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## Killing Kinetics of Simian Immunodeficiency Virus-Specific CD8<sup>+</sup> T Cells: Implications for HIV Vaccine Strategies

This information is current as of August 9, 2022.

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*J Immunol* 2007; 179:4571-4579; ;  
doi: 10.4049/jimmunol.179.7.4571  
<http://www.jimmunol.org/content/179/7/4571>

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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Killing Kinetics of Simian Immunodeficiency Virus-Specific CD8<sup>+</sup> T Cells: Implications for HIV Vaccine Strategies<sup>1</sup>

Erik Rollman,\* Miranda Z. Smith,\* Andrew G. Brooks,\* Damian F. J. Purcell,\* Bartek Zuber,<sup>†</sup> Ian A. Ramshaw,<sup>‡</sup> and Stephen J. Kent<sup>2\*</sup>

Both the magnitude and function of vaccine-induced HIV-specific CD8<sup>+</sup> CTLs are likely to be important in the outcome of infection. We hypothesized that rapid cytolysis by CTLs may facilitate control of viral challenge. Release kinetics of the cytolytic effector molecules granzyme B and perforin, as well as the expression of the degranulation marker CD107a and IFN- $\gamma$  were simultaneously studied in SIV Gag<sub>164–172</sub> KP9-specific CD8<sup>+</sup> T cells from *Mane-A\*10*<sup>+</sup> pigtail macaques. Macaques were vaccinated with either prime-boost poxvirus vector vaccines or live-attenuated SIV vaccines. Prime-boost vaccination induced Gag-specific CTLs capable of only slow (after 3 h) production of IFN- $\gamma$  and with limited (<5%) degranulation and granzyme B release. Vaccination with live-attenuated SIV resulted in a rapid cytolytic profile of SIV-specific CTLs with rapid (<0.5 h) and robust (>50% of tetramer-positive CD8<sup>+</sup> T cells) degranulation and granzyme B release. The cytolytic phenotype following live-attenuated SIV vaccinations were similar to that associated with the partial resolution of viremia following SIV<sub>mac251</sub> challenge of prime-boost-vaccinated macaques, albeit with less IFN- $\gamma$  expression. High proportions of KP9-specific T cells expressed the costimulatory molecule CD28 when they exhibited a rapid cytolytic phenotype. The delayed cytolytic phenotype exhibited by standard vector-based vaccine-induced CTLs may limit the ability of T cell-based HIV vaccines to rapidly control acute infection following a pathogenic lentiviral exposure. *The Journal of Immunology*, 2007, 179: 4571–4579.

Research shows that CD8<sup>+</sup> T cells help partially control HIV-1 infection in humans and SIV infection in macaques. Depletion of CD8<sup>+</sup> T cells results in a brisk rise in viremia in SIV-infected macaques (1–5). Considerable focus now concentrates on the induction of HIV-specific CD8<sup>+</sup> T cell immunity with novel vector-based vaccine strategies delivering Ags intracellularly to the MHC class I processing pathways. Many current approaches are studying heterologous prime-boost vaccinations with combinations of DNA, adenovirus, and poxvirus recombinants in advanced clinical trials. Although many vector-based approaches have demonstrated partial efficacy in non-human primate studies, live-attenuated virus (LAV)<sup>3</sup> vaccine using SIV have consistently shown more efficient protection in macaque studies (6–8). LAV vaccines using HIV are currently perceived to be too dangerous for clinical trials (9); however, LAV vaccines provide an excellent model to study potentially effective immunity to SIV in macaques (10).

Most studies of CD8<sup>+</sup> T cell immunity in macaque models and human trials have analyzed the magnitude of T cell immunity after

vaccination and following challenge, commonly by measuring IFN- $\gamma$  expression by ELISPOT or intracellular cytokine staining (ICS) techniques (11, 12). The phenotype and functional capacity of CD8<sup>+</sup> T cells are also important determinants of the effective T cell immunity. Betts et al. (13) recently showed in typical 6-h ICS assays that the ability of CD8<sup>+</sup> T cells to secrete multiple cytokines and chemokines and express the degranulation marker CD107a (“polyfunctional” T cells) correlates with long-term control of HIV in humans.

The capacity of CD8<sup>+</sup> T cells to very rapidly lyse virus-infected cells is likely to be the most critical function of HIV/SIV-specific T cells (14–17). Viral vector-based vaccines can efficiently prime CD8<sup>+</sup> T cells, but Ag expression is typically short-lived and the specific T cell populations contract to low levels late after vaccination (18). A substantial delay of activation, expansion, and potentially cytolytic function of these cells exists following virus challenge (19, 20). We recently showed that the baseline prechallenge levels, and rates of expansion, of recombinant DNA and poxvirus vaccine-induced SIV-specific T cells correlate with control of wild-type virus at an immunodominant Gag epitope (21). Memory CD4<sup>+</sup> T cells are irreparably lost during the early days of acute SIV infection of macaques (22), before the efficient expansion of CD8<sup>+</sup> T cells generated by typical vector-based vaccines. The remaining levels of memory CD4<sup>+</sup> T cells predict long-term survival in vaccinated macaques (23, 24). Ameliorating or limiting the early immunological damage during acute infection via efficient CD8<sup>+</sup> T cell killing of virus-infected cells may therefore be crucial to effective T cell-based HIV vaccine strategies.

Immunity induced by live-attenuated SIV vaccines much more efficiently quashes early viral replication following SIV challenge compared with vector-based vaccination (6–8, 10). Protection afforded by LAV vaccines using SIV could not be transferred with

\*Department of Microbiology and Immunology, University of Melbourne, Victoria, Australia; <sup>†</sup>Mabtech, Nacka, Sweden; and <sup>‡</sup>John Curtin School of Medical Research, Australian National University, Canberra, Australia

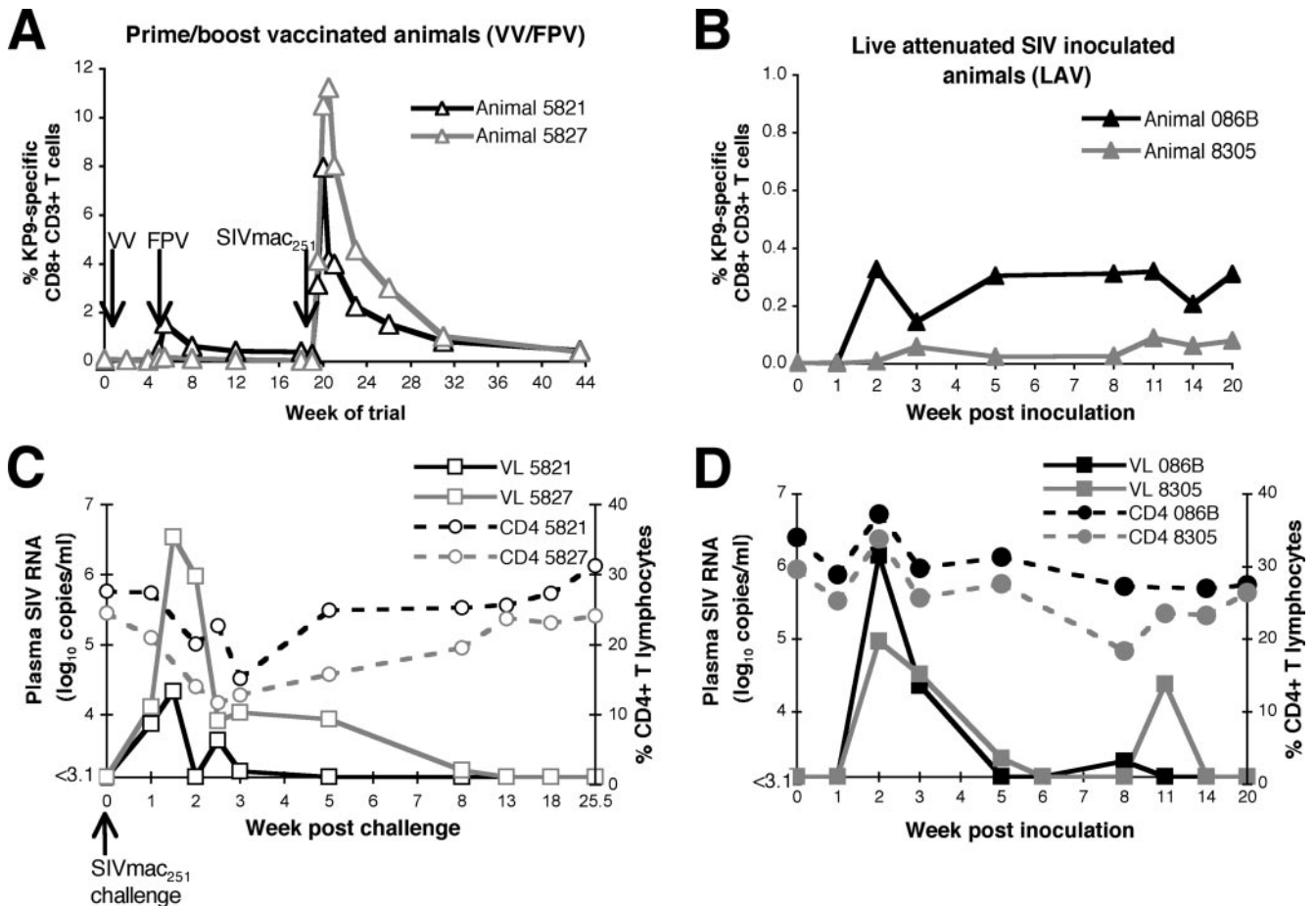
Received for publication July 17, 2007. Accepted for publication July 20, 2007.

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<sup>1</sup> This work was supported by Grants 299907 and 251654 from the Australian National Health and Medical Research Council, by the Australian Centre for HIV and Hepatitis Virology Research, and by a 2005-2527 Swedish Research Council Fellowship.

<sup>2</sup> Address correspondence and reprint requests to Prof. Stephen J. Kent, Department of Microbiology and Immunology, University of Melbourne, Victoria 3010, Australia. E-mail address: skent@unimelb.edu.au

<sup>3</sup> Abbreviations used in this paper: LAV, live-attenuated virus; FPV, fowlpoxvirus; VV, vaccinia virus; ICS, intracellular cytokine staining; LTR, long terminal repeat.



**FIGURE 1.** Evaluation of the kinetics of cytolysis between vaccination and infection determined by CD8<sup>+</sup> T cell tetramer frequency, CD4 counts, and viral load. **A**, The frequency of SIV KP9-specific tetramer-positive CD8<sup>+</sup>CD3<sup>+</sup> T cells plotted over time for VV/FPV-vaccinated animals (5821 and 5827) subsequently challenged with SIV<sub>mac251</sub> on week 18. **B**, The tetramer frequency found in the live-attenuated SIV-vaccinated (LAV) animals (8305 and 086b). **C** and **D**, Viral load (VL) and CD4 count (%CD4<sup>+</sup> T lymphocytes) over time in the same four animals. CD4 counts are the percentage of CD3<sup>+</sup>CD4<sup>+</sup> T cells divided by all cells in the lymphocyte gate  $\times 100$ .

passive Abs (25). Effective T cell immune responses may be primed to kill infected cells and control viremia in animals inoculated with live-attenuated SIV vaccines. The rapidity with which SIV-specific CD8<sup>+</sup> T cells degranulate effector molecules such as granzyme B and perforin upon cognate Ag exposure was therefore studied in macaques vaccinated with either a recombinant poxvirus-based vaccine strategy or a live-attenuated SIV vaccine.

## Materials and Methods

### Animals, vaccination, and SIV challenge

Juvenile *Macaca nemestrina* were free from HIV-1/SIV/simian retrovirus infection and anesthetized with ketamine (10 mg/kg i.m.) before procedures. Experiments were approved by the University of Melbourne and Commonwealth Scientific and Industrial Research Organisation Livestock Industries Animal Experimentation and Ethics Committees.

We prospectively studied four *Mane-A\*10*<sup>+</sup> pigtail macaques. Two macaques were vaccinated with a prime-boost regimen of recombinant vaccinia virus (VV) and fowlpoxvirus (FPV) both expressing wild-type SIV Gag as previously described (26). The pigtail macaques were MHC typed for *Mane-A\*10* using sequence-specific primer PCR and confirmed by Reference Strand-mediated Conformational Analysis as previously described (27–29). We previously showed that a VV/FPV prime-boost regimen in *Mane-A\*10*<sup>+</sup> macaques induces ~1% KP9-specific CD8<sup>+</sup> T cells in blood following vaccination (26). The FPV used for this study also coexpressed the human 4-1BBL molecule as our prior murine studies showed coexpression of this molecule enhances immunologic memory (30). The animals were vaccinated 4 wk apart i.m. with  $2 \times 10^8$  PFU of each poxvirus construct (see Fig. 1).

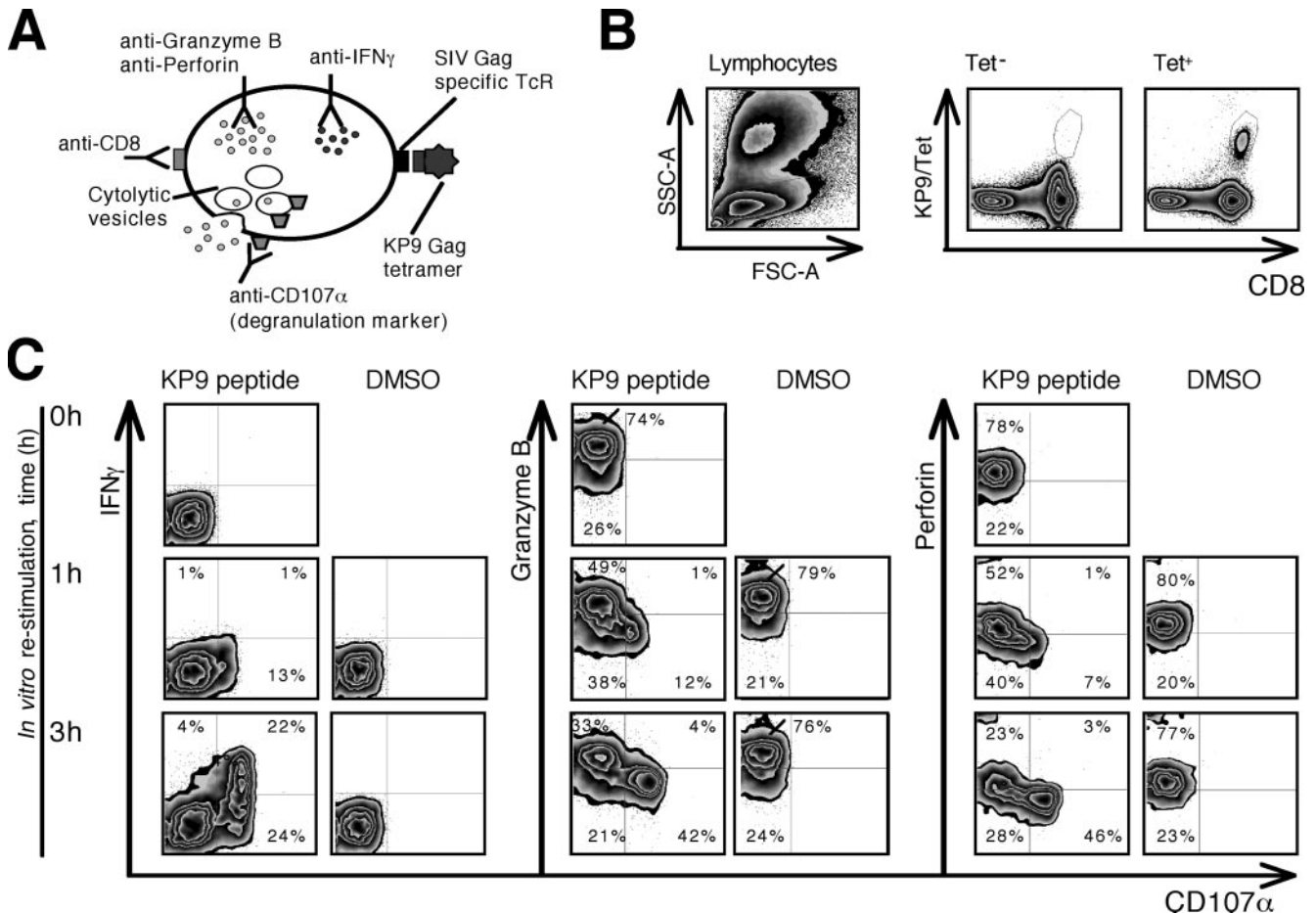
The two VV/FPV-vaccinated macaques were challenged i.v. with SIV<sub>mac251</sub> (40 TCID<sub>50</sub>/ml) 14 wk after the last vaccination as previously described (31). SIV plasma viremia was quantified by reverse transcriptase real-time PCR on an ABI 7700 machine as described (12), except a TaqMan probe was used instead of a molecular beacon to detect fluorescence.

To compare the kinetic profile of KP9-specific responses, we inoculated an additional two *Mane-A\*10*<sup>+</sup> pigtail macaques with a live-attenuated SIV vaccine. The LAV inoculation used a previously reported *nef*-long terminal repeat (*nef*/LTR)-deleted SIV<sub>mac239</sub> provirus (SIV<sub>sbbcΔ3Δ5</sub>) modeled on the deleted HIV-1 strains present in a linked cohort of long-term slow progressors termed the Sydney Blood Bank Cohort (8, 32). SIV<sub>sbbcΔ3Δ5</sub> expresses wild-type SIV Gag and contains an LTR deletion at both ends of the provirus to avoid recombination to wild type (33). The SIV<sub>sbbcΔ3Δ5</sub> was administered as proviral DNA; 75 μg inoculated half i.m. and half into an inguinal lymph node. Our previous studies have shown similar virus/inoculation systems to be a reliable vaccination method, and as with other studies of live *nef*-deleted SIV, efficacious in controlling acute viremia following wild-type SIV<sub>mac251</sub> challenge (8, 10).

### The tetramer degranulation assay

The tetramer degranulation assay is essentially a combination of a CD8<sup>+</sup> T cell tetramer staining assay and an ICS assay (12, 27) similar to previous work (34). In brief, 0.2 ml of fresh whole blood was cocultured with the anti-CD107a allophycocyanin mAb conjugate (catalog no. 624076; BD Biosciences) following restimulation with 1 μg/ml immunodominant CD8<sup>+</sup> T cell epitope (KP9, SIV Gag<sub>164–172</sub>) or medium alone (see Fig. 2). Whole blood restimulation was performed in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich), 5 μg/ml monensin (Sigma-Aldrich), 1 μg/ml anti-CD28 (catalog no. 340975; BD Biosciences), and 1 μg/ml anti-CD49d (catalog no. 340976; BD Biosciences). Parallel 0.2 ml of whole blood





**FIGURE 2.** The kinetic tetramer degranulation assay. *A*, Macaque whole blood is stimulated directly ex vivo with the dominant SIV Gag peptide in the presence of labeled anti-CD107a mAb. The lymphocytes are subsequently stained with a SIV Gag KP9-specific tetramer and anti-CD8-conjugated mAb followed by intracellular staining for IFN- $\gamma$  and granzyme B or perforin. *B*, Side scatter area (SSC-A) and forward scatter area (FSC-A) are used to gate on lymphocytes. Only tetramer-positive CD8<sup>+</sup> T cells were analyzed for levels of IFN- $\gamma$ , granzyme B, and perforin. *C*, As an illustration of the assay, whole blood from a vaccinated and SIV-challenged animal (5827, week 5 postchallenge, week 23) was restimulated in vitro with KP9 peptide for variable time (0, 1, and 3 h) and stained for combinations of IFN- $\gamma$ /granzyme B/CD107a or IFN- $\gamma$ /perforin/CD107a. Restimulation with DMSO (the same concentration as used to dissolve the peptide) or culture medium (data not shown) was used as negative control and showed limited degranulation/cytokine expression of the tetramer-positive CD8<sup>+</sup> T cells over time.

samples were set up in 5 ml of FACS tubes, either not incubated ( $t = 0$ , control) or incubated at 37°C with 5% CO<sub>2</sub> for variable times. At 1, 3, or 5 h after incubation was initiated, the reactions were stopped by washing with ice-cold PBS, followed by staining with a MHC class I-specific tetramer (*Mane-A\*10/KP9-PE*) and anti-CD8-PerCP (catalog no. 347314; BD Biosciences). For selected experiments in which there was a rapid cytolytic profile, we also stimulated blood with KP9 peptide for 30 min. RBC lysis (BD lysing solution, catalog no. 349202) and cell permeabilization (permeabilization solution, catalog no. 340973; BD Biosciences) preceded the staining with anti-IFN- $\gamma$  PE-Cy7 (catalog no. 557643; BD Biosciences), anti-granzyme B FITC (clone GB11; Mabtech) or anti-perforin FITC (clone Pf-344; Mabtech). The samples were fixed with 2% formaldehyde and acquired on a six-color LSRII flow cytometer (BD Biosciences) within 24 h of staining. Analyses of cryopreserved PBMC were performed on cells thawed on the same day as stimulation/staining. Data were analyzed if the number of KP9-specific CD8<sup>+</sup> T cells acquired exceeded 100 events.

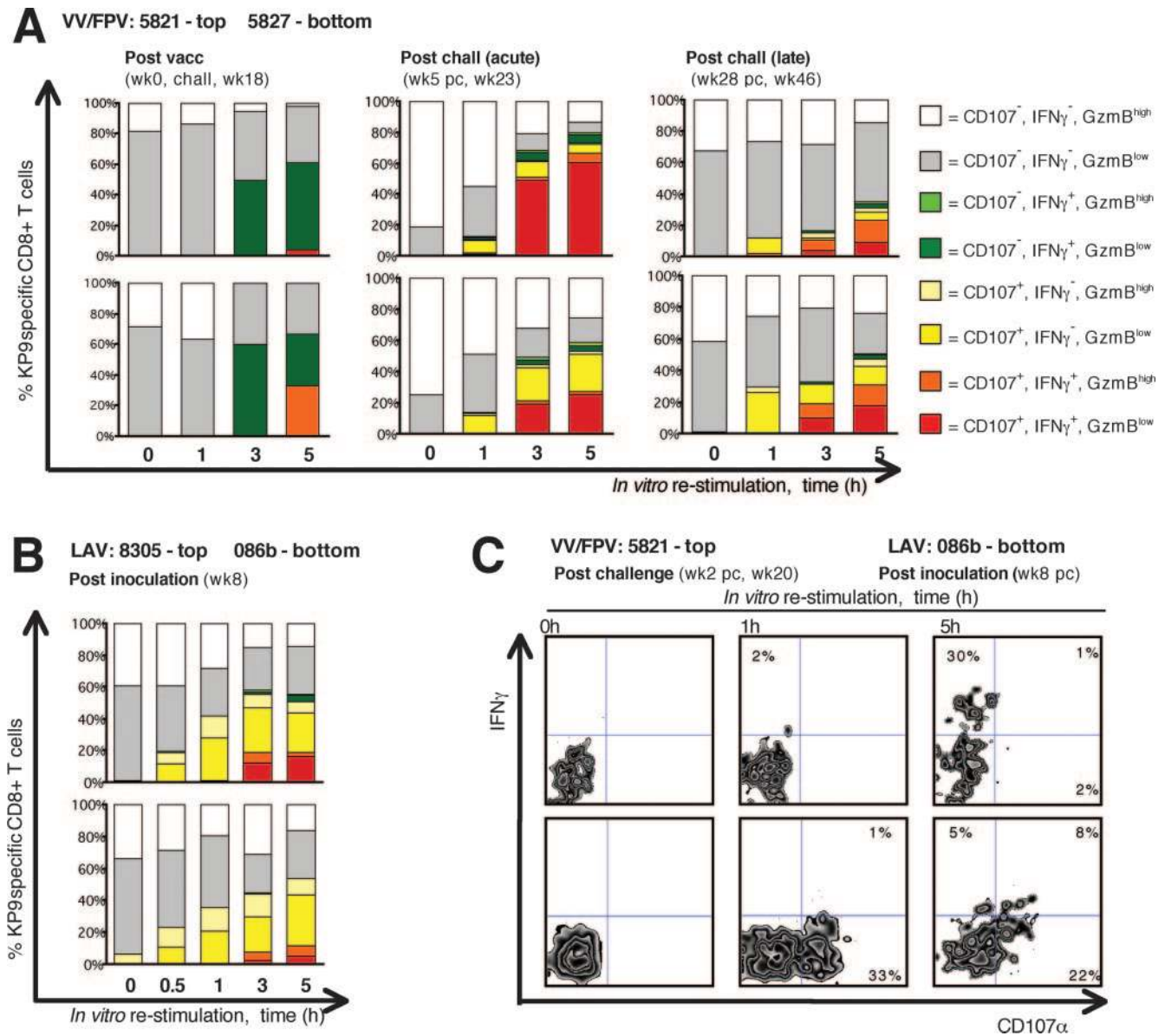
#### Lymphocyte phenotyping for memory markers

Staining for memory markers was conducted as described elsewhere (35, 36). Whole blood was stained with the *Mane-A\*10/KP9* tetramer and anti-CD3 PerCP (BD, clone SP34-2; BD Biosciences), anti-CD8 PE-Cy7 (clone SK1; BD Biosciences) in combination with anti-CD28 allophycocyanin (clone 28.2; BD Biosciences), and anti-CD27 FITC (clone M-T271; BD Biosciences) or anti-CD45RA FITC (clone 5H9; BD Biosciences). Erythrocytes were lysed using FACS lysing solution (BD Biosciences), the remaining cells were fixed in 1% formaldehyde, and the samples acquired as described.

## Results

### *SIV vaccination and infection of pigtail macaques*

To evaluate whether the kinetics of cytotoxicity were different between vaccination and infection, we prospectively studied the cytotoxic phenotype of viral-specific CD8<sup>+</sup> T cells in four *Mane-A\*10*<sup>+</sup> macaques. *Mane-A\*10* restricts an immunodominant SIV Gag epitope, KP9, for which we have an MHC class I tetramer (28). Two macaques were vaccinated with a VV/FPV prime-boost regimen and subsequently challenged with wild-type SIV, and two were inoculated with a live-attenuated SIV provirus. The prime-boost regimen consisted of priming with VV expressing SIV Gag and boosting with FPV expressing both SIV Gag and the costimulatory molecule 4-1BBL. VV/FPV prime-boost regimens have shown promise in mouse and macaque studies for the induction of T cell immunity (26, 37, 38). This regimen induced reasonable levels of KP9-specific CD8<sup>+</sup> T cells shortly after the FPV boost (0.2–1.6%) that waned over the 14 wk before SIV<sub>mac251</sub> i.v. challenge (Fig. 1A). The SIV challenge resulted in substantial recall of KP9-specific CD8<sup>+</sup> T cells, peaking at 7.9–11.3%. There was an early peak of plasma SIV viremia that was subsequently controlled to <10<sup>3</sup> copies/ml within 5–8 wk after challenge (Fig. 1C). There



**FIGURE 3.** VV/FPV vaccination induces a slow CD8<sup>+</sup> T cell cytolytic phenotype, whereas live-attenuated SIV inoculation induces a rapid profile. *A*, The intracellular stained tetramer-positive CD8<sup>+</sup> T cells in the kinetic tetramer degranulation assay can be divided into eight different degranulation/activation CD8<sup>+</sup> T cell populations. These profiles show both the cytolytic phenotype and the timely kinetics of the response. There is a much more pronounced “killer phenotype” (CD107<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>, Gzmb<sup>low</sup>) in blood samples obtained after SIV challenge as compared with following vaccination. The cytolytic phenotype kinetics change dramatically in VV/FPV vaccination compared with a subsequent SIV challenge both in the early (acute) and the chronic (late) stage of infection. *B*, The cytolytic profile from the two live-attenuated SIV-inoculated animals (8305 and 086b) shows a rapid degranulation at week 8 postinoculation with limited IFN- $\gamma$  expression. *C*, A VV/FPV-vaccinated animal (5821) has clearly different kinetics of degranulation (CD107a) and degree of cytokine induction (IFN- $\gamma$ ) as compared with a live-attenuated SIV-vaccinated (LAV) animal (086b).

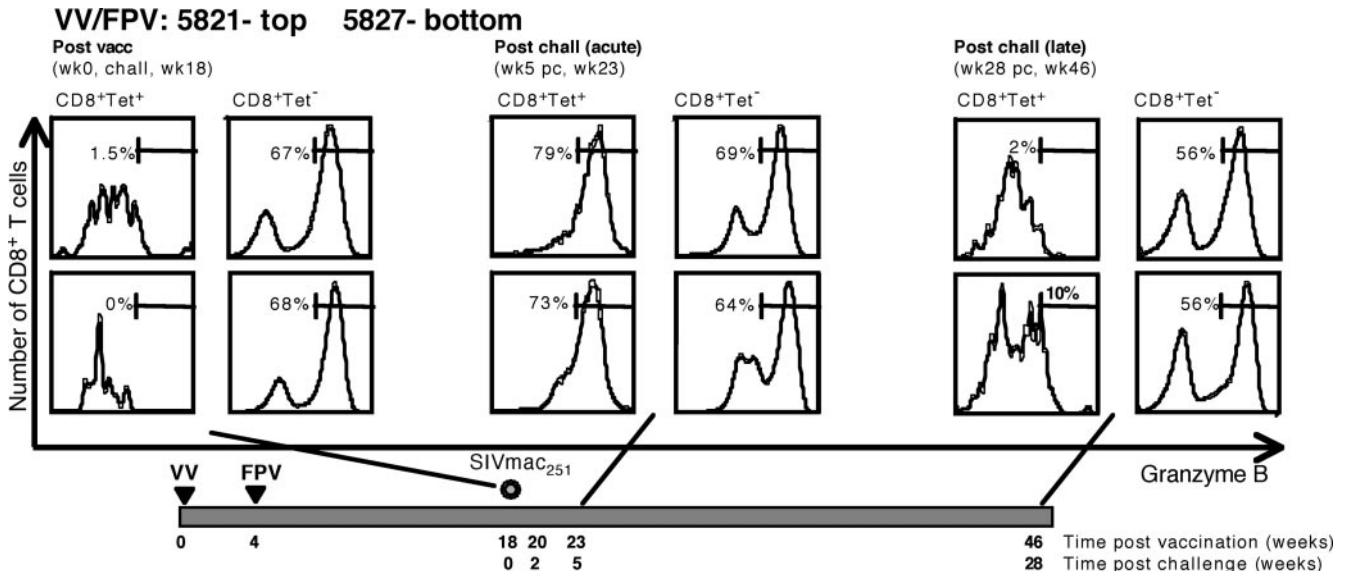
was a 50% loss of CD4<sup>+</sup> T cells during acute infection that recovered to normal levels by week 11 postchallenge, and levels have remained normal (>20% of CD3<sup>+</sup> lymphocytes) out to 29 wk following challenge.

Two *Mane-A\*10*<sup>+</sup> pigtail macaques were inoculated with a *nef*/LTR-deleted SIV provirus, which we previously showed results in a stable live-attenuated SIV infection (8). Both macaques became infected with low postacute viral load levels and stable CD4<sup>+</sup> T cell levels (Fig. 1*D*). Both macaques generated KP9-specific CD8<sup>+</sup> T cells, albeit with lower frequencies (0.1–0.3% of CD8<sup>+</sup> T cells) than the prime-boost-vaccinated animals either at the peak postvaccination time point or postchallenge (Fig. 1*B*).

#### The kinetic tetramer degranulation assay

To study the kinetics of the cytolytic potential of SIV-specific CTLs after both SIV vaccination and infection, we developed a kinetic whole blood cytolytic phenotype assay. This assay simultaneously quantifies the intracellular expression of IFN- $\gamma$ , surface expression of the degranulation marker CD107a, and release of intracellular granzyme B or perforin on KP9-specific CD8<sup>+</sup> T cells after parallel in vitro stimulation of whole blood for various times (0.5–5 h) with or without KP9 peptide.

To validate the assay, we studied a SIV Gag vaccinated *Mane-A\*10*<sup>+</sup> pigtail macaque 5 wk after SIV challenge, a time of high numbers of KP9-specific cells and T cell-mediated control of acute



**FIGURE 4.** Ex vivo levels of intracellular granzyme B. The immediate ( $t = 0$  h) levels of ex vivo granzyme B in KP9/tetramer-positive CD8<sup>+</sup> T cells (CD8<sup>+</sup>Tet<sup>+</sup>) compared with the bulk CD8<sup>+</sup> T cell population (CD8<sup>+</sup>Tet<sup>-</sup>) after vaccination (week 18), early (week 23), and late (week 46) postchallenge (pc). SIV<sub>mac251</sub> = SIV macaque clone 251.

infection (Fig. 2). Following in vitro stimulation, we simultaneously studied the expression of IFN- $\gamma$ , the degranulation marker CD107a, and the release of granzyme B and perforin over a maximum of 5 h. Surface expression of the degranulation marker CD107a and loss of intracellular granzyme B and perforin were detected as early as 1 h after in vitro stimulation with the KP9 peptide (Fig. 2C). This detection indicates there was potential for rapid activation of cytolytic functions after cognate Ag stimulation in this setting. We performed additional experiments after only 0.5 h of incubation, which showed similar data to that obtained after 1-h incubation. We also performed an experiment with a 7-h incubation that showed similar data to a 5-h incubation (data not shown). We therefore focused future work on 1–5 h of ex vivo Ag exposure.

#### *Kinetics of expression of cytotoxicity markers on CTLs and prime-boost vaccination and SIV challenge*

We studied whether the rapidity of SIV-specific expression and release of cytotoxic markers was similar after both vaccination with the live vector VV/FPV regimen and after pathogenic SIV<sub>mac251</sub> challenge. We serially studied the simultaneous kinetics of Ag-driven IFN- $\gamma$ , granzyme B, and CD107a expression on KP9-specific CD8<sup>+</sup> T lymphocytes in fresh whole blood. The KP9-specific cells can be divided into eight separate populations depending on the combinations of IFN- $\gamma$ , CD107a, and granzyme B expression the cells exhibit (Fig. 3A). Maximally functional cells up-regulate expression of IFN- $\gamma$  and CD107a and have lost granzyme B (Fig. 3A). Control unstimulated samples had no expression of IFN- $\gamma$  or CD107a on KP9-specific CD8<sup>+</sup> T cells after either vaccination or SIV challenge (data not shown).

After VV/FPV vaccination there was no expression of IFN- $\gamma$  or CD107a, or granzyme B release after 1 h of stimulation with KP9 peptide (Fig. 3A). By 3 h there was IFN- $\gamma$  expression but no CD107a expression. Only after 5 h was CD107a expression detected, and only in one of the two vaccinated animals. There was no accompanying granzyme B release, although levels of intracellular granzyme B were low at baseline.

In contrast, in fresh blood taken 5 wk after SIV challenge of the same animals, there was significant (10–15%) CD107a expression

and granzyme B release in the KP9-specific CD8<sup>+</sup> T cells within 1 h of KP9 Ag stimulation ex vivo. By 3–5 h after stimulation, 65–85% of cells expressed CD107a and/or had released granzyme B. Late after SIV challenge (28 wk), a similar pattern of early expression of CD107a and granzyme B release was observed in both animals, although the cytolytic phenotype of the cells was less marked than during acute infection (Fig. 3A).

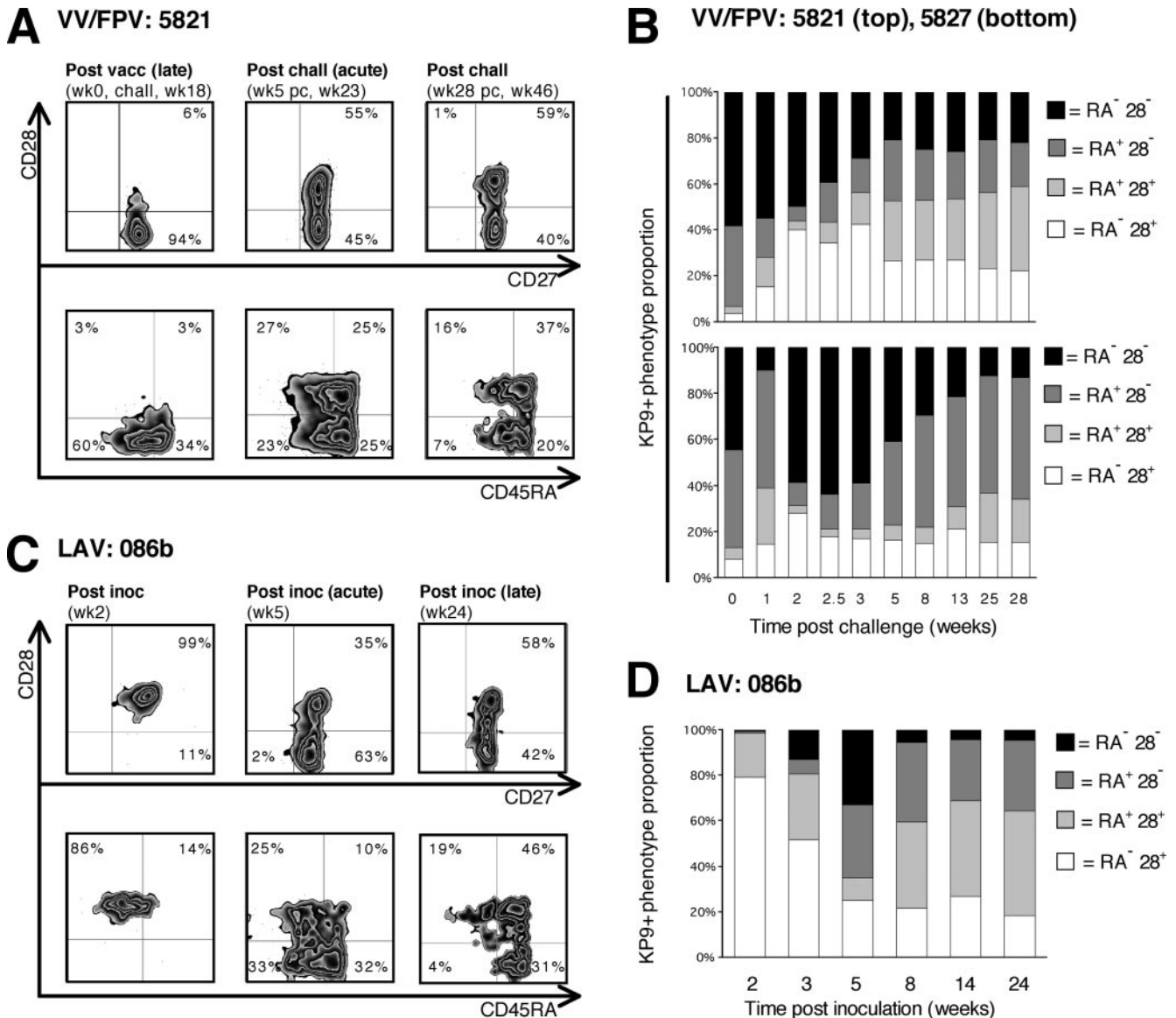
#### *Cytolytic phenotype kinetics after live-attenuated SIV inoculation*

The slow cytolytic phenotype kinetics after vector-based vaccination and rapid kinetics postchallenge coincident with control of viremia suggested that ongoing antigenic stimulation from infection could facilitate a “readiness” for rapid killing by CTLs. We therefore studied the cytolytic phenotype kinetics over time on serial fresh blood samples from animals inoculated with a live-attenuated SIV vaccine. The cytolytic phenotype kinetics in both animals was rapid, with expression of CD107a and release of granzyme B within 0.5–1 h (Fig. 3B and data not shown). Interestingly, there was only a much small proportion of KP9-specific CD8<sup>+</sup> T cells expressing IFN- $\gamma$  in the LAV-inoculated animals either 5 or 8 wk after inoculation (15% and 10% in the two animals at week 5, and 24% and 10% at week 8). In contrast, the prime-boost-vaccinated animals had much higher proportions of KP9-specific CD8<sup>+</sup> T cells expressing IFN- $\gamma$ , either postvaccination (61% and 62% in the two animals) or 5 wk postchallenge (73% and 25%) after in vitro stimulation. There is a clear difference in the cytolytic phenotype comparing vaccination with VV/FPV to LAV as further illustrated in Fig. 3C.

#### *Loading of granzyme B in tetramer-positive cells postvaccination and postchallenge with SIV*

As an indication of the killing capacity of the KP9-specific CD8<sup>+</sup> T cells, we first analyzed baseline levels of intracellular granzyme B without in vitro Ag stimulation. There was a striking difference in intracellular levels of granzyme B levels in ex vivo KP9-specific CD8<sup>+</sup> T cells in the same animals following either prime-boost vaccination or following SIV challenge (Fig. 4). In the two VV/FPV animals,  $\leq 1.5\%$  of the KP9-specific CD8<sup>+</sup> T cells expressed





**FIGURE 5.** The CD8<sup>+</sup> T cell memory phenotype. *A*, CD28/CD27 and CD28/CD45RA expression on tetramer-positive CD8<sup>+</sup> T cells changes over time in a VV/FPV-vaccinated animal (5821). *C*, The same profile on an animal (086b) inoculated with a live-attenuated SIV vaccine. *B* and *D*, Kinetics of the memory phenotype showing CD28/CD45RA expression after SIV<sub>mac251</sub> infection of VV/FPV-immunized animal 5821 and 5827 (*B*) and the same analysis for the animal 086b after live-attenuated SIV inoculation (*D*).

high levels of granzyme B after vaccination. Early after SIV challenge in the same animals, 75–80% of KP9-specific CD8<sup>+</sup> T cells had high levels of granzyme B. Later in the chronic state of infection the granzyme B levels of the KP9-specific CD8<sup>+</sup> T cells contracted to 35–40%. These findings, observed in fresh blood samples assayed at different time points were confirmed in frozen PBMC from separate time points, thawed, and stained on the same day (data not shown). A similar infection-induced loading of intracellular perforin within KP9-specific CD8<sup>+</sup> T cells occurred following SIV infection (data not shown). The two animals inoculated with a live-attenuated SIV vaccine showed high levels (70–80%) of KP9-specific T cells loaded with high levels of granzyme B at weeks early after inoculation (data not shown).

#### Memory phenotyping of KP9-specific CD8<sup>+</sup> T cells

The differing cytolytic phenotype kinetics of KP9-specific CD8<sup>+</sup> T cells before and after SIV infection suggested these cells may differ in their expression of memory and cell differ-

entiation markers. We therefore serially studied expression of CD28, CD27, and CD45RA over time on KP9-specific CD8<sup>+</sup> T cells before and after infection in the prime-boost animals and after live-attenuated SIV infection. The costimulatory molecules CD28 and CD27 have been shown to down-regulate in humans as cells differentiate (36), and are typically absent on late-differentiated memory T cells. CD45RA is expressed on naive cells, but can be re-expressed on memory cells in both humans and macaques (39). In combination with CD28, CD45RA expression can be used to further distinguish memory subsets (35). After prime-boost vaccination and challenge in animals 5821 and 5827, there was minimal loss of CD27 expression, although CD28 expression reduced over time after vaccination and was regained in a significant proportion of KP9-specific cells after challenge (Fig. 5, *A* and *B*, and data not shown). CD45RA/CD28 coexpression increased at later times after challenge in the tetramer-positive CD8<sup>+</sup> T cells, concurrent with a decrease in the CD45RA/CD28 double negative

population (Fig. 5B). After inoculation of the live-attenuated SIV-vaccinated animals the memory phenotype initially (week 2) showed high frequencies of CD28/CD27 double positive cells with low CD45RA expression, a phenotype clearly different to that observed at the same time point following challenge of the VV/FPV-vaccinated animals (Fig. 5, C and D). The later phenotypes in the LAV-inoculated animals show a similar proportion of CD45RA<sup>+</sup> cells in comparison to the late time points in the VV/FPV-challenged animals.

## Discussion

No vaccines have yet been able to induce broadly neutralizing HIV Abs. Designing vaccines that generate and maintain CD8<sup>+</sup> T cells that have the capacity to rapidly kill virus-infected cells will therefore be critical in preventing the massive memory CD4<sup>+</sup> T cell depletion that accompanies unchecked acute HIV infection and foreshadows eventual AIDS (22, 24). T cell immunity, particularly to Gag, is broadly linked to better control of HIV infection in humans and SIV infection in macaques (40, 41). A more accurate picture of the most desirable characteristics of specific CD8<sup>+</sup> T cells is emerging. CD8<sup>+</sup> T cells with the ability to secrete multiple cytokine and chemokines and degranulate effector molecules upon cognate Ag recognition are tightly correlated to control of HIV viremia in humans (13). A small window of opportunity exists to modulate acute HIV infection and prevent the massive loss of CD4<sup>+</sup> T cells (22). It has recently been shown that Gag is rapidly processed for T cell recognition (15) and that, together with the high fitness costs associated with escape at Gag (42, 43), probably underlies the utility of Gag-specific CD8<sup>+</sup> T cells in HIV infection (40).

We show in this study that prime-boost vaccination with recombinant poxviruses induces high levels of SIV-specific CD8<sup>+</sup> T cells in macaques that readily express the cytokine IFN- $\gamma$  but only slowly degranulate cytolytic effector molecules after cognate Ag recognition before SIV exposure. The KP9/Gag-specific CD8<sup>+</sup> T cells induced by prime-boost vaccination are poorly loaded with preformed intracellular granzyme B and perforin and therefore cannot rapidly release large amounts of these effector molecules. We confirmed these findings of poor loading of granzyme B and slow killing capacity of CTLs in stored PBMC samples from the VV/FPV-vaccinated animals at earlier time points (weeks 12–14) postvaccination (data not shown). Although it was not always possible to acquire a large number of KP9-specific cells at multiple time points from the small quantities of frozen PBMC available, we did detect slow cytotoxic phenotypes from animals receiving DNA and FPV prime-boost vaccinations as well as other animals receiving VV/FPV vaccinations (data not shown), confirming our data using fresh samples.

Upon acute SIV challenge of prime-boost-vaccinated animals, intracellular levels of granzyme B and perforin increase and these molecules are rapidly released *in vitro* with cognate Ag stimulation, along with expression of CD107a and IFN- $\gamma$ . The acquisition of this rapidly cytolytic phenotype of the KP9-specific cells is temporally linked to the resolution of viremia after acute infection in both vaccinated animals. However, a significant peak of viremia occurred after challenge (mean 5.4 log<sub>10</sub> copies/ml) that resulted in a transient depletion of half of all peripheral CD4<sup>+</sup> T cells. Based on other reports, there was likely a much larger depletion of central memory CD4<sup>+</sup> T cells in the gastrointestinal tract and probably effective seeding of latent reservoirs (22, 44). Latent HIV reservoirs do not undergo significant decay over time (45). More efficient vaccination strategies are needed to control acute levels of viremia.

A remarkable feature of live-attenuated SIV vaccine is the efficient control of acute SIV replication during the first few weeks after challenge in vaccinated animals compared with other vaccine modalities (10). Several potentially effective cellular immune responses have been identified in live-attenuated SIV-vaccinated animals and subjects with attenuated HIV-1 strains, such as the induction of SIV-specific CD4<sup>+</sup> T cells and generation of neutralizing Abs (10, 46, 47). Nonimmune mechanisms have also been postulated to facilitate the effectiveness of retrovirus in LAV vaccines (48, 49).

Although several potentially effective immune responses are induced by LAV, we found that live-attenuated SIV consistently results in low levels of CTLs, but the CTLs that are present have a much more rapid cytolytic phenotype. These findings accord with more effective control of acute SIV infection of macaques by SIV-based LAV vaccine compared with vector-based vaccines (10). Furthermore, other strategies that result in prolonged Ag exposure or replication, such as recombinant HSV (50, 51) or VV (52) expressing SIV or HIV-1 Ags may more closely mimic LAV. It would be highly informative to expand our findings reported in this study on larger cohorts of SIV-vaccinated macaques and HIV-vaccinated humans, directly comparing promising vaccine candidates with prolonged Ag expression.

There was an interesting difference in the levels of IFN- $\gamma$  expressed in KP9-specific CTLs in prime-boost-vaccinated animals compared with CTLs from the live-attenuated SIV-inoculated animals. IFN- $\gamma$  expression has become the most common method to detect T cell immunity in trials of HIV vaccines. Almost all KP9-specific CTLs from prime-boost-vaccinated animals expressed IFN- $\gamma$  after 5 h of culture both before and after SIV challenge. In contrast, a much more limited proportion of KP9-specific CTLs from the LAV-inoculated animals expressed IFN- $\gamma$ , despite almost all the CTLs expressing CD107a and releasing granzyme B. We confirmed this surprising result in standard ICS assays in which we did not simultaneously measure CD107a and granzyme expression (data not shown). These results suggest that killing capacity is not always mirrored by IFN- $\gamma$  expression. Assessing successful T cell-based vaccine strategies for the killing capacity of CTLs induced may provide important clues to the correlates of immunity.

There was a strong link between virus exposure (SIV<sub>mac251</sub> challenge or LAV inoculation) and the presence of a rapidly cytolytic phenotype in all four macaques, suggesting viral exposure and presence of viral Ag may drive this effector CTL phenotype. Betts et al. (53) have shown *in vitro* that peptide concentrations influence the ability of CD8<sup>+</sup> T cells to degranulate effector molecules and secrete cytokines. A clear future challenge is to assess whether inducing and maintaining a rapidly effective CTL phenotype with safer HIV vaccines is achievable and protective.

Effector CD8<sup>+</sup> T cells have been shown to degranulate more rapidly than effector or central memory cells in the mouse lymphocytic choriomeningitis virus model (54). Memory phenotypes in pigtail macaques have not previously been studied. Although limited by the low number of animals included, our longitudinal findings highlight a few characteristics of memory phenotypes in this emerging animal model. There is a dramatic difference in KP9-specific phenotypes between VV/FPV and LAV infection, and these phenotypes clearly change over time. The predominantly CD28<sup>-</sup> phenotypes that present early after challenge (weeks 0–2) in the VV/FPV-vaccinated animals contrast strongly with the predominantly CD28<sup>+</sup> populations in the LAV-inoculated animals at similar time points. Over time, the phenotypic differences between the VV/FPV and LAV animals decrease, with similar levels of CD28<sup>+</sup> phenotypes seen. Notably, CD27 expression appears unchanged over the course of vaccination/infection, unlike what has



been described in human infection (36). We speculate that the rapid degranulation observed 2 wk after LAV inoculation is associated with the CD45RA<sup>-</sup>/CD28<sup>+</sup> population that predominates at this time. In contrast, the CD45RA<sup>-</sup>/CD28<sup>+</sup> population is virtually undetectable in the VV/FPV vaccinated animals when the slow cytolysis profile is observed. Further studies in larger cohorts of animals using expanded combinations of phenotypic markers, such as CD95, are suggested by these studies (39).

In summary, we described the cytolytic phenotype kinetics of SIV-specific CTLs induced by prime-boost vaccination and live-attenuated virus inoculation. The functional characteristics of the CTLs were very different, with a much more rapid cytolytic phenotype, and less IFN- $\gamma$  expression, in LAV inoculated animals than prime-boost immunized animals. These differences may have an important influence on the rapid control of acute infection upon virus exposure and therefore the design and evaluation of HIV vaccines.

## Acknowledgments

We thank R. De Rose, S. Alcantara, J. Stephany, J. Stewart, and J. Lin for excellent technical assistance and D. Boyle and B. Coupar for assistance with the FPV vaccines.

## Disclosures

The authors have no financial conflict of interest.

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