Cytokine Expression, Natural Killer Cell Activation, and Phenotypic Changes in Lymphoid Cells from Rhesus Macaques during Acute Infection with Pathogenic Simian Immunodeficiency Virus

LUIZ D. GIAVEDONI,1,2* M. CRISTINA VELASQUILLO,1 LAURA M. PARODI,1 GENE B. HUBBARD,2,3 AND VIDA L. HODARA1

Department of Virology and Immunology,1 Department of Laboratory Animal Medicine,3 and Southwest Regional Primate Research Center,2 Southwest Foundation for Biomedical Research, San Antonio, Texas 78245

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We studied the innate and adaptive immune system of rhesus macaques infected with the virulent simian immunodeficiency virus isolate SIVmac251 by evaluating natural killer (NK) cell activity, cytokine levels in plasma, humoral and virological parameters, and changes in the activation markers CD25 (interleukin-2R [IL-2R] α chain), CD69 (early activation marker), and CD154 (CD40 ligand) in lymphoid cells. We found that infection with SIVmac251 induced the sequential production of interferon-α/β (IFN-α/β), IL-18, and IL-12. IFN-γ, IL-4, and granulocyte-macrophage colony-stimulating factor were undetected in plasma by the assays used. NK cell activity peaked at 1 to 2 weeks postinfection and paralleled changes in viral loads. Maximum expression of CD69 on CD3−CD16+ lymphocytes correlated with NK cytotoxicity during this period. CD25 expression, which is associated with proliferation, was static or slightly down-regulated in CD4+ T cells from both peripheral blood (PB) and lymph nodes (LN). CD69, which is normally present in LN CD4+ T cells and absent in peripheral blood leukocyte (PBL) CD4+ T cells, was down-regulated in LN CD4+ T cells and up-regulated in PBL CD4+ T cells immediately after infection. CD8+ T cells increased CD69 but not CD25 expression, indicating the activation of this cellular subset in PB and LN. Finally, CD154 was transiently up-regulated in PBL CD4+ T cells but not in LN CD4+ T cells. Levels of antibodies to SIV Gag and Env did not correlate with the level of activation of CD154, a critical costimulatory molecule for T-cell-dependent immunity. In summary, we present the first documented evidence that the innate immune system of rhesus macaques recognizes SIV infection by sequential production of proinflammatory cytokines and transient activation of NK cytotoxic activity. Additionally, pathogenic SIV induces drastic changes in the levels of activation markers on T cells from different anatomic compartments. These changes involve activation in the absence of proliferation, indicating that activation-induced cell death may cause some of the reported increase in lymphocyte turnover during SIV infection.

The immune system of higher vertebrates consists of innate and adaptive components. Innate immunity exhibits immediate recognition and response without prior sensitization. Cells of the innate immune system (i.e., monocytes/macrophages, natural killer [NK] cells, and polymorphonuclear leukocytes) recognize pathogen-associated molecular patterns and activate events such as phagocytosis, induction of the synthesis of antimicrobial peptides, expression of inflammatory and effector cytokines and chemokines, induction of nitric oxide synthase in macrophages, and expression of costimulatory molecules on antigen-presenting cells. The adaptive immune system uses somatically generated antigen receptors that are clonally distributed on T and B lymphocytes. Generally, adaptive immune recognition in the absence of innate immune recognition results in inactivation of lymphocytes that express receptors involved in the identification events (20). Thus, innate immune responses have critical consequences in adaptive immune responses.

Little is known of the contribution of the innate immune system during infection with the human immunodeficiency virus (HIV). Based on similarities of biologic and genetic features, simian immunodeficiency virus (SIV) infection of rhesus macaques provides the best animal model of HIV infection and AIDS. Accordingly, this animal model is critical for the elucidation of mechanisms of pathogenesis and for the development of vaccines and antiviral therapies (12). As with almost all viral infections, the innate immune system is thought to be the first component of the immune system that recognizes SIV infection. However, few studies have methodically analyzed the changes induced in cell phenotype and cytokine levels by SIV infection. Recent studies have demonstrated that SIV infection results in a generalized increase in lymphocyte turnover (23) and that the primary site for viral replication is activated memory CD4+ T cells that are present in the intestinal lamina propria (46). Although cellular changes are not that dramatic at this early stage in peripheral lymphoid tissue, peripheral blood (PB) and lymph nodes (LN) still reflect the pathologic changes induced by the viral infection and are readily available for longitudinal studies.

To analyze changes in the activation state of cells from the innate and adaptive immune system after SIV infection, we evaluated NK activity, cytokine levels in plasma, and changes in activation markers on lymphoid cells of rhesus macaques after infection with pathogenic SIVmac251. We found the sequential appearance in plasma of interferon-α/β (IFN-α/β) interleukin-18 (IL-18) and IL-12, whereas IL-4, IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) remained undetectable. We also found transient activation of NK cells during the peak of viral replication, and this activation

* Corresponding author. Mailing address: Southwest Foundation for Biomedical Research, P.O. Box 760549, San Antonio, TX 78245-0549. Phone: (210) 258-9603. Fax: (210) 670-3310. E-mail: Lgiavedo@icarus.sfbr.org.
not was predictive of disease progression. Finally, we observed that after SIV infection, both CD4+ and CD8+ T cells became activated in the absence of markers for proliferation, suggesting that the increased turnover of these cells reflects activation-induced cell death rather than differential compartmentalization.

**MATERIALS AND METHODS**

**Infection of rhesus macaques.** Four colony-bred, weight- and age-matched adult male rhesus macaques (Macaca mulatta) seronegative for simian type D retrovirus and simian leukemia virus antigens were used in this experiment. The animals were used and cared for in accordance with the American Association for Accreditation of Laboratory Animal Care Guidelines. The macaques were used and cared for in accordance with the American Association for Accreditation of Laboratory Animal Care Guidelines. The macaques were inoculated intravenously with 1 ml of RFP1 1640 containing 100% tissue culture infective doses (TCID50) of SIVmac251. The animals were euthanized when they showed three or more of the following clinical symptoms: (i) weight loss greater than 10% in 2 weeks or 30% in 2 months; (ii) chronic diarrhea that was unresponsive to treatment; (iii) infections that were unresponsive to antibiotic treatment; (iv) inability to maintain body heat or fluids without supplementation; (v) persistent, marked hematologic abnormalities including lymphopenia, anemia, thrombocytopenia, or neutropenia, and (vi) persistent, marked splenomegaly or hepatomegaly.

Cell cultures. Cells, CMX-174 cells, rhesus peripheral blood mononuclear cells (PBMCs), and LN cells were used for SIV isolation and propagation. These cells and the NK-sensitive human erythroleukemia cell line K562 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 50 U/ml of penicillin, 50 mg/ml of streptomycin, and 2 mM of L-glutamine (Cellgro Mediatech, Herndon, Va.) per ml. Human A549 cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum and antibiotics.

SIVmac251 , a pathogenic biological isolate, was provided kindly by J. Allan (Southwest Foundation for Biomedical Research, San Antonio, Tex.); the virus was propagated in rhesus PBMCs and subjected to titration determination in CMX-174 cells. Erythroleukemia cells (EMV) used for the assay of IFN-γ in plasma, was propagated in human A549 cells.

**Measurements in plasma.** Plasma p27 antigenemia was measured by a commercial SIV core antigen capture enzyme-linked immunosorbent assay (ELISA) (Coulter Corp., Hialeah, Fla.) as instructed by the manufacturer (sensitivity of 30 pg/ml). The levels of GM-CSF, IL-4, IL-12, and IFN-γ were determined using commercial ELISA kits (Immunotech for GM-CSF and IL-4, and Cytoscreen Monkey IFN-γ and IL-12 from BioSource, Camarillo, Calif.). The limits of detection were 5 pg/ml for GM-CSF and IL-4 and 4 pg/ml for IFN-γ and IL-12. The levels of IL-18 in plasma were determined with an ELISA kit kindly provided by H. Okamora (Hyogo College of Medicine and Hayashibara Corp.) as described previously (41). The sensitivity of this assay was 10 pg/ml.

IFN activity in plasma was determined by measuring the inhibition of the cytopathic effect caused by EMCV infection in A549 cells (15). Plasma samples were diluted threefold in DMEM. Aliquots (50 µl) of these dilutions were plated in 96-well plates and 105 A549 cells in 100 µl of DMEM with 10% fetal calf serum were added to each well. After 24 h of incubation, the cells were washed and incubated with 10 µg/ml of p27 protein in EMV. Units of antiviral activity were expressed as the reciprocal of the dilution of plasma that afforded 50% protection against EMCV infection.

**LN biopsies.** Peripheral LN (axillary and/or inguinal) were obtained by transcutaneous biopsy under ketamine HCl anesthesia (10 mg/kg, injected intramuscularly) (Parke-Davis, Morris Plains, N.J.) before and at 2, 4, 12, and 24 weeks postinfection (p.i.). The LN were divided axially into two fractions for single-cell preparation and pathologic analysis. Lymphocyte suspensions were obtained by mechanical teasing of tissues. The fractions for pathologic analysis were fixed in 10% buffered formalin, processed conventionally, cut at 5 µm, and stained with hematoxylin and eosin (H+E) for routine histologic examination. Tissues were examined blinded for changes in lymphoid architecture and cellularity.

**Cell-associated viral loads.** Cell-associated virus, latent or productive, was assayed in whole blood or LN cells for the SIV major core protein (p27) by ELISA (19). Cultures were recorded as positive for virus when p27 antigen was detected at two consecutive CEM time points. End-point cultures were maintained and tested for 4 weeks before being scored as negative. Titers were calculated by the method of Reed and Muench (29) and expressed as TCID50 per 106 cells.

**Lymphocyte phenotyping.** Phenotypic characterization of lymphocytes in PB and LN was performed by flow cytometry using three-color direct immunofluorescence. Surface marker expression on CD3+ cells was assessed at 100% light scatter. Briefly, 96-well ELISA plates (Immulon II; Dynex Technologies) were coated overnight with 10 µg/ml of the indicated antigens. Plates were disrupted with 1% Triton–phosphate-buffered saline (PBS), and 0.5 ml of the viral preparation was added to each well. Plates were washed four times with culture medium and adjusted to a concentration of 5 × 105 cells/ml 24 h prior to testing to ensure that they were in log phase and not in a metabolically toxic environment. The cells were washed once with phenol red-free RPMI 1640 supplemented with 2.5% heat-inactivated fetal calf serum (cRPMI-2.5). K562 target cells were resuspended in cRPMI-2.5 at 2 × 106 cells/ml and labeled with 10 µM calcein-AM for 30 min at 37°C in room air. The cells were washed four times in culture medium and adjusted to a concentration of 5 × 105 cells/ml. Rhesus PBMC were washed twice with cRPMI-2.5. Cytotoxicity assays were carried out in round-bottom microculture plates. Effector cells were plated at serial dilutions of the stock preparation to give effector-to-target cell ratios of 45:1, 15:1, 5:1, and 1:1. Target cells were added, and the plates were incubated for 4 h at 37°C in an atmosphere of 95% air–5% CO2. Target cells were also incubated in medium alone and with 2% Triton (Sigma) for estimations of spontaneous and maximum release. Aliquots of 110 µl of supernatant were removed from each well and transferred to 96-well flat-bottom microtiter plates (Microfluor-Black; Dynex Technologies, Chantilly, Va.) for reading calcein fluorescence in each well using a fluorometer with a 485-nm excitation filter and an emission filter setting of 530/20. The levels of calcein fluorescence were subtracted from the values of the maximum, spontaneous, and percentage of killing; the level of killing for each condition was calculated as (experimental fluorescence – spontaneous fluorescence) × 100 (maximum fluorescence – spontaneous fluorescence). To calculate lytic units for each animal at each time point, the effector-to-target ratio was adjusted by considering the percentage of CD3+ CD8+ lymphocytes in the PBMC population. Graphs were prepared by plotting the corrected effector-to-target ratio in log scale on the abscissa and the percent killing on the ordinate, and the number of effector cells that killed 10% of the target cells was calculated by extrapolation. Finally, this value was used to calculate the number of lytic units per 106 NK cells.

**Analysis of the humoral immune response of rhesus macaques.** Plasma samples were analyzed for the presence of antibodies reactive to SIV envelope glycoprotein using a competitive ELISA. For this assay, a viral preparation from a viral preparation of SIVmac239 concentrated by 20% sucrose cushion centrifugation. The protein content of the viral preparation was determined with the protein quantification kit (Bio-Rad, Hercules, Calif.). Competitive ELISA antibodies were titrated as previously described (8, 31). Briefly, 96-well ELISA plates (Immulon II; Dynex Technologies) were coated with 0.5 µg of concanaval A (ConA; Sigma) per well. Virus particles were disrupted with 1% Triton–phosphate-buffered saline (PBS), and 0.5 µg of viral protein/well was adsorbed overnight at 4°C. The plates were washed four times with washing buffer (0.15 M NaCl, 0.05% Tween 20). Nonfat milk in PBS (5%; Blotto) was added to block unreacted ConA binding sites. The plates were shaken for 90 min at 37°C. Aliquots (100 µl) of serial fourfold dilutions of monkey plasma (starting 1:100) were added to the wells. Plasma samples were incubated at 37°C, shaken for 1 h, and washed, and peroxidase-conjugated anti-monkey immunoglobulin G (Kierkegaard) was added for a 1-h incubation with shaking at 37°C. After washing, 200 µl of TM-Blue (Sigma) in 1% perborate buffer (50 mM NaH2PO4, 25 mM citric acid, 19.5 mM NaBO3) was added to each well. After color development, the reaction was stopped with 50 µl of 2 N sulfuric acid and the optical density at 450 nm (OD450) was measured in an automated plate reader. End point titers were determined as the dilution that generated an OD450 twice the value of the blank.

For the anti-p27 antibody ELISA, the disrupted, envelope-depleted viral preparation was added to ELISA plates previously coated with anti-p27 antibodies (19) and incubated overnight at 4°C. Serial fourfold dilutions of monkey plasma (starting 1:100) were added to the wells. Plasma samples were incubated at 37°C, shaken for 1 h, and washed, and peroxidase-conjugated anti-monkey immunoglobulin G (Kierkegaard) was added for a 1-h incubation with shaking at 37°C. After washing, 200 µl of TM-Blue (Sigma) in 1% perborate buffer (50 mM NaH2PO4, 25 mM citric acid, 19.5 mM NaBO3) was added to each well. After color development, the reaction was stopped with 50 µl of 2 N sulfuric acid and the optical density at 450 nm (OD450) was measured in an automated plate reader. OD450 values were determined as the dilution that generated an OD450 twice the value of the blank.

**Determination of anti-SIVgp160 antibody avidity.** The antibody avidity index values of plasma antibodies to the SIVmac239 envelope glycoproteins were determined using the Rafter assay (Rafter et al. 1996). ELISA. This method, as previously described (8), measures the resistance of antibody-envelope glycoprotein immune complexes. Briefly, plasma samples were diluted to produce an OD450 of
1 to 1.5 in the ConA ELISA procedure. Following plasma incubation, the plates were treated three times for 5 min each with PBS (pH 7.4) or a solution of 8 M urea in PBS. This treatment was followed by incubation with peroxidase-conjugated anti-monkey immunoglobulin G (1:5,000). After color development, the reaction was stopped with 50 µl of 2 N sulfuric acid and the OD450 was measured in an automated plate reader. The avidity index was then calculated from the ratio of the absorbance obtained with urea treatment to that with PBS and then multiplied by 100.

Statistical analyses. Correlation analysis was performed by using the Pearson product moment correlation coefficient. Baseline and follow-up data were compared using the paired t test or Wilcoxon matched-pairs test, according to the type of distribution of the variables.

RESULTS

Four adult rhesus macaques (identification numbers 863, 868, 876, and 880) were inoculated intravenously with 1 ml of RPMI 1640 containing 100 TCID50 of the pathogenic isolate SIVmac251. Blood samples and peripheral LN were obtained periodically before and after infection. All animals became infected, and virus was isolated from PBMC by 1 week p.i. (data not shown). SIV infection of rhesus 880 progressed rapidly, and the animal was euthanized at 20 weeks p.i. due to severe immunodeficiency. Postmortem examination of this animal showed profound lymphoid depletion in the LN, spleen, and intestinal tract, adenovirus pancreatitis and gastritis with protozoal colonization, and necrotizing hepatitis. Rhesus 876 also developed immunodeficiency and died at 32 weeks p.i. (data not shown). SIV infection of rhesus 880 progressed rapidly, and the animal was euthanized at 20 weeks p.i. due to severe immunodeficiency. Postmortem examination of this animal showed profound lymphoid depletion in the LN, spleen, and intestinal tract, adenovirus pancreatitis and gastritis with protozoal colonization, and necrotizing hepatitis. Rhesus 876 also developed immunodeficiency and died at 32 weeks p.i. Rhesus 863 and 868 became chronically infected and had moderate lymphadenopathy but did not show signs of immunodeficiency throughout the course of this experiment (32 weeks). Serial histological analysis of peripheral LN obtained from the rapid progressors 876 and 880 showed a rapid onset of lymphadenopathy, characterized by destruction of the LN architecture, absence of germinal centers, and profound lymphoid depletion. The results of pathologic analysis of LN from the slow progressors 863 and 868 were not remarkably different from those found for the rapid progressors during the first 4 weeks p.i. However, LN obtained from these animals at later time points showed the concomitant presence of areas of hyperplasia and active germinal centers and zones of moderate lymphoid depletion (data not shown).

Changes associated with innate immunity. Plasma samples were analyzed for the presence of IL-12, IL-18, SIVp27, IFN-γ, IL-4, and GM-CSF by specific ELISA and for IFN-induced antiviral activity by a biological assay. We determined that IL-18 is usually detected at low levels (200 to 400 pg/ml) in uninfected animals. However, after infection with SIV, increments in the level of IL-18 in plasma were observed in all animals during the first 2 to 3 weeks p.i., with those in two macaques reaching concentrations of 3,000 pg/ml or higher (Fig. 1A, 868 and 880). In contrast to IL-18, the levels of IL-12 did not change immediately after infection (Fig. 1B). For rhesus 863, 868, and 876, the IL-12 levels reached a maximum by 3 to 4 weeks p.i. and then gradually declined. Rhesus 880 had unusually high levels of IL-12 in plasma before challenge, and these levels dropped continuously until the time of death. The extent of viral replication, as measured by the concentration of SIV p27 in plasma, coincided with that of IL-18, reaching a peak by 2 weeks p.i. (Fig. 1C). However, there was no correlation between IL-18 and SIV p27 levels for individual animals.
Rhesus 880 had the highest level of SIV p27 during the peak of viral replication; antigen was always detectable after that, and it increased to higher levels at the onset of AIDS. The level of antiviral activity in plasma, measured as the ability of plasma to prevent EMCV-mediated cytopathic effects on A459 cells, is an indicator of the presence of IFN-γ and/or IFN-α/β (Fig. 1D). This antiviral activity was undetectable in all macaques before infection. However, all animals showed the transient appearance of antiviral activity by 1 week p.i., which was no longer detectable in three macaques by 4 weeks. The exception was rhesus 880, which had continuously increasing values of antiviral activity until the time of necropsy. The other rapid progressor, rhesus 876, became positive again after 8 weeks p.i. Interestingly, the levels of IL-4, IFN-γ, and GM-CSF in plasma remained below the limit of detection of the respective assays at all time points (data not shown). The inability to detect IFN-γ in the same samples by a very sensitive ELISA, combined with the lack of reduction in antiviral activity after combining plasma with a neutralizing antibody to IFN-γ, points to IFN-α/β as being responsible for the antiviral activity found in plasma.

The activation of NK cells was studied by a fluorogenic cytotoxicity assay on K562 cells and by the determination of the percentage of CD69+/− NK cells. The specific killing activity of NK cells increased immediately after SIV infection, reaching a peak by 2 weeks p.i. and decreasing afterward (Fig. 2A). For rhesus 880, NK cytotoxicity was absent after the initial peak, whereas for the other three animals, a secondary increase in activity was observed after 20 weeks p.i. Similarly, the percentage of CD69+ NK cells increased after infection, reaching a peak by 2 weeks p.i. (Fig. 2B). After the initial peak, a constant increase was observed for the rapid progressors 876 and 880, whereas rhesus 863 and 868 had relatively constant values. There was a good correlation between the values of NK killing activity and CD69+ NK cells during the first 12 weeks p.i. (Fig. 2C, \( R^2 = 0.89637, P = 0.04 \)).

**Changes in activation markers of T cells after SIV infection.**

Lymphocytes from PB and lymphoid organs were analyzed by cell surface staining and flow cytometry for cell subset composition and for expression levels of CD25, CD69, and CD154. The absolute number of B cells in the PB dropped during the first 2 to 3 weeks of infection, remained generally at lower levels than the values before infection, and increased dramatically at the time of AIDS for the rapid progressors (rhesus 876 and 880). In LN, however, the proportion of B cells increased at 2 weeks p.i. and then returned to preinfection levels, except for rhesus 880, which had escalating values of B cells (Fig. 3, top panels). For CD4+ T cells, the absolute numbers in PB increased immediately after infection for most animals and remained relatively constant. Macaque 880 showed a progressive decline in the percentage of CD4+ T cells (data not shown), but there was a remarkable lymphocytosis by 16 and 20 weeks p.i. that resulted in increased absolute numbers of CD4+ T cells and B cells. In LN, the percentage of CD4+ T cells declined abruptly by 2 weeks p.i. and continued to decrease more slowly afterward for all animals (Fig. 3, second row). For CD8+ T cells, SIV infection resulted in an increase in the absolute number of PB that varied considerably from animal to animal, whereas this increase was discrete in LN. Macaque 880 had a sharp decline in cellularity and the content of CD8+ T cells in LN at the time of death (Fig. 3, third row), which may explain the sharp increase in the percentage of B cells. Finally, the proportion of NK cells slightly increased after infection and varied considerably from animal to animal and for each animal individually (Fig. 3, bottom row, left panel). The percentage of total T cells (CD4+ and CD8+) in LN decreased over the course of infection. For the rapid progressor 880, T cells, which comprised 90% of all lymphoid cells in the LN before infection, declined to 30% at the time of death (Fig. 3, bottom row, right panel).

**Activation markers on T cells isolated from PB or from LN were modified after infection with SIVmac251.** Proliferating CD4+CD25+ T cells from PB peaked by 1 week p.i. and then showed a slight decrease in absolute number. The proportion of CD4+CD25+ T cells in LN did not show the same increase p.i.; instead, the rapid progressors showed a steady decline in the proportion of cells expressing the IL-2Rα chain (Fig. 4, A). For CD4+ T cells, the absolute numbers in PB increased immediately after infection for most animals and remained relatively constant. Macaque 880 showed a progressive decline in the percentage of CD4+ T cells (data not shown), but there was a remarkable lymphocytosis by 16 and 20 weeks p.i. that resulted in increased absolute numbers of CD4+ T cells and B cells. In LN, the percentage of CD4+ T cells declined abruptly by 2 weeks p.i. and continued to decrease more slowly afterward for all animals (Fig. 3, second row). For CD8+ T cells, SIV infection resulted in an increase in the absolute number of PB that varied considerably from animal to animal, whereas this increase was discrete in LN. Macaque 880 had a sharp decline in cellularity and the content of CD8+ T cells in LN at the time of death (Fig. 3, third row), which may explain the sharp increase in the percentage of B cells. Finally, the proportion of NK cells slightly increased after infection and varied considerably from animal to animal and for each animal individually (Fig. 3, bottom row, left panel). The percentage of total T cells (CD4+ and CD8+) in LN decreased over the course of infection. For the rapid progressor 880, T cells, which comprised 90% of all lymphoid cells in the LN before infection, declined to 30% at the time of death (Fig. 3, bottom row, right panel).

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top row). The absolute number of activated CD4+ CD69+ T cells in PB reached a peak at 1 week p.i., declined afterward to levels similar to the ones before challenge, and had a secondary increase after 16 weeks p.i. Conversely, the percentage of CD4+ CD69+ T cells in LN declined by 2 to 4 weeks p.i. and slowly returned to preinfection levels. The rapid progressor 880 showed almost no CD4+ CD69+ T cells in circulation and a sharp increase in the number of these cells in LN at the time of death (Fig. 4, second row). The absolute number of CD4+ T cells expressing CD154 in PB reached a maximum at 2 weeks p.i., coinciding with the peak of viremia, and returned to preinfection levels by 4 weeks. The percentage of CD4+ CD154+ T cells in LN did not change drastically after the initiation of the infection, with the exception of rhesus 876, which showed
an unusually large number of CD4$^+$ CD154$^+$ T cells on the day of infection (Fig. 4, bottom row).

Changes for CD25 expression on CD8$^+$ T cells were slightly different from those on CD4$^+$ T cells. A smaller absolute number of CD8$^+$ T cells expressed CD25 in PB, and the changes after infection were not statistically significant. Similarly, the percentage of CD8$^+$ T cells expressing CD25 in LN was smaller than the percentage of CD4$^+$ CD25$^+$ T cells, and there was no significant variation after infection (Fig. 5, top row). However, the number of activated CD8$^+$ CD69$^+$ T cells increased substantially in both PB and LN after infection (Fig. 5, bottom row).

Humoral immune response to infection. The presence of antibodies directed at SIV gp160 and Gag in infected macaques was evaluated by antigen-specific ELISA. Purified virus was used as a source of gp160 for plates coated with ConA and as a source of Gag for plates coated with a murine MAb specific for SIV p27. Antibodies to SIV gp160 were detected by 4 weeks p.i. in all animals. However, these antibodies were transient for rhesus 880 and were no longer detected by 8 weeks p.i. Macaques 863 and 868 mounted a strong humoral response, while rhesus 876 had a constant, low-titer anti-gp160 antibody production (Fig. 6A). The humoral immune response to SIV Gag had a different outcome. The rapid progressor, rhesus 880, failed to make antibodies, whereas rhesus 876 had a transient low-titer humoral response that became undetectable by 20 weeks p.i. Rhesus 863 and 868 mounted strong anti-Gag humoral responses, with titers for 863 being more than 1 order of magnitude higher than the ones for 868 (Fig. 6B). The avidity of the anti-SIV gp160 antibodies was analyzed with samples obtained 16 and 20 weeks p.i. Although higher in anti-SIV gp160 ELISA titer at 20 weeks p.i., the avidity for rhesus 868...
antibodies was slightly lower than that observed for macaque 863. Coincidental with its low titer, the gp160 avidity of rhesus 876 antibodies was very poor at both time points (Table 1).

**DISCUSSION**

**Innate immune responses of rhesus macaques to SIV infection.** The innate immune system reacts to microbial invaders with the production of cytokines and the activation of its cellular components. Cytokines that are produced by cells of the innate immune system include IFN-α/β, transforming growth factor β, tumor necrosis factor, IL-1, IL-6, IL-10, IL-12, IL-15, and IL-18. A characteristic innate cytokine response to viral infections is the early production of IFN-α/β (4). As shown in this study, infection with SIV also results in early production of IFN-α/β, which precedes the detection of SIVp27 in plasma.

**FIG. 5.** Changes in the level of expression of activation markers on CD8 T lymphocytes from rhesus macaques in PB (total cell number) and LN (percentage of the CD8 T-cell population) after infection with SIVmac251.

**FIG. 6.** Humoral immune response of rhesus macaques infected with SIVmac251. (A) Anti-gp160 antibodies titer were determined on ELISA plates treated with ConA and detergent-disrupted SIVmac239. (B) Anti-Gag antibodies titer were determined on ELISA plates treated with an anti-SIVp27 murine MAb and detergent-disrupted SIVmac239.
and usually becomes undetectable after the peak of viremia (Fig. 1). In general, we have observed that high levels of IFN-\(\alpha/\beta\) correlate with high viral loads and that persistent presence of IFN-\(\alpha/\beta\) in plasma is associated with rapid disease progression or onset of AIDS (reference 14 and this study). The triggering event for the release of IFN-\(\alpha/\beta\) appears to be the interaction between the mannose receptor of PB dendritic cells and the glycosylated viral envelope protein (22). It has been shown that IFN-\(\alpha\) has a potent in vitro antiviral effect on SIV by blocking steps between attachment and reverse transcription (42). However, as we show in this study, IFN-\(\alpha\) does not seem to be sufficient to control SIV infection in vivo in the absence of other antiviral immune mechanisms. For example, the constant high levels of IFN-\(\alpha/\beta\) in rhesus 880 were not effective in limiting SIV replication.

Another important cytokine is IL-12, or NK cell-stimulatory factor, a heterodimeric cytokine produced by phagocytic cells of the innate immune system (monocytes, macrophages, and neutrophils) and B cells (44). The ELISA for IL-12 used in this study detects the inducible p40 component that constitutes the biologically active p70 heterodimer. IL-12 exerts its biological activity in T and NK cells, inducing the production of IFN-\(\gamma\), enhancing the generation of cytotoxic cells, and stimulating antigen-activated lymphocytes. The production of IL-12 in response to infections represents an important functional link between effector cells of innate resistance (phagocytic and NK cells) and effector cells of adaptive resistance (T and B lymphocytes). However, production of IL-12 has been detected in some but not all viral infections (10, 27) and can be experimentally blocked by IFN-\(\alpha/\beta\) (5). Similarly, our study shows that during SIV infection, the levels of IL-12 in plasma do not increase until the levels of IFN-\(\alpha/\beta\) drop (Fig. 1). Likewise, as in the case of the rapid progressor 880, increasingly higher levels of IFN-\(\alpha/\beta\) were accompanied by decreasing levels of IL-12.

IL-18, or IFN-\(\gamma\)-inducing factor, is another proinflammatory cytokine produced by monocytes/macrophages, keratinocytes, cells from the zona reticularis and zona fasciculata of the adrenal cortex, and brain microglia and astrocytes (9, 26). We determined the concentration of this cytokine by an ELISA that detects primarily the biologically active molecule (41). Because of its synergistic effect with IL-12 on activation of NK and T cells, it has been suggested that IL-18 is also an important link between the innate and adaptive immune systems (26). Nevertheless, the role of IL-18 during viral infections is not well known. A recent report demonstrated that the in vitro infection of macrophages with influenza A virus resulted in the production of IL-18 in the absence of IL-12 (35). Similarly, we show in this study that SIV infection of rhesus macaques results in rapid production of IL-18, which seems to follow the appearance of IFN-\(\alpha/\beta\) and precede the production of IL-12. Although the pattern was similar for all animals, we could not find a correlation between peak levels of IL-18 and either viremia or levels of IFN-\(\alpha/\beta\) in plasma.

NK cells contribute to resistance during the early phases of many viral infections, and it has been postulated that they may influence the selection and activation of an appropriate type of adaptive immunity (32). During a typical in vivo viral infection, NK cells are activated by IFN-\(\alpha/\beta\), IL-12, IL-18, and other cytokines, as well as by a variety of viral glycoproteins (16). When virus-specific cytotoxic T-cell responses start to develop, NK cell activity declines and returns to preinfection levels. Therefore, it has been proposed that NK cells might influence cytotoxic T lymphocyte responses either by providing a differentiation signal to CD8\(^+\) cytotoxic T-lymphocyte precursors or by stimulating CD8\(^+\)T-cell proliferation (16). We observed that infection with SIV resulted in increased NK cytotoxicity that reached a peak by 2 weeks p.i., coincidental with the peak of viremia. This increment in innate cytotoxicity correlated inversely with antigenemia levels. Interestingly, the cytotoxic activity of NK cells was not apparently affected by the levels in plasma of IL-12 and IL-18, cytokines that have very potent in vitro NK-activating activity. The peak of NK cytotoxic activity preceded the increment of IL-12, and there was no correlation between IL-12 concentration and cytotoxic activity. Another unusual observation was the lack of correlation between NK activation and levels in plasma of cytokines that are known to be produced by activated NK cells, such as IFN-\(\gamma\), tumor necrosis factor alpha, GM-CSF, M-CSF, IL-2, IL-3, IL-5, and IL-8 (43). More importantly, NK cell-produced IFN-\(\gamma\) has been shown to act as a critical antiviral mediator against several viruses (4, 28). Interestingly, we did not find detectable levels of IFN-\(\gamma\) or GM-CSF in plasma during the first weeks of SIV infection, even at the peak of NK cell cytotoxicity. This finding is in agreement with the low levels of IFN-\(\gamma\) mRNA found in PBMC of cynomolgus macaques infected with SIVmac251 (2). Conversely, our findings do not preclude the local rather than systemic production of IFN-\(\gamma\) in lymphoid tissues of infected macaques, as has been demonstrated by others (7, 50). However, because CD3\(^+\) CD16\(^+\) CD56\(^+\) NK cells are not usually found in macaque LN (reference 39 and our own observations), CD3\(^+\) T cells must be the main producers of IFN-\(\gamma\) in these tissues.

The CD69 antigen is one of the earliest markers expressed on all activated T, B, and NK lymphocytes following stimulation by a variety of mitogenic agents. Recent in vitro studies have shown that IL-12 induces increase in CD69 expression only on NK cells (48) and that CD69 expression on NK cells identifies cells in a state of postfunction anergy, not cells that are preactivated and ready to function (11). In our in vivo observations in macaques infected with SIV show that NK cells up-regulate CD69 before the peak of IL-12 and that this CD69 up-regulation correlates very well with NK cell cytotoxicity during the first 8 to 12 weeks of infection. The second wave of CD69 up-regulation in NK cells, during the chronic stage of infection, is a reflection of the activation state seen for all lymphoid cells. An interesting observation is that the peak of NK activity seems to be short-lived and is not necessarily coincident with the appearance of cytotoxic T lymphocytes. Although we did not determine the presence of SIV-specific cytotoxic T lymphocytes in our infected macaques, it has been demonstrated that the induction of these cells requires help by CD4\(^+\) T-cell (1, 30, 37). For example, the rapid progressor 880 failed to make antibodies against SIVGag, an antigen for which CD4\(^+\) T help is required (24), and most probably did not elicit anti-SIV cytotoxic T lymphocytes; however, this animal still demonstrated a peak of NK activity that coincided with the peak of viremia and resulted in a transient reduction in antigenemia. More indirect evidence for the role of NK cells in primary SIV infection comes from experiments in which macaques were depleted of their CD8\(^+\) lymphocytes (36). The experimental elimination of CD8\(^+\) cells in rhesus macaques at

### Table 1. Avidity of anti-SIVgp160 antibodies

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<thead>
<tr>
<th>Macaque</th>
<th>Avidity of antibodies at:</th>
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<tbody>
<tr>
<td></td>
<td>16 wk p.i.</td>
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<tr>
<td>863</td>
<td>85</td>
</tr>
<tr>
<td>868</td>
<td>81</td>
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<tr>
<td>876</td>
<td>38</td>
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<td>880</td>
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* Percentage of antibody binding in the presence of 8 M urea.
the time of SIV exposure resulted in uncontrolled SIV replication and rapid disease progression. However, the antibody used for those experiments recognized the CD8 \( \alpha \) chain, which is present in CD8\( \alpha \beta \) T cells as well as in CD8\( \alpha \) NK cells. Taken together, there is an indication that NK cells contribute to the initial containment of primary SIV infection but are ineffective in the absence of an appropriate cytotoxic T lymphocyte response.

In summary, we show that the innate immune system of rhesus macaques reacts to SIV infection with the sequential production of IFN-\( \alpha/\beta \), IL-18, and IL-12. NK cells are activated by IFN-\( \alpha/\beta \), reach their maximum cytotoxic activity at the time of peak viremia, and contribute to the initial immune containment of infection.

**Adaptive immune responses of rhesus macaques to SIV infection.** Dramatic changes in the number and phenotype of the cells that constitute the adaptive immune system can be seen early after infection with SIV. Whether these reductions in cell numbers represent actual disappearance of cells or redistribution to other body compartments is still a matter of debate (33). Initially, the virus replicates preferentially in activated memory CD4\(^+\) T cells that are present in the intestinal lamina propria, which becomes rapidly depleted of these cells (46). Our sequential analysis of SIV-infected macaques shows that by 2 weeks p.i., a similar type of CD4\(^-\) T-cell depletion is noticeable in peripheral lymphoid tissue but not in PB, even though depletion of peripheral CD4 T cells and B cells has been associated with rapid disease progression in SIV- and SHIV-infected macaques (13, 40). We observed a slight reduction in levels of circulating B cells, but we did not find a correlation between this decline and disease progression or antibody production. Interestingly, the percentage of B cells in LN did not change drastically with infection. For the rapid progressors, the combined pathological examination and analysis of the cell composition of the LNs showed a loss of LN architecture, T-cell depletion, and internal redistribution of B cells from germinal centers to other areas of the LN.

In general, T-cell responses to viruses are modulated substantially during systemic infections. There is an induction phase associated with a massive virus-specific CD8 T-cell response, an apoptosis phase during which the T cells become sensitized to activation-induced cell death, a silencing phase during which the T-cell number and activation state are reduced, and, finally, a memory phase associated with the very stable preservation of virus-specific memory cytotoxic T lymphocyte precursors (47). However, these phases are not clearly present during an unresolved, chronically active viral infection. Several studies have reported an increased turnover for lymphocytes (CD4 and CD8 T, B, and NK cells) in SIV-infected macaques (23, 34). This increase in the rate of cell proliferation and death has been linked to general cell activation, direct cell killing induced by the virus, and/or apoptosis. CD69 is usually undetectable on the plasma membrane of resting PBMCs but is rapidly expressed on antigen- or mitogen-stimulated T, B, and NK lymphocytes (3). In T cells constantly exposed to antigen, such as T cells in the germinal center and pericortical zone of the tonsils and LNs, the expression of CD69 is continuous (48). In our study, the preinfection percentage of CD69\(^+\) CD4\(^+\) T cells in LN was around 30% of all CD4 T cells, whereas in PB it was only 3%. The activation marker CD25, the \( \alpha \)-chain component of the high-affinity IL-2 receptor, is also expressed on stimulated T and B cells, but its expression is delayed with respect to CD69, it is more stable, and it is an indication of cell proliferation (21). CD69 expression is an early biochemical event in cell signaling that does not necessarily reflect T-cell proliferation under all conditions (6). For example, activation of CD4 T lymphocytes with *Staphylococcus* enterotoxin B resulted in incorporation of 5-bromodeoxyuridine in only a fraction of CD69\(^+\) cells, whereas all CD25\(^+\) cells incorporated 5-bromodeoxyuridine (21). Other examples of accumulation of CD25\(^-\) CD69\(^+\) lymphocytes include tumor-infiltrating lymphocytes from patients suffering from cervical carcinoma (38) and superantigen-stimulated T cells (18). Similarly, we observed in SIV-infected macaques an increased accumulation of CD4 and CD8 T cells expressing CD69 but not CD25. These data suggest increased activation with reduced proliferation, which could result in increased activation-induced cell death and in the high turnover rates observed during SIV infection.

Recent studies in murine and primate models have raised some questions on the concept of bystander activation by demonstrating that at the peak of some primary and secondary immune responses to viral infection, 50 to 70% of the activated CD8\(^+\) T cells are virus specific (17, 25). However, our study shows that the rapid progressors 876 and 880 had dramatic increases in the numbers of activated CD69\(^+\) CD8 T cells in LN after infection (Fig. 5). As discussed above, considering that these animals did not elicit stable SIV/Gag-specific antibodies (Fig. 6) and that CD4 T-cell help is critical for the generation of anti-SIV gag antibody and SIV-specific cytotoxic T lymphocytes, one could infer that the activated CD8 T-cell population of these macaques had very few SIV-specific cytotoxic T lymphocytes. That is, these CD8 T cells were most probably a cytokine-activated bystander population.

In summary, we demonstrate for the first time that infection with SIV results in the sequential plasmatic accumulation of IFN-\( \alpha/\beta \), IL-18, and IL-12, and in transient activation of NK cell cytotoxicity. This innate immune response is not sufficient to control the initial infection, and rapid progressors that fail to mount an adaptive immune response show increasing levels of IFN-\( \alpha/\beta \). Infection also results in a rapid and transient activation of CD4 T cells in PB but not in lymphoid tissues, whereas the activation of CD8 T cells occurs in all tissues. The inability of the immune system to clear the viral infection completely leads to a chronic inflammatory process that results in dysregulation of both the innate and adaptive immune systems. Some of the mechanisms that contribute to this state of generalized activation and increased lymphocyte turnover are direct viral cytopathology and activation-induced cell death.

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**REFERENCES**


