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Ig-like Transcript 7, but Not Bone Marrow Stromal Cell Antigen 2 (Also Known as HM1.24, Tetherin, or CD317), Modulates Plasmacytoid Dendritic Cell Function in Primary Human Blood Leukocytes

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The Ig-like transcript (ILT) 7 is a surface molecule selectively expressed by human plasmacytoid dendritic cells (pDCs). ILT7 cross-linking suppresses pDC activation and type I IFN (IFN-I) secretion following TLR7/9 engagement. The bone marrow stromal cell Ag 2 (BST2, aka HM1.24, tetherin, or CD317) is expressed by different cell types upon exposure to IFN-I and is a natural ligand for ILT7. In this study, we show that ILT7 expression decreased spontaneously in pDCs upon in vitro culture, which correlates with pDC differentiation measured as increased side scatter properties and CCR7 expression. TLR7/9 ligands, as well as HIV, induced BST2 upregulation on all tested cell types except T cells, which required TCR stimulation to respond to TLR9L-induced IFN-I. IFN-γ, IL-4, IL-10, and TNF-α had only marginal effects on BST2 expression in blood leukocytes compared with TLR9L. Preincubation with ILT7 cross-linking Ab inhibited IFN-I production in PBMCs treated with TLR7/9L or HIV, whereas BST2 blockade did not affect IFN-I responses even when BST2 upregulation was further boosted with TCR agonists or immunoregulatory cytokines. Our data indicate that BST2-mediated ILT7 cross-linking may act as a homeostatic regulatory mechanism on immature circulating pDC, rather than a negative feedback for activated mature pDCs that have downregulated ILT7.

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Plasmacytoid dendritic cells (pDCs) are a subpopulation of blood leukocytes that play a key role in the innate immune response against viral infections. Blood pDCs are precursors of the immature pDCs that patrol tissues and mucosal areas for the presence of pathogens, and that can mature into fully functional pDCs upon recognition of pathogen-associated molecular patterns (1, 2). pDCs express endosomal TLR7 and TLR9 (1, 2). TLR7 and TLR9 are respectively triggered by ssRNA and unmethylated CpG-rich DNA, which are characteristic of most viral genomes (2). Thus, pDCs are directly activated by the engulfed viral pathogens, and they produce large amounts of type I IFN (IFN-I; IFN-α and IFN-β) in response to viral stimuli (1). IFN-I exert antiviral activity by inducing intracellular restriction factors that interfere with viral replication (3), and by promoting apoptosis of potentially infected cells (4). Furthermore, IFN-I contribute to shape the adaptive immune response by promoting maturation of other APCs and favoring the differentiation of CD4 T cells toward a Th1 phenotype. Activated pDCs also express high levels of the tryptophan (Trp)-catabolizing enzyme IDO, which exerts powerful immunoregulatory activity and plays a critical role in the maintenance of immune tolerance (5).

The tight regulation of pDC responses is critical to allow the smooth transition from an innate immune response to an Ag-specific T cell–mediated immune response (1, 6). Studies conducted in murine models have shown that T cell responses to immunization can be enhanced if the TLR9 ligand (TLR9L) CpG oligodeoxynucleotide (ODN) is administered locally at the site of immunization (7), whereas Ag-specific T cell responses are inhibited by an IDO-dependent mechanism upon systemic administration of TLR9L in the same immunization setting (7). Furthermore, prolonged pDC stimulation with TLR9L or TLR7L had deleterious effects on lymphoid tissue architecture, lymphocyte populations, and both cell-mediated and humoral immune responses in mice (8, 9). In humans, dysregulated pDC activation contributes to suppress immune responses during chronic pathologic conditions, such as cancer and chronic infections (10–13). In particular, during pathogenic HIV infection, pDC activation is thought to contribute to several aspects of chronic immune activation and immune exhaustion, such as T cell apoptosis, dysfunction, and phenotypic activation (14–18); systemic diffusion of the infection via chemoattraction of CCR5+ CD4 T cells (19); and alteration of the Th17/regulatory T cell balance (18, 20, 21). Additionally, in nonhuman primate models of SIV infection, persistent upregulation of IFN-stimulated genes (ISGs) beyond the acute phase is observed only in pathogenic infection of nonnatural hosts (rhesus macaques), and not in nonpathogenic infection of...
natural host nonhuman primates (sooty mangabeys and African green monkeys) (22, 23). Thus, physiologic mechanisms that limit IFN-I production may be dysfunctional during HIV infection, and fail to drive the contraction of innate immune responses.

The Ig-like transcript (ILT) 7 (CD85g; LILRA4) was identified as a surface molecule selectively expressed by pDC (24). ILT7 is expressed in association with the FcεR1γ chain, and cross-linking of ILT7 results in a FcεR1γ-transduced circuit, involving Src and Syk kinases and activation of ITAM signaling, which limits pDC activation following TLR7 or TLR9 engagement (25). The bone marrow stromal cell Ag 2 (BST2, HM1.24, tetherin, CD317) has been recently identified as a natural ligand for ILT7 (26). BST2 is a homodimeric surface protein encoded by an ISG, and its expression can be induced in several different cell types (27, 28).

In vivo expression profiling studies have revealed that BST2 is expressed at different levels in specialized cells in a variety of human tissues, including hepatocytes, pneumocytes, plasma cells, monocytes, and vascular endothelium (29). BST2 is also known as tetherin, due to its ability to interfere with the release of enveloped viruses, such as HIV-1, by binding newly formed virions ( tethering action) and promoting their endocytosis and degradation in intracellular compartments (30, 31). In addition, mouse studies have shown that BST2 can be used as a target for Ag delivery to pDC, which can induce efficient T cell immunity after TLR stimulation (32). Engagement of ILT7 by BST2 has been suggested to suppress pDC activation (26). Thus, BST2 may play a critical role in the physiologic regulation of pDC-mediated IFN-I responses. Upon activation, pDCs produce high levels of IFN-I, which may induce BST2 upregulation in surrounding cells; BST2 may then interact with ILT7-expressing pDCs, causing downregulation of IFN-I, and return to the original resting condition.

We tested the dynamics of BST2 and ILT7 expression and regulation in primary human blood leukocytes and their role in modulating pDC activation. We found that ILT7 is rapidly downregulated in pDCs upon in vitro culture and differentiation, and that ILT7 cross-linking inhibits IFN-α production following stimulation with TLR9L or HIV. However, BST2 blockade did not enhance IFN-α responses in vitro, even when BST2 upregulation was induced in virtually all circulating leukocytes, raising doubts on the innate host nonhuman primates (sooty mangabeys and African green monkeys) (22, 23). Thus, physiologic mechanisms that limit IFN-I production may be dysfunctional during HIV infection, and fail to drive the contraction of innate immune responses.

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Materials and Methods

Leukocyte isolation and culture

Leukoreduction system chambers from healthy blood-bank donors were obtained from the North London Blood Transfusion Service. PBMCs were isolated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, Poole, U.K.) and cultured at 2 × 10⁶ cells/ml in RPMI 1640 (PAA Laboratories, Pasching, Austria), 10% FBS (Sigma-Aldrich), and 1% Pen-Strep-Glut (Sigma-Aldrich). Stimulation of PBMCs with TLR ligands, HIV, cytokines, and TCR agonist

PBMCs were cultured in presence or absence of specific stimuli for different periods of time, depending on the experimental setting, as described in Results. The TLR9 ligand (TLR9L) CpG ODN type A (Invivogen, San Diego, CA) was used at 0.75 μM final concentration. The TLR7 ligand (TLR7L) R848 (Imiquimod; Invivogen) was used at 5 μg/ml final concentration. HIV-1MN/CEMx174 was obtained from the AIDS and Cancer Research Laboratories, Pasching, Austria), 10% FBS (Sigma-Aldrich), and 1% Pen-Strep-Glut (Sigma-Aldrich).

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Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL). Pairwise comparisons between control and stimulated cells were performed using nonparametric Wilcoxon sign rank test. Changes in measured parameters over time in kinetic experiments were analyzed using Friedman’s two-way ANOVA by ranks, and pairwise comparisons were subjected to Dunn’s post hoc correction for multiple analyses. The p values <0.05 were considered statistically significant.

Results

ILT7 expression is limited to pDCs and is downregulated during in vitro culture

Expression of ILT7 has been reported to be strictly limited to pDCs (24). Our analysis of freshly isolated primary PBMCs from...
healthy donors confirmed the selective expression of ILT7 by pDCs (Fig. 1A, 1B). As expected, overnight culture resulted in partial loss of pDCs (Supplemental Fig. 1A) and reduction of BDCA2 expression (Supplemental Fig. 1A). ILT7 expression decreased progressively during in vitro culture, following a linear profile during the first 6 h of culture (R² = 0.9625; Fig. 1C), and was dramatically reduced after overnight culture (Fig. 1D). The decrease in ILT7 was observed both when the frequency of ILT7⁺ pDCs (Fig. 1C, 1D) and ILT7 mean fluorescence intensity (MFI) (Supplemental Fig. 1B, 1C) were considered. ILT7 downregulation appeared to be due to a generalized decrease of surface expression on all pDCs, rather than the selective reduction in one subpopulation of pDCs (Supplemental Fig. 1B). Stimulation with TLR9L or HIV did not prevent ILT7 downregulation, despite promoting pDC activation as measured by upregulation of the activation marker CD83 (Fig. 1D).

ILT7 downregulation is associated with morphologic and phenotypic changes consistent with differentiation of blood pDC precursors

In vitro culture results in spontaneous differentiation of pDC precursors into immature pDCs, which can be activated in presence of adequate stimulation. We tested whether ILT7 downregulation was associated with changes in the morphology and surface molecule expression pattern of pDCs. The reduction in ILT7 expression was associated with an increase in side scatter (SSC) properties of pDCs after overnight culture of unsorted PBMCs (Fig. 2), indicating augmented intracellular complexity, granularity, and dendritic morphology. TLR7L appeared to induce a more rapid downregulation of ILT7, which was expressed at significantly lower levels compared with untreated and TLR9L- and HIV-treated cells after 6-h culture. Conversely, TLR9L and HIV, but not TLR7L, induced a more rapid increase in SSC compared with media alone, as indicated by significantly higher SSC after 6-h culture. The change in pDC morphology occurred independent of whether PBMCs were cultured in media only or with TLR7/9L or HIV (Fig. 2). Furthermore, expression of the chemokine receptor CCR7, associated with homing to secondary lymphoid tissues such as lymph nodes, spontaneously increased in pDCs during in vitro culture (Fig. 2B) in media alone, and was further enhanced when PBMCs were cultured in presence of TLR7/9L or HIV (Fig. 2B). Conversely, a mild increase in the expression of the pDC activation marker CD83 was observed only transiently at 6-h culture in unstimulated PBMCs, whereas it was higher and persistent in pDCs from TLR7/9L- or HIV-treated PBMCs (Fig. 2). ILT7 downregulation was also associated with increased expression of the costimulatory molecules CD40, CD80, and CD86 in pDCs after overnight incubation with TLR9L, but not in media alone (Supplemental Fig. 1D, 1E).

These data collectively suggest that ILT7 downregulation is associated with a process of pDC differentiation, characterized by increased morphological complexity and CCR7 expression, but not with activation and full maturation, epitomized by increased expression of the activation marker CD83 and costimulatory molecules (CD80, CD86, and CD40), which occurred only following TLR stimulation.

**BST2 expression is upregulated following PBMC incubation with TLR7/9L, but is not sufficient to suppress IFN-α production via ILT7 cross-linking**

We tested the expression of BST2 on freshly isolated PBMCs and on leukocytes cultured overnight in presence or absence of TLR7/9L or HIV. TLR9L, TLR7L, and HIV directly activate pDC and induce IFN-α production, which is a known inducer of BST2 (28). Among freshly isolated PBMCs, only monocytes (CD14⁺) expressed constitutively high levels of BST2, intermediate levels were observed in myeloid DCs (mDCs) and B cells, whereas a minor portion of pDCs and T cells tested positive for BST2 (Fig. 3A, Supplementary Fig. 2A). In vitro overnight culture of PBMCs with TLR7L, TLR9L, or HIV induced different degrees of BST2 upregulation depending on the cell types analyzed (Fig. 3B, Supplementary Fig. 2B). Thus, pDCs became highly positive for BST2 when PBMCs were cultured with TLR7L, TLR9L, or HIV (Fig. 3B, Supplementary Fig. 2B). mDCs showed a reduction of

**FIGURE 1.** ILT7 expression is limited to pDCs and is downregulated following in vitro culture. (A) Flow cytometry dot plots showing the gating strategy used to identify pDC (CD14⁻ BDCA2⁺ CD123⁺), monocytes (CD14⁻), mDCs (CD19⁻ CD14⁻ CD3⁻ BDCA1/BDCA3⁺), B cells (CD14⁻ CD19⁻ CD3⁻), and T cells (CD14⁻ CD19⁻ CD3⁻). (B) Flow cytometry histograms showing ILT7 expression on different cell populations gated as in (A); thin lines indicated fluorescence-minus-one (FMO) controls, and thick lines indicate ILT7 staining; for both (A) and (B), one example of experiments performed on n = 6 independent donors is shown. (C) Frequency of ILT7⁺ pDCs in function of time; each dot represents median of n = 5 donors for a specific time point; lines indicate linear regressions of medians and IQRs. **p < 0.01 compared with 0 h (Friedman’s test with Dunn’s correction for multiple pairwise comparisons).** (D) Change in the frequency of ILT7⁺ pDCs (circles) and CD83⁺ pDCs (triangles) over time in presence or absence of the TLR9L Cpg ODN or HIV; medians and IQRs are shown (n = 3); p values below (D) indicate Friedman’s test results for changes over time in each condition.
BST2 expression after overnight culture in the absence of stimuli \((p = 0.0001; \text{compare Fig. 3A and 3B})\), but treatment of PBMCs with TLR7/9L or HIV resulted in a significant upregulation of BST2 on mDCs compared with untreated control (Fig. 3B, Supplemental Fig. 2B). B cells partially upregulated BST2 when PBMCs were treated with either TLR7/9L or HIV (Fig. 3B, Supplemental Fig. 2B). T cells showed minor alterations of BST2 expression, which tested significant only after stimulation with TLR7L or HIV (Fig. 3B, Supplemental Fig. 2B). Because the frequency of BST2+ monocytes approached 100\% even in the absence of stimulation \((p = 0.0001; \text{compare Fig. 3A and 3B})\), but treatment of PBMCs with TLR7/9L or HIV resulted in a significant upregulation of BST2 on mDCs compared with untreated control (Fig. 3B, Supplemental Fig. 2B). B cells partially upregulated BST2 when PBMCs were treated with either TLR7/9L or HIV (Fig. 3B, Supplemental Fig. 2B). 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As expected, BST2 upregulation was inhibited on all cell types when IFNAR2 was blocked by preincubation of PBMCs with 5 \(\mu\)g/ml anti-IFNAR2 Ab for 30 min before stimulation with TLR7/9L or HIV (data not shown). These data are consistent with the regulation of BST2 expression by IFN-\(\iota\)-produced by TLR7/9-stimulated pDCs, and corroborate the findings by Homann et al. and Bego et al. (35, 36) showing BST2 regulation by TLR agonists and IFN regulatory factor 7, respectively.

We tested the effect of one ILT7-specific (17G10.2) and one BST2-specific Ab (26F8) on TLR9L-induced IFN-\(\iota\) production in PBMCs. Clone 17G10.2 has been used in plate-bound form to cross-link ILT7 and suppress pDC activation (25), whereas 26F8 was reported to block BST2-ILT7 interaction (26). Based on the observed upregulation of BST2 on different cell types upon exposure of PBMCs to TLR7/9L and HIV, we expected 26F8 to increase IFN-\(\iota\) responses by inhibiting BST2-ILT7 interactions. Stimulation with TLR9L, TLR7L, and HIV induced statistically significant increases in IFN-\(\iota\) measured in culture supernatants after overnight culture (Fig. 3D). Preincubation (30 min before stimulation) with the ILT7 cross-linking Ab 17G10.2 significantly inhibited TLR9L- and HIV-induced IFN-\(\iota\); conversely, the BST2-blocking Ab 26F8 had no detectable effect on IFN-\(\iota\) production (Fig. 3D). The inhibitory effect of 17G10.2 was detectable already 6 h after stimulation of PBMCs with TLR9L, whereas 26F8 did not show any effect on TLR9L-induced IFN-\(\iota\) production at any of the time points considered (Fig. 3E). Both 26F8 and 17G10.2 are mouse IgG1 isotype, and no effect was observed when isotype control Abs were used (data not shown).

Because pDC activation leads to upregulation of the immunosuppressive enzyme IDO and increased catabolism of the essential

**FIGURE 2.** ILT7 downregulation is associated with pDC precursor differentiation. (A) Flow cytometry contour plots showing ILT7 expression in comparison with SSC properties (SSC; upper panels) and CD83 expression (lower panels) of pDCs in freshly isolated PBMCs (black contours in all plots) and in PBMCs cultured overnight (SSC, red contours; CD83, blue contours) in presence or absence of TLR9L, TLR7L, or HIV; one example of experiments performed on \(n = 6\) independent donors is shown. (B) Summary graphs showing (from top to bottom) frequency of ILT7+ pDCs, fold change in SSC, frequency of CCR7+ pDCs, and frequency of CD83+ pDCs in freshly isolated PBMCs and PBMCs cultured in control media alone or in presence of TLR9L, TLR7L, or HIV; SSC were normalized against measurements on fresh cells; medians and IQRs are shown \((n = 6)\); \(^p < 0.05\), \(^{**}p < 0.01\) (Friedman’s test with Dunn’s correction for multiple pairwise comparisons), \(^p < 0.05\) compared with untreated control at the same time point (Wilcoxon sign rank test).
amino acid Trp into the Kyn pathways (5, 12, 20), we tested whether ILT7 cross-linking or BST2 blockade influenced TLR7/9L- and HIV-induced IDO activity. The ratio between Kyn and Trp (Kyn:Trp), a well-accepted marker of IDO activity (34), was measured by HPLC in culture supernatants. Similar to IFN-α, TLR9L, TLR7L, and HIV all induced statistically significant increases in Kyn:Trp (Fig. 3F). Interestingly, cross-linking of ILT7 by preincubation with 17G10.2 did not reduce TLR9/7L-induced Kyn:Trp, but resulted in a statistically significant inhibition of HIV-induced IDO activity.

No effect on IFN-α production or IDO activity was observed after preincubation with 26F8 even when the Ab was used at concentrations up to 10 μg/ml.

The complete lack of biologic effect of 26F8 prompted us to test its reactivity with the extracellular portion of BST2 in a controlled in vitro system. We used fluorescently labeled (PE) 26F8 to detect BST2 expression by flow cytometry on 293T cells, which were transfected or not with wild-type BST2 or BST2-bearing specific mutations, as well as mock-transfected 293T cells, which were treated with rIFN-α. Efficient staining was observed using as little as 3 μg/ml 26F8-PE in rIFN-α–treated 293T cells and 293T cells transfected with wild-type BST2 (BST2WT), but not in mock-transfected untreated cells (Fig. 3G). Furthermore, 26F8-PE efficiently stained 293T cells transfected with BST2 10-12A and BST2Y6,8A, which bear mutations in the intracellular region of BST2, as well as BST2ΔGPI, which lacks the extracellular membrane anchor (Fig. 3G). The 293T cells transfected with monomorphic BST2CA also stained positive for 26F8-PE, albeit showing reduced staining on a per cell basis as measured by MFI. Finally, BST2F123P mutated in the coiled-coil extracellular region, pre-
sumably the region responsible for ILT7 binding, showed no re-
activity whatsoever with 26F8-PE.

Collectively, these data indicate that BST2 blockade does not affect pDC activation in unsorted PBMC cultures stimulated with either TLR7/9L or HIV, and that this ineffectiveness is not due to lack of reactivity of the 26F8 Ab with the extracellular portion of BST2.

**TLR9L-induced BST2 expression on T cells, but is not sufficient to suppress IFN-α production via ILT7 cross-linking**

To test whether BST2 expression on T cells could be upregulated following direct TCR-dependent stimulation, we cultured PBMCs with stimulating anti-CD3 Ab (HIT3a) in presence or absence of TLR9L. When used alone, TLR9L and HIT3a induced a limited increase in BST2 expression on CD4 and CD8 T cells, which tested statistically significant only for CD4 T cells (Fig. 4A). Interestingly, when TLR9L and HIT3a were used together, we observed a significant upregulation of BST2 expression by both CD4 and CD8 T cells, indicating a synergistic effect of TCR stimulation and TLR7/9L-induced IFN-I signaling (Fig. 4A). Activating Ab specific for CD28 (CD28.2), used as control, did not synergize with TLR9L.

We investigated the mechanism responsible for the synergy between HIT3a and TLR9L. We first tested whether PBMC stimulation with HIT3a increased the expression of IFNAR2 on T cells, and found no increase in the receptor’s expression following TLR9L stimulation with HIT3a increased the expression of IFNAR2 on T cells, which tested statistically significant only for CD4 T cells (Fig. 4A). Interestingly, when TLR9L and HIT3a were used together, we observed a significant upregulation of BST2 expression by both CD4 and CD8 T cells, indicating a synergistic effect of TCR stimulation and TLR7/9L-induced IFN-I signaling (Fig. 4A). Activating Ab specific for CD28 (CD28.2), used as control, did not synergize with TLR9L.

Because incubation of PBMCs with TLR9L and TCR-activating HIT3a Abs induced high BST2 expression on T cells (Fig. 4C), we tested whether TLR9L-induced IFN-α production may be inhibited in these conditions. PBMCs were cultured overnight with different combinations of HIT3a, TLR9L, and 26F8 (CD28.2 was used as a control for HIT3a), and IFN-α was measured in culture supernatants by ELISA. Reduction of TLR9L-induced IFN-α was observed in any condition analyzed (Fig. 4C), suggesting that, even under conditions of maximum BST2 expression, T lymphocytes do not downregulate pDC activation by engaging ILT7.

**IL-10 downregulates IFN-α production in response to TLR9L in a BST2/ILT7-independent manner**

We investigated the effect of a panel of cytokines on BST2 expression on different cell types and their effect on TLR9L-induced BST upregulation. We chose IFN-γ, IL-4, IL-10, and TNF-α as examples of Th1, Th2, immunosuppressive, and proinflammatory cytokines, respectively. PBMCs from healthy donors were cultured overnight in presence or absence of TLR9L and/or stimulating CD3-specific Ab (HIT3a), and pretreated or not with blocking Abs against IFNAR2 and/or against IFNGR1; each symbol represents results from one independent donor (n = 3) for both CD4 and CD8 T cells (empty and solid symbols, respectively). (C) IFN-α was measured by ELISA in supernatants from PBMCs cultured overnight in presence or absence of different combinations of TLR9L, HIT3a, and CD28.2, pretreated or not with the BST2-specific blocking Ab 26F8; medians and IQRs of experiments from n = 6 independent donors are shown. In (A) and (C), *p < 0.05, **p < 0.01 compared with control.

**FIGURE 4.** Effect of TCR engagement on BST2 expression in T cells and effect on TLR9L-induced IFN-α production. (A) Frequencies of BST2+ CD4 and CD8 T cells among PBMCs cultured overnight in presence or absence of different combinations of TLR9L, stimulating CD3-specific Ab (HIT3a), and stimulating CD28-specific Ab (CD28.2); medians and IQRs of experiments from n = 6 independent donors are shown. (B) Frequencies of BST2+ CD4 and CD8 T cells among PBMCs cultured overnight in presence or absence of TLR9L and/or stimulating CD3-specific Ab (HIT3a) and pretreated or not with blocking Abs against αIFNAR2 and/or against αIFNGR1; each symbol represents results from one independent donor (n = 3) for both CD4 and CD8 T cells (empty and solid symbols, respectively). (C) IFN-α was measured by ELISA in supernatants from PBMCs cultured overnight in presence or absence of different combinations of TLR9L, HIT3a, and CD28.2, pretreated or not with the BST2-specific blocking Ab 26F8; medians and IQRs of experiments from n = 6 independent donors are shown. In (A) and (C), *p < 0.05, **p < 0.01 compared with control.
IL-4, and TNF-α (~50, 40, and 10%, respectively; Fig. 5B). Unexpectedly, preincubation with IL-10 further enhanced the positive effect of TLR9L on BST2 upregulation on monocytes (Fig. 5B). Preincubation of PBMCs with any of the cytokines tested did not affect BST2 upregulation in other cell types after stimulation of PBMCs with TLR9L (Fig. 5B).

Because we observed an enhancement of TLR9L-mediated BST2 upregulation on monocytes (Fig. 5B), we tested whether BST2/ILT7-mediated suppression of IFN-α production would be favored in presence of IL-10. Stimulation with TLR9L induced significant IFN-α production in untreated PBMCs or PBMCs that were pretreated with IFN-γ, IL-4, or TNF-α, but not IL-10 (Fig. 5C). In addition, IFN-α production in response to TLR9L was significantly lower in cells pretreated with IL-10 or TNF-α compared with untreated cells, but the defects were not corrected by preincubation with 26F8 (Fig. 5C). These data indicated that both the immunosuppressive cytokine IL-10 and the proinflammatory TNF-α exert inhibitory activity on IFN-α production by TLR9L-stimulated pDCs, but this inhibitory activity is not mediated by BST2-ILT7 interaction.

**Discussion**

The critical function played by pDCs in the early phases of antiviral immune responses bears the risk of an excessive and uncontrolled activation of IFN-I and IDO and subsequent immune dysregulation. Persistent pDC overactivation has been shown to have deleterious effects on the immune function in murine models (8, 9), and is thought to be a key contributor to the immunopathogenesis of HIV infection (11). ILT7 is a pDC-specific surface receptor that, when cross-linked, exerts potent inhibitory activity on pDC activation (25). The only known natural ligand for ILT7 is the IFN-I–regulated surface protein BST2, better known for its ability to prevent the release of HIV particles from infected cells, which is counteracted by the HIV-1 accessory protein Vpu (26, 31). It has been hypothesized that, upon production of IFN-I by pDCs, surrounding cells may upregulate BST2, which may in turn suppress pDC activation by cross-linking ILT7, providing the negative feedback necessary to prevent potentially deleterious pDC overactivation (26, 27).

Our data collectively argue against a role for BST2 in regulating pDC activity, particularly IFN-α production. Thus, we were unable to enhance pDC responses by blocking BST2 using the 26F8 Ab. This same Ab has been used to successfully inhibit BST2/ILT7 interactions in other experimental settings, such as ILT7-reporter systems (26), but not in unsorted human PBMCs, which represent a more physiologically relevant setting. Conversely, our data indicate that preincubation with the ILT7 cross-linking Ab 17G10.2 exerted inhibitory activity on TLR9L- and HIV-induced IFN-α production, as well as HIV-induced IDO activity. One possible explanation for the lack of biologic effect of 26F8 may be the incomplete blocking of BST2. However, in BST2-transfected HEK 293T cell lines, fluorescently labeled 26F8 stained 96% of
cells, indicating efficient binding to the vast majority of expressed protein. Furthermore, by using HEK 293T cells transfected with a panel of BST2 mutants, we confirmed that 26F8 binds to the extracellular coiled-coil region of the molecule, which is most likely the region of interaction with ILT7.

An alternative explanation for the inability of 26F8 to enhance IFN-α responses is that BST2 expression levels in PBMCs are insufficient to cause ILT7 cross-linking. Ligand-receptor interaction involving pDCs are more likely to occur either in trans with T cells or in cis within the pDC itself, rather than with other APCs. However, we found that only monocytes, among freshly isolated PBMCs, express constitutively high levels of BST2. Upon stimulation of IFN-α production with TLR7/9L or HIV, all cell types analyzed showed upregulation of BST2 to high levels, with the exception of T cells. Conversely, T cells required direct TCR engagement and subsequent IFN-γ production in addition to TLR9L-induced IFN-α to achieve maximum BST2 expression. However, even in conditions in which BST2 expression was induced in virtually all major cell types, we were unable to enhance IFN-α responses using the BST2-blocking Ab 26F8.

Because innate immune responses may be modulated by secreted cytokines present in the environment, we tested whether BST2-mediated inhibition of pDC activation may rely on secondary signals provided by proinflammatory or immunoregulatory cytokines. We found that only IL-10 and, to a much lesser extent, TNF-α exerted inhibitory activity on TLR9L-induced IFN-α production. This effect was consistent with both the well-known anti-inflammatory activity of IL-10 (37–39) and our surprising observation that IL-10 enhanced TLR9L-induced upregulation of BST2 on monocytes. However, BST2 blockade with 26F8 was ineffective at either improving or fully restoring IL-10–inhibited IFN-α responses. The lack of BST2-mediated pDC regulation in this setting is also supported by the fact that IFN-γ inhibited BST2 expression on monocytes, whereas IL-4 inhibited BST2 expression on both monocytes and mDCs. However, neither IFN-γ nor IL-4 affected TLR9L-induced IFN-α production. Finally, TNF-α inhibited IFN-α production, albeit very mildly, despite causing a slight reduction in BST2 expression in monocytes.

We cannot exclude that ILT7/BST2 interactions occur in vivo, possibly in lymphoid tissues, and that such interactions are simply not reproducible in culture in vitro. However, our data showed that ILT7, albeit exclusively expressed by pDCs among freshly isolated PBMCs, is rapidly downregulated upon in vivo culture, independently of stimulation with HIV or TLR7/9L. Our results only partially resemble the decrease in ILT7 expression observed by Cao et al. (25), who described a slow reduction in ILT7 MFI in human pDCs, even in presence of IL-3 or TLR9L, but retention of a large population of ILT7+ pDCs after up to 72 h in vitro culture. Conversely, we found that ILT7 downregulation is extremely rapid, and results in a pDC population that is homogeneously negative for ILT7, independent of whether pDC were activated or not. The reasons for this discrepancy remain unclear. However, our data suggest that ILT7 downregulation is not a consequence of pDC activation, but rather a spontaneous change occurring during blood pDC differentiation. Thus, circulating blood pDCs are in an immature state and have the potential to differentiate into mature pDCs with full Ag-presenting function that can be found in lymphoid tissues (40, 41). When cultured in vitro, freshly isolated blood pDCs differentiate into immature pDCs, which can then mature into fully competent APCs in response to adequate stimulation (1, 2, 40, 41). Furthermore, pDCs can directly migrate from blood to inflamed lymph nodes through high endothelial venules (42). The observed downregulation of ILT7 appears to be part of the blood pDC differentiation process and was indeed accompanied by an increase in the cellular morphological complexity, epitomized by augmented SSC properties, and by increased expression of the lymph node homing marker CCR7. Conversely, expression of the activation marker CD83 showed only a modest and transient increase after 6 h of culture in media alone, and returned to the original level unless the pDC-activating stimuli TLR7/9L or HIV were added to the culture. Thus, the differentiation of freshly isolated blood pDCs in vitro appears to occur in two phases, as follows: an initial spontaneous step characterized by a morphological change, expression of homing receptors and ILT7 downregulation, and a second step triggered by TLR7/9 stimulation, which promotes full maturation and IFN-α production. In light of these findings, it is tempting to speculate that ILT7 may act as a control mechanism to prevent or limit the activation of immature pDCs in the peripheral blood, rather than as a negative feedback on mature activated pDCs.

Benlahrech et al. (43) have recently shown that ILT7 expression is reduced in pDCs from HIV-infected patients when viral replication is not efficiently controlled by therapy. Based on our findings, it is possible that the downregulation of ILT7 observed during HIV infection is symptomatic of partial or incomplete pDC differentiation due to chronic stimulation. Consistent with this hypothesis, Sabado et al. (44) reported that pDC and mDC relocation to lymphoid tissues may occur already during primary HIV infection, and persists throughout the course of disease. However, reports on IFN-α secretion by pDCs in lymphoid tissues during chronic HIV infection showed contrasting results. For example, although IFN-α and upregulation of ISGs have been reported in tissues from HIV+ patients (45, 46), Nascimbeni et al. (47) showed that pDCs in the spleen of HIV-infected patients have an immature phenotype and do not contribute to the increased IFN-α production. The question may be raised as to whether ILT7 downregulation may contribute to chronic pDC activation during HIV infection by allowing a lower activation threshold or an enhanced response to chronic stimulation.

A number of questions arise and remain to be answered. Further studies are required to test whether ILT7 expression varies among pDCs in different anatomic locations, including mucosal and lymphoid tissues. Also, the role of BST2 in regulating pDC activation via ILT7 remains unclear, and it is possible that other unidentified ligands promote ILT7 cross-linking on circulating pDCs. Finally, the potential contribution of the ILT7 impairment to chronic viral infections or inflammatory disease, as well as the exploitation of this regulatory system for therapeutic purposes, needs to be further evaluated.

Disclosures
The authors have no financial conflicts of interest.

References
ILT7 and BST2 in PRIMARY BLOOD LEUKOCYTES


