Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction

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Plasmacytoid dendritic cells (pDCs) produce copious type I interferon (IFN) upon sensing nucleic acids through Toll-like receptor (TLR) 7 and TLR9. Uncontrolled pDC activation and IFN production are implicated in lymphopenia and autoimmune diseases; therefore, a mechanism controlling pDC IFN production is essential. Human pDCs specifically express an orphan receptor, immunoglobulin-like transcript 7 (ILT7). Here, we discovered an ILT7 ligand expressed by human cell lines and identified it as bone marrow stromal cell antigen 2 (BST2; CD317). BST2 directly binds to purified ILT7 protein, initiates signaling via the ILT7–FcεRIγ complex, and strongly inhibits production of IFN and proinflammatory cytokines by pDCs. Readily induced by IFN and other proinflammatory cytokines, BST2 may modulate the human pDC’s IFN responses through ILT7 in a negative feedback fashion.

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In humans and other mammals, plasmacytoid dendritic cells (pDCs) are specialized immune cells that selectively express Toll-like receptor (TLR) 7 and TLR9, which are key endosomal sensors of microbial and self-RNA or DNA, respectively (Jarrossay et al., 2001; Kadowaki et al., 2001; Liu, 2005; Gilliet et al., 2008). Activation of TLR7 or TLR9 by nucleic acids in pDCs triggers signal transduction, leading to rapid and robust secretion of type I IFN, inflammatory cytokines, and chemokines (Colonna et al., 2004; Honda and Taniguchi, 2006; Kawai and Akira, 2006; Piqueras et al., 2008). Activation of TLR7 or TLR9 by nucleic acids in pDCs triggers signal transduction, leading to rapid and robust secretion of type I IFN, inflammatory cytokines, and chemokines (Colonna et al., 2004; Honda and Taniguchi, 2006; Kawai and Akira, 2006; Piqueras et al., 2008). The TLR-induced IFN response is regulated by several immunoreceptor tyrosine-based activation motif (ITAM)-bearing signaling receptors on pDCs (Novak et al., 2004; Fuchs et al., 2005; Blasius et al., 2006a; Cao et al., 2006, 2007; Röck et al., 2007; Cho et al., 2008; Gilliet et al., 2008). One such receptor is ILT7, a member of the Ig-like transcript (ILT) family (also known as leukocyte Ig-like receptors) found in humans and primates (Brown et al., 2004).

ILTs, which are expressed by a variety of immune cell types, are comprised of a group of inhibitory receptors bearing immunoreceptor tyrosine-based inhibitory motifs and a group of stimulatory receptors that signal through their association with adaptor molecules containing ITAM (Brown et al., 2004). ILT7, also known as LILRA4 and CD85g, contains four extracellular Ig-like domains and a positively charged residue within the transmembrane region, allowing ILT7 to form a receptor complex with a signaling adaptor FcεRIγ. Uniquely expressed by human pDCs, ILT7 suppresses TLR7/9-induced type I IFN secretion by pDCs when cross-linked by anti-ILT7 mAbs that trigger signaling activation (Cao et al., 2006; Brown et al., 2004).

Abbreviations used: BST2, bone marrow stromal cell antigen 2; ILT, Ig-like transcript; ITAM, immunoreceptor tyrosine-based activation motif; MOI, multiplicity of infection; pDC, plasmacytoid DC; TLR, Toll-like receptor.
RESULTS
Human cancer cells express novel ILT7 ligand
Previously, we constructed an NFAT-GFP reporter cell line expressing ILT7 and FcεRIγ that expresses GFP in response to ILT7 surface ligation (Cao et al., 2006). To detect the presence of ILT7 ligands (ILT7-L), we used these cells to screen virus-infected cells and a large panel of human tumor cell lines. Co-culture of the ILT7 reporter cells with human breast carcinoma MDA-MB-468, MCF7, and T47D cells, but not with MDA-MB-231 or ZR-75-1 cells, induced GFP expression by the reporter cells (Fig. 1 A). We further tested several human ovarian, colon, melanoma, glioma, and lung cancer cell lines and found that two melanoma lines, WM35 and Me938, were able to similarly activate the ILT7 reporter cells (Fig. 1 B). Other transformed cell lines, such as HEK293, Vero, CHO, Cos7, and Jurkat, were unable to stimulate the ILT7 reporter cells (unpublished data).

As the breast cancer line T47D is most potent in triggering ILT7, we further characterized these cells. The ability of T47D cells to activate the reporter cells is ILT7 dependent because NFAT-GFP reporter cells expressing only the signaling adaptor FcεRIγ, and not ILT7, were not activated (Fig. 1 A). In addition, the induction of GFP was completely abolished by a neutralizing anti-ILT7 mAb (Fig. 1 C). Pretreatment with IFN-α and TNF-α enhanced the ability of the breast cancer MDA-MB-468 cells to activate the ILT7 reporters (Fig. 1 D), suggesting that ILT7-L expression is regulated by immune responses.

ILT7 activation by cancer cells requires direct cell–cell contact as cells cultured in separate chambers of a transwell dish fail to induce GFP (unpublished data). Although the two other ILT family members, ILT2 and ILT4, bind to the classical and nonclassical MHC class I ligands (MHC-I) ligands (Navarro et al., 1999; Brown et al., 2004), the putative ILT7-L appears to be unrelated to MHC class I or class II because antibodies against human MHC class I or class II did not block the ability of T47D cells to activate the ILT7 reporter cells (unpublished data), and both the MHC class I–expressing cell lines (e.g., Jurkat and MDA-MB-231) and the MHC class II–expressing cell lines (e.g., EBV-transformed B cells) were unable to activate ILT7 reporter cells (unpublished data).

Identification of ILT7-L
To facilitate the identification of the putative ILT7-L, we immunized mice with T47D (ILT7-Lneg) and MDA-MB-231 (ILT7-Lpos) cells and obtained multiple hybridoma clones that bound specifically to T47D cells, but not to MDA-MB-231 cells. To identify mAbs that specifically recognize ILT7-L, we further screened the hybridoma clones for the ability to block T47D-induced ILT7 reporter cell activation. Two such clones (26F8 and 28G4) were identified (Fig. 2 A).

Using flow cytometry, we found that these two mAbs stained the breast tumor cell lines MDA-MB-468, MCF7, and T47D, which activated ILT7 reporter cells, but minimally reacted with breast tumor cell lines MDA-MB-231 and ZR-75-1, which failed to activate ILT7 reporters (Fig. 2 B). Other cell lines (e.g., HEK293, Vero, CHO, and Cos7) that failed to activate the ILT7 reporter cells were not stained by either 26F8 or 28G4 (unpublished data). In addition, these two mAbs, but not the isotype-matched control mAb, strongly inhibited the ability of the tumor cell line T47D to activate the ILT7 reporter cells in a dose-dependent fashion (Fig. 2 C). The two mAbs recognize nonoverlapping epitopes, because one did not block the binding of the other to T47D cells, as determined by flow cytometry (unpublished data). Nevertheless, 26F8 and 28G4 mAbs immunoprecipitated three similar protein bands in T47D cells, but not in MDA-MB-231 cells (Fig. 2 D), suggesting the presence of cellular proteins as the potential ligand for ILT7.

To identify the ligand for ILT7, 26F8 and 28G4 mAbs were used to screen a human cDNA library. Both antibodies specifically recognized the gene product of human BST2 (CD317). BST2 is a 180-aa glycoprotein that was initially identified as a membrane protein expressed by bone marrow stromal cells and later shown to be expressed by plasma cells and multiple types of cancer cells (Ohtomo et al., 1999; Kupzig, 2003; Walter-Yohrling et al., 2003). BST2 is reportedly expressed on many different types of cells after exposure to IFN-α (Blasius et al., 2006b; Neil et al., 2008; Van Damme et al., 2008).

BST2 is an ILT7 ligand
The 26F8 and 28G4 mAbs stained the human BST2 cDNA-transfected HEK293 cells, but not mock-transfected cells, by flow cytometry (Fig. 3 A). In addition, recombinant ILT7 protein directly bound to a recombinant BST2-GST fusion protein, but not GST protein, in a dose-dependent manner (Fig. 3 B). Specific interaction between recombinant BST2-Fc and ILT7-Fc with an estimated affinity of $\sim 10^{-6}$ M was detected by surface plasma resonance with Biacore, an interaction that was completely neutralized by 26F8 mAb (Fig. 3 C).

Furthermore, rBST2-GST fusion protein, but not GST alone, strongly activated the ILT7 reporter cells (Fig. 4 A). The specificity of the BST2–ILT7 interaction was demonstrated by the findings that rBST2-GST failed to induce GFP expression in ILT7-negative reporter cells and that BST2-induced GFP expression in the ILT7 reporter cells was abrogated by
activate the ILT7 reporter was blocked by neutralizing antibodies against either ILT7 or BST2 (Fig. 4B). These data identify BST2 as a physiological ligand that specifically binds and activates ILT7.

Figure 1. Human tumor cell lines express a potential ligand for ILT7. (A) Five human breast cancer cell lines were co-cultured with either ILT7+ NFAT-GFP reporter cells or parental NFAT-GFP reporter cells. The percentages of GFP-positive reporter cells were analyzed. Data are representative of four independent experiments. (B) Human carcinoma cells were co-cultured with ILT7+ NFAT-GFP reporter cells. The percentages of GFP-positive reporter cells were analyzed. The categories of the cancer lines are as follows: OVCAR-3, SKOV-3, and DOV-13 (ovarian); HT-29 and HCT-116 (colon); WM35, WM239, MEL526, MEL624, MEL888, and MEL938 (melanoma); U87, LN229, and SNB19 (glioblastoma); A549 (lung cancer). Data are representative of two independent experiments. (C) T47D cells were co-cultured with ILT7+ NFAT-GFP reporter cells in the presence of 1 µg/ml of control IgG1 or anti-ILT7 mAb. The percentages of GFP-positive reporter cells were plotted. Data are representative of four independent experiments. (D) Breast cancer MDA-MB-468 cells were first cultured for 5 d in the presence of medium, 5 ng/ml of TNF-α or 500 U/ml of IFN-α, and then co-cultured with NFAT-GFP reporter cells. The percentages of GFP-positive reporter cells were analyzed. Data are representative of four independent experiments. Error bars represent the mean ± the SEM.
BST2 negatively regulates the innate immune functions of human pDCs

As previously shown, antibody cross-linking of ILT7 can induce prominent calcium influx in primary pDCs as a result of ITAM-mediated FceRIγy signaling (Cao et al., 2006; Cho et al., 2008). Similarly, a rBST2-Fc protein, but not a control Fc protein alone, induced calcium mobilization in human pDCs, which depends on the function of Syk (Fig. 5 A).

Because mAb cross-linking of ILT7 inhibits the immune response of pDCs, we investigated the effect of BST2 on TLR-induced cytokine responses by pDCs. Freshly isolated pDCs from human peripheral blood were preincubated with plate-bound rBST2-Fc protein or plate-bound Fc protein for 30 min, and were then challenged with influenza virus (Flu) or CpG, which trigger TLR7 and TLR9, respectively. rBST2 protein suppressed the secretion of IFN-α and TNF-α (Fig. 5 B), as well as the transcription of type I IFN subtypes, including IFNα1, IFNα4, IFNα8, and IFN-β, plus IL-6, by pDCs (Fig. 5 C) when activated by TLR agonists. In contrast, BST2 did not alter expression of co-stimulatory molecules, such as CD80 and CD86, by pDCs (Fig. 5, C and D). The effect of BST2-mediated IFN suppression was abolished when ILT7 was neutralized with anti-ILT7 mAb, but not with control IgG1, indicating a direct engagement between ILT7 and rBST2 (Fig. 5 E). Lastly, pDCs co-cultured with HEK293 cells expressing an HA-tagged BST2 secreted reduced levels of IFN-α in response to Flu virus when compared with pDCs in contact with untransfected HEK293 cells (Fig. 5 F), suggesting that the BST2–ILT7 interaction modulates pDCs’ TLR-induced IFN responses.

BST2 expression by human cells and by pDCs

To understand the prevalence of BST2 expression on human cells in response to IFN treatment, we cultured embryonic...
kidney HEK293 cells, dermal fibroblast line NHDF cells, human umbilical vein endothelial line HUVEC cells, and keratinocyte line HaCat cells in the absence or presence of IFN-α, and then analyzed BST2 surface expression by flow cytometry (Fig. 6 A). Although these cells minimally express BST2 under normal conditions, IFN-α treatment resulted in significant surface BST2 expression in all of them, which is consistent with the reported IFN-mediated BST2 induction (Blasius et al., 2006b; Neil et al., 2008; Van Damme et al., 2008).

Interestingly, resting mouse pDCs prominently express BST2 (Blasius et al., 2006b), but lack the expression of a direct orthologue of ILT7 (Brown et al., 2004). However, low amounts of surface BST2 were detected on human pDCs that were freshly isolated or cultured with either TLR ligands or cytokines (Fig. 6 B), although BST2 transcripts were found elevated in TLR-activated pDCs (unpublished data). To investigate a potential cis interaction between ILT7 and BST2, we transduced ILT7 and FcεRIγ into a human Burkitt’s lymphoma cell line expressing endogenous BST2. Interestingly, expression of the ILT7–FcεRIγ complex, but not BDCA2–FcεRIγ, another human pDC-specific receptor complex, reduced the levels of surface BST2 (Fig. 6 C). Thus, ILT7–BST2 cis interaction likely results in BST2 internalization and might have functional consequences in human pDCs.

**DISCUSSION**

Here, we identify BST2 as a physiological ligand for a human pDC-specific receptor ILT7. pDCs play a critical role in antiviral innate immune responses by secreting large quantities of IFN-α/β. However, the type 1 IFN responses immediately after viral infection are short lived; if not, massive and prolonged IFN exposure will damage hematopoiesis, leading to lymphopenia (Lin et al., 1998; Kamphuis et al., 2006), and increase the risk of autoimmunity (Gota and Calabrese, 2003). Hence, a mechanism ensuring a specific and transient IFN response to viruses is critical to minimize the possibility of lymphopenia and autoimmune diseases in the host. As BST2 is well known to be robustly induced on the surface of various types of cells after exposure to IFN and other proinflammatory cytokines via STAT activation (Ohtomo et al., 1999; Blasius et al., 2006b; Neil et al., 2008; Van Damme et al., 2008), the BST2–ILT7 interaction, therefore, likely serves as an important negative feedback mechanism for preventing prolonged IFN production after viral infection (Fig. 7).

Intracellular TLRs have limited ability to discriminate host versus foreign nucleic acids (Haas et al., 2008). Several host factors, including anti-DNA antibodies, antimicrobial

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**Figure 3. BST2 directly binds to ILT7.** (A) HEK293 cells transiently transfected with mock control or BST2 cDNA were analyzed by Western blotting for BST2 protein expression. Transfected cells were stained with 26F8 mAb and analyzed by flow cytometry. The staining profile with IgG isotype-matched control mAb is shown in the shaded area. Staining with 28G4 mAb produced identical results (not depicted). Data are representative of five independent experiments. (B) Plate-coated GST or BST2-GST were incubated with different concentrations of recombinant ILT7-Fc and HRP-conjugated anti–human Fc. Shown is absorption at OD 450 nm for each sample after addition of Tetramethyl benzidine (TMB) substrate. Data are representative of three independent experiments. (C, top) Recombinant BST2-Fc, precoated on the surface of Biacore sensor chips, was mixed with different concentrations of injected recombinant ILT7-Fc. The kinetic response data after subtracting the value from precoated Fc are shown. Also shown are control responses with Fc alone or buffer. (bottom) Precoated recombinant BST2-Fc was premixed with buffer, control IgG1, or anti-BST2 mAb 26F8 and then exposed to injected recombinant ILT7-Fc. The kinetic response data after subtracting the value from precoated Fc are shown. Data are representative of two independent experiments.
peptide LL37, or the nuclear DNA-binding protein HMGB1, alone or in combination, facilitate entry of self-DNA into the endosomes of pDCs, where they trigger TLR9 to induce type 1 IFN responses (Lande et al., 2007; Marshak-Rothstein and Rifkin, 2007; Tian et al., 2007). Similarly, autoantibody–self small nuclear ribonucleoprotein complexes can activate TLR7 through FcγRII to induce IFN (Vollmer et al., 2005; Savarese et al., 2006). This might lead to the constitutive activation of pDCs, which contributes to the autoimmune pathology of systemic lupus erythematosus and psoriasis. It will be of further interest to study if the BST2–ILT7–mediated controlling mechanism is breached in patients with systemic lupus erythematosus and psoriasis (Blanco et al., 2001; Lande et al., 2007; Marshak-Rothstein and Rifkin, 2007), which may provide an opportunity to develop therapeutics to down-modulate pDC activation during autoimmune disease.

Interestingly, BST2 represents the first non–MHC class I–type ligand for a member of the ILT receptor family (Brown et al., 2004). By sequence analysis, human receptor ILTs are divided into two separate groups—five inhibitory ILTs containing intracellular immunoreceptor tyrosine-based inhibitory motifs and six stimulatory ILTs that would couple with ITAM-bearing adaptors. The inhibitory ILTs, e.g., ILT2 and ILT4, recognize proteins encoded by many MHC class I alleles and UL18, which is a MHC-like molecule associated with human cytomegalovirus (Chapman et al., 1999). However, the ligands for the stimulatory group of ILTs are largely unknown (Cosman et al., 1997; Brown et al., 2004). Structural analysis revealed significant differences in MHC class I–binding sites between the inhibitory and stimulatory ILTs (Shiroishi et al., 2006), suggesting distinct ligand recognition by the two groups of ILTs. Here, we used a NFAT–GFP reporter cell system that reliably senses ILT7 surface ligation in the presence of an ILT7 ligand, which led to the positive identification of BST2, a glycoprotein unrelated to the MHC molecules. BST2 protein has a unique topology, containing an N-terminal transmembrane domain and a C-terminal GPI anchor, and locates within the lipid raft microdomains on plasma membrane (Kupzig, 2003). Further structural analysis of the binding interface between BST2 and ILT7 may shed light on the structural basis of ligand recognition by ILT7 and stimulatory ILTs. The receptor–NFAT reporter system should be also useful for the identification of the relevant ligands for other ILT orphan receptors and a better understanding of ILT’s biological function.

As the innate immune system has widely adopted “paired receptors” to exert tight regulatory control, many of the inhibitory receptors participate in self tolerance by recognizing ubiquitously expressed endogenous molecules, whereas stimulatory receptors may recognize “alert” molecules that are up-regulated after viral infection or immune activation (Lanier, 2008; Yamada and McVicar, 2008). For example, Ly49A, a prototype inhibitory Ly49 receptor expressed on mouse NK cells, recognizes endogenous MHC-I molecule peptide LL37, or the nuclear DNA-binding protein HMGB1, alone or in combination, facilitate entry of self-DNA into the endosomes of pDCs, where they trigger TLR9 to induce type 1 IFN responses (Lande et al., 2007; Marshak-Rothstein and Rifkin, 2007; Tian et al., 2007). Similarly, autoantibody–self small nuclear ribonucleoprotein complexes can activate TLR7 through FcγRII to induce IFN (Vollmer et al., 2005; Savarese et al., 2006). This might lead to the constitutive activation of pDCs, which contributes to the autoimmune pathology of systemic lupus erythematosus and psoriasis. It will be of further interest to study if the BST2–ILT7–mediated controlling mechanism is breached in patients with systemic lupus erythematosus and psoriasis (Blanco et al., 2001; Lande et al., 2007; Marshak-Rothstein and Rifkin, 2007), which may provide an opportunity to develop therapeutics to down-modulate pDC activation during autoimmune disease.

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Figure 5. BST2 activates primary pDCs and inhibits IFN and cytokine production by pDCs. (A) pDCs were incubated with anti-ILT7, recombinant Fc, or BST2-Fc proteins and then analyzed for calcium influx. pDCs pretreated with 5 µM of Syk inhibitor were also analyzed. Data are representative of three independent experiments. (B) The amounts of secreted cytokines from pDCs cultured with plate-bound Fc or BST2-Fc are shown. pDCs were activated overnight with either 0.2 µM of CpG 2216 or MOI 6 of Flu. Data are representative of five independent experiments with ten donors. (C) The levels of gene transcripts from pDCs cultured with purified Fc or BST2-Fc are shown. The relative expression of each gene was normalized with S18 and calculated against the value obtained from normal total PBMCs. Data are representative of two independent experiments. (D) BST2 does not affect co-stimulatory molecule expression by pDCs. pDCs cultured with plate-bound Fc or BST2-Fc and then activated with 0.2 µM of CpG 2216 for 48 h. Surface levels of CD80 and CD86 are shown. Data are representative of two independent experiments. (E) pDCs preincubated with medium, 20 µg/ml of IgG1 or 20 µg/ml of neutralizing ILT7 mAb were cultured overnight with plate-bound Fc or BST2-Fc in the presence of CpG 2216. The amounts of secreted IFN-α were measured by ELISA. Percent of BST2-mediated IFN-α suppression was calculated as percent ratio of IFN-α ([Fc minus BST2-Fc]/[Fc]) and plotted. Data are representative of two independent experiments with four donors. (F) The amounts of secreted IFN-α from Flu-challenged pDCs cultured with HEK293 with or without surface HA-tagged BST2 are shown. Data are representative of two independent experiments.
H-2D<sup>d</sup> (Karlhofer et al., 1992), whereas stimulatory NK receptor Ly49H binds to glycoprotein M157 encoded by mouse cytomegalovirus (Arase et al., 2002; Smith et al., 2002). Ly49P recognizes the H-2D<sup>d</sup>-restricted mouse cytomegalovirus–infected cells (Desrosiers et al., 2005), and NKG2D interacts with ligands with structural homology with MHC class I, including RAE-1α–ε, H60, and MULT1 (Cerwenka et al., 2000; Diefenbach et al., 2001, 2003; Carayannopoulos et al., 2002), expression of which is under control of TLR activation, viral infections, and stress (Lodoen et al., 2003; Hamerman et al., 2004; Nice et al., 2009). In human, NKG2D engages with MHC class I-like molecules MICA and MICB (Bauer et al., 1999), inducible by cell stress, and ULBP1–4 (Cosman et al., 2001; Chalupny et al., 2003), expression of which is influenced by TLR activation or viral infections (Ebihara et al., 2007). The fact that BST2 is a gene product whose expression is under control of inflammatory signals is consistent with the notion that stimulatory receptors may regulate immune functions by interacting with inducible or pathogen–associated ligands.

Like NK cells, pDCs express both inhibitory receptors and stimulatory receptors. Human pDC receptors ILT7, BDCA2, high-affinity Fc receptor for IgE, and NKp44, which all signal through an ITAM-mediated pathway, exert negative effects on the pDC's IFN response to TLR activation (Dzionek et al., 2001; Novak et al., 2004; Fuchs et al., 2005; Cao et al., 2006, 2007). Similarly, activation of Siglec-H, in association with the ITAM-bearing DAP12 subunit, reduces type I IFN production by mouse pDCs in vitro and in vivo (Blasius et al., 2006a). On the other hand, Ly49Q, an inhibitory receptor for H-2D<sup>d</sup> (Karlhofer et al., 1992), whereas stimulatory NK receptor Ly49H binds to glycoprotein M157 encoded by mouse cytomegalovirus (Arase et al., 2002; Smith et al., 2002). Ly49P recognizes the H-2D<sup>d</sup>-restricted mouse cytomegalovirus–infected cells (Desrosiers et al., 2005), and NKG2D interacts with ligands with structural homology with MHC class I, including RAE-1α–ε, H60, and MULT1 (Cerwenka et al., 2000; Diefenbach et al., 2001, 2003; Carayannopoulos et al., 2002), expression of which is under control of TLR activation, viral infections, and stress (Lodoen et al., 2003; Hamerman et al., 2004; Nice et al., 2009). In human, NKG2D engages with MHC class I-like molecules MICA and MICB (Bauer et al., 1999), inducible by cell stress, and ULBP1–4 (Cosman et al., 2001; Chalupny et al., 2003), expression of which is influenced by TLR activation or viral infections (Ebihara et al., 2007). The fact that BST2 is a gene product whose expression is under control of inflammatory signals is consistent with the notion that stimulatory receptors may regulate immune functions by interacting with inducible or pathogen–associated ligands.

Like NK cells, pDCs express both inhibitory receptors and stimulatory receptors. Human pDC receptors ILT7, BDCA2, high-affinity Fc receptor for IgE, and NKp44, which all signal through an ITAM-mediated pathway, exert negative effects on the pDC's IFN response to TLR activation (Dzionek et al., 2001; Novak et al., 2004; Fuchs et al., 2005; Cao et al., 2006, 2007). Similarly, activation of Siglec-H, in association with the ITAM-bearing DAP12 subunit, reduces type I IFN production by mouse pDCs in vitro and in vivo (Blasius et al., 2006a). On the other hand, Ly49Q, an inhibitory

**Figure 6.** Analysis of BST2 expression and potential BST2–ILT7 cis-interaction. (A) HEK293, NHDF, HUVEC, and HaCat cells, cultured in the absence or presence of 500 units/ml of IFN-α for 48 h, were stained with anti-BST2 mAb 26F8. Staining with isotype-matched control Ig is shown in the gray shaded area. Data are representative of three independent experiments. (B) Fresh isolated pDCs from peripheral blood or cells activated with various stimuli for 48 h were analyzed for surface BST2 expression by flow cytometry. Data are representative of two independent experiments. (C) Human Burkitt's lymphoma Namalwa cells transduced with different pDC receptor complexes, i.e., BDCA2/FceR1y or ILT7/FceR1y, were analyzed for surface BST2 and ILT7 expression. Data are representative of three independent experiments.
ELISA plates were coated with 10 µg/ml of BST2–ILT7 interaction mediated regulation of IFN-I responses (bottom) In a tumor environment where BST2 is endogenously expressed, the consequence of ILT7 engagement of ILT7 might be bidirectional in a cancer model of BST2–ILT7 mediated regulation of IFN-I responses. (top) By sensing viral infection, pDCs can rapidly and rigorously produce large amounts of IFN-I via TLR7 or TLR9 activation. IFN-I then may induce the neighboring cells to express cytokines, and the cells were lysed and RNA subjected to RT-PCR analysis, as previously described (Cao et al., 2006). Human TNF-α was purchased from Sigma-Aldrich. ILT7 reporter cell assay. ELISA plates were coated with 10 µg/ml of protein in PBS or at the concentration specified. 10⁶ cancer cells or transfected HEK293 cells were seeded in 24-well plates the day before the experiment. After 10⁴ NFAT–GFP reporter cells were added to the ELISA plate or the cell monolayer, the plates were spun at 100 g for 2 min. After overnight culture, cells were subjected to flow cytometric analysis to measure GFP expression.

Figure 7. A proposed model of BST2–ILT7 mediated regulation of pDC innate immune responses. (top) By sensing viral infection, pDCs can rapidly and rigorously produce large amounts of IFN-I via TLR7 or TLR9 activation. IFN-I then may induce the neighboring cells to express BST2, which in turn engages with ILT7 on pDCs to down-regulate the magnitude of IFN and cytokine responses in a negative feedback manner. (bottom) In a tumor environment where BST2 is endogenously expressed, infiltrating pDCs may be functionally suppressed to elicit normal IFN response to TLR ligands as a result of BST2–ILT7 interaction.

MATERIALS AND METHODS

Reagents and cells. HEK293 cells were grown in high-glucose DME supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin. Breast cancer cells and mouse B2B4 NFAT-GFP reporter cells (Cao et al., 2006) were grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics. NHDF and HUVEC cells (Lonza) were cultured in Clonetics Fibroblast Cell Medium FGM-2 and Endothelial Cell Basal medium, respectively, supplemented with growth factors following the manufacturer’s recommendations. HaCaT cells were provided by S. Ullrich (M.D. Anderson Cancer Center, Houston, TX) and cultured in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Namalwa cells were cultured as previously described (Cao et al., 2007). Human TNF-α was purchased from Peprotech. IFN-α was purchased from Sigma-Aldrich.

BST2 activation of human primary pDCs. The institutional review board for human research at the M.D. Anderson Cancer Center approved the use of human blood samples for this study. Primary human pDCs were isolated from blood using a negative selection kit (Miltenyi Biotec) and sorted by flow cytometry as CD3-CD11c+CD14-CD15-CD16-CD19-CD56-CD4+CD123+ cells. pDCs were preincubated with plate-bound control Fc protein or BST2-Fc protein captured with 10 µg/ml of F(ab)2 goat anti-human IgG Fc (Jackson ImmunoResearch Laboratories) on an ELISA plate for 30 min before stimulation with 0.2 µM of CpG 2216 (Sigma-Geniosy) or heat-inactivated influenza virus PR8 at a multiplicity of infection (MOI) of 6. 18 h later, the supernatants were harvested and analyzed for cytokines, and the cells were lysed and RNA subjected to RT-PCR analyses, as previously described (Cao et al., 2006). To neutralize ILT7 in this assay, pDCs were preincubated with 20 µg/ml of anti-ILT7 (clone 17.2; Cao et al., 2006), washed, and then incubated with plate-bound Fc or BST2-Fc and stimulated with CpG. In parallel, control pDCs were preincubated with medium or 20 µg/ml of IgG1, washed, and stimulated similarly on Fc- or BST2-Fc–coated plates. To perform pDC and BST2-transfectant cell co-culture, pDCs were preincubated with inactivated influenza virus (MOI = 8) for 30 min, and cells were pelleted by centrifugation for 5 min at 500 g and added to parent HEK293 or BST2–HA–transfected HEK293 monolayers. Cells were packed briefly by centrifuging at 100 g for 2 min. 18 h later, the supernatants were harvested and analyzed for cytokine secretion. To study calcium influx, purified control Fc protein or BST2–Fc protein was incubated with pDCs in the presence of goat anti-human Fc (ab) (Jackson ImmunoResearch Laboratories). Dye loading and flow cytometric analysis were performed as previously described (Cao et al., 2006).

Anti–ILT7-L mAb generation. 6–8-wk-old BALB/c mice were immunized with T47D and MDA-MB-231 cells by the alternate footpad method (Cao et al., 2006). Hybridoma clones secreting mAbs that specifically stained T47D cells, but not MDA-MB-231 cells, were expanded. They were further screened for their ability to block T47D-induced GFP expression from ILT7 reporter cells. mAbs 26F8 (IgG1) and 28G4 (IgG2a) were affinity purified and fluorochrome conjugated using mAb conjugation kits (Invitrogen).

cDNA library screening. A library of human full-length cDNA clones was purchased from OriGene Technologies, Inc. Plasmid DNA was prepared using the Wizard plus miniprep kit (Promega). For transfection, HEK293 cells were seeded at 10⁴ cells/well in 96-well plates. 0.6 µl of Fugene 6 (Roche) was added to 15 µl of Opti-MEM (Invitrogen) and mixed with 10 µl of DNA from individual cDNA clone. After 15 min at room temperature, the mixture was added to cells. 48 h later, cells were centrifuged
BST2 transfection and expression analysis. HEK293 cells were transfected with an expression plasmid containing the full-length human BST2 cDNA (Open Biosystems) with lipofectamine (Invitrogen). 48 h later, cells were either lysed for Western blot analysis using a BST2-specific rabbit polyclonal antibody (FabGennix, Inc.) or subjected to staining with fluorochrome-conjugated anti–ILT7-L mAbs. HEK293 cells stably expressing BST2-HA were transfected with plasmids encoding ILT7-Fc, selected with Zeocin, and sorted for high HA expression by flow cytometry.

Generation of recombinant ILT7 and BST2 fusion protein. The extracellular domain of ILT7 or BST2 were cloned into an expression vector containing a mutated GPI anchor (Arase et al., 2002). HEK293 cells were transiently transfected with the expression plasmids or the empty vector to produce recombinant protein, which was purified by using a Protein A column (GE Healthcare). The extracellular domain of BST2 (excluding GPI anchor) was reconstituted as a GST–fusion protein, which was stably expressed in CHO-K1SV cells and purified by glutathione–Sepharose affinity chromatography (GE Healthcare).

Surface plasma resonance analysis. Surface plasma resonance was performed using a Biacore 3000 (GE Healthcare). BST2–Fc and Fc were covalently immobilized onto flow cells of a CMS sensor chip by amine coupling according to the manufacturer’s instructions. ILT7–Fc, Fc, and BST2–Fc were injected at 20 µl/min. To block the interactions between BST2 and ILT7, 100 mM Hepes (pH 7.4), 150 mM NaCl, and 0.005% P20 were passed over the chip at 20 µl/min. After injection of ILT7–Fc (120 µg/ml), IgG1 (120 µg/ml), or buffer was injected over BST2–Fc–coated sensor chip before injection of ILT7–Fc (120 µg/ml).

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