



**HAL**  
open science

## **The EBI2 receptor is coexpressed with CCR5 in CD4+ T cells and boosts HIV-1 R5 replication**

Adeline Guigues, Sandrine Gimenez, Clément Mettling, Damien Maurel, Etienne Doumazane, Laurent Prézeau, Vincent François, Pierre Corbeau

### ► **To cite this version:**

Adeline Guigues, Sandrine Gimenez, Clément Mettling, Damien Maurel, Etienne Doumazane, et al.. The EBI2 receptor is coexpressed with CCR5 in CD4+ T cells and boosts HIV-1 R5 replication. *AIDS. Official journal of the international AIDS Society*, 2024, 38 (10), pp.1449-1459. <10.1097/QAD.0000000000003931>. <hal-04748558>

**HAL Id: hal-04748558**

**<https://hal.science/hal-04748558v1>**

Submitted on 22 Oct 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization



## 1 **Abstract**

2 **Objective.** CCR5, a G protein-coupled receptor (GPCR), is used by most HIV strains as a  
3 coreceptor. In this study, we looked for other GPCRs able to modify HIV-1 infection.

4 **Design.** We analyzed the effects of one GPCR coexpressed with CCR5, EBI2, on HIV-1  
5 replicative cycle.

6 **Methods.** We identified GPCRs expressed in primary CD4+CCR5+ T cells by multi-RT-  
7 qPCR. We studied GPCR dimerization by FRET technology. Cell lines expressing EBI2  
8 were established by transduction with HIV vectors. HIV-1 entry was quantified with virions  
9 harboring  $\beta$ -lactamase fused to the viral protein vpr, early and late HIV-1 transcriptions by  
10 qPCR, NF $\kappa$ B nuclear activation by immunofluorescence and transfection, and viral  
11 production by measuring p24 concentration in culture supernatant by ELISA.

12 **Results.** We showed that *EBI2* is naturally expressed in primary CD4+CCR5+ T cells, and  
13 that CCR5 and EBI2 heterodimerize. We observed that this coexpression reduced viral entry  
14 by 50%. The amount of HIV reverse transcripts was similar in cells expressing or not EBI2.  
15 Finally, the presence of EBI2 induced the translocation of NF $\kappa$ B and activated HIV-1 genome  
16 expression. Globally, the result was a drastic HIV-1 R5, but not X4, overproduction in *EBI2*-  
17 transduced cells.

18 **Conclusions.** EBI2 expression in CD4+CCR5+ cells boosts HIV-1 R5 productive infection.  
19 As the natural ligand for EBI2 is present in blood and lymphoid tissues, the constant EBI2  
20 activation might increase HIV replication in CD4+ T cells. It might be of interest to test the  
21 effect of EBI2 antagonists on the residual viral production persisting in patients aviremic  
22 under treatment.

23 **Keywords:** GPCR, CCR5 dimerization, HIV-1 entry, reverse transcription, HIV-1  
24 transcription

## 1 Introduction

2 Most HIV strains use the chemokine receptor CCR5 as a coreceptor in addition to the CD4  
3 molecule. These are referred to as R5 strains. <sup>[1]</sup>. Their gp120 envelope binds to CD4 and  
4 thereafter to CCR5. This allows the next step in HIV life cycle, the fusion between virus and  
5 target cell membranes. In addition, gp120 binding to CCR5 triggers a signal that boosts HIV  
6 reverse transcription and viral promoter activation <sup>[2,3]</sup>.

7 CCR5 belongs to the family of G protein-coupled receptors (GPCR). GPCR may interact with  
8 each other physically and/or functionally <sup>[4]</sup>. On one hand some GPCR heterodimerize, and  
9 this may result in a modification of their cell surface density, of the way they interact with  
10 their ligands, and/or of their signaling. On the other hand, triggering a GPCR may reduce the  
11 capability of another GPCR to be activated, a phenomenon called heterodesensitization. As  
12 CCR5 cell surface density, CCR5-gp120 affinity and CCR5 signaling modulate HIV life  
13 cycle <sup>[2,3,5-7]</sup>, we looked for GPCR that could interfere with these CCR5 characteristics. Here  
14 we unraveled that the GPCR Epstein-Barr virus-induced gene 2 (EBI2) is coexpressed with  
15 CCR5 in primary CD4+CCR5+ T cells and macrophages.

16 *EBI2*, also called *GPR183*, was identified in 1993 as a gene activated by Epstein-Barr virus in  
17 a Burkitt lymphoma cell line <sup>[8]</sup>. In 2011, 7 $\alpha$ ,25-dihydroxycholesterol (7 $\alpha$ ,25-OHC) was  
18 identified as an endogenous ligand for EBI2 <sup>[9,10]</sup>. EBI2 participates with the chemokine  
19 receptors CCR7, CXCR4 and CXCR5 in the regulation of B cell homing in secondary  
20 lymphoid tissues <sup>[11,12]</sup>. Naïve B cells, particularly if they are activated, display high EBI2 cell  
21 surface densities that drive them to outer follicular areas. By contrast, downregulation of  
22 EBI2 cell surface expression drives B cells to central follicular areas <sup>[11,12]</sup>. EBI2 has also  
23 been involved in B cell activation and proliferation <sup>[13]</sup>. Additionally, EBI2 is expressed in  
24 monocytes/macrophages, dendritic cells, T cells and astrocytes. The interaction between

1 EBI2 and  $7\alpha,25$ -OHC has been involved in the splenic homing of dendritic cells <sup>[14, 15]</sup>. EBI2  
2 also downregulates type I interferon responses in plamacytoid dendritic cells <sup>[16]</sup>. In  
3 macrophages, EBI2 stimulation leads to calcium mobilization and cell migration <sup>[17, 18]</sup>.  
4 Moreover, CD4+ T differentiation into Th17 cells is driven by the agonistic effect of  $7\alpha,25$ -  
5 OHC on RAR-related orphan receptor gamma t (ROR $\gamma$ t) <sup>[19]</sup> and EBI2 stimulation has been  
6 involved in multiple sclerosis <sup>[20]</sup>. Here we show that the coexpression of EBI2 with CCR5  
7 modifies HIV replication cycle.

## 1 **Results**

### 2 *Identification of EBI2 as a GPCR coexpressed with CCR5 in CD4+ T cells and macrophages.*

3 In a search for GPCR coexpressed with CCR5 in CD4+ T cells, we sorted with magnetic  
4 beads and specific antibodies CCR5+CD4+ T lymphocytes from the blood of healthy donors  
5 and quantified by multi-RT-qPCR the expression of 361 *GPCR* genes as previously described  
6 [21]. We did the same with macrophages differentiated in vitro from the circulating monocytes  
7 of the same donors. We observed that among the *GPCR* genes expressed in peripheral  
8 CD4+CCR5+ T cells the *EBI2* gene was highly transcribed, *EBI2* transcripts being 16-fold  
9 more abundant than *CCR5* transcripts in these HIV target cells (Table 1). *EBI2* was also  
10 coexpressed with *CCR5* in macrophages but *EBI2* transcripts were 2-fold less abundant than  
11 *CCR5* transcripts in these myeloid cells (Figure 1).

12 *EBI2 forms heteromers with CCR5 in the outer cell membrane.* When a GPCR  
13 heterodimerizes with another GPCR, some of its characteristics may be modified, including  
14 its cell surface density, its ligand affinity, and its signaling [22-26]. For *CCR5*, all these  
15 characteristics determine HIV-1 R5 replicative cycle. Therefore, we analyzed by time-  
16 resolved FRET (tr-FRET) whether *EBI2* was able to form heteromers with *CCR5* using a  
17 method giving a direct assessment of receptor cross-interaction at the plasma membrane of  
18 living cells (21). To this aim, receptors were fused at their N-terminal extracellular domain  
19 with the genetically encoded tags SNAP or CLIP, that allow specific labelling with  
20 fluorescent substrates. Thereafter, SNAP-*EBI2* and CLIP-*CCR5* encoding plasmids were co-  
21 transfected in 293T cells, and the corresponding receptors were labeled by adding non-  
22 permeant fluorophores in the culture medium. The tr-FRET signal between the cell surface  
23 tagged-receptors was measured, as well as the fluorescence signal from each fluorophore. We  
24 observed a saturating tr-FRET signal between SNAP-*EBI2* and CLIP-*CCR5*, typical of a

1 specific interaction in the outer membrane (Fig. 2). Conversely, Figure 2 shows a non-  
2 saturating tr-FRET signal between CCR5 and the beta-2 adrenergic receptor ( $\beta$ 2AR) (24),  
3 indicating random collisions between these two receptors known not to interact with each  
4 other (23). As a positive control, CLIP-CCR5 was found to form homomers with SNAP-  
5 CCR5 (Figure 2) as previously shown (25).

6 *Establishment of HIV-1 R5-infectible cell lines expressing EBI2.* To explore the consequences  
7 of EBI2 expression on HIV-1 replicative cycle, we created CD4+CCR5+ cell lines expressing  
8 or not the *EBI2* gene. To this aim, we first cloned the *EBI2* and the *EGFP* genes separated by  
9 an IRES sequence into an HIV-1 transfer vector. Then we produced HIV-1 particles able to  
10 deliver this construct, and transduced CCR5- and CD4-positive human osteosarcoma HOS  
11 cells or lymphoid MT4 cells with these particles. As a negative control, we transduced the  
12 same cells with a *LacZ* gene. We then verified by flow cytometry that EGFP was expressed in  
13 the HOS (Fig. 3A) and MT4 (Fig. 3B) cells transduced with the EBI2-IRES-EGFP vector. We  
14 also checked that EBI2 transduction did not modify CCR5 expression (Figure 3C). To  
15 ascertain that EBI2 was functional at the surface of these cells, we monitored cell culture  
16 impedance in presence or absence of the EBI2 ligand  $7\alpha,25$ -OHC. As shown in Figure 3D and  
17 3E, addition of  $7\alpha,25$ -OHC specifically modified the impedance of cells transduced with  
18 *EBI2* but not in cells transduced with *LacZ* ( $0.153 \pm 0.014$  versus  $0.035 \pm 0.011$  units,  $p < 10^{-4}$ ).  
19 4).

20 *EBI2 expression reduces HIV entry.* As heterodimerization may modify the interactions  
21 between the GPCR involved in the heteromer and their ligands, we tested whether the  
22 presence of EBI2 modified R5 envelope-CCR5 interaction and thereby the efficiency of HIV-  
23 1 R5 entry. To this aim, we used an assay measuring the delivery of an enzyme,  $\beta$ -lactamase,  
24 in the course of MT4 cell infection by HIV-1 virions harboring this enzyme fused to the viral  
25 protein Vpr (Vpr-Blam). The presence of the enzyme in the target cells was measured by

1 quantifying in flow cytometry the transformation of a substrate, CCF2-MA, into a fluorescent  
2 product by  $\beta$ -lactamase. As shown in Figure 4A, the presence of EBI2 decreased HIV-1 R5  
3 entry efficiency. In three independent experiments, this inhibition was  $48.4 \pm 4.9\%$  (mean  $\pm$   
4 SEM). By comparison, preincubation of the MT4 cells with soluble CD4 before infection to  
5 inhibit viral envelope binding to CD4 resulted in a  $90.7 \pm 5.0\%$  (mean  $\pm$  SEM) inhibition.

6 *Effect of EBI2 expression on HIV RNA reverse transcription.* As CCR5 signaling boosts HIV  
7 RNA reverse transcription <sup>[27]</sup>, we tested the hypothesis that EBI2 signaling might have the  
8 same effect. To this aim, we infected HOS cells transduced with *EBI2* or *lacZ*, with  
9 replication defective R5 virions and monitored the appearance of early and late reverse HIV  
10 transcripts in these cells. The amount of early ( $0.067 \pm 0.006$  and  $0.070 \pm 0.003$  arbitrary  
11 units,  $p = 0.601$ ) and late ( $0.012 \pm 0.001$  and  $0.019 \pm 0.003$  arbitrary units,  $p = 0.263$ )  
12 transcripts were similar 3 days later in EBI2- and LacZ-expressing cells, respectively (Fig.  
13 4B). This result suggests that EBI2 expression increases early reverse transcription efficiency  
14 in target cells, thus compensating for its inhibitory effect on viral entry.

15 *EBI2 expression induces NFkB activation.* Next, we tested the hypothesis that EBI2 signaling  
16 might activate the transcription factor NFkB. To this aim, we examined by  
17 immunofluorescence whether EBI2 expression associated with  $7\alpha,25$ -OHC stimulation  
18 resulted in NFkB nuclear translocation in Hela cells transduced with *EBI2* or *lacZ* (Fig. 4C).  
19 Whereas NFkB translocation to the nucleus was provoked by TNF $\alpha$  in both cell types, it was  
20 only induced by  $7\alpha,25$ -OHC in EBI2-expressing cells. To confirm this result, both cell types  
21 were transfected with a *luciferase* gene driven by a promoter containing or not NFkB sites,  
22 and stimulated or not with  $7\alpha,25$ -OHC or TNF $\alpha$  used as a positive control (Fig. 4D-E). In  
23 absence of NFkB binding sites,  $7\alpha,25$ -OHC induced *luciferase* activation neither in *lacZ*- nor

1 in *EBI2*-transduced cells (Fig. 4D). By contrast, in presence of NFκB binding sites, 7α,25-  
2 OHC induced luciferase expression in *EBI2*- but not in *lacZ*-transduced cells (Fig. 4E).

3 *EBI2* signaling activates *HIV-1* promoter. As NFκB translocation is known to activate the  
4 HIV promoter LTR, we checked whether *EBI2* signaling was able to induce the transcription  
5 of an LTR-driven reporter gene. To this aim, we transduced Hela-LTR.Luc cells, stably  
6 transfected with an LTR fused to a *luciferase* gene<sup>[28]</sup>, with *EBI2* or *lacZ*, incubated these  
7 cells with increasing concentrations of 7α,25-OHC (0.2 to 2 μM), and monitored *luciferase*  
8 transcription. As shown in Figure 5A, the *EBI2* ligand induced luciferase expression in *EBI2*-  
9 expressing cells but not in *LacZ*-expressing cells ( $1.90 \pm 0.14$  versus  $0.84 \pm 0.02$  fold increase  
10 for 0.2 μM,  $p = 0.002$ ). TNFα, used as a positive control, induced luciferase expression in  
11 *EBI2*+ and *EBI2*- cells. To ascertain that the effect of 7α,25-OHC was mediated by *EBI2*  
12 signaling, we repeated the experiment after having incubated or not the cells with the *EBI2*  
13 specific antagonist NIBR189. Again 7α,25-OHC activated the LTR in *EBI2*-expressing cells  
14 but not in *LacZ*-expressing cells even at 0.01 μM ( $1.41 \pm 0.04$  versus  $1.00 \pm 0.03$  fold  
15 increase,  $p = 0.001$ ), and this effect was inhibited by the *EBI2* antagonist (Fig. 5B).

16 *EBI2* expression increases *HIV-1* R5 replication. As *EBI2* signaling activates the HIV  
17 promoter, we analyzed whether it could amplify HIV-1 R5 production. To this purpose we  
18 infected CD4- and CCR5-positive HOS cells transduced with *EBI2* or *lacZ* with a replicative  
19 HIV-1 R5 strain. *EBI2*-positive cells produced much more virus than the negative control  
20 *lacZ*-positive cells (Fig. 6A). As soon as day 4, p24 production was 2-fold higher in *EBI2*-  
21 than in *LacZ*-transduced cells ( $8.9 \pm 0.6$  versus  $4.5 \pm 0.3$  ng/mL, respectively,  $p = 0.003$ ). At  
22 day 11 the ratio was 11:1. As 25-hydroxycholesterol has been shown to block membrane  
23 fusion between viruses, including HIV, and target cell with an IC<sub>50</sub> of 1μM independently of  
24 *EBI2*<sup>[29]</sup>, we tested the effect of 7α,25-OHC on *EBI2*-transduced HOS cell infection.

1 Interestingly,  $7\alpha,25$ -OHC further increased HIV-1 R5 replication at  $0.2\mu\text{M}$ , a concentration  
2 sufficient to activate HIV LTR (Fig. 5A), but not to block viral fusion <sup>[29]</sup>, but inhibited it at  
3  $1\mu\text{M}$  (Fig. 6B). This effect was prevented by the pre-incubation of the cells by the EBI2  
4 antagonist NIBR189 (Fig. 6C). HIV-1 R5 replication was also increased in *EBI2*-transduced  
5 MT4 cells as compared with *LacZ*-transduced MT4 cells ( $1480 \pm 49$  and  $719 \pm 135$  ng of  
6 p24/mL at day 7, respectively,  $p = 0.006$ , Fig. 6D). In the same line, R5 HIV-1 replication in  
7 PBMC was reduced by the presence of NIBR189 (Fig.6E). At day 8, NIBR189-treated PBMC  
8 produced  $46 \pm 34$  ng/mL, whereas untreated cells produced  $123 \pm 26$  ng/mL of p24 antigen ( $p$   
9  $= 0.036$ , Fig.6E).

10 To test whether this effect was specific of HIV-1 R5 strains or was also observed with HIV-1  
11 X4 strains, we exposed HOS cells expressing or not EBI2 to defective virions harboring a  
12 *luciferase* gene and pseudotyped with an R5, X4, or Vesicular Stomatitis Virus envelope  
13 (VSV-G). After exposition to 10ng of R5 defective virions, the amount of virus produced, as  
14 evaluated by luciferase activity, was higher in *EBI2*-transduced cells than in *LacZ*-transduced  
15 cells ( $240,000 \pm 10,000$  versus  $100,000 \pm 20,000$  units,  $p < 0.001$ , Fig. 6F). By contrast, *EBI2*-  
16 transduced cells exposed to 10ng of X4 defective virions produced the same quantity of virus  
17 as *LacZ*-transduced cells ( $40,300 \pm 608$  versus  $40,000 \pm 500$  units,  $p = 0.545$ , Fig. 6G). This  
18 absence of difference was also observed with lower (2ng) and higher (50ng) viral input (Fig.  
19 6G). Likely, *EBI2*- and *LacZ*-transduced cells infected with 1ng, 5ng, 10ng ( $2,066,700 \pm$   
20  $152,700$  versus  $1,900,000 \pm 300,000$  units,  $p = 0.439$ ) or 15ng of defective virions  
21 pseudotyped with VSV-G envelope produced equivalent quantities of virus (Fig. 6H).

22

## 1 Discussion

2 Here we show that EBI2 dimerizes with CCR5. R. Barroso et al. have reported the  
3 heterodimerization of EBI2 with another chemokine receptor, CXCR5<sup>[30]</sup>. Of note, this  
4 dimerization resulted in a decrease in the affinity of the chemokine CXCL13 for CXCR5.  
5 Likewise, CCR5-EBI2 dimerization might reduce the affinity of CCR5 ligands, including  
6 HIV envelope, for CCR5. This could explain why we observed that EBI2 expression  
7 diminished the efficiency of HIV R5 entry into CCR5-positive cells. By contrast, EBI2  
8 expression seemed to increase early HIV DNA reverse transcription, compensating for its  
9 inhibitory effect on viral entry. This effect of EBI2 expression on reverse transcription might  
10 be a consequence of the fact that CCR5 signaling triggered upon HIV-1 R5 envelope binding  
11 to CCR5 increases HIV DNA reverse transcription, as previously reported by our group<sup>[3]</sup>.  
12 CCR5-EBI2 dimerization might amplify this phenomenon.

13 In addition, we show that EBI2 signaling activates NFkB, and thereby HIV-1 LTR and finally  
14 virus replication. Of note, this effect is specific of R5 strains since it is not observed with X4  
15 strains or with viruses pseudotyped with a VSV envelope. It is therefore logical to assume that  
16 CCR5-EBI2 dimerization is also responsible for a signal triggered upon R5 envelope binding  
17 to CCR5 which boosts LTR activation via NFkB.

18 Interestingly, S.-Y. Liu et al. have reported that 25-OHC, at the higher concentration of 1mM,  
19 inhibited HIV-1 entry at the fusion step by interfering with the target cell membrane<sup>[29]</sup>. Of  
20 note, they show the same effect with other enveloped virus as Ebolavirus, Nipahvirus, or Rift  
21 Valley fever virus, but not with Adenovirus, a non-enveloped virus. This aligns with our  
22 present observation that 1mM of 7 $\alpha$ ,25-OHC reduces the productive HIV-1 infection of HOS  
23 cells. These concentrations are much lower than the concentrations at which 7 $\alpha$ ,25-OHC  
24 interferes with enveloped virus-cell fusion<sup>[29]</sup>.

1  $7\alpha,25$ -OHC is present in human plasma, at a concentration of 0.9 nM<sup>[31]</sup> for an IC<sub>50</sub> of 0.8  
2 nM on EBI2<sup>[10]</sup>. EBI2 ligands have also been detected in many lymphoid and nonlymphoid  
3 tissues<sup>[32]</sup>. Moreover,  $7\alpha,25$ -OHC and its precursors are thought to spread from cell to cell<sup>[33]</sup>.  
4 In vivo, EBI2-positive cells might be constantly activated by these ligands. Consequently, the  
5 predominant *in vivo* effect of  $7\alpha,25$ -OHC might be to constantly favor HIV postentry steps in  
6 CD4+ T cells rather than to inhibit the fusion step of viral life cycle. This is probably  
7 particularly true in secondary lymphoid tissues where stromal cells express the two enzymes  
8 required for  $7\alpha,25$ -OHC synthesis, cholesterol 25-hydroxylase and 25-hydroxycholesterol 7-  
9 hydroxylase (CYP7B1)<sup>[34]</sup>. The presence of the EBI2 natural ligand  $7\alpha,25$ -OHC in the culture  
10 might be responsible for an EBI2 signaling boosting the retroviral reverse transcription. The  
11 origin of  $7\alpha,25$ -OHC may be the fetal bovine serum added to the culture medium, since  
12  $7\alpha,25$ -OHC is present in culture cells<sup>[35]</sup>. In line with this model, we have observed a  
13 tendency towards an increase in LTR activation in the transfection assays performed in  
14 cultured EBI2-expressing cells. Accordingly, it would be interesting to monitor the effect of  
15 EBI2 antagonists on the residual viral production persisting in most patients aviremic under  
16 treatment<sup>[36]</sup>. Moreover, given that oxysterols, including  $7\alpha,25$ -OHC, bind to ROR $\gamma$ t and  
17 thereby promote Th17 differentiation<sup>[19]</sup>, EBI2 antagonists should also dampen the  
18 pathogenic immune activation observed in virologic responders<sup>[37]</sup>.

19 Just as CXCR5-EBI2 dimerization impairs the interaction between CXCR5 and its ligand,  
20 CXCL13<sup>[30]</sup>, it is possible that CCR5-EBI2 interaction decreases the affinity of EBI2 for its  
21 natural ligands and thereby alters EBI2 physiological functions. In addition, HIV gp120  
22 binding to CCR5 dimerized with EBI2 might further exacerbate this phenomenon.  
23 Consequently, in viremic patients the role of EBI2 may be perturbed in CD4- and CCR5-  
24 positive cells. EBI2 signaling has been reported to inhibit type I interferon production in  
25 plasmacytoid and myeloid dendritic cells<sup>[16]</sup>. As HIV may infect dendritic cells via CCR5,

1 gp120-CCR5 binding could alleviate this inhibition and participate in the type I interferon  
2 overproduction observed in the course of HIV infection <sup>[38]</sup>. EBI2 has also been shown to  
3 drive the positioning of splenic dendritic cells in marginal zone bridging channels, where they  
4 encounter blood-borne antigens <sup>[14, 15]</sup>. Impairment of this positioning consecutive to gp120-  
5 CCR5-EBI2 interaction could result in deficient CD4+ T cell activation and humoral immune  
6 response. This dysfunction might be further increased by the fact that EBI2 also guides T  
7 follicular helper T cell in secondary lymphoid organs towards the interface of the follicle and  
8 T zone, where they interact with dendritic cells <sup>[39]</sup>. Macrophages also express EBI2, in  
9 addition to CD4 and CCR5, and the enzymes necessary for  $7\alpha,25$ -OHC synthesis, including  
10 cholesterol 25-hydroxylase and CYP7B1 <sup>[17]</sup>. In these cells, EBI2 activation induces calcium  
11 flux and chemotaxis <sup>[17, 18, 40]</sup>. Thus, macrophage activity could similarly be impaired by  
12 circulating soluble or viral gp120.

13 Last, our data unveil the possibility that EBI2 agonists could be latency reversing agent  
14 candidates in HIV-1 infection.

15

## 1 **Methods**

2 *Cells.* CD4<sup>+</sup> human osteosarcoma HOS cells, human embryonic kidney 293T cells, HeLa-  
3 LTR.Luc cells <sup>[28]</sup> were cultivated in DMEM supplemented with 10% fetal bovine serum,  
4 glutamin and penicillin/streptomycin. MT4 cells were cultivated in DMEM supplemented in  
5 the same way.

6 *Quantitative multi-RT-PCR.* CD4+CCR5+ T cells (2 millions) were sorted using magnetic  
7 beads coupled to antibodies (Dynabeads Pan Mouse IgG, Invitrogen). Macrophages were  
8 obtained by *in vitro* differentiation with GM-CSF of peripheral blood mononuclear cells  
9 (PBMC) isolated by low density gradient centrifugation. RNAs were extracted with Qiamp  
10 RNA blood (Qiagen) where an RNase free DNase treatment was included. cDNA was  
11 synthesized with the high-capacity cDNA archive kit (Applied Biosystems). Quality of sample  
12 was controlled by Q-PCR for correct GAPDH/CCR5 ratio. cDNA from 3 donors were mixed  
13 in equimolar amounts and 750 ng of this mixture were loaded on a 384-well microfluidic  
14 cards pre-loaded with primers for 361 different GPCR and 14 housekeeping genes as positive  
15 control (TaqMan Low Density Arrays from Applied Biosystems). Q-PCR was realized on an  
16 ABI Prism 7900HT Sequence Detection System.

17 *Time-resolved FRET.* The DNA fragments for *CCR5* and *EBI2* were obtained by PCR and  
18 cloned in pRK5-HA-SNAP or pRK5-FLAG-CLIP, resulting in a fusion of a signal peptide, an  
19 hemagglutinin (HA) or a FLAG tag, the SNAP or CLIP enzyme <sup>[41]</sup>, and the receptor from the  
20 second codon to the STOP codon. The SNAP- $\beta_2$ AR construct has been previously described  
21 <sup>[42]</sup>. 293T cells were cotransfected with 4 ng of CLIP-CCR5 and increasing amount (1 to 25  
22 ng) of SNAP-CCR5, SNAP-EBI2 or SNAP-  $\beta_2$ AR, plated at 10<sup>5</sup> cells/well in a poly  
23 ornithine-coated 96-well plate for 30 hours, and then labeled for 2 hours at 37°C with BC-  
24 Lumi4-Tb (1  $\mu$ M) and BG-Green (0.3  $\mu$ M) in TagLite (TL) buffer (Cisbio Bioassays). After

1 washing, the emission signals from the BC-Lumi4-Tb and the BG-Green fluorophores were  
2 recorded at 620 nm and 520 nm, respectively, after excitation at 337 nm and 485 nm on a  
3 time-resolved fluorimeter (PHERAstar FS, BMG Labtechnologies). In the same wells, tr-  
4 FRET signal was measured at 520 nm between 60 and 400  $\mu$ s after laser excitation at 337 nm.  
5 *Flow cytometry.* Cells ( $2 \times 10^5$ ) were incubated with PE-Cy5-conjugated anti-CCR5  
6 monoclonal antibody (mAb) 2D7 (Pharmingen), or anti-human EBI2 mAb 10272-100 (R&D)  
7 for 1h on ice at 10  $\mu$ g/mL, followed by a 1:100 dilution of FITC-conjugated F(ab')<sub>2</sub> fragment  
8 goat anti-mouse IgG (H+L, Beckman Coulter) after washing, or with an isotype control. Cells  
9 were then washed, fixed in CellFIX (Becton Dickinson) and analyzed on a FACScalibur  
10 (Becton Dickinson).

11 *Transduction.* HIV transfer vectors that express *CCR5* or *LacZ* were produced as described <sup>[3]</sup>.  
12 The HIV transfer vector harboring *EBI2* was obtained by cloning *EBI2* cDNA into the  
13 pWPXL lentiviral vector. Transductions were performed as previously described <sup>[3]</sup>.

14 *Infection assays with replicative virus.* HOS or MT4 cells were exposed to 10 ng/mL of p24  
15 equivalent of the R5 strain Ad8, washed and cultured in supplemented DMEM or RPMI-1640  
16 medium, respectively. Cell number was adjusted twice a week, and viral production was  
17 evaluated by measuring p24 antigen in culture supernatant (Innotest HIV Antigen mAb kit,  
18 Fujirebio, Ingen).

19 *HIV entry assay.* Virions harboring  $\beta$ -lactamase were produced by cotransfecting 3 millions  
20 of 293T cells per plate with 15  $\mu$ g of the HIV proviral DNA pNL4.3 env-luc+, the R5  
21 envelope plasmid pCMV-Ad8 (6  $\mu$ g), a packaging plasmid (pAX2, 2  $\mu$ g), and the plasmid  
22 pMM310 encoding a *Vpr* gene fused to a  *$\beta$ -lactamase* gene (7  $\mu$ g). Virus preparations were  
23 concentrated by ultracentrifugation on 4% sucrose (1h30, 26,000 rpm, 4°C). MT4 cells were  
24 then exposed for 3.5 hours at 37°C to 500 ng of R5 Vpr-Blam virus. Cells were then washed

1 and loaded with the CCF2-AM LiveBLAzer™ FRET-B/G Loading Kit (Life Technologies)  
2 in the presence of 15 mM Probenecid (Sigma). Cells were incubated for 1 h at room  
3 temperature in CO<sub>2</sub>-independent medium (Gibco), washed and fixed. The CCF2-AM and the  
4 cleaved CCF2-AM fluorescences (excitation at 405nm, emission at 520 nm and 447 nm,  
5 respectively) were measured by flow cytometry on a MACSQuant analyser 10 (Mylteni).

6 *Real-time PCR detection of HIV transcripts.* To produce replication-defective HIV virions,  
7 293T cells were co-transfected with the pNL4.3-env<sup>-</sup>-luc plasmid and with the pCMV-Ad8-  
8 Env (PNAS 2002). HOS cells were exposed to 70 ng of p24 equivalent of these virions, and  
9 lysed 24 hours later. DNA from early and late transcripts was amplified as described <sup>[3]</sup>.  
10 Genomic CCR5 DNA was used for normalization.

11 *LTR activation assays.* HeLa-LTR.Luc cells were transduced with *EBI2* or *LacZ*, exposed or  
12 not to 10ng/ml of TNF $\alpha$  for 6 hours and lysed. Alternatively, HOS cells transduced with *EBI2*  
13 or *LacZ* were transiently transfected by using lipofectamine Plus (Invitrogen) with the LTR-  
14 luc reporter plasmid pL274 <sup>[43]</sup>, exposed or not to 10ng/ml of TNF $\alpha$  for 6 hours and lysed.  
15 Luciferase activity was then measured in cell extracts using a commercial assay (Promega).  
16 To test the role of NF $\kappa$ B in *EBI2* signaling induced gene expression, HeLa cells were co-  
17 transfected with either a lacZ (pHRTlacZ) or *EBI2* (pCDNA3-*EBI2*) expressing plasmid and  
18 a plasmid containing the reporter *luciferase* gene driven a promoter containing ((Igk)3-  
19 conaluc) or not (conaluc) NF $\kappa$ B binding sites <sup>[44]</sup> with lipofectamin2000 (Invitrogen)  
20 according to the manufacturer recommendation. At day 3, the cells were incubated or not with  
21 either 50ng/ml TNF $\alpha$  or 10 $\mu$ M 7 $\alpha$ ,25-OHC for 6 hours and the cells were harvested for  
22 luciferase activity quantification with the luciferase assay system kit (Promega).

23 *Immunofluorescence assay.* HOS cells transduced with *EBI2* or *LacZ* were exposed or not for  
24 2 hours to 10 ng/ml TNF $\alpha$  and labeled for 45 min with an NF $\kappa$ B p65 antibody (F-6, Santa

1 Cruz), followed by a secondary fluorescent anti-mouse antibody (A546), and finally with  
2 Dapi at 5 µg/ml for nucleus staining.

3 *One-round infection assay.* 293T cells were cotransfected with the pNL4.3-env<sup>-</sup>-luc (AIDS  
4 Reagent Program) plasmid, containing the entire HIV-1 genome deleted in the *envelope* gene  
5 and harboring a *luciferase* reporter gene fused to *nef*, and either with the pCMV.AD8-Env  
6 plasmid coding for the R5 envelope of HIV-1 prototype AD8, with the pDOL plasmid coding  
7 for the X4 envelope of HIV-1 prototype NL4.3, or with the pMD.G plasmid encoding the G  
8 protein of the VSV-G at a molecular ratio of 2:1 to produce replication-defective HIV virions  
9 pseudotyped with an R5, X4 or VSV-G envelope, respectively. HOS cells were then plated (8  
10 × 10<sup>4</sup> cells per well in a 24-well plate) and exposed to the indicated amount of defective  
11 virions for 18 h, washed, and cultured. At Day 3, luciferase activity was measured by  
12 luminometry (Luciferase Assay System, Promega).

13 *Statistical analysis.* Values are expressed as the mean ± SEM. Differences were analyzed with  
14 the one-tailed unpaired Student's *t* test.

15

## 16 **Acknowledgements.**

17 AG, SG, and CM contributed to the design of the flow cytometry, cell transduction,  
18 infection, HIV entry, RT, LTR activation, and immunofluorescence assays, and acquired,  
19 analyzed and interpreted data. DM and ED contributed to the conception of the FRET  
20 experiments. VF and PC contributed to the conception and design of the study, analyzed  
21 and interpreted data. PC wrote the first draft of the manuscript.

## 1 **References**

- 2 1. Feng Y, Broder CC, Kennedy PE, Berger EA. **HIV-1 entry cofactor: functional cDNA**  
3 **cloning of a seven-transmembrane, G protein-coupled receptor.** *Science* 1996; 272:872-877.
- 4 2. Abbas W, Herbein G. **Plasma membrane signaling in HIV-1 infection.** *Biochimica et*  
5 *biophysica acta* 2014; 1838(4):1132-1142.
- 6 3. Lin Y-L, Mettling C, Portales P, Reynes J, Clot J, Corbeau P. **Cell surface CCR5 density**  
7 **determines the postentry efficiency of R5 HIV-1 infection.** *Proc Natl Acad Sci USA* 2002;  
8 99(24):15590-15595.
- 9 4. Pin JP, Prezeau L. **Allosteric modulators of GABA(B) receptors: mechanism of action**  
10 **and therapeutic perspective.** *Current neuropharmacology* 2007; 5(3):195-201.
- 11 5. Lin Y-L, Mettling C, Portales P, Réant B, Clot J, Corbeau P. **G-protein signaling**  
12 **triggered by R5 human immunodeficiency virus type 1 increases virus replication**  
13 **efficiency in primary T lymphocytes.** *J Virol* 2005; 79(12):7938-7941.
- 14 6. Reynes J, Baillat V, Portalès P, Clot J, Corbeau P. **Relationship between CCR5 density**  
15 **and viral load after discontinuation of antiretroviral therapy.** *JAMA* 2004; 291(1):46.
- 16 7. Reynes J, Portales P, Clot J, Corbeau P. **CD4+ T cell surface CCR5 density and virus**  
17 **load in persons infected with Human Immunodeficiency Virus type 1.** *J Infect Dis* 2000;  
18 182:1285-1286.
- 19 8. Birkenbach M, Josefsen K, Yalamanchili R, Lenoir G, Kieff E. **Epstein-Barr virus-**  
20 **induced genes: first lymphocyte-specific G protein-coupled peptide receptors.** *Journal of*  
21 *virology* 1993; 67(4):2209-2220.
- 22 9. Hannedouche S, Zhang J, Yi T, Shen W, Nguyen D, Pereira JP, et al. **Oxysterols direct**  
23 **immune cell migration via EBI2.** *Nature* 2011; 475(7357):524-527.
- 24 10. Liu C, Yang XV, Wu J, Kuei C, Mani NS, Zhang L, et al. **Oxysterols direct B-cell**  
25 **migration through EBI2.** *Nature* 2011; 475(7357):519-523.

- 1 11. Gatto D, Paus D, Basten A, Mackay CR, Brink R. **Guidance of B cells by the orphan G**  
2 **protein-coupled receptor EBI2 shapes humoral immune responses.** *Immunity* 2009;  
3 31(2):259-269.
- 4 12. Pereira JP, Kelly LM, Xu Y, Cyster JG. **EBI2 mediates B cell segregation between the**  
5 **outer and centre follicle.** *Nature* 2009; 460(7259):1122-1126.
- 6 13. Niss Arfelt K, Barington L, Benned-Jensen T, Kubale V, Kovalchuk AL, Daugvilaite V,  
7 et al. **EBI2 overexpression in mice leads to B1 B-cell expansion and chronic lymphocytic**  
8 **leukemia-like B-cell malignancies.** *Blood* 2017; 129(7):866-878.
- 9 14. Gatto D, Wood K, Caminschi I, Murphy-Durland D, Schofield P, Christ D, et al. **The**  
10 **chemotactic receptor EBI2 regulates the homeostasis, localization and immunological**  
11 **function of splenic dendritic cells.** *Nature immunology* 2013; 14(5):446-453.
- 12 15. Yi T, Cyster JG. **EBI2-mediated bridging channel positioning supports splenic**  
13 **dendritic cell homeostasis and particulate antigen capture.** *eLife* 2013; 2:e00757.
- 14 16. Chiang EY, Johnston RJ, Grogan JL. **EBI2 is a negative regulator of type I interferons**  
15 **in plasmacytoid and myeloid dendritic cells.** *PloS one* 2013; 8(12):e83457.
- 16 17. Preuss I, Ludwig MG, Baumgarten B, Bassilana F, Gessier F, Seuwen K, et al.  
17 **Transcriptional regulation and functional characterization of the oxysterol/EBI2 system**  
18 **in primary human macrophages.** *Biochemical and biophysical research communications*  
19 2014; 446(3):663-668.
- 20 18. Rutkowska A, O'Sullivan SA, Christen I, Zhang J, Sailer AW, Dev KK. **The EBI2**  
21 **signalling pathway plays a role in cellular crosstalk between astrocytes and**  
22 **macrophages.** *Scientific reports* 2016; 6:25520.
- 23 19. Soroosh P, Wu J, Xue X, Song J, Sutton SW, Sablad M, et al. **Oxysterols are agonist**  
24 **ligands of ROR $\gamma$  and drive Th17 cell differentiation.** *Proceedings of the National*  
25 *Academy of Sciences of the United States of America* 2014; 111(33):12163-12168.

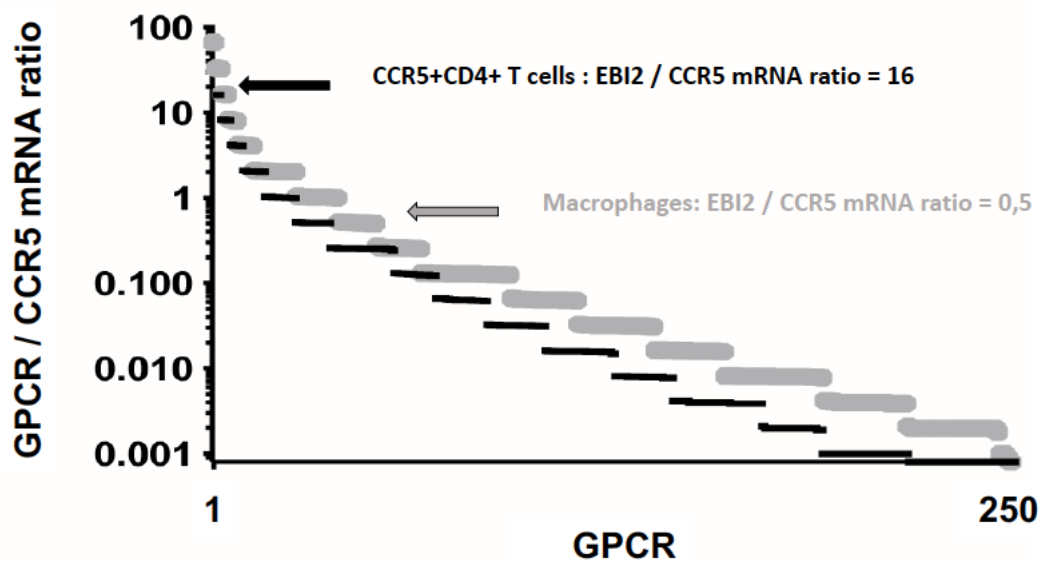
- 1 20. Wanke F, Moos S, Croxford AL, Heinen AP, Graf S, Kalt B, et al. **EBI2 Is Highly**  
2 **Expressed in Multiple Sclerosis Lesions and Promotes Early CNS Migration of**  
3 **Encephalitogenic CD4 T Cells.** *Cell reports* 2017; 18(5):1270-1284.
- 4 21. Duquenne C, Gimenez S, Guigues A, Viala B, Boulouis C, Mettling C, et al. **Reversing**  
5 **HIV latency via sphingosine-1-phosphate receptor 1 signaling.** *AIDS* 2017; 31(18):2443-  
6 2454.
- 7 22. George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, et al. **Oligomerization of mu- and**  
8 **delta-opioid receptors. Generation of novel functional properties.** *The Journal of*  
9 *biological chemistry* 2000; 275(34):26128-26135.
- 10 23. Hillion J, Canals M, Torvinen M, Casado V, Scott R, Terasmaa A, et al. **Coaggregation,**  
11 **cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2**  
12 **receptors.** *The Journal of biological chemistry* 2002; 277(20):18091-18097.
- 13 24. Jordan BA, Devi LA. **G-protein-coupled receptor heterodimerization modulates**  
14 **receptor function.** *Nature* 1999; 399:697-700.
- 15 25. Mellado M, Rodriguez-Frade JM, Vila-Coro AJ, Fernandez S, Martin de Ana A, Jones  
16 DR, et al. **Chemokine receptor homo- or heterodimerization activates distinct signaling**  
17 **pathways.** *The EMBO journal* 2001; 20(10):2497-2507.
- 18 26. Pfeiffer M, Koch T, Schroder H, Laugsch M, Hollt V, Schulz S. **Heterodimerization of**  
19 **somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and**  
20 **desensitization.** *The Journal of biological chemistry* 2002; 277(22):19762-19772.
- 21 27. Lin Y-L, Mettling C, Réant B, Clot J, Corbeau P. **CCR5 Signaling-Induced ERK1/2**  
22 **Activation Boosts R5 HIV-1 Replication in Primary Mononuclear Cells.** *Conference on*  
23 *Retroviruses and Opportunistic Infections* 2005; Abstract 278.

- 1 28. Treand C, du Chene I, Bres V, Kiernan R, Benarous R, Benkirane M, et al. **Requirement**  
2 **for SWI/SNF chromatin-remodeling complex in Tat-mediated activation of the HIV-1**  
3 **promoter.** *The EMBO journal* 2006; 25(8):1690-1699.
- 4 29. Liu SY, Aliyari R, Chikere K, Li G, Marsden MD, Smith JK, et al. **Interferon-inducible**  
5 **cholesterol-25-hydroxylase broadly inhibits viral entry by production of 25-**  
6 **hydroxycholesterol.** *Immunity* 2013; 38(1):92-105.
- 7 30. Barroso R, Martinez Munoz L, Barrondo S, Vega B, Holgado BL, Lucas P, et al. **EBI2**  
8 **regulates CXCL13-mediated responses by heterodimerization with CXCR5.** *FASEB*  
9 *journal : official publication of the Federation of American Societies for Experimental*  
10 *Biology* 2012; 26(12):4841-4854.
- 11 31. Iuliano L, Crick PJ, Zerbinati C, Tritapepe L, Abdel-Khalik J, Poirot M, et al. **Cholesterol**  
12 **metabolites exported from human brain.** *Steroids* 2015; 99(Pt B):189-193.
- 13 32. Kelly LM, Pereira JP, Yi T, Xu Y, Cyster JG. **EBI2 guides serial movements of**  
14 **activated B cells and ligand activity is detectable in lymphoid and nonlymphoid tissues.**  
15 *Journal of immunology* 2011; 187(6):3026-3032.
- 16 33. Cyster JG, Dang EV, Reboldi A, Yi T. **25-Hydroxycholesterols in innate and adaptive**  
17 **immunity.** *Nature reviews Immunology* 2014; 14(11):731-743.
- 18 34. Yi T, Wang X, Kelly LM, An J, Xu Y, Sailer AW, et al. **Oxysterol gradient generation**  
19 **by lymphoid stromal cells guides activated B cell movement during humoral responses.**  
20 *Immunity* 2012; 37(3):535-548.
- 21 35. Mutemberezi V, Guillemot-Legris O, Muccioli GG. **Oxysterols: From cholesterol**  
22 **metabolites to key mediators.** *Progress in lipid research* 2016; 64:152-169.
- 23 36. Palmer S, Maldarelli F, Wiegand A, Bernstein B, Hanna GJ, Brun SC, et al. **Low-level**  
24 **viremia persists for at least 7 years in patients on suppressive antiretroviral therapy.**

- 1 *Proceedings of the National Academy of Sciences of the United States of America* 2008;  
2 105(10):3879-3884.
- 3 37. Younas M, Psomas C, Reynes J, Corbeau P. **Immune activation in the course of HIV-1**  
4 **infection: Causes, phenotypes and persistence under therapy.** *HIV medicine* 2016;  
5 17(2):89-105.
- 6 38. Lehmann C, Harper JM, Taubert D, Hartmann P, Fatkenheuer G, Jung N, et al. **Increased**  
7 **interferon alpha expression in circulating plasmacytoid dendritic cells of HIV-1-infected**  
8 **patients.** *Journal of acquired immune deficiency syndromes* 2008; 48(5):522-530.
- 9 39. Li J, Lu E, Yi T, Cyster JG. **EBI2 augments Tfh cell fate by promoting interaction**  
10 **with IL-2-queenching dendritic cells.** *Nature* 2016; 533(7601):110-114.
- 11 40. Eibinger G, Fauler G, Bernhart E, Frank S, Hammer A, Wintersperger A, et al. **On the**  
12 **role of 25-hydroxycholesterol synthesis by glioblastoma cell lines. Implications for**  
13 **chemotactic monocyte recruitment.** *Experimental cell research* 2013; 319(12):1828-1838.
- 14 41. Doumazane E, Scholler P, Zwier JM, Trinquet E, Rondard P, Pin JP. **A new approach to**  
15 **analyze cell surface protein complexes reveals specific heterodimeric metabotropic**  
16 **glutamate receptors.** *FASEB journal : official publication of the Federation of American*  
17 *Societies for Experimental Biology* 2011; 25(1):66-77.
- 18 42. Maurel D, Comps-Agrar L, Brock C, Rives ML, Bourrier E, Ayoub MA, et al. **Cell-**  
19 **surface protein-protein interaction analysis with time-resolved FRET and snap-tag**  
20 **technologies: application to GPCR oligomerization.** *Nature methods* 2008; 5(6):561-567.
- 21 43. Wagschal A, Rousset E, Basavarajaiah P, Contreras X, Harwig A, Laurent-Chabalier S, et  
22 al. **Microprocessor, Setx, Xrn2, and Rrp6 co-operate to induce premature termination of**  
23 **transcription by RNAPII.** *Cell* 2012; 150(6):1147-1157.
- 24 44. Munoz E, Courtois G, Veschambre P, Jalinot P, Israel A. **Tax induces nuclear**  
25 **translocation of NF-kappa B through dissociation of cytoplasmic complexes containing**

- 1 **p105 or p100 but does not induce degradation of I kappa B alpha/MAD3.** *J Virol* 1994;
- 2 68(12):8035-8044.
- 3

1  
2  
3  
4  
5  
6  
7



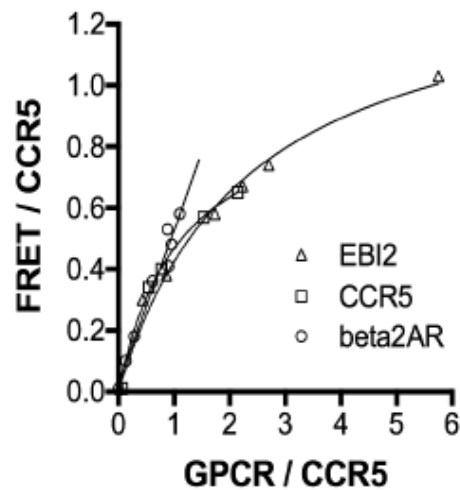
8  
9  
10  
11  
12  
13

**Figure 1. Comparative expression of *CCR5* and other *GPCR* genes in *CCR5+CD4+* T cells and macrophages.** GPCR mRNA were quantified by multi-RT-qPCR in *CCR5+CD4+* T cells sorted from peripheral blood (black) and in cultured macrophages (grey). The ratio between *CCR5* mRNA and other GPCR mRNA is shown in the y axis.

1

2

3



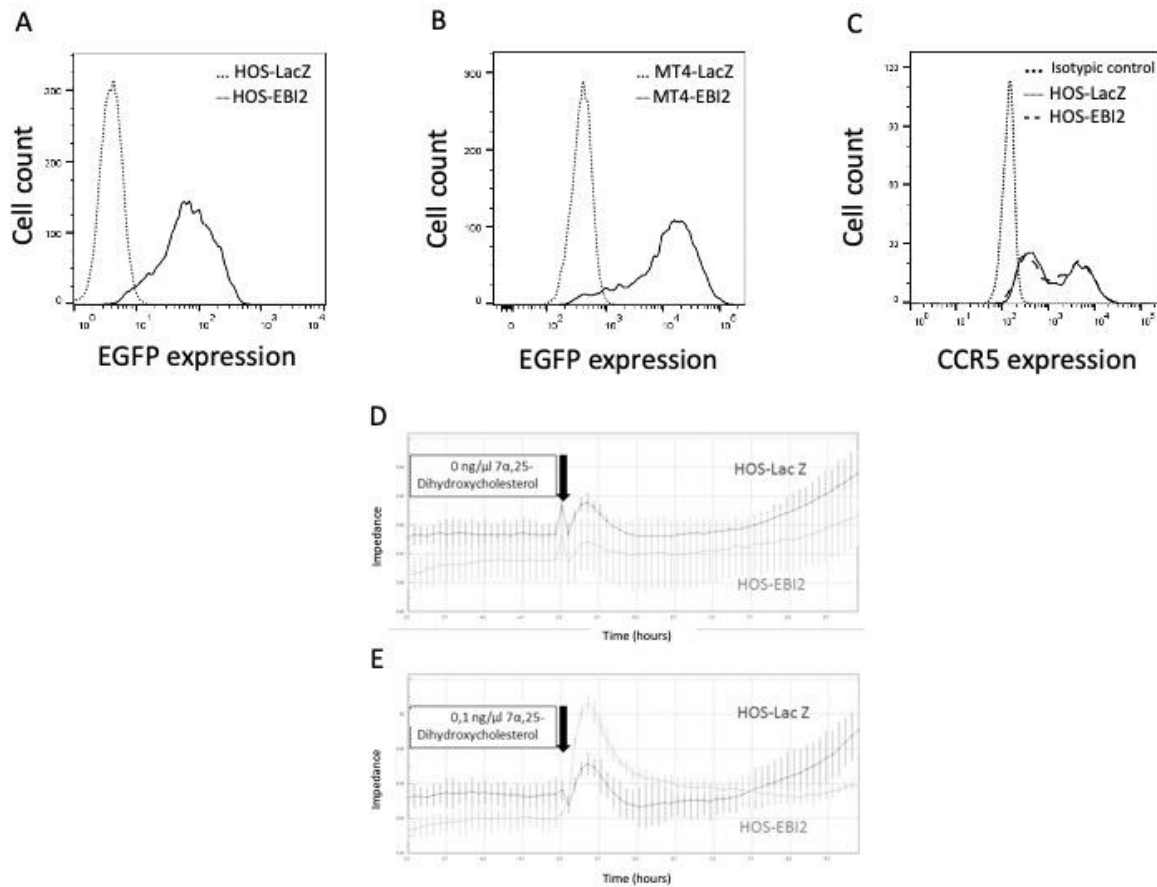
4

5 **Figure 2.** CCR5 forms heteromers with EBI2. Cells were cotransfected with a constant  
6 amount of CLIP-CCR5 plasmid and increasing amount of the SNAP-EBI2, SNAP-CCR5, or  
7 SNAP- $\beta$ 2AR plasmid, and labeled with the corresponding fluorophores. Tr-FRET signal  
8 normalized with the CLIP-CCR5 expression signal (FRET / CCR5) is represented in function  
9 of SNAP-EBI2 ( $\Delta$ ), SNAP-CCR5 ( $\square$ ) or SNAP- $\beta$ 2AR (O) / CLIP-CCR5 expression ratio  
10 (GPCR / CCR5). Tr-FRET signal is expressed by the  $\Delta$ ratio = (520/620)samples –  
11 (520/620)background. The background signal corresponds to cells expressing SNAP- and  
12 CLIP-tagged proteins labeled with the same fluorophores. The curves were obtained by non-  
13 linear regressions with  $R^2 = 0.9921$  and  $0.9875$  for SNAP-CCR5 and SNAP-EBI2,  
14 respectively, and by a linear regression for SNAP- $\beta$ 2AR (p value < 0.0001).

15

16

1



2

3

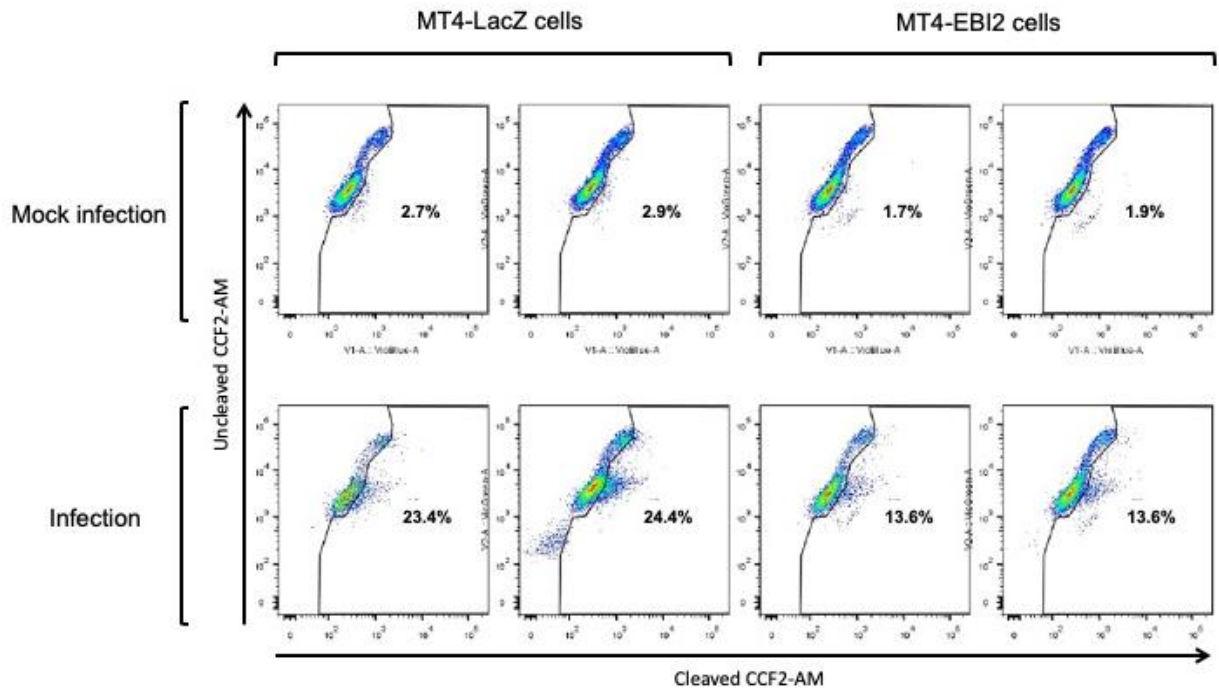
4 **Figure 3. EBI2 expression in HOS and MT4 cells.** (A) Transgene expression in HOS cells.  
 5 EGFP fluorescence of HOS cells transduced with *EBI2-EGFP* (—) or *LacZ* (...). (B)  
 6 Transgene expression in MT4 cells. EGFP fluorescence of MT4 cells transduced with *EBI2-*  
 7 *EGFP* (—) or *LacZ* (...). (C) CCR5 expression in HOS cells transduced with *EBI2*. HOS cells  
 8 transduced with *EBI2-EGFP* (—) or *LacZ* (---) were indirectly labeled with a CCR5 antibody  
 9 or an isotypic control (...). (D, E) EBI2 functionality at the surface of *EBI2-EGFP* transduced  
 10 HOS cells. HOS cells transduced with *EBI2-EGFP* (thin line) or *LacZ* (thick line) were  
 11 exposed (E) or not (D) to 7 $\alpha$ ,25-OHC (arrow), and impedance was monitored over time.

1

2

3

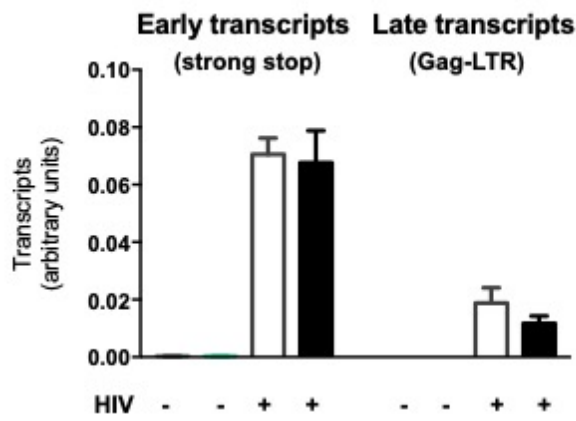
4 **A**



5

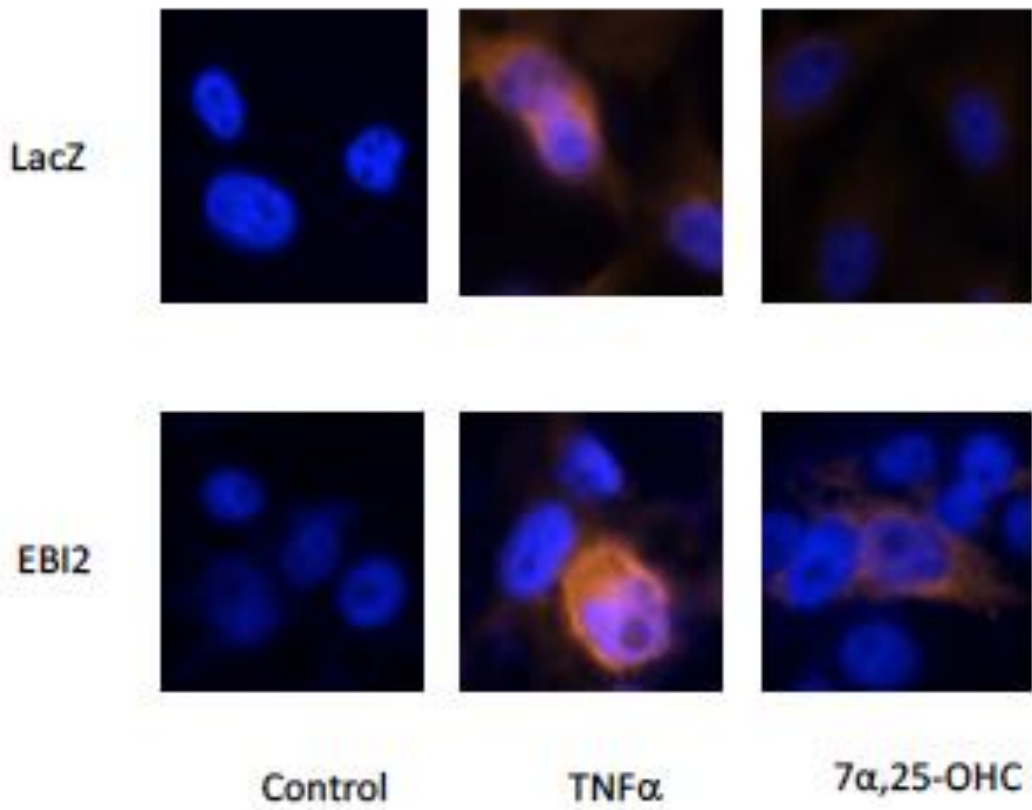
6

7 **B**

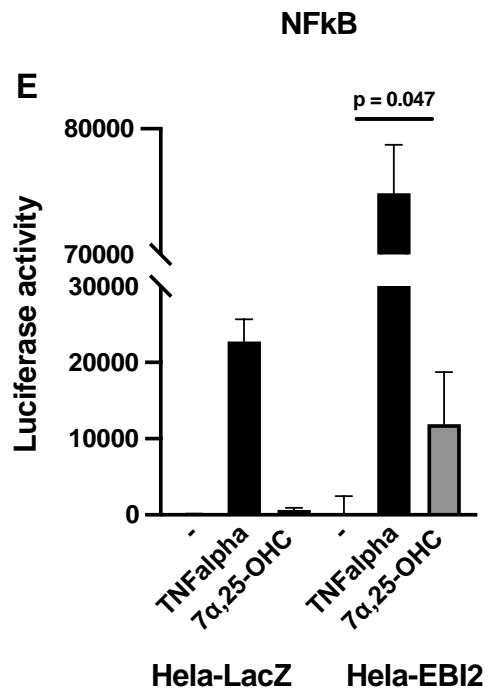
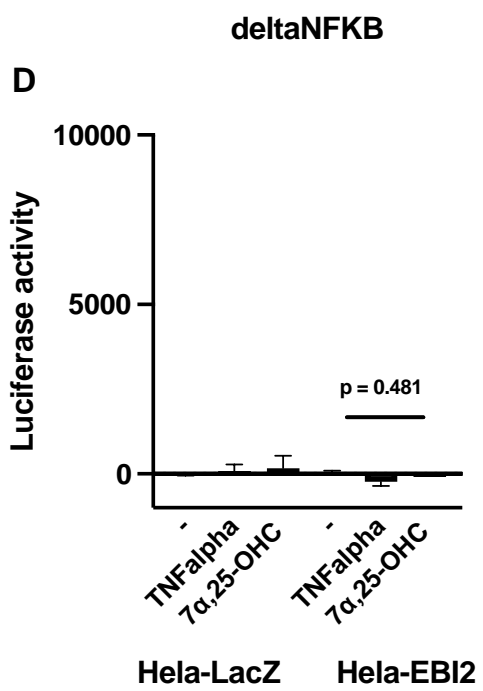


8

1 C



2



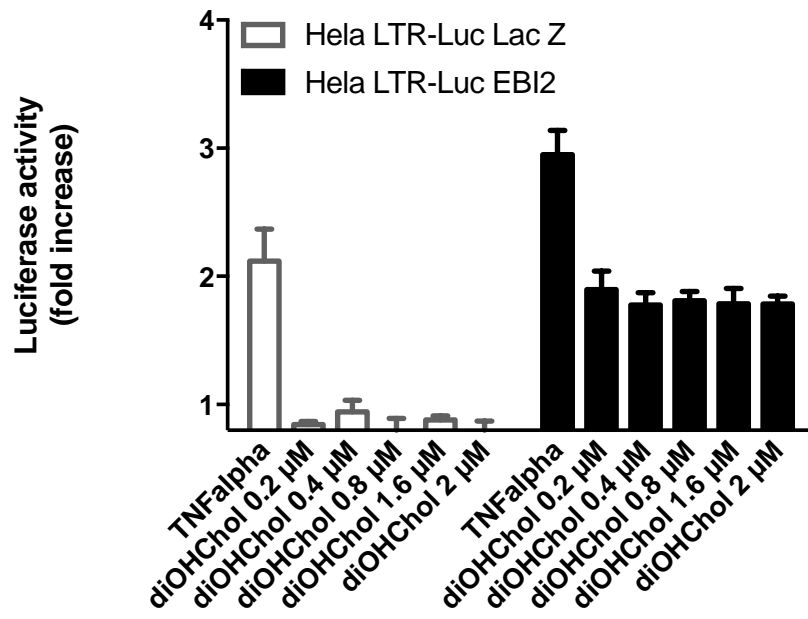
3

1 **Figure 4. EBI2 expression reduces HIV entry.** (A) MT4 cells transduced with *LacZ* or  
2 *EBI2-EGFP* and cultured in duplicate were exposed or not to R5 HIV-1 particles containing  
3 Vpr-Blam, and to the CCF2-AM substrate. The fluorescence induced by CCF2-AM cleavage  
4 was then evaluated by flow cytometry, as a marker of viral entry. Representative experiment  
5 out of three experiments. (B) Effect of EBI2 expression on HIV RNA reverse transcription.  
6 HOS cells transduced with *LacZ* (open histograms) or *EBI2-EGFP* (closed histograms) were  
7 exposed or not to an R5 HIV-1 strain. The amount of early (left hand) and late (right hand)  
8 transcripts was determined by quantitative PCR. (C-E) EBI2 signaling activates NFkB. (C)  
9 HeLa cells transduced with *EBI2* or *LacZ* were exposed or not (control) to the EBI2  
10 ligand  $7\alpha,25$ -OHC or to TNF $\alpha$ . Thereafter, NFkB p65 was labeled with a specific antibody  
11 and analyzed in fluorescence microscopy. (D, E) HeLa cells transduced with *EBI2* (Hela-  
12 *EBI2*) or *LacZ* (Hela-*LacZ*) were transfected with a plasmid encoding luciferase under  
13 the control of a promoter harboring (NFkB, E) or not ( $\Delta$ NFkB, D) NFkB binding sites,  
14 and exposed or not (-) to  $7\alpha,25$ -OHC or to TNF $\alpha$ . Thereafter, luciferase activity was  
15 measured.

16

1

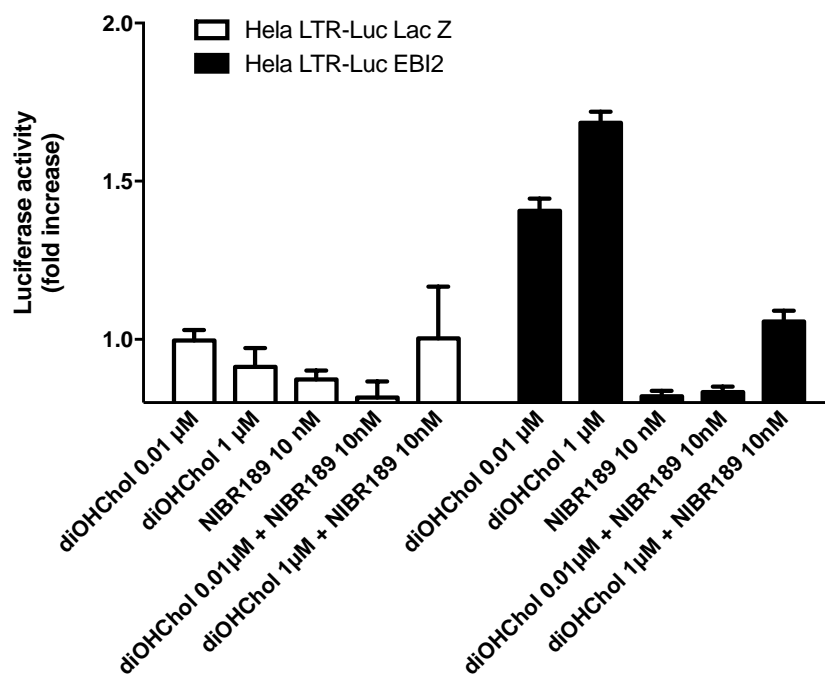
A



2

3

B



4

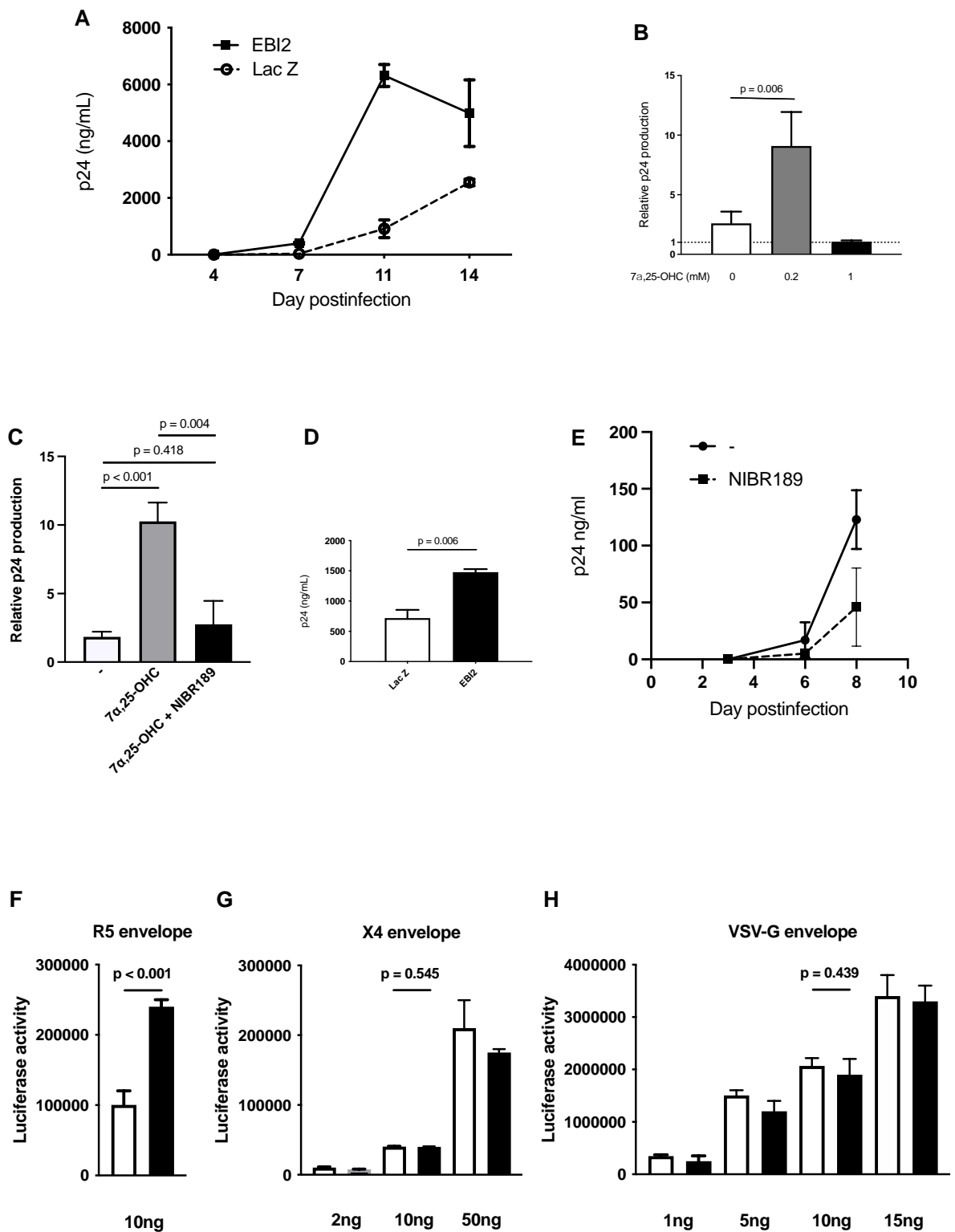
5

6 **Figure 5. EBI2 signaling activates HIV-1 LTR.** (A) HeLa cells stably transfected with a7 *luciferase* gene driven by an HIV-1 LTR and transduced with *LacZ* (open histograms) or

1 *EBI2-EGFP* (closed histograms) were exposed or not (negative control) to various  
2 concentrations of  $7\alpha,25\text{-OHC}$  or to  $\text{TNF}\alpha$  (positive control). Thereafter, luciferase activity  
3 was measured. The increase in luciferase activity as compared with the negative controls  
4 (absence of stimulus) is represented. (B) The same experiment was performed with two  
5 different concentrations of  $7\alpha,25\text{-OHC}$ , and after having or not preincubated the cells with the  
6 *EBI2* antagonist NIBR 189. The increase in luciferase activity as compared with the negative  
7 controls (absence of stimulus) is represented.

8

1

2  
3  
4

5 **Figure 6. EBI2 expression increases HIV-1 R5 replication.** (A) EBI2 expression increases  
6 HIV-1 R5 replication in HOS cells. HOS cells transduced with *LacZ* (O) or *EBI2-EGFP* (■)

1 were exposed to an R5 HIV-1 strain and cultured. Viral production was monitored over  
2 time by measuring p24 concentration in the cell supernatant. (B) Effect of  $7\alpha,25$ -OHC on  
3 HIV-1 R5 infection of EBI2-expressing cells. HOS cells transduced with *LacZ* or *EBI2-EGFP*  
4 were exposed to an R5 HIV-1 strain and cultured in the presence of the indicated  
5 concentrations of  $7\alpha,25$ -OHC. Viral production was monitored by measuring p24  
6 concentration in the cell supernatant at day 7. The ratio p24 produced by EBI2-positive  
7 cells : p24 produced by *LacZ*-positive cells at day 7 postinfection is represented. (C) The  
8 effect of  $7\alpha,25$ -OHC on HIV-1 R5 infection of EBI2-expressing cells is inhibited by an EBI2  
9 antagonist. Same experiment as in (B) using 0.2mM  $7\alpha,25$ -OHC, with or without pre-  
10 incubation with the EBI2 antagonist NIBR189. (D) EBI2 expression increases HIV-1 R5  
11 replication in MT4 cells. MT4 cells transduced with *LacZ* (open histogram) or *EBI2-EGFP*  
12 (closed histogram) were exposed to an R5 HIV-1 strain and cultured. Seven days later,  
13 p24 concentration was measured in the cell supernatant. (E) PBMC, preincubated (---) or  
14 not (—) with the EBI2 antagonist NIBR189, were exposed to an R5 HIV-1 strain and  
15 cultured in presence or absence of the antagonist. Viral production was monitored over  
16 time by measuring p24 concentration in the cell supernatant. (F-H) One-round infection  
17 of *LacZ* (open box) or *EBI2* (closed box) -transduced HOS cells exposed to the indicated  
18 amount of defective HIV-1 virions pseudotyped with an R5 (F), X4 (G), or VSV-G (H)  
19 envelope and expressing luciferase. Luciferase activity was measured to quantitate HIV  
20 infection.