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Early Cytokine and Chemokine Gene Expression in Lymph Nodes of Macaques Infected with Simian Immunodeficiency Virus Is Predictive of Disease Outcome and Vaccine Efficacy

WEIPING ZOU,[1] ANDREW A. LACKNER,[2] MEREDITH SIMON,[2] INGRID DURAND-GASSELIN,[1] PIERRE GALANAUD,[1] RONALD C. DESROSIERS,[2] AND DOMINIQUE EMILIE[1]*

Institut Paris-Sud sur les Cytokines, INSERM U131, 92140 Clamart, France,[1] and New England Regional Primate Research Center, Harvard Medical School, Southborough, Massachusetts 01772[2]

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Competitive PCR was used to evaluate the expression of cytokine, granzyme B, and chemokine genes in lymph nodes of macaques recently infected with the simian immunodeficiency virus (SIV) pathogenic molecular clone SIVmac239 ($n = 16$), the nonpathogenic vaccine strain SIVmac239Δnef ($n = 8$), and the nonpathogenic molecular clone SIVmac1A11 ($n = 8$). For both SIVmac239 and its *nef*-deleted derivative, strong expression was observed as early as 7 days postinfection for interleukin 1β (IL-1β), IL-6, tumor necrosis factor alpha, gamma interferon, and IL-13. The levels of gene induction were equally intense for both viruses despite a lower viral load for SIVmac239Δnef compared with that for SIVmac239. However, the nature of the cytokine network activation varied with the viral inocula. Primary infection with SIVmac239 was characterized by a higher level of IL-4, IL-10, MIP-1α, MIP-1β, MCP-1, and RANTES gene expression and a lower level of IL-12 and granzyme B gene expression compared with infection with SIVmac239Δnef. Thus, infection with *nef*-deleted SIV was associated with a preferential Th1 versus Th2 pattern of cytokine production. Infection with SIVmac1A11 was characterized by a delayed immune response for all markers tested. The unique patterns of cytokine and chemokine gene expression in lymph nodes correlated nicely with the pathogenic potential of the SIV strains used as well as with differences in their ability to serve as protective vaccines.

Both viral and host factors likely contribute to differences in rates of disease progression in human immunodeficiency virus (HIV)-infected individuals. However, it is difficult to determine the respective contributions of these factors in HIV-infected patients. Several recent studies have thus begun to address this question during the first few weeks after infection with the simian immunodeficiency virus (SIV)-infected macaque (SIVmac) model (2, 3, 8, 20, 27, 33, 48, 51). These studies have shown that several of the retroviral auxiliary genes are essential for SIV pathogenicity. Of particular interest is the *nef* gene. Macaques inoculated with *nef*-deleted variants of SIVmac have persistently low virus loads, and the disease does not progress (32). Furthermore, prior infection with *nef*-deleted SIV can protect macaques against subsequent challenge with pathogenic virus (17). Conversely, the substitution of only one amino acid in *nef* at position 17 of SIVmac239 results in an acutely pathogenic virus (20, 21, 51). Another attenuated virus, SIVmac1A11, provided only weak protection against subsequent pathogenic SIV challenge (35, 41).

To evaluate the role of the host immune response in the evolution of the disease, the pattern of cytokine production has been recently analyzed with SIV-infected macaques (2, 3). These studies have shown that genes of proinflammatory cytokines are expressed by peripheral blood cells early after infection and that the expression of T-lymphocyte-derived cytokine genes by these cells differs between wild-type SIV and a *nef*-truncated derivative. Whether such findings reflect the immunological events in lymphoid organs is unknown, but studies of HIV-infected patients have shown that cytokine network deregulation may differ between blood and lymph nodes. For example, gamma interferon (IFN-γ) is mainly produced by $CD8^+$ T lymphocytes in lymphoid organs (22, 28), whereas it is produced by $CD4^+$ T lymphocytes in the blood (25).

Determination of cytokine production in lymphoid organs, the primary site of interaction between the retrovirus and the immune system, would, however, be of great value for our understanding of the mechanism of protection or pathogenicity associated with each viral strain. It would allow evaluation of the intensity of the immune response. For SIV infection and other models of viral infection, the prognosis of the disease is probably dependent on the viral replication rate relative to the antiviral immune response, especially during the initial days postinfection (p.i.). In addition, different cytokine profiles may greatly influence the eventual outcome. For example, studies of HIV-infected patients led to the suggestion that increased production of interleukin 4 (IL-4) may be associated with a poor prognosis (11, 12). Similarly, decreased production of IL-12 with preserved or increased production of IL-10, by impairing cell-mediated immunity, may predict an unfavorable outcome (4, 13, 14, 45). However, these suggestions result from in vitro studies and studies with blood cells.

We therefore used the SIVmac model to investigate differences in cytokine gene expression in vivo. The intensity and the quality of the response were evaluated in lymphoid organs of SIV-infected macaques with competitive PCR to monitor the level of expression of several genes coding for various cytokines, chemokines, and granzyme B. To assess the possible contribution of this immune response to the tempo of disease progression and to the protective effect of nonpathogenic strains, we compared levels of cytokine gene expression induced by molecular clones of SIV which varied in their pathogenic potential and ability to serve as a vaccine.

* Corresponding author. Mailing address: INSERM U131, Institut Paris-Sud sur les Cytokines, 32 rue des Carnets, 92140 Clamart, France. Phone: (33.1) 46.32.12.08. Fax: (33.1) 46.32.79.93.

TABLE 1. Animal, virus, and times of tissue collection

Virus group (no. of animals)	No. of animals with tissue collected at wk p.i.:					
	1	2	3	8	13	23
Group 1						
SIVmac239 (8)	2	2	2	2		
SIVmac239Δnef (8)	2	2	2	2		
Group 2						
SIVmac239 (8)		2		2	2	2
SIVmac1A11 (8)		2		2	2	2

MATERIALS AND METHODS

Animals and viral infections. Peripheral lymph nodes from 32 male rhesus macaques (*Macaca mulatta*) infected with distinct molecular clones of SIV were included in this study. All animals were housed in accordance with the standards of the American Association for Accreditation of Laboratory Animal Care. The investigators adhered to the *Guide for the Care and Use of Laboratory Animals* (30a). Before use, the animals were negative for antibodies to HIV-2, SIV, type D retrovirus, and simian T-cell leukemia virus type 1.

Animals were intravenously inoculated in two groups (Table 1). The first group consisted of 16 animals: 8 inoculated with SIVmac239 and 8 inoculated with SIVmac239Δnef. Each of the 16 animals received 50 ng of SIVp27 (approximately 2.10^4 50% tissue culture infective doses). Viral p27 antigen was assayed by antigen capture (Coulter Corp., Miami, Fla.). SIVmac239 is the prototype pathogenic molecular clone of SIV (31). SIVmac239Δnef is a nonpathogenic derivative of SIVmac239 with a 182-bp deletion in the *nef*-unique region (32) and provides excellent protection against infection with pathogenic strains of SIV (17). At various time points throughout the study, two animals inoculated with each of these viruses were killed by intravenous sodium pentobarbital overdose (1, 2, 3, and 8 weeks p.i.).

The second group of animals consisted of 16 animals inoculated with 10^4 50% tissue culture infective doses of SIVmac239 ($n = 8$) or SIVmac1A11 ($n = 8$). Similar to SIVmac239Δnef, SIVmac1A11 is nonpathogenic and is related to SIVmac239 (39, 40). However, the sequence changes in SIVmac1A11 are scattered throughout the genome (36). Two animals inoculated with each of these viruses were killed by intravenous overdose of sodium pentobarbital at 2, 8, 13, and 23 weeks p.i. Details of virus isolation, host immune response, lymphoid histopathology, viral distribution by in situ hybridization, and a semiquantitative assessment of viral load based on numbers of in situ hybridization-positive cells in the tissues of all of these animals have been published with the exception of the eight animals inoculated with SIVmac239Δnef (33, 51, 53). As a negative control, a lymph node was collected from an uninfected macaque.

Virus isolation. Peripheral blood was collected prior to inoculation and 3, 7, 14, 21, 35 and 50 days p.i. from animals in group 1 and at weeks 1, 2, 4, 6, 8, 11, 13, 21, and 23 from animals in group 2. Infectious virus was isolated from blood by cocultivation of 5×10^6 CEMX174 cells with at least 10^6 peripheral blood mononuclear cells (PBMCs) as described previously (18, 31, 43). Culture supernatants were tested every 2 weeks for 8 weeks for SIV by p27 capture enzyme-linked immunoassay (Coulter Corp.). Cultures were considered positive if supernatants yielded 10 ng of SIV p27 per ml or more at two consecutive time points.

For the animals in group 1, quantitative viral cultures were also performed as described previously (27). Briefly, serial threefold dilutions were performed in duplicate, beginning with 10^6 PBMCs. PBMC dilutions were cocultured with 10^5 CEMX174 cells in a volume of 1 ml. Cultures were split 1:2 twice weekly until day 21, when the cultures were assayed for virus production by enzyme-linked immunoassay for SIV p27 (Coulter Corp.). Results are expressed as the minimum number of cells/10^6 PBMCs required to isolate virus.

Localization of SIV-infected cells by in situ hybridization. In situ hybridization was performed with formalin-fixed paraffin-embedded sections. The DNA probe used was labeled with digoxigenin-11-dUTP by random priming (Boehringer Mannheim, Indianapolis, Ind.) as previously described (30). Sections were examined microscopically and scored semiquantitatively on a scale of 0 to 4 as follows: the absence of positive cells was given a score of 0; the presence of 1 to 5 positive cells per section was given a score of 1; the presence of 5 to 10 positive cells per ×10 field was given a score of 2; the presence of 10 to 15 positive cells per ×10 field was given a score of 3; and the presence of more than 15 positive cells per ×10 field was given a score of 4.

Cytokine gene expression by competitive PCR. Competitive PCR to assay cytokine RNA was performed as previously described (24, 57). Briefly, frozen tissues were lysed in RNAzol (Bioprobe, Montreuil, France), treated with DNase (Boehringer Mannheim), extracted with phenol-chloroform, and precipitated in ethanol. cDNAs were synthesized with oligo(dT) primers. β-Actin cDNAs were first quantified by competitive PCR with a series of concentrations of a β-actin competitor plasmid (6) added to fixed amounts of cDNAs. Next, competitive PCR for cytokine cDNAs was performed with cDNA preparations containing 10^7 molecules of β-actin cDNA and one of a series of amounts of a cytokine competitor plasmid (6). A colorimetric assay was used for quantification of amplified products, either from the cDNA or from the competitor (57). This method involved recognition on streptavidin-coated dishes of amplified products containing a biotinylated oligonucleotide and digoxigenin-coupled nucleotides, followed by an enzyme-linked immunosorbent assay with an antidigoxigenin monoclonal antibody. The sequences of oligonucleotides used for amplification and of the biotinylated oligonucleotides used to assay amplified products are available upon request.

RESULTS

SIV isolation, quantitation, and detection in situ. All animals inoculated with SIVmac239 and SIVmac239Δnef were viremic within the first week p.i. as shown in Fig. 1 and as previously published (33, 51). Animals inoculated with SIVmac1A11 were all viremic by week 2, but viremia was transient. This is a consistent feature of infection with SIVmac1A11 (33, 40, 42).

While animals inoculated with both the pathogenic molecular clone SIVmac239 and the nonpathogenic *nef*-deletion variant SIVmac239Δnef had virus that was readily recovered from their PBMCs there were marked differences in viral load. Animals inoculated with SIVmac239 had at least 1 log more virus in PBMCs than animals inoculated with SIVmac239Δnef. By 50 days p.i., this difference had increased to 3 logs. The viral loads in animals inoculated with SIVmac239 and SIVmac239Δnef were consistent with previous observations (17, 27, 30, 32).

To determine if the higher viral load in the peripheral blood of animals inoculated with pathogenic SIVmac239 versus nonpathogenic SIVmac239Δnef was reflected in tissues, a semiquantitative assessment of viral load in axillary lymph node was performed by in situ hybridization. Lymph nodes from animals inoculated with SIVmac239 consistently had more SIV-positive cells than those from animals inoculated with either SIVmac239Δnef or SIVmac1A11 (Table 2 and Fig. 2). The number of virus-infected cells remained high in animals inoculated with SIVmac239 through 8 weeks p.i., while animals inoculated with either of the nonpathogenic clones had few if any positive cells at this or later time points. After week 8 p.i., the number of virus-positive cells in lymph nodes of animals

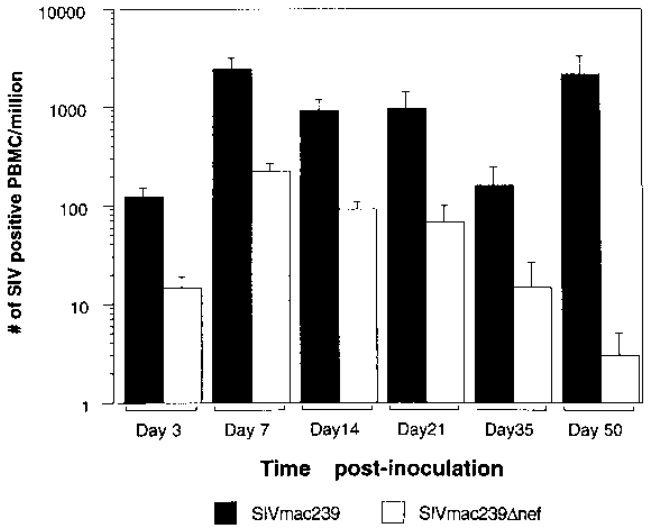

FIG. 1. Cell-associated virus loads in peripheral blood from macaques infected with SIVmac239 and SIVmac239Δnef from days 3 to 50 p.i. Results are expressed as means ± standard errors of viral loads. For both SIVmac239-infected and SIVmac239Δnef-infected macaques, results were obtained from eight animals for days 3 and 7, from six animals for day 14, from four animals for day 21, and from two animals for days 35 and 50.

TABLE 2. In situ hybridization-positive cells in axillary lymph node after infection with pathogenic and nonpathogenic molecular clones of SIV

Virus	Animal	Hybridization score at wk p.i.[a]:					
		1	2	3	8	13	23
SIVmac239	250-93	2					
	277-93	4					
	23894		2				
	23830		NA				
	285-93		2				
	388-93		4				
	406-93			4			
	441-93			4			
	24231				2		
	24255				3		
	364-83				4		
	434-93				3		
	24289					1	
	24219					0	
	24242						1
	24263						1
SIVmac239Δnef	243-93	2					
	493-93	2					
	251-93		2				
	484-93		2				
	400-93			2			
	478-93			2			
	442-93				1		
	492-93				0		
SIVmac1A11	24120		0				
	24777		1				
	24029				1		
	24209				1		
	24062					0	
	24172					0	
	24091						0
	24272						0

[a] Results of in situ hybridization were quantified as follows: 0, no positive cells; 1, 1 to 5 positive cells per section; 2, 5 to 10 positive cells per ×10 field; 3, 10 to 15 positive cells per ×10 field; 4, more than 15 positive cells per ×10 field; NA, not available.

inoculated with SIVmac239 decreased coincidentally with increased immune response as shown previously (33).

Expression of proinflammatory cytokine genes in lymph nodes of macaques infected with different clones of SIV. The expression of the genes coding for the proinflammatory cytokines IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) was measured in lymphoid tissues of macaques inoculated with the various clones of SIV. Fourteen days p.i., axillary lymph nodes were collected from four macaques infected with the prototype pathogenic molecular clone SIVmac239 and from macaques infected with the nonpathogenic clones SIVmac239Δnef ($n = 2$) and SIVmac1A11 ($n = 2$). Cytokine gene expression in the lymph nodes was evaluated with a competitive PCR. Results varied according to the clone of SIV.

The highest level of IL-1β, IL-6, and TNF-α gene expression was observed in lymphoid tissues from macaques infected with SIVmac239, in which the levels ranged from 7.10^2 to 4.10^3 molecules of cytokine mRNA per 10^7 molecules of β-actin mRNA (Fig. 3). The levels of expression of the three genes were slightly lower in macaques infected with SIVmac239Δnef. In contrast, the level was extremely low in SIVmac1A11-infected macaques, about 2 logs fewer IL-1β and IL-6 mRNA molecules than in SIVmac239-infected macaques.

Expression of the granzyme B gene in lymph nodes of macaques infected with different clones of SIV. To evaluate the intensity of the immune reaction developing in lymphoid tissues in SIV-infected macaques, we next measured the expression of the granzyme B gene. This gene is induced in cytotoxic cells following their activation, and it is expressed at a high level in lymphoid organs of HIV-infected patients (19). Again, the level of expression varied according to the clone of SIV. It was higher in macaques infected with SIVmac239Δnef than with SIVmac239. The lowest level was observed in animals inoculated with SIVmac1A11 (Fig. 4). This indicates a strong activation of the cytotoxic cell compartment, either cytotoxic T lymphocytes (CTL) or NK cells, in lymph nodes of macaques infected with SIVmac239Δnef.

Balance between Th1-type and Th2-type cytokines in lymph nodes of macaques infected with different clones of SIV. The level of gene expression of the Th1-type cytokine IFN-γ and of the Th2-type cytokines IL-4 and IL-13 was then evaluated. The level of expression of the IFN-γ gene was higher in macaques infected with the pathogenic SIVmac239 than in those infected with the nonpathogenic clones. The opposite pattern was observed for the expression of the IL-13 gene (Fig. 5). However, these differences were moderate. This was not the case for the expression of the IL-4 gene. The number of mRNA molecules coding for the IL-4 gene ranged between 10^2 and 10^3 per 10^7 β-actin mRNA molecules in macaques infected with SIVmac239 (Fig. 5). This level of expression was in the same range as that of the IFN-γ gene in the same animals. It was at least 20 times higher than in macaques infected with the nonpathogenic clones, in which no IL-4 gene expression was detected. Therefore, although infection with SIVmac239Δnef induces a strong immune activation, there is in this case a lack of a critical Th2 component to this response.

Balance between IL-12 p40 and IL-10 mRNA expression in lymph nodes of macaques infected with different clones of SIV. The development of cell-mediated immunity is largely dependent on the equilibrium between IL-12 and IL-10, two cytokines mainly produced by macrophages. To evaluate this equilibrium, we monitored the expression of the IL-10 and IL-12 p40 genes (Fig. 6). The levels of IL-10 gene expression in the three groups of macaques overlapped, although the mean value was higher in macaques infected with SIVmac239. Expression of the IL-12 p40 gene was strongest in lymph nodes from macaques infected with SIVmac239Δnef and lowest in lymph nodes from macaques infected with the pathogenic SIVmac239; expression differed by approximately 1 log between the two groups of animals.

Therefore, comparison of cytokine gene expression 14 days p.i. in macaques infected with three different molecular clones of SIV shows that primary infection with nonpathogenic, protective SIVmac239Δnef is specifically associated with undetectable expression of IL-4 and with strong expression of IL-12 p40 and granzyme B, which are all expected to be associated with a better prognosis and with an efficient anti-SIV immune response.

Kinetics of cytokine gene expression during primary SIV infection. The results obtained 14 days p.i. (above) may have reflected different kinetics of cytokine gene expression. We therefore compared the previous parameters in lymph nodes collected 7, 14, and 21 days after infection with either pathogenic SIVmac239 or nonpathogenic SIVmac239Δnef (Fig. 7). Each lymph node was obtained from a different animal. The differences in cytokine gene expression observed on day 14 were stable during the first 3 weeks of infection. Interestingly, these differences were already present on day 7, indicating that the nature of the immune response induced by the various

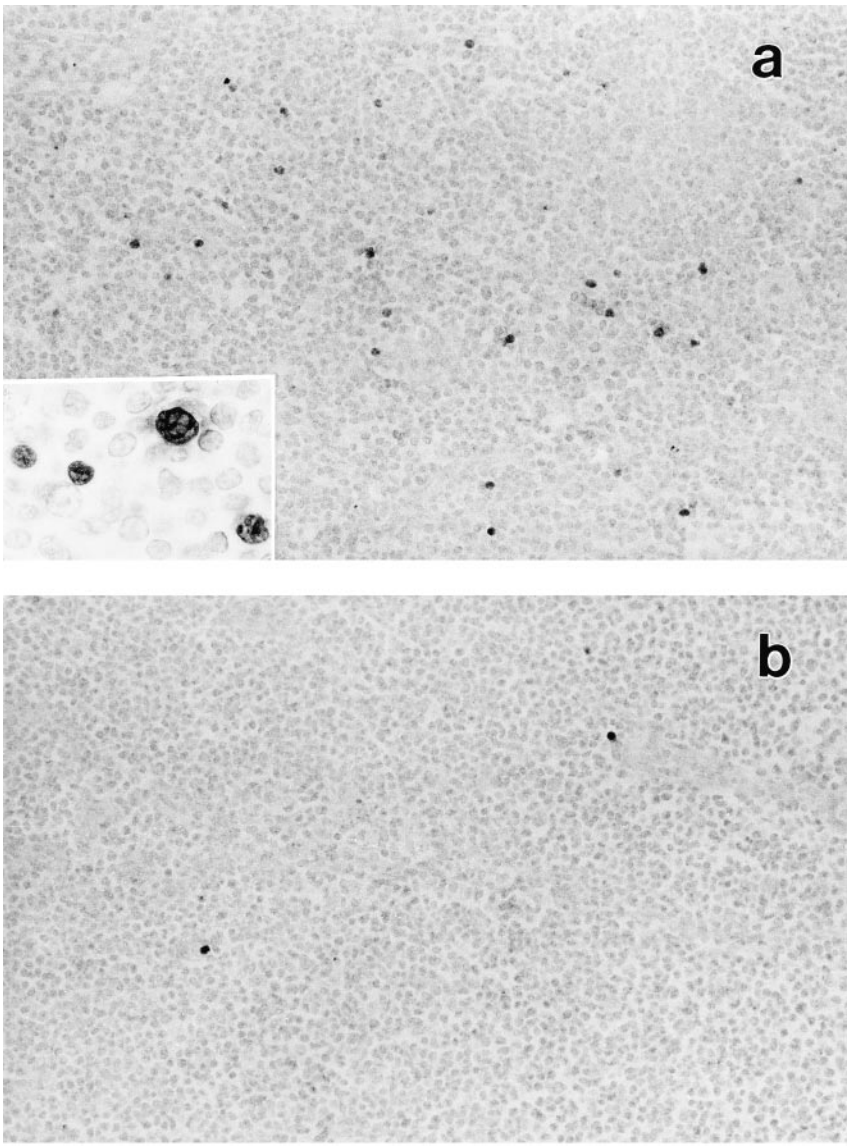

FIG. 2. In situ hybridization for SIV in axillary lymph nodes from macaques infected with equal doses of SIVmac239 (a) and SIVmac239Δnef (b) at 7 days p.i. Note the many infected cells (enlarged in inset) in the section from the animal infected with SIVmac239, compared with relatively few infected cells in the animal infected with SIVmac239Δnef. a and b, ×265; inset, ×800.

strains of SIV is determined during the first days of the infection.

Comparison of cytokine gene expression between the primary phase and the chronic phase of SIV infection. The pattern of cytokine gene expression in lymphoid organs observed during primary SIV infection with the pathogenic SIVmac239 differed from what we and others previously described for the chronic phase of HIV infection, characterized by a low level of IL-4 gene expression (23, 28). This led us to compare the profile of cytokine gene expression between primary infection and the chronic phase of SIV infection. To this end, we studied lymphoid tissues obtained at various intervals between 14 days and 23 weeks after SIVmac239 infection. Each lymph node studied was obtained from a different animal. For comparison, we also studied lymphoid tissues from macaques infected with SIVmac1A11.

There was a shift of IL-4 gene expression between weeks 8 and 13 in macaques infected with SIVmac239. The number of IL-4 mRNA molecules dropped from approximately 5×10^2 molecules per 10^7 β-actin mRNA molecules on week 8 to less than 10 on weeks 13 and 23. This decrease was unique to IL-4, because it was noted for none of the other markers studied (Fig. 8).

The opposite evolution of IL-4 gene expression was observed in macaques infected with the nonpathogenic SIVmac1A11 clone. Whereas no IL-4 gene expression was detected up to week 8 p.i., strong expression was detected on weeks 13 and 23 p.i. However, this delayed increase was not restricted to IL-4, because the expression of most other markers also increased with time after infection with this strain (Fig. 8).

Chemokine gene expression in lymphoid organs of SIV-infected macaques. Finally, we studied expression of the genes coding for the chemokines MIP-1α, MIP-1β, MCP-1, RANTES, and IL-8 in lymphoid tissues from the macaques infected with

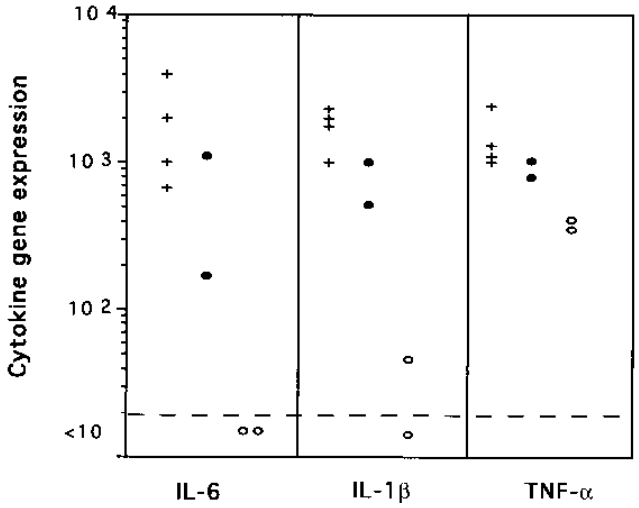

FIG. 3. Expression of proinflammatory cytokine genes in SIV primary infection. The level of expression of IL-6, IL-1β, and TNF-α genes was evaluated 14 days after infection with SIVmac239 (+), SIVmac239Δnef (●), or SIVmac1A11 (○). Results are expressed as the number of cytokine mRNA molecules per 10^7 β-actin mRNA molecules. The number of IL-6, IL-1β, and TNF-α mRNA molecules in the control lymph node <10 for each gene.

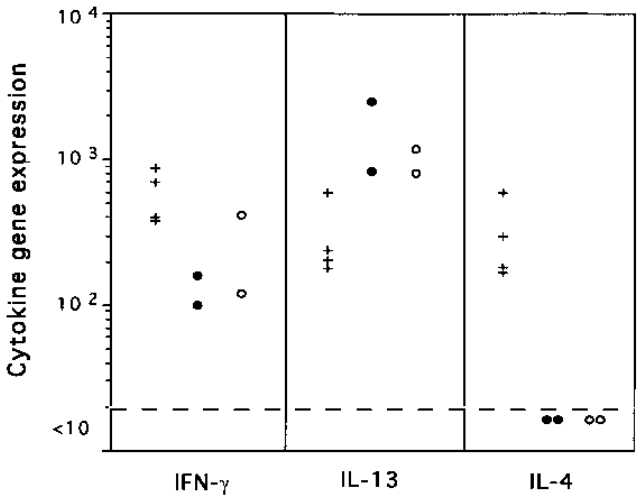

FIG. 5. Expression of Th1- and Th2-type cytokine genes in SIV primary infection. The level of expression of cytokine genes was evaluated for IFN-γ, IL-13, and IL-4 14 days after infection with SIVmac239 (+), SIVmac239Δnef (●), or SIVmac1A11 (○). Results are expressed as the number of cytokine mRNA molecules per 10^7 β-actin mRNA molecules. The numbers of IFN-γ, IL-13, and IL-4 mRNA molecules in the control lymph node were 20, 200, and less than 10, respectively.

pathogenic or nonpathogenic clones. In all samples, at all times p.i., and for all molecular clones of SIV, expression of the IL-8 gene remained below 40 mRNA molecules per 10^7 β-actin mRNA molecules.

A very strong expression of the genes coding for MIP-1α, MIP-1β, and MCP-1 was detected in lymph nodes from macaques infected with SIVmac239. This expression increased as early as day 7 p.i. and remained high up to week 23. RANTES gene expression was also increased from day 7 p.i. but decreased transiently on weeks 8 and 13 (Fig. 9). Results were quite different with the nonpathogenic clones. In macaques infected with SIVmac239Δnef, expression of each of the four chemokines was clearly lower than in SIVmac239-infected macaques from day 7 to day 21. In macaques infected with SIVmac1A11, expression of the four genes was initially undetectable. Expression was first detected on week 8 and then progressively increased. However, expression of MIP-1α, MIP-1β, and MCP-1 was still 1 log lower than in SIVmac239-infected macaques at the last time point studied, week 23 (Fig. 9).

DISCUSSION

In this work, we analyzed the expression of cytokine genes in lymph nodes from macaques recently infected with various strains of SIV to investigate why these molecular clones exhibit different pathogenicity and immunogenicity.

As early as day 7 p.i. with the pathogenic molecular clone SIVmac239, a number of genes were strongly expressed in

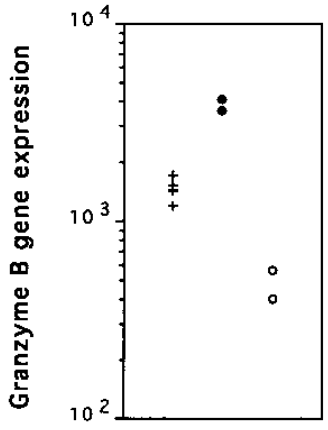

FIG. 4. Expression of the granzyme B gene in SIV primary infection. The level of expression of the granzyme B gene was evaluated 14 days after infection with SIVmac239 (+), SIVmac239Δnef (●), or SIVmac1A11 (○). Results are expressed as the number of granzyme B mRNA molecules per 10^7 β-actin mRNA molecules. The number of granzyme B mRNA molecules in the control lymph node was 60.

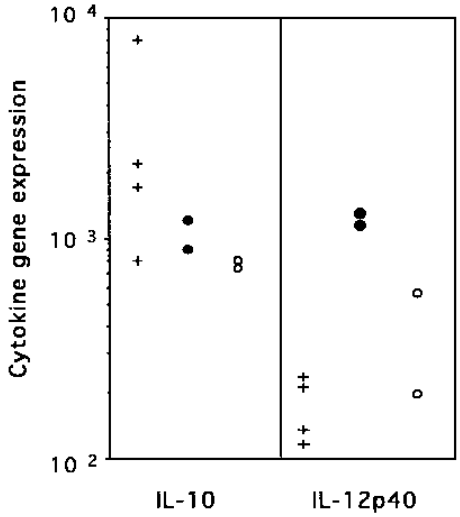

FIG. 6. IL-10 and IL-12 p40 gene expression in SIV primary infection. The level of expression of the IL-10 and of the IL-12 p40 genes was evaluated 14 days after infection with SIVmac239 (+), SIVmac239Δnef (●), or SIVmac1A11 (○). Results are expressed as the number of cytokine mRNA molecules per 10^7 β-actin mRNA molecules. The numbers of IL-10 and of IL-12 p40 mRNA molecules in the control lymph node were 160 and <10, respectively.

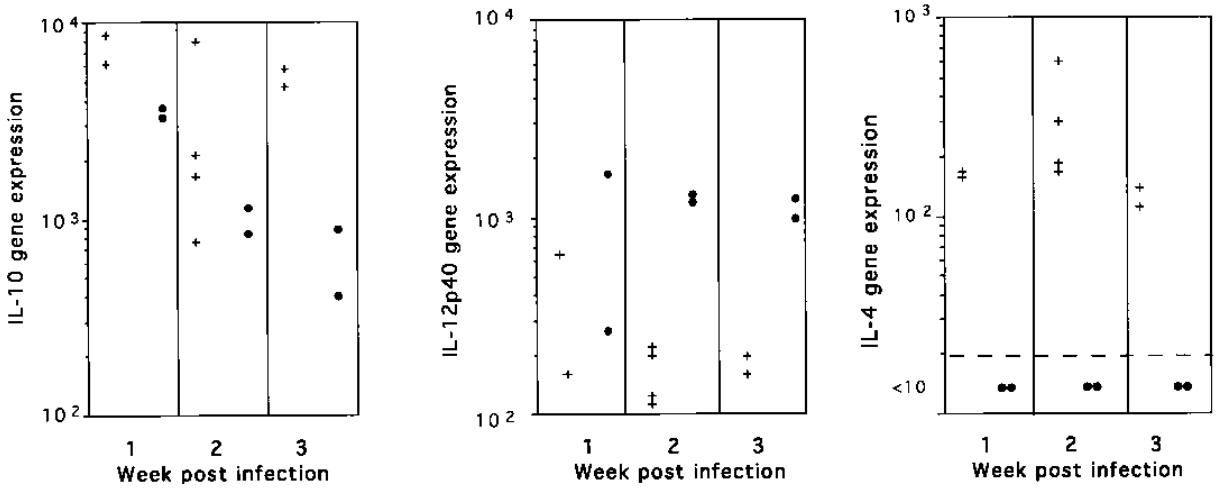

FIG. 7. Kinetics of cytokine gene expression in SIV primary infection. The level of gene expression was evaluated for several cytokines and for granzyme B on days 7, 14, and 21 after infection with SIVmac239 (+) or SIVmac239Δnef (●). Results are expressed as the number of cytokine mRNA molecules per 10^7 β-actin mRNA molecules.

lymphoid tissues: genes coding for proinflammatory cytokines (IL-6, IL-1β, and TNF-α), for T-lymphocyte-derived cytokines (IFN-γ, IL-13, and IL-4), for a cytotoxic cell enzyme (granzyme B), for IL-12 and IL-10 (two activation markers of antigen-presenting cells), and for chemokines (MIP-1α, MIP-1β, MCP-1, and RANTES). The profile of gene expression was stable during primary infection, from day 7 to week 8, indicating that both the intensity and the nature of the immune response induced by SIV were determined within the first days after infection. This is in agreement with recent findings showing that a strong and specific CTL response emerges within days of SIV (26, 48, 56) or HIV (5, 50) infection. In both cases, this immune response rapidly leads to a selection of T lymphocytes expressing a restricted repertoire (9, 46). Our findings show that this early immune response is also reflected at the level of cytokine and granzyme B gene expression in lymphoid organs.

The rapidity of the immune response was also evident in animals inoculated with the nonpathogenic SIVmac239Δnef. In these animals, the intensity of the immune response observed 14 days after infection was of the same order of magnitude as that in animals inoculated with SIVmac239. The level of gene expression in lymph nodes of SIVmac239Δnef-infected animals was slightly lower for the proinflammatory cytokines, but stronger for granzyme B and IL-12 p40. As for the wild-type SIVmac239, immune activation quickly followed infection, because both the intensity and the pattern of gene expression were stable from day 7 to day 21 p.i. The similar intensities of the immune responses induced by SIVmac239 and its *nef*-deleted counterpart have already been noted for

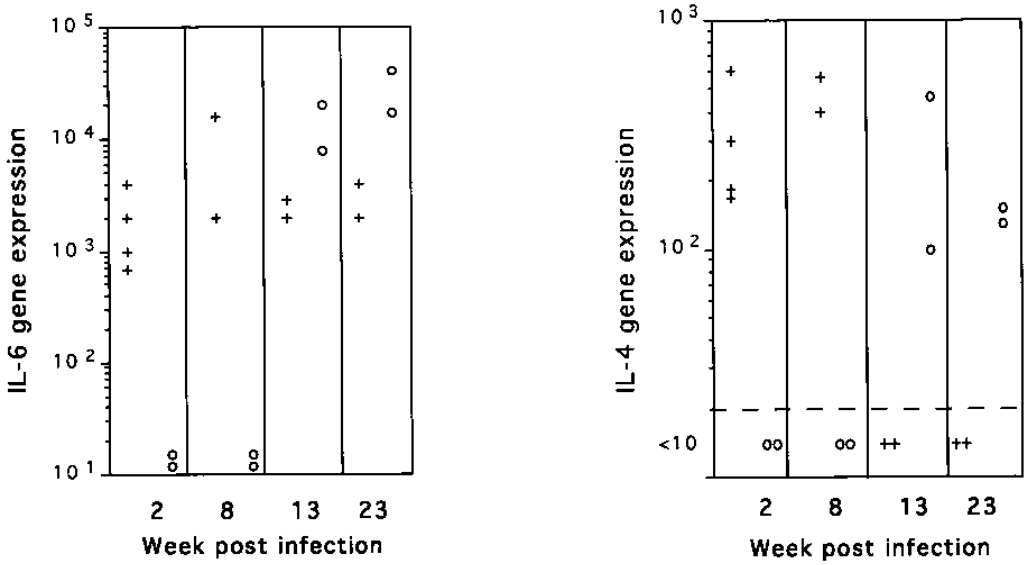

FIG. 8. Shift of cytokine gene expression between SIV primary infection and the chronic phase of the disease. The level of gene expression was evaluated for several cytokines and for granzyme B from week 2 to week 23 after infection with SIVmac239 (+) or SIVmac1A11 (○). As shown for IL-6, all markers except IL-4 remained increased in SIVmac239-infected macaques from week 2 to week 23. Only IL-4 gene expression dropped in these animals between weeks 8 and 13. Results are expressed as the number of cytokine mRNA molecules per 10^7 β-actin mRNA molecules.

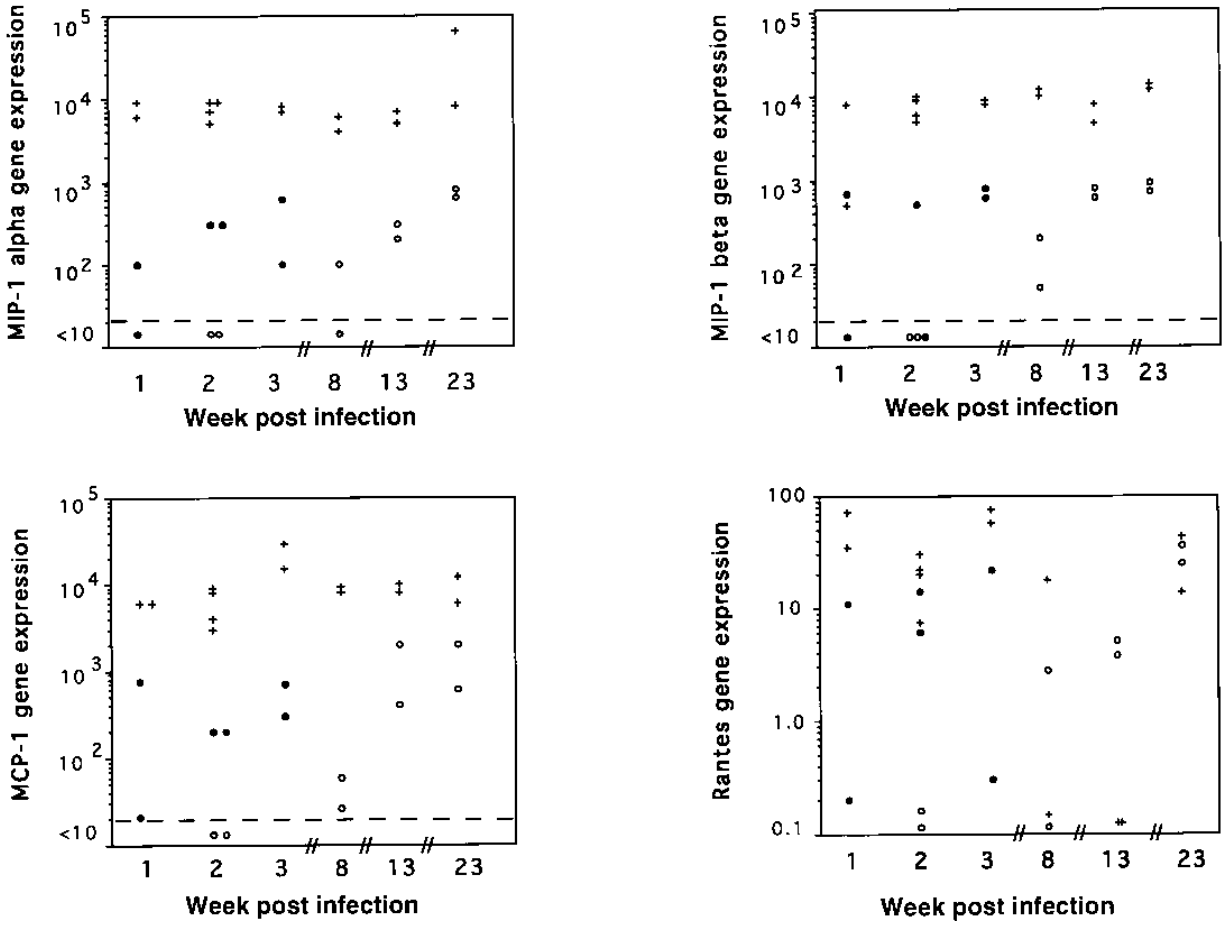

FIG. 9. Expression of chemokine genes in SIV primary infection. The level of expression of chemokine genes was evaluated for MIP-1α, MIP-1β, MCP-1, and RANTES on different days after infection with SIVmac239 (+), SIVmac239Δnef (●), or SIVmac1A11 (○). Results for MIP-1α, MIP-1β, and MCP-1 (evaluated by competitive PCR) are expressed as the number of cytokine mRNA molecules per 10^7 β-actin mRNA molecules. Expression of RANTES (evaluated with a semiquantitative PCR) is expressed in arbitrary units. The numbers of MIP-1α, MIP-1β, and MCP-1 mRNA molecules in the control lymph node were <10, 20, and 20 per 10^7 β-actin mRNA molecules, respectively. No RANTES gene expression was detected in the control lymph node.

anti-SIV antibody production: although there is variation between individuals, especially for those infected with SIVmac239, the mean levels and kinetics of anti-SIV antibody production are not significantly different for SIVmac239 and SIVmac239Δnef (32). These similar intensities of the immune reaction contrast with the large difference in viral loads in peripheral blood and lymphoid organs. Indeed, the level of SIV replication is much lower after infection with SIVmac239Δnef than with wild-type SIVmac239 (reference 32 and this work). The favorable outcome of SIVmac239Δnef infection may therefore be due not only to the virus being attenuated (which is obviously important), but also to the strong immune response it is able to induce. This would also explain why this attenuated strain provides excellent protection against subsequent challenge with a pathogenic SIV (17).

It is also possible that the different disease evolution in animals inoculated with SIVmac239 or its Δnef derivative results in part from a qualitatively different immune response. The natures of the immune response induced by the two clones, in particular IL-4 gene and chemokine gene expressions, were indeed very different. Up to week 8, IL-4 gene expression in animals infected with SIVmac239 was strong and of the same order of magnitude as those of other T-lymphocyte-derived cytokine genes such as IFN-γ or IL-13. In contrast, IL-4 gene expression was undetectable during primary infection in all macaques infected with the *nef*-deleted variant. A smaller difference was also observed between the two groups of macaques for IL-12 p40 gene expression: it was approximately 10 times lower in animals infected with the wild-type strain than in those infected with the *nef*-deleted derivative. This difference of IL-12 p40 gene expression may actually be secondary to that of IL-4 gene expression, because IL-4 decreases IL-12 p40 gene expression (10, 54).

It has been reported from in vitro studies performed with Jurkat cells that *nef* may selectively downregulate Th1-type cytokine production (16). Our in vivo findings do not support this, because we detected higher rather than lower expression of the IFN-γ gene in SIVmac239-infected macaques than in macaques infected with the *nef*-deleted derivative. Possibly, *nef* has different effects in vivo and in vitro. Alternatively, IFN-γ gene expression in lymphoid organs of infected animals may originate mainly from $CD8^+$ T lymphocytes, as shown during HIV infection (22, 28), and such cells may not be sensitive to the inhibitory effect of *nef* on Th1-type cytokine production.

Our findings for lymphoid tissues also differ from those of Benveniste et al. (3) who showed that PBMCs from macaques infected with a *nef*-truncated SIVmac251 derivative expressed T-lymphocyte-derived cytokines, whereas PBMCs from ma-

caques infected with pathogenic SIVmac251 did not. This has been suggested to reflect an early immune deficiency induced by the pathogenic virus (3). This was unexpected in view of the early anti-SIV CTL activation and anti-SIV antibody production previously reported in macaques infected with pathogenic SIV (26, 32, 48, 56). It also contrasts with the enlargement of lymphoid organs observed during the course of SIV primary infection. All of these findings suggest that there is a potent and efficient anti-SIV immune response in animals infected with pathogenic SIV. In primary SIV infection of macaques, as in primary human HIV infection (29), data for the blood compartment may not accurately reflect events in lymphoid organs.

Cytokines may regulate SIV or HIV replication directly. Proinflammatory cytokines, and especially TNF-α, upregulate HIV replication in vitro. IL-4 directly stimulates HIV replication in T lymphocytes (44), and it decreases the production by $CD8^+$ T lymphocytes of soluble mediators inhibiting HIV replication (34). Cytokines may also affect the rate of emergence of the immune defect, by modulating the efficacy of the antiviral immune response or the rate of destruction of $CD4^+$ T lymphocytes. This particularly applies to IL-12 and IL-10. In HIV-infected patients, IL-12 restores cell-mediated immunity and prevents programmed cell death of $CD4^+$ T lymphocytes, whereas IL-10 has the opposite effects (4, 13, 14, 45). The efficacy of the antiviral cytotoxic cell response is also critical for controlling the infection. During primary infection with SIVmac239, IL-4 gene expression was much stronger and proinflammatory cytokine gene expression was slightly stronger than with the *nef*-deleted variant. In contrast, expression of both the granzyme B and IL-12 genes was lower. Each of these differences, either large or moderate, is expected to be associated with more rapid disease progression in SIVmac239- than in SIVmac239Δnef-infected animals.

The largest difference between the two groups of animals was observed for IL-4 gene expression. Thus, this expression may depend on the presence or absence of a functional *nef* gene. SIV may activate the T helper lymphocyte it infects, and *nef* itself may contribute to this process. This has been shown for the *nef* gene of SIVmac239/YEnef, a derivative of SIVmac239 with two amino acid changes in *nef*. This SIVmac239 derivative causes lymphocyte activation and proliferation in vitro and in vivo (20, 51). It has been suggested that other forms of the *nef* gene-coded protein, less active than that of the SIVmac239/YEnef, also have this effect on infected cells (1, 20, 52). HIV preferentially replicates in IL-4-producing, Th0 or Th2 helper T lymphocytes (37). If this also applies for SIV, it could explain why SIVmac239 infection stimulates IL-4 gene expression, and why a functional *nef* gene is required for this effect. Were this the case, a vicious cycle would be established during primary SIV infection, in which IL-4 production induced by SIV stimulates SIV replication. Such a scenario would explain why an early increase in Th2-type markers such as immunoglobulin E and soluble CD30 levels in serum in HIV infection indicates a poor prognosis (47, 49). The increase in these Th2-type markers may directly reflect the pathogenicity of the infectious strain rather than a preexisting bias of the immune responsiveness of the host.

The strong IL-4 gene expression observed with SIVmac239 up to week 8 p.i. contrasts with the low or undetectable IL-4 gene expression in lymph nodes of both HIV-infected patients during the chronic phase of the disease (23, 28, 58) and SIVmac239-infected macaques after week 13 p.i. (this work). This shift in IL-4 gene expression could result from the efficient control of SIV replication. Indeed, the viral load in lymph nodes of SIVmac239-infected macaques drops substantially between weeks 8 and 13 (reference 33 and this work). A relationship between viral load and IL-4 production is in agreement with a direct role for SIV in the induction of IL-4 gene expression, as previously suggested. It is also possible that preferential replication of SIV in Th0 or Th2 T lymphocytes results in a selective loss of these cell populations.

A strong induction of chemokine gene expression was only observed in macaques infected with pathogenic SIV and not in those infected with its *nef*-deleted derivative. Of the five chemokine genes we studied, four (those coding for MIP-1α, MIP-1β, MCP-1, and RANTES) were upregulated in SIVmac239-infected macaques, whereas expression of the IL-8 gene was unaffected. The strong induction of chemokine gene expression may be a direct consequence of the high rate of viral replication in lymph nodes of SIVmac239-infected macaques. Indeed, in vitro experiments have shown that infection of macrophages with HIV stimulates the production of MIP-1α, but not that of IL-8 (7). The low expression of the chemokine genes in macaques infected with SIVmac239Δnef may thus reflect the lower viral load in these animals. The level of chemokine gene expression may also result from that of other cytokines, because RANTES production by macrophages is positively regulated by IL-4 (38).

The contribution if any of this deregulation of chemokine gene expression on the course of primary infection is unclear. It may affect viral replication: in vitro studies have shown that MIP-1α, MIP-1β, and RANTES can downregulate HIV replication in T lymphocytes (15), whereas MCP-1 has the opposite effect (55). Alternatively, by recruiting circulating immune cells and activating them in lymphoid organs, chemokines may amplify retroviral spread.

The two different nonpathogenic clones of SIV induced different immune responses. SIVmac239Δnef induced a strong and rapid immune response, whereas SIVmac1A11 induced a delayed immune response. This was most apparent for granzyme B gene expression and for proinflammatory cytokine gene expression, both virtually undetectable in lymph nodes of SIVmac1A11-infected animals before week 13 p.i. This delayed immune response after SIVmac1A11 infection has already been reported in an analysis of anti-SIV antibody production (33). It may be due to a low viral load during primary infection (reference 33 and this work) and/or to poor immunogenicity. The poor immunogenicity of SIVmac1A11 was evident in earlier vaccination studies; whereas SIVmac239Δnef is fully protective against a subsequent challenge with pathogenic SIV, SIVmac1A11 is not and only attenuates the severity of subsequent infection (40). This lack of protective effect of SIVmac1A11 is clearly consistent with the weakness of the immune response it induces.

This study, analyzing the immune response in lymphoid organs of SIV-infected macaques, extends previous work showing that *nef*-deleted variants of SIV initiate a protective immune response. Unlike other attenuated strains of SIV, including SIVmac1A11, SIVmac239Δnef strongly stimulates the immune system to a degree in the range of that of pathogenic SIVmac239 despite much lower viral loads. However, the quality of the immune response induced by the Δnef variant differs from that of the wild-type strain, because it meets all criteria expected to be associated with better protection against disease. The most appealing result of this study is the large difference in IL-4 gene expression and chemokine gene expression induced by SIVmac239 and its *nef*-deleted variant. This difference may reflect an important functional effect of *nef* on T-lymphocyte activation, and this property may be a critical determinant of SIV and HIV pathogenicity.

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