

β -Chemokine production in macaques vaccinated with live attenuated virus correlates with protection against simian immunodeficiency virus (SIVsm) challenge

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Simian immunodeficiency virus (SIV) uses the CCR5 chemokine receptor as the main co-receptor to enter CD4⁺ cells. RANTES, MIP-1 α and MIP-1 β have been suggested as the major human immunodeficiency virus-suppressor factors produced by CD8⁺ T-cells. The aim of this study was to investigate the CD8⁺ T-cell production of anti-viral factors and of β -chemokines in six cynomolgus macaques vaccinated with live attenuated SIVmac8 in relation to protection against infectious intrarectal SIVsm challenge. Three of the vaccinated animals were completely protected and one was partially protected against the challenge virus. Interestingly, these monkeys showed higher *in vitro* anti-viral CD8⁺ cell suppressor activity and β -chemokine production both before and after vaccination as compared to the infected monkeys. The results indicate that β -chemokines may play a role in protective immunity but also that genetic and/or environmental factors may influence their production.

Replication of human immunodeficiency virus type 1 (HIV-1) has been shown to be suppressed by a soluble factor secreted by activated CD8⁺ T-cells via a non-lytic mechanism (Walker & Levy, 1989). This T-cell anti-viral factor (CAF) appears early after HIV infection (Mackewicz *et al.*, 1994) and is maintained in asymptomatic individuals but seems to decline as HIV-infected patients progress to AIDS (Landay *et al.*, 1993). However, the efficiency of CAF differs among individuals (Walker *et al.*, 1989). The HIV-1 anti-viral factor is also

active against different strains of HIV-2 and simian immunodeficiency virus (SIV) (Walker *et al.*, 1991). CD8⁺ lymphocytes from SIV-infected monkeys can block SIV replication *in vitro* (Kannagi *et al.*, 1988) and CD8⁺ cells from HIV-2-infected baboons can control HIV-2 replication (Blackbourn *et al.*, 1997).

The β -chemokines RANTES (regulated upon activation, normal T-cell expressed and secreted), MIP-1 α (macrophage inflammatory protein) and MIP-1 β have recently been suggested to be the major HIV-suppressive factors produced by CD8⁺ T-cells (Cocchi *et al.*, 1995). However, interleukin-16 and macrophage-derived chemokine (Baier *et al.*, 1995; Pal *et al.*, 1997), together with so far unidentified factors, account for some of the T-cell-mediated suppression *in vitro* (Rubbert *et al.*, 1997).

It is now well-documented that different HIV isolates differ in cellular tropism and, in addition to CD4, they use different co-receptors for entry into target cells. Macrophage-tropic viruses use CCR5, a ligand for RANTES, MIP-1 α and MIP-1 β (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996), and CXCR4, a ligand for stromal cell-derived factor 1, is the co-receptor for T-cell-tropic viruses (Feng *et al.*, 1996; Oberlin *et al.*, 1996). Besides CCR5 and CXCR4, CCR2b and CCR3 have been identified as co-receptors for HIV-1 entry into CD4⁺ cells (Choe *et al.*, 1996; Doranz *et al.*, 1996). In contrast to HIV-1 both macrophage-tropic and T-cell-tropic strains of SIV use CCR5 for fusion and not CXCR4 (Chen *et al.*, 1997; Hill *et al.*, 1997). SIV strains can also infect cells lacking CCR5 by using other recently identified co-receptors. BOB and Bonzo are closely related to the chemokine receptor family and are shown to facilitate SIV virus entry *in vitro* (Alkhatib *et al.*, 1997; Deng *et al.*, 1997).

The importance of CCR5 as a co-receptor for HIV-1 *in vivo* was indicated by the finding that some multiply exposed individuals resistant to HIV-1 had a homozygous defect in their CCR5 gene (Liu *et al.*, 1996; Samson *et al.*, 1996). Furthermore, deletion of one CCR5 gene allele appears to

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Table 1. Suppressive effect on SIV replication and chemokine production of CD8⁺ cell culture fluid in relation to outcome of intrarectal SIVsm challenge of macaques vaccinated with SIVmacC8

The cells were collected 2–3 weeks after vaccination while SIVmac C8 could still be isolated from the PBMCs. The β -chemokines were analysed in duplicate on at least three different occasions. The percent inhibition was determined by an in-house HIV-2/SIV antigen capture ELISA. The assay was performed in triplicate at least three times. The average maximum antigen concentration without inhibition was 2500 pg, equivalent to approximately 375 pg p24 antigen/ml.

Monkey	Outcome of SIV challenge	Inhibition (%)	Chemokine production (pg/ml)		
			RANTES	MIP-1 α	MIP-1 β
C15	Protected	52	715	414	13 380
C17	Protected	55	941	394	6 440
B177	Protected	45	1 442	531	8 980
B194	Partially protected	33	1 146	512	12 740
C29	Infected	31	556	167	4 520
C30	Infected	9	198	240	2 880

protect against HIV disease progression (Meyer *et al.*, 1997). These findings suggest that blocking of the CCR5 receptor by chemokines *in vivo* might be beneficial. Induction of β -chemokines by prophylactic or therapeutic vaccines might be a useful complement to other immune mechanisms. In a vaccination experiment by Lehner *et al.* (1996) protection against infectious challenge with SIVmac of macaques was associated with significantly increased levels of CD8 suppressor factor and the β -chemokines RANTES and MIP-1 β .

In our previous vaccine experiments using whole inactivated HIV-2 (Putkonen *et al.*, 1994), native HIV-2 gp125 (Nilsson *et al.*, 1995) or recombinant HIV-2-canarypox virus (Andersson *et al.*, 1996) we have shown complete protection against challenge with infectious HIV-2 in 30–50% of the animals. The protective efficacy of the vaccines was correlated neither to humoral immunity as determined by titres of binding antibodies, neutralizing antibodies and antibodies active in antibody-dependent cellular cytotoxicity nor to cellular immunity as determined by the presence of HIV-2-specific T-cell proliferative responses and cytotoxic T-lymphocytes. Humoral and cellular immunity were demonstrable at variable frequency and levels in both protected and infected animals. However, other studies have implicated the role of neutralizing antibodies or cytotoxic T-lymphocytes in controlling HIV or SIV infection (reviewed in Haigwood & Zolla-Pazner, 1998; Gotch *et al.*, 1997).

The objective of this investigation was to determine the antiviral activity of CD8⁺ cell supernatants and production of β -chemokines in phytohaemagglutinin (PHA)-stimulated CD8⁺ cells before and after vaccination of six cynomolgus monkeys with an attenuated SIVmac 32H molecular clone pC8 (SIVmac C8) and to study any correlation with protection against infectious SIVsm challenge, since we had not been able to correlate protection with neutralizing antibodies or CTL, as reported in our previous study (Nilsson *et al.*, 1998).

Four of the monkeys (C15, C17, C29, C30) were challenged 8 months after vaccination intrarectally with 10 monkey infectious doses (MID₅₀) of SIVsm when they were virus-isolation-negative but PCR-positive, along with four naive control animals. The other two monkeys (B177, B194) were challenged after 16 months, as were two unimmunized controls. The experiment is described by Nilsson *et al.* (1998). Briefly, three of the monkeys (C15, C17, B177) were completely protected against SIVsm challenge as determined by negative virus isolation and discriminative PCR. Monkey B194 was partially protected during the first year of follow up, as it showed replication of the challenge virus only at 2 and 4 weeks post-inoculation. Monkeys C29 and C30 and the controls were repeatedly virus-isolation- and PCR-positive for the challenge virus. The six control macaques were not included in the present study.

CAF was produced by enriched CD8⁺ cells after 3 days stimulation with PHA (Difco). Culture fluids were collected every 2 days thereafter as described by Mackewicz *et al.* (1995). CAF content in the CD8⁺ cell culture fluids was assayed by testing for anti-SIV activity in an acute infection microtitre assay as described earlier (Lehner *et al.*, 1996). The culture supernatants collected on day 7 were screened by an HIV-2/SIV antigen assay (Thorstensson *et al.*, 1991). The ability of CD8⁺ cell supernatant from SIVmacC8-vaccinated monkeys to suppress the replication of SIV in purified CD4⁺ monkey PBMCs is shown in Table 1. The culture fluids collected after SIVmacC8 immunization from the completely protected monkeys suppressed the SIV replication by 45–55% as compared with those induced by CD8⁺ cell supernatant obtained from the partially protected B194, and the infected monkeys (C29, C30) which were clearly lower (33, 31 and 9% respectively) (Table 1). Although the number of animals investigated in this study is small, our results showed that all macaques vaccinated with live attenuated SIVmacC8 vaccine

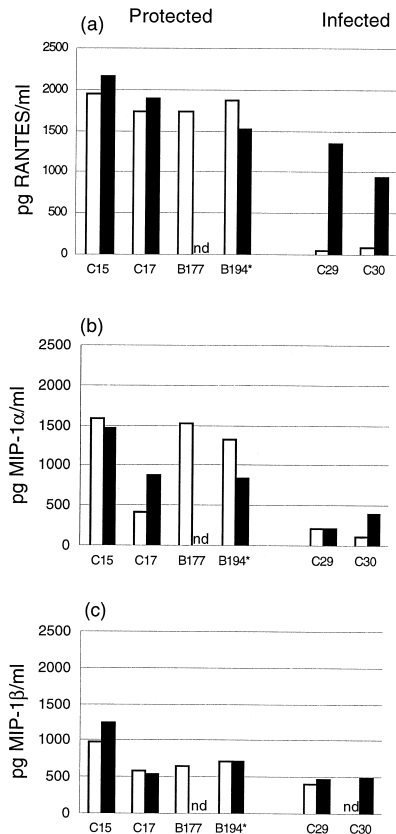


Fig. 1. Chemokine production of PHA-stimulated CD8⁺-enriched peripheral blood lymphocytes from monkeys before (white bars) and 6–8 months after (black bars) vaccination with live attenuated SIVmac C8 vaccine. (a) RANTES, (b) MIP-1 α , (c) MIP-1 β . Monkey B194 was partially protected as it controlled the replication of challenge virus during the first year of follow up (*). nd, Not determined.

which were resistant to infectious SIV challenge had higher *in vitro* anti-viral suppressor activity than the animals that became infected. Suppressor cell activity could only be determined in supernatants of cells collected 2–3 weeks after inoculation with SIVmacC8 due to the lack of cellular material.

To determine the possible role of RANTES, MIP-1 α and MIP-1 β in suppression of SIV replication in monkey CD4⁺ cells, we measured the concentrations of chemokines in the culture supernatants used in the suppressor cell assay by use of reagents from R&D Systems. Higher concentrations of each of the three β -chemokines were generated in the CD8⁺ cell culture supernatants of all of the completely or partially protected monkeys (C15, C17, B177, B194) as compared with animals which became infected after SIV challenge (P 0.06, determined by the Mann-Whitney U test for non-parametric observations) (C29, C30) (Table 1). We showed an association between high levels of RANTES, MIP-1 α and MIP-1 β and percentage suppression of SIV infection, suggesting that the β -chemokines were the major suppressive factors. However, by neutralizing the β -chemokines with antibodies others have demonstrated that the HIV-suppressive effects of soluble

factors produced by activated CD8⁺ cells cannot be explained solely by the β -chemokines (Kinter *et al.*, 1996; Paliard *et al.*, 1996; Rubbert *et al.*, 1997). Several studies have shown that CD8⁺ suppressor factors (Blackbourn *et al.*, 1997; Ennen *et al.*, 1994; Kannagi *et al.*, 1988; Landay *et al.*, 1993; Levy *et al.*, 1996; Mackewicz *et al.*, 1994; Walker & Levy, 1989; Walker *et al.*, 1991) as well as β -chemokines (Cocchi *et al.*, 1995; Kinter *et al.*, 1996; Mackewicz *et al.*, 1997; Pal *et al.*, 1997) can control virus replication *in vitro*. Our present results as well as some previous findings indicate that these factors also play an important role *in vivo*. Indeed, significant increases in the levels of CD8 suppressor factor, RANTES and MIP-1 β were associated with protection against rectal SIVsm challenge in macaques immunized with SIV envelope and core vaccine (Lehner *et al.*, 1996) or with HIV-1 envelope vaccine (Heeny *et al.*, 1998). High CD8⁺ T-cell-dependent virus suppression correlated with low virus load in macaques able to control their SIV infection *in vivo* (Abimiku *et al.*, 1997).

To study the effect of live attenuated SIVmacC8 vaccine on β -chemokine production the concentrations of RANTES, MIP-1 α , and MIP-1 β before and after vaccination were quantified in CD8⁺ cell culture supernatants collected and tested at the same time and under identical conditions. In general the CD8⁺ cell production of β -chemokines was very similar before and 6–8 months after vaccination (Fig. 1). However, increased level of RANTES was observed in the two monkeys (C29, C30) producing the lowest amount of RANTES before vaccination. Increased CD8⁺ cell production of MIP-1 α after vaccination was observed in two animals (C17, C30), while MIP-1 β production was unchanged in all the investigated monkeys. However, it is possible that we did not investigate the chemokine production at the optimal time-points after vaccination since it has been shown that infection with attenuated SIV clones induces low transient expression of chemokine genes (Zou *et al.*, 1997).

It is noteworthy that the six macaques vaccinated with live attenuated SIVmacC8 showed significantly increased MIP-1 β production 2–3 weeks after immunization as compared to the levels obtained before vaccination and at the time of challenge (compare Table 1 and Fig. 1). Whether the increased production was induced by the vaccine or only reflected differences in capacity of the CD8⁺ T-cells to proliferate in response to PHA is unclear. It is intriguing that the increased production was only in the concentration of MIP-1 β and appeared to be transient. All animals completely or partially protected against infectious SIVsm challenge showed higher production of β -chemokines after vaccination as compared to the monkeys that became infected (Fig. 1). Surprisingly, higher CD8⁺ cell-dependent β -chemokine production was observed in the protected macaques before they were vaccinated and without any obvious pre-selection of animals. The mitogen-induced CD8⁺ cell production of β -chemokines was also investigated in 33 naive macaques prior to inclusion in any HIV/SIV experiments (Fig. 2). The median levels were 771, 342 and 799

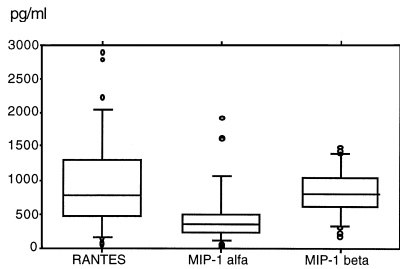


Fig. 2. β -Chemokine production of PHA-stimulated CD8⁺-enriched peripheral blood lymphocytes from 33 naive cynomolgus macaques. The three naive monkeys showing the highest RANTES and MIP-1 α production were among the animals which resisted infectious SIVsm challenge after vaccination.

pg/ml RANTES, MIP-1 α and MIP-1 β , respectively. Substantial variations in β -chemokine production were observed between different monkeys. Interestingly the four naive monkeys showing the highest RANTES production were among the animals which resisted infectious SIVsm challenge after vaccination with live attenuated virus (Table 1). Three of these four animals also produced the highest concentrations of MIP-1 α *in vitro*.

Apparently β -chemokines and HIV/SIV-specific immune responses have synergistic effects, since high chemokine levels were not sufficient to protect the macaques from infection with the vaccine inoculum. Furthermore among more than 150 monkeys tested we have not seen any naive animal being protected from an infectious challenge with HIV-2 or SIV given intravenously or intrarectally (Andersson *et al.*, 1996; Nilsson *et al.*, 1995; Putkonen *et al.*, 1994; our unpublished observations). In these experiments ≥ 10 MID₅₀ have been used to ensure infection of all monkeys. If lower challenge doses were used the relative role of β -chemokines may have become apparent. One control monkey with very high levels of CD8 suppressor factor and chemokine production by lymph node cells as well as by PBMCs was reported to be protected against a high dose (25 MID₅₀) of SIV given intrarectally (Lehner *et al.*, 1996).

Our results suggest that genetic or environmental factors influence the production of β -chemokines and that this may help in generating HIV/SIV-suppressive responses upon the first exposure to HIV/SIV. Resistance to HIV and SIV has been associated with high levels of β -chemokines in humans (Furci *et al.*, 1997) and in the macaque model (Lehner *et al.*, 1996), respectively.

Several studies have shown HIV/SIV-specific cellular immunity in repeatedly exposed but persistently uninfected individuals and monkeys (Shearer & Clerici, 1996; Putkonen *et al.*, 1997). Infection with HIV increases the mitogen-induced production of β -chemokines by PBMCs, CD8⁺ and CD4⁺ cells (Mackewicz *et al.*, 1997), but also HIV-1 p24 antigen-specific induction of β -chemokines and IFN- γ has been demonstrated in CD4⁺ T-cells of long-term non-progressors (Rosenberg *et al.*,

1997). High levels of β -chemokines are also secreted by CD4⁺ T-cells in response to an HIV-1 *env*-specific peptide in multiply HIV-1 exposed but persistently uninfected individuals (Furci *et al.*, 1997). Thus, it seems that live vaccines may induce protective chemokine production.

Sexual transmission of HIV occurs mainly by macrophage- or dual-tropic virus strains which preferentially use the CCR5 co-receptor (Zaitseva *et al.*, 1997). Therefore induction of immune responses aiming at blocking the virus entry mediated by CCR5 should be considered in future HIV vaccine development. Thus, vaccines able to induce a Th1-type immune response should be favourable as they may have the potential to induce β -chemokine production besides induction of virus-specific immune mechanisms. Further studies are needed to determine the relative role of the β -chemokines and the specific immune responses for protection and how to obtain the best synergistic effects of these protective mechanisms.

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