Plasmacytoid dendritic cell–specific receptor ILT7–FcεRIγ inhibits Toll-like receptor–induced interferon production

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Immunoglobulin-like transcripts are a family of inhibitory and stimulatory cell surface immune receptors. Transcripts for one member of this family, ILT7, are selectively expressed in human plasmacytoid dendritic cells (pDCs). We demonstrate here that ILT7 protein associates with the signal adapter protein FcεRIγ to form a receptor complex. Using an anti-ILT7 monoclonal antibody, we show that ILT7 is expressed specifically on human pDCs, but not on myeloid dendritic cells or other peripheral blood leukocytes. Cross-linking of ILT7 resulted in phosphorylation of Src family kinases and Syk kinase and induced a calcium influx in freshly isolated pDCs, which was blocked by Src family and Syk kinases inhibitors, thus indicating the activation of an immunoreceptor-based tyrosine activation motif–mediated signaling pathway. ILT7 cross-linking on CpG or influenza virus-stimulated primary pDCs inhibited the transcription and secretion of type I interferon and other cytokines. Therefore, the ILT7–FcεRIγ receptor complex negatively regulates the innate immune functions of human pDCs.

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Plasmacytoid DCs (pDCs) are a distinct population of DCs in the peripheral blood and secondary lymphoid organs and are characterized by their plasma cell–like morphology and unique surface receptor phenotype (1). These cells play an important role in innate antiviral immunity by rapidly secreting abundant type I IFNs (IFNα, β, ω, λ) after exposure to various DNA and RNA viruses (1, 2). Type I IFNs produced by pDCs promote the function of NK cells, B cells, T cells, and myeloid DCs (mDCs) during the initial immune response (3–5). After activation, pDCs differentiate into a unique type of mature DCs, capable of directing T cell responses with considerable flexibility (3, 4). Thus, pDCs represent a critical link between innate and adaptive immune responses.

The unique ability of pDCs to sense and respond rigorously to microbes by rapidly producing large amounts of type I IFN is underlined by their expression, in contrast with mDCs and other immune cells, of a selective set of toll-like receptors (TLRs), in particular TLR7 and TLR9 (6). Recent studies have revealed an intracellular multiprotein complex that likely includes TLR9/7–MyD88–IRAK1/4–TRAF6–IRF7 and a complicated spatiotemporal signaling scheme in pDCs (7, 8). Because both TLR7 and TLR9 are located in the endosomal compartment of pDCs, how these cells sense the external microenvironment by surface receptors has remained elusive. We, therefore, performed a global gene expression analysis of human pDCs, in comparison with the other major human immune cell types. Human pDCs selectively express ILT7 (also named CD85g and LILRA4) transcripts as well as IL-3R (CD123) and BDCA-2, as previously reported (9–11).

ILT7 is a member of the immunoglobulin-like transcripts (ILTs), or leukocyte immunoglobulin-like receptor (LIR) gene family (12), which comprises at least 13 loci. ILTs are predominantly expressed on the surface of

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myelomonocytic cells, including macrophages and DCs. Although the extracellular Ig domains are responsible for ligand binding, the residues within the transmembrane and cytoplasmic domains define two functional classes of ILTs: the inhibitory ILTs contain the immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the cytoplasmic domain, whereas the activating ILTs lack any intrinsic signaling motifs and rely on association with transmembrane adapter proteins bearing immunoreceptor-based tyrosine activation motif (ITAM). Certain ILTs, such as ILT2 and ILT4, bind to classical and nonclassical MHC class I proteins (13). The ITIM-containing ILT2 inhibits signaling through the TCR in T cells (14) and enhances the inhibitory effects of killer cell Ig–like receptors (KIRs) in NK cells (13). In contrast, ILT1 associates with FcεRIγ and activates eosinophils to release cytotoxic granule proteins, cytokines, and lipid mediators (15).

ILT7 encodes a surface receptor that is preferentially transcribed by human pDCs. This molecule contains four extracellular Ig domains and has a positively charged residue within the transmembrane region, which potentially allows it to associate with membrane-anchored adapter proteins. In this study, we report that ILT7 and FcεRIγ form a receptor complex that is specific for human pDCs and transduces ITAM-mediated signals that negatively modulate TLR-induced type I IFN production by human pDCs.

RESULTS AND DISCUSSION

ILT7 mRNA is specifically expressed by pDCs

To determine the expression profile of ILT7 in human leukocytes, we searched our established expression database, which included the major immune cell types in peripheral blood. Strikingly, ILT7 transcripts were expressed abundantly and exclusively by human pDCs (Fig. 1 A). pDCs also expressed ILT2 and ILT3; however, these receptors were also expressed by other cell types (Fig. 1 A). To verify this finding, we performed quantitative RT-PCR analysis on several cell types from multiple healthy donors. Consistently, human pDCs uniquely expressed ILT7 mRNA (Fig. 1 B).

ILT7 uses FcεRIγ as an adapter

The positively charged arginine residue at position 449 is located within the predicated transmembrane segment of the ILT7 protein. To look for the adapter proteins interacting with ILT7, we examined the expression of the transmembrane

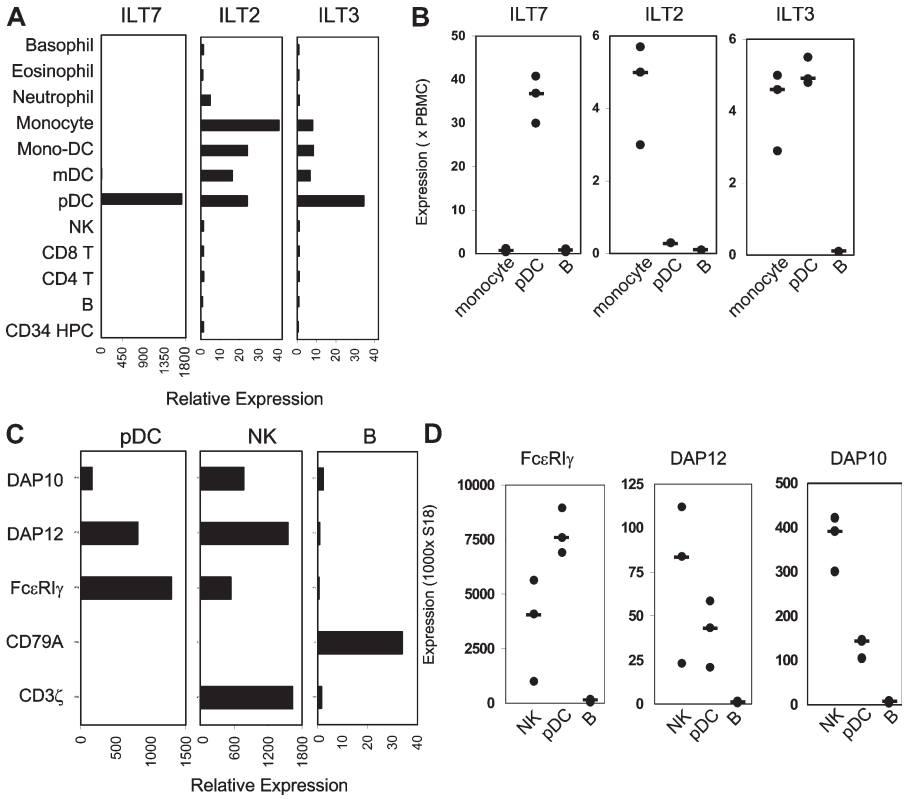

Figure 1. Human pDCs preferentially express ILT7 and three transmembrane signaling adapters. (A) The relative expression of ILT family members on peripheral blood leukocytes was compared by plotting the values extracted from the gene expression database. A value <1 indicated the absence of gene expression. (B) The relative gene expression of ILTs on different cell types from three healthy donors was determined by quantitative RT-PCR analysis. The expression was normalized with the level of total PBMCs. The median expression is marked by a horizontal bar. (C) The expression levels of known transmembrane signaling adapters in pDCs were plotted using the values extracted from the gene expression database. (D) The relative expression of FcεRIγ, DAP12, and DAP10 was determined from three healthy donors by quantitative RT-PCR analysis. The expression was normalized with S18 and multiplied by 1,000. The median expression is marked by a horizontal bar.

adapters in human pDCs from the expression database (Fig. 1 C). Although lacking the expression of ITAM-bearing components found in TCR and B cell receptors (BCRs), pDCs expressed two ITAM-bearing adapters (i.e., FcεRIγ and DAP12). DAP10, a non-ITAM adapter, which signals via a YINM motif permitting activation of PI3 kinase, was also transcribed in pDCs (Fig. 1 C). We further confirmed the expression of these adapters by RT-PCR analysis of three healthy donors (Fig. 1 D).

To determine which adapter pairs with ILT7, we used an "adapter trap" reporter cell system in which mouse BaF/3 pro–B cells were stably transfected with FcεRIγ, DAP12, or DAP10. ILT7 stabilized the surface expression of FcεRIγ, but neither DAP12 nor DAP10 (Fig. 2 A). Similarly, FcεRIγ enhanced the cell surface expression of ILT7 (Fig. 2 A). As a positive control for DAP10 and DAP12, we used a mouse NKG2D variant that could pair with either DAP12 or DAP10 (16). Additionally, ILT7 and FcεRIγ were coimmunoprecipitated from a lysate of BaF/3 cells transfected with HA-tagged ILT7 and FLAG-tagged FcεRIγ (Fig. 2 B). Thus, our results demonstrate that ILT7 associates with the ITAM-containing signaling adapter FcεRIγ to form a stable receptor complex.

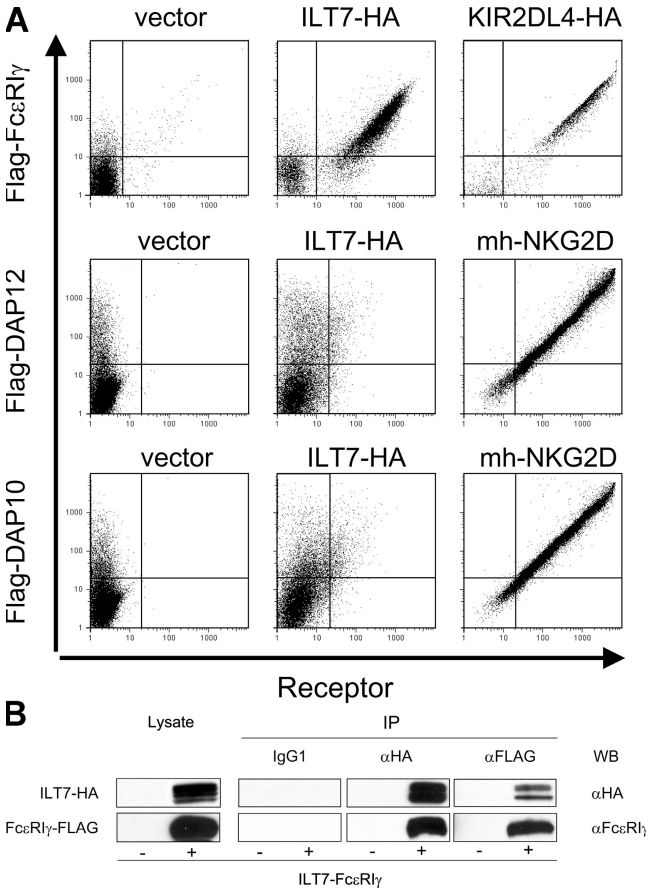

Figure 2. ILT7 associates with FcεRIγ. (A) BaF/3 cells stably transfected with FLAG-FcεRIγ (top), -DAP12 (middle), or -DAP10 (bottom) were transduced with HA-ILT7 (center column) or with the vector alone (left column). Cells were stained for FLAG and HA and analyzed by flow cytometry. HA-tagged human KIR2DL4 or a mouse NKG2D variant was transduced into the BaF/3 cells as positive controls (right column). (B) Lysate from parental BaF/3 cells or cells expressing ILT7-HA and FcεRIγ-FLAG was precipitated with IgG1, anti-HA, or anti-FLAG mAbs. Western blot (WB) was performed with anti-HA or anti-FcεRIγ Ab. A fraction of the original lysate was run as control. Notably, ILT7-HA ran as two bands, likely reflecting differentially processed isoforms.

ILT7 is a pDC-specific receptor

To confirm the expression of the ILT7 protein, we generated a mAb that recognizes a mouse T cell line transfected with human ILT7 (Fig. 3 A). In total PBMCs, anti-ILT7 mAb stained a rare cell population of non–T, non–B, nonmonocyte, and non–NK cells (unpublished data). When double stained with mAb against pDC marker BDCA2, almost all the ILT7$^+$ cells were BDCA2^{high} (Fig. 3 B), suggesting that the circulating blood pDCs preferentially express ILT7. When activated by CpG or treated with IL-3, pDCs expressed lower levels of ILT7 (Fig. 3 C), consistent with the reported down-regulation of ILT7 mRNA in activated pDCs (10, 11).

ILT7 triggers ITAM signaling

To reveal the cellular signals transduced by ILT7, we introduced a human ILT7–FcεRIγ complex into a mouse T cell hybridoma line that contained an intracellular NFAT–GFP construct (Fig. 4 A). Cross-linking ILT7 with immobilized anti-ILT7 mAb resulted in GFP expression, indicating that ILT7-FcεRIγ is able to activate NFAT, similar to the effect of TCR activation. The immobilized isotype-matched control antibody did not activate the cells under the same conditions (Fig. 4 A). When the same experiment was conducted in NFAT-GFP reporter cells expressing ILT7 alone (without FcεRIγ), cross-linking ILT7 did not induce GFP expression, which illustrated again the requirement of FcεRIγ for ILT7 to signal (unpublished data).

To evaluate the signaling events in primary human pDCs induced by ILT7 activation, we cross-linked ILT7 on freshly isolated pDCs and analyzed the phosphorylation status of two key types of protein tyrosine kinases (PTKs) by Western blot analysis (Fig. 4 B). After ITAM-mediated activation, two core tyrosine residues within the ITAMs are phosphorylated by PTKs of the Src family kinases. The tyrosine-phosphorylated ITAMs associate with the Src homology 2 (SH2) domains of spleen tyrosine kinase (Syk)-family kinases to initiate the well-orchestrated cascade of downstream events. Human pDCs express several members of the Src family kinases and Syk, but not ZAP70 (unpublished data). Shortly after ILT7 activation, both Src family kinases and Syk were phosphorylated (Fig. 4 B), indicating the onset of ITAM-mediated signaling in pDCs. In contrast, cross-linking with neither the isotype-matched control antibody nor the mAb against BDCA4, another surface molecule expressed on pDCs, phosphorylated these kinases under the same conditions.

Next, we analyzed one of the important cellular activation events that occurs downstream of ITAM-mediated signaling: calcium influx. Cross-linking of ILT7 effectively triggered prominent intracellular calcium mobilization in

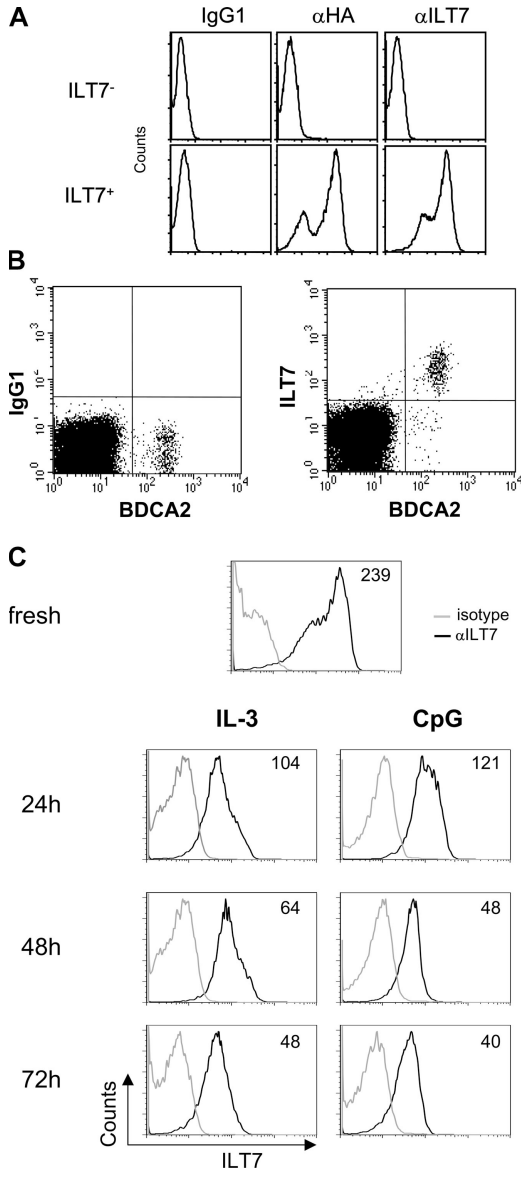

Figure 3. Anti-ILT7 mAb specifically stains peripheral blood pDCs. (A) Mouse 2B4 T cell hybridoma expressing human FcεRIγ (top) was transduced with HA-ILT7 (bottom). The cells were stained with an isotype-matched control Ab (IgG1), anti-HA, or anti-ILT7 mAb. (B) Total PBMCs were stained with IgG1 (left) or anti-ILT7 (right) and anti-BDCA2 mAb. Representative results from the analysis of multiple healthy donors are shown. (C) Surface ILT7 was measured on freshly isolated, IL-3–treated, or CpG-treated pDCs at various times after culture. Mean fluorescence intensity of each sample is indicated (top right).

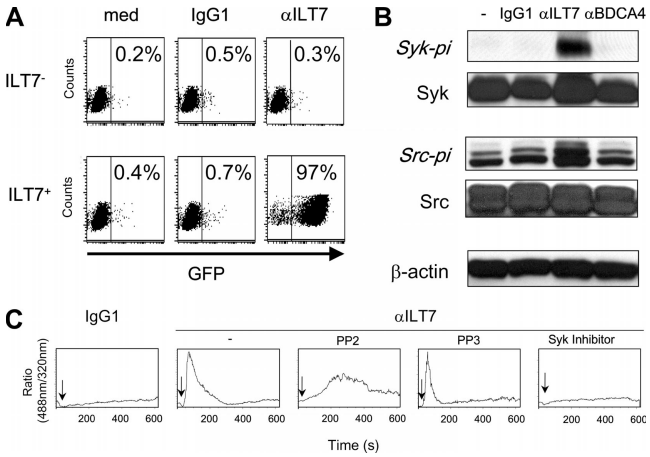

Figure 4. Cross-linking of ILT7/FcεRIγ activates ITAM-mediated signaling. (A) 2B4 T cell hybridoma expressing FcεRIγ and ILT7 was cross-linked with mouse IgG1 or anti-ILT7 mAb and analyzed for NFAT-activated GFP expression. Cells cultured in medium alone were also analyzed. (B) Freshly isolated human pDCs were cross-linked with mouse IgG1, anti-ILT7, or anti-BDCA4 mAb and the cell lysates were analyzed by Western blotting. (C) The kinetics of intracellular calcium flux in human pDCs when cross-linked (arrow) by control (IgG1) or anti-ILT7 Ab. As indicated, cells were preincubated for 30 min with 50 μM of PP2, 50 μM of PP3, or 10 μM of Syk inhibitor (EMD Biosciences) before cross-linking.

human primary pDCs (Fig. 4 C). This activity was greatly reduced by PP2, a compound that interferes with the function of Src family kinases, but was not affected by an inactive control compound PP3 (Fig. 4 C). In addition, an inhibitor specific to Syk kinase completely abolished the intracellular calcium activity in ILT7–cross-linked pDCs (Fig. 4 C). Therefore, the ILT7–FcεRIγ complex is capable of activating the ITAM pathways in human pDCs.

ILT7–FcεRIγ inhibits pDCs' TLR responses

An important role of the ILT molecules is to modulate the function of other immune receptors (12). pDCs expressing TLR9 and TLR7 respond to CpGs and viruses by producing large amounts of type I IFN, as well as other proinflammatory cytokines (6). We examined how ILT7 triggering influences TLR responses in human pDCs. When ILT7 was cross-linked, pDCs stimulated by the TLR9 ligand CpG oligonucleotide (ODN) produced less IFNα and TNFα, in comparison with medium control or isotype-matched control Ab (Fig. 5 A). However, ILT7 cross-linking did not affect the surface maturation phenotype of pDCs, as measured by CD80 and CD86 expression (Fig S1, available at http://www.jem.org/cgi/content/full/jem.20052454/DC1). Cross-linking of surface BDCA4 under the same conditions did not alter the TLR responses (Fig. 5 A), consistent with a published report (17). As a positive control, preincubation of pDCs with anti-BDCA2 mAb (5 μg/ml) dramatically reduced the TLR-mediated responses. Interestingly, ILT7 cross-linking effectively reduced TLR response even after pDCs were preexposed to CpG up to 1 h, which was analogous to the effects of BDCA2 cross-linking (Fig. 5 B). Moreover, when we activated pDCs with inactivated influenza virus (Flu), a ligand for TLR7 (18), ILT7 cross-linking similarly inhibited the production of both IFNα and TNFα (Fig. 5 A).

To confirm this observation, we examined the intracellular IFNα and TNFα levels in the CpG-activated pDCs that were cross-linked under similar conditions. Consistently, ILT7 cross-linking specifically decreased the amount of intracellular IFNα as well as TNFα (Fig. 5 C). Last, we

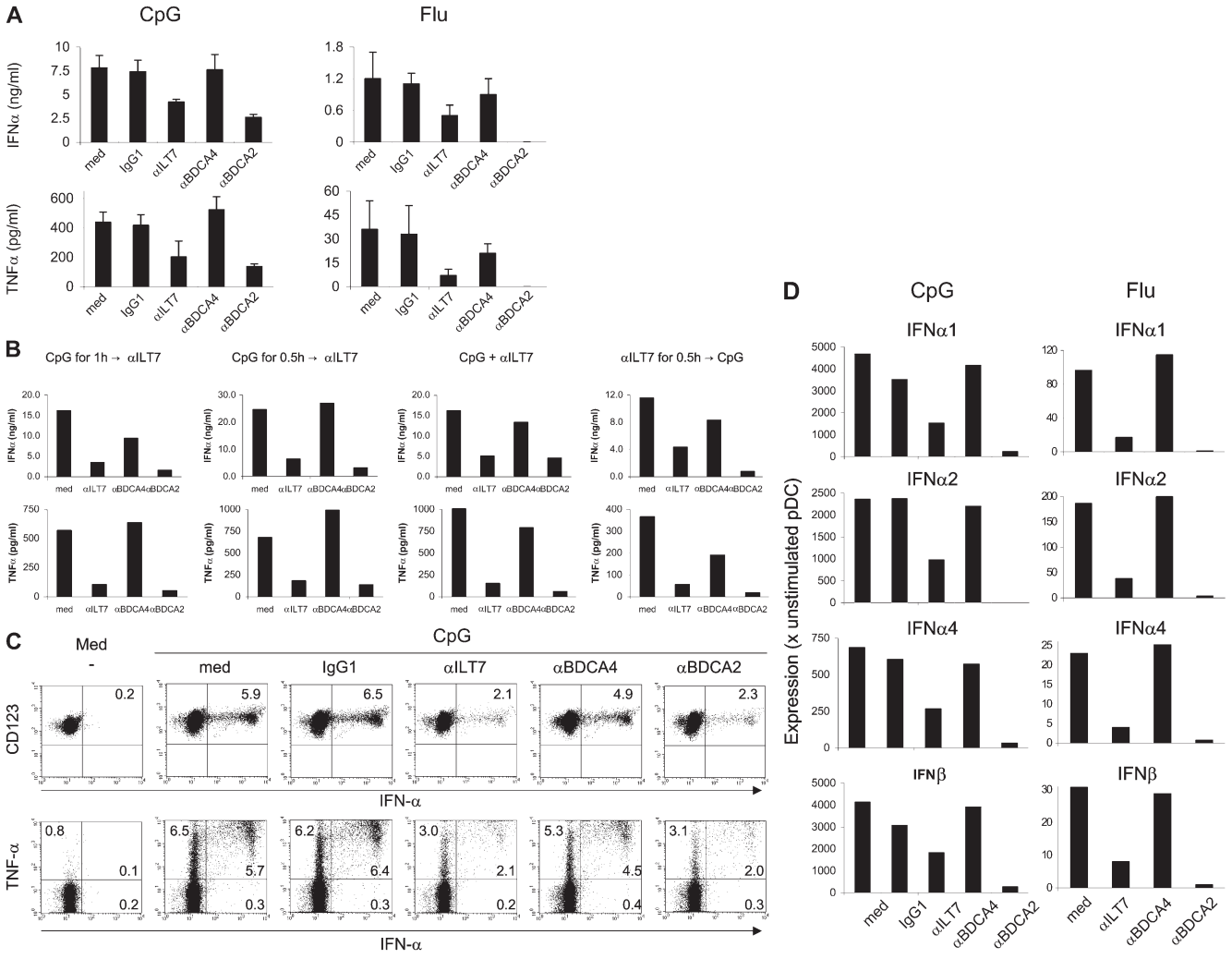

Figure 5. ILT7 cross-linking inhibits TLR responses by pDCs. (A) Human pDCs were cross-linked with various Abs before stimulation by CpG (left) or Flu virus (right). The levels of the secreted IFNα and TNFα in a triplicate assay from a representative donor are shown. (B) Purified pDCs were cultured with CpG for 1 h (left) or 30 min (second panel from left) at 37°C before being cross-linked. Alternatively, CpG was added at the same time as (second panel from right) or 30 min after (right) cells were cross-linked by various mAbs. The amounts of IFNα and TNFα in the supernatant after 18 h of culture are shown. (C) The amounts of IFNα and TNFα were determined by intracellular staining of CpG-activated pDCs. The percentage of the double-positive cell population is indicated. (D) The amounts of type I IFN transcripts were determined by quantitative RT-PCR analysis. The expression is shown as the relative level of transcription compared with unstimulated pDCs.

evaluated the amount of type I IFN transcripts from both CpG and Flu-activated pDCs that were cross-linked with anti-ILT7 or control mAbs. ILT7 cross-linking consistently reduced the transcription of all four subtypes of type I IFN analyzed (i.e., IFNα1, IFNα2, IFNα4 and IFNβ) (Fig. 5 D). Thus, ILT7 activation by mAb cross-linking significantly down-regulated the TLR-induced IFN and cytokine responses in pDCs.

We have shown that the ILT7–FcεR1γ complex represents the first human pDC-specific ITAM-containing receptor complex. Cross-linking of the surface ILT7–FcεR1γ complex on pDCs elicited activating signals, including phosphorylation of Src family kinases and Syk kinase, and induced a robust intracellular calcium mobilization. However, rather than enhancing the production of cytokines induced by stimulation of TLR, ILT7 functioned as a negative regulator of these TLR-mediated responses. Inhibition of TLR-induced human pDC activation by ITAM-receptor signaling has been documented in several prior studies. For example, cross-linking of the high affinity IgE receptor, which signals via FcεRIγ, also inhibits CpG-induced type I IFN production by human pDCs (19, 20). Additionally, Fuchs et al. reported that cross-linking NKp44, a receptor signaling through the ITAM-bearing DAP12 adapter, inhibited CpG-induced IFN-α production (20, 21). In mice, mAb cross-linking of Siglec-H, another DAP12-associated receptor, reduced type I IFN production by pDCs in vitro and in vivo (22). These findings support and extend the prior studies reporting that

ITAM-mediated signaling can suppress the response of mouse macrophages to TLR ligands in vivo and in vitro (22, 23).

It is not clear how ITAM and TLR pathways intersect at the molecular level in human pDCs. Schroeder et al. demonstrated that IgE receptor cross-linking inhibited TLR9 expression in pDCs, which may explain in part the reduced CpG responses by these cells (19). Alternately, one possible mechanism may involve a bifunctional role of the ITAM of FcεRIγ. Specifically, under different conditions, FcεRIγ may function as an activating molecule by recruiting Syk, or alternatively act as an inhibitory receptor by recruiting SHP-1 and subsequently impairing protein tyrosine phosphorylation in the absence of sustained receptor aggregation (23, 24). Similarly, the downstream adapter protein LAB/NTAL may play either positive or negative roles in regulating FcεRIγ-mediated signaling under different conditions (25, 26). It will be important to examine in detail the biochemical mechanism of this inhibitory effect by ITAMs on TLR signaling.

pDCs are critically responsible for the massive and rapid type I IFN production elicited by viral infections. Controlling the type and the magnitude of this response is important in resolving disease without harming healthy tissue. Elevated levels of IFNα and up-regulated expression of IFN-responsive genes were found in the peripheral blood of systemic lupus erythematosus patients, where pDCs were presumably activated by chromatin-containing immune complexes (27). Therefore, the effective inhibition of pDCs and down-regulation of their constitutive type I IFN production, possibly through the ILT7–FcεRIγ receptor complex, may present a plausible therapeutic approach.

MATERIALS AND METHODS

Establishment of human leukocyte expression database. This study was approved by the institutional review board for human research at the M.D. Anderson Cancer Center. Human peripheral DCs, B cells, T cells, monocytes, and monocyte-derived DCs were prepared as described previously (28). $CD56^+CD4^-CD14^-CD19^-CD69^-$ NK cells were isolated by flow cytometry. Neutrophils, eosinophils, basophils, and blood $CD34^+$ hematopoietic progenitor cells (HPCs) were isolated as described previously (29). RNA preparation and microarray hybridization was performed as described previously (27, 28). The Positional Dependent Nearest Neighbor model (30) was used to estimate the gene expression values from the probe intensity values. The final expression output was normalized with the numerical value of one representing the estimated threshold of basal expression.

Quantitative RT-PCR analysis. Reverse transcription and quantitative PCR was performed as described previously (27, 28). Oligonucleotide primers used are listed in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20052454/DC1).

Cloning and expression of ILT7 and transmembrane adapters. ILT7 was cloned from pDC cDNAs. A retroviral vector expressing ILT7 with an HA tag was used to transduce BaF/3 cells expressing FLAG-tagged human FcεRIγ, DAP12, or DAP10 (16).

Generation of ILT7 mAb and PBMC staining. 6–8-wk-old BALB/c mice were immunized with ILT7–FcεRIγ-transfected 2B4 cells. Hybridoma clones secreting mAb that specifically stained $ILT7^+$ cells were established. mAb 17G10.2 against ILT7 (IgG1) was purified and fluorochrome conjugated using mAb-labeling kits obtained from Invitrogen. PBMCs were stained with anti-BDCA2–FITC (Miltenyi Biotec) and Alexa Fluor A647–labeled anti-ILT7 mAb.

NFAT-GFP reporter assay. HA-tagged ILT7 was transduced into the 2B4 NFAT-GFP reporter cells (H. Arase, Osaka University, Osaka, Japan) with or without mouse FcεR1γ. The cells were cultured on Ab-coated plates (10 μg/ml) for 16 h and analyzed for GFP expression.

Western blot analysis of protein tyrosine phosphorylation. pDCs isolated using BDCA4 cell isolation kit (Miltenyi Biotec) were incubated with mouse IgG1 (eBioscience), anti-ILT7, or anti-BDCA4 mAb (IgG1; Miltenyi Biotec) and cross-linked with $F(ab')_2$ goat anti–mouse IgG (Jackson ImmunoResearch Laboratories) for 2 min. Western blot was performed with an anti-phospho–Src family Ab, anti-phospho–Syk Ab, anti-nonphospho–Src family Ab, anti-Syk Ab (Cell Signaling Technology), and anti–β-actin mAb (Sigma-Aldrich).

Calcium influx assay. pDCs preloaded with Fluo4- and Fura Red-AM (Invitrogen) were cross-linked as described in the previous paragraph and analyzed on a FACSAria (BD Biosciences) and by FlowJo software (Tree Star).

ILT7 cross-linking pDCs on pDCs in culture. pDCs isolated by flow cytometry ($CD4^+CD11c^-CD3^-CD14^-CD16^-CD19^-CD56^-$) were incubated in the Ab-coated wells (10 μg/ml) for 30 min before stimulation with 0.1 μM of CpG 2216 or heat-inactivated Flu at a multiplicity of infection of 3, unless otherwise specified. Cells and supernatants were harvested 18 h later for RT-PCR and ELISA. ELISA kits used were human IFNα (Bender MedSystems) and TNFα (R&D Systems). Intracellular cytokine staining was performed after 6 h of culture with CpG as described previously (28). Cells were stained with anti–IFN-α–FITC Ab (Chromaprobe), anti-CD123–PE Ab (eBioscience), and anti-TNFα–allophycocyanin Ab (BD Biosciences).

Online supplemental material. Fig. S1 shows that ILT7 cross-linking does not affect pDC maturation. Surface CD80 and CD86 expression was measured by flow cytometry on pDCs cultured with CpG or IL-3. Table S1 lists all the oligonucleotide primers used in this study. Fig. S1 and Table S1 are available at http://www.jem.org/cgi/content/full/jem.20052454/DC1.

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