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Mucosal HIV-1 Pox Virus Prime-Boost Immunization Induces High-Avidity CD8+ T Cells with Regime-Dependent Cytokine/Granzyme B Profiles

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The quality of virus-specific CD8+ CTL immune responses generated by mucosal and systemic poxvirus prime-boost vaccines were evaluated in terms of T cell avidity and single-cell analysis of effector gene expression. Intranasal (I.N.) immunization regimes generated higher avidity CTL responses specific for HIV KβGag197–205 (amino acid sequence AMQMLKETI; H-2Kb binding) compared with i.m. immunization regime. Single-cell RT-PCR of KβGag197–205-specific mucosal and systemic CTL revealed that the cytokine and granzyme B expression profiles were dependent on both the route and time after immunization. The I.N./i.m.-immunized group elicited elevated number of CTL-expressing granzyme B mRNA from the genitomucosal sites compared with the i.m./i.m. regime. Interestingly, CTL generated after both I.N. or i.m. immunization demonstrated expression of Th2 cytokine IL-4 mRNA that was constitutively expressed over time, although lower numbers were observed after I.N./i.m. immunization. Results suggest that after immunization, Ag-specific CTL expression of IL-4 may be an inherent property of the highly evolved poxvirus vectors. Current observations indicate that the quality of CTL immunity generated after immunization can be influenced by the inherent property of vaccine vectors and route of vaccine delivery. A greater understanding of these factors will be crucial for the development of effective vaccines in the future. The Journal of Immunology, 2007, 178: 2370–2379.

Virus-specific CD8+ CTL play a pivotal role in the control of many acute and chronic viral infections (1–4) including HIV-1 (5–7). Depletion of CD8+ lymphocytes from rhesus macaques during chronic SIV infection can result in a rapid increase in viremia, which can subsequently be controlled by reintroduction of SIV-specific CD8+ T cells (8). The genitoreal mucosa is the primary site of HIV-1 viral entry and predominant site of HIV-1 replication and CD4+ T cell depletion (9). Indeed, significant CD4+ T cell depletion occurs at the mucosa well before that observed in the peripheral circulation. Therefore, vaccines capable of inducing HIV-specific CTL in local mucosa could better control viral replication within local tissue and perhaps prevent systemic dissemination of the virus. Studies have demonstrated that mucosal CTL can control a mucosal viral challenge, whereas systemic CTL are ineffective against mucosal challenge (10).

Currently, a range of vaccine strategies has been tested in various animal models for HIV-1. Many vaccines strategies that co-express molecules to enhance T cell responses to HIV Ags have also been tested (11–14). Although the majority of these vaccines elicit CTL responses, neither initial virus growth nor the establishment of chronic infection is prevented, but a reduction in chronic viral load has been observed (7, 15–19). It is now evident that the route of vaccine delivery plays an important role in the induction of T cell immunity (20). However, there is little information available on the qualitative differences between CTL responses generated by these different vaccines, or delivery routes in terms of CTL avidity or cytokine/granzyme profiles. Although low-avidity CTL are incapable of effector function at low concentrations of Ag, high-avidity CTL are capable of recognizing low concentrations of Ag/peptide and possess an increased functional ability (21, 22). A compelling body of evidence suggest that high-avidity CTL are more effective in viral or tumor clearance compared with low-avidity T cells (23–25). Recently, Belyakov et al. (7) have shown that a mucosal peptide prime/poxvirus boost-immunization regime can generate high-avidity mucosal CTL that are more capable of controlling systemic dissemination of virus in rhesus macaques compared with systemic vaccination. Thus, a much greater understanding of how route of immunization can influence correlation between TCR avidity and protection against HIV-1 will enable the development of better vaccines in the future.

In this study, to determine the quality of immune responses generated by the poxvirus prime-boost vaccines, we have used several poxvirus prime-boost (AE fowlpox virus (FPV)/AE vaccinia virus (VV)) vaccine strategies and compared the levels of mucosal and systemic HIV-specific CD8+ T cell responses generated at early and late stages of secondary response in terms of their T cell avidity. We have also characterized regime-specific cytokine and granzyme B expression profiles of Gag KβGag197–205-positive T cells at a single-cell level to 1) unravel the nature of these T cells and 2) possibly identify traits that are unique to mucosal vaccination.

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2 Address correspondence and reprint requests to Dr. Charani Ranasinghe, Division of Immunology and Genetics, John Curtin School of Medical Research, Australian National University, P.O. Box 334, 2601 Canberra, Australia. E-mail address: Charani.Ranasinghe@anu.edu.au

3 Abbreviations used in this paper: FPV, fowlpox virus; VV, vaccinia virus; I.N., intranasal; DEPC, diethyl pyrocarbonate; CT, cycle threshold.

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In parallel, we have also evaluated the cytokine profiles generated in the total CD8+ T cell population by RT-PCR and FACS analysis.

Materials and Methods
Recombinant poxvirus vaccines
The AE FPV contained modified AE clade gag, pol, env, rev, and tat genes, and AE VV contained modified gag and pol genes as described elsewhere (26–28).

Immunization of mice
Pathogen-free 5- to 6-wk-old female BALB/c (H-2d) mice were obtained from the Animal Breeding Establishment, John Curtin School of Medical Research (JCSMR). All animals were maintained and used in accordance with animal ethics guidelines. Mice (n = 4 or 5) were primed and boosted with 1 × 107 PFU rVV-expressing AE clade HIV-1 Ags (under mild methoxyfluorane anesthesia) 2 wk apart using different immunization regimens: 1) intranasal (I.N./i.m., purely mucosal; 2) I.N./I.N., purely mucosal; and 3) i.m./i.m., purely systemic. The I.N. rFPV or rVV was given in a final volume of 10–25 l, where i.m. immunization was delivered in a 100 l volume. Before each immunization, the rFPV or rVV was diluted in PBS and sonicated 30–40 s to obtain a homogeneous viral suspension. Note that most of these experiments were repeated at least three times.

Sample collections and preparation of lymphocytes
To measure systemic and mucosal T cell responses, mice were sacrificed at different time intervals (3 days to 13 wk) postbooster immunization, spleen and genitourinary lymph nodes (iliac lymph nodes) were removed, and cell suspensions were prepared in complete RPMI 1640.

IFN-γ ELISPOT assay
HIV-specific T cell responses were measured by IFN-γ capture ELISPOT assay as described elsewhere (20). The cells were stimulated for 20–24 h in the presence of HIV-specific 15-mer overlapping Gag peptide pool supplied by the National Institutes of Health AIDS Research and Reference Reagent Program or the immunodominant H-2Kd binding AMQMLKETI, 9-mer Gag peptide (synthesized at the Bio-Molecular Resource Facility at JCSMR, Canberra, Australia) (29, 30). In these assays, Con A (Sigma-Aldrich) was used as the positive control and unstimulated cells as negative controls. The spot-forming units were counted using an ELISpot BioReader-4000 (Biosys). Results are expressed as 1 × 105 T cells and represent the average of the duplicate or triplicate value. Unstimulated cell counts were subtracted from each sample set before plotting the data.

Tetramer staining and single-cell sorting
Allophycocyanin-conjugated KβGag97–205 tetramers were synthesized at the Bio-Molecular Resource Facility at JCSMR. The tetramer staining was performed as described previously (20). Briefly, 2–5 × 106 splenocytes or genitourinary lymphocytes were stained with anti-CD8 FITC Ab (BD Pharmingen) and allophycocyanin-conjugated KβGag97–205 tetramer. Splenic and genitourinary lymph node-derived lymphocytes from unimmunized animals were used as background controls.

Single-cell cDNA synthesis and single-cell RT-PCR
The 96-well plates containing the single cells were kept on ice, and 5 µl of cDNA buffer (Sensiscript RT kit; Qiagen) containing 0.5 mM dNTP (Promega), 125 ng of oligo(dT) (Promega), 0.5 nM sperridine (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), 100 µg/ml tRNA, and 0.125 µl of Sensiscript Reverse Transcriptase (Sensiscript RT kit; Qiagen) was added immediately to each cell, and the plates were centrifuged at 2500 rpm for 3 min and incubated at 37°C for 110 min to synthesize the cDNA and stored at −20°C until use (31, 32).

Nested PCR was performed using HotStar Taq Master mix (Qiagen) with 5 pmol of forward and reverse cytokine primers indicated in Table I. The L32 ribosomal protein mRNA (housekeeping mRNA) was used as the positive control to monitor the presence of a cell in each well and the quality of cDNA synthesis. Appropriate positive controls for primes and negative controls were also used at all times. The PCR 1 and PCR 2 were performed, one cycle of 95°C for 15 min, to activate the HotStar Taq (Qiagen), followed by 35 cycles for 95°C 20 s, 55°C 20 s, and 72°C 30 s.

Table I. Primers used in single-cell-nested PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense Primer Sequence 5’–3’</th>
<th>Antisense Primer Sequence 5’–3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ FWD1/REV1</td>
<td>A7GAGA6GCACTGAGACCTGACCAC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IFN-γ FWD2/REV2</td>
<td>CTGTGA6GCACTGAGACCTGACCAC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
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<tr>
<td>TNF-α FWD1/REV1</td>
<td>AGCA6GCACTGAGACCTGACCACAC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>TNF-α FWD2/REV2</td>
<td>GTCA6GCACTGAGACCTGACCACAC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IL-2 FWD1/REV1</td>
<td>CCTATAC6GCACTGAGACCTGACCAC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IL-2 FWD2/REV2</td>
<td>GTCA6GCACTGAGACCTGACCACCA</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IL-4 FWD1/REV1</td>
<td>GCACTCGAC6GCACTGAGACCTGACC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
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<tr>
<td>IL-4 FWD2/REV2</td>
<td>C6GCACTCGAC6GCACTGAGACCTG</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IL-10 FWD1/REV1</td>
<td>GCT6GCACTCGAC6GCACTGAGACCT</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IL-10 FWD2/REV2</td>
<td>GCT6GCACTCGAC6GCACTGAGACCT</td>
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<tr>
<td>IL-13 FWD2/REV2</td>
<td>GCT6GCACTCGAC6GCACTGAGACCT</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
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<tr>
<td>Gran B FWD1/REV1</td>
<td>CGACTCGAC6GCACTGAGACCTGACC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>Gran B FWD2/REV2</td>
<td>CGACTCGAC6GCACTGAGACCTGACC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
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<tr>
<td>L32 FWD1/REV1*</td>
<td>6GCACTCGAC6GCACTGAGACCTGAC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>L32 FWD2/REV1*</td>
<td>CGACTCGAC6GCACTGAGACCTGAC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
</tbody>
</table>

*, L32 REV1 was reused in the second round. FWD, Forward; REV, reverse; Gran, granzyme.

Table II. Primers used in real-time PCR

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Sense Primer Sequence 5’–3’</th>
<th>Antisense Primer Sequence 5’–3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>CCTGAGACGACAGTCAGAGATTTAC</td>
<td>TCCAGACGAGCTGACCTTTTCC</td>
</tr>
<tr>
<td>IL-4</td>
<td>GG6GCACTCGAC6GCACTGAGACCTG</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IL-6</td>
<td>AGAG6GCACTCGAC6GCACTGAGACCT</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IL-10</td>
<td>AG6GCACTCGAC6GCACTGAGACCTG</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IL-13</td>
<td>GCAACTAC6GCACTGAGACCTGACCAC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IL-18</td>
<td>GACTCGAC6GCACTGAGACCTGAC</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>ATGAGACGACAGTCAGAGATTTAC</td>
<td>TCCAGACGAGCTGACCTTTTCC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CAT6GCACTCGAC6GCACTGAGACCT</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
<tr>
<td>L3-2</td>
<td>CT6GCACTCGAC6GCACTGAGACCTG</td>
<td>TCA6GACGAGCTGACTTTTCC</td>
</tr>
</tbody>
</table>
Tetramer dissociation assay

The dissociation assay was performed as described elsewhere (22). Briefly, $2 \times 10^6$ cells from each sample were aliquoted into a round-bottom 96-well plate and were stained with FITC-CD8 and allophycocyanin-Gag KdGag197–205 as described previously. The plate was configured to have six time points per sample (0–60 min). A total of $50 \mu g/ml$ H-2Kd-competitive Ab (BD Pharmingen) was added to each well to prevent tetramer rebinding, and plates were incubated at 37°C with 5% CO2. At each time point, aliquots were transferred to ice-cold FACS buffer to stop the reaction, washed, and resuspended in 100 l of FACS buffer containing 0.5% paraformaldehyde. These samples were analyzed on a flow cytometer.

FIGURE 1. Immunization regime-specific systemic T cell responses. Two weeks after poxvirus prime-boost, splenocytes from BALB/c (H-2d) ($n = 4$ mice/group) were stimulated with 9-mer Gag peptide as described in Materials and Methods and T cell responses were measured by IFN-γ ELISPOT (A). The unstimulated cells from each sample were used as the background control, and this value was subtracted from each sample before plotting the data. The data represent mean ± SD of three experiments. Responses between L.N./I.N.–I.N./i.m. (*) and L.N./I.N.–i.m./i.m. (**) were significantly different; $p < 0.05$. At 14 days prime-boost, the percentage of KdGag197–205-specific CD8+ T cell response was measured in spleen (B). The y-axis represents the tetramer-positive cells per 106 total T cells. The unimmunized animals were used as background controls. The data represent mean ± SE. Immunization regime-specific systemic and mucosal CD8+ T cell responses were measured over time (C). The percentage of KdGag197–205-positive CD8+ T cells measured in spleen (black lines) and genitorectal nodes (gray lines) at 3, 7, and 14 days after prime-boost immunization. These data represent a pooled value and are representative of three experiments.

FIGURE 2. Immunization route and T cell avidity. BALB/c (H-2d) mice ($n = 4$/group) were immunized I.N./I.N. (gray line), I.N./i.m. (black dotted line), and i.m./i.m. (black line), and at 7 (A) and 14 days (B) after AE VV boost the percentage of KdGag197–205-positive CD8+ splenocyte loss (dissociation) was measured as described in Materials and Methods. The day 7 data represent a pooled value and are representative of three experiments. The day 14 data represent individual animals with mean ± SD. I.N./i.m.–i.m./i.m. (*, $p < 0.005$) and L.N./I.N.–i.m./i.m. (**, $p < 0.001$) that are highly significant. These experiments were repeated over three times.

Tetramer dissociation assay

The dissociation assay was performed as described elsewhere (22). Briefly, $2 \times 10^6$ cells from each sample were aliquoted into a round-bottom 96-well plate and were stained with FITC-CD8 and allophycocyanin-Gag KdGag197–205 as described previously. The plate was configured to have six time points per sample (0–60 min). A total of $50 \mu g/ml$ H-2Kd-competitive Ab (BD Pharmingen) was added to each well to prevent tetramer rebinding, and plates were incubated at 37°C with 5% CO2. At each time point, aliquots were transferred to ice-cold FACS buffer to stop the reaction, washed, and resuspended in 100 μl of FACS buffer containing 0.5% paraformaldehyde. These samples were analyzed on a flow cytometer.

FIGURE 3. I.N./i.m. regime-specific KdGag197–205-positive spleen and genitorectal node sort profiles. At 14 days following AE VV booster immunization, KdGag197–205-specific spleen (gate R1 = 9.89%), genitorectal (gate R3 = 5.89%) lymph nodes, and KdGag197–205-negative spleen (gate R2), genitorectal (gate R4) lymph nodes were single-cell sorted into Eppendorf 96-well plates, snap frozen immediately to prevent RNA degradation, and stored at −80°C until RT-PCR. Note that first, the single-cell precision was confirmed by sorting single microbeads onto a glass slide and confirming by microscopy, and also sorting the KdGag197–205-specific cells a second time to ensure purity of the cells. (The percentage of KdGag197–205-positive cells is represented as a percentage of total CD8+ T cells).
FACSCalibur flow cytometer (BD Biosciences) using CellQuest Pro analysis software (BD Biosciences).

**mRNA extractions from primary CD8\(^+\) T cells**

CD8\(^+\) T cells were isolated from unimmunized, I.N./i.m., and i.m./i.m. immunized mice using the Dynal negative isolation kit (Dynal Biotech) according to the manufacturer’s instructions. Fifty percent of selected cells were stimulated with 15-mer Gag peptide pool for 16–20 h, while others were kept untreated for the same length of time. From these cells mRNA was extracted using Tri-Reagent (Sigma-Aldrich), and cell pellets were resuspended in a final volume of 20 \(\mu\)l diethyl pyrocarbonate (DEPC)-treated water (Ambion). To each sample, 2U of DNaseI (Roche) and first-strand buffer (Invitrogen Life Technologies) was added and incubated at 37°C for 30 min to degrade any contaminating DNA followed by incubation for 5 min at 75°C to inactivate DNaseI. The concentration of total mRNA was measured using the Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop), and samples were aliquoted and stored at −70°C until required.

**cDNA syntheses from primary CD8\(^+\) T cells**

From each sample 1 \(\mu\)g mRNA was converted into cDNA. To each sample, 2 \(\mu\)l of master mix containing 10 mM dNTPs and 100 \(\mu\)M oligo(dT)s were added, and each sample was made up to a final volume of 15 \(\mu\)l using DEPC-treated H\(_2\)O and incubated at 37°C for 30 min to degrade any contaminating DNA followed by incubation for 5 min at 75°C to inactivate DNaseI. The concentration of total mRNA was measured using the Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop), and samples were aliquoted and stored at −70°C until required.

**Real-time PCR**

Real-time PCR master mix contained 5 pmols of forward and reverse primers (Proligo; Table II), 10 \(\mu\)l of 2\(\times\) SYBR Green (Applied Biosystems), and DEPC-treated H\(_2\)O to a final volume of 20 \(\mu\)l per reaction. A total of 50 ng of cDNA (in a 2 \(\mu\)l volume) from each sample was aliquoted into a Thermo-Fast 96-well detection plate (ABgene), and 18 \(\mu\)l of master mix was added to each well. Real-time PCR was performed as cycle of 50°C, 2 min, 95°C, 10 min followed by 40 cycles of 95°C, 15 s, 60°C, 1 min, using an ABI Prism 7700 Sequence Detection System (PerkinElmer/Applied Biosystems). All reactions were performed in duplicate, and to ensure that single products were obtained after each reaction the melting curves of the primers were also tested by dissociation runs.

The amplification plots obtained for each sample were analyzed using 7700 software, and the corresponding dissociation curves were checked to ensure that each primer pair only yielded one product with one melting point. The fold change in mRNA expression was calculated using the comparative cycle threshold (CT) method using the following formula: fold change = \(2^{-\Delta\Delta CT}\), where 2 represents a constant reaction efficiency and \(\Delta\Delta CT\) is defined as (CT\(_{target} - CT_{reference}\))stimulated – (CT\(_{target} - CT_{reference}\))unstimulated. The reference value is equivalent to the CT value of L32 ribosomal protein mRNA, which was the housekeeping mRNA.

**Intracellular cytokine staining**

A total of 1–2 \(\times\) 10\(^6\) lymphocytes was stimulated overnight in the presence of immunodominant H-2K\(^b\) binding AMQMLKETI, 9-mer Gag peptide...
and then for a further 4–5 h in the presence of 2 μM monensin, and stained as described previously (20). After stimulation, cells were surface stained with anti-CD8 allophycocyanin or FITC and with anti-CD4 PerCP (BD Pharmingen). These cells were fixed and permeabilized before staining with anti-mouse IFN-γ, FITC, TNF-α PE, IL-2 allophycocyanin or IL-10 PE (BD Pharmingen). Samples were analyzed using a four-color FACSCalibur flow cytometer (BD Biosciences), and results were analyzed using CellQuest Pro software (BD Biosciences). Unstimulated cell counts were used as the background control, and where appropriate these values were subtracted from each sample before plotting the data (see Fig. 5).

Statistics and analysis of data
Where appropriate, SE or SD was calculated and p values were determined using a two-tailed, two sample equal variance Student’s t test.

Results
Evaluating CTL responses generated by mucosal and systemic immunization regimes

The KdGag197–205-specific CTL response was compared following AE FPV prime AE VV boost-immunized mice (n = 4–5) generated by either systemic (i.m./i.m.), mucosal (I.N./I.N.), and combination mucosal and systemic (I.N./i.m.) immunization regimes 14 days following the booster immunization (20). Intramuscular/i.m. immunization regimes induced the highest number of KdGag197–205-positive single splenic and genitorectal lymph nodes, with the greatest numbers being induced at days 7 and 14 with I.N./i.m. immunization (Fig. 1B). As previously described (20), i.m./i.m. immunization appeared to have peaked at day 3 with a decline in the proportion of KdGag197–205-specific CTL by day 14 after immunization.

Evaluating the avidity of CD8+ KdGag197–205-specific CTL responses generated after mucosal or systemic immunization regimes

First, to evaluate the avidity of KdGag197–205-specific CTL generated after either mucosal or systemic immunization, splenocytes from I.N./I.N., I.N./i.m., and i.m./i.m.-immunized mice were used in a tetramer dissociation assay as described previously (22). Intramuscular/i.m. immunization resulted in a KdGag197–205-specific CTL population that demonstrated a faster tetramer dissociation rate (i.e., lower avidity) compared with similar CTL populations obtained from either I.N./I.N. or I.N./i.m. immunization regimes (Fig. 2A). By day 14 after immunization, KdGag197–205-specific CTL induced after i.m./i.m. immunization regimen recorded the fastest tetramer dissociation, I.N./i.m. immunization regime elicited intermediary levels of tetramer dissociation, and I.N./I.N. immunization demonstrated the slowest tetramer dissociation (Fig. 2B). These data clearly demonstrate that mucosal immunization can generate HIV-specific CTL of higher avidity than the i.m./i.m. immunization regime (I.N./I.N., i.m./i.m; p < 0.001).

Characterization of cytokine profiles of KdGag197–205-positive genitourinary lymph node and splenic CD8+ T cells

To assess whether the tetramer-positive cells from the I.N./i.m. and i.m./i.m. immunization strategies were functionally different, we compared the mRNA expression of ex vivo-derived splenic and genitourinary lymph node KdGag197–205-specific CTL (Fig. 3) by single-cell multiplex RT-PCR. We examined these cells at days 3, 7, and 14 time points after AE VV booster immunization. The expression of IFN-γ, TNF-α, IL-2, IL-4, and IL-13 mRNA for each single CTL was assayed using a nested PCR strategy (Table I).

At 3 days after prime-boost immunization or early stage of secondary response, KdGag197–205-specific splenic CTL from the I.N./i.m. group elicited a cytokine expression profile IL-4 > IFN-γ > TNF-α > IL-13 > IL-10 (Fig. 4A), whereas the i.m./i.m. regime showed >30% of the cells expressing the cytokine IFN-γ, with a profile of IFN-γ > IL-4 > TNF-α > IL-10 and no IL-13 (Fig. 4B). At day 3 after AE VV boost, a greater proportion
served in a small proportion of K\textsuperscript{Gag\_197–205}\textsuperscript{specific} CTL derived from the I.N./i.m. immunization (Fig. 4, compare A and B).

By day 7 after secondary boost, similar frequencies of IFN-\(\gamma\)\textsuperscript{+} K\textsuperscript{Gag\_197–205}\textsuperscript{specific} CTL were found after i.m./i.m. (Fig. 4C) and I.N./i.m. immunization (Fig. 4D) for both splenic and genitorrectal lymph node populations. In contrast, the number of splenic and genitorrectal lymph node-derived CTL producing TNF-\(\alpha\) was significantly less after i.m./i.m. immunization compared with the I.N./i.m.-immunized group (Fig. 4, compare C and D). The hierarchy of mRNA expression within K\textsuperscript{Gag\_197–205}\textsuperscript{specific} CTL from I.N./i.m. immunization was IFN-\(\gamma\) > IL-4 \(\geq\) TNF-\(\alpha\) > IL-10 > IL-13. There was an increase in the frequency of IL-13\textsuperscript{+} CTL at day 7 compared with day 3 after i.m./i.m. immunization (Fig. 4, compare B and D).

At day 14, similar proportions of K\textsuperscript{Gag\_197–205}\textsuperscript{specific} CTL were IFN-\(\gamma\)\textsuperscript{+} (>80%) for all three immunization regimes. The frequency of TNF-\(\alpha\)\textsuperscript{+} CTL decreased to <1% (compared with >10% at day 7) in the I.N./i.m.-immunized mice with little change in the frequency of TNF-\(\alpha\)\textsuperscript{+} CTL from the i.m./i.m. regime (Fig. 4E). Interestingly, 14 days after booster immunization major differences were observed in the IL-10 mRNA expression in K\textsuperscript{Gag\_197–205} splenocytes obtained from the I.N./i.m. group (Fig. 4E), showing an increase in IL-10-producing cells to 45%, compared to 7 days <10% (Fig. 4C) or to the other two immunization regimes (Fig. 4F). Expression of low amounts of IL-10 by CD8\textsuperscript{+} T cells after I.N./i.m. poxviral prime boosting, but not i.m./i.m. regime, was also confirmed by intracellular cytokine staining and FACS analysis. IL-2 mRNA was not detected in K\textsuperscript{Gag\_197–205}\textsuperscript{specific} CTL populations from any of the immunized groups.

Moreover, the expression profile of IL-4 did not alter significantly over time (prime only, 3, 7, or 14 days) in splenocytes and lymphocytes obtained from the I.N./i.m. and i.m./i.m. groups. In contrast, expression of IL-4 in the I.N./I.N. group was relatively low compared with the other two regimes. Data indicate that the poxviral prime-boost immunization can generate tetramer-positive as well as tetramer-negative CD8\textsuperscript{+} T cells that are able to constitutively express IL-4 mRNA. Whether this expression relates to protein production is yet to be determined.

The heterogeneity of cytokine mRNA expression was determined within single K\textsuperscript{Gag\_197–205}\textsuperscript{specific} CTL was evaluated for all immunization regimes at the various time points after AEVV boosting (Fig. 5). The I.N./i.m. group showed increased frequency of

### FIGURE 6

Granzyme B profile in tetramer-specific single T cells. BALB/c mice (4–5/group) were immunized I.N./i.m. (A and B) and i.m./i.m. (A). At 7 (A) and 84 (B) days prime-boost immunization, K\textsuperscript{Gag\_197–205}\textsuperscript{positive} cells (■) and dimer-negative (III) CD8\textsuperscript{+} spleen and genitorrectal node cells were single-cell sorted into 96 wells, and cDNA was synthesized as described in Materials and Methods. In each cell, the presence of IFN-\(\gamma\), IL-4, and granzyme B mRNA transcripts were evaluated using nested PCR (total of 48 cells). Graph (A) represents I.N./i.m. (■), i.m./i.m. (■), and tetramer-negative cells obtained from I.N./i.m. (III). Graph (B) represents I.N./i.m. tetramer-positive splenocytes (■) and genitorrectal nodes (□) at 84 days after boost.

### FIGURE 7

Real-time PCR of total CD8\textsuperscript{+} T cells obtained from unimmunized, i.m./i.m., i.m./I.N., and I.N./I.N.-immunized mice. Fourteen days after AE poxvirus prime-boost immunization (n = 4–5 mice/group), negatively isolated CD8\textsuperscript{+} splenocytes were stimulated with 15-mer AE clade overlapping Gag peptide pool for 16–20 h and mRNA levels were measured by real-time PCR as indicated in Materials and Methods. The graphs represent the fold increases of IFN-\(\gamma\) (A), TNF-\(\alpha\) (B), and IL-2 (C) in stimulated compared with unstimulated cells. Data are representative of three experiments.
Assessment of granzyme B profile in K dGag197–205-specific groups compared with the I.N./I.N. group.

Multiple mRNAs in CTL derived from the I.N./i.m. and i.m./i.m. earlier time points, with a higher frequency of CTL expressing multiple cytokine mRNAs by day 14 compared with was an increase in the frequency of KdGag197–205-specific CTL genitorectal node populations than the splenocytes (Fig. 5). There were an increase in the frequency of IFN-γ/TNF-α, IFN-γ/IL-10, TNF-α/IL-4, and IL-10/IL-4. These differences were more prominent in the genitoreal node populations than the splenocytes (Fig. 5). There was an increase in the frequency of K dGag197–205-specific CTL expressing multiple cytokine mRNAs by day 14 compared with earlier time points, with a higher frequency of CTL expressing multiple mRNAs in CTL derived from the I.N./i.m. and i.m./i.m. groups compared with the I.N./I.N. group.

Assessment of granzyme B profile in K d Gag197–205-specific systemic and mucosal-immunized CTL

The expression of granzyme B mRNA was assessed for K d Gag197–205-specific CTL at 7 and 84 days following I.N./i.m. and i.m./i.m. prime-boost immunizations (Fig. 6). The number of tetramer-positive cells producing granzyme B mRNA in the genitoreal lymphocytes from the I.N./i.m.-immunized animals was much higher (71%) than that found in splenocytes (38%) (Fig. 6). In contrast, i.m./i.m. immunization elicited higher granzyme B production in splenocytes (58%) than genitorectal lymphocytes (29%) (Fig. 6). The tetramer-negative population from the I.N./i.m.-immunized animals also showed a small proportion of cells producing granzyme B, where the expression was higher in mucosal than splenocytes (18 vs 6%) (Fig. 6A), probably reflect activated CTL with other specificities (i.e., to the vector).

Quantitation of regime-specific cytokine mRNA profiles in CD8+ T cells by real-time PCR

At 14 days following prime-boosting IL-2, IL-4, IL-6, IL-10, IL-13, IL-18, IFN-γ, and TNF-α, mRNA expression patterns were also measured in the “total HIV-specific CD8+ T cell population” after in vitro stimulation for 16–20 h with AMQMLKETI Gag peptide and cytokines IFN-γ and TNF-α were measured by intracellular staining. The FACS plots represent CD8+ T cells (gated on CD8+ T cells) expressing IFN-γ (A) and TNF-α (B). The values in the upper right quadrants indicate the percentage of CD8+ T cells producing IFN-γ or TNF-α, where corresponding unstimulated background value was subtracted. Note that only the i.m./i.m.-unstimulated group is represented to show the general background poststimulation. The y-axis indicate the CD8-allophycocyanin channel and x-axis the IFN-γ-FITC or TNF-α-PE channels. Data are representative of >3 experiments.

However, both I.N/i.m. and the purely systemic regimes showed a hierarchy of expression IFN-γ > TNF-α > IL-2, and the purely mucosal regime showed a different mRNA expression profile of IFN-γ > IL-2 > TNF-α (Fig. 7).

Identification of regime-specific cytokine profiles in T cells by intracellular cytokine staining

To determine whether the mRNA expression also correlated with the protein expression profiles, intracellular cytokine staining was performed as described in Materials and Methods. The data indicate that at 14 days, the IFN-γ production in stimulated CD8+ splenocytes was regime specific, showing i.m./i.m. ≥ I.N/i.m. > I.N/i.N. (Fig. 8A) expression profile after 16 h of stimulation with AMQMLKETI Gag peptide. Although the number of cells producing both TNF-α was higher in the I.N/i.m.-immunized group, no significant differences were observed between the other two groups (Fig. 8B). However, after overnight culture, a high background expression of TNF-α in both stimulated and nonstimulated cells was visible compared with the unimmunized group (Fig. 8B).

Similar observations were made with TNF-α ELISPOT assays (C. Ranasinghe, unpublished observations), suggesting that the poxvirus regime enhances the overall TNF-α production in these T cells. Interestingly, none of these groups showed CD8+ T cells producing the cytokine IL-2 after overnight stimulation with peptide. This observation was further substantiated by IL-2 ELISPOT assay, which showed extremely low numbers of T cells producing IL-2 upon stimulation with the same peptide pool (C. Ranasinghe, unpublished observations).

Discussion

Recently, several studies have indicated that poxvirus prime-boost immunization strategies can generate robust T cell immunity to vaccine Ags in animal models (2, 33). Our recent findings indicate that of the mucosal and systemic immunization regimes tested, the I.N. AE FPV prime i.m. AE VV boosting can generate the highest mucosal and systemic CTL immune responses in BALB/c mice (20). In this study, we have observed that in I.N/i.m. and i.m./i.m.-immunized animals, the level of K d Gag197–205-specific systemic T cells peaked at 14 days after the boost, although Ag-specific iliac lymph node CTL responses in the I.N/i.m. and i.m./i.m.-immunized groups considerably differed over time. These
produced, single-cell cytokine profiling of these CD8+ immunization regimes had any correlation with the cytokines they test whether the differences in T cell avidity observed in these high-avidity CTL. Limited Ag dose at the priming site resulted in the selection of distinct functional avidities and viral clearance abilities (21, 34). Moreover, recently it has been documented that anatomical location (i.e., spleen vs mucosa) can play an inductive role in memory T cell differentiation (35). We postulate that the differences in T cell avidity observed could be linked to the 1) mode of Ag uptake and presentation by nasopharynx-associated lymphoid tissue via M (microfold) cells and dendritic cells, 2) initial Ag strength at the priming site, and 3) milieu they induce (34, 36). In summary, the limited Ag dose at the priming site resulted in the selection of high-avidity CTL.

It has been shown that one of the best evaluation methods of recently activated T cells is the detection of cytokines (37). Hence, to test whether the differences in T cell avidity observed in these immunization regimes had any correlation with the cytokines they produced, single-cell cytokine profiling of these CD8+ T cells were performed. Early after I.N./i.m. immunization, IL-4 mRNA was more prevalent compared with IFN-γ mRNA in Ag-specific CTL, and another type 2 cytokine, IL-13, was also detected. In humans, IL-4 and IL-13 genes are located in tandem within a cluster of cytokine genes on the same chromosome (38). Hence, these genes could be regulated by similar transcription factors or common distal promoters, which could explain the detection of both of these Th2 cytokines in HIV-specific tetramer-positive CD8+ T cells (39). The results also indicated that the cytokine profiles were regime and time dependent, and the IFN-γ production in these cells may be controlled by the interplay of IL-4/IL-13 and IL-2 expression. At 14 days, tetramer-positive T cells from the I.N./LN.-immunized regime expressed low levels of IL-4 and these cells were found to be higher in avidity, although the I.N./i.m. and i.m./i.m.-immunized groups were found to constitutively express IL-4 mRNA (≤20%) and generated lower avidity T cells compared with the I.N./LN. group. These observations suggest that the Th2 cytokine milieu (IL-4/IL-13) may impact the avidity of these T cells.

Current results clearly indicate that the tetramer-positive T cell population is heterogeneous for cytokine mRNA profiles. Hence, the IL-4-producing cells could be a subset of CD8+ T cells that have a unique function, which could be inherent to poxvirus infection. We have also identified a subset of CD8+ T cells that express IL-4 after a natural mouse-pox infection (C. Ranasinghe and I. A. Ramshaw, unpublished observations). The notion that IL-4 production by Ag-specific CTL is pathogen dependent is supported by the finding that influenza A virus-specific CTL do not express IL-4 (S. Turner, personal communication). Previous studies by our group have shown that an expression of mouse IL-4 by recombinant ectromelia virus generates immune responses that lack virus-specific CTL activity in mice (40). In another study, inhibition of IL-13 expression was shown to enhance protection against viral infection (41). Hence, we postulate that 1) the induction of IL-4/IL-13 expression in CD8+ T cells could be a mechanism that has evolved by particular viruses such as poxviruses to evade the host immune system (42), and 2) some of the various cytokine/chemokine inhibitors encoded by poxviruses (43, 44) may be responsible for diverting CD8+ T cells to produce IL-4. These factors should be taken into consideration when poxviruses are used as vaccine vectors.

Kelso and Groves (45) have demonstrated that under type 2 polarizing conditions in an in vitro culture system, CD8+ T cells are able to express IL-4. Recently, they have shown that these IL-4-producing T cells can be subdivided into CD8+high and CD8+low populations according to the type 1 or 2 cytokine milieu and the weak or strong Ag pressure they encounter, respectively, during development (46). Compared with the CD8+high T cells, the CD8+low T cells also elicited a reduced cytolytic activity (47). IL-4-producing CD8+ T cell subsets that show reduced CTL activity was also reported in HIV-1-infected individuals (48). In this study following the first I.N. or i.m. AE-FPV encounter, the primary tetramer-positive CD8+ T cells were found to produce IL-4 or and IFN-γ (data not shown). These findings further substantiate the fact that naive CD8+ T cells can undergo changes to suite the environment and hence steer subsets of cells to coexpress type 2 cytokines, in contrast to the well-accepted CD8+ polarization theory.

Tetramer-positive T cells obtained from all three immunization regimes showed a lack of cytokine IL-2 mRNA transcripts. Nevertheless, when total CD8+ T cells from immunized animals were stimulated with the full-length Gag 15-mer overlapping peptide pool (which also contained the immunodominant AMQMLKETI Gag sequence) for 16–20 h, enhancement of IL-2 mRNA was detected in a regime-dependent manner; I.N./I.N. > I.N./i.m. > i.m./i.m. However, IL-2 protein was undetected at this time point. It has been recognized that kinetics of IL-2 expression upon external stimuli is relatively different to other cytokines such as IFN-γ (F. Shannon, personal communication). Our recent observations also reveal that after 15-mer overlapping Gag peptide pool stimulation, much higher levels of IL-2 and TNF-α mRNA can be detected at 2–3 h compared with 16–20 h (C. Ranasinghe unpublished data). This again supports the notion that cytokine expression should be evaluated in a time-dependent manner because kinetics are known to depend upon the length, strength, and dose of Ag exposure (37, 49) plus the number of cells producing the cytokine (50). Intriguingly, the expression of IL-2 has also been implicated in T cell avidity. It has been shown that low Ag exposure favor IL-2 expression by high-avidity CD8+ T cells (22), whereas high Ag dose was associated with CD8+low T cells that expressed IL-4 (46). We observed little IL-2 production by Ag-specific CTL after various immunization regimes and increased IL-4 production. Given the fact that high Ag load induces low-avidity CTL, we postulate that the systemic delivery (i.m./i.m.) of recombinant poxvirus may possibly create a high Ag milieu in vivo, thus skewing the relative immune response toward low avidity CD8+ cell subsets expressing IL-4 over IL-2 compared with mucosal delivery.

According to the immunization regimes tested, the number of tetramer-positive cells producing granzyme B differed in the mucosal and systemic compartments. The genitorectal cells from I.N./i.m. immunization mice showed twice the number of cells producing granzyme B compared with mice immunized by the pure systemic immunization regime, whereas i.m./i.m.-immunized animals elicited higher proportions of splenocytes producing granzyme B. Previously, we have also shown that i.m. recombinant
DNA prime/intrarectal rFPV boosting can significantly reduce plasma viremia against a pathogenic vaginal SHIV(SF162P3) challenge compared with i.m. recombinant DNA/i.m. rFPV immunization regime (19). Recently, Belyakov et al. (7) have also shown that mucosal immunization can protect rhesus macaques from a pathogenic intrarectal SHIV-k2 challenge. The current observations further substantiate that the mucosal immunization can induce higher numbers of iliac lymph node cytotoxic CD8+ T cells compared with a systemic immunization and this may be reflective of their capacity to resist virus challenge at this site. Interestingly, only a small number of CD8+ cells present at these sites showed expression of perforin, whether this is a consequence of a vaccination regime, expressed HIV Ags, or the use of poxvirus vectors is unclear at this stage. However, in another study, a lower level of perforin expression was found in the same model system (8). These observations suggest that the mucosal immunization can induce higher numbers of iliac lymph node cytotoxic CD8+ T cells compared with a systemic immunization and this may be reflective of their capacity to resist virus challenge at this site.

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