PNAS PNAS PNAS

Soluble factors from T cells inhibiting X4 strains of HIV are a mixture of β chemokines and RNases

Fiorenza Cocchi[a,b], Anthony L. DeVico[a,b], Wuyuan Lu[a,c], Mikulas Popovic[a], Olga Latinovic[a,d], Mohammad M. Sajadi[a,b], Robert R. Redfield[a,b], Mark K. Lafferty[a,d], Massimo Galli[e], Alfredo Garzino-Demo[a,d], and Robert C. Gallo[a,b,1]

[a]Institute of Human Virology and Departments of [b]Medicine, [c]Biochemistry and Molecular Biology, and [d]Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201; and [e]Section of Infectious Diseases and Immunopathology, Department of Clinical Sciences, Luigi Sacco', University of Milan School of Medicine, 20157 Milan, Italy

Contributed by Robert C. Gallo, February 22, 2012 (sent for review July 19, 2011)

T-cell-derived soluble factors that inhibit both X4 and R5 HIV are recognized as important in controlling HIV. Whereas three β chemokines, regulated-on-activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, and MIP-1β, account for the suppression of R5 HIV by blockade of HIV entry, the major components responsible for the inhibition of X4 HIV strains have not been identified previously. We identify these factors primarily as a mixture of three β chemokines [macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC), and I-309] and two RNases (angiogenin and RNase 4) of lesser potency and show that in a clade B population, some correlate with clinical status and are produced by both CD4^{+} and CD8^{+} T cells (chemokines, angiogenin) or only by CD8^{+} T cells (RNase 4). The antiviral mechanisms of these HIV X4-suppressive factors differ from those of the previously described HIV R5-suppressive β chemokines.

antiretroviral | lentivirus

Cell-mediated immunity is important in the control of persistent HIV replication (1–5). In part, the mechanisms involve soluble factors. More than 20 y ago, Levy et al. discovered that an unidentified substance(s) released from CD8^{+} T cells, which they called CD8^{+} antiviral factor (CAF), suppressed replication of both R5 and X4 HIV-1 strains (6). High levels of CAF activity were found in individuals, called long-term nonprogressors (LTNPs), who control HIV infection in the absence of antiretroviral therapy. This suggested that such factor(s) may be important in delaying or preventing disease progression (7). Our group identified the β chemokines, macrophage inflammatory protein (MIP)-1α, MIP-1β, and regulated-on-activation normal T-cell expressed and secreted (RANTES), as major mediators of the CD8 suppressive activity against R5 HIV-1 isolates (8) that depend upon CC chemokine receptor (CCR)5 for entry (9), and, later, we and others showed that levels of some correlated with protection against infection, as well as with an asymptomatic clinical status in HIV infection (10–14). Our group and others also reported that CD4^{+} T cells secreting antiviral CCR5 ligands are self-protected against infection with R5 virus during the primary immune response in vitro (15–18). Therefore, it is clear that the suppressive soluble anti-HIV R5 factors are a collection of β chemokines, which are derived from both CD4^{+} and CD8^{+} cells.

On the other hand, it is also known that X4 HIV isolates, which use CXC chemokine receptor (CXCR)4 as a coreceptor for entry into target cells (9) are inhibited by soluble factors released from activated T cells (18–20). However, the identity of immunological anti-HIV X4 factors has remained elusive until now. Although a few factors have been described with X4 anti-HIV activity, we show here that they make up only a small portion of the total activity, and we identify the major components of the anti-X4 activity released by T cells as the β chemokines I-309, macrophage-derived chemokine (MDC), and thymus and activation-regulated chemokine (TARC), together with RNase 4 and angiogenin. The neutralization of endogenous production of these chemokines by primary CD8^{+} and CD4^{+} cells enhanced the replication of X4 HIV-1 isolates in CD4^{+} T cells. Moreover, a combination of neutralizing antibodies (NAbs) against the β chemokines and the placental ribonuclease inhibitor abrogated the anti-X4 activity of CD8-derived supernatants from some HIV[+] asymptomatic individuals. In summary, our study suggests that these factors are secreted by primary T cells at effective antiviral concentrations, suggesting their relevance as HIV suppressor factors. The study of their role in protection against HIV-1 is of clear future interest.

Results

Identification of the Anti-X4 T-Cell-Derived Soluble Factors MDC, TARC, I-309, RNase 4, and Angiogenin. To identify the factor(s) that suppress X4 HIV strains, we first examined primary cells from LTNPs for total anti-X4 HIV soluble activity. In all subjects analyzed, we observed strong inhibition (64–88%) against the X4 isolate HIV-1_{IIIB} with supernatants from both CD4^{+} and CD8^{+} T cells (Table S1). To obtain a more reproducible source of noncytolytic X4 HIV suppressor activity, we developed HTLV-1 immortalized CD8^{+} and CD4^{+} T-cell lines from these LTNPs. One CD8^{+} T-cell line (LTNP-J) and one CD4^{+} T-cell line (LTNP-3) that consistently released the highest levels of noncytolytic X4 HIV suppressor activity were adapted to grow in serum-free medium, and the conditioned media (CM) from these cultures were used for purification. The CM were passed over a heparin-affinity column, and fractions containing anti-HIV X4 activity were purified by reverse-phase HPLC.

From LTNP-J, we identified two separate HPLC fractions (fractions 26 and 28) (Fig. 1*A*) with HIV-1-suppressor activity. Each contained a single major peak, and SDS/PAGE revealed two protein species of 16 and 8 kDa (Fig. 1*B*). The stained bands were excised and eluted, and peptides subjected to proteolytic digestion and identified by mass spectrometry. The 8-kDa species contained a mixture of the β chemokines TARC, I-309, and MDC. Using alternative protein purification protocols (21), we, again, identified MDC and TARC as the major components of the anti-X4 activity present in one HPLC fraction (profile not shown). NAbs against TARC and MDC abrogated the antiviral activity present in the fraction (Fig. 1*C*). The 16-kDa band contained a mixture of two proteins belonging to the ribonuclease A (RNase A) superfamily, angiogenin and RNase 4. The pure forms of each of these molecules had antiviral activity as shown below. A fraction with suppressive activity obtained from LTNP-3 CM was similarly analyzed. A single protein species of 12-kDa was isolated from an HPLC fraction with anti-X4 activity and identified as a glycosylated form of I-309 (HPLC profile not shown). I-309 NAb reduced the anti-X4 activity present in the fraction by 67% (Fig. 1*D*).

Author contributions: F.C. designed research; F.C., O.L., M.K.L., and A.G.-D. performed research; W.L. contributed new reagents/analytic tools; F.C., A.L.D., M.P., M.M.S., R.R.R., M.G., and A.G.-D. analyzed data; and F.C., A.G.-D., and R.C.G. wrote the paper.

The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. E-mail: rgallo@ihv.umaryland.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202240109/-/DCSupplemental.

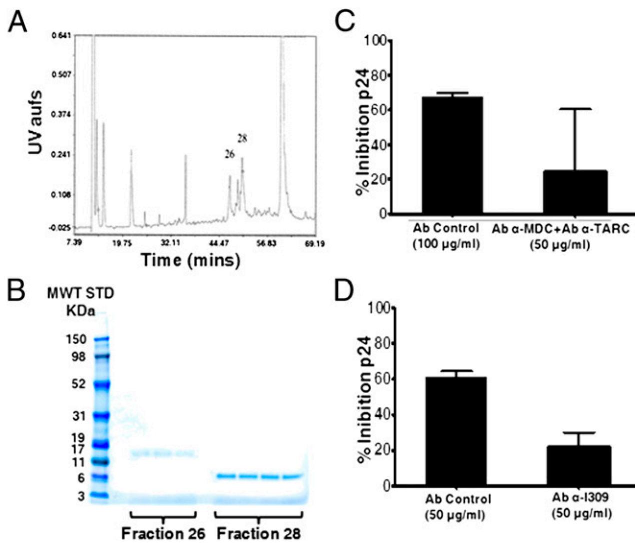

Fig. 1. Identification of MDC, TARC, I-309, RNase 4, and angiogenin as anti X4 soluble factors. (*A*) HPLC profile of heparin eluate from serum-free culture of an HTLV-1 immortalized CD8$^+$ T-cell line from a LTNP patient. Two separate fractions (fractions 26 and 28), each containing a single major peak, showed strong HIV-1 suppressor activity. (*B*) SDS/PAGE of two protein species of 16 and 8 kDa that were isolated from fractions 26 and 28, respectively. Analysis of the 16-kDa band revealed a mixture of two proteins belonging to the ribonuclease A (RNase A) superfamily, angiogenin, and RNase 4. Analysis of the 8-kDa band showed a mixture of the chemokines MDC, TARC, and I-309. (*C* and *D*) Neutralization of the anti-X4 activity present in HPLC fractions containing TARC and MDC (*C*) or I-309 (*D*). HPLC fractions were pretreated with NAbs against TARC and MDC or I-309. Subsequently, HPLC pretreated fractions were added to peripheral blood mononuclear cells (PBMCs) infected with HIV-1_{IIIB} and then added to the cultures every 48 h. HPLC fractions treated with control antibodies, used at the same concentration as the neutralizing antibodies, were tested in parallel. The levels of HIV-1 replication were tested at day 5 after infection by p24 antigen capture on cell-free supernatants. (Error bars: SD.)

I-309, MDC, and TARC Production by CD8$^+$ and CD4$^+$ T Cells from HIV$^+$ Asymptomatic Individuals, AIDS Patients, and Uninfected Donors. To determine whether chemokine production correlated with clinical status, supernatants from phytohemagglutinin (PHA)-activated enriched CD4$^+$ and purified CD8$^+$ T cells were analyzed by ELISA for the production of I-309, MDC, and TARC. We compared chemokine production in three different groups: (*i*) uninfected controls; (*ii*) asymptomatic HIV$^+$ patients, which included LTNPs (subjects that despite ≥7 y of documented HIV infection maintained CD4$^+$ T cells >600 cells/μL and low viral loads) and natural viral suppressors (NVSs) [HIV$^+$ individuals without HIV-related disease who maintained plasma HIV RNA < 400 copies/mL for at least 2 y (22)]; and (*iii*) a group of untreated HIV$^+$ individuals who progressed to AIDS with CD4$^+$ T cells <200 cells/μL and high viral loads. The NVS group controls HIV replication in the absence of antiviral therapy and is similar to the group called elite HIV controllers (23). All HIV$^+$ individuals analyzed were infected with HIV clade B. None was receiving antiretroviral therapy.

Production of I-309, MDC, and TARC by autologous CD4$^+$ T cells was much greater than that of parallel cultures of CD8$^+$ T cells (Fig. 2), and CD4$^+$ T cells from asymptomatic infected subjects produced higher levels of TARC ($P = 0.2$), I-309 ($P = 0.1$), and MDC ($P = 0.01$) than did those from uninfected controls (Fig. 2 *A*–*C*). Production of both MDC ($P = 0.002$) and TARC ($P = 0.01$) (Fig. 2 *A* and *B*) but not I-309 ($P = 0.09$) (Fig. 2*C*) by CD4$^+$ T cells from asymptomatic HIV$^+$ subjects was also significantly greater than that from AIDS patients. The lack of strong statistical significance for differences in I-309 levels may reflect variation in glycosylation that might hinder detection. There were no significant differences in production of the chemokines by CD8$^+$ T cells between any of the groups (Fig. 2 *E*–*G*).

Production of RNase 4 and Angiogenin. Potent HIV suppressor activity was recovered from the CD8$^+$ HTLV-1 immortalized cell line LTNP-J, as described above, and identified as a mixture of two proteins belonging to the RNase A superfamily, angiogenin and RNase 4 (Fig. 1 *A* and *B*). In addition to their common RNase activity, members of the RNase A superfamily have been reported previously to inhibit HIV infection (24). We measured RNase 4 in primary CD8$^+$ and CD4$^+$ T cells from 14 normal donors, 10 LTNPs, 9 NVSs, and 8 AIDS subjects. RNase 4 mRNA signal was detected only in CD8$^+$ T cells from uninfected controls (50%), LTNPs (60%), and NVSs (78%) and from 25% of AIDS patients. The differences were not significant, but a trend toward a significant difference was observed between the NVSs and AIDS patients ($P = 0.056$) (Table S2). Angiogenin production was greater from CD4$^+$ T cells than CD8$^+$ and was greater from both cell types with cells derived from asymptomatic HIV-infected individuals ($P = 0.076$ and $P = 0.67$, respectively) compared with those from uninfected controls and those produced by AIDS patients ($P = 0.004$ and $P = 0.0005$, respectively) (Fig. 2 *D* and *H*).

To be certain that natural antigens (instead of PHA) also induced these activities, we tested HIV antigens including myristoylated p17, p24, and gp120 with peripheral blood mononuclear cells (PBMCs) from four NVS subjects. All were active but varied among the subjects tested (Fig. S1).

Interaction of CD28 on T cells with its ligands CD80 and CD86 on antigen-presenting cells is required for the induction of many cellular immune responses (25). We analyzed CD28-induced secretion of MDC, TARC, I-309, and angiogenin in PBMCs from three uninfected donors and from CD4$^+$ T cells of two NVSs. Stimulation of antigen and CD28 receptors on PBMCs and CD4$^+$ T cells by immobilized monoclonal antibodies (mAbs) to CD3 and CD28 induced the secretion of the three X4 β chemokines and angiogenin. The levels of MDC, TARC, I-309, and angiogenin were similar in CD28-costimulated and PHA-activated PBMC cultures (Table S3).

Protective Role of Endogenous I-309, MDC, TARC, and Angiogenin Against Infection of CD4$^+$ T Cells by X4 HIV Isolates. We investigated the effects of endogenous I-309, MDC, and TARC on the replication of X4 HIV isolates in PBMCs from normal donors (Fig. 3 *A*–*C*). Because these β chemokines are secreted primarily by CD4$^+$ T cells, we also studied the self-protective effects in these cells (Fig. 3*D*) and in a CD4$^+$ T-cell line (LTNP3) (Fig. 3*E*). When a mixture of NAbs against I-309, MDC, and TARC was added to PBMCs or CD4$^+$ T cells infected with the X4 isolate HIV-1_{IIIB} or with two primary X4 isolates, SI04 and SI05, viral replication was increased compared with that of cultures treated with control antibody, as determined by p24 antigen levels. There was no significant increase in viral replication when the chemokine NAbs were used individually. The same results were obtained when LTNP3 was infected with HIV-I_{IIIB} in the presence of autologous (CM) treated with NAbs against the β chemokines or antibody controls. The CM contained high levels of I-309 (200 ng/mL), MDC (247 ng/mL), and TARC (26 ng/mL) and lower levels of angiogenin (2 ng/mL), as measured by ELISA. After infection, the CD4$^+$ T cells were cultured in the presence of autologous CM treated with NAbs or antibody controls every 72 h. The levels of p24 antigen in the control cultures were almost undetectable (0.3 ng/mL) 10 d after infection. Treatment with a combination of NAbs against I-309, MDC, and TARC markedly enhanced virus replication as measured by p24 (8 ng/mL), whereas treatment with only two NAbs against MDC and TARC gave modest enhancement (Fig. 3*E*). Individual antibodies (against I-309, MDC, TARC, or angiogenin) alone only modestly effected HIV-1_{IIIB} replication. The addition of antibody against angiogenin to the β-chemokine antibody mixture did not increase the effect on viral replication (Fig. S2). We also analyzed the role played by the X4 β chemokines and angiogenin in CD4-derived supernatants from two HIV-1 asymptomatic individuals (NVSs) and from an allogeneic uninfected subject (Fig. 3*F*). CD4-derived supernatants were treated with a combination of NAbs against I-309, MDC, TARC, and angiogenin or with a mixture of control antibodies and then added to PBMCs infected with HIV-1_{IIIB}. The

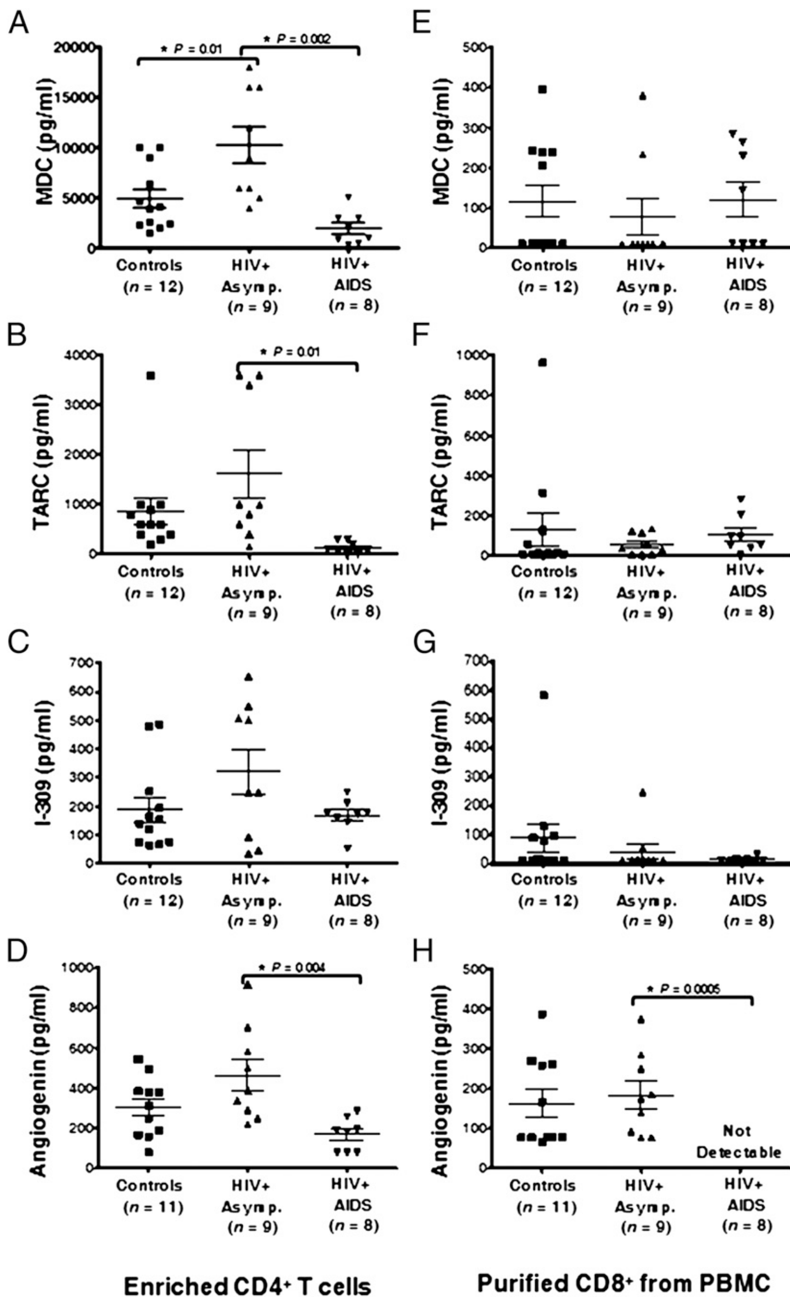

Fig. 2. Production of MDC, TARC, I-309, and angiogenin. Secretion of MDC, TARC, I-309, and angiogenin was tested in enriched CD4+ T cells (*A–D*) and in purified CD8+ T cells (*E–H*) from normal donors (solid squares), asymptomatic HIV+ subjects (solid triangles), and subjects with AIDS (inverted solid triangle). Cell culture supernatants of PHA-stimulated CD4+ and CD8+ T cells were collected on day 9 and analyzed by ELISA for the presence of I-309, MDC, TARC, and angiogenin. The indicated *P* value was generated from *t* tests. (Error bars: SD.)

neutralization of the β chemokines together with angiogenin abrogated the anti-X4 activity of CD4-derived supernatants from NVS 9, NVS 11, and a normal donor by 73%, 50%, and 65% respectively.

Antibodies Against I-309, MDC, and TARC in Combination with Placental RNase Inhibitor Neutralize the CD8-Derived HIV-Suppressive Activity. We evaluated the role of the endogenous anti-X4 β chemokines angiogenin and RNase 4 in primary CD8-derived supernatants from six HIV-1 asymptomatic subjects (one LTNP and five NVS) that exhibited strong inhibition of X4 virus replication (Fig. 4 *A* and *B*). CD8-derived supernatants were treated with placental RNase inhibitor or with control buffer alone and then were added to PBMCs infected with HIV-1_{IIIB}. The RNase inhibitor showed a moderate reduction of the anti-X4 activity of supernatants from NVS 13 (46%) and NVS 5 (34%) and a somewhat lesser effect on supernatants from other subjects (Fig. 4*A*), suggesting that RNase 4 played an appreciable role in the anti-X4 activity of all CD8-derived supernatants analyzed. A combination of NAbs against I-309, TARC, and MDC plus RNase inhibitor abrogated the anti-X4 activity of CD8-derived supernatants from NVS 13, LTNP-J, NVS 9, and NVS 35 (60%, 74%, 82%, and 77%, respectively). NVS 5 and NVS 10 supernatants were slightly less sensitive to neutralization (51% and 38% blocking, respectively) (Fig. 4*B*).

Dose-Dependent Inhibition of HIV Replication by I-309, MDC, TARC, RNase 4, and Angiogenin. We used recombinant human (rh) chemokines to determine the dose dependency and potency of the antiviral activities of MDC, TARC, and I-309. We previously showed that MDC inhibited both X4 and R5 infection of PBMCs (26). We again found a dose-dependent inhibition by MDC of HIV-1 replication in PBMCs acutely infected with the X4 isolate HIV-1_{IIIB}, confirming previous results (Fig. 5*A*). If the mechanism of the anti-HIV effect of MDC involves its receptor, CCR4 (9), and subsequent signaling events, then it would be expected that a dose-dependent inhibition of X4 viruses would also be observed with

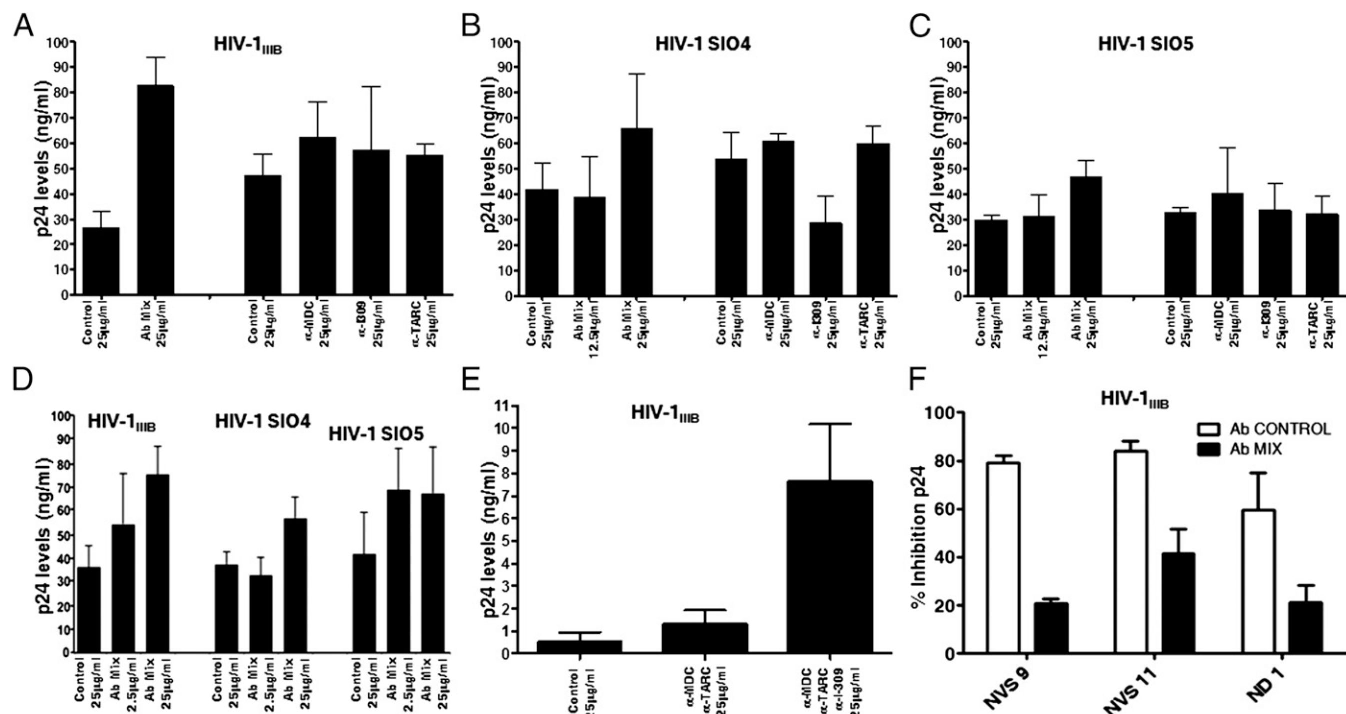

Fig. 3. Neutralization of the X4 β chemokines and angiogenin reduced the inhibition of HIV replication by supernatants from PBMCs, CD4$^+$ T cells, and an immortalized CD4$^+$ T-cell line, LTNP-3. PHA-activated PBMCs (*A*–*C*) and enriched CD4$^+$ T cells (*D*) from normal donor cells were infected in vitro with X4 HIV-1 isolates. After infection, NAbs against I-309, MDC, and TARC alone, or in combination (Ab Mix), were added to the cell cultures every 72 h. The HTLV-1 immortalized CD4$^+$ T-cell line LTNP-3 (*E*) was infected with HIV-1$_{IIIB}$ in the presence of its own conditioned media (CM) treated with either NAbs against the β chemokines or isotype controls. CD4-derived supernatants from two NVS and an allogeneic uninfected donor (ND 1) (*F*) were pretreated with a combination of NAbs against I-309, MDC, TARC, and angiogenin (Ab Mix) at the concentration of 25 μg/mL each. Subsequently, supernatants (50% vol/vol) were added to PBMCs infected with HIV-1$_{IIIB}$ and replaced with new treated supernatants every 72 h. The cultures were tested for HIV-1 p24 antigen at day 5 or 10 after infection. Isotype control antibodies were tested at the same concentrations used for the NAbs. Cultures treated with control antibodies showed the same levels of p24 as cultures treated with RPMI medium 1640 alone. (Error bars: SD.)

TARC, which also uses CCR4 as a receptor (9), and this was indeed the case (Fig. 5*A*). The similarity of results between MDC and TARC suggest that the mechanism involves G protein-related signaling events, which is the subject of current studies. The X4 antiviral activity of I-309, a ligand for CCR8 (9), was greater than that of either MDC or TARC (Fig. 5*A*). Infection of PBMCs with the R5 HIV-1$_{BaL}$ isolate was also inhibited by I-309, MDC, and TARC in a dose-dependent manner (Fig. 5*B*).

When the HIV-suppressive effect of synthetic RNase 4 on the replication of HIV-1$_{IIIB}$ was investigated in PBMCs, a dose-dependent inhibition of HIV replication was observed (Fig. 5*C*). In agreement with a previous study reporting the antiviral activity of angiogenin against an HIV-1 isolate using predominantly the CXCR4 coreceptor (24), rh angiogenin suppressed replication of the X4 HIV-1 SIO5 isolate in PBMCs in a dose-dependent manner (Fig. 5*D*).

MDC, TARC, and I-309 Inhibit X4 Viral Replication in PBMCs at a Postfusion Level. CCR4, the receptor for MDC and TARC, and CCR8, the receptor for I-309, when overexpressed in cell lines, appear to support infection by a variety of HIV-1 strains (9). Nevertheless, the role of CCR4 and its ligands MDC and TARC in infection of PBMCs is not clear, and the role of CCR8 and its ligand I-309 has not been investigated. To determine whether CCR4 and CCR8 ligands affect fusion in PBMCs, we used an X4 HIV pseudotype virus expressing a β lactamase-Vpr (BlaM-Vpr) fusion protein. Fusion of virions containing BlaM-Vpr with PBMCs can be detected by cleavage of a green fluorogenic substrate previously loaded into target cells to generate blue fluorescence. I-309, MDC,

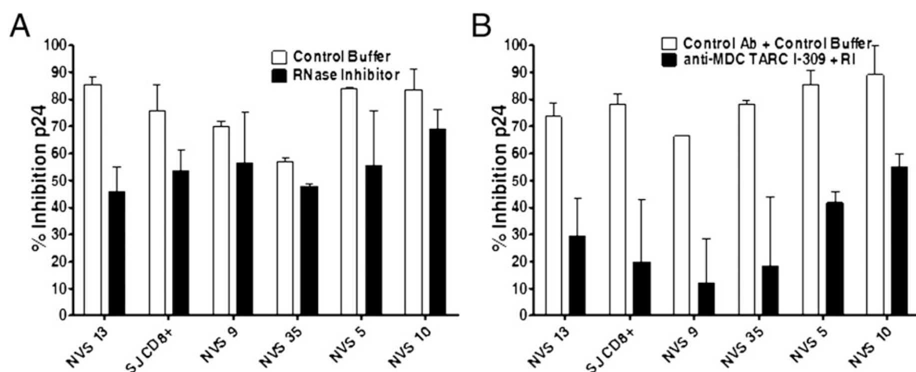

Fig. 4. Contribution of I-309, MDC, TARC, angiogenin, and RNase 4 to the CD8-derived anti-X4 activity in PBMCs. CD8-derived supernatants were pretreated with 200 units of ribonuclease inhibitor alone (*A*) or in combination with NAbs against I-309, MDC, and TARC at the concentration of 25μg/mL each (*B*). Subsequently, supernatants (50% vol/vol) were added to PBMCs infected with HIV-1$_{IIIB}$ and replaced with new treated supernatants every 48 h. Cultures treated with the control buffer and with the same concentrations of isotype control antibodies were tested in parallel. The cultures were tested for HIV-1 p24 antigen at day 5 after infection. (Error bars: SD.)

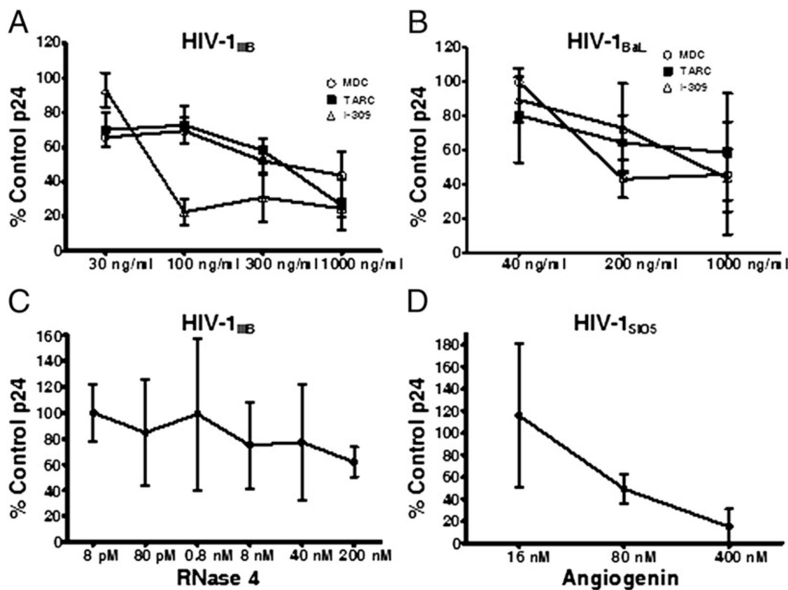

Fig. 5. Antiviral activity of MDC, TARC, I-309, angiogenin, and RNase 4. Rh MDC, TARC, I-309 (*A* and *B*), synthetic RNase 4 (*C*), and rh angiogenin (*D*) were tested in PBMCs infected with the X4 isolates HIV-1_{IIIB} and SIO5 or with the R5 HIV-1_{BaL} isolate. After infection, PBMCs were cultured in the presence of serial dilutions of each chemokine. PBMCs were pretreated with extracellular RNases (RNase 4 and angiogenin) for 1 h before infection and then were cultured in the presence of serial dilutions of each RNase. Cultures without inhibitors were tested in parallel. Inhibitors were added every 48 h to the cell culture. The level of HIV replication was tested at day 5 after infection by p24 antigen capture. (Error bars: SD.)

and TARC (625 nM) did not inhibit fusion of PBMCs with virions pseudotyped with the X4-tropic HXB2 envelope, whereas the fusion inhibitors C52L peptide (0.5 μM) and T-20 (1 μM) completely abrogated fusion (Fig. 6). Our results are in agreement with a previous report showing no effect of MDC on fusion of X4 viruses in PBMCs (27) and, again, suggest that the antiviral activity depends upon intracellular events and differs fundamentally from that of the anti-R5 β chemokines.

Discussion

Although the dominant role of CCR5-binding chemokines as CD8-derived and CD4-derived HIV-suppressive factors has been widely confirmed (11–13), it is also clear that $CD8^+$ and $CD4^+$ T cells produce a complex array of molecules with antiviral activity, some of which are active against the X4 isolates of HIV-1 and awaited discovery (19). Our goal here was to identify the HIV suppressor factor(s) that selectively suppress X4 HIV strains. We show that the β chemokines MDC, TARC, and I-309, together with angiogenin and RNase 4, are naturally produced by primary T cells at effective antiviral concentrations and appear to represent the major components of the anti-X4 activity released by T cells, suggesting their importance as HIV suppressor factors. This is underscored by their higher expression levels in primary PHA-activated T cells from HIV^+ asymptomatic compared with HIV^+ symptomatic individuals. The enhanced replication of X4 HIV strains by neutralization of the endogenous β chemokines I-309, MDC, and TARC suggests a protective role for these chemokines against infection of $CD4^+$ T cells by X4 viruses, as has previously been described for the antiviral CCR5 ligands that can protect HIV-antigen specific $CD4^+$ T cells against R5 viruses (15–18). A number of posttranslational modifications, including NH_2- and COOH-terminal proteolytic processing and glycosylation, have been described for natural chemokines, with a variety of consequences for their biological activity (28). The finding that rh β chemokines showed modest antiviral effects compared with the suppressor activity of the endogenous molecules may reflect differences between rh and native proteins in posttranslational modifications improving their antiviral activity. Naturally truncated MDC has been reported to inhibit HIV infection (26), and posttranslational modification of MDC, such as NH_2-terminal truncation, enhances its antiviral activity against both X4 and R5 HIV-1 strains (29). Members of the chemokine receptor family, in combination with CD4, allow HIV-1 entry into cells. CCR5 and CXCR4 are the primary coreceptors for M-tropic and T-tropic HIV-1 strains, respectively (9), and their ligands inhibit infection by blocking interaction of the coreceptor with HIV gp120. The role of CCR4 and CCR8 in PBMC infection remains unclear. To address this issue, we examined the effect of the CCR4 ligands MDC, TARC, and of the CCR8 ligand I-309 on fusion of X4 HIV-1 strains and PBMCs, using the BlaM Vpr assay, which detects fusion of virions with biologically relevant target cells such as PBMCs. Our data show that MDC, TARC, and I-309 inhibit X4 viral replication at a postfusion level, and the mechanism, therefore, differs fundamentally from that of the previously described R5-specific chemokines. In a previous study, the β chemokine MIP-3α, the cognate ligand of CCR6 (30), also inhibited R5 and X4 HIV-1 isolates in PBMCs at a postentry stage of HIV-1 replication. The inhibitory activity depended upon expression of the host restriction factor apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) (31). As with MIP-3α, the mechanism of the activity we report here may involve G protein-related signaling events. These results do not exclude the possibility that under different conditions other antiviral factors yet to be identified may further contribute to the anti-HIV activity of T cells. Further studies on the regulation of their expression and their exact mechanism(s) of action could lead to preventative and/or therapeutic approaches to HIV infection, as was the case following the discovery of β-chemokine inhibitors of R5 HIV (8, 32).

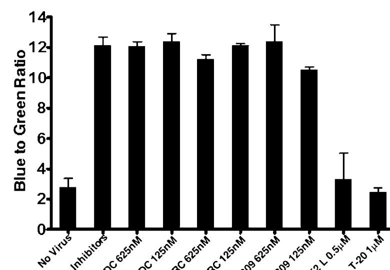

Fig. 6. Effect of MDC, TARC, and I-309 on fusion of the X4 HIV-1 HXB2. PBMCs were pretreated with MDC, TARC, and I-309 for 1h at 37 °C, whereas T20 and C52L were added just before infection with HXB2 containing BlaM-Vpr fusion protein (MOI, 0.3). Cells were washed and cultured in the presence of each inhibitor. Control cultures without any inhibitor or virus were tested in parallel. PBMCs were loaded with a green fluorescent substrate, CCF4/AM, and turned blue upon virus fusion, as a consequence of the substrate cleavage by BlaM-Vpr chimeric proteins. Blue to green cell ratio was read on a BioTek Synergy HT plate reader. (Error bars: SD.)

Materials and Methods

HIV+ Subjects and Control Cases. Heparinized peripheral blood samples were obtained from HIV-1-infected subjects at different stages of disease progression. We analyzed 19 asymptomatic HIV infected subjects (10 LTNP, 9 NVS) and 8 AIDS subjects. The only selection criteria were to ensure that there was representation of patients with AIDS vs. those without AIDS. All infected cases were without treatment with antiretroviral drugs at the time of the study. HIV-1 seronegative healthy donors (14 subjects) were analyzed in parallel. All samples were collected after informed consent (approved by the Institutional Review Board of the University of Maryland) and by using standard protocols.

Cell Isolation. PBMCs were obtained from Ficoll-histopaque gradient separation (Amersham Pharmacia). T cells were isolated from PBMCs by negative selection with an enrichment mixture of mAbs (StemCell Technologies) according to the instructions of the manufacturer. See *SI Materials and Methods* for detailed methods.

Infectivity Assay. Unfractionated PBMCs or enriched CD4+ T cells (CD8-depleted PBMCs) from normal donors previously activated with PHA (1 μg/mL) (Abbott Laboratories) for 3 d were acutely infected with the X4 isolates HIV-1_{IIIB}, HIV-1 SIO4, and HIV-1 SIO5 by using the same titer for all three viruses [multiplicity of infection (MOI), 0.01] for 1 h at 37 °C. Cells were washed twice with PBS to remove unabsorbed virus, resuspended in complete RPMI medium 1640 containing rh IL-2 (10 ng/mL) (R&D Systems), and plated at a concentration of 1×10^5 cells/well in 96-well flat-bottom microtiter plates in triplicate. The supernatant from the cultures was harvested on day 5 after infection and tested for HIV-1 replication by p24 ELISA.

Immortalization in Vitro with HTLV-1. Human CD8+ and CD4+ T lymphocytes, purified from the peripheral blood of LTNPs, were activated in vitro with purified PHA at 1 μg/mL, before cocultivation with the HTLV-1 producer cell line MT-2 (33) previously irradiated with 10,000 rad. All of the HTLV-1 immortalized T-cell lines obtained depended on exogenous IL-2 for their growth.

Protein Purification. Media from LTNP-J- and LTNP-3-transformed T-cell lines exhibiting strong anti-X4 activity were passed over a heparin-affinity column, as described previously (26). Eluate fractions were dialyzed and tested for anti-HIV activity using an acute infection assay. The only divergence from the protocol (26) was that the heparin column was eluted with a NaCl gradient (0–2.0 M), and the fractions collected showing anti-X4 activity were concentrated with a Millipore prep/scale tangenital flow filtration cartridge with a 100-, 30-, and 10-kDa nominal molecular mass exclusion limit before being processed with HPLC.

Cell Metabolism and Proliferation Assays. Cell metabolism was determined by MTS assay (Promega) following the provided protocol.

Production of MDC, TARC, I-309, and Angiogenin by CD4+ T and CD8+ T Lymphocytes. Cells were cultured in RPMI medium 1640 complete medium in the presence of PHA (1 μg/mL) and rh IL-2 (10 ng/mL) at a cell concentration of 2×10^6 cells/mL. After 9 d of PHA stimulation, supernatants were collected and stored at −80 °C until analyzed for β-chemokine and angiogenin levels by ELISA (Quantikine Kits; R&D Systems) according to the instructions of the manufacturer.

Detection of RNase 4 RNA Signal. RNase 4 RNA signal was detected in CD8+ and CD4+ T cells by RT-PCR. Products were analyzed by gel electrophoresis. See *SI Materials and Methods* for detailed methods.

HIV-1 Virion Fusion Assay in PBMCs. We used a BlaM (β-lactamase) entry assay (34) to detect and measure virus cell fusion in PBMCs. See *SI Materials and Methods* for detailed methods.

RNase 4 Synthesis. Human RNase 4 was chemically synthesized via a three-segment native chemical ligation reaction (35, 36). See *SI Materials and Methods* for detailed methods.

Statistical Analysis. For categorical independent variables, differences between groups were assessed by using Student *t* test and the Fisher Exact *t* test using Graph Pad software.

ACKNOWLEDGMENTS. We thank G. Melikian (Emory University) for the fusion assay reagents; M. Reitz [Institute of Human Virology (IHV), University of Maryland, School of Medicine (SOM)] for comments on the manuscript; the University of Virginia Biomolecular Research Facility for mass spectrometry; L. Sun, W. Yuan, G. Jandu, S. Reid, B. Taylor, and L. Robinson (IHV, University of Maryland, SOM) for technical assistance. This study was supported by National Institute of Allergy and Infectious Diseases (NIAID) Grant R21AI065330-02. M. Lafferty was a trainee under NIAID Institutional Training Grant T32AI007540, and L. Sun was supported by National Institute of Neurological Disorders and Stroke Grant R01NS066842.

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