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Role of β -Chemokines in Suppressing HIV Replication

Fiorenza Cocchi *et al.* (1) found that three different β -chemokines (RANTES, MIP-1 α , and MIP-1 β) produced by CD8 $^{+}$ T lymphocytes suppress human immunodeficiency virus (HIV) replication in peripheral blood mononuclear cells (PBMC). Moreover, neutralizing antibodies to all three chemokines eliminate the activity against HIV detected in CD8 $^{+}$ cell supernatants (1). They conclude that these chemokines are responsible for the CD8 $^{+}$ cell anti-HIV

activity described in our studies (2–4).

During our attempts to identify CD8 $^{+}$ cell antiviral factors (CAFs) that could mediate CD8 $^{+}$ cell anti-HIV activity, several cytokines—including the interferons α and β , the chemokine IL-8, TGF- β , TNF- α , and the β -chemokines reported by Cocchi *et al.*—were identified as having antiviral activity (3–5) (see below). None of these cytokines, however, has been present in consistent or sufficient amounts to be CAF.

In addition, neutralizing antibodies to these cytokines have not affected the extent of the anti-HIV activity that we have detected in CD8 $^{+}$ cell culture fluids (3–5).

In our evaluation of the β -chemokines, we found that the concentrations of RANTES, MIP-1 α , and MIP-1 β in CD8 $^{+}$ cell supernatants [as measured by enzyme-linked immunosorbent assay (ELISA)] did not correlate with the anti-HIV activity detected in our assays (Fig. 1). Culture fluids with high anti-HIV activity (6) had concentrations of these chemokines from 0.1 to 4 ng/ml, and culture fluids lacking antiviral activity showed similar concentrations. Moreover, none of these three chemokines, even when used together, inhibited HIV-1_{SF2} replication in purified CD4 $^{+}$ cells at the concentrations found in supernatants of CD8 $^{+}$ cells nor at the concentrations reported by Cocchi *et al.* (1) (Fig. 2A). This virus is one used in our measurement of CAF antiviral activity (3, 4, 6, 7).

When these chemokines were used with a variety of freshly isolated viruses, different sensitivities were noted (see examples, Fig. 2, B to D). Some were highly sensitive to the chemokines (for example, SV), some were resistant, and others showed an intermediate pattern. Cell antiviral factors show antiviral activity against all these viruses. As expected, a mixture of neutralizing antibodies to the three β -chemokines, at quantities similar to those cited (1), did not block the antiviral activity of CD8 $^{+}$ cell supernatants against acute HIV-1_{SF2} repli-

Fig. 1. RANTES, MIP-1 α , and MIP-1 β in CD8 $^{+}$ cell culture fluids that were positive (hatched bars) or negative (open bars) for CAF antiviral activity. Chemokine concentrations in randomly selected CD8 $^{+}$ cell culture fluids were measured by ELISA (Quantikine kits, R&D Systems, Minneapolis, Minnesota) in duplicate according to the manufacturer's instructions. CAF activity of each fluid was previously determined as described (4, 6, 7). Data shown are representative of nine CAF-positive and -negative fluids. Fluids were collected on days 7 through 12 from cultured CD8 $^{+}$ cells from asymptomatic HIV-1-infected individuals. This time period is optimal for CAF production in vitro (4).

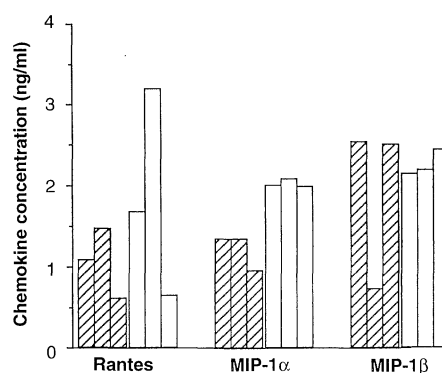


Fig. 2. Effect of recombinant chemokines on HIV-1 replication in CD4 $^{+}$ cells. (A) CD4 $^{+}$ cells acutely infected with HIV-1_{SF2} (4). (B through D) Primary isolates of HIV-1 obtained from asymptomatic long-term survivors (B) SV, (C) NB, or (D) EM were cultured in triplicate in the presence of a mixture of the recombinant human chemokines RANTES, MIP-1 α , and MIP-1 β (R&D Systems). The concentrations of the chemokines tested were 500 ng/ml (●), 50 ng/ml (▲), 5 ng/ml (◆), and no chemokine added (■). The cultures were passed every 2 days and replenished with fresh chemokines at each passage. Reverse transcriptase (RT) activity was measured in culture fluids from the indicated time-points as described (4).

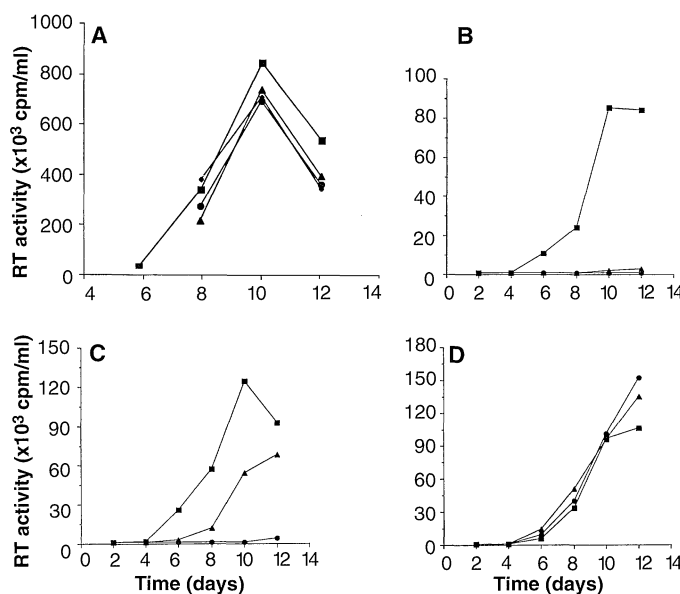


Table 1. Effect of neutralizing antibodies to β -chemokine on the ability of CAF-containing fluids to suppress HIV-1 replication in CD4 $^{+}$ lymphocytes. A 50% dilution of CAF-containing culture fluid, or the medium control fluid, was left untreated or pretreated with control antibodies (Ab) or a mixture of antibodies specific for the β -chemokines for 40 min at room temperature before addition to HIV-1_{SF2}-infected CD4 $^{+}$ lymphocytes. Control antibody was nonspecific goat polyclonal (R&D Systems); anti-chemokine antibody mix consisted of goat polyclonal neutralizing antibodies specific to the human chemokines, RANTES (100 μ g/ml), MIP-1 α (50 μ g/ml), and MIP-1 β (100 μ g/ml) (R&D Systems). Reverse transcriptase (RT) activity shown indicates the average peak of HIV-1_{SF2} replication (at day 10) in triplicate cultures. Fluids contained β -chemokine levels similar to those shown in Fig. 1. Culture fluids receiving the antibodies to chemokine showed a complete elimination of the chemokines as detected by ELISA (5).

Antibody treatment	RT activity ($\times 1000$ cpm/ml)	
	CAF-containing fluid	Control fluid
Untreated	1952	942
Control Ab	1740	923
Chemokine-Ab mix	1881	903

Table 2. Effect of neutralizing antibodies to β -chemokines on CAF-mediated suppression of a β -chemokine-sensitive HIV-1 isolate. As described in Table 1, a 50% dilution of two CAF-containing culture fluids was pretreated with control antibody or with a mixture of neutralizing antibodies to RANTES, MIP-1 α , and MIP-1 β before addition to HIV-1_{SV}-infected CD4⁺ cells. The effect of these treatments on the percentage of suppression of HIV replication by the CAF-containing medium relative to control medium-treated cells is presented. Virus replication in the control culture receiving no CAF was about 150,000 cpm of RT activity per milliliter of culture fluid. The chemokine levels in CAF fluid 1 were 689, 132, and 520 pg/ml for RANTES, MIP-1 α , and MIP-1 β , respectively. For CAF fluid 2, we selected a CD8⁺ cell culture supernatant with high chemokine levels, which were 14,161, 14,519, and 11,450 pg/ml for RANTES, MIP-1 α , and MIP-1 β , respectively. Fluids receiving the anti-chemokine antibodies showed complete elimination of the chemokines as measured by ELISA.

Antibody	Suppression of HIV production (%)	
	CAF 1	CAF 2
Neutralizing Ab	55	72
Control	46	96

cation in purified CD4⁺ cells (Table 1), and HIV-1_{SF33} replication in the Jurkat cell-derivative 1G5 cell line (5). In the latter case, the suppressive effects of CAF on HIV-induced long terminal repeat (LTR) driven transcription of luciferase (7) were also evaluated in the presence of the antibody mixture (8). CAF-containing fluids inhibited luciferase production by 84%, and the neutralizing antibodies to the chemokines showed no effect on this antiviral effect of the CAF-containing fluid (81% suppression) (5). The chemokines showed no effect on the LTR-driven production of luciferase (5). We have also found that CAF blocks phorbol 12-myristate 13-acetate (PMA) activation of HIV-LTR transcription in the 1G5 line (5); the chemokines show no effect in this system (5).

We evaluated a chemokine-sensitive HIV isolate (SV) by the neutralization assay. This virus was suppressed by CAF-containing fluid, even in the presence of the mixture of neutralizing antibodies to the three chemokines (Table 2). The partial elimination of suppressing activity observed with treatment of CAF 2 with the antibodies suggests that the high concentrations of β -chemokines present in this CAF fluid (Table 2) could be contributing to the antiviral activity measured.

In summary, some β -chemokines exhibit anti-HIV activity in vitro against certain primary isolates. However, as observed with interferons, IL-8, TGF β , and TNF α , these cytokines are not primarily responsible for

the noncytotoxic antiviral activity we observed with CD8⁺ cells (3). Production of CAF is highest in asymptomatic individuals and decreases with progression to disease (3). RANTES, MIP-1 α , and MIP-1 β are not present in higher concentrations in CD8⁺ cell culture fluids from HIV-infected individuals who are long-term survivors as compared with those fluids from individuals in whom the disease is progressing (5). They do not show the broad antiviral activity of CAF. Moreover, they do not appear to suppress HIV transcription, as do CAF and CD8⁺ cells when they are added to infected CD4⁺ cells (7). Nevertheless, recent observations about the β -chemokines (9) further support the value of studying natural immune factors against HIV infection.

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6. CD8⁺ cell culture supernatants were prepared by our standard procedure (4). In brief, CD8⁺ cells were obtained from PBMC recovered from asymptomatic HIV-infected individuals. These cells were stimulated for 3 days with antibodies to CD3 attached to immunomagnetic beads. Cells were removed, washed, and cultured for 2 weeks in AIM-V serum-free medium (Gibco, Gaithersburg, MD) with 1% antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). Fluids were collected every 2 days and stored at -70°C. At the completion of the cell cultivation, the individual fluids recovered were filtered and tested for anti-HIV activity against acute infection of purified normal CD4⁺ cells with HIV-1_{SF2}. In this CAF assay, 1000 TCID₅₀ of HIV-1_{SF2} were inoculated into purified CD4⁺ cells (3×10^6 cells). After 1 hour, cells were trypsinized to remove input virus, and culture fluid was added. The culture fluids were removed every 2 days and assayed for viral reverse transcriptase activity. Fresh control fluid or the CAF-containing fluid mixed with fresh medium at a 1:1 ratio was added to the cultures. Those fluids showing anti-HIV activity ($\geq 50\%$ suppression) were pooled, filtered, and used for the assays described in this comment. All CAF-negative fluids that were used showed less than 10% suppression of HIV replication. The CD8⁺ cell fluids were also tested for antiviral activity with the use of acute infection of the 1G5 cell line with HIV-1_{SF33} (7). The culture fluids were assayed for viral reverse transcriptase activity, and extracts from the cells were examined for luciferase activity (7). Results were compared with those obtained with control culture fluids.
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8. The 1G5 cell line has a single, stably integrated copy of an HIV LTR linked to a luciferase gene [E. Aguilar-Cordova *et al.*, *AIDS Res. Human Retroviruses* **10**, 295 (1994)]. Infection of this cell line with HIV-1_{SF33}

induces an increase (≥ 100 -fold) in luciferase production, presumably as a result of transactivation by HIV Tat expressed during infection.

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Response: Mackewicz *et al.* contend that the C-C chemokines RANTES, MIP-1 α , and MIP-1 β that we have recently identified as major components of the HIV-suppressive soluble activity released by both primary and immortalized CD8⁺ T cells (1) are not the so-called "CAF" they have investigated in the course of the last 10 years (2). Within 6 to 8 months of the publication of our report (1), a large body of evidence has accumulated that confirms the relevance of chemokines to the physiology of HIV infection. Indeed, a series of new, fundamental advances in our understanding of HIV infection has directly stemmed from the initial connection between HIV and chemokines (3).

It is encouraging that Mackewicz *et al.* now acknowledge that RANTES, MIP-1 α , and MIP-1 β can totally suppress HIV infection even at doses as low as 0.005 μ g/ml (figure 2B of the comment), in contradiction with their recent statement that "only at high levels (0.5 to 1.0 μ g ml⁻¹)" do these chemokines "show some anti-HIV activity" (4). Some of the other experiments described by Mackewicz *et al.* (figure 2A and table 1) were performed with T cell line tropic viruses (HIV-1_{SF2} and HIV-1_{SF33}), which we have previously shown to be insensitive to RANTES, MIP-1 α , and MIP-1 β (1). This lack of sensitivity is determined by critical changes in the V3 domain of the gp120 envelope glycoprotein (5). The low-level "CAF" activity detected against these isolates—that is, only about 50% RT suppression (table 1 of the comment)—must therefore result from factors other than such chemokines. These "other factors" may include some of the known HIV-suppressive cytokines produced by CD8⁺ T cells [the role of most of these factors was not rigorously excluded with the acute infection test, which is performed with T cell line tropic strains, but only with the endogenous trans-well test (6), which is commonly performed with NSI strains] and the C-X-C chemokine SDF-1 (the newly identified CXCR4-ligand). Nevertheless, it cannot be excluded that other, still unidentified factors (probably cytokines) might also play a role, particularly in a nonspecific manner—for example, by altering T cell activation or metabolism. With regard to results with a " β -chemokine-sensitive" HIV-1 isolate (table 2 of the comment), many other laboratories have already con-

firmed that RANTES, MIP-1 α , and MIP-1 β are major components of the HIV-suppressive activity against primary HIV and SIV isolates, which are those commonly assayed in the classical test of viral suppression, the endogenous test. Of note, the CAF-1 fluid, which showed only a modest suppressive activity (less than 50%), had the lowest content of RANTES, MIP-1 α , and MIP-1 β . Mackewicz *et al.* also find low levels of chemokine production by purified CD8⁺ T cells (figure 1 of the comment). Although the experimental systems are difficult to compare in the absence of sufficient technical details, these results are not consistent with those reported by us (1) and others (3, 7).

The "CAF" theory is founded on two major postulates that, until the positive identification of the factor, cannot be subjected to a rigorous scientific scrutiny: first, that the HIV-suppressive activity produced by CD8⁺ T cells results from a single factor; second, that all the different tests used to study "CAF" (endogenous, acute infection, and transcriptional) measure the activity of the same suppressive factor. The evidence thus far accumulated seems to contradict both of these assumptions.

With regard to the first postulate, CD8⁺ T cells produce a complex cocktail of factors, some of which have a well-documented

HIV-suppressive activity. For example, the HIV-suppressive effect could be abrogated in our system only when a combination of antibodies against all the different suppressive factors present in the cocktail was used (1). Thus, previous results obtained with the use of a single cytokine-neutralizing antibody at a time (6) should be critically reevaluated.

With regard to the second postulate, it is increasingly evident that different assay systems measure different suppressive factors (or different sets of suppressive factors); an example is the selectivity of chemokines against different biological subtypes of HIV (RANTES, MIP-1 α , and MIP-1 β for NSI isolates; SDF-1 for SI isolates). The concept of two easily distinguishable suppressive activities was implicit in previous results obtained by Levy and his colleagues, who observed that CD8⁺ T cells derived from healthy seronegative individuals, unlike those from HIV-seropositive patients, suppress only in the endogenous test (mostly NSI viruses), but not in the acute infection test (SI viruses) (8). The best explanation for these findings is that CD8⁺ T cells from uninfected people release a more limited complement of HIV-suppressive factors.

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