Phenotypic and kinetic analysis of effective simian–human immunodeficiency virus-specific T cell responses in DNA- and fowlpox virus-vaccinated macaques

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Abstract

Although T cell immunity is important in the control of HIV-1 infection, the characteristics of effective HIV-specific T cell responses are unclear. We previously observed protection from virulent SHIV challenges in macaques administered priming with DNA vaccines and boosting with recombinant fowlpox viruses expressing shared SIV Gag antigens. We therefore performed a detailed kinetic and phenotypic study of the T cell immunity induced by these vaccines prior to and following SHIV challenge utilizing intracellular cytokine staining. Pigtail macaques vaccinated intramuscularly with DNA/recombinant fowlpox virus exhibited a coordinated induction of first Gag-specific CD4 T cell responses and then a week later Gag-specific CD8 T cell responses following the fowlpox virus boost. Overall, the magnitude and timing of the peak CD8 T cell responses following challenge was significantly associated with reductions in SHIV viremia following pathogenic challenge. After pathogenic lentiviral challenge, virus-specific effector memory T cells derived from animals controlling SHIV infection recognized a broad array of epitopes, expressed multiple effector cytokines and rapidly recognized virus-exposed cells ex vivo. These results shed light on some of the requirements for T cells in the control of pathogenic lentiviral infections.

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Introduction

The development of an effective vaccine against HIV-1 is one of humanity’s greatest challenges. Generating effective immunity to HIV is hampered by the virus’s capacity to evade innate, humoral and cell-mediated immunity, the likely need to generate effective mucosal immunity and the lack of a precise immune correlate of protection (Nabel, 2001; Stratov et al., 2004).

Although induction of broad neutralizing antibody to HIV by vaccines would be ideal, this has not been achievable to date. Many current HIV-1 vaccine research efforts endeavor to primarily induce effective HIV-specific T cell immunity. CD8+ cytotoxic T lymphocytes (CTLs) are important in controlling HIV-1 infection of humans and SIV/SHIV infection in macaques and generally correlate with protective immunity in macaque-SHIV studies (Barouch et al., 2000; Borrow et al., 1994; Koup et al., 1994; Ogg et al., 1998). There are several strategies to induce high-level T cell immunity, including priming with DNA with or without boosting with recombinant viral/bacterial vectors or cytokines. Promising data have been obtained in non-human primate models, showing protection from AIDS and ≥100-fold reductions in SHIV viral load following the induction of SHIV-specific T cell immunity with DNA/poxvirus prime/boost vaccination regimens (Amara et al., 2001; Dale et al., 2004a). DNA prime/poxvirus boost vaccine regimens have provided less durable protection against SIV in macaques (Horton et al., 2002).

HIV-specific T cell immunity involves both CD4 and CD8 T cells. The absence of functional HIV-specific T cells in HIV-infected humans is recognized as a key defect in enabling more efficient control of viremia (Rosenberg et
the six animals in group 1 of trial 1 vaccinated with our most successful vaccination regimen demonstrated a remarkably coordinated T cell immunity in individual animals for their surface marker phenotype, the number of epitopes recognized, the cytokine secretion profile and their ability to recognize intact virions of the T cell responses.

Results

Vaccination and SHIV challenge studies

We studied the kinetics and phenotype of SIV/HIV in pigtail macaques immunized with DNA prime/rFPV boost vaccine regimens involved in three separate studies (Dale et al., 2004a; De Rose et al., 2005; Kent et al., submitted). Two of these studies involved SHIV challenge, the first examining 30 animals receiving combinations of IM DNA and rFPV vaccinations and a highly pathogenic intrarectal CXCR4-utilizing SHIVΔmne229 challenge (Dale et al., 2004a). The second study involving immunization of 20 macaques with combinations of IM and mucosally delivered DNA and rFPV vaccines followed by a moderately pathogenic CCR5-utilizing SHIVSF162P3 challenge. Animals involved in the 3rd study were immunized IM with various doses of HIV-1 DNA and rFPV vaccines and were studied for the cytokine-secretion profile of T cells described below (De Rose et al., 2005). A summary of the regimens and outcomes is shown in Table 1.

Based on our previous studies (Dale et al., 2004b; Kent et al., 1998), we anticipated the most successful vaccination regimens would be those that used IM priming with two or three DNA vaccinations followed by one or more rFPV boosts. This prediction proved true (Table 1). In trial 1, the six animals vaccinated IM utilizing two DNA priming and with non-cytokine expressing rFPV boosting (group 1) induced peak SIVgag-specific CD8+ T lymphocytes capable of expressing IFNγ ranging from 0.3% to 4.7% of all CD8+ T cells, early after the rFPV boost, as previously reported (Dale et al., 2004a). After DNA vaccine priming alone, CD8 and CD4 T cell responses were low (<0.3%) or undetectable.

Kinetics of Gag-specific T cell responses in blood following prime/boost vaccination

We first studied the kinetic relationship of the SIVgag-specific CD4 and CD8 T cell responses in detail early after the rFPV boost vaccine in fresh peripheral blood samples from the six animals in group 1 of trial 1 vaccinated with our most effective, DNA/rFPV prime/boost regimen. Five of the six vaccinated animals demonstrated a remarkably coordinated induction of Gag-specific T cell immune responses (see Fig.
1A) with an early CD4 T cell response 1 week after the rFPV boost (mean 1.0%; range 0.2–2.1%) followed by a larger (week 2) CD8 T cell response (mean 2.4%; range 0.9–4.7%).

To confirm this kinetic relationship of the virus-specific CD4 and CD8 T cell response, we again studied the temporal association of SIV Gag-specific T cell responses in the four animals vaccinated IM with the same DNA and rFPV vaccines in trial 2. The pattern of an initial Gag-specific CD4 T cell response followed by a CD8 T cell response was repeated (Fig. 1B). In trial 2, a second boost with rFPV resulted in only a modest increase in SIVgag-specific CD8 T cells, which did not reach the level obtained from the initial boosting, likely due to immunity to the rFPV vector (Dale et al., 2004b).

**Kinetics of Gag-specific T cell responses following SHIV challenge**

We then studied the kinetics of recall Gag-specific T cell responses following SHIV challenge. Following pathogenic challenge with CXCR4-utilising SHIV <sup>mm229</sup>, the six animals in group 1 of trial 1 vaccinated IM twice with DNA and boosted with rFPV preserved peripheral CD4 T cells and had lower peak and set point plasma SIV viral loads than control animals (Dale et al., 2004a). A large anamnestic SIV Gag-specific CD8 T cell response (mean of 11.7%) was present in these animals. This response peaked 3 weeks after challenge and 1 week after the peak of the plasma SHIV RNA (mean 6.67 log<sub>10</sub> copies/mL; Fig. 1C). We also studied the kinetics of T cell immunity in the animals vaccinated IM with DNA and rFPV in trial 1 following the SHIV<sub>sf162p3</sub> challenge. A similar pattern was seen in the four animals in group 1 of trial 2 challenged with CCR5-utilising SHIVSF162P3. Peak plasma SIV viral load was 5.50 log<sub>10</sub> copies/mL at week 3, closely followed by peak Gag-specific CD8 T cell responses of 7.5% at week 3.5 (data not shown). Taken together, these kinetic data are consistent with previous data on the role of CD8 T cells in control of acute infection (Borrow et al., 1994; Koup et al., 1994).

Post-challenge SIV Gag-specific CD4 T cell responses were much lower than the CD8 T cell responses and there was no temporal relationship to the peak viral load or SHIV-specific CD8 T cell responses (Fig. 1C).

**Relationship of CD8 T cell immunity after challenge with virologic outcome**

The temporal relationship between the Gag-specific CD8 T cell response and peak viral load in group 1 animals in

<table>
<thead>
<tr>
<th>Trial</th>
<th>Group</th>
<th>Vaccination regime&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Route of vaccine (DNA/FPV)</th>
<th>n</th>
<th>Animal ID numbers</th>
<th>Peak Gag-specific CD8/CD4 T cell responses&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Challenge virus</th>
<th>Reduction in acute SHIV VL&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>1 1</td>
<td>2 DNA/FPV</td>
<td>IM/IM&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6 17105, 4247, 4277, 4290, 4295, 4296</td>
<td>2.4/0.97</td>
<td>SHIV&lt;sub&gt;mm229&lt;/sub&gt;</td>
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<tr>
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<tr>
<td>3 1</td>
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<td>6 4241, 4297, 4299, 4301, 4386, 4523</td>
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<tr>
<td>3 3</td>
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<td>IM/IM</td>
<td>12&lt;sup&gt;e&lt;/sup&gt; 200B, 1D52, 710D, 1302, 6820, 4C05, 2976, 3560, 0C18, 5D2F, 505D, 207F</td>
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<td>Nil</td>
<td>N/A</td>
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<sup>a</sup> Refers to number of DNA primes and FPV boost vaccines. All vaccines were administered at 3–4 weekly intervals.

<sup>b</sup> Mean % T cell response in group.

<sup>c</sup> Mean reduction in peak SHIV plasma RNA (log<sub>10</sub> copies/mL plasma) in group compared to control group.

<sup>d</sup> IM = intramuscular; IR = intrarectal; IN = intranasal; IVag = intravaginal.

<sup>e</sup> All animals received the same HIV-1 subtype AE DNA and rFPV vaccines at varying doses.
both SHIV challenge trials 1 and 2 suggested that there may be a correlation between either the timing or strength of T cell immunity and outcome of challenge. We therefore analyzed the timing and magnitude of the peak CD8 T cell response in comparison to the peak level of acute SHIV viremia. To broaden and improve power for these analyses we studied all 50 animals (including controls) challenged with either SHIV$_{mn229}$ or SHIV$_{SF162P3}$ across both trials 1 and 2.

The strength of the peak peripheral blood CD8 T cell post-challenge was indeed significantly inversely correlated with the peak SHIV viral load across the 50 animals infected in both trials 1 and 2 with the SHIV challenge viruses (Fig. 2A). We then also assessed whether the timing of the post-challenge peak CD8 immunity affected the peak level of SHIV viremia post-challenge. Animals with a very early (week 1) or late (>4 weeks) peak peripheral blood CD8 T cell response following SHIV challenge were less likely to
control the peak SHIV viremia, with a mean 0.88 log_{10} copies/mL higher level of plasma SHIV RNA compared to animals with peak SIV Gag-specific CD8 T cell responses that peaked at weeks 2–3 (Fig. 2B).

**Phenotype of Gag-specific T cells**

To further characterize effective T cell immunity to SIV/HIV in macaques, we then performed additional experiments on the phenotype, breadth, cytokine secretion profile and ability of T cell responses to recognition of whole virions. For these analyses, we studied some of the animals vaccinated with the DNA/FPV vaccine regimens (group 1 of trials 1 and 2) associated with high levels of peripheral T cell immunity and good outcomes from SHIV challenge.

To first determine the phenotype of the effector CD8 T cells to SIV Gag assisting in the control of SHIV viremia, we studied the surface expression of a series of molecules associated with an effector/memory phenotype in an ICS assay on PBMC from animals in group 1 of trial 1. The β-chain of CD8 is specific to CD3+8+ T lymphocytes. CD8^{hi} T cells are more characteristic of naïve cells, with increased expression in cord blood and larger V_{β} repertoire or they express markers of central memory (CCR7, CD62L and CD28), while CD8^{lo} T cells display an effector memory phenotype with increased levels of activation and cytotoxic molecules and express low levels of CD62L and CCR7 but high levels of surface CD95 (Konno et al., 2002; Schmitz et al., 1998). The vast majority of the responding CD8 T cells were confined to the CD8^{lo}-expressing cells (Fig. 3A). These effector SIVgag-specific CD8 T cells were phenotypically CD45RA negative and CD95 positive (Fig. 3B). To study CD62L expression, a marker of effector T cells (Kaech et al., 2002), PBMC were sorted for CD62L expression (high or low) prior to ICS assay to obviate CD62L down regulation during the 7 h in vitro incubation for the ICS assay. CD8 T cells producing IFNγ in response

![Fig. 2. Post-challenge correlation between peak SHIV plasma viral load and gag-specific CD8 T cell IFNγ responses in PBMC. All 50 control and vaccinated macaques receiving the various DNA and rFPV regimens in both trial 1 and trial 2 (see Table 1) were included in these analyses. (A) The peak viral load is inversely correlated with the peak IFNγ response (P = 0.003; R-squared 0.18). (B) The peak viral load correlates with the timing of the peak IFNγ response (P = 0.014, t test).](image-url)
to SIV Gag following SHIV challenge were confined to the sorted cells that were CD62L negative (0.34% Gag-specific CD8 T cells cf. 0.05% for CD62Lhi; Fig. 3C).

**Differential cytokine secretion of Gag-specific T cells**

IFNγ is only one of several effector cytokines likely to be important in the control of lentivirus infections, with evidence that TNFα and IL-2 are potentially important mediators of effective control of these infections (McKay et al., 2002; Pantaleo and Koup, 2004). We therefore studied the differential cytokine profile of Gag-specific T cells in DNA/rFPV-vaccinated macaques (Fig. 4).

In 9 of 11 blood samples studied in animals in group 1 of trials 1 and 2, DNA/rFPV vaccine-induced Gag-specific CD4 and CD8 T cells simultaneously produced both IFNγ and TNFα (Fig. 4A). We then also studied the kinetics of the simultaneous production of IL-2, IFNγ and TNFα after the
rFPV in two animals vaccinated with HIV-1 expressing DNA and rFPV vaccines in trial 3. IL-2 was also produced by Gag-specific T cells although we did not co-stain for all three cytokines in the same tube. Nevertheless, the production of all three cytokines by Gag-specific CD4 and CD8 T cells followed the same temporal pattern after rFPV boosting when studied kinetically in macaques vaccinated with HIV-1 DNA and rFPV vaccines in trial 3, although the production of IL-2 was shorter-lived (Fig. 4B). A similar pattern was observed for both animals studied and this was duplicated even when individual epitopes were tested, as opposed to a pool of peptides (data not shown).

There were, however, interesting exceptions to this general observation that IFNγ secretion from Gag-specific T cells paralleled that of other cytokines. In 2 of 11 animals studied, Gag-specific CD8 T cells primarily liberated TNFα and not IFNγ (Fig. 5). After the rFPV boosting, DNA-primed animals 4245 and 4381 (from trial 2, group 1, respectively) demonstrated minimal or no (0.02% and 0.03%, respectively) CD8+ T cells expressing IFNγ in response to SIV Gag but did demonstrate TNFα responses of 0.27% and 0.42%, respectively. In these two animals, the Gag-specific CD4 T cell responses secreted both IFNγ and TNFα. Even after SHIV challenge, macaque 4245, which controlled SHIVSF162P3 plasma viremia to <3.2log10 copies/mL by 11 weeks after challenge, failed to mount Gag-specific CD8+ T cells expressing IFNγ, although Gag-specific CD4 T cells expressing IFNγ were again detected. Although anecdotal, this observation suggests that secretion of TNFα by SHIV-specific T cells may, at least in some animals, facilitate control of viremia.

**Breadth of SHIV-specific CD4 and CD8 T cell responses**

Macques receiving DNA/rFPV vaccination (primarily expressing shared SIV Gag/Pol antigens) are able to develop effective CD8 and CD4 T cell responses following SHIV challenge virus (Dale et al., 2004a). Responses were detected against multiple SIV/HIV proteins in many animals, including peptide pools derived from HIV-1 Env, SIV Pol and Nef as well as SIV Gag, although responses directed against Gag were the strongest, presumably reflecting better priming of Gag-specific T cells by the Gag-expressing vaccines (data not shown).

Sufficient data were generated in many animals to study, in detail, the breadth of epitopes recognized within the Gag protein. Utilizing truncated sets of overlapping 15mer peptides, at least 19 different CD8 and 18 different CD4 epitopes were detected (Fernandez et al., 2005). An example of epitope mapping in a macaque with a broad and robust Gag-specific T cell response 4277 (group 1, trial 1) is shown in Fig. 6. This macaque recognized a minimum of seven epitopes (three CD8 and four CD4 epitopes) on initial screening with pools of five peptides. Of the seven epitopes identified (including the peptide 41 epitope which had been detected in other macaques), six of these T cell responses were narrowed to single 15mer peptides or to a pair of overlapping 15mer peptides. We have recently restricted one of the peptide epitopes (peptide 41) to a novel pigtail macaque MHC class I molecule ManeA*10 (Smith et al., 2005) and work on MHC restriction of other epitopes is ongoing.

Overall, the breadth of the T cell response across IM vaccinated macaques in group 1 of trials 1 and 2 averaged...
3.3 Gag epitopes per animal in peripheral blood. In contrast, only 2 of the 10 controls across both trials 1 and 2 had a sufficient Gag-specific T cell response after challenge to map T cell epitopes, and in both of these animals recognition of only a single 15mer peptide each was detected.

Recognition of whole SIV by CD8 and CD4 T cells

We then studied the Gag-specific CD4 and CD8 T cell immune responses induced by DNA/rFPV vaccination for their ability to recognize SIV/HIV in its native conformation, rather than just as individual 15mer peptides. Using conformationally intact whole inactivated SIV, we investigated the time required in vitro for T cells in whole blood to respond to processed whole SIV virions. The time taken to process and present SIV was evaluated by adding BFA, which blocks intracellular processing of whole antigen for presentation by MHC class I to CD8 T cells, at varying time intervals after exposure of antigen. A macaque, 4290 from group 1 in trial 1, which had a favorable outcome from SHIV challenge with persistent high levels of both CD8 and CD4 Gag-specific T cell immunity was studied for these experiments.

Within 30 min antigen-presenting cells in peripheral blood processed and began to present epitopes of the whole virus on their surface for recognition by circulating CD8 T cells (Fig. 7A). CD8 T cell expression of intracellular IFNγ in response to whole inactivated SIV peaked with a 2-h processing time prior to the addition of BFA. The CD8 T cell responses to the SIV Gag peptide pool were 10-fold greater than that to whole SIV, presumably reflecting the higher molarity of peptide epitopes available. Unlike responses to whole SIV, CD8 T cell responses to the Gag peptide pool are comparatively unaffected by addition of BFA at time zero. This suggests that the responses to the whole inactivated SIV particle are not due to contaminating free Gag peptide fragments. The CD4 T cell responses to the whole inactivated SIV parallels, and is nearly equivalent to, the CD4 T cell response to free Gag peptides, indicating that alternative processing of class II antigens from the whole inactivated SIV for CD4 recognition does not require such extensive intracellular processing (Fig. 7B).

Discussion

Protection from disease, at least in the short term, is observed following pathogenic SHIV challenge of macaques using prime/boost vaccine regimens (Dale et al., 2004a; Robinson et al., 1999; Shiver et al., 2002). Several previous studies have either measured SHIV-specific T cell immune responses by IFNγ ELISpot, which is unable to readily phenotype effector cells, or focus primarily on measuring the magnitude of the CD8 T cell response to a single immunodominant peptide by MHC class I tetramer analysis (Barouch et al., 2000). Vaccines based on single viral epitopes have been relatively inefficient in protecting macaques against SIV challenge (Yasutomi et al., 1995) and there is increasing evidence for CTL escape from narrowly directed CD8 T cell immunity (O’Connor et al., 2002). Further defining the phenotype and kinetics of protective CD4 and CD8 T cell immune responses may assist rational improvement of vaccine design.

The systematic study of SIV Gag-specific CD4 and CD8 T cells by ICS following rFPV boost in standard IM regimens of DNA and rFPV vaccinations in 10 macaques across two trials revealed an interesting pattern. We observed a coordinated induction of first SIV Gag-specific CD4 T cell immune responses, followed by larger peaks of CD8 T cell immune responses. Whether this relationship between CD4 and CD8 T cell immune responses also occurs with other viral proteins remains to be determined and likely to depend in part on the efficiency of vaccination, epitopes
encoded by each protein and MHC haplotype of the host. However, such a temporal relationship should be desirable in maintaining memory levels of CD8 T cell responses, given evidence that CD4 T cell help is critical in the expansion of CD8 + CTLs upon re-encounter with antigen (Janssen et al., 2003). Janssen et al. demonstrated that mice depleted of CD4 T cells prior to secondary encounter with antigen can still mount CD8 + CTL responses provided CD4 help was available during the initial encounter with antigen during immunization. This is of critical importance given that acute HIV infection is tropic for CD4 T cells rendering them dysfunctional (Douek et al., 2002).

The kinetics and magnitude of the anamnestic Gag-specific T cell response following SHIV challenge was also intriguing. For these studies we examined the relationship of peak CD8 T cell responses following SHIV challenge with peak acute SHIV viremia levels in 50 control and vaccinated animals across two trials. The timing (weeks 2–3) and magnitude of the peak Gag-specific CD8 T cell response following challenge were significantly associated with improved control of SHIV viremia across the two SHIV challenge studies performed. Optimal generation and expansion of virus-specific CD8 T cell responses following encounter with pathogenic lentiviruses is likely to be an important determinant of the outcome of infection. Interestingly, there was no clear relationship between the recall of SHIV-specific CD4 T cell responses and control of peak viremia, possibly because these cells are readily infected, and likely rendered dysfunctional, by primate lentiviruses (Douek et al., 2002).

The phenotype and cytokine of Gag-specific CD8 T cells following challenge was further probed by studying individual macaques vaccinated with DNA and rFPV regimens associated with successful outcomes of SHIV challenge. As expected, the phenotype was that of an effector/memory T cell, having the surface cell phenotype of CD8\textsuperscript{hi}, CD45RA negative, CD95\textsuperscript{hi} and CD62L negative. The proportion of SIVgag-specific cells in the CD8\textsuperscript{lo} fraction was approximately twofold higher than that in the CD3/CD8\textsuperscript{a} fraction. Additional surface markers such as CCR7 would have been useful to confirm this phenotype but were not available for pigtail macaque cells, although CD8\textsuperscript{lo} cells are typically CCR7 negative (Konno et al., 2002). Generally, all three cytokines studied (IFN\textgamma, TNF\alpha...
and IL-2) were expressed by Gag-specific T cells, both after vaccination and after challenge. Expression of IL-2 was more short lived in both CD4 and CD8 T cells after the rFPV boost compared to IFNγ and TNFα expression, consistent with their effector/memory phenotype (Konno et al., 2002). IL-2 expression is likely to drive expansion of the virus-specific T cells and may wane as vaccine-expressed antigen levels decline. More prominent IL-2 expression from virus-specific T cells is a feature of central memory cells (Konno et al., 2002; Sallusto et al., 1999) and may result in more durable levels of T cell immunity.

There were exceptions observed to the general rule that multiple cytokines are co-expressed by Gag-specific T cells. Two of 11 animals studied predominantly expressed TNFα and not IFNγ, and one of these animals achieved full control of viral replication. Although requiring confirmation in larger studies, this observation suggests that TNFα expression may be sufficient for effective immunity and perhaps an improved surrogate marker for effective T cell immunity. However, we consistently noted mildly higher levels of background TNFα expression from T cells in unstimulated blood (data not shown), compared to IFNγ expression, which limits the sensitivity of TNFα expression in studying antigen-specific cells.

A large breadth of SIV-specific CD4 and CD8 T cell responses was also observed following SHIV challenge of IM DNA/rFPV-vaccinated macaques. A broad T cell response is likely to be more important than a large narrowly focussed one. Rapid recognition of whole virions by both CD4 and CD8 T cell responses prior to release of progeny viruses should be critical in effectively controlling viremia (Yang et al., 1996, 1997). We studied T cell recognition of whole processed virions. SIV-specific CD8 T cells induced by DNA/rFPV vaccination begin to secrete IFNγ within 30 min, indicating rapid processing of whole inactivated SIV virions within antigen-presenting cells. SIV-specific CD4 T cells do not require processing of SIV through the endoplasmic reticulum and can express IFNγ when BFA is added simultaneously with the antigen. This rapid recognition of virus exposed cells by SIV-specific CD8 and CD4 T cells is likely to be an important factor in the control of virus replication. Future studies could address the comparative rapidity of recognition of virus-infected cells by specific T cell responses to more accurately define the requirements for protective immunity.

In summary, this study of DNA/rFPV-vaccinated macaques found a coordinated induction of broad CD4 and CD8 T cell responses. Recall of Gag-specific CD8 T cell immune responses correlated with reductions in SHIV viremia following pathogenic challenge. The broad T cell responses induced were of an effector-memory phenotype, capable of expressing multiple effector cytokines and rapidly recognized virus-exposed cells. Taken together, these results illustrate some of the potentially complex requirements of effective T cell immunity to HIV-1.

Materials and methods

Animal experimentation

Studies were performed on juvenile pigtail macaques (Macaca nemestrina). All experimentation was approved by relevant ethics committees under previously described conditions (Dale et al., 2002, 2004a).

Vaccine protocols

Three separate pigtail macaque vaccine trials studying DNA/rFPV prime/boost vaccination were undertaken using various protocols as previously described (Dale et al., 2004a; Kent et al., 1998) (see Table 1). Briefly, trial 1 involved two intramuscular DNA vaccine priming and boosting with a single intramuscular rFPV vaccine (both encoding shared SIVmac239 Gag/Pol proteins), followed by rectal challenge with highly pathogenic SHIV329 which results in reductions in SHIV viremia and retention of CD4 T cells (Dale et al., 2002, 2004a; Kent et al., 2002). Trial 2 involved three DNA priming vaccinations then two boosting vaccinations with rFPV (again encoding shared SIV proteins), followed by vaginal challenge with moderately pathogenic SHIVSF162P3 (Harouse et al., 1999, 2001), which also resulted in reduction in SHIV viral load in comparison to control unvaccinated animals (S. Kent et al., manuscript submitted). Trial 3 involved three intramuscular DNA priming vaccinations and a single intramuscular rFPV boosting, both encoding HIV-1 subtype AE proteins, without viral challenge (De Rose et al., 2005).

DNA vaccines

DNA vaccine, pHIS-SHIV-B, encoded full-length unmutated SIVmac239 Gag and Pol, HIV-1NL4-3, Tat, Rev and Vpu and the 5’ one third of HIV-1AD8 Env (Dale et al., 2004a). Genes were inserted into vector pHIS-64 (Dr. Heather Davis, Coley Pharmaceuticals) behind the human cytomegalovirus immediate–early promoter. Plasmid vector pHIS-64 has kanamycin resistance, bovine growth hormone polyA termination signal and 14 primate-optimized Cpg immunostimulatory sequences. Empty vector plasmid DNA, pHIS, served as a control vaccine. Plasmid pHIS-SHIV-B (1mg/dose IM) for immunization was prepared by QIAGEN (Hilden, Germany), control DNA vaccine pHIS was prepared using the EndoFree Plasmid Giga kit (QIAGEN).

Recombinant fowlpox virus vaccines

Construction of the FPV vaccines has been described (Boyle et al., 2004; Dale et al., 2004a). Briefly, FPVgag/pol, expressing SIV Gag/Pol, was constructed by inserting the promoter-SIV gag/pol PCR amplicon into pKG10aSIVgag-pol for insertion into FPV-M3 at the F6,7,9 site. PCR primers used the FPV early/late promoter and an early
transcription terminator for insertion into fowlpox virus vector FPV-M3. HIV-1 93TH254 env (mutated to remove the middle third) was amplified from plasmid vector pCH34AEnv(m) and inserted into a separate FPV-M3 vector at the REV site. Recombinants were selected on the basis of co-expression of the Escherichia coli gpt gene and plaque purified on the basis of co-expression of the E. coli beta-galactosidase gene. FPV vaccines were prepared in saline.

Antigens

Antigens studied for T cell responses were primarily 15mer peptides overlapping by 11 amino acids spanning SIVmac239 Gag, Pol and Nef, HIV-1MN Env, all kindly obtained from the NIH AIDS Research and Reference Reagent Repository. For trial 3 we obtained homologous subtype AE HIV-1 9TH253 Gag 15mer peptides (Auspep, Parkville, Australia). Peptides were solubilized in pure DMSO at high concentrations (1 mg peptide/10–40 μL DMSO) and peptides for each antigen pooled. 1 μg/mL of each 15mer peptide within the pools was used for the ICS assay. To assess immune responses to intact virions we used whole aldrithiol-2 inactivated SIVmne (Arthur et al., 1998; Assay. To assess immune responses to intact virions we used whole aldrithiol-2 inactivated SIVmne (Arthur et al., 1998; assay. To assess immune responses to intact virions we used whole aldrithiol-2 inactivated SIVmne (Arthur et al., 1998; Dale et al., 2004a) (10 μg/mL, kindly provided by Dr J Lifson, National Cancer Institute, Frederick, MD) and the relevant microvesicle control preparation derived from the same cell line used to grow the virus.

Intracellular cytokine staining

Intracellular cytokine staining (ICS) assay was performed as previously described (Dale et al., 2004a; Maecker et al., 2001). Briefly, in the presence of co-stimulatory molecules CD28 and CD49d (BD Sciences), whole blood or mononuclear cell preparations were incubated with pools of 15mer peptides or whole inactivated SIV. Brefeldin A (BFA; Sigma; final concentration 10 μg/mL) was added to prevent protein export from the endoplasmic reticulum. Incubation times were generally terminated after 7 h, the last 5 h in the presence of BFA, although this varied in some experiments. Cells were then stained with surface antibodies including anti-CD4 FITC and PerCP, anti-CD3 PE, anti-CD8 PerCP and APC, anti-CD45RA PE, anti-CD95 FITC, anti-CD62L PE (BD Biosciences, Pharmingen, San Diego, CA) or CD8β PE (Immunotech, Marseille, France), then lysed and permeabilized as per standard protocols. Intracellular staining was then performed with anti-IFNγ APC, anti-IL-2 APC and/or anti-TNF-α FITC (BD) and fixed prior to acquisition via FACSort (BD) and analyzed using cell quest software (BD).

Determining SHIV-specific T cells by ICS

Gating on T lymphocytes was performed using forward and side scatter criteria and combined this with surface expression of CD3, based on the nadir of a CD3 histogram. Two-dimensional dot plots (x-axis: CD8; y-axis: IFNγ) were then formed and responses calculated using quadrant lines. The vertical quadrant line (defining CD3/CD8+ cells) was placed along the margin where the density of clearly CD8+ cells started to reduce, so as to be confident that all cells included in the quadrant were truly CD8+, although very similar percentages were obtained when the vertical quadrant line was placed at the nadir of a CD8+ cell histogram (not shown). The horizontal quadrant line (defining IFNγ-producing cells) was placed within approximately 10 channel values above the margin where the density of CD8+ cells had clearly reduced. Analyses generally were performed after collecting approximately 100,000 lymphocytes. This produced a final CD3/CD8+ count of approximately 20,000 events; counts of less than 1000 CD3/CD8 (or CD3/CD4) events were excluded from analysis.

Validation of true positive antigen-specific responses

Validation of the ICS assay in M. nemestrina was performed by studying background levels of IFNγ production in the first 101 negative control samples; one (1%) sample was excluded from our statistical validation of the cut-off for a true positive. This sample had a background of 1.88% (i.e., 48-fold above the mean background level) and was 5.7-fold larger than the next largest background level of 0.33%. From these 100 samples used for final statistical analysis, 81% of negative control samples had a background ≤0.05%, the mean background being 0.04% (range 0.00–0.33%; standard deviation 0.05%). Ninety-six (96%) of the samples had a background level within 2 standard deviations of the mean. We determined that a true positive was an antigen-specific result more than two standard deviations above the background level (i.e., approximately 0.10% or threefold above background). To be confident of a true positive in samples with backgrounds greater than 0.05%, we determined that IFNγ production in antigen-specific samples needed to be >0.10% above background and threefold above background.

Background ICS results were consistent throughout the studies in naïve, vaccinated and SHIV-infected macaques; that is, 80–90% of samples had background levels <0.05% and only 1–2% of samples had very high backgrounds (>10-fold above average background).

Cell sorting to determine CD62L status

Sorting for cells for the expression of CD62 ligand (CD62L) was performed on a MoFlo flow cytometer (Dako cytometry, Fort Collins, USA). Briefly, fresh PBMC were stained with CD62L-PE (at 4 °C for 30 min) and CD62L-positive and -negative cells sorted into separate tubes before proceeding to Gag antigen stimulation.
References


