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The role of type-1 and type-2 T-helper immune responses in HIV-1 vaccine protection

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Abstract: The dichotomy of type-1 and type-2 T-helper (Th) immune responses is thought to be an obstacle to develop Human immunodeficiency virus-type-1 (HIV-1) vaccines capable of inducing effective cellular as well as humoral immune responses. *Macaca mulatta* were immunized using two different HIV-1_{sf2} envelope vaccine strategies, based on either immune-stimulating complexes (ISCOM) or chimeric Fowlpox (FP) vaccines. One month following the third immunization all animals were heterologously challenged with simian/human immunodeficiency virus ($SHIV_{sf13}$). Vaccinated monkeys, which were protected had the highest levels of both type-1 and type-2 HIV-1 specific T-helper cell (Th) responses in addition to the highest homologous and heterogenous virus neutralizing antibodies. To determine how long Th responses persisted and if they correlated with protection, animals were re-challenged after waiting for four months without re-boosting. Macaques which maintained the highest gp120-specific type-1 (IFN-γ) responses were protected, while there was evidence of viral clearance in two others. These findings demonstrate the importance of both or mixed type-1 and type-2 Th responses in HIV-1 vaccine induced immunity while suggesting a possible role of persistent type-1 responses in maintaining protective immunity over time.

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Introduction

An understanding of the types of immune responses important for protection from HIV infection and the development of AIDS may possibly facilitate development of an effective HIV vaccine [2,3,6,13,15,17]. In the course of the last 6 years a number of vaccine efficacy studies in chimpanzees have been performed and have evaluated efficacy to homologous as well as heterologous HIV-1 challenges [2,6,13,15]. The single most consistent HIV-1 specific immune response that correlated with protection from cell-free infection in that model has been HIV-1 neutralizing antibody titers (VNT) [2,6,11,13,15,16]. From these studies, data on T helper or other cell-mediated immune (CMI) responses were generally not available [19]. Studies in the SIV rhesus model have been somewhat more revealing about the possible role CMI responses may play in protective immunity to AIDS viruses. SIV vaccines have been shown to protect against infection following challenge with primary PBMCs from a rhesus monkey with AIDS [20]. Protection correlated with MHC class I sharing between donor and recipients and the presence of CTL, implicating MHC class I restricted CTL responses in protection in that setting [20]. In another study macaques vaccinated with recombinant pox viruses expressing SIVnef were either protected from SIV infection or had reduced virus load, the level of protection correlating with the CTL precursor frequency [14]. More recently protective mucosal immunity was shown to be elicited by targeted iliac lymph node immunization. Protection from SIV infection was found to correlate with an increase in the number of iliac lymph node secretory IgA (sIgA) antibody secreting cells, CD8 suppressor factor and the β-chemokines Rantes and MIP-1 [23].

Owing to the intracellular and extra cellular nature of HIV infection it has been proposed that truly

effective HIV vaccines will need to induce both Th1 and Th2 type responses to effect cellular (for instance CTL responses) as well as humoral [virus neutralizing antibodies/secretory IgA (sIgA)] arms of the immune system, respectively. Furthermore, HIV-1 vaccines should induce broad and durable immune responses, which can be maintained for long periods of time. It has been proposed that a Th1 response would promote the necessary cellular immune responses, but the objective of achieving a coordinated and balanced cellular (type-1) and humoral (type-2) response is thought to be difficult to overcome because of cytokine cross regulation [26], which tends to bias one type of Th response versus the other [30,31]. The role of T-helper responses in the induction of immunity in HIV vaccine efficacy studies in nonhuman primates has been poorly studied, and it remains controversial if both types of T-helper responses can be induced and which types of responses are correlated with vaccine protection. We set out to determine if both type-1 as well as type-2 T-helper responses could be induced by HIV immunization with either one of two different HIV-1 vaccine strategies and to investigate the type of T-helper responses, which correlated with vaccine efficacy in a nonhuman primate model system.

Materials and methods

Comparison of two HIV-1_{sf2} envelope candidate vaccine strategies was undertaken in *Macaca mulatta* (rhesus monkeys). HIV-1_{sf2} recombinant FP, or ISCOM vaccines or controls, were administered at 0, 6, and 16 weeks to a total of 12 rhesus monkeys. The HIV-1 ISCOM vaccination strategy was designed to induce immune responses to multiple, independent epitopes; neutralizing (VN) antibodies to the V2 and V3 regions and cytotoxic T-cell (CTL) to env and gag epitopes as recently described [9]. The first two injections consisted of monomeric, recombinant HIV-1_{sf2} gp120 and p24 coupled to ISCOMs. In addition, at 6, and 16 weeks animals were immunized with synthetic peptides representing the V2 (IRDKI QKENA LFRNLC) and V3 (NNNTR KSIYI GPGRAC) regions coupled to Flu Pr8 ISCOMs [25,27]. In contrast, a live chimeric HIV-1_{sf2} gp160 FP [28] vaccine was expected to induce antibodies to conformational epitopes expressed on multimeric glycoproteins, as well as inducing CTL by restricted replication in host cells. Controls consisted of ISCOM-flu (animals 9258 and BB70) or the wild-type (wt) FP-vector alone (animals BB102 and 9219).

Two weeks after the second and third immunizations freshly isolated peripheral blood mononuclear cells (PBMC) were monitored for antigen specific T-helper cell responses with an enzyme-linked immunosorbent (ELI)spot assay to enumerate the number of gp120 specific cytokine [interleukin-4 (IL-4), interleukin-2 (IL-2) and interferon- (IFN-γ)] secreting cells (Fig. 1) [12,34].

Serum anti-gp120 and VNT were performed as described [8,10]. Blanks represent samples not available. Gp120 specific cytokine responses minus background in the absence of antigen are expressed as the arithmetic mean ± SEM of cytokine ELIspots per 4×10^5 PBMC [12,34]. LP (given as stimulation index (SI) and counts per minute (cpm), given the working range of the β-plate reader in this laboratory) and CTL responses as previously [20]. CTL based on greater than 10% specific lysis on autologous peptide pulsed target cells are represented by the letter of the peptide pools (overlapping 15 mers) giving positive responses. Post challenge virology was based on PCR [20] as represented as positive (+) or negative (–), and quantitative virus isolation (vi) given us the number of virus producing cells in 10×10^6 PBMC [2,4,6,11,13,15,16]. Animals negative by both assays after three consecutive samples were classified as protected.

High affinity multi-well plates were coated with 10 mg/ml peptides (15–16 mer; partially overlapping) and plasma or sera samples were screened after 1:100 dilution in DB (dilution buffer, 0.1% BSA in PBST, 137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 4 mM Na_2HPO_4, and 0.05% Tween 20, pH 7.2). Recombinant $gp160_{SF2}$ oligopeptides (MRC, UK) at a concentration of 10 mg/ml in 50 ml coating buffer (CB, 15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 0.1% Thimerosal, pH 9.6) were adsorbed for 18 hours on 96 wells, flat bottom, high-binding capacity microtiter plates (Cova-Link, Nunc, Naperville, IL).

Plates were saturated for one hour at 37°C with 200 ml/well of 1% BSA in PBST. 50 ml of macaque sera or plasmas, diluted 1:100 in DB were subsequently added to each well and further incubated for one hour at 37°C; 50 ml of horseradish peroxidase conjugated rabbit anti-monkey IgG (Sigma Chemical Co., St. Louis, MO), diluted 1:10,000 in DB were added to each well and further incubated for one hour at 37°C. Extensive washing with PBST was performed after each incubation. 100 ml of the colorimetric substrate 1,2-orthophenylendiamine solution (OPD, 0.08% in citric acid-phosphate buffer pH 5.0) and 1.7 ml of 37% H_2O_2 were added. After 20 min incubation at room temperature in the dark, the colorimetric reaction was stopped with 50 ml 2 M H_2SO_4. The absorbance of each well was read at 492 nm with a Titertek Multiskan spectrophotometer (Labsystem). Samples were assayed in duplicate).

The $SHIV_{sf13}$ molecular clone was constructed based on the HIV-1_{sf13} molecular clone [7] in a simi-

lar fashion as the $SHIV_{sf33}$ chimeric [24]. A virus stock produced on rhesus PBMC was titrated IV in mature rhesus monkeys to determine the monkey infectious dose [5]. Animals in this study were given approximately 30 monkey IV infectious doses at each challenge.

Results

The HIV-1 vaccinated ISCOM group had the highest gp120 specific T-helper responses throughout the study. High levels of gp120 specific INF-γ secreting cells were observed after the second immunization with a return to baseline at week twelve (Fig. 1). A booster effect was subsequently observed 2 weeks after the third immunization in which peptides only were used. Significant responses were not seen in other groups (Fig. 1). The number of antigen specific IL-2 secreting cells observed in this assay are typically a log lower in number due to the inherent kinetics of this cytokine making comparative analysis difficult. However, significant but low responses were observed only in the HIV-1 ISCOM vaccinated group at week 18. The last ISCOM immunization which consisted of gp120 V2 and V3 peptides alone failed to boost the numbers of gp120 specific IL-4 secreting cells. The IL-4 responses continued to fall and thus resembled the lymphocyte proliferation kinetics (Fig. 1). In contrast in the HIV-1 FP vaccinated group, the magnitude of their T-helper responses was much lower and the pattern of their gp120 specific IL-4 and lymphocyte proliferation (LP) responses was different. LP responses were not significant and the number of gp120 specific IL-4 secreting cells did not rise until week 12, 6 weeks after the second immunization. They had declined when observed 2 weeks after the third immunization (Fig. 1).

Quantitatively the humoral response as determined by antibody titers to gp120 [8,10] reached their highest levels in the HIV-1 ISCOM vaccinated group 2 weeks after the second immunization and were slightly boosted by the peptide ISCOM immunization but did not reach the magnitude of the initial peak (Fig. 1). In this respect, therefore, they resembled the declining IL-4 responses and contrasted with the increasing INF-γ and IL-2 responses found in this group.

An increase in the number of INF-γ secreting cells (in contrast with IL-4) in the HIV-1 ISCOM vaccinated animals after peptide boosting suggested that this strategy may selectively boost the Th1 response, which in turn may have a qualitative influence on the resulting immune responses. Western blot analysis was used to confirm the general specificity of the antibody response to gp120 (data not shown). However, to document the fine specificity of the humoral response we assayed the binding of antibodies to a series of overlapping 15 mer peptides [8,10] the $HIV\text{-}1_{sf2}$ envelope glycoproteins (Fig. 2). There was very little variability between the responses of monkeys, which had been boosted with peptides. Their sera had high levels of activity to the V2 and V3 regions (peptide numbers 18 and 32), with low levels elsewhere. In contrast, the $HIV\text{-}1_{sf2}$ FP vaccinated animals showed a more heterogeneous response, both in terms of individual variation and in binding to peptides outside the putative neutralizing epitopes (Fig. 2) [8,10]. To determine if the V2 and V3 peptides had also elicited a T-helper response, we enumerated the number of cytokine secreting cells in response to these specific peptides as compared to gp120 in each individual animal immediately prior to challenge. The most marked responses were observed in the HIV-1 ISCOM vaccinated group with respect to peptide specific IFN-γ secretion. Three out of four animals made significant IFN-γ responses while the fourth produced an IL-2 response to both peptides (data not shown). Interestingly, the only IL-4 specific responses to peptides were observed to the V2 peptide and again, only in the ISCOM group.

HIV-1gp120 specific IFN-γ , IL-2, and IL-4 T-helper responses were observed in each of the four HIV-1 ISCOM vaccinated animals with the intensity of each cytokine response being consistent for each individual animal in this group, in contrast to the HIV-1 FP vaccinated animals (Table 1). The quality of the T-helper responses prior to challenge generally correlated with the magnitude and breadth of the specific humoral responses. As summarized in Table 1, all of the HIV-1 ISCOM vaccinated animals developed the high homologous $HIV\text{-}1_{sf2}$ neutralizing titers and in addition developed heterologous neutralizing titers to $HIV\text{-}1_{sf13}$ ranging between 1/160 and 1/320. In comparison, the HIV-1 FP vaccine induced poor T-helper responses and a heterologous neutralization response reflected in low antibody titers to $HIV\text{-}1_{sf13}$. An association with gp120 specific, cytokine production, LP responses, and neutralizing antibodies seemed apparent in most cases. $HIV\text{-}1_{sf2}$FP vaccinated monkeys did not produce significant Th1 or Th2 like gp120 specific cytokine responses (Table 1).

With regard to HIV-1 specific CTL responses, we examined the ability of lymphocytes from vaccinated monkeys to lyse autologous lymphocytes pulsed with peptides consisting of overlapping 15 mers spanning the entire gp120 and gag. Pools of peptides were used to identify CTL in bulk culture as we have previously described for SIV vaccinated macaques [20]. Similar to our previous findings, in this group of outbred *Macaca mulatta*, CTL re-

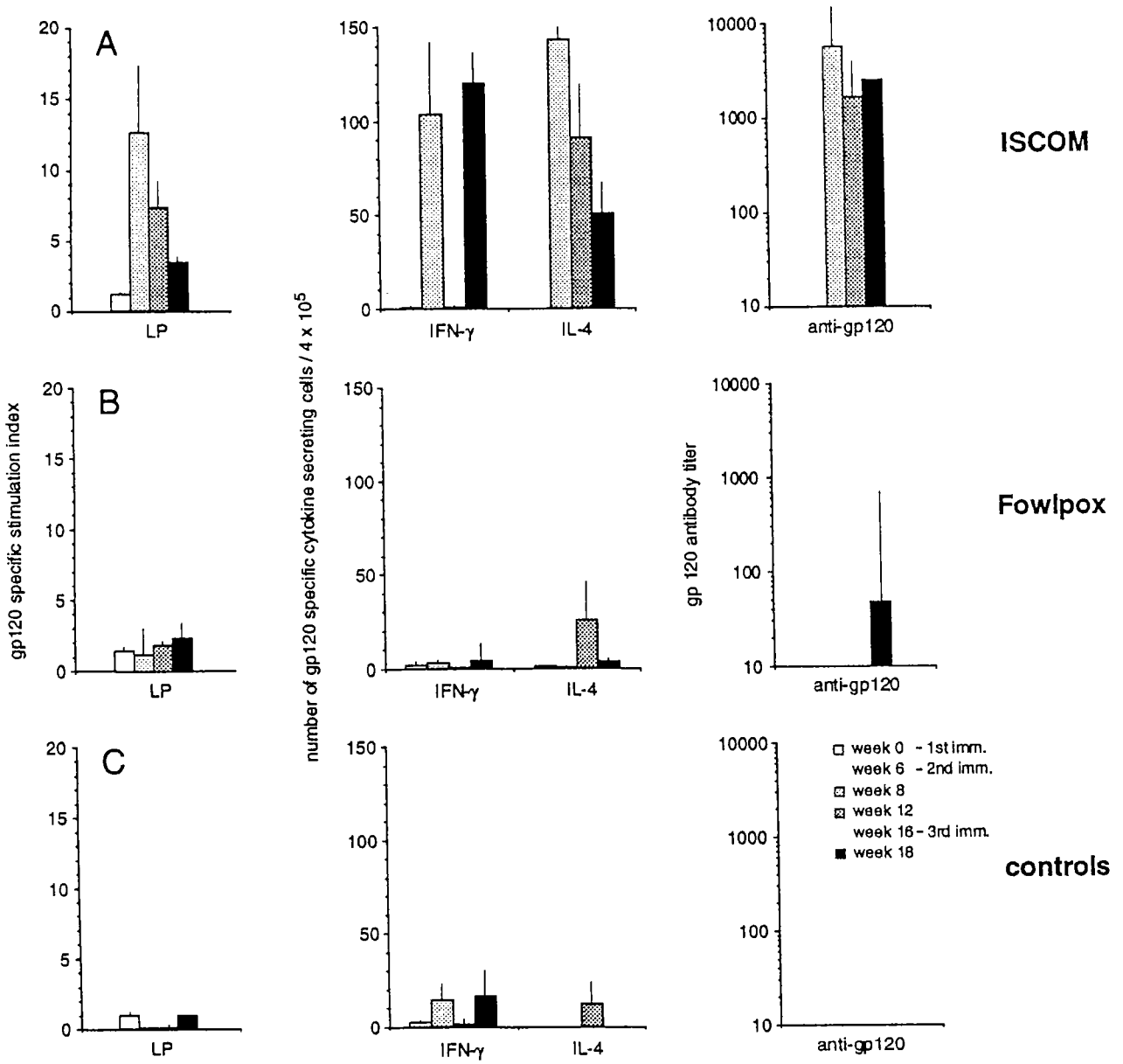

Fig. 1. Comparison of immune responses over time. Data are plotted as the mean of four animals ± SEM per group, HIV-1_{sf2} ISCOM (A), HIV-1_{sf2}FP (B), and controls (C) (Controls = 2 ISCOM-flu + 2 wtFP vaccinated animals). All animals were immunized at 0, 6 and 16 weeks with samples taken at 0, 8, 12, and 18 weeks. Lymphocyte proliferation (LP) to gp120 is expressed as the SI. The number of gp120 specific cytokine secreting cells/4x10^4 PBMC are shown for each sampling point for IFN-γ and IL-4. Samples for IL-2 measurement were not available (n.a.) from weeks 8 and 12. The HIV-1_{gp120} specific antibody titers were determined by ELISA [8,10]. The HIV-1_{sf2} ISCOM group (A) has in general greater gp120 specific responses. HIV-1 specific responses are generated much slower and are predominantly IL-4 and antibody responses.

sponses were found to some peptide pools at different time points and not in all ISCOM vaccinated animals. In this study CTL responses were detected two weeks after the second and third immunizations in animals BB85 and 9251, respectively, out of the four animals which had consistent strong T-helper responses. The presence of detectable CTL responses did not appear to correlate with a biased type-1 or type-2 cytokine response (Table 1).

Four weeks after the last immunization all animals were simultaneously challenged heterologously with an in vivo titered stock of $SHIV_{sf13}$ propagated on rhesus PBMC. All HIV-1 ISCOM vaccinated monkeys were completely protected from infection as rigorously determined by nested PCR on sequential samples, quantitative virus isolation (Fig. 3) and serological follow-up (data not shown). In order to determine which immune responses were important for protective immunity, the specific immune responses assayed immediately prior to challenge

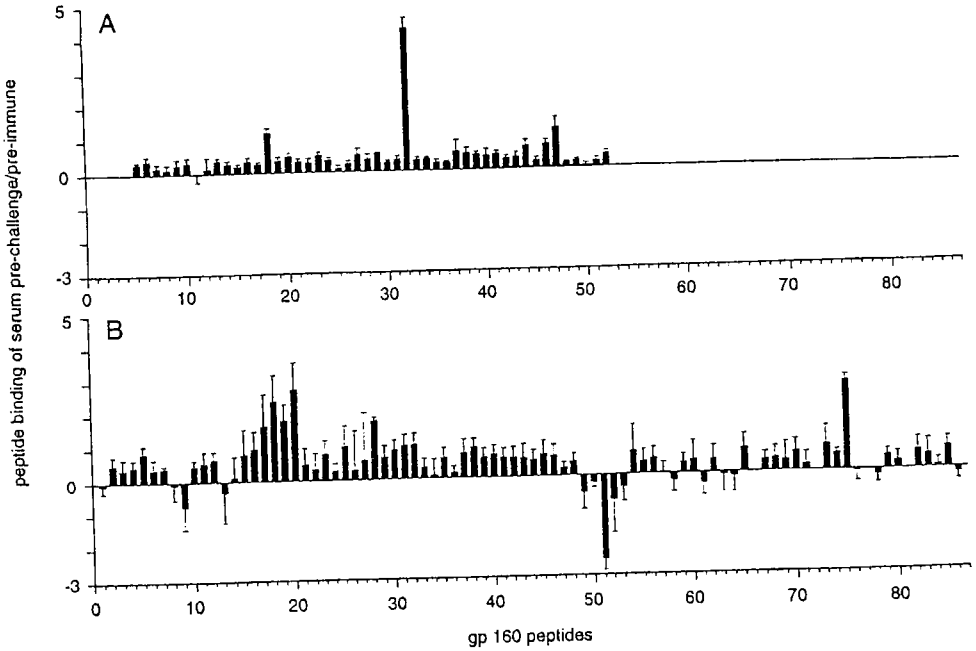

Fig. 2. Antibody specific peptide analysis comparing the ISCOM (A) and Fowlpox (B) vaccines. Data represent the pre-challenge humoral immune response of macaques 2 weeks after the third inoculation with HIV-$1_{sf2\ env/gag}$ ISCOM or live, HIV-$1_{sf2\ env}$ recombinant, Fowlpox virus. Antibodies against partially overlapping 15 mer peptides were measured at 492 nm by an enzyme-linked immunoadsorbent assay (ELISA). *Pre*-challenge/pre-immune ratios of peptide binding were calculated for each animal (vaccines 9263, BB85, 9251, and 9111 for ISCOM and 4044, 9264, BB98, and BB108 for Fowlpox; controls 9258, BB70 for ISCOM and BB102, 9219 for Fowlpox). Average values of respective controls (n=2) were subtracted from each of the four vaccines. Differences were plotted against peptide numbers as means ± SEM (represented by error bars, n=4). The antibody binding profile of ISCOM vaccinated animals showed three main peaks, two of which correspond to peptide 18 and 32, which are comprised in the V2 and V3 regions (A). Conversely the profile exhibited by Fowlpox vaccinated macaques was more complex (B), with higher variability among the animals and a less specific response to individual peptides, probably due to the interference of the conformational antibodies elicited by the vector (data not shown).

were examined for correlation with complete protection from infection or protection from virus load. An important and clear correlation was apparent with the number of gp120 specific IFN-γ and IL-4 producing cells, virus neutralizing and anti-gp120 antibody titers (Fig. 4). Comparison of the number of gp120 specific IFN-γ and IL-4 (Fig. 4) secreting cells with humoral responses immediately prior to challenge revealed a strong correlation between animals which were protected from infection versus those which were not. In those HIV-1 vaccinated animals which were not protected there appeared to be a vaccine effect (fewer virus infected PBMC) compared to the number of virus producing PBMCs in infected controls (Fig. 3). Furthermore, HIV-1 vaccinated animals which became infected but which did not develop detectable heterologous neutralizing responses to HIV-1_{sf13} had numbers of infected PBMCs in circulation similar to control animals. However, the most significant observation was found in animals, which were protected from infection after initial challenge in which there appeared to be an important correlation between both HIV-1 specific type-1 and type-2 cytokine responses in the induction of protective immunity (Fig. 4).

To determine which type of Th responses may play a role in enduring immunity we waited four months without re-boosting the protected animals and re-exposed them to a second challenge [5,7,24]. Two of

Table 1. Summary of immune responses before the first challenge 4 weeks post-immunization and outcome of virus challenge. Immune responses were determined from samples taken 2 weeks after the third immunization except for CTL responses which were assayed throughout the entire immunization period

Vaccine	Fowlpox	Control	ISCOM
Infection status after 1st challenge	++ (4/4)	++++ (4/4)	– (0/4)
Immune responses prior to challenge			
Anti-gp120	++	–	++++
Epitope specificity	+	–	+++
Homologous NAb	+++	–	++++
Heterologous NAb	+	–	+++
Th1 responses	+	–	+++
Th2 responses	+	–	+++
CTL responses	0/4	0/4	2/4

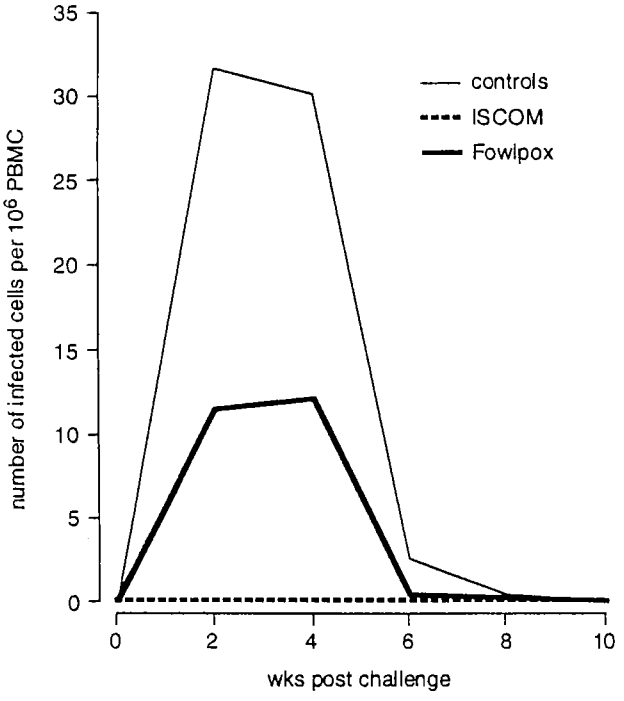

Fig. 3. Results of first challenge four weeks after the third immunization. Quantitative virus isolation following challenge revealed high mean virus loads as compared to the mean virus loads of the Fowlpox-SF2 immunized animals, which became infected. None of the ISCOM immunized animals became infected as determined by virus isolation and PCR.

the four HIV-1 ISCOM vaccinated animals which were protected from the primary heterologous challenge still possessed low but specific IFN-γ responses to gp120 5 months after the last immunization and four months after the first challenge. Type-2 (IL-4) responses had dropped to background by this point (Fig. 5). Four new non-vaccinated controls also tested had background responses. At approximately 5 months after the last immunization the protected animals received a second challenge with the same challenge stock. Within two weeks after this second challenge all four controls became PCR and virus isolation positive, while all four vaccines remained negative. Four weeks after this challenge two of the vaccinated animals became PCR positive, one of which (9251) remained virus isolation negative and was only transiently PCR positive, while the other (BB85) was only virus isolation positive with a low number (n=13) of infected PBMC at one time point (week four) (Fig. 5). The results of this rechallenge suggested that effective enduring immunity correlated with persistence of Th1 responses. Furthermore, data demonstrated that two vaccinated animals cleared the infection after challenge (Fig. 5).

Discussion

These data demonstrate that subunit HIV-1 vaccine efficacy can be achieved by inducing both HIV-specific type-1 and type-2 Th cytokine responses and also suggests that despite the development of strong humoral immunity that Th1 memory may be maintained. Furthermore these findings provide insight into the immune correlates of protective immunity to HIV-1 infection, the issue of preferential cellular or humoral immunity and the dichotomy of the

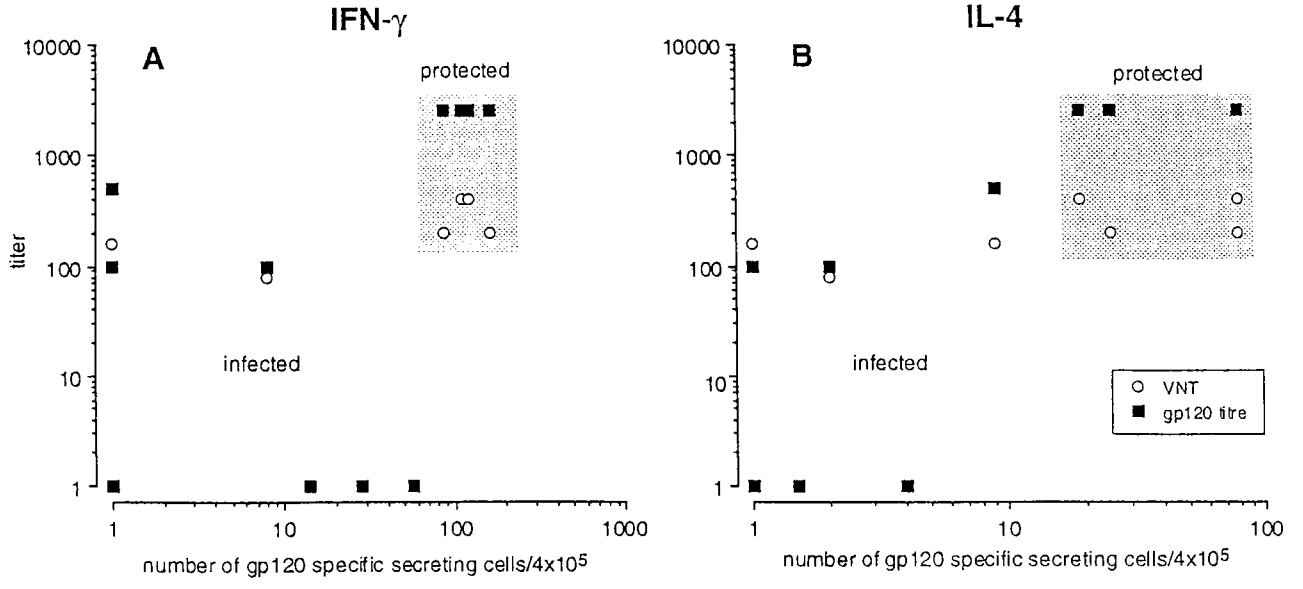

Fig. 4. Correlation of the anti-gp120 antibody and/or virus neutralizing titers plotted against the number of gp120 specific IFN-γ (**A**) or IL-4 (**B**) secreting cells prior to the first challenge. In animals protected from vaccine challenge a correlation of anti-gp120 and VNT is found with both the number of IFN-γ and IL-4 secreting cells. Protected animals have high numbers of gp120 specific IFN-γ or IL-4 secreting cells and high VNT, anti-gp120 titers.

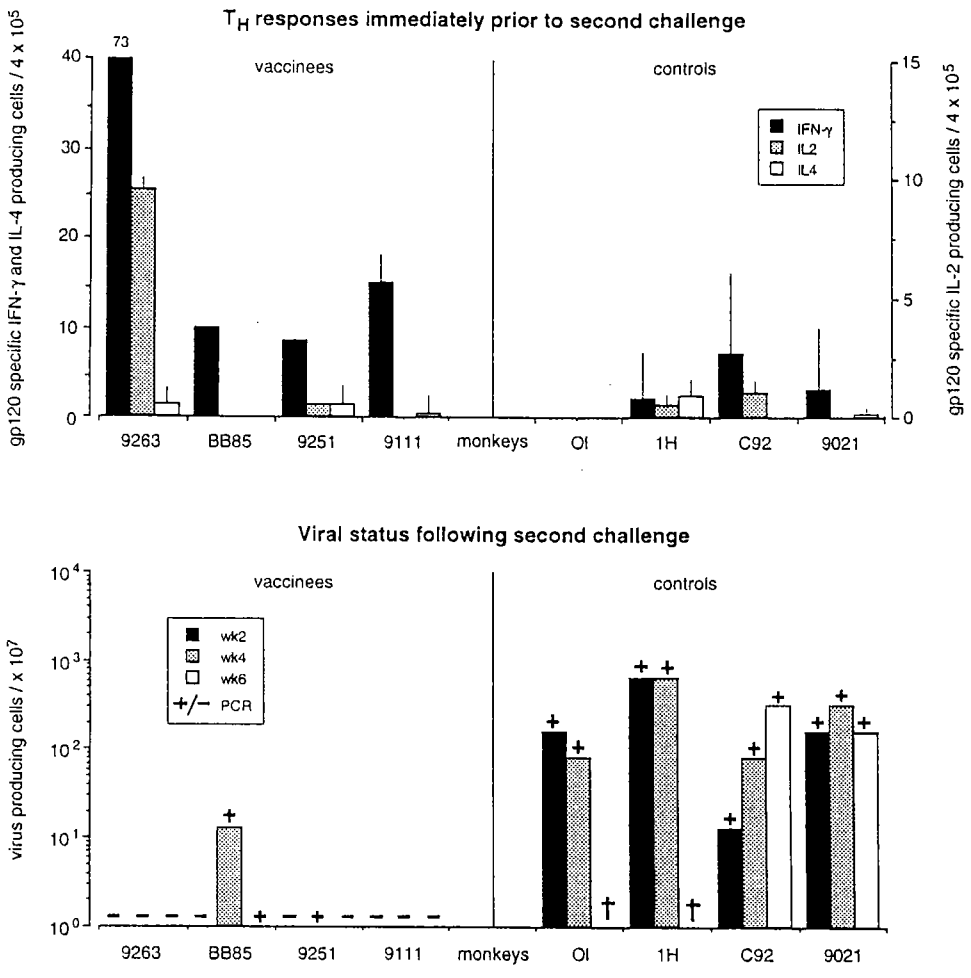

Fig. 5. HIV-1_{gp120} specific T-helper cytokine producing cells prior to second challenge (A) and viral status post-second challenge (B). IFN-γ and IL-4 scale (left hand side) has been decreased (0 to 40), IL-2 uses the same scale (right hand side). A decline in all gp120 specific cytokine secreting cells are observed. Results following second challenge (B) are shown as the PCR result (+ or -) above each time point and the number of virus producing cells × 10^{17} depicted quantitatively. († = euthanized). Controls had much higher virus loads than vaccinees (virus only isolated from one time point from vaccinees). Those with highest gp120 specific IFN-γ secreting cells remained free of infection.

underlying Th1-Th2 responses. Data from various pre-clinical vaccine efficacy studies in chimpanzees have clearly pointed to the important role of neutralizing antibodies in the absence of CTL responses [2,6,11,13,15,16]. In contrast, the absence of a correlation with neutralizing antibodies, and a positive correlation with CTL responses in some SIV vaccine studies [14,20] and CTL and/or other CMI responses in naturally exposed seronegative persons [30,31] point to cell mediated immune responses to HIV as being important in protective immunity. Furthermore, in other studies despite the presence of both virus specific VN and CTL responses, protection was not achieved [21], emphasizing the importance of the quality of optimal immune responses especially with regard to the nature of underlying T-helper responses in the generation of protective immunity.

One of the most effective vaccine strategies tested in the rhesus model has been the live attenuated SIV vaccine approach. This strategy has been criticized owing to safety issues. However, it has been an important system for proof of principle [19]. We have recently reported on the use of the live attenuated approach to demonstrate protection from SHIV infection in rhesus monkeys [4] but, despite intensive study by many laboratories immune correlates associated with this type of vaccination have not been identified. However, a recent study evaluating type-1 and type-2 cytokine responses in macaques infected with live attenuated SIV found highly elevated expression of IFN-γ, IL-2, and IL-4, while levels in animals infected with pathogenic SIV were lacking or absent [1]. Those observations together with findings that T-helper responses remain intact in HIV-1 infected chimps [18], which can resist heterologous HIV-1 challenge [32], strongly suggest a role of T-helper responses in generating protective immune responses in a vaccine context.

The results of this study provide a step toward the development of a safe and effective HIV-1 vaccine. The demonstration of subunit HIV-1 vaccine efficacy in this relatively new SHIV animal model provides proof of its important role in preclinical HIV-1 vaccine development. For future studies new generation SHIV chimerics with increased pathogenic potential are being developed and tested [22,29], eventually allowing for more vigorous chal-

lenge studies with chimeric SHIVs containing of divergent HIV-1 envelopes. This study provides new insight into the immune correlates of HIV-1 vaccine efficacy and may lead to a better understanding of the types of T-helper responses important for protective immunity to HIV-infection. Utilizing a clinically applicable HIV-1 ISCOM vaccine preparation, data were obtained supporting the importance of generating a combination of both type-1 and type-2 T-helper responses for the coordinated induction of specific cellular and humoral responses to generate protective immunity. Studies are underway to evaluate the immunogenicity and efficacy of this HIV-1 ISCOM vaccine candidate in a mucosal study in macaques. Further studies are needed to 1) further define and expand an optimal combination of epitopes to qualitatively broaden HIV immunity to a diverse number of isolates and 2) to better understand mechanisms of generating and maintaining optimal Th memory responses [12,33,34].

Acknowledgments

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