectal swab samples in solution were spun, filtered and concentrated down to approximately half of the starting volume. The samples were examined for blood contamination and measured for semiquantitative evaluation of hemoglobin. Purified IgG (DBM5) from a SIV-infected macaque (provided by M. Roederer, VRC, NIH) was used as the positive control to calculate SIV antibody concentration. Positive controls for each antigen were tracked via a Levy Jennings Plot. Specific activity was calculated from the total macaque IgG levels and the SIV-specific concentrations. Antibodies against native V1/V2 epitopes were quantitated by binding assays against native SIV V1/V2 antigens expressed as gp70-fusion proteins related to the CaseA2 antigen used in the RV144 correlate study (provided by A. Pinter). These proteins contained the glycosylated, disulfide-bonded V1/V2 regions of SIVmac239, SIV_{mac251} and SIV_{smE660} (corresponding to AA 120–204 of HXB2 Env), fused to residue 263 of the SU (gp70) protein of Fr-MuLV.

REAGENTS AND SURFACE PLASMON RESONANCE

SIV-V2 peptide was synthesized by JPT Peptide Technologies GmbH, Berlin, Germany. The peptide was allowed to fold and cyclize under thermodynamic control at high dilution, and the purity was determined to be greater than 90% by high-performance liquid chromatography and mass spectrometry. The amino acid sequence of the SIV V2 peptide is based on the SIVsmE543-3 V2 domain from GenBank accession number U72748. The SIV-V2 peptide sequence contains an N-terminal biotin tag and the sequence is as follows:

GF SIV_{smE543}

CIKNNSCAGLEQEPMIGCKFNMTGLKRDKKIEYNETWYSRDLICEQPANGSESKCY.

GF SIV_{mac251} full

CIAQNNCTGLEQEQMISCKFNMTGLKRDKTKEYNETWYSTDLVCEQGNSTDNESRCY.

CM5 chips and the Biacore amine coupling kit were purchased from GE Healthcare, Piscataway, NJ, USA. Streptavidin was purchased from Invitrogen, Grand Island, NY. Affinity purified goat anti-monkey IgG and IgA (gamma-chain- or alpha-chain-specific) antibody was purchased from Rockland Immunochemicals, Gilbertsville, PA.

Surface plasmon resonance (SPR) measurements were conducted with a Biacore T200 using the CM5 chip, as described previously[63]. Streptavidin was immobilized onto the chip using the amine coupling kit, as directed by the immobilization wizard packaged within the T200 control software. 6,700 reported response units (RU) of 1 µM streptavidin in 20 mM sodium formate, pH 4.2 (10 min contact time, 10 µl/min flow rate) was immobilized. The biotinylated peptide was prepared at a concentration of 1 µM in 20 mM TRIS, pH 7.4 and allowed to flow (at 10 µl/min) over the streptavidin-coated surface of flow cell 4, until 3,500 RU of SIV V2 peptide were captured.

The mucosal swabs were thawed on ice and centrifuged at 16,100 rcf, 4 °C, for 5 min. The

supernatant was diluted tenfold in TBS, pH 7.4, and then analyzed on the Biacore. The diluted mucosal samples were passed over the chip surface at a flow rate of 30 µl/min for 3 min, followed by a 5-min dissociation period. A 20 µg/ml solution of affinity-purified, gamma-chain-specific goat anti-monkey IgG or IgA antibody was passed over the peptide-coated, Ig-bound surface for 2 min at a flow rate of 10 µl/min. After a 70-s dissociation period, the chip surface was regenerated and data were analyzed, as previously described, using the BIAevaluation 4.1 software[63]. The RU for the IgG-specific or IgA-specific values are the difference between the average value of a 5-s window taken 60 s after the end of the anti-IgG or anti-IgA injection and the average value of a 5-s window taken 10 s before the beginning of the anti-IgG or anti-IgA injection. The data (RU) will be presented in the results chapter as dot plots for individual mucosal samples.

To determine total IgG antibodies in the mucosal samples, anti-IgG immobilization on a CM5 chip was performed using 100 nM of unconjugated gamma-chain-specific goat anti-monkey IgG (Rockland, Inc., Gilbertsville, PA) in 20 mM sodium acetate, pH 4.2, with a 5-min contact time and 10 µl/min flow rate, resulting in the immobilization of 9,100 RU. Centrifuged mucosal samples (diluted 1:10) were passed over the chip surface at a flow rate of 30 µl/min for 3 min, followed by a 5-min dissociation period. The relative amount of monkey IgG was determined using the same secondary injection and analysis strategy described above.

Chemstrips were used to determine the blood contamination in mucosal samples. 10 µl of the mucosal supernatant sample was spotted onto a Chemstrip 5 OB Urine Test Strip (Roche, cat. #11893467-160). After 60 s, any change in color was recorded for comparison to the manufacturer's color chart.

REAGENTS AND SURFACE PLASMON RESONANCE FOR CYCLIC V2 PEPTIDES BY BIACORE 4000 FOR HUMAN SAMPLES

Reagents:

Goat-Anti-human-IgG (gamma chain) Binding Sites (cat# 617-101-012 Lot#: 33560)

Amine coupling kit (cat#: BR-1000-50, lot#: 2077015, GE Healthcare)

The CM7-S series chip (cat#: 28-9538-28, lot#: 10239700, GE Healthcare)

Sodium acetate pH 4.5 (cat#: 28-9538-28, lot#: 20278962011, GE Healthcare)

Hepes buffer (10 mM Hepes, 150 mM NaCl pH 7.4): lot#: CH#20160321 (self-prepared)

Streptavidin (Cat#: SNN10001, lot#: 1634103A, Life Tech)

HIV peptides:

Peptide synthesis by JPT, Germany, HPLC and LCMS quality control

1. Cyclic biotin V2.AE.92TH023 (lot#: 16716_1, bath#: 120911A5, JPT)

CSFNMTTELRDKKQKVHALFYKLDIVPIEDNTSSSEYRLINC

2. Cyclic biotin V2.B.CaseA2 (lot#: 16816_1a, bath#: 101011A2, JPT)

CSFNITTSIRDKVQKEYALFYKLDIVPIDNPKNSTNYRLISC

3. Cyclic biotin V2.B (MN) (lot#: 22038_1, batch#: 011111A2W1013, JPT)

CSFNITTSIGDKMQKEYALLYKLDIEPIDNDSTSYRLISC

4. Cyclic biotin V2.C.1086 (lot#: 24653_6, bath#: 1012114HS-Ksch2, JPT)

CSFKATTELKDKKHKVHALFYKLDVVPLNGNSSSSGEYRLINC

Sample preparation:

To deactivate the complements and remove lipid contents, sera (105 µL) were heated at 56°C for 45 min followed by centrifugation at 16,000 x g at 4°C for 20 min. The supernatants were collected and stored at -80°C for Biacore assay.

Sample analysis:

The immunogenicity assessment for cyclic V2 peptides was conducted using Biacore 4000 system. The immobilizations were completed in 10 mM Hepes and 150 mM NaCl pH 7.4

using a standard amine coupling kit. The CM7-S series chip surface was activated with a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M N-hydroxysuccinimide (NHS) for 600 s. Then 1 µM Streptavidin in 10 mM sodium acetate pH 4.5 was immobilized for 720 s (11,400 12,300 RU). The immobilized surface was then deactivated by 1.0 M ethanolamine-HCl pH 8.5 for 600 s. Spot 3 in each flow cell was left unmodified to serve as a reference. After the surface deactivation, 2.5 µM cyclic biotinylated peptide of AE.92TH023. V2 (cbV2_AE): 4,700 - 4,800 RU, B.MN. V2 (cbV2.MN): 4,000 RU, B.caseA2. V2 (cbV2.BcaseA2): 5,200 – 5,400 RU, C.1086. V2 (cbV2.C): 4,900 RU was captured. Following the surface preparation, the heat-inactivated plasma was diluted (1:40) in 10 mM Hepes, 150 mM NaCl pH7.4. The diluted plasma was injected onto the captured surface for 300 s followed by a 30 s dissociation period. The bound antibodies were then enhanced by the injection of a secondary antibody, goat anti-human IgG antibody (30 µg/ml) for 360 s. To regenerate the bound surface, 175 mM HCl was injected for 60 s. Each sample was tested in quadruplicate for each peptide at a rate of 10 Hz and analysis temperature at 25°C. All sample injections were conducted at a flow rate of 10 µl/min. Data analysis was performed using Biacore 4000 Evaluation software 4.1 with double subtractions for unmodified surface and buffer for blank.

PHENOTYPIC ANALYSES OF PLASMABLASTS

The frequency of PBs was measured in the blood of 23 macaques vaccinated with ALVAC–SIV + gp120 MF59 and 22 macaques vaccinated with the ALVAC–SIV + gp120 alum regimen, before vaccination and at 7 d after the last immunization. Not less than 10 million cells at a time were stained with Ax700 anti-CD3 (SP34-2; cat. #557917, 1:80 dilution), anti-CD14 (M5E2; cat. #557923, 1:50 dilution), anti-CD16 (3G8; cat. #557920, 1:85 dilution), anti-CD56 (B159, cat. #557919, 1:50 dilution), PECy7 anti-CD21 (B-ly4; cat. #561374, 1:85 dilution), PE anti-Ki67 (B56, cat. #556027, 1:25 dilution), PerCPCy5.5 anti-

IgM (G20-127; cat. #561285, 1:50 dilution); Qdot605 anti-IgG (G18-145; cat. #562025, 1:50 dilution) and PECF594 anti-CXCR3 (1C6; cat. #562451, 1:50 dilution) from BD Biosciences; PECy5-CD19 (J3-119; cat. #IM2643U, 1:30 dilution, Beckman Coulter); 650-CD20 (2H7; cat. #95-0209, 1:50 dilution, eBiosciences); FITC-CD38 (AT-1; cat. #10415, 1:250 dilution, StemCell); BV421-CD39 (MOCP-21; cat. #328214, 1:50 dilution, BioLegend); IgA-Texas Red (polyclonal; cat. #2050-07, 1:50 dilution, SouthernBiotech) and APC-α4β7 (cat. #11718, 1:50 dilution, NIH AIDS Reagent Program, Division of AIDS, NIAID). Cells were permeabilized with Cytofix/Cytoperm (BD Biosciences). Acquisition was performed on LSRII (BD Biosciences), and data were analyzed by FlowJo software (TreeStar). PBs were gated as lineage-negative ($CD3^{-}CD14^{-}CD16^{-}CD56^{-}$) $CD20^{+}CD21^{-}Ki67^{pos/high}$ ($CD38^{pos/high}$ $CD39^{+}$) [82]. Cells in this gate were positive for IgG, IgA and IgM, thus validating this panel to detect both PBs and plasma cells in macaques. The frequency of PBs expressing CXCR3 or α4β7 was calculated.

RV135 AND RV132 STUDY DESIGN AND IMMUNOGENS

The frequency of PBs expressing CXCR3 or α4β7 was assessed also in PBMCs collected at 2 weeks after the final immunization from 17 volunteers vaccinated with ALVAC–HIV + gp120 alum (RV135) and 17 vaccinated with ALVAC–HIV+ gp120 MF59 regimen (RV132). Both RV135 and RV132 studies had a double-blind placebo-controlled phase 2, and were approved by the appropriate Institutional Review Boards, specifically by four ethical-review committees in Thailand and the United States ((i) Ministry of Public Health Ethical Review Committee of Research in Human Subjects (MoPH EC) —FWA00001953; (ii) Institutional Review Board, Royal Thai Army Medical Department (IRBRTA)—FWA00001813; (iii) Mahidol University Central Institutional Review Board (MU-CIRB)—FWA 00000926; (iv) Office of Research Protections, Human Research Protection Office (ORP HRPO)) and by

the National AIDS Commission of Thailand. Volunteers were healthy, HIV-negative Thai adults, and written informed consent was obtained. The vaccines used in those trials were ALVAC–HIV (vCP1521): Recombinant canary-pox vector expressing *gag* and protease subtype B (LAI) and Env-gp120 CRF01_AE (92TH023) linked to the transmembrane-anchoring portion of subtype B gp41 (LAI) genes (Sanofi Pasteur), bivalent HIV-1 gp120 SF2 50 μg (subtype B) and gp120 CM235 100 μg (CRF01_AE) adjuvanted with MF59 (Novartis) and AIDSVAX B/E: bivalent gp120 MN 300 μg (subtype B) and gp120 A244 300 μg (CRF01_AE) adjuvanted with alum (Global Solutions for Infectious Diseases, GSID, South San Francisco, CA) [83, 84].

In the human samples, B cells were defined as $CD20^{+}CD19^{+}$ $CD27^{+}$. PBMCs were collected from vaccines before and 2 weeks after the last immunization.

PHENOTYPIC AND FUNCTIONAL ANALYSIS OF MUCOSAL NK-CELLS.

Rectal biopsies were obtained 1 week after the last immunization (week 25). Mononuclear cells were isolated from rectal biopsies. For the phenotypic characterization, 3×10^{6} cells were used, and flow cytometry staining was carried out for cell-surface and intracellular molecules using standard protocols. After this, anti-human fluorochrome-conjugated mAbs known to cross-react with rhesus macaques were used for the staining: AlexaFluor 700 anti-CD3 (SP34-2, cat. #557917, 0.2 mg/ml), APCCy7 anti-CD8 (SK1, cat. #561945, 5 μl) PerCPCy5.5 anti-CD4 (L200; cat. #552838, 5 μl) from BD Biosciences (San Jose, CA); V450 anti-TNF-α (Mab11; cat. #502920, 5 μl), NKp44 (P44-8; cat. #325110, 5 μl), PECy5 anti-CD40L (24-31; cat. #310808, 5 μl) 605NC anti-IL-2, (17H12; cat. #500331, 50 μg/ml), 605NC anti-CD20 (2H7, cat. #302334, 50 μg/ml) from BioLegend (San Diego, CA); PE-CY7 anti-IL-17 (eBio64DEC17; cat. #25-7179-42, 0.125 μg), FITC anti-CD107a (eBioH4A3; cat. #11-1079-42, 5 μl, 0.5 μg) from eBioscience (San Diego, CA); PE anti-NKG2A (Z199; cat.

#PN A60797, 5 µl) from Beckman Coulter (Fullerton, CA). The yellow and aqua LIVE/DEAD viability dyes (Invitrogen, cat. #L34959, 1 µl) were used to exclude dead cells. At least 50,000 $CD3^-$ singlet events (were acquired on a LSR II (BD) and analyzed using FlowJo Software (TreeStar). Mucosal NK cell were gated on live lymphocytes, which were negative for CD20 and CD3 and further classified on the basis of the expression of NKG2A and NKp44 surface markers [85, 86].

HIV PATIENTS

Patients were selected from our HIV outpatient clinic and underwent informed written consent. In order to characterize and study the humoral response to the envelope we enrolled patients belonging to two distinct groups:

1. **CHRONIC HIV INFECTION, PRE-ART (CHI).** These patients have been infected with HIV for at least six months and are naïve for antiretroviral therapy.
2. **LONG TERM NON-PROGRESSORS.** These patients have been infected with HIV for at least eight year, naïve for antiretroviral therapy, not affected by CD4 loss and. The $CD4^+$ cell count for this patients has been determined over 250 cells/µl at the enrollement. Absence of clinical symptoms.

All patients shared the same exclusion criteria:

- Age below 18 years
- Serology positive for HIV-2
- Previous administration of experimental vaccines aimed to elicit HIV-specific immunization

  Peripheral blood was collected during follow up visits. Thirty milliliters were drawn in one single phlebotomy.

Ethylenediaminetetraacetic acid–anticoagulated blood that was collected and processed by centrifugation to separate plasma. Peripheral blood mononuclear cells were harvested by Ficoll hypaque centrifugation, washed, counted and stored as cell pellets or cryopreserved in FBS-DMSO freezing medium. All samples were stored at -80°C, with the exception of live cells, kept in liquid nitrogen.

Virological data and history of HIV infection were collected from clinical charts and gathered in a dedicated database (Table 1).

PT_ID	DOB	age	sex	Risk factor/Transmission	Diagnosis date	Years from Dx	HIV-1 Clade	VL setpoint	VL at the enrollment	CD4 nadir	CD4 nadir (%)	CD4 at the enrollment	CD4 enrollment (%)	enrollment	group
1	10/11/79	34	M	Unprotected Sex	8/18/14	2.955556	B	1097000	1097000	523	9.2	523	9.2	8/21/14	CHI
2	2/22/51	64	M	Not know	7/1/15	1.955556	B	4000000	4000000	458	13	458	13	7/16/15	CHI
3	5/15/91	22	M	MSM	2/10/14	2.955556	B	102600	102600	666	30.4	666	30.4	4/2/14	CHI
4	9/24/90	24	M	MSM	5/19/15	1.955556	B	77320	14360	544	28.9	929	32	6/23/15	CHI
5	11/29/72	42	M	IDU	10/1/13	3.955556	B	427700	427700	227	14	227	14	10/24/13	CHI
6	7/20/88	27	M	MSM	11/1/12	4.955556	B	220000	90760	245	17.8	245	17.8	5/6/13	CHI
7	1/16/86	30	M	MSM	8/1/16	0.955556	B	460000	460000	145	10.3	145	10.3	9/7/16	CHI
8	9/4/88	28	M	MSM	6/10/16	0.955556	CRF12_BF	58470	58470	522	30.4	522	30.4	7/11/16	CHI
9	8/21/74	42	M	Unprotected Sex	9/17/16	0.955556	B	65500	65500	385	25	385	25	9/28/16	CHI
10	12/12/67	48	M	Unprotected Sex	7/1/16	0.955556	B	21290	21290	474	19.7	474	19.7	9/19/16	CHI
11	8/23/63	53	M	Unprotected Sex	8/13/16	0.955556	B	95350	95350	159	13.1	159	13.1	9/8/16	CHI
12	4/4/72	44	M	Unprotected Sex	9/15/16	0.955556	F1	1259000	1259000	55	4.7	55	4.7	9/15/16	CHI
1	11/13/66	47	M	Unprotected Sex	1/1/07	9.955556	B	2000	1042	249	21.5	289	25.2	2/17/12	LTNP
2	11/4/57	57	M	IDU	1/1/85	31.95556	B	38	41	381	34	1002	31.1	22/12/14	LTNP
3	7/2/61	55	M	IDU	1/1/90	26.95556	?	<37	<37	601	29	659	29.3	10/21/16	LTNP
4	5/13/68	45	M	Unprotected Sex	1/1/07	9.955556	B	1400	1062	419	27.8	610	34.6	11/19/15	LTNP
5	7/8/68	45	M	Blood transfusion	1/1/85	31.95556	?	<37	<37	493	28.2	756	25.1	11/14/13	LTNP
6	12/11/59	53	M	IDU	1/1/91	25.95556	B	4000	968	531	27.1	666	22.8	4/16/13	LTNP
7	9/18/68	47	F	IDU	1/1/87	29.95556	B	<37	<37	666		724		5/19/15	LTNP
8	3/16/64	52	M	Unprotected Sex	1/1/07	9.955556	B	400	563	377	32	484	34.8	9/21/16	LTNP
9	5/25/77	39	M	MSM	1/1/04	12.95556	B	4000	4751	467	30	564	36	5/24/16	LTNP
10	6/16/75	40	M	Unprotected Sex	1/1/02	14.95556	A1	200	284	647	25.5	909	24.7	7/4/15	LTNP
11	10/16/61	51	M	IDU	1/1/85	31.95556	B	100	390	489	26	489	26	7/31/12	LTNP
12	7/12/67	38	M	Unprotected Sex	1/1/02	14.95556	F1	<37	<37	1001	11	2177	12.8	8/31/15	LTNP
13	2/17/69	46	F	IDU	1/1/98	18.95556	B	<37	<37	386	28.2	490	32	12/1/15	LTNP
14	5/9/61	53	F	IDU	1/1/89	27.95556	B	2400	5340	532	53.2	588	53.2	11/4/14	LTNP

Table 1 | Epidemiological, clinical, immunological and virological characteristics of patients

ELISA FOR LINEAR MN PEPTIDES MAPPING FOR HUMAN PLASMA

For HIV MN pepscan, we coated 1ug/well peptide in 100ul 50mm sodium bicarbonate pH 9.6 overnight at 4 deg c in Nunc MaxiSorp plates, emptied wells and blocked with 200ul/well SuperBlock Blocking Buffer in PBS (Thermo Scientific) in PBS for one hr at room temperature, added 100ul of 1:50 or 1:10 sample in Sample Diluent (AVIOQ, Inc) per well and incubated at 37C for one hour, washed plates 5 times with PBS with .05% tween 20, added 100ul per well peroxidase labeled affinity purified antibody to Human IgG produced in goat (KPL) at a dilution of 1:120,000 in Dilsm II and incubated at 37C for one hour, washed plate 5 times as before, added 100ul per well K-Blue Aqueous Substrate (Neogen) and inc at rt 30min, stopped reaction with 100ul per well 2N sulfuric acid(Cole-Parmer) and read at 450nm using Molecular Devices E-max plate reader.

STATISTICAL ANALYSIS

The Mann–Whitney–Wilcoxon test was used to compare continuous factors between two groups. Correlation analyses were performed using the Spearman rank correlation method with exact permutation *P* values calculated. The number of challenges before viral acquisition was assessed using the log–rank test of the discrete–time proportional hazards model. The Wilcoxon signed–rank test was applied to changes in PB levels from prevaccination to postvaccination. Data statistical analysis was performed using GraphPad Prism, a 2D graphing and statistics software published by GraphPad Software.

RESULTS

VACCINE EFFICACY

We divided 54 rhesus macaques in two vaccine groups. The animal were randomized and controlled for the major histocompatibility complex (MHC)-I, age, weight and gender. All macaques were primed with ALVAC–SIV (week 0 and week 4) and boosted with ALVAC–SIV coupled with the gD-g p120 M766 and gD-gp120 CG7V (week 12 and week 24) formulated either in alum (group 1 *n* = 27) or MF-59 (group 2 *n* = 27). (Fig. 3). The gp120 proteins used in the study were different in amino acid sequence in order to mirror what have been done in the RV144. This clinical trial, in fact, used the gp120 clades E and B (known also as Circolating Recombinant Form 01_AE) matching the circulating clades in Thailand [42].

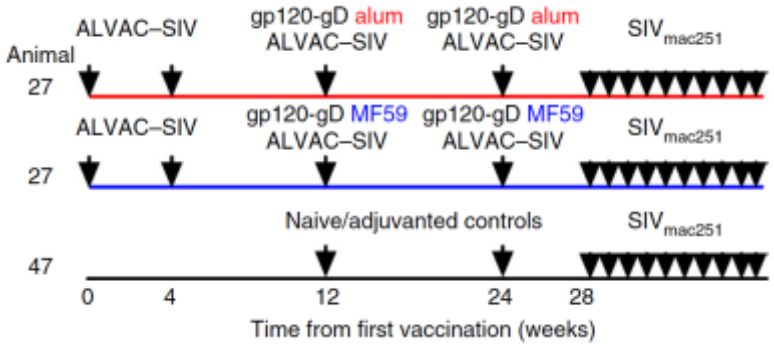

Fig. 3 | Study design. Animals were immunized with ALVAC (vCP2432) expressing SIV genes *gag-pro* and *gp120TM*, derived from the founder variant of SIV_{mac251} designated M766r and boosted with the SIV_{mac251} M766 gp120-gD (referred to in the text as gp120) and SIV_{smE660} gp120-gD CG7V (referred to in the text as gp120) proteins formulated in alum (200 mg) or MF59 (100 mg).

The amount of protein used was 200 ug in the alum group and 100 ug in the MF59 group (dose sparing ajuvant). Fourty-seven unvaccinated macaques were used as controls (24 concurrent and 23 historical) and matched vaccinated animals (weight, gender and MHC-I alleles). The study was powered to compare the relative vaccine efficacy in vaccinated macaques with placebo controls, but not to compare vaccine efficacy between the two

regimens. We challenged animals intrarectally weekly with ten repeated low doses of SIV_{mac251}, 4 weeks after the final immunization, at week 28. The time of challenge was chosen to model early exposure after vaccination, given the fact that enrolled volunteers in Thai Trial might have been exposed to HIV either during the vaccination or immediately after the end of the immunization. We observed a reduced risk of SIV_{mac251} acquisition relative to unvaccinated controls only in the group of animals vaccinated with ALVAC–SIV + gp120 alum (log–rank test; P = 0.020, vaccine efficacy of 44% at each challenge) (Fig. 4a). The infection rate in the group immunized with the ALVAC–SIV + gp120 MF59 vaccine strategy was not different from the infection rate observed in the placebo control (log–rank test, P = 0.562) (Fig. 4b) We observed no evidence of enhancement or viral suppression in the plasma (Fig. 4c,4d) of the vaccinated macaques that became infected in either arm of the study. The two vaccine regimens did not prevent the loss of CD4+ T cells in macaques that became infected (Fig. 4e).

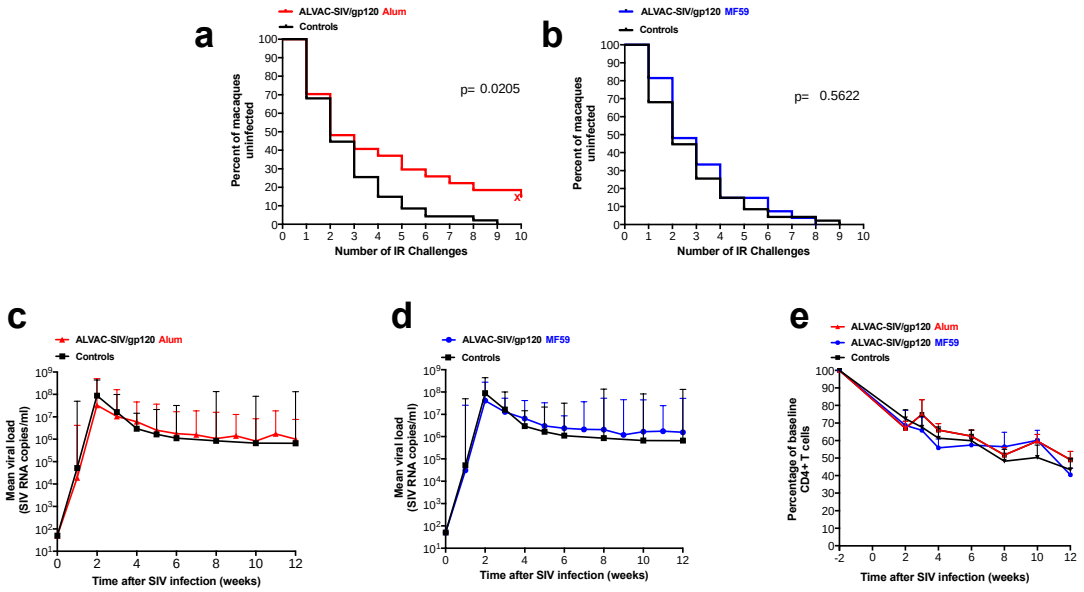

Fig. 4 | a, b. SIV_{mac251} acquisition. The number of intra-rectal (IR) exposures before viral acquisition was assessed in the **(a)** alum and **(b)** MF59 groups, relative to the controls, using the log-rank test of the discrete-time proportional hazard model. Study design and vaccine efficacy. **c, d.** SIV RNA levels in the plasma. Error bars show logarithmic mean ± s.d. of animals vaccinated with alum (**c**) or MF59 (**d**), and all historical and concurrent control animals (n = 47). **e.** Percentage of CD4+ T cell changes in the blood over time (weeks) (mean ± s.e.m.).

HUMORAL RESPONSES AND THE ROLE OF gp120-cV2

In order to scan the humoral response and to understand its role in the reduced risk of acquisition, the titers of specific antibodies (IgG and IgA) were investigated in both groups of animals. Overall the vaccination with ALVAC–SIV + gp120 MF59 induced higher levels of systemic binding antibody to SIV Env proteins than alum, both IgG (Fig. 5a, 5b,5c,5f) and IgA (Fig. 5d, 5e).

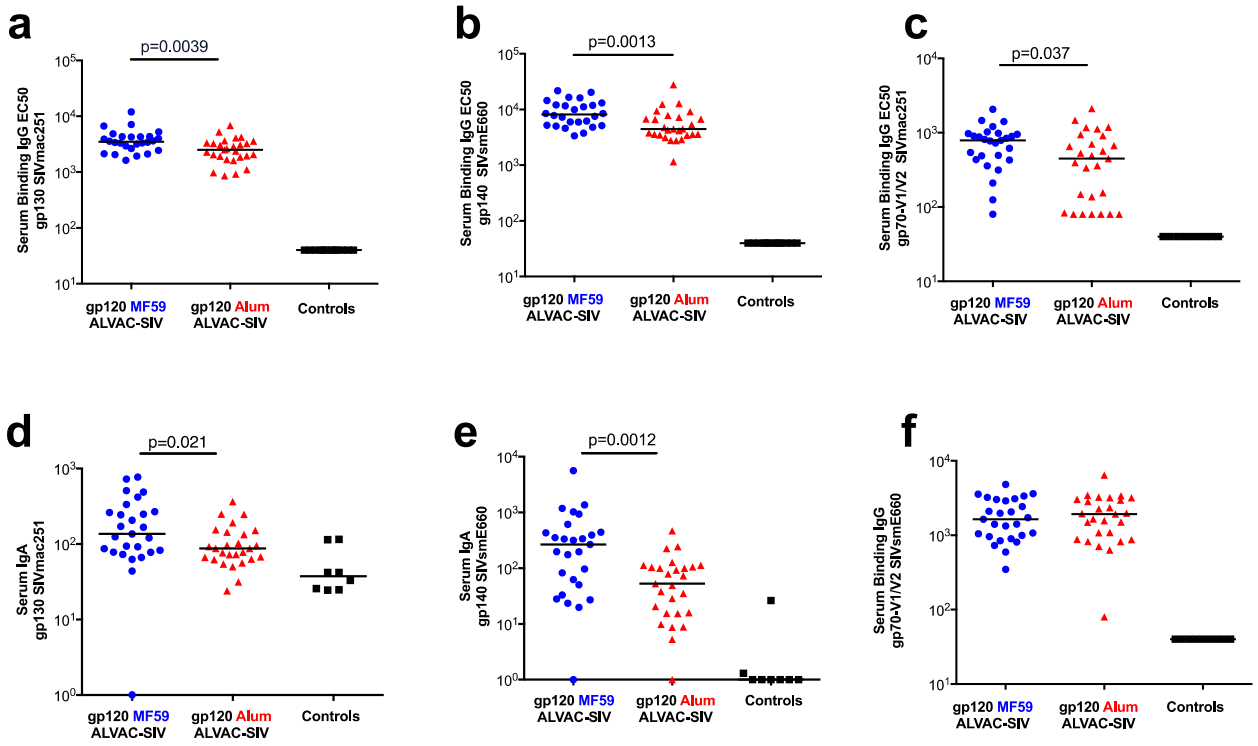

Fig. 5 | All vaccinated animals in the alum group are in red (n = 27) and those from the MF59 (n = 27) and controlgroup (n = 8) are in blue and black respectively. **a.** IgG titers in serum against the gp130 of SIV_{mac251} and **b.** the gp140 SIV_{smE660}. **c.** Serum IgG titers to the gp70-V1/V2 scaffold of SIV_{mac251}. **d.** IgA titers in serum against the gp130 of SIV_{mac251} and **e.** the gp140 $SIVsm_{E660}$ **f.** Serum IgG titers to the gp70-V1/V2 scaffold of SIV_{smE660}.

Similarly the levels of binding antibodies in the rectal mucosa were higher in the MF59 group compared to the alum group (Fig. 6a, 6b, 6c, 6d).

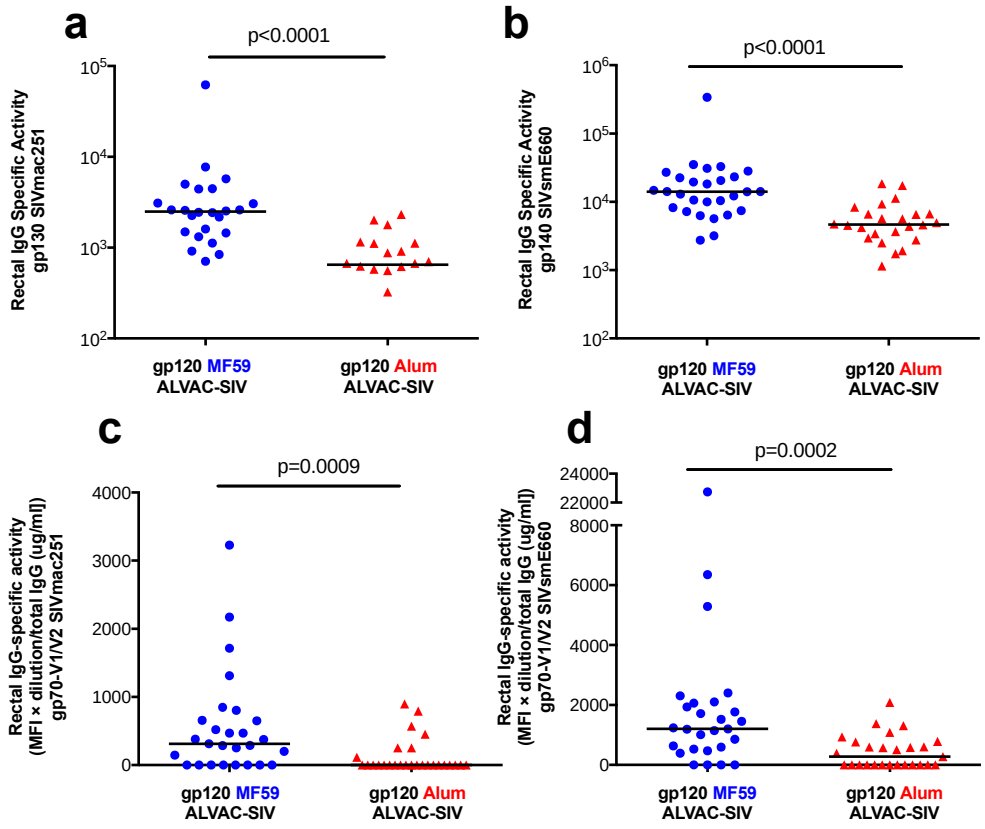

Fig. 6 | Env-specific IgG responses in mucosa. The alum group is in red (n = 27) and the MF59 in blue (n = 27) . **a.** Specific activity of rectal IgG-binding antibodies to the gp130 of SIV_{mac251} and **b.** to the gp140 SIV_{smE660}. **c.** Specific activity of IgG-binding antibodies to the gp70-V1/V2 scaffold of SIV_{mac251}. and **d.** to the gp70-V1/V2 of SIV_{smE660}

Notably, although the levels of IgG to cyclic V2 (cV2) were higher in the serum of the MF59 group than in that of the alum group (Fig. 7a, 7b), cV2 IgG was higher in rectal secretions from the alum group than in those from the MF59 group (Fig 7c, 7d), Importantly, among the alum-treated macaques, the mucosal cV2 IgG response was associated with a decreased risk of SIV acquisition (log–rank test, $P = 0.0018$) (Fig 7e). By contrast, rectal IgG antibodies

to cV2 were correlated with an increased risk of SIV_{mac251} acquisition in the MF59 group (log–rank test, $P = 0.0016$) (Fig 7f).

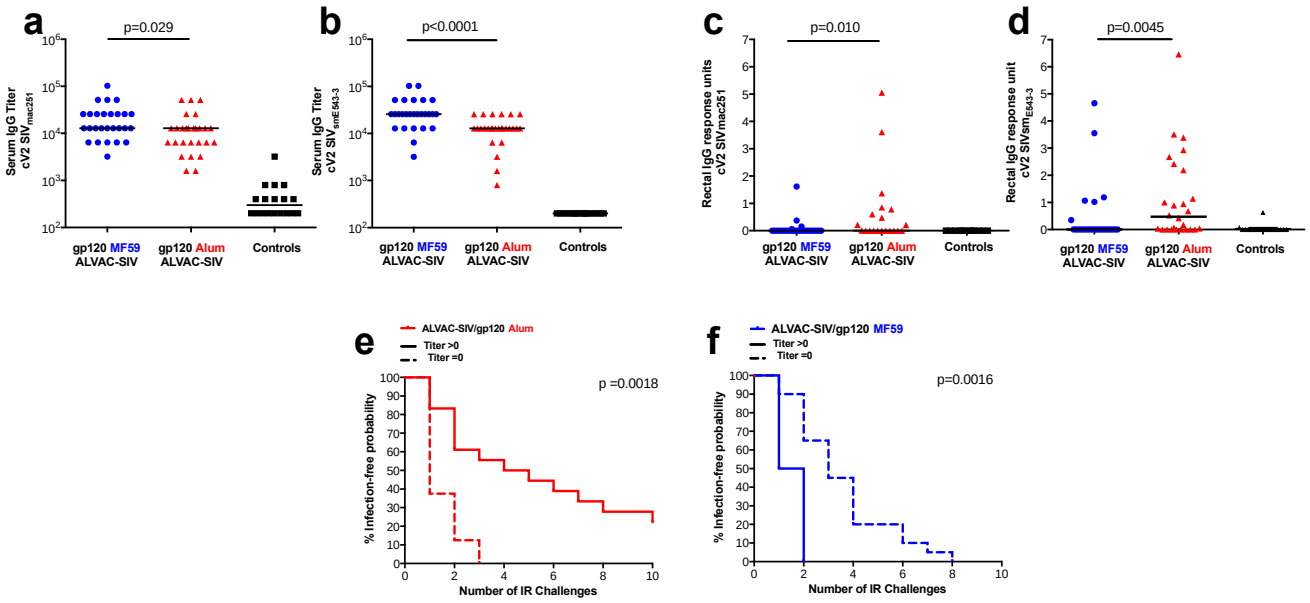

Fig. 7 | Serum and rectal IgG to cyclic V2 and SIV_{mac251} acquisition **a.** Serum IgG to cV2 of SIV_{mac251} **b.** Serum IgG to cV2 of $SIV_{sme543.}$ **c.** Rectal IgG to cV2 of SIV_{mac251} **d.** Rectal IgG to cV2 of $SIV_{sme543.3}$ **e.** SIV_{mac251} acquisition in vaccinated animals that had post-vaccination rectal IgG response units to cV2 of SIV_{smE543} >0 (solid lines) or 0 (dotted lines) in the alum group **f.** SIV_{mac251} acquisition in vaccinated animals that had post-vaccination rectal IgG response units to cV2 of SIV_{smE543} >0 (solid lines) or 0 (dotted lines) in the MF59 group

PLASMABLASTS

The difference in the distribution of the antibody response against cV2 in plasma and mucosal sites induced us to study the homing marker receptors on the surfaces of early plasma-cells precursors, plasmablasts (PBs). PBs are short-lived, highly proliferating, antibody-secreting, plasma-cell precursors, which are found in peripheral blood upon antigen exposure and migrate toward tissues according to their homing markers expression [87]. The presence of interferon-γ (IFN-γ), a hallmark of inflamed tissue, increases the expression on PBs of the chemokine receptor CXCR3 and of CXCL10 (ligand of CXCR3) by cells located in inflammatory sites. On the other hand, the role of surface integrins, such

as α4β7, has emerged as crucial to understand PBs migratory patterns to mucosal site and the induction of local immunity [88]. The levels of α4β7 and of the chemokine receptor CXCR3 were measured before and after immunization on total PB subsets (**Fig. 8**).

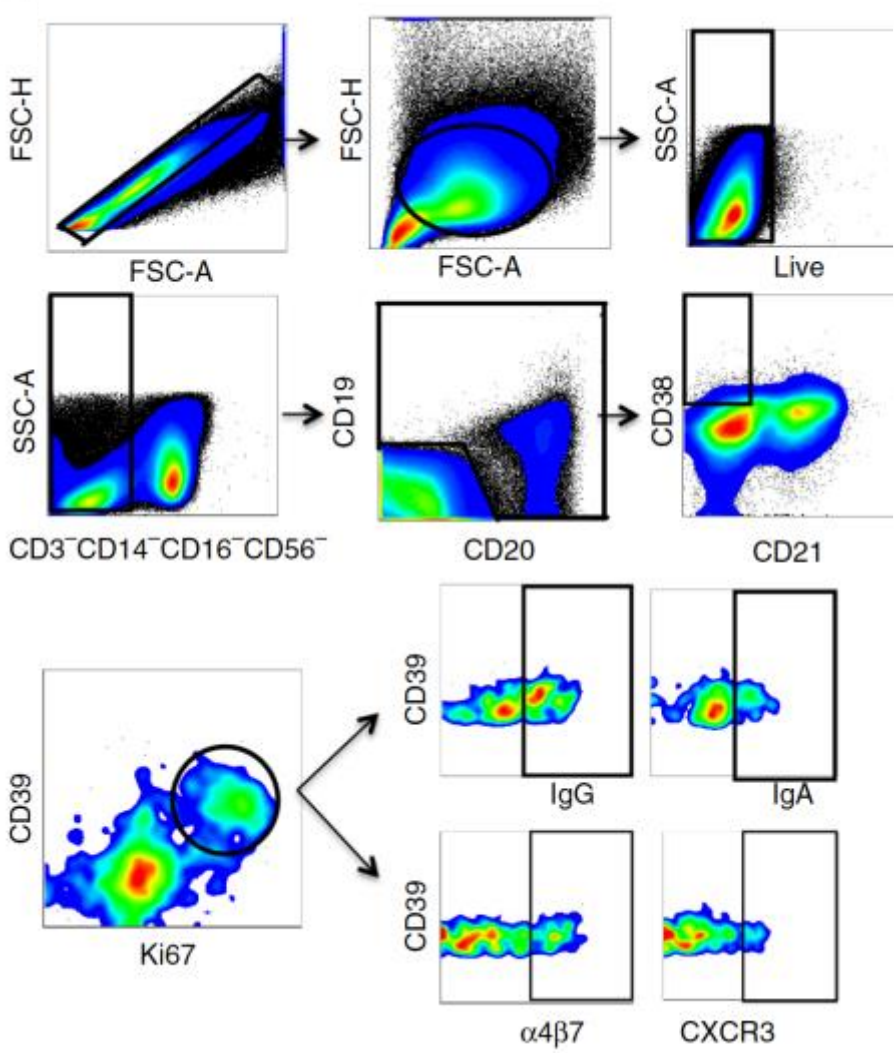

Fig. 8 | Phenotypic characterization of PBs. Plots represent the gating strategy, where gating is represented by squares or circles and the arrows represent the parent-to-daughter population flow for each line.

Alum adjuvant did not significantly alter the expression of either molecule (Fig. 9a, 9b), as compared to pre-vaccination specimens, whereas MF59 did (Fig. 9a, 9b), resulting in a significantly higher level of α4β7+ PBs in the alum group than in the MF59 group ($P = 0.20$) (Fig. 9c). MF59 was also associated with a significant increase ($P < 0.001$) above the baseline in the number of CXCR3+ PBs in macaques (Fig. 9b) resulting in a significantly

higher level of CXCR3 expression on PBs in MF59 group than in the alum group ($P = 0.007$) (Fig.9d).

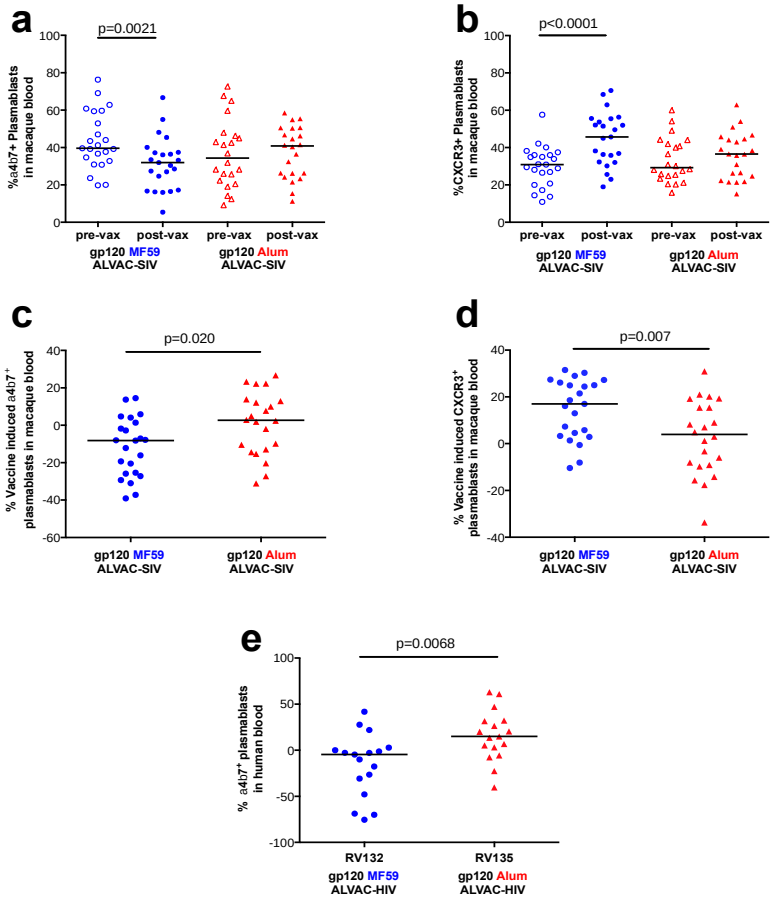

Fig. 9 | Adjuvant associated differences in PBs homing markers in blood. **a.** Percentage of α4β7+ and **b.** CXCR3+ PBs in macaques' blood (MF59 n = 23; alum n = 22 animals) before and one week after the last immunization. **c.** Frequency of vaccine-induced (post – pre) α4β7+ or **d.** CXCR3 PBs **e.** Frequency of vaccine-induced α4β7+ PBs in the blood of 17 humans enrolled in the RV132 and RV135 HIV-vaccine trials. Blood was collected before and at 14 d from last vaccination (Wilcoxon signed–rank test).

The relevance of these findings in humans was tested by measuring the frequency of vaccine-induced $\alpha4\beta7^+$ and $CXCR3^+$ PBs in the blood of vaccinees enrolled in the RV135 and RV132 trials, which used an ALVAC (vCP1521)–HIV + rgp120 (MN/A244) that was adjuvanted either with alum or MF59, respectively [83]. We observed similar differences, consistent with the macaque results, in α4β7 levels on circulating PBs in immunized humans (Fig.9e).

NATURAL KILLER CELLS

The ALVAC-HIV/AIDSVAX-B/E RV144 vaccine trial showed an estimated efficacy of 31%. RV144 secondary immune correlate analysis demonstrated that the combination of low plasma anti-HIV-1 Env IgA antibodies and high levels of antibody-dependent cellular cytotoxicity (ADCC) inversely correlate with infection risk. One hypothesis is that the observed protection in RV144 is partially due to ADCC-mediating antibodies [89]. One of the function of Natural Killer cells (NK-cells) to mediate ADCC. CD16 (FcγRIII) expressed on their cellulare membrane recognize and bind to the Fc portion of the antibody bounded on the surface of a pathogen-infected target cell. Once the link between NK and infected cells, through the antibody, happen, NK-cells start to release cytotoxic cytokines [90]. For this reason mucosal (NK) cells that express NKG2A and NKp44 [85, 86] receptors were studied in the two groups of animals. Rectal biopsies were analyzed at one week after the last immunization for the frequency and function of NK cells by flow cytometry (Fig. 10).

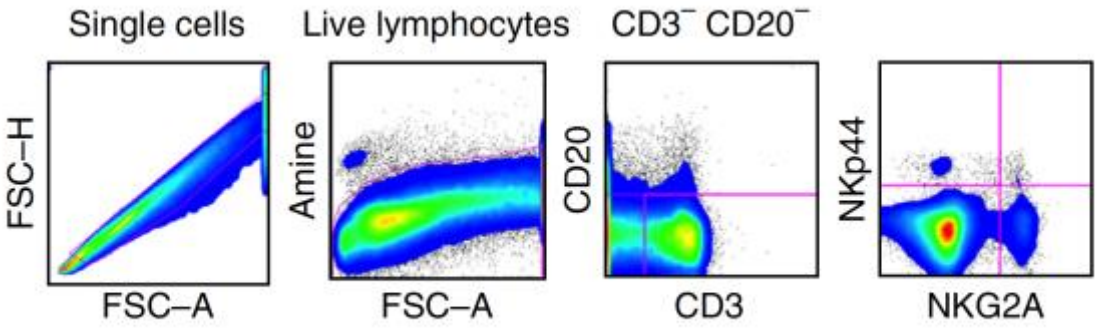

Fig. 10 | Representative flow cytometric plots defining ILCs (Innate Lymphoid Cells) in the rectal mucosa of rhesus macaques. ILCs were identified using a side-scatter versus forward-scatter gate and phenotypically defined as CD3−CD20− and NKG2A+ or NKp44+ cells, or as NKG2A−NKp44−cells. Purple line defines the gates used to calculate CD20−CD3− cells and NKp44+NKG2A+ cells.

Vaccination with both adjuvants increased the frequency of mucosal NKp44+ cells, and decreased that of NKp44−NKG2A− cells, as compared to the unvaccinated controls (Fig. 11a, 11b). The stimulation of mucosal mononuclear cells with overlapping peptides that encompass the entire SIV_{mac251} gp120 proteome revealed a higher proportion of NKp44+IL-17-producing cells in the alum group relative to MF59 recipients (Fig. 11c). Among the features associated with acquisition in this group of cells IL-17+ NK cells (% Env NKp44+IL-17+) were associated with a reduced risk of acquisition in the multivariate analysis (R = 0.377; data not shown).

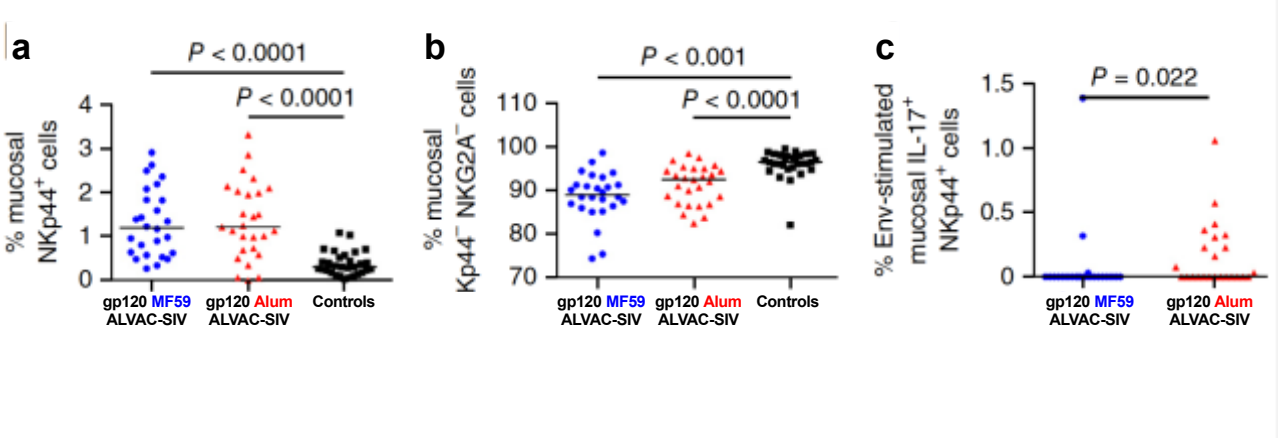

Fig. 11 | Adjuvant associated differences in NK subtype in rectal mucosa**.** Relative frequency of NKp44+ **b.** and NKp44- NKG2A-. **c.** Frequency of NKp44+ cells producing IL-17 after Env-stimulation (alum, n = 24; MF59, n = 22).

PATIENT CHARACTERISTICS

The characteristics of the participants belonging to the different groups are shown in Table 2. The mean age was 43.3 years. The majority of patients were male (88%). Baseline CD4+ T-cell counts were not different between pre-ART CHI and LTNP. Significantly different were the copies of RNA-HIV in the plasma at the Setpoint and at the enrollment between the two groups.

Characteristic mean (range)	CHI (n=12)	LTNPs (n=14)
Age	38.1 (22-64)	47.7 (39-57)
Male Gender	100%	78.50%
YY since diagnosis	2.03 (0.9-2.9)	21.3 (9.9 - 31.9)
HIV-RNA copies at Setpoint (RNA copies/ml)	657,019 (21,290-4.000,000)	1,615 (<37-4,000)
HIV-RNA copies at the enrollment (RNA copies/ml)	641,002 (14360 - 4000000)	1137 (<37-4,751)
CD4 count at Nadir (cells/ul)	366 (55-666)	517 (249-1,001)
CD4 count at enrollment (cells/ul)	399 (55-929)	743 (289-2,177)

Table 2 | Characteristics of study population

IMMUNE CORRELATES OF CONTROL

The importance of the antibodies against the V2 of gp120 in plasma and tissue has been underlined by many studies in humans and macaques in which HIV/SIV infection was prevented with a vaccine or controlled after ART interruption [43, 73, 91, 92]. We enrolled 14 LTNP and 12 Pre-ART CHI patients mainly clade B (85%) and we characterized the V2 response in plasma. First we studied the plasma reactivity against V2 of HIV gp120 linear overlapping 15-mers peptides of MN (clade B) isolate (Fig. 12a). Only 1 out of 12 CHI patient showed a low response against peptide 24 (Fig. 12b) . 4 out of the 14 LTNP showed a stronger wider response against the MN V2 gp120 regions (Fig.12c)

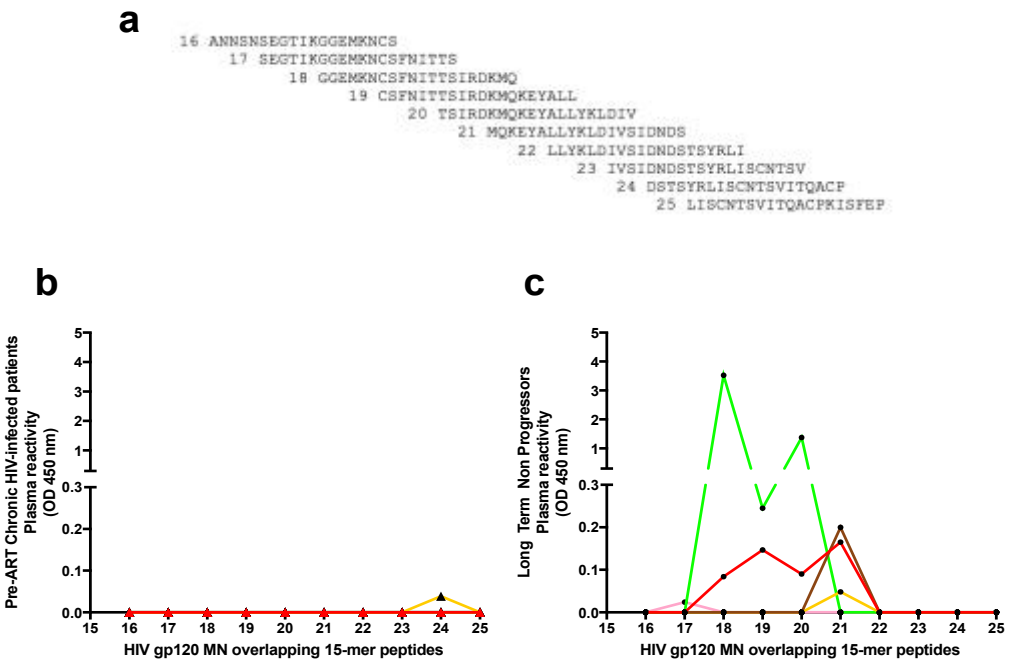

Fig. 12 | a. MN clade B V2 gp120 overlapping 15-mer peptides **b.** response of pre-ART CHI patients plasma to MN clade B V2 gp120 overlapping 15-mer peptides (n=12) **c.** response of LTNPs plasma to MN clade B V2 gp120 overlapping 15-mer peptides (n=14)

We then investigated the role of the conformation of the V2 of clade B (MN and Case A). We tested the plasma of the LTNPs and the pre-ART CHI patients with cyclic V2 (cV2) of gp120 isolated from MN (clade B). Although the levels of reaction to the linear peptides were higher in the LTNPs compared to the pre-ART CHI patients, the response was not significantly higher. The Plasma Enhance (RU) to cV2 of MN (clade B) (RU) of the LTNPs was significantly higher compared to that of the pre-ART CHI patients (P = 0.0012) (Fig. 13a). Interestingly the reactivity of the LTNPs was higher also against other isolate cV2 of the same clade (Case A) and other clades (clade AE, Th023) (respectively P = 0.05 and P < 0.0001) but not against the isolate 1086 of clade C (Fig. 13b, 13c, 13d).

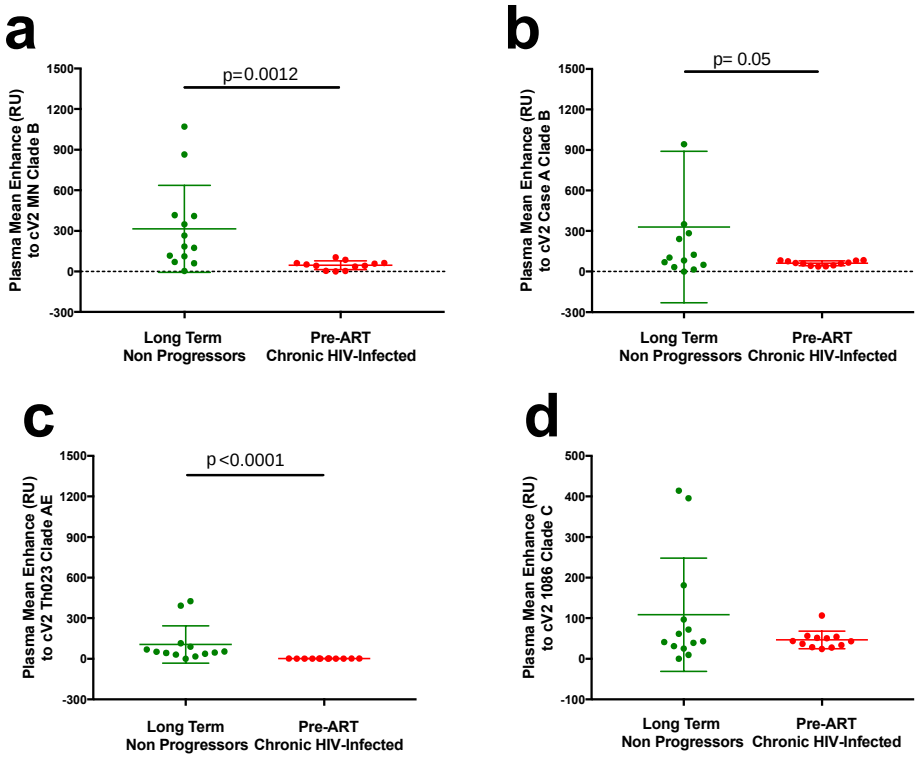

Fig. 13 | Response of LTNPs (green n=13) and pre – ART CHI patients (red n=12) against cV2 of **a.** MN clade B **b.** Case A clade B **c.** Th023 clade AE **d.** 1086 clade C

Even more interestingly, the plasma reactivity to cV2 against the clade B isolates of all the patients (85% clade B) was inversely correlated with the viral load measured at the same time point (*P* =0.0021 and *P* =0.0136 for the two clade B isolates) (Fig. 14a, 14b).

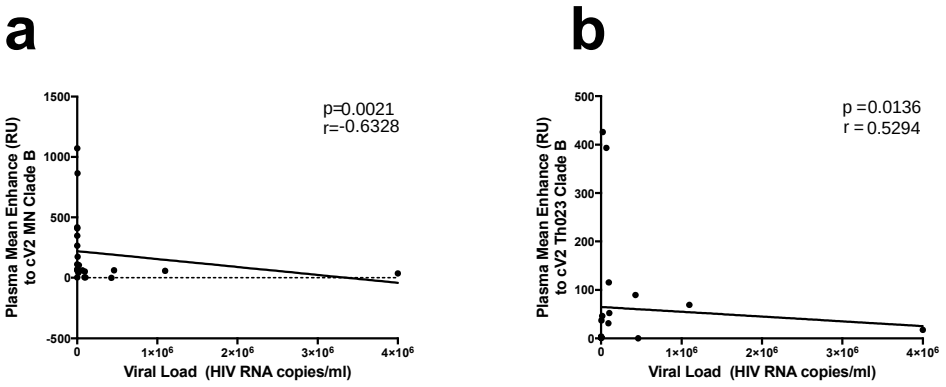

Fig. 13 | Correlation between plasma reactivity to cV2 of the two clade B isolates and the viral load in the plasma measured at the same time. **a.** MN clade B **b.** Case A clade B

DISCUSSION

In this study, a prime-boost ALVAC/SIV-gp120/Alum vaccine strategy was used to immunize a group of Indian rhesus macaques. The aim of this study was to recapitulate the results of the HIV vaccine phase III clinical trial RV144, also known as Thai Trial, conducted in Thailand. In the RV144, ALVAC/HIV-gp120/Alum was administer on 16,000 volunteers and the regimen achieved moderate protection. We designed this study in order to re-test the vaccine efficacy and investigate the immune correlates of protection in rhesus macaques. In November 2016, in South Africa, started the phase III clinical trial HVTN 702. In this regimen the immunogens and schedule of the RV144 vaccine regimen have been adjusted in order to: increase the immunogenicity, match the circulating HIV clade in South Africa (clade C), and to achieve a durative protection. Very importantly, the gp120 boost, in HVTN 702, is combined with MF59 instead of Alum. Our macaque study was also designed in order to explore the potential efficacy of MF59 by immunizing a second group of animals with ALVAC-gp120/MF59. In our study, ALVAC-gp120 vaccine regimen adjuvanted in alum resulted in a significant level of protection (44% at each challenge) in Indian rhesus macaques challenged intrarectally with low doses of SIV_{mac251}, thus recapitulating the results of RV-144 clinical trial conducted in humans. Consistently, the animal model used in this study is predictive of the results observed in humans. On the contrary, the MF59-adjuvanted vaccine regimen did not protect from SIV acquisition upon rectal exposure of low doses of SIV_{mac251}. However, MF59 was demonstrated to be highly immunogenic, because of its capability of eliciting higher antibody titers against gp120 protein as compared to alum. Nevertheless, the immunoglobulin titer against cyclic V2 peptide of gp120 in rectal mucosa was higher in the alum-adjuvanted group of animals. In the RV144 trial, the estimated efficacy of a vaccine regimen against human immunodeficiency virus type 1 (HIV-1) was 31.2%. In the RV144, the IgG antibody binding to scaffolded V1V2 Env were associated with protection [43, 44]. We recapitulate this immune correlate of protection in macaques: in the Alum group in fact, the mucosal cV2 IgG response was associated with a decreased risk of SIV acquisition (log–rank test, $P = 0.0018$). Since these antibodies were found in the rectal mucosa and were associated with antibody-dependent cell-mediated cytotoxicity activity

(ADCC) in RV144 trial, we study the homing markers on plasma-cells precursors, PBs and we characterized NK-cells in the rectal mucosa that potentially can mediate ADCC. PBs directed towards the gut mucosa were identified by the expression of α4β7 integrin on their surface, whereas PBs migrating to inflammatory sites were characterized according to their expression of CXCR3 chemokine receptor. Indeed, while α4β7 binds to MadCAM-1 and addresses cells to the gut mucosal layer, CXCR3 plays a major role in enabling the cells to target the inflammatory sites. Before immunization the frequency of blood circulating PBs from both groups of animals positively correlated with the expression of the α4β7 integrin (data not shown), indicating the higher the number of PBs, the higher the expression of α4β7. These findings suggest that PBs trafficking is regulated according to a homeostatic pattern, whereby under basal condition (i.e., without immunization) circulating PBs migrate towards the gut mucosa, which constitutes a portal of entry for the virus. In the alum-adjuvanted group no significant alterations of the pattern of homing-marker expression were found after vaccination. On the contrary, the MF59-adjuvanted vaccine regimen resulted in a lower expression of α4β7 and a concurrent higher expression of CXCR3 on circulating PBs surface. Consistently, when vaccine-induced PBs were considered, the alum group showed a higher frequency of α4β7 than the MF59 group, and concurrently the MF59 group showed a significant increase in the expression of CXCR3. All these evidences appear to confirm that there is a natural homing homeostasis of blood circulating PBs, which is not altered by the ALVAC/SIV-gp120/Alum regimen and is profoundly affected by the ALVAC/SIV-gp120/MF59. Indeed, the MF59-adjuvanted vaccine seems to impair the traffic of PBs, skewing them to the inflammatory sites. Furthermore, in preliminary studies it was shown that MF59 adjuvant is a more potent immunogen and elicits a strong inflammatory response, diverting some subsets of immune cells towards the main sites of inflammation. In this scenario, the increase of CXCR3 and the decrease of α4β7 on circulating PBs plasma membranes strongly suggests that the MF59 may be able to alter the trafficking of PBs away from the gut mucosa where they are naturally headed to. Importantly, we observed and confirmed similar differences, consistent with the macaque results, in α4β7 levels on

circulating PBs in humans immunized in the RV135 and RV132 trials, which used an ALVAC (vCP1521)–HIV + rgp120 (MN/A244) that was adjuvanted either with alum10 or MF59, respectively. Next, we studied mucosal ILCs (Innate Lymphoid Cells) including natural killer (NK) cells that express NKG2A and NKp44 receptors. Within the mucosal ILC population, NKG2A+ cells mediate cytotoxicity, whereas NKp44+ cells produce IL-17, IL-22 and B cell–activating factor (BAFF), thereby promoting epithelial integrity and mucosal homeostasis. NKp44−NKG2A− is a less-defined ILC population that produces cytokines, including IFN-γ. Vaccination with both adjuvants increased the frequency of mucosal NKp44+ cells, and decreased that of NKp44−NKG2A− cells, as compared to the unvaccinated controls. The stimulation of mucosal mononuclear cells with overlapping peptides that encompass the entire SIV_{mac251} gp120 proteome revealed a higher proportion of NKp44+IL-17-producing cells in the alum group relative to MF59 recipients. NKp44+IL-17-producing cells are ILCs that regulate mucosal integrity and that are functionally impaired in HIV infection. Correlative analysis in the alum group demonstrated associations between the risk of SIV_{mac251} acquisition and the frequency of mucosal NKp44+IL-17+ as well as with the level of mucosal IgG to cyclic V2. Strikingly, within the MF59 group, the mucosal level of IgG to V2 was correlated with an increased risk of virus acquisition, which suggests that antibodies with seemingly similar specificities carry functional differences. The importance of the antibodies against the V2 region of gp120 in plasma and tissue has been underlined by studies in humans and macaques in which HIV infection was respectively prevented or controlled without ART [42, 73] . This response has been associated with HIV prevention in human in the RV144 and confirmed through the results described in this study in macaque model. Furthermore, the V2 response has been identified recently as immune correlate of control in SIV-infected animals treated with monoclonal antibodies to α4β7 that achieved a sustained virological control after ART interruption [73]. Interestingly, only 30-50% of HIV patient (treated or non treated with ART) present low levels of V1V2 antibody response [93]. To date, sustained virological control without ART has not been achieved in human. Based on these observations we studied the V2 response in those HIV patients that naturally

control the course of HIV infection: the LTNPs. We studied a total of 26 patients belonging to two different groups: 12 pre-ART CHI patients and 14 LTNPs. We tested the plasma of the pre-ART CHI pantients and of the LTNPs to the overlapping peptides that encompass the entire V2 region of the gp120 protein of the MN clade B sequence and the cyclic form of the same antigen. We also tested the plasma of the patients to the cyclic form of cross-clades HIV V2 gp120 antigens. Our data suggest that LTNPs have a significantly higher response to V2 of the gp120 homologous clade compared to the pre-ART CHI patients. Interestingly, this response reached statistical significativity also when measured to the V2 of non-homologous clade (such as clade AE). Notably, this response was inversely correlated to the HIV plasma viral load. The functions of these antibodies and their relevance in controlling other HIV clades has to be explored in future studies. Overall our data underline the importance of the V2 region of the gp120 protein of HIV as a target for immunological pressure. Our data confirm the HIV vaccine efficacy achieved in human by the immunization with a prime/boost approach with an ALVAC-HIV + gp120-Alum vaccine (RV144, Thai Trial) in a SIV_{mac251} model. In our study we demonstrated the importance of the antibodies to V2 of the gp120 in prevention from SIV mucosal acquisition as demonstrated in the RV144 trial. We also found novel immune features (IL-17-producing NK-cells and PBs directed to mucosal site) that might have contribute in protection. We also confirmed part of these responses in humans vaccinated with an RV144-like regimen underlying the importance of animal models. The role of antibody to V2 region of SIV/HIV have been recently invigorated by their description in a rhesus macaque study in which substained virologic control was achived after ART suspension. We studied antibody to the V2 region of gp120 for the first time in a group of LTNPs comparing their quantity and their ability to bind cross clades antigens with the ones of HIV chronic infected individuals. Plasma antibodies to V2 were stastistically higher in the LTNP group compared to the HIV chronic infected group. Interestingly, antibodies to V2 in the LTNP group were also able to bind cross clades antigens. Understanding the mechanism in which these antibody might contribute to mediate protection from HIV/SIV acqusition and HIV/SIV control will require further studies**.** Boost

the immune response in order to target V2 might increase the level of protection in future HIV/SIV vaccine studies and might contribute to achieve susbstained HIV control in HIV/SIV infected subjects.

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“Keep your eyes lifted and your head turning. The search for knowledge is in our genes. It was put there by our distant ancestors who spread across the world, and it's never going to be quenched. To understand and use it sanely a vastly larger population of scientifically trained people like you it is required.”

E.O. Wilson