Transcriptional Profiling of Experimental CD8+ Lymphocyte Depletion in Rhesus Macaques Infected with Simian Immunodeficiency Virus SIVmac239

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CD8+ T cells inhibit virus replication in SIV-infected rhesus macaques. However, it is unclear to what extent the viral suppression mediated by CD8+ T cells reflects direct killing of infected cells as opposed to indirect, noncytolytic mechanisms. In this study, we used functional genomics to investigate noncytolytic mechanisms of in vivo viral suppression mediated by CD8+ lymphocytes. Eight chronically SIVmac239-infected rhesus macaques underwent CD8+ lymphocyte depletion, and RNA from whole blood was obtained prior to depletion, during the nadir of CD8+ cell depletion, and after CD8+ lymphocyte numbers had rebounded. We observed significant downregulation of the expression of genes encoding factors that can suppress SIV replication, including the CCR5-binding chemokine CCL5/RANTES and CCL4 and several members of the tripartite motif-containing (TRIM) family. Surprisingly, we also noted a strong, widespread downregulation of α- and β-defensins with anti-HIV activity, which are not expressed by CD8+ T cells. After cessation of depleting antibody treatment, we observed induction of a transcriptional signature indicative of B lymphocyte activation. Validation experiments demonstrated that animals during this period had elevated levels of B cells coupled with higher expression of the proliferative marker Ki67, indicating that CD8+ depletion triggered a potent expansion of B cell numbers. Collectively, these data identify antiviral pathways perturbed by in vivo CD8+ T cell depletion that may contribute to noncytolytic control of SIV replication.

The global spread of the human immunodeficiency virus (HIV) pandemic, currently affecting over 30 million individuals worldwide, emphasizes the urgency to develop a safe and effective vaccine. However, fundamental obstacles remain at the level of the basic biology of the interaction between HIV and the human immune system (1–3). Due to the current absence of immunogens that can elicit HIV-specific broadly neutralizing antibodies (4–6), numerous vaccine strategies have been proposed that are based on antiviral cellular immunity (7).

Virus-specific T cell responses, in particular those mediated by CD8+ cytotoxic T lymphocytes (CTLs), confer protection against many viral infections by favoring both viral clearance and resistance to reinfection (8, 9). Classical studies suggested a role for CTL responses in the control of HIV in vivo by demonstrating that major histocompatibility complex (MHC)-restricted CD8+ cells from seropositive individuals were capable of lysing autologous cells loaded with HIV antigen by vaccinia virus transduction (10, 11). Here, several lines of evidence have bolstered a model in which CD8+ T cells mediate control of virus replication during both HIV infection of humans and simian immunodeficiency virus (SIV) infection of rhesus macaques (RMs). First, the postpeak decline of viremia in acute HIV infection is coincident with the expansion of HIV-specific CD8+ T cells (12, 13). Second, during acute and chronic HIV/SIV infection, immunologic pressure mediated by HIV/SIV-specific CD8+ T cells is manifested by viral escape mutations (14). Third, a clear association between certain MHC class I alleles and reduced disease progression during both HIV infection of humans and SIV infection of RMs has been demonstrated (15, 16). Fourth, HIV-1-infected individuals with multifunctional HIV-1-specific T cells progress less rapidly than those with limited T cell functionality (17).

Perhaps the most convincing evidence for a direct effect of CD8+ lymphocytes on suppressing HIV/SIV replication came from a series of elegant studies in which these cells were depleted in vivo in SIV-infected RMs. Initial work demonstrated that antibody-mediated in vivo depletion of CD8+ lymphocytes is consistently associated with increased plasma viremia (18–20). Following these studies, similar in vivo experimental approaches yielded the observations that depletion of CD8+ T cells directly led to (i) a loss of host control of live attenuated SIVΔ nef viruses (LAVs) (21), (ii) SIV recrudescence after initial control due to early antiretroviral therapy (ART) treatment (22), (iii) partial loss of challenge virus suppression in Δ nef LAV-vaccinated RMs, and, importantly (23), (iv) poorer survival and increased CD4+ T cell loss during SIVmac infection of RMs (24). These studies powerfully demonstrated a causative, rather than correlative, relationship between CD8+ T cells and SIV replication. While it has been broadly assumed that the primary mechanism by which CTLs exert this control is by the killing of infected target cells, this model has not been formally demonstrated in vivo.
Recent evidence has suggested that additional, noncytolytic mechanisms may also contribute to the control of HIV/SIV replication \textit{in vivo} (25). Studies conducted by us and others have shown that depletion of CD8$^+$ lymphocytes in SIV-infected RMs followed by ART treatment did not alter the life span of productively SIV-infected cells or impact viral decay kinetics compared to undepleted animals (26, 27). In addition, decay rates of wild-type and escape mutant virus were found to be similar during the acute phase of simian-human immunodeficiency virus (SHIV) infection of macaques (28). Suppression of HIV replication by CD8$^+$ T cells via noncytolytic mechanisms that inhibit virus transcription was first observed by Levy and colleagues in 1986 (29, 30), although the molecular mechanisms underlying this antiviral activity have remained elusive (31–33). Potential noncytolytic mechanisms by which CD8$^+$ T cells may suppress HIV/SIV replication include blocking virus spread from cell to cell by releasing soluble factors, such as CCR5-binding chemokines (i.e., MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5), that act as competitive inhibitors of CCR5-mediated virus entry (34, 35), as well as the release of other potential antiviral factors (i.e., interferons [IFNs] and cytokines). To date, however, there is a lack of data describing the noncytolytic mechanisms utilized by CD8$^+$ T cells to control virus \textit{in vivo}.

To define potential noncytolytic mechanisms by which CD8$^+$ lymphocytes control \textit{in vivo} SIV infection, we performed microarray-based gene expression profiling on RMs depleted of CD8$^+$ lymphocytes during chronic infection. To date, this is the first report using high-throughput genomics to characterize the effects of \textit{in vivo} CD8$^+$ lymphocyte depletion on the transcriptional profile of circulating leukocytes. We observed that depletion of CD8$^+$ lymphocytes resulted in the reduction of transcripts for several soluble anti-HIV factors, including β-chemokines. Surprisingly, the class of transcripts exhibiting the most dramatic suppression was not chemokines, but those encoding defensins, which have anti-HIV activity but are not expressed by CD8$^+$ T cells. In addition, during the repopulation of CD8$^+$ T cells, we observed significant proliferation and expansion of B cells, a feature previously undescribed in the macaque/CD8 depletion model. Overall, these results map out the predominant noncytolytic antiviral pathways controlled by CD8$^+$ lymphocytes during \textit{in vivo} SIV infection.

MATERIALS AND METHODS

Animals. Ten rhesus macaques (Macaca mulatta) of Indian origin (6 of which were ManuA*01, distributed 3 per group) were intravenously (i.v.) infected with 3,000 50% tissue culture infective doses (TCID$_{50}$) of SIVmac239, as originally reported by Klatt et al. (26). RNA samples from eight macaques were selected for this study based on sample quality. All animals were housed at the Yerkes National Primate Research Center and maintained in accordance with NIH guidelines. These studies were approved by the Emory University Institutional Animal Care and Use Committee.

CD8$^+$ lymphocyte depletion, antiretroviral therapy, and plasma viral load assay. As described previously, SIV-infected RMs included in this study were treated i.v. with 4 mg/kg of body weight/day of OKT8F monoclonal antibody (MAb) for three consecutive days to deplete CD8$^+$ cells (26). Reverse transcriptase inhibitors 9-R-(2-phosphonooxypropyl)-adenine (PMPA; tenofovir) and beta-2,3-dideoxy-3-thia-5-fluorocytidine (FTC; emtricitabine) were provided by Gilead Sciences and administered to each animal intramuscularly (i.m.) (30 mg/kg/animal/day each) for 28 days. To achieve sufficient statistical power but reduce animal usage, we employed a reciprocal experiment design (outlined in Fig. 1 and in reference26). Briefly, half the animals were administered CD8-depleting MAb at day 58, followed by 28 days of ART starting at day 63 (“early” group), while the “late” half also commenced ART at day 63 but received no depleting MAb. At day 177, the experiment was repeated with the groups reversed: the late animals received CD8-depleting MAb treatment, followed by both groups initiating 28 days of ART at day 182. RNA samples for the “depletion” analysis were obtained at days 63 and 182, prior to ART initiation. Levels of plasma SIVmac239 were monitored using a quantitative real-time PCR (qPCR) assay, as previously described (2).

RNA purification, array hybridization, and hemoglobin blocking. Total RNA purification and microarray hybridization were performed as described previously (36). Briefly, 2.5 ml of venous blood was collected into Paxgene blood RNA tubes (Becton Dickinson, San Diego, CA) and stored at −80°C. Total RNA was purified with Paxgene Blood RNA kits (Qiagen) according to the manufacturer’s protocol with on-column DNase digestion. The RNA quantity was estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE), and RNA integrity was assessed by Agilent Bioanalyzer capillary electrophoresis; all samples had an RNA Integrity Number (RIN) score of 8.8 or higher. Purification and hybridization of RNA samples were performed by single operators in balanced blocks to minimize experimental bias according to standard microarray experimental design. Samples were hybridized to Affymetrix GeneChip Rhesus Macaque Genome Arrays (Affymetrix, Santa Clara, CA). The Affymetrix 2-cycle cDNA synthesis protocol was used to amplify 0.5 μg of total RNA; inhibition of nonspecific binding by hemoglobin transcripts was afforded by including a set of 5 peptide nucleic acid (PNA) oligonucleotides (Bio-Synthesis Inc.) specific for regions of hemoglobin α and β mRNA in the reverse transcription cocktail, as described previously (36).

Microarray data analysis. After scanning, gene expression intensity measurements for individual hybridizations were exported as Affymetrix CEL files. CEL files from individual hybridizations were preprocessed using the robust multichip average (RMA) algorithm with median polish summarization. RMA and subsequent analyses were performed using Partek Genomics Suite software v6.4 (Partek Inc., St. Louis, MO). To determine genes statistically changed after CD8$^+$ cell depletion or during reexpansion we employed a statistical model that combined the previously unpublished depletion/expansion data described here with data from prior work. Briefly, two-factor analysis of variance (ANOVA) was used to determine the significance of probe set intensity changes after SIVmac239 infection over multiple time points, as follows: day −5, “preinfection”; day 0, “baseline”; day 9; day 14; day 31, “predepletion”; days 63 and 182, “depletion”; days 70 and 189, “rebound”; day 70, early group, “post-ART”; and samples from >224 days, “chronic” (false discovery rate [FDR]-adjusted P value of less than 0.0335477). Data derived from CEL files for days −5, 9, 14, 31, and >224 have been previously published (36). In addition to significance determined by ANOVA, probe sets from either the depletion or rebound time points were tested post hoc versus the predepletion time point, and those with significant FDR (<0.021411, depletion; <0.00190135, rebound) and a change versus predepletion greater than ±1.5-fold were judged as differential. Lastly, because samples taken in the rebound phase were during the period when animals were receiving ART, we identified genes regulated by ART alone in group B (fold change, ±1.5; P < 0.00032252) and removed 84 ART-responsive probe sets from the rebound gene list. For calculation of the postdepletion fold change for group B, we assessed whether RNA samples from day 31 or day 171 would be more appropriate for use as a baseline by comparing the calculated expression profiles to those obtained for group A with day 31. We observed that group B profiles calculated using day 31 had better correlation with group A profiles (see Fig. S1 in the supplemental material). To confirm that changes in gene expression were consistent between groups A and B, we examined the fold change of several genes determined to be significantly different during depletion between groups and found them to be similar to fold changes calculated by using groups A and B combined (see Table S1 in the supplemental material). Probe set identifiers (IDs) were annotated using v.32 of the Affymetrix Rhesus Gene Chip annotation supplemented with additional IDs from version 3 of the annotation pro-
A
CD8+ Lymphocyte Depletion Schedule

<table>
<thead>
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<th>Days post-infection</th>
<th>CD8</th>
<th>CD3+CD8+ T cells</th>
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</thead>
<tbody>
<tr>
<td>31</td>
<td></td>
<td>OKT8 + ART</td>
</tr>
<tr>
<td>58</td>
<td></td>
<td>ART alone</td>
</tr>
<tr>
<td>70</td>
<td></td>
<td>OKT8 + ART</td>
</tr>
<tr>
<td>172</td>
<td></td>
<td>ART alone</td>
</tr>
<tr>
<td>189</td>
<td></td>
<td>OKT8 + ART</td>
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B
Microarray Analysis Design

C

D

FIG 1 Experimental design of genomic analysis of CD8 depletion in SIVmac239-infected rhesus macaques. (A) Idealized kinetics of the viral load in CD8-depleted animals described by Klat et al. (26). Rhesus macaques were infected with SIVmac239 and administered OKT8F MAb i.v. at days 58 and 177 for 3 days. (B) Study design and RNA-sampling schedule. Five days after OKT8F treatment (days 63 and 182), animals were either administered ART (PMPA plus FTC) for 28 consecutive days or left untreated as controls. Whole-blood samples for microarray analysis were drawn at days 0, 10, 14, and 31 (predepletion, groups A and B); 63 and 182 (postdepletion, groups A and B); and 70 and 182 (CD8 rebound phase, groups A and B). As indicated, gene expression fold changes in blood during CD8 depletion were defined as the average ratio of intensity measurements at day 63 or 182 divided by those at day 31; fold changes during CD8 rebound were calculated as the mean ratio of probe set intensities at day 70 or day 189 divided by those at day 31. The black and red lines denote the depletion of CD3+CD8 T cells in the early and late phases of the study. (C and D) Plasma viral loads (C) and levels of CD3+CD8+ T cells in the blood (D) of SIV-infected macaques over the course of the study. CD3+CD8+ T cells were gated on live lymphocytes. The periods in which animals underwent CD8 depletion and ART are denoted by gray and dashed boxes, respectively. A subset of the data shown in Fig. 1C and D was published previously [26].

Real-time PCR. Quantitative real-time PCR on total RNA was performed as previously described (37); briefly, 300 to 400 ng of RNA was reverse transcribed in 20-μl reaction mixtures using 6.25 μM dN6 random hexanucleotide primer (Applied Biosystems, Foster City, CA); 50 mM Tris-HCl, pH 8.3; 10 mM dithiothreitol (DTT); 3 mM MgCl2; 5 mM hexanucleotide primer (Applied Biosystems, Foster City, CA); 50 mM Tris-HCl, pH 8.3; 10 mM dithiothreitol (DTT); 3 mM MgCl2; 75 mM KCl; 500 μM dATP, dGTP, dTTP, and dCTP; and 200 U SuperScript II RNase H− reverse transcriptase at 42°C for 1 h. CDNA was then stored at −20°C until the qPCRs were performed using an ABI-Prism 7900HT Sequence Detection System (Applied Biosystems) and SYBR green PCR Master Mix (Applied Biosystems). Each qPCR was performed in a volume of 10 μl with 0.2 μl of CDNA, 250 nM primer pair, and 5 μl of SYBR green PCR MasterMix in ABI-Prism optical 386-well plates. A logarithmic dilution of CDNA was used to generate a standard curve for each primer, and gene expression fold changes were calculated using the standard curve method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an endogenous standard to normalize samples. Fold change differences were calculated by dividing the normalized postdepletion sample quantity by the normalized predepletion sample quantity. Primers were purchased from Integrated DNA Technologies (Coraville, IA). The primer sequences used were as follows: GAPDH-forward, 5′-GCA CCA CCA ACT GCT TAG CAC-3′, and GAPDH-reverse, 5′-TCT TCT GGG TGG CAG TGA TG-3′; MNPIA-forward, 5′-CAG GAC CAG ATT CCA ACA GAC AAC-3′, and MNPIA-reverse, 5′-TCT TCC CAT GTA GAA GCA GGT TCC-3′; MNP2-forward, 5′-CTC AGG CTA AGT CAC TCC AGG AAA-3′, and MNP2-reverse, 5′-GAG TAG GAC TCA CGC CTC AAA CAA-3′. Because the rhesus α-defensin family is composed of short, highly homologous proteins, we conducted BLAST searches of the MNP primers against the rhesus macaque genome; the MNPIA primer set also likely amplifies rhesus MNPI, and the MNP2 set likely amplifies rhesus MNPI/S567.

Immunophenotyping of T and B lymphocytes. To quantitate changes in T and B cell numbers, multiparametric flow cytometry was performed as described previously (26). Whole blood was incubated with antibodies against surface markers CD20 (ECD, clone B9E9; Beckman Coulter), CD3 (Alexa 700, clone SP34-2; BD Pharmingen), CD8 (Pacific Orange, clone RPA-T8; BD Pharmingen), CD4 (phycoerythrin [PE]-Cy5.5, clone OKT4; ebioscience), HLA-DR (PE-Cy7, clone M5E2; BD Pharmingen), CD14 (PE-Cy7, clone M5E2; BD Pharmingen), CD123 (PE, clone 9F5; BD Pharmingen), CD11c (allophycocyanin [APC], clone S-HCL-3; BD Pharmingen), CD16 (Pacific Blue, clone 3g8; Beckman Coulter), Aqua Live/Dead amine dye-AmCyan (Invitrogen). Erythrocytes were subsequently lysed using BD FACs Lyse. Samples were stained for intracellular Ki67 (fluorescein isothiocyanate [FITC], clone B26; BD Pharmingen) using the CytoFix/Perm Kit (BD Pharmingen). Acquisition was performed on an LSRII flow cytometer, and at least 100,000 lymphocytes were obtained for each sample. Analysis of flow cytometry data was performed using FlowJo software (version 9.2; TreeStar). Representative gating of CD3-CD20- B lymphocytes and Ki67 is shown in Fig. S2 in the supplemental material.
Microarray data accession number. The microarray data set was submitted to the GEO repository according to MIAME (Minimum Information about a Microarray Experiment) standards (GEO accession number GSE37834).

RESULTS

Study design. A summary of the experimental design and an idealized representation of the kinetics of the viral load and CD8+ T cell levels in peripheral blood observed in the original study of the effect of CD8+ lymphocyte on the calculated in vivo life span of productively infected cells (26) is depicted in Fig. 1A. During ART treatment, the slopes of the decay of the plasma viral load were equivalent between CD8-depleted and undepleted animals, supporting a model in which the presence of CD8 T cells does not affect the life span of infected cells and suggesting a larger role for noncytolytic mechanisms of viral control in vivo. In the current study, we used genome-wide transcriptional profiling to identify potential noncytolytic mechanisms by which CD8 T cells suppress viral replication in vivo. To this end, we used microarray data derived from blood samples obtained at 5 days post-CD8+ lymphocyte depletion (“depletion”) and during the reappearance of CD8+ lymphocytes after the cessation of antibody treatment (“rebound”). To estimate gene expression fold changes during depletion or rebound, RMA-preprocessed log_{10} intensity measurements for individual animals were subtracted by that animal’s log_{10} intensity measurement at day 31 (“predepletion”); the logarithmic mean of log_{10} differences from individual animals were used to estimate the gene expression fold changes for individual genes (Fig. 1B). To improve the statistical power, we combined expression profiles from RMs in both the early and late groups. To determine whether it would be appropriate to use RNA samples taken just before depletion (day 171) as a baseline for the late group, we examined the viral loads and CD8+ T cell counts of individual animals (Fig. 1C and D). While the CD8+ T cell count was relatively stable in the late group leading up to depletion, we noted that the plasma viral load at day 171 was 3-fold lower than at day 31. Comparison of postdepletion gene expression in group B showed that profiles calculated using day 31 as a baseline were better correlated with group A profiles than those using group B day 171 measurements (see Fig. S1 in the supplemental material).

Detection of genes differentially expressed during antibody-mediated CD8+ lymphocyte depletion. To investigate the impact of CD8+ lymphocyte depletion on the transcriptional profile of circulating leukocytes of SIV-infected RMs, we isolated RNA from whole blood 5 days after animals were treated with OKT8F CD8+ lymphocyte-depleting monoclonal antibody. Flow cytometry confirmed the near-complete depletion of CD8+ T cells (99.97% and 99.95% average depletion in peripheral blood in groups A and B, respectively, as previously reported [26]). Of note, the OKT8F antibody can also deplete other non-T cell subsets expressing the CD8 molecule (i.e., NK, NKT, and γδ T cells). The statistical model to determine differentially expressed genes during CD8+ lymphocyte depletion is described in detail in Materials and Methods, but briefly, we used three criteria: (i) a significant FDR-corrected \( P \) value determined by 2-factor ANOVA across all time points (\( P < 0.0335477 \)), (ii) a significant \( P \) value determined by post hoc pairwise analysis of probe set intensities at the depletion time point (day 63/day 182) versus the predepletion samples (day 31), and (iii) a 1.5-fold change in expression between the post-depletion and predepletion time points (Fig. 1B). Based on this analysis, we observed that CD8- lymphocyte depletion was associated with a total of 2,662 differentially expressed probe sets (1,825 upregulated and 837 downregulated). A comprehensive list of genes with significantly increased or decreased expression during CD8- cell depletion is contained in Table S2 in the supplemental material. We identified the major immunological pathways exhibiting perturbation in gene expression in CD8+ lymphocyte-depleted SIV-infected RMs using multiple ontology tools (Ingenuity; DAVID) and review of the literature. The list of genes detected as upregulated during CD8 lymphocyte depletion was enriched for transcripts associated with regulating innate immunity and viral recognition. In contrast, the major immunological pathways enriched in the downregulated gene list were involved in TCR and NK cell signaling, likely due to the removal of CD8-bearing cells from the blood.

We then examined individual genes modulated by CD8+ lymphocyte depletion, with a focus on genes involved in immunoregulatory pathways. As expected, we observed a striking down-regulation for probe sets specific for both the α and β chains of the CD8 molecule, as well as those targeting the highly CD8+ lymphocyte-specific granzyme B and H genes, underscoring the accuracy of the assay for detection of the loss of CD8+ lymphocytes (Fig. 2). Granzymes A, K, and M, which have been associated with CD8+ T lymphocyte-mediated lysis, as well as NK cell-mediated lysis, were also observed to have significantly lower mRNA levels after OKT8F-mediated depletion. A number of transcripts associated with NK cell function were also observed to be significantly downregulated, likely reflecting the ability of the OKT8F clone to deplete NK cells (Fig. 2). Overall, these data indicate a consistent downregulation of several genes central to CD8+ T lymphocyte and NK cell function, demonstrating the ability of the microarray assay to detect the loss of CD8+ T cells based on transcript levels. Although it was not tested in the original study, these data indicate that the OKT8F treatment also substantially depleted NK cells.

We also noted a significant upregulation in multiple interferon-stimulating genes (ISGs) (Fig. 2), which has been demonstrated in previous studies by us and others to be a sensitive and reliable indicator of SIV viremia, even in the absence of detectable levels of type I IFN (references 38 and 37 and unpublished data). Although the expression of ISGs prior to depletion was significantly increased compared to preinfection, experimental CD8+ lymphocyte depletion resulted in further elevation, likely in response to the increase in viremia (0.7 to 2.2 log units in plasma) (26). Interestingly, we noted upregulation of the probe sets for certain interferon-inducible retroviral host restriction factors, such as BST2/tetherin and tripartite motif-containing 5 (TRIM5), TRIM25, TRIM56, and several other TRIMs not known to be associated with antiviral activity (39, 40). The upregulation of TRIMs and antiviral molecules was not universal; four TRIM family members (TRIM2, TRIM10, TRIM15, and TRIM59) exhibited decreased expression (Fig. 2). Collectively, these data indicate that CD8- lymphocyte depletion was associated with markedly reduced expression of CD8+ lymphocyte-specific genes and a relative increase of a number of type I ISGs. In addition, we observed reduced expression of several retroviral host restriction factors following CD8+ lymphocyte depletion.

β-Chemokines are significantly downregulated upon depletion of CD8+ T cells. Recent work has suggested that the \( \text{in vivo} \) antiviral activity of CD8+ lymphocytes may, at least in part, be mediated by noncytolytic mechanisms, such as the production and release of soluble antiviral factors (25). Consistent with this
hypothesis, we noted that CD8+ lymphocyte depletion was associated with significantly reduced expression of the CCR5-binding chemokines CCL5/RANTES and CCL4/MIP1α (Fig. 2), both of which are capable of inhibiting HIV-1 infection at the entry level (34) and are known to be produced by activated CD8+ T cells (41, 42). In contrast, we did not observe any significant downregulation of CCL3/MIP1α (Fig. 2).

Widespread suppression of defensin mRNA during CD8 depletion. In contrast to the modest downregulation of β-chemokine genes, we observed that genes encoding rhesus α-defensins 1 and 2 (MNP1A and MNP2) and the precursor for rhesus α-defensin 3 were strongly and significantly downregulated upon CD8+ lymphocyte depletion, with mean changes of −5.7-fold and −5.4-fold, respectively (Fig. 3A). We also observed significantly reduced expression levels of genes encoding demidefensin 1/rhesus theta defensin 1B (RTD1B) (−4.7-fold) and demidefensin 3/RTB1A (−4.6-fold) (Fig. 3A), the rhesus β-defensin DEFB121 (−2.0-fold), and rhesus θ-defensin 1a/RTD1A (−6.7-fold). Comprehensive tables of genes with increased or decreased expression during CD8+ cell depletion are contained in Table S2 in the supplemental material. To confirm the decrease of α-defensin mRNA observed in the microarray analysis, we performed real-time PCR to quantify mRNA levels for rhesus macaque α-defensin 1A/MNP1A and for α-defensin 2/MNP2. We observed that mRNA levels for both genes were significantly lower in samples collected after depletion of CD8+ lymphocytes (P = 0.0156) than in predepletion samples, validating the microarray data (Fig. 3B and C). The average changes observed were 72- and 26-fold reduction after depletion for α-defensin 1A/MNP1A and α-defensin 2/MNP2, respectively, with both genes observed to be decreased in seven out of eight RMs (Fig. 3B to D).

Since the major producers of α-defensins in blood are neutrophils, we next investigated whether CD8+ lymphocyte depletion was associated with changes in the neutrophil count that can explain the observed down modulation of α-defensins. We found that while two RMs showed decreases in the overall neutrophil numbers upon CD8+ lymphocyte depletion, levels in the remaining six animals were relatively unperturbed (Fig. 3E). Several reports in the literature have demonstrated that, in humans, CD8+ T cells do not produce α-defensins (33, 43–45). To examine the expression of α-defensins in RM s, we performed qPCR using defensin-specific primers on RNA from CD3+CD8+ and CD3−CD8+ cells purified using flow cytometric sorting. While defensin mRNA was slightly higher in the CD3+CD8+ population than in CD3−CD8+ cells, transcripts were barely above the threshold of detection for both (data not shown). No significant correlation was detected between the viral load and the levels of defensin mRNA measured by qPCR (data not shown). Since neutrophils were not significantly affected by the CD8+–depleting antibody, the observed downregulation of these molecules after CD8+ lymphocyte depletion may be related to the removal of other CD8+ lymphoid subsets, such as NK and CD8+γδ T cells (33, 44, 46, 47). Collectively these data demonstrate that experimental depletion of CD8+ lymphocytes induces a dramatic decrease in the expression of defensins, an important class of soluble antiviral factors.

Reexpansion of CD8+ lymphocytes is associated with induction of B cell genes. After cessation of OKT8F antibody administration, CD8+ lymphocytes reexpanded in blood and tissues, reaching levels of approximately 80% of baseline within 12 to 13 days (26). To identify genes differentially expressed during CD8+ lymphocyte rebound, we used the same statistical approach as for the depletion analysis described above, with the pairwise comparison (FDR < 0.05) and fold change (1.5-fold) determined using expression measurements from the predepletion time point (31 days postinfection [p.i.]) and the rebound interval (70/189 days p.i., 12 days after MAb treatment was stopped). Genes observed to be differentially expressed by ART alone were also excluded. Using these criteria, 1,224 probe sets were identified as differentially expressed during CD8+ lymphocyte depletion and rebound; this decrease in ISG levels was largely expected due to the drop in the viral load (Fig. 4). Interestingly, a large number of genes that were differentially expressed during CD8+ lymphocyte depletion and rebound were associated with induction of B cell genes. The expression of genes encoding B cell receptors and molecules, such as IgG, IgA, and IgM, as well as B cell activation markers and cytokines, was significantly increased during CD8+ lymphocyte rebound (Fig. 4). These findings suggest that CD8+ lymphocyte depletion and rebound may contribute to the reconstitution of B cell function in RMs following ART.
of probe sets specific for CD19, CD20/MSA4A1, and components of the B cell receptor (CD79A and CD79B) were significantly upregulated during the rebound phase (Fig. 4). Additionally, probe sets specific for immunoglobulins and several other genes regulating B cell responses were found to be upregulated during the CD8⁺/H11001 lymphocyte rebound phase, including BANK1, AICDA, and CD22. Individual immunoregulatory genes detected as significantly changed are summarized in Fig. 4, and the full lists of genes with altered expression during the reexpansion of CD8⁺ T lymphocytes can be found in Table S3 in the supplemental material.

**Depletion and repopulation of CD8 T cells after depletion are accompanied by an expansion of B cells.** The vast induction of genes encoding B cell receptor signaling and regulatory molecules during the period when CD8⁺ T cells were depleted and then recovered from depletion (Fig. 4) suggested that an expansion of B cells was occurring during CD8⁺ T lymphocyte depletion and re-
bound. To test this hypothesis, we first measured the levels of B cells in whole blood during the period between day −40 and day 105 of the study in the group A RMs (n = 5), which encompassed pre- and post-SIV infection, CD8− lymphocyte depletion and rebound, and the postrebound phase. We found that during the depletion of CD8− lymphocytes, the mean percentage of CD20+ B cells increased from 17% at day 45 to 30% at day 63 and continued to increase during the rebound phase to 40% at day 77 before decreasing to 19% at day 105 (see Fig. S3 in the supplemental material). However, measurement of B cell levels as a percentage of lymphocytes was confounded by the fluctuating levels of CD8− lymphocytes due to SIV infection and, more pronouncedly, by the antibody-mediated depletion. Therefore, we examined the absolute levels of circulating B cells per microliter of blood and found that the number of B cells was significantly increased after rebound (day 70) compared to predepletion levels (day 56) (Fig. 5) (P = 0.009). To determine if the expansion of B cells was related to increased levels of B cell proliferation following CD8− lymphocyte depletion, we next measured the fraction of B cells expressing the cell cycle marker Ki67. A representative flow plot of our gating strategy for this analysis is shown in Fig. S2 in the supplemental material. Before CD8− lymphocyte depletion (i.e., day 56 p.i.), the mean fraction of CD20+ Ki67+ B cells was 7.5%, which was similar to preinfection levels of 5.3% (day −40) and 5.7% (day 0). After depletion, the fraction of CD20+ Ki67+ B cells increased to 15.2% (day 63; P = 0.013) before dropping to 9.4% at day 64 (P = 0.039). More dramatic increases in the fraction of CD20+ Ki67+ B cells were observed during the CD8− lymphocyte rebound phase, with average levels of 20.1% (day 66; P = 0.001) and 21.0% (day 70; P = 0.006). Collectively, these data indicate that during antibody-mediated CD8− lymphocyte depletion and repopulation, B cells undergo a significant expansion accompanied by increased levels of proliferation. To gain insight into the potential mechanisms driving B cell expansion, we examined the changes in interleukin 7 (IL-7) and IL-21 mRNAs during rebound as measured by microarray. While one probe set specific for IL-7 was upregulated during rebound (see Table S3 in the supplemental material), four others showed no change; no differential expression was observed for IL-21. Previously, we reported a modest decrease in plasma IL-7 during CD8− lymphocyte depletion (26); however, IL-7 levels during the rebound phase were observed to be similar to baseline predepletion levels.

**DISCUSSION**

CD8+ lymphocytes mediate a strong *in vivo* antiviral effect in SIV-infected RMs. While it is generally accepted that control of viral replication is due to a combination of cytolytic and noncytolytic mechanisms, very little is known about which noncytolytic pathways are important *in vivo*. In an effort to elucidate the molecular mechanisms underlying this antiviral activity, we examined gene expression at a genome-wide level during *in vivo* experimental CD8− lymphocyte depletion in SIV-infected RMs. While

![Graph showing gene expression](image-url)
in vivo depletion of specific immune cell subtypes using monoclonal antibodies has become a key experimental tool to study immune responses in nonhuman primates, to the best of our knowledge, this is the first report utilizing high-throughput genomic technology to characterize the effect of CD8$^+$ lymphocyte depletion during SIV infection of RMs. One major caveat of this study, however, is that because the assay was performed on whole blood from which CD3$^+$ CD8$^+$ T cells, as well as a large fraction of NK and NKT cells, were actively removed, observed changes in gene expression could be theoretically attributed either to system-wide gene expression changes or to the loss and/or change in distribution of cells themselves (as demonstrated by the observed decrease in expression in genes encoding CD8 proteins). On the other hand, in the case of genes that are absent or expressed at very low levels in CD8$^+$ T cells (e.g., α-defensins), our study presented a unique opportunity to conduct more accurate validation and interpretation of the data.

Previous data from several groups demonstrated that in vivo CD8$^+$ lymphocyte depletion in RMs causes increases in SIV loads. More recently, however, two independent studies indicated that the kinetics of viral decay after ART were identical in CD8$^+$ lymphocyte-depleted and control animals, suggesting that the life span of productively infected cells was not affected by the presence of cytotoxic T lymphocytes (26, 27). Given this finding, we hypothesized that CD8$^+$ T cells would contribute to viral control using additional, noncytolytic mechanisms, such as the induction of innate antiviral molecule expression or through secretion of soluble inhibitory factors. Surprisingly, we observed that CD8$^+$ lymphocyte depletion was followed by a widespread upregulation of antiviral molecules induced by type I IFN, which was most likely related to the observed increase in viral replication.

Interestingly, depletion of CD8$^+$ lymphocytes also resulted in significant reduction in the expression of the β-chemokines CCL5/RANTES and CCL4/MIP1β. This reduction can be attrib-
puted to the massive loss of both CD8^+ T cells and NK cells. In our previous study (26), we detected a trend toward a decrease in plasma CCL3/MIP1α and CCL5/RANTES. The production of these chemokines, which are capable of inhibiting SIV/HIV by competing for binding sites on the CCR5 receptor by activated CD8^+ T cells, is well documented (41). However, our analysis indicates that the depletion of CD8^+ T cells resulted in a major loss of mRNA production in these cytokines. The role of β-chemokines in HIV/SIV control has been well described in vitro. While a similar activity would be predicted in vivo, multiple studies have examined potential correlation between endogenous β-chemokine levels, β-chemokine gene polymorphisms, and the viral burden in HIV-infected patients, with conflicting and inconclusive results (48). More clear-cut in vivo evidence for the ability of CCR5 interference to reduce the viral load has been demonstrated in human and nonhuman primate studies using Maraviroc and similar CCR5-directed fusion inhibitors (49, 50). In our view, the data presented in the current study provide evidence of a direct relationship between CD8^+ T cell-mediated chemokine production and control of the viral load in vivo.

We also noted downregulation of several intracellular restriction factors and viral inhibitory molecules after CD8^+ lymphocyte depletion, including TRIM10 (51), TRIM15 (52), and TRIM59 (39). CD8 depletion also upregulated expression of three TRIM family members, two of which (TRIM25 and TRIM56) have been recently demonstrated to act as intracellular sensors for viral components (53, 54), making it likely that their upregulation was part of the antiviral response responsible for the observed enhanced expression of ISGs. Collectively, the observed reduction in expression of retroviral host restriction factors and intrinsic antiviral molecules in the absence of CD8^+ T cells may allow more efficient replication of the virus.

CD8^+ lymphocyte depletion coincided with a strong and consistent decrease in the expression of α- and θ-defensins, which was intriguing, as CD8^+ T cells have been definitively shown to lack endogenous defensin expression. Of note, θ-defensins are expressed as functional proteins with antiviral properties in macaques, while their human orthologues contain a premature stop codon that produces immature protein products (55). The potential role of defensins in HIV infection has been well studied, revealing that the anti-HIV activity of α-defensins (as well as that of RTDs in RMs and synthetic retrocytins in humans) (56), occurs by multiple mechanisms, including direct contact and disruption of the virus particle by interfering with gp120-CD4 interactions (57, 58). In contrast, β-defensins inhibit early events in HIV reverse transcription (57, 58). A role for defensins in the CD8^+ T cell-mediated control of HIV replication was originally reported by Zhang and colleagues, who proposed that medium cultures of CD8^+ T cells from long-term nonprogressors (LTNPs), but not progressors, contained α-defensins, which were responsible for most of the CD8^+ antiviral activity (47). Subsequent work, however, demonstrated that α-defensin proteins were not synthesized in CD8^+ T cells but rather in granulocytic feeder cells (33, 44, 45).

Recently, α-defensins have been linked to protection from SIV disease in two other in vivo models: (i) α-defensin expression is induced by SIV infection in jejunal Paneth cells of SIV-infected RMs and inversely correlates with disease progression (59); (ii) α-defensins are detectable in the lymph nodes of acutely SIV-infected sooty mangabeys, which do not progress to AIDS despite chronic, life-long viremia (36). The current finding that CD8^+ depletion results in a global reduction in defensin expression in whole blood suggests that CD8^+ lymphocytes play a role in maintaining or orchestrating defensin expression. The observation that neutrophil counts were not affected by CD8^+ lymphocyte depletion suggests that the observed decrease in defensin expression reflects a system-wide transcriptional repression. Recent work has demonstrated that γδ T cells are able to modulate the production of α-defensins in granulocytes (60) and β-defensins in epithelial cells (61). A few studies have reported that CD3^+ CD8^+ cells (NK and γδ T cells) produce defensins, and while we failed to detect significant levels of defensin mRNA, it remains formally possible that a portion of the observed decrease in defensin expression is in part related to their depletion (62–64). Attempts to elucidate the cellular source of defensin production during SIV infection are under way. Regardless of the source, this study provides evidence that perturbations of defensin expression are linked to increased viremia during in vivo SIV infection.

We also characterized gene expression during the reexpansion of CD8^+ T cells after anti-CD8 (αCD8) MAb administration was stopped. The downmodulation of genes encoding CD8 chains and granzymes observed during the depletion phase was not present during the rebound phase, indicating that their expression had returned to baseline. Similarly, the perturbations in gene expression that were seen during CD8^+ lymphocyte depletion (i.e., ISG induction and decreases in β-chemokines) also returned to baseline during the rebound phase, providing additional evidence that the CD8^+ lymphocyte depletion, and not an extraneous variable, was the stimulus driving the observed changes in gene expression. Gene expression changes during CD8^+ lymphocyte rebound was particularly enriched with upregulation of immunoglobulin genes and those involved in B cell differentiation. The observed upregulation of B cell-specific genes was associated with a significant increase in the total level of circulating B cells, as well as the fraction of B cells that are actively proliferating, as measured by Ki67 expression. Given the substantial viral recrudescence that follows CD8^+ lymphocyte depletion, this B cell expansion may be driven by three stimuli: (i) the introduction of the OKT8F MAb as a foreign antigen, (ii) the lack of competition by CD8^+ lymphocytes for homeostatic cytokines and anatomic niches, and (iii) the increase in overall SIV antigenic burden. In addition, B cell proliferation may occur in response to an outgrowth of new viral variants that may emerge in the absence of selective pressure by CD8^+ T cells. Current work in our laboratory is aimed at characterizing SIV diversity during experimental CD8^+ lymphocyte depletion using deep-sequencing technology.

Collectively, these results indicate that the increase in viral load that follows depletion of CD8^+ T cells in SIV-infected RMs is associated with an overall reduction in the expression of genes encoding several important classes of viral inhibitory proteins, which include host restriction factors, β-chemokines, and defensins. These data cannot provide any quantitative assessment of the relative contributions of these potential antiviral mechanisms as opposed to the well-established role of direct cytolytic activity of CD8^+ T cells. Rather, the goal of this work was to identify the primary potential noncytolytic pathways acting in vivo. Some pathways, such as the β-chemokines, have been reported in the literature several times, and our data are confirmatory in this regard (65). However, the observation of pervasive defensin downregulation was unexpected, given that CD8^+ T cells themselves do not produce defensins, and it suggests that the contribution of
defensins to controlling virus may be greater than previously appre-
ciated. Further studies of the noncytolytic mechanisms con-
tributing to suppression of the viral load will hopefully lead to
novel concepts for vaccine design and immunotherapy against
HIV infection.

ACKNOWLEDGMENTS
This work was supported by NIH grant RO1-AI90797 to G.S. and by Office
of Research Infrastructure Programs P51OD11107 and (formerly) NCCR
P51RR169. We are grateful for the contributions of Jim Else, Tracey Meeker,
Stephanie Ehner, Mirko Piaridini, Greg Tharp, Ben Isett, and Don
Baldwin.

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