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The Role of Endogenous IL-18 in HIV-1 Production in Infected Peripheral Blood Mononuclear Cells

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인간면역 결핍 바이러스로 감염된 말초 혈액 단핵구 세포에서 바이러스 생성에 대한 체내 인터루킨 18의 역할

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최 희 정

목 적 : 인간 면역 결핍바이러스(HIV-1) 감염시 많은 싸이토카인 들에 의해 생성이 조절되고 있고, 그 중 외부에서 주입된 인터루킨18은 말초혈액 단핵구 세포에서 바이러스를 억제하는 것으로 알려져있다. 이 연구에서는 HIV-1 생성에 미치는 체내 IL-18의 역할을 조사해보고자 하였다.

방 법 : 정상인의 말초혈액 단핵구 세포를 분리한 뒤 HIV-1을 감염시켜 인터루킨 18의 특이적 억제제인 인터루킨 결합 단백질(IL-18BP)을 처리한 뒤 4일간 배양 후, 세포를 분해시킨 뒤 HIV-1 생성은 p24 효소면역검사법으로, 인터페론 감마와 TNF는 전기화학형광법으로 측정하였다.

결 과 : 말초혈액 단핵구세포를 바이러스로 감염시킨 직후 IL-18BP 처리시 바이러스 생성을 40%까지 감소시켰다($p<0.01$). 이와 달리, 세포를 감염시키기 전 2일동안 IL-18BP로 먼저 처치한 뒤 4일 배양후 바이러스 생성을 0.8~2.4배 증가시켰다. 모든 실험에서, IL-18B의 효과는 인터페론 감마 합성과는 무관하였다.

결 론 : 이 연구는 체내 인터루킨 18이 HIV-1 생성에 영향을 준다는 것을 실험적으로 입증하였다. 세포 감염후의 체내 인터루킨 18의 존재는 바이러스 생성을 증가시키지만, 감염전부터 있던 체내 인터루킨 18의 활동에 의해 바이러스가 억제되었다.

중심 단어 : 인터루킨 18 · 인터루킨 18결합단백 · HIV-1.

Introduction

Interleukin(IL)-18 is an interferon γ (IFN-γ) inducing factor important in the generation of the T helper type 1(Th1) response[1-3]. The Th1 response likely contributes to human immunodeficiency virus type 1(HIV-1) containment *in vitro* and *in vivo*[4-6]. In a previous report, we showed that exogenous IL-18 added to peripheral blood mononuclear cells(PBMC) immediately after infection with either monocyte(M)- or T cell(T)-tropic virus inhibited HIV-1 production in 4-day cultures. This IL-18 inhibition was mediated through induction of IFN-γ synthesis and by reduced cell surface CD4 ex-

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pression[7]. Since HIV-1 suppression was also observed in 4-day infected cultures when IL-18 was added exclusively during the 2 days prior to infection with virus, the IL-18 inhibitory effect was likely operative during the early phases of PBMC infection.

IL-18 binding protein (IL-18BP) is a constitutively expressed and secreted soluble protein, which was purified from human urine and sequenced[8]. IL-18BP binds and neutralizes IL-18 and specifically reduces its various biological activities[8]. Having established an HIV-1 inhibitory effect for exogenously added IL-18 in infected PBMC cultures, we used IL-18BP to examine the HIV-1 effect of endogenous IL-18 synthesized by the PBMC.

Results

1. IL-18BP specifically neutralizes the biological activity of IL-18 in U1 cells

To assess the effect of endogenous IL-18 *in vitro*, we used recombinant human IL-18BP in these investigations. IL-18BP is a naturally occurring specific IL-18 inhibitor[8]. To verify that IL-18BP specifically neutralized IL-18 biological activity, we used U1 cells. In previous studies, we showed that IL-18 induced HIV-1 production in this chronically infected cell line, and HIV-1 induction involved activation of p38 mitogen-activated protein kinase (MAPK)[9][10]. U1 cells were cultured for 2 days in medium alone, stimulated with either IL-18 or with a hyperosmotic concentration of NaCl, or cultured with each stimulus in the presence of 50nM IL-18BP (Fig. 1). Cells exposed to medium alone for 2 days produced 262 ±45pg/ml p24 antigen (Ag). Cells stimulated using IL-18 at 0.5nM had increased 2-day p24 Ag levels of 2,750±723pg/ml, a mean 9.5-fold increase compared to cultures conducted in medium alone. In cultures stimulated using 0.5nM IL-18 in the presence of 50nM IL-18BP, the mean IL-18-induced HIV-1 fell by 95% (from 2,750±723pg/ml to 397±64pg/ml). We previously demonstrated an HIV-1-stimulating effect for NaCl-induced hyperosmolarity[9]. The addition of 60mM NaCl increased p24 to 1,650±160pg/ml, a 5.3-fold increase compared to medium alone. HIV-1 stimulation using hyperosmotic NaCl was not significantly affected by the presence of

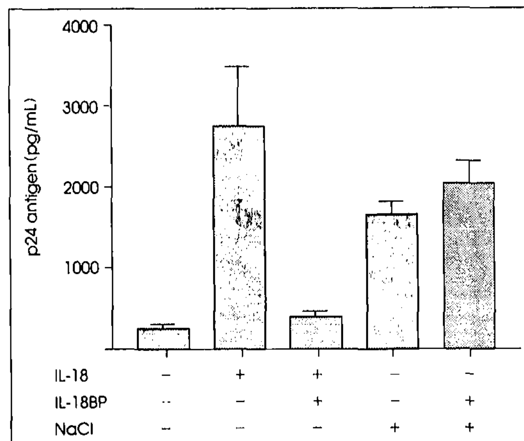

Fig. 1. IL-18BP specifically neutralizes IL-18 biological activity in U1 cells. U1 cells were cultured in medium alone, or were stimulated with a 0.5nM concentration of IL-18 or with the addition of 60 mM NaCl, as indicated. IL-18BP (50nM final concentration) was added 1h before each stimulus in cultures conducted with both a stimulus and IL-18BP. After 2 days of incubation, the cultures were lysed and HIV-1 p24 antigen concentrations measured. The results are presented as mean ± SEM p24 antigen concentrations from three separate experiments.

50nM IL-18BP (2,038±283pg/ml p24 Ag). These results (Fig. 1) show that IL-18BP has no effect on osmotic stress-induced HIV-1 production.

2. IL-18BP added to PBMC after infection with HIV-1 inhibits virus production

Previously, we showed that exogenous IL-18 added to PBMC after infection with HIV-1 significantly inhibited p24 production in 4-day cultures[7]. To extend these observations, we examined the role of post-infection endogenous IL-18 in HIV-1 production. In Fig. 2A, PBMC were infected with T-tropic HIV-1, and the infected cells were cultured in the absence (spontaneous) or in the presence of IL-18BP. After 4 days of culture, spontaneous p24 Ag concentration was 696±88pg/ml, which was set at 100%. IL-18BP at 0.05, 0.5, 5, or 50nM reduced 4-day p24 Ag levels by 11%, 8%, 12%, or 40%, respectively, compared to spontaneous (medium alone) cultures (Fig. 2A). Fifty nM IL-18BP significantly inhibited p24 production compared to spontaneous cultures. In contrast to the p24 results, none of the IL-18BP concentrations tested affected the mean IFN-γ levels (compared to

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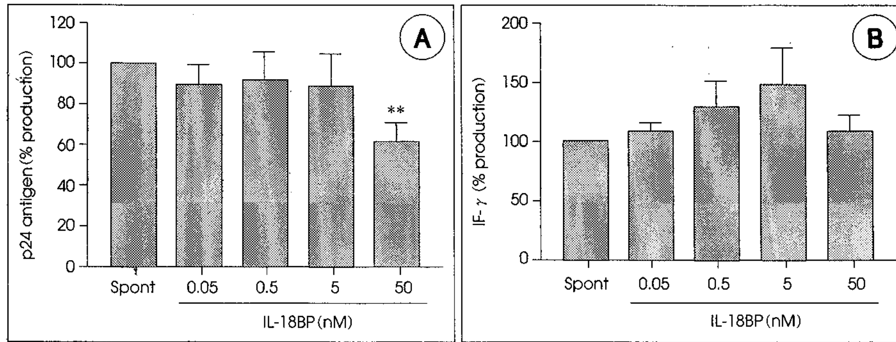

Fig. 2. IL-18BP added after PBMC infection inhibits HIV-1 production. After PBMC were infected with T-tropic HIV-1, a time 0 sample was obtained, and the PBMC were incubated for 4 days in the absence (spontaneous, spont) or in the presence of IL-18BP. Concentrations of the added IL-18BP are shown under the horizontal axes. After 4 days, concentrations of p24 antigen (A) and IFN-γ (B) were determined in the lysed cultures. The results for each donor were calculated as percent p24 production, and percent IFN-γ production, with the values obtained in spontaneous cultures set at 100%. Results are presented as mean±SEM percent production in PBMC from seven donors. ** : $p<0.01$ compared to 100% (spontaneous) using ANOVA.

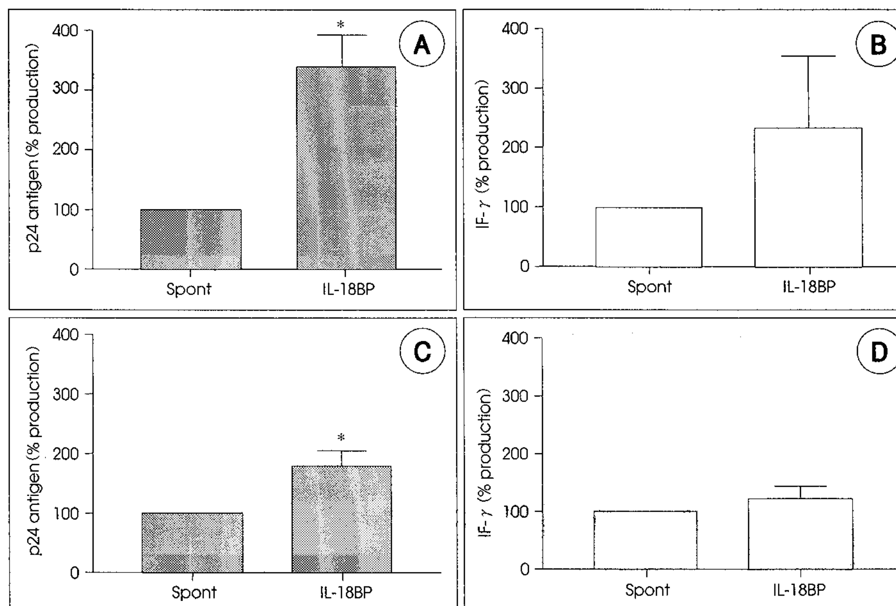

Fig. 3. Pre-incubation of PBMC with IL-18BP augments HIV-1 production in 4-day cultures. During the 2 days prior to infection with HIV-1, PBMC were incubated in the absence (spontaneous, spont), or in the presence of 50nM IL-18BP. PBMC from three donors were infected with M-tropic HIV-1 (A and B), and PBMC from three different donors were infected with T-tropic virus (C and D). After PBMC infection with HIV-1, a time 0 aliquot of freshly infected cells was obtained. The infected PBMC were then incubated for 4 days without exogenous IL-18BP. The concentrations of p24 antigen (A and C) and of IFN-γ (B and D) were determined in the lysed cultures. Results are presented as mean±SEM percent p24 antigen production or IFN-γ production (compared to spontaneous, set at 100%) in the lysed PBMC cultures. * : $p<0.05$, compared to 100% (spontaneous) by Student's t-test.

spontaneous) measured in the same PBMC cultures (Fig. 2B).

We examined IL-18BP for effects on HIV-1-infected PBMC viability and proliferation. Using both trypan blue exclusion and formazan production, no IL-18BP effect was observed on viability or on cellular proliferation in 4-day infected PBMC cultures in three separate experiments (data not shown).

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3. IL-18BP added to PBMC prior to infection with HIV-1 increases virus production

We demonstrated that exogenous IL-18 inhibited HIV-1 production in infected PBMC when IL-18 was added for 2 days prior to infection[7]. We expanded these studies to determine the effect of blocking endogenous IL-18 using IL-18BP in PBMC under similar conditions. PBMC isolated from individual donors were incubated in medium alone, or incubated with 50nM IL-18BP for 2 days prior to infection with HIV-1. After infection and washing, infected PBMC were cultured in medium alone (no IL-18BP) for 4 days. Results for PBMC infected with M-tropic HIV-1 are depicted in Figs. 3A, B. Spontaneous 4-day cultures contained 6,858±4,618pg/ml p24 Ag (set at 100% in Fig. 3A). Two days of pre-infection exposure to IL-18 BP resulted in a 2.4-fold increase in p24 Ag after 4 days of culture, which achieved statistical significance ($p<0.05$). In Fig. 3B, IFN-γ concentrations were measured in the same cultures shown in Fig. 3A. IL-18BP increased IFN-γ production by 1.3-fold compared to spontaneous cultures (254±169pg/ml IFN-γ, set at 100%), which was not statistically significant.

Results for similar experiments using PBMC infected with T-tropic HIV-1 are shown in Figs. 3C, D. Fig. 3C shows that 2 days of exposure of PBMC to 50nM IL-18BP prior to infection increased 4-day p24 Ag production by 0.8-fold ($p<0.05$), compared to spontaneous cultures (16,217±10,570pg/ml p24, set at 100%). In the same samples, spontaneous IFN-γ production was unaffected by the presence of IL-18BP in the cultures (Fig. 3D).

4. IL-18BP does not affect expression of HIV-1 co-receptors

In prior studies, we showed that exogenous IL-18 reduced expression of the cell-surface HIV-1 co-receptor CD4 in PBMC[7]. We analyzed cell surface expression of the HIV-1 co-receptors CD4 and CCR5 in uninfected PBMC by flow cytometry. PBMC were incubated for 4 days in the absence or in the presence of 50nM IL-18BP. Neither the expression of CD4, nor that of CCR-5, was affected by IL-18BP in three separate experiments (data not shown).

5. Soluble TNF receptor p55(sTNFRp55) added to PBMC after infection with HIV-1 does not affect virus production

Neutralizing IL-18 by adding IL-18BP to HIV-1-infected PBMC after infection with virus inhibited 4-day p24 production (Fig. 2A). These results indicate an HIV-1-inducing effect of endogenous IL-18. Since IL-18 and TNF-α activate similar signal transduction molecules following receptor engagement[11-15], endogenous TNF-α may also stimulate HIV-1 production in these cultures. To assess the effect of endogenous TNF-α on HIV-1 production, we cultured HIV-1-infected PBMC in the absence, or in the presence, of TNF-α blockade using neutralizing amount of sTNFRp55. Fig. 4 shows the results from these experiments, conducted using aliquots of the same PBMC shown in Fig. 2. The PBMC were infected with T-tropic virus and cultured after infection without (spontaneous, set at 100%), or with 10μg/ml sTNFRp55 for 4 days. As shown, exposure of the infected PBMC to sTNFRp55 did not significantly affect HIV-1 production in the 4-day cultures. The biological activity of the sTNFRp55 was established by showing

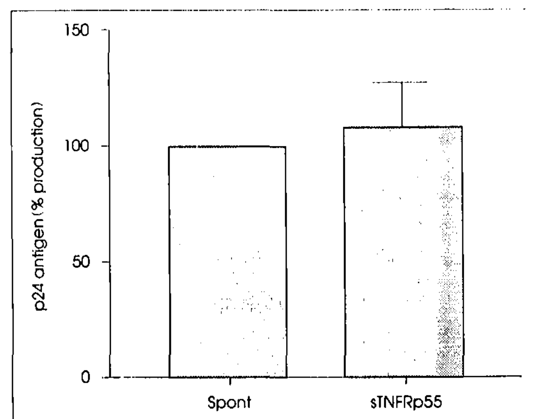

Fig. 4. sTNFRp55 added after PBMC infection does not affect HIV-1 production. Aliquots of the T-tropic HIV-1-infected PBMC from the same seven donors shown in Fig. 2A were used. Immediately after infection, a time 0 sample of infected cells was collected for each donor, and the PBMC were cultured in the absence (spontaneous, spont), or in the presence of 10μg/ml sTNFRp55. After 4 days, the percent production of p24 antigen was determined in the culture lysates (spontaneous cultures set at 100%). Results are shown as mean +SEM.

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that this TNF inhibitor reversed TNF-α-induced HIV-1 production in U1 cells in three separate experiments (data not shown).

Discussion

To investigate the role of endogenous IL-18 in HIV-1 production in primary cells, we used recombinant human IL-18BP. First, we assessed the specificity of the IL-18BP effect using U1 cells. IL-18 increases HIV-1 production in chronically infected U1 cells[9]. As shown in Fig. 1, IL-18BP at a 100-fold molar excess, nearly completely neutralized IL-18-induced HIV-1 production. In contrast, IL-18BP did not affect HIV-1 production in response to hyperosmolar stress due to excess NaCl[13][16]. Therefore, IL-18BP is a highly specific inhibitor of IL-18-induced p24.

We recently demonstrated that exogenous IL-18 added to PBMC after infection with HIV-1 inhibited viral production measured after 4 days of culture[7]. This IL-18 inhibitory effect was associated with increased IFN-γ production. Using neutralizing anti-IFN-γ antibody, we showed that the IL-18-induced IFN-γ was responsible for the IL-18 inhibitory effect of HIV-1. With these results as background, we predicted that IL-18BP-induced IL-18 neutralization after cells were infected with virus would increase HIV-1 production in 4-day infected PBMC cultures. Also, since our prior investigations demonstrated significant IL-18-induced IFN-γ synthesis, we anticipated an IL-18BP-induced reduction in IFN-γ concentration. Surprisingly, IL-18BP inhibited HIV-1 production (Fig. 2A), indicating that endogenous IL-18 functioned as an HIV-1 inducer in these experiments. Furthermore, the HIV-1 inhibition was not associated with any significant change in IFN-γ production in IL-18BP-containing cultures.

What accounts for this discrepancy between the HIV-1 suppressive effect of exogenous IL-18, and the HIV-1 stimulatory effect of endogenous IL-18? After cells are infected with HIV-1, there may be a balance between a direct IL-18 HIV-1 stimulatory effect (Fig. 2), and an indirect IL-18 suppressive HIV-1 effect mediated by the synthesis of IFN-γ[7]. The inability of endogenous IL-18 blockade to affect IFN-γ synthesis (Fig. 2B) indicates that endogenous IL-18 did not affect IFN-γ production. The amount of endogenous IL-18 in the infected PBMC cultures may have been insufficient to activate signaling pathways responsible for IFN-γ synthesis, and the unopposed IL-18 direct effect stimulated HIV-1 production. Using exogenous IL-18, the HIV-1 suppressive effect of the induced IFN-γ may supercede the direct HIV-1-stimulating activity of endogenous IL-18. A direct HIV-1 inducing effect for IL-18 has been reported elsewhere. In chronically HIV-1-infected promonocytic U1 cells, IL-18 induced HIV-1 (Fig. 1), and IL-18-induced NF-κB activation contributed to this increased virus production[9]. It is noteworthy that U1 cells do not produce IFN-γ in response to IL-18 exposure (our unpublished observation), and this may, in part, explain the IL-18 stimulatory effect on HIV-1 production in these cells.

Previously, we showed that exogenous IL-18 added to PBMC during the 2 days before HIV-1 infection inhibited p24 production measured 4 days after infection. Inhibition was observed despite the absence of exogenous IL-18 added after infection with virus[7]. Using similar conditions, blockade of endogenous IL-18 biological activity using IL-18BP during the 2 days before HIV-1 infection augmented virus production. This effect was observed when PBMC were infected with either M- or T-tropic virus (Fig. 3A, C, respectively). These data indicate that endogenous IL-18 activity prior to infection of cells with virus was antiretroviral. It is interesting that the IL-18BP-induced augmentation in HIV-1 production was more pronounced in cells infected with the M-tropic virus (Fig. 3A) than with the T-tropic virus (Fig. 3C). Since the PBMC used for M-tropic virus infection were different from the PBMC used for T-tropic virus infection, a direct comparison between IL-18BP effect and virus strain is not possible. However, our results imply enhanced HIV-1-suppressive effect of endogenous IL-18 in cells infected with M-tropic virus.

The results shown in Fig. 3, and those of our prior studies[7], suggest that endogenous or exogenous IL-18 inhibits HIV-1 production during the early stages of viral infection. However, there are two important differences between the IL-18BP-induced effect, and that of exogenous IL-18 previously reported. First, exogenous IL-18 induced significant IFN-γ production compared to

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spontaneous cultures, whereas IL-18BP did not significantly change IFN-γ levels (Fig. 3B, D)[7]. This observation was unexpected, since we anticipated decreased IFN-γ production due to neutralization of endogenous IL-18 using IL-18BP. The amount of endogenous IL-18 produced by the PBMC may have been too small to induce significant IFN-γ. Therefore, blockade of IL-18 activity using IL-18BP would not affect IFN-γ production. Since IL-18BP did not affect IFN-γ production, it is suggested that endogenous IL-18 possesses an HIV-1-suppressive effect which is independent of IFN-γ. Second, blockade of endogenous IL-18 using IL-18BP did not affect cell surface CD4 HIV-1 co-receptor expression in the present investigations (data not shown), whereas exogenous IL-18 reduced CD4 expression in our prior studies[7]. Thus, endogenous IL-18 inhibited HIV-1 by a mechanism unrelated to down-regulation of cell surface CD4. Exogenous IL-18-induced down-regulation of CD4 was probably mediated by intermediate IL-18-induced IFN-γ. This hypothesis is supported by studies showing that exogenous IFN-γ down-regulated cell surface CD4 molecules[17]. Since IL-18BP did not affect IFN-γ synthesis, this may explain the lack of IL-18BP effect on CD4 expression. Overall, both endogenous and exogenous IL-18 inhibit PBMC HIV-1 production when present prior to infection with virus, even though several biological effects of exogenous and endogenous IL-18 differ.

Reports examining signaling events initiated by IL-18 have demonstrated similarities with TNF-α-induced signaling. For example, IL-18 and TNF-α can each activate MAPKs and p56^{lck} signaling molecules[11-15)18]. TNF-α stimulates HIV-1 production in U1 cells and in a T cell line[19)20], and TNF-α-induced HIV-1 increase was associated with enhanced binding of activated NF-κB to the HIV-1 long terminal repeat[11)19-21]. We have previously shown that IL-18, like TNF-α, induced HIV-1 production in U1 cells, and the increase was associated with enhanced NF-κB activation[9]. On the other hand, differences in the biological activities of IL-18 and TNF-α have been reported. These differences are underscored by the dissimilar phenotypes of IL-18 and TNF-α-deficient mice[2)22)23]. Given these considerations, it is unclear whether the HIV-1 effects of endogenous IL-18 and of endogenous TNF-α would be similar or discordant. PBMC first infected with T-tropic HIV-1 were cultured in the presence of IL-18-inhibiting IL-18BP (Fig. 2), or with TNF-α-neutralizing concentrations of the TNF-α-inhibitor sTNFRp55 (Fig. 4). Since the same HIV-1-infected PBMC donors were used for the IL-18BP containing cultures and for the sTNFRp55 containing cultures, the results can be directly compared. While IL-18 blockade reduced 4-day HIV-1 production, TNF-α blockade did not alter HIV-1 production (each compared to HIV-1 in spontaneous cultures). Therefore, endogenous IL-18 originating from the infected PBMC had a stimulatory role on HIV-1, which contrasted with an absent HIV-1 effect of endogenous TNF-α. These results demonstrate different roles for endogenous IL-18 and endogenous TNF-α after HIV-1 infection in PBMC cultures.

The study of IL-18 function in HIV-1 infected patients is in its earliest stages. Torre et al[24] showed that increased IL-18 levels correlate with the clinical stage of HIV-1 disease. This report establishes endogenous IL-18 production in the presence of HIV-1 infection in patients, supporting the biological relevance of our *in vitro* study. However, it is uncertain whether increased endogenous IL-18 associated with HIV-1 disease progression is adaptive or maladaptive. Furthermore, the effect of induced IL-18 during HIV-1 infection may be a function of induced IFN-γ synthesis, and whether IL-18 is present before of after cells are infected with virus.

In conclusion, using specific IL-18 neutralization with IL-18BP, we discovered a role for endogenous IL-18 in HIV-1 production in infected PBMC. In PBMC already infected with HIV-1, endogenous IL-18 stimulated virus production. On the other hand, uninfected PBMC exposed to endogenous IL-18, followed by infection with HIV-1, revealed an inhibitory IL-18 effect. This pre-infection IL-18 inhibitory effect indicated IL-18 suppression operative during the early stages of viral infection. None of the HIV-1 effects due to endogenous IL-18 were attributable to changes in IFN-γ synthesis. The amount of IL-18 produced endogenously may have been insufficient to induce IFN-γ. This contrasted with our prior studies demonstrating significant IFN-γ synthesis in response to exogenous IL-18[7]. Therefore, although IL-

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18 is a known IFN-γ inducing factor, the effects of endogenous IL-18 we observed are likely independent of intermediate IFN-γ synthesis. Other investigators have also described IFN-γ independent IL-18 activities[25)26)]. The effects of endogenous IL-18 inhibition were not replicated by blocking endogenous TNF-α activity, underscoring differences in function between these two cytokines. IL-18, with both pro-inflammatory and Th-1-inducing activities, has unique effects on HIV-1 production.

Materials and Methods

1. Reagents

Recombinant human IL-18 was supplied by Dr. Tariq Ghayur (BASF Corp., Worcester, MA). Ultrapure crystalline NaCl (Aldrich, St. Louis, MO) was dissolved in pyrogen-free water at a stock concentration of 2M. The solution was autoclaved for 1h to inactivate pyrogens, as previously described.16 Recombinant human IL-18BP was purified to homogeneity from a Chinese hamster ovary cell line (Serono, Randolph, MA)[8)]. The sTNFRp55 is a construct of two recombinant TNF receptor extracellular p55 molecules linked by polyethylene glycol (Amgen, Boulder, CO). T-tropic HIV-1 strain A018A was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (NIAID)[27)]. A clinically-derived M-tropic HIV-1 isolate was provided by Daniel R. Kuritzkes (University of Colorado Health Sciences Center)[28)]. These two viral strains were expanded in HIV-1-negative PBMC and titered by end-point infectivity[29)30)]. Medium for U1 cell cultures consisted of RPMI 1640 medium (Mediatech, Herndon, VA) containing 2.5mM L-glutamine, 25mM HEPES, 10% (vol/vol) heat-inactivated foetal calf serum (FCS, Life Technologies, Grand Island, NY), and 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). R3 medium consisted of RPMI 1640 medium containing 2.5mM L-glutamine, 25mM HEPES, 20% (vol/vol) FCS, 5% (vol/vol) IL-2 (Hemagen, Waltham, MA), and 100U/ml penicillin and 100 μg/ml streptomycin.

2. U1 cells

U1 cells were obtained from the NIH AIDS Research and Reference Reagent Program, NIAID. U1 cells are derived from U937 promonocytic cells infected with HIV-1 which contain two integrated copies of proviral HIV-1 DNA[31)]. When in log phase growth, U1 cells were pelleted and resuspended in fresh medium at 2×10^6 per ml and the cell viability was assessed using trypan blue exclusion (>95% viable for all experiments). All cultures were conducted in 24-well polystyrene tissue culture plates (BD Falcon, Franklin Lakes, NJ) using cells initially added at 1×10^6 per ml in a final volume of 0.5ml. U1 cells were cultured in medium alone (control), in the presence of stimulus alone, or with IL-18BP added 1 hr prior to stimulus. After 2 days of culture (37℃, 5% CO_2), Triton-X-100 (1% vol/vol final concentration) was added to each culture and the lysed cultures frozen at −70℃ until assay.

3. Isolation and infection of PBMC

These studies were approved by the Institutional Review Board of the University of Colorado Health Sciences Center, and informed consent was obtained. Thirty milliliter aliquots of plateletpheresis residual blood were collected from healthy volunteers, layered over 20ml of Histopaque 1077 (Sigma, St. Louis, MO) in 50ml polypropylene tubes (BD Falcon), and centrifuged at room temperature for 30min at $400\times g$[13)]. The PBMC layer was aspirated and washed twice in calcium- and magnesium-free phosphate buffered saline (Life Technologies).

For experiments designed to assess the HIV-1 effect of IL-18BP and sTNFRp55 added to PBMC after infection with virus, freshly-obtained PBMC were incubated for 48h with R3 medium containing additional 5% (vol/vol) IL-2 and 3.3 μg/ml PHA. PBMC were then infected with T-tropic virus using 250 tissue culture infectious doses $(TCID)_{50}$ of virus per 1×10^6 cells. Virus was added to cells in each tube for 3h at 37℃, 5% CO_2 as described[13)]. After infection and washing, a 250 μl aliquot of infected PBMC was obtained, an additional 250 μl R3 medium containing Triton-X-100 (1% vol/vol final concentration) was added, and this time 0 sample was frozen at −70℃ until assay. Two-hundred fifty μl of the remaining infected PBMC were then placed into

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wells of a 24-well polystyrene tissue culture plate. Each well then received an additional 250 μl of R3 medium alone (spontaneous cultures), 250 μl of medium containing IL-18BP (0.05, 0.5, 5, or 50nM final concentration), or 250 μl of medium containing sTNFRp55 (10 μg/ml final concentration). Cultures were incubated at 37℃ in 5% CO_2 for 4 days, after which Triton-X-100 (1% vol/vol final concentration) was added to each well and the cultures were frozen (−70℃) until assay.

Separate experiments were designed to assess the effect of PBMC pre-incubated with IL-18BP prior to infection with HIV-1. For these studies, uninfected cells obtained from each donor were suspended at 1×10^6 per ml in R3 medium supplemented with an additional 5% (vol/vol) IL-2 and 3.3 μg/ml PHA (Sigma). PBMC suspensions from each donor were divided into two separate 5ml polypropylene tubes in medium alone, or in medium containing 50nM IL-18BP. These two tubes were incubated for 2 days (37℃, 5% CO_2) prior to infection with HIV-1. After incubation, an aliquot of PBMC suspension from each 5ml tube was removed for cell count and assessment of cell viability using trypan blue exclusion (>95% for each experiment). PBMC from each of the two 5ml tubes were then pelleted and infected with M-tropic virus as described above. The infected PBMC were washed in R3 medium to remove unbound virus. The infected PBMC in each tube were then resuspended at 2×10^6 per ml using fresh R3 medium. A single 250 μl aliquot of infected PBMC suspension was transferred from each of the two tubes into separate 1.5ml polypropylene centrifuge tubes. Two hundred fifty μl of R3 medium containing Triton-X-100 (1% vol/vol final concentration) were added to each of these samples and the samples frozen at −70℃. These samples were designated time 0. Two hundred fifty μl of the remaining infected PBMC suspension from each of the two tubes were then transferred into separate wells of a 24-well polystyrene tissue culture plate, along with an additional 250 μl of R3 medium. No exogenous IL-18BP was added. Cultures were incubated (37℃, 5% CO_2) for 4 days, after which Triton-X-100 (1% vol/vol) was added to each culture-containing well, and the cultures were frozen (−70℃) until assay.

Using PBMC from separate donors, experiments were performed using the same protocol described above, except that the cells were infected with T-tropic HIV-1.

4. Measurement of p24 Ag and cytokines.

Quantification of HIV-1 p24 Ag was performed using an ELISA kit with a lower limit of detection of 7.8pg/ml (Coulter Corp., Miami, FL). IFN-γ was quantified using electrochemiluminescence (ECL)[32)33)]. The ECL-determined concentration of IFN-γ was measured using an Origen Analyzer and special software (Igen, Gaithersburg, MD), with a lower limit of detection of 12pg/ml. The presence of 1% vol/vol Triton-X-100 did not affect the levels of p24 Ag or IFN-γ measured using ELISA or ECL.

5. Cell viability

A toxic or cell-proliferative effect of IL-18BP was assessed in 4-day HIV-1-infected PBMC cultures. PBMC were infected with T-tropic HIV-1 as described above and aliquoted into wells in a 96-well polystyrene tissue culture plate at 1×10^6 cells per ml in a final volume of 200 μl. Infected PBMC were cultured in the absence (spontaneous) or in the presence of 50nM IL-18BP, and the cultures were incubated for 4 days at 37℃, 5% CO_2. After incubation, each PBMC-containing well received 20 μl of CellTitier 96 AQueous One Solution Reagent (Promega, Madison, WI). Using this reagent, the capacity of mitochondrial dehydrogenase in viable cells to reduce tetrazolium compound to formazan was quantified[34)]. After 2h of incubation, the amount of color in the cultures was measured using an ELISA reader (Optical density, determined at 490nm). The optical density values measured in spontaneous and in IL-18BP-containing cultures were compared.

6. Flow cytometry

After isolated PBMC were cultured in R3 medium supplemented with an additional 5% (vol/vol) IL-2 and 3.3 μg/ml PHA for 2 days, PBMC were washed and resuspended in fresh R3 medium. The PBMC were incubated at 1×10^6 cells/ml in R3 medium alone, or in medium containing 50nM IL-18BP for 4 days as described above except without HIV-1 infection. Aliquots of 4-day PBMC cultures received fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 monoclonal

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antibody (Mab, BD PharMingen), or phycoerythrin (PE)-conjugated anti-human CCR5 Mab (BD PharMingen). After the PBMC were incubated with these Abs, each of the aliquots received 7-aminoactinomycin D, a marker used to assess cell viability. The viable PBMC were then analyzed using an EPICS XL flow cytometer (Beckman Coulter) as previously described[7].

7. Statistical analysis

All data are presented as the mean ± SEM, and mean differences were considered significant for $p<0.05$. PBMC p24 and IFN-γ results were converted to percent production. For experiments conducted in each PBMC donor, p24 measured at time 0 (T=0 sample) was subtracted from the p24 value measured in each additional sample. The percent p24 production in each sample was calculated using the following formula :

$$\frac{(\text{sample p24 concentration}) - (\text{T=0 p24 concentration})}{(\text{spontaneous p24 concentration}) - (\text{T=0 p24 concentration})} \times 100\%$$

Using this calculation, the p24 measured in each spontaneous culture was set at 100%. The percent IFN-γ production was calculated similarly. Group means were compared using ANOVA with Fisher's Least Significant Difference, except where indicated in the text. For experiments assessing 2-day pre-incubation with IL-18BP or sTNFRp55, the data were compared using Student's *t*-test.

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