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Systemic Lupus Erythematosus Immune Complexes Increase the Expression of SLAM Family Members CD319 (CRACC) and CD229 (LY-9) on Plasmacytoid Dendritic Cells and CD319 on CD56<sup>dim</sup> NK Cells

Niklas Hagberg,* Jakob Theorell,† Heinrich Schlums,‡ Maija-Leena Eloranta,* Yenan T. Bryceson,† and Lars Rönnblom*

Patients with systemic lupus erythematosus (SLE) display an activated type I IFN system due to unceasing IFN-α release from plasmacytoid dendritic cells (pDCs) stimulated by nucleic acid–containing immune complexes (ICs). NK cells strongly promote the IFN-α production by pDCs; therefore, we investigated surface molecules that could be involved in the pDC–NK cell cross-talk. In human PBMCs stimulated with RNA-containing ICs (RNA-ICs), the expression of the signaling lymphocyte activation molecule (SLAM) family receptors CD319 and CD229 on pDCs and CD319 on CD56<sup>dim</sup> NK cells was selectively increased. Upregulation of CD319 and CD229 on RNA-IC–stimulated pDCs was induced by NK cells or cytokines (e.g., GM-CSF, IL-3). IFN-α–producing pDCs displayed a higher expression of SLAM molecules compared with IFN-α–pDCs. With regard to signaling downstream of SLAM receptors, pDCs expressed SHIP-1, SHP-1, SHP-2, and CSK but lacked SLAM-associated protein (SAP) and Ewing’s sarcoma-activated transcript 2 (EAT2), indicating that these receptors may act as inhibitory receptors on pDCs. Furthermore, pDCs from patients with SLE had decreased expression of CD319 on pDCs and CD229 on CD56<sup>dim</sup> NK cells, but RNA-IC stimulation increased CD319 and CD229 expression. In conclusion, this study reveals that the expression of the SLAM receptors CD319 and CD229 is regulated on pDCs and NK cells by lupus ICs and that the expression of these receptors is specifically altered in SLE. These results, together with the observed genetic association between the SLAM locus and SLE, suggest a role for CD319 and CD229 in the SLE disease process. The Journal of Immunology, 2013, 191: 2989–2998.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of immune complexes (ICs) that deposit in tissue and cause inflammation (1). Such SLE ICs are typically formed by autoantibodies and nucleic acid–containing autoantigens, which are released by dying cells (2). SLE ICs promote tissue inflammation and immune activation through several mechanisms, most importantly by complement activation and FcγR triggering of immune cells (3). In response to SLE ICs, macrophages and neutrophils produce proinflammatory cytokines and proteolytic enzymes (4), whereas plasmacytoid dendritic cells (pDCs) produce type I IFN (5). The DNA- or RNA-containing SLE ICs (interferogenic ICs) are internalized by pDCs through FcγRIIA and trigger TLR7 or TLR9 activation, ultimately leading to IFN-α secretion (5). A continuous activation of pDCs by interferogenic ICs is a prominent feature of SLE and causes an increased expression of type I IFN–induced genes (an IFN signature) in both circulating cells (6, 7) and affected tissues (8). An IFN signature has also been observed in other systemic autoimmune diseases that exhibit interferogenic ICs (9, 10), suggesting that an activation of the type I IFN system may be important in the development of an autoimmune disease process. This assumption is also supported by the observation that treatment of infectious or malignant diseases with IFN-α can trigger the development of several autoimmune diseases (11, 12).

Previous studies showed that NK cells interact with pDCs to potently enhance IFN-α production upon stimulation with viruses, synthetic oligonucleotides, or RNA-containing ICs (RNA-ICs) (13–15). Mechanistically, NK cells promoted RNA-IC–induced IFN-α secretion by pDCs via soluble factors, such as MIP-1β, and via LFA-1–dependent cell–cell interactions. In addition to IFN-α, the secretion of several cytokines and chemokines implicated in the pathogenesis of SLE (e.g., IFN-γ, IL-6, IL-8, MIP-1β, and TNF-α) was also enhanced in RNA-IC–stimulated pDC–NK cell cocultures (15). Consequently, pDC–NK cell cross-talk could be important in promoting sustained type I IFN production and the inflammatory response in systemic autoimmune diseases.

In the current study, we aimed to identify cell surface molecules of possible importance in the interaction between pDCs and NK cells when activated by RNA-ICs. By screening pDCs and NK cells for molecules that were regulated by RNA-ICs, consisting of SLE-IgG and U1snRNP particles, we identified two members of the signaling lymphocyte activation molecule (SLAM) family that
were strongly upregulated on pDCs and NK cells by RNA-ICs: CD319 (SLAMF7, CRACC, CS1) and CD229 (SLAMF3, LY9). In humans, the SLAM family of receptors includes seven members: CD150 (SLAMF1), CD48 (SLAMF2), CD229, CD244 (SLAMF4, 2B4), CD84 (SLAMF5), CD352 (SLAMF6, NTB-A), and CD319) encoded by a locus on chromosome 1 (16). With the exception of CD48, which interacts with CD244, all of the SLAM members interact via homophilic interactions. The SLAM receptors have important immunomodulatory effects, and the SLAM locus has been genetically associated with human SLE and mouse models of SLE (17–21). Therefore, we investigated the mechanism for the regulation of CD319 and CD229 and the expression of signaling molecules downstream of these receptors in pDCs and NK cells. Finally, the expression of all SLAM family members in pDCs and NK cells was compared between healthy individuals and patients with SLE.

Materials and Methods

Cells
PBMCs were isolated from healthy blood donors or patients with SLE (Rheumatology Unit, Uppsala University Hospital) by density-gradient centrifugation. For the comparison of SLAM expression on PBMCs from healthy individuals and patients with SLE, PBMCs were resuspended in 90% FCS and 10% DMSO and stored at −80°C pending flow cytometry analysis. NK cells and pDCs were isolated by negative selection, according to the manufacturer’s instructions (NK or pDC cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany), and monocyte-depleted PBMCs were prepared using CD14 MicroBeads (Miltenyi Biotec). Isolated pDCs and NK cells were routinely >95% BDCA-2+ and CD3−, respectively. If not otherwise stated, cells were cultured in 96-well V-bottom plates (Nunc, Roskilde, Denmark) using macrophage serum–free medium supplemented as CD3, CD229 (HLy9.1.25; BioLegend), CD244 (C1.7; BioLegend), CD84 (HLy9.1.25; BioLegend), and 2G7 (eBioscience) (version 7.6 and 9.4; TreeStar, Ashland, OR).

For flow cytometry, fluorochrome-conjugated mAbs to CD3 (HIT3a or SK7), CD14 (Mpd9), CD56 (NCAM16.2), CD19 (HIB19) (all from BD Biosciences, San Jose, CA), CD8 (B5; Invitrogen), CD11c (Bu15; BioLegend, San Diego, CA), BDCA-2 (AC141), and BDCA-4 (AD5-17F6) (both from Miltenyi Biotec) were used to identify different cell types, as specified. A fluorescent, fixable dead cell marker (LIVE/DEAD near IR; Invitrogen) was used to exclude dead cells from the analysis. Fluorescein-conjugated mAbs to CD69 (TIP1.55.3; Beckman Coulter, Indianapolis, IN), CD150 (A12[7D4]; eBioscience, San Diego, CA), CD48 (MEM102; BioLegend), CD229 (HLy9.1.25; BioLegend), CD244 (C1.7; BioLegend), CD84 (CD84.1.21 [BioLegend] and 2G7 [eBioscience]), NTB-A (#292811; R&D Systems, Minneapolis, MN), and CD319 (16.2; BioLegend) were used to determine surface expression. If not otherwise stated, pDCs were defined as CD3− CD14+ CD56− CD48−CD229−CD319−, whereas expression of CD84, CD229, and CD319 was increased on both pDCs and NK cells. After RNA-IC stimulation, in freshly isolated PBMCs, nearly all pDCs expressed CD48, CD229, and CD319, whereas expression of CD319 and CD229 expression is increased on both pDCs and NK cells.

Western blot analysis
Isolated pDCs or NK cells were resuspended in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, and 1% Triton X-100) supplemented with protease inhibitor mixture (Pierce, Rockford, IL). After lysis, cells were centrifuged at 4°C for 10 min at 14,000 × g. Proteins were separated by SDS-PAGE (NuPAGE; Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) by Western blotting. A rat anti–SLAM-associated protein (SAP) mAb (XLP27:295; Miltenyi Biotec) and a rabbit mAb to SHP-1 (EPR5519; Epitomics, Burlingame, CA), followed by HRP-conjugated goat anti-rabbit IgG (H+L) (F(ab′)2 fragments) (Invitrogen) were used to detect SAP. Rabbit polyclonal Abs to Ewing’s sarcoma-activated transcript 2 (EAT2), SHIP-1, SHP-2, CSK (Proteintech Group, Chicago, IL), and a rabbit mAb to SHP-1 (EPR5519; Epitomics, Burlingame, CA), followed by HRP-conjugated goat anti-rabbit IgG (H+L) (Invitrogen), were used to detect the indicated signaling molecules.

Results

CD319 and CD229 expression is increased on both pDCs and NK cells after RNA-IC stimulation
In a screen of receptors and ligands potentially involved in the RNA-IC–induced pDC–NK cell cross-talk, we examined expression of 42 surface molecules (summarized in Supplemental Table 1) on pDCs and CD56dim and CD56bright NK cells in PBMCs cultured with or without RNA-ICs for 6 h.

As expected, strong upregulation on pDCs and NK cells was seen for the early activation marker CD69 (median fluorescence intensity [MFI] was increased 37- and 7-fold on pDCs and CD56dim NK cells, respectively), confirming that RNA-ICs are potent activators of both pDCs and NK cells (Fig. 1A). Remarkably, in addition to CD69, CD319 showed a strong increase in surface expression on both pDCs and CD56dim NK cells (2.7- and 2.2-fold mean increase in MFI, respectively, Fig. 1A). In contrast, RNA-ICs did not induce an increase in expression of CD69 or CD319 on B cells or T cells (Supplemental Fig. 1A).

The finding of dynamic regulation of CD319 expression, a receptor belonging to the immunomodulatory SLAM receptor family associated with SLE, prompted us to examine the expression of all seven SLAM family members on pDCs and NK cells before and after RNA-IC stimulation. In freshly isolated PBMCs, nearly all pDCs expressed CD48, CD229, and CD319, whereas expression of CD84 and NTB-A was lower.

CD319 and CD229 expression is increased on both pDCs and NK cells after RNA-IC stimulation

Concentration of IFN-α in culture supernatants was determined using a dissociation-enhanced lanthanide fluoroimmunoassay, as previously described (24).

For detection of intracellular IFN-α, PBMCs depleted of monocytes (1 × 10^6/well) were stimulated with RNA-ICs for 9 h, with the addition of GolgiPlug (BD Biosciences) after 6 h. Cells were spun down and stained for surface markers, fixed in 1% paraformaldehyde, and permeabilized with 0.5% saponin (Sigma-Aldrich) before being stained with allophycocyanin-conjugated anti-human IFN-α (LT27:295; Miltenyi Biotec) and analyzed by flow cytometry.

Stimulation of cells
SLE IgG was purified by protein G chromatography from a patient serum containing autoantibodies to Sm1, Sm2, U1-RNP, A, U1-RNP C, ribosomal P Ag, histone, and dsDNA (2). U1snRNP particles were purified from HeLa cells, as previously described (23).

IFN-α detection
Concentration of IFN-α in culture supernatants was determined using a dissociation-enhanced lanthanide fluoroimmunoassay, as previously described (24).
expressed a similar profile of SLAM molecules as did pDCs, but they displayed higher levels of NTB-A, lower levels of CD84, and expressed CD244. Of note, in contrast to CD56dim NK cells, CD56bright NK cells expressed very low levels of CD319 (Fig. 1B, 1C). Upon RNA-IC stimulation, we found that, in addition to CD319, the expression of CD229 was increased on pDCs (1.9-fold) and CD56bright NK cells (2.0-fold) (Fig. 1D). In contrast, none of the SLAM receptors was regulated by RNA-ICs on B cells or T cells (Supplemental Fig. 1B).

To assess the dynamics of the regulation of CD319 and CD229, PBMCs were stimulated with medium or RNA-ICs and analyzed at different time points. In pDCs, the increased expression of CD319 and CD229 started 3 h after RNA-IC stimulation and continued for the duration of the study (Fig. 1E). In CD56dim NK cells, the expression of CD319 decreased during the first 3 h in both unstimulated and RNA-IC–stimulated cultures. This decrease continued in unstimulated cultures, whereas there was increased CD319 expression in RNA-IC–containing cultures. The CD229 expression in CD56bright NK cells decreased during the time course studied; after 6 h only a very low percentage was positive for CD229 (data not shown). Therefore, the increased MFI after RNA-IC stimulation only resulted in a minor increase in CD229+ CD56bright NK cells; therefore, we chose not to study this regulation further.

In summary, stimulation of PBMCs with RNA-IC induces a rapid and prominent increase in the expression of the SLAM family members CD319 and CD229 on pDCs, as well as CD319 on CD56dim NK cells.

**RNA-ICs induce CD319 and CD229 via both FcγR and TLR stimulation**

Next, we asked whether the ability to stimulate the increased expression of CD319 and CD229 on pDCs and NK cells was specific for interferogenic ICs or whether it could be triggered by ICs without RNA or DNA content. Stimulation of PBMCs with HAIG from healthy donors also induced CD319 and CD229 expression on pDCs and CD319 on CD56dim NK cells, but the induction by RNA-ICs was significantly higher (Fig. 2A; p = 0.0001, p = 0.03, and p = 0.005, respectively). Because the synthetic TLR9 agonist ODN2216 also induced expression of these molecules (Fig. 2A), it seemed likely that the effect from RNA-ICs was a combination of FcγR stimulation and endosomal TLR stimulation.
To verify TLR involvement in the regulation of CD319 and CD229, cells were treated with chloroquine prior to RNA-IC stimulation. Interfering with endosomal TLR signaling by chloroquine partially blocked the RNA-IC-induced upregulation of CD319 and CD229 on pDCs (Fig. 2B; *p = 0.03 and *p = 0.03, respectively), whereas the difference observed on NK cells was not significant (*p = 0.06). As expected, chloroquine treatment did not affect HAIG-mediated induction of CD319 and CD229, but it completely blocked ODN2216-mediated induction of CD319 and CD229 (Fig. 2B). Furthermore, preincubation with cycloheximide or actinomycin D completely blocked the upregulation of CD319 and CD229 on pDCs and NK cells stimulated by RNA-ICs (Fig. 2C), demonstrating the requirements for both transcription and translation for CD319 and CD229 expression.

Together, these results show that both FcγR stimulation and endosomal TLR ligation induce CD319 and CD229 expression on pDCs. Moreover, both de novo transcription and translation are required to induce these molecules.

**CD319 expression is upregulated directly on CD56dim NK cells and indirectly on pDCs by RNA-ICs**

To investigate the requirements for the increased expression of CD319 and CD229 on pDCs and NK cells, we stimulated purified cells with RNA-ICs. Surprisingly, when isolated pDCs were stimulated neither CD319 nor CD229 was upregulated (Fig. 3A). Because the stimulation of NK cells by RNA-ICs. Surprisingly, when isolated pDCs were stimulated neither CD319 nor CD229 was upregulated (Fig. 3A). Because the stimulation of NK cells by RNA-ICs.

To further dissect the mechanisms for the increased expression of CD319 and CD229, we stimulated purified pDCs or NK cells with RNA-ICs, HAIG, or ODN2216. HAIG did not induce CD319 or CD229 on pDCs, whereas it was as efficient as RNA-ICs on CD56dim NK cells, and this increase was even larger than that seen on CD56dim NK cells in RNA-IC-stimulated PBMCs (Fig. 3A).

Thus, together these data suggest that ligation of FcγRs on NK cells and stimulation of endosomal TLRs in pDCs induces the expression of CD319 on NK cells and CD319/CD229 on pDCs, respectively. Moreover, the increased expression of these molecules seems to be further enhanced by a bidirectional cross-talk between pDCs and NK cells.

To investigate whether soluble factors secreted by NK cells were important for the regulation of CD319 and CD229, isolated pDCs were stimulated with RNA-ICs in the presence of supernatants from RNA-IC–stimulated NK cells or unstimulated NK cells. Supernatants from RNA-IC–stimulated NK cells significantly enhanced the expression of CD319 and CD229 on RNA-IC–stimulated pDCs (Fig. 3C, *p = 0.03 for both), whereas supernatants from unstimulated NK cells had no effect (*p = 0.38 and *p = 0.63, respectively), indicating that secreted soluble factors were needed for the regulation of CD319 and CD229. Of note, in comparison with NK cell supernatants, NK cells induced a significantly higher expression of CD319 and CD229 (*p = 0.03 for both).

RNA-IC stimulation of pDC–NK cell cocultures induces the production of multiple cytokines that can affect both pDCs and NK cells (15). To investigate which cytokines could be responsible for the increased expression of CD319 and CD229, purified pDCs were cultured with or without RNA-IC stimulation and the addition of a selection of recombinant cytokines, which are either produced in pDC–NK cell cocultures (15) or known to have a stimulatory effect on pDCs (25). A weak increase in CD319 and CD229 expression on pDCs was seen after the addition of rTNF-α, rGM-CSF, and rIL-3 (Fig. 3D). However, when pDCs were stimulated with RNA-ICs together with GM-CSF or IL-3, an equally strong increase in CD319 and CD229 expression was seen as when NK cells were used (Fig. 3D). To investigate whether CD319 and CD229 could be induced in the absence of RNA-ICs, pDCs were stimulated with combinations of GM-CSF, IFN-α, and IFN-γ. A combination of GM-CSF and IFN-α or IFN-γ was as potent an inducer of CD319 and CD229 as was RNA-ICs in combination with GM-CSF (Fig. 3E). On CD56dim NK cells, expression of CD319 was not affected by GM-CSF or IL-3, but it was increased strongly following IFN-α or IL-12/IL-18 stimulation. This effect was seen even in the absence of RNA-ICs (Fig. 3D).

In conclusion, RNA-ICs, IFN-α, and IL-12/IL-18 induce the expression of CD319 directly on CD56dim NK cells, whereas pDCs require a combination of stimuli, such as RNA-ICs together with NK cells, RNA-ICs together with cytokines (e.g., GM-CSF or IL-3), or multiple cytokines (e.g., GM-CSF and IFN-α) to increase their expression of CD319 and CD229.
IFN-α–producing pDCs have higher CD319 and CD229 expression

Because the expression of CD319 and CD229 was upregulated by interferogenic ICs, we asked whether there was a difference in surface expression of SLAM molecules between the pDCs that produced IFN-α and the pDCs that did not. Because monocytes inhibit IFN-α production by pDCs (14), PBMCs were depleted of monocytes before stimulation with RNA-ICs. After stimulation, pDCs were evaluated for intracellular IFN-α and SLAM family member expression. The frequency of pDCs that produced IFN-α
after RNA-IC stimulation was 31.5 ± 7.3% (mean ± SD). Nearly all IFN-α⁺ pDCs expressed CD319, CD229, and CD84, whereas only a subpopulation of the IFN-α⁺-producing pDCs expressed NTB-A (Fig. 4A). Interestingly, the IFN-α⁺-producing pDCs had a significantly higher expression of CD319, CD229, and NTB-A compared with the IFN-α⁻ pDCs, as determined by the MFI (Fig. 4B).

Thus, following RNA-IC stimulation, the IFN-α⁺-producing pDCs are characterized by a higher expression of CD319, CD229, and NTB-A compared with IFN-α⁻ pDCs.

pDCs do not express the activating, but do express the inhibitory, adaptor molecules of SLAM receptors

Given the strong regulation of CD319 and CD229 by interferogenic ICs and their increased expression on IFN-α⁺-producing pDCs, we next investigated whether CD319 and/or CD229 affected IFN-α production. To this end, CD319 or CD229 was cross-linked on RNA-IC–stimulated pDCs through plate-bound mAbs. Anti-BDCA-2 mAb, which inhibits IFN-α production by pDCs (26), was used as a control. Although BDCA-2 cross-linking strongly inhibited IFN-α production, cross-linking of CD319 or CD229 had no effect (Fig. 5A).

To better understand the function of CD319 and CD229 on pDCs, we investigated the downstream-signaling molecules of these receptors. The signaling through SLAM receptors is mediated via the activating SH2 domain–containing adaptor molecules SAP and EAT2 (27). Although NK cells are known to express both, the expression of SAP and EAT2 has not been studied in pDCs. To determine whether pDCs expressed SAP and EAT2, Western blots of whole-cell lysates from isolated pDCs and NK cells were performed. No expression of SAP was observed in pDCs, whereas a strong expression was seen in NK cells (Fig. 5B). EAT2 was highly expressed in NK cells but barely detectable in pDCs. A similar weak signal from EAT2 was seen from all cell types tested (data not shown) and probably reflects background staining.

In the absence of activating adaptor molecules, SLAM receptors were suggested to signal via inhibitory SH2 domain–containing adaptor molecules (e.g., SHIP-1, SHP-1, SHP-2, and CSK) (27). Western blots of whole-cell lysates from purified pDCs and NK cells revealed that both cell types expressed these molecules (Fig. 5C).

FIGURE 4. IFN-α⁺⁻ and IFN-α⁺⁺ pDCs differ in SLAM family member expression. (A and B) Expression of indicated SLAM family members on IFN-α⁺⁻ and IFN-α⁺⁺ pDCs in RNA-IC–stimulated CD14-depleted PBMCs was determined by flow cytometry. Data from four healthy volunteers are presented as frequency (%) in (A) and MFI in (B). pDCs were defined as CD3⁺CD11c⁺CD14⁻CD19⁻CD56⁻HLA-DR⁺BDCA-4⁺ cells. Statistical analysis was performed using the paired t test.

In summary, cross-linking of CD319 or CD229 alone on pDCs does not influence IFN-α production. Furthermore, pDCs lack expression of SAP and EAT2 but express SHIP-1, SHP-1, SHP-2, and CSK.
Altered expression of CD319 and CD229 on pDCs and NK cells from patients with SLE

Given the potent regulation of SLAM receptors on pDCs and NK cells by RNA-ICs in vitro, and the fact that patients with SLE have similar interferogenic ICs in circulation, we compared the expression of all SLAM receptors on pDCs and NK cells from patients with SLE and healthy individuals.

Patients with SLE have a reduced frequency of circulating pDCs (24), and two of the patients in this cohort were excluded from the analysis of pDCs because they had too few cells (<100 events) to evaluate. Like pDCs from healthy individuals, almost all SLE pDCs expressed CD48, CD229, and CD319 (data not shown). However, pDCs from patients with SLE had a significantly lower expression of CD84 and CD319 ($p = 0.04$ and $p = 0.001$, respectively) (Fig. 6A). A slightly lower expression of CD229 was also seen on SLE pDCs, but the difference was not statistically significant ($p = 0.102$).

We observed a decreased expression of CD229 and an increased expression of CD150 on CD56$^{\text{dim}}$ NK cells from SLE patients ($p = 0.002$ and $p = 0.005$, respectively), whereas CD56$^{\text{bright}}$ NK cells had an increased expression of CD319 and CD48 ($p = 0.01$ and $p = 0.009$, respectively).

Finally, PBMCs from patients with SLE were stimulated with RNA-ICs, and the regulation of CD319 and CD229 on pDCs and CD56$^{\text{dim}}$ NK cells was compared with that of healthy donors.

Similar to cells from healthy donors, pDCs and CD56$^{\text{dim}}$ NK cells from patients with SLE responded to RNA-IC stimulation with an increased expression of CD319 and CD229 on pDCs and CD319 on CD56$^{\text{dim}}$ NK cells (Fig. 6B).

Thus, patients with SLE have an altered expression of several SLAM receptors, including both CD319 and CD229, on pDCs and NK cells.

FIGURE 6. Altered expression of SLAM family receptors on immune cells from patients with SLE. (A) PBMCs were stained for SLAM family receptors and analyzed by flow cytometry. Expression (MFI) of indicated SLAM family receptor on pDCs, CD56$^{\text{dim}}$ NK cells, and CD56$^{\text{bright}}$ NK cells, as specified. Live single-cell lymphocytes were gated according to viability stain and forward light scatter/side scatter characteristics. pDCs were gated as CD14$^{-}$CD3$^{-}$CD19$^{-}$CD16$^{-}$CD56$^{-}$BDCA-2$^{+}$CD4+, and NK cells were gated as CD14$^{-}$CD3$^{-}$CD19$^{-}$CD56$. Data from 19 healthy donors (HD) and 19 patients with SLE are shown for pDCs, and data from 19 healthy donors (HD) and 21 patients with SLE are shown for CD56$^{\text{dim}}$ and CD56$^{\text{bright}}$ NK cells. Horizontal lines denote the median value. (B) PBMCs from six healthy donors (HD) or five patients with SLE were cultured for 6 h, with or without the addition of RNA-IC, and expression of CD319 and CD229 was determined by flow cytometry on pDCs and CD56$^{\text{dim}}$ NK cells. Data from two independent experiments are presented as fold increase in MFI in RNA-IC-stimulated cells relative to cells cultured in medium. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Mann–Whitney $U$ test.
Discussion

SLAM family receptors are important immunomodulatory receptors involved in the cross-talk between a number of immune cells (16). The SLAM locus is genetically associated with SLE (17–21), and recent data suggest involvement of SLAM receptors in the pathogenesis of lupus (28–30). In the current study, we found that the expression of the SLAM family receptors CD319 and CD229 was dynamically regulated on pDCs and CD56^dim NK cells by RNA-ICs typically found in lupus. Importantly, there was no general upregulation of all SLAM molecules on pDCs and NK cells or an increase in SLAM family receptor expression on T and B cells by RNA-ICs. Thus, lupus ICs seem to selectively regulate the expression of certain SLAM molecules on two cell types, which together have the capacity to produce large amounts of type I IFN after activation.

Interferogenic ICs, such as RNA-ICs, have the capacity to activate both FcγRIIA and endosomal TLRs. We showed that signaling induced by engagement of both the IgG component and the nucleic acid–containing autoantigen in the RNA-ICs contributed to the increased expression of CD319 and CD229 on pDCs and CD319 on CD56^dim NK cells. When the mechanisms behind the regulation of CD319 and CD229 were investigated further, we found that transcription and translation were required. Experiments using isolated or mixed populations of purified pDCs and NK cells stimulated with RNA-ICs revealed that the upregulation of CD319 and CD229 on pDCs by RNA-ICs required NK cells. This effect was partially mediated by soluble factors produced by NK cells, because supernatant from RNA-IC–stimulated NK cells increased CD319/CD229 expression. RNA-ICs in combination with GM-CSF or IL-3, or GM-CSF together with IFN-α increased CD319/CD229 expression. RNA-ICs in combination with GM-CSF or IL-3, or GM-CSF together with IFN-α or IFN-γ was an equally strong inducer of CD319 and CD229 on pDCs as NK cells and RNA-ICs. These results demonstrate that the regulation of at least some SLAM molecules on pDCs is complex and involve several pathways that need to act in concert to trigger an increased expression. In support of this conclusion, a previous study showed that the combination of HSV and IL-3 is necessary to upregulate CD319 in pDCs (31). Whether the induction of SLAM molecules is coupled to maturation of pDCs to efficient Ag-presenting dendritic cells is unknown, but this clearly is a possibility given the fact that both IL-3 and GM-CSF are stimuli for pDC differentiation (25).

In contrast to pDCs, purified NK cells activated by RNA-ICs exhibited a strong increase in CD319 expression on CD56^dim NK cells. This increase was even more pronounced than the increase in CD319 among CD56^dim NK cells in PBMC cultures. Consequently, inhibitory cells present in the PBMC population may suppress the upregulation of SLAM molecules on NK cells, possibly via the release of reactive oxygen species, TNF-α, and PGE_2, which suppress NK cell function (14). We also noted that IFN-α and IL-12/IL-18 could upregulate CD319 on CD56^dim NK cells, representing an interesting mechanism whereby inflammation may augment NK cell recognition of, and activation by, hematopoietic cells. Notably, RNA-ICs or proinflammatory cytokines increased the CD319 expression on NK cells to comparable levels. Thus, it appears that at least partially different signaling pathways lead to CD319 expression in pDCs and NK cells. Furthermore, we noted that IL-12/IL-18 increased the expression of CD319 on CD56^bright NK cells (data not shown). These cytokines are well-known activators of NK cells (32), and one mechanism by which both IL-12/IL-18 and RNA-ICs might contribute to increased NK cell cytotoxicity is by upregulation of CD319.

The pDC population is heterogeneous with respect to the expression of several molecules and the capacity to produce IFN-α. To clarify whether there was any difference in the expression of different SLAM molecules between pDCs with and without the ability to synthesize IFN-α, we stimulated the cells with RNA-ICs and investigated SLAM expression and IFN-α content among the pDCs. This experiment revealed that the IFN-α–producing pDCs displayed higher expression of CD319 and CD229, as well as NTB-A, compared with IFN-α− cells. It is well known that only FcγRIIA-expressing pDCs have the capacity to produce IFN-α in response to interferogenic ICs (33, 34), and the differences in SLAM expression between IFN-α+ and IFN-α− pDCs could be due to the lack of FcγRIIA/TLR-7 activation of the latter population. Thus, our observation may indicate that induction of IFN-α production and increased SLAM molecule expression by RNA-IC stimulation of pDCs are linked processes in the activation of pDCs, although this needs to be formally proven.

On NK cells, CD319 and CD229 act as coactivating receptors, which results in increased cytotoxicity against targets cells expressing CD319 and CD229 (35, 36), whereas, on B cells, ligation of CD319 induces proliferation and cytokine production (37). In contrast, no study of the function of SLAM receptors on pDCs has been reported. When investigating the potential role of CD319 and CD229 on pDC function, we noted that cross-linking of these receptors by immobilized mAbs did not affect the RNA-IC–induced IFN-α secretion in isolated pDCs. This finding does not necessarily exclude the involvement of CD319 and CD229 in regulating IFN-α production, because SLAM receptors function as coreceptors. Thus, CD319 and CD229 may be dependent on concomitant engagement of other activating or inhibitory receptors to exert their effect. Although such receptors are well characterized on NK cells, the identity of these receptors on pDCs remains to be determined. Blocking experiments might be a way to circumvent the need for coligation of other receptors, but the lack of Abs that effectively block the homophilic binding of CD319 or CD229 regrettably precludes these experiments. Given these circumstances, we cannot exclude a function for CD319 and CD229 in regulating IFN-α production.

With the exception of CD48, the SLAM molecules contain intracellular immuno-tyrosine switch motifs that bind and signal via the SH2 domain–containing adaptor molecules SAP and EAT2 (27). CD319 differs from the other SLAM family receptors in that it only recruits EAT2 (38). SAP is expressed in T cells, NK cells, NKT cells, and perhaps some B cells, whereas EAT2 expression has been described in NK cells, dendritic cells, and macrophages (27). The presence of SAP or EAT2 has not previously been investigated in pDCs. When isolated pDCs and NK cells were examined for SAP and EAT2, no SAP and little to no EAT2 were detected in pDCs. In contrast, and as expected, high levels of both SAP and EAT2 were detected in NK cells. Ligation of CD319 in NK cells deficient in EAT2 was shown to abolish the activating effect of CD319 and result in inhibition of NK cell cytotoxicity (39). Furthermore, ligation of CD319 on CD4+ T cells or monocytes, which naturally lack EAT2 expression, inhibits Ag-induced proliferation and LPS-induced production of TNF-α, respectively (39, 40). The inhibitory effect of SLAM receptors in cells lacking SAP and EAT2 probably can be explained by the fact that, in the absence of these molecules, SLAM receptor signaling couples to inhibitory adapter proteins, such as SHIP-1, SHP-1, SHP-2, and CSK (27). CD319 differs from the other SLAM family receptors in that it contains RIIA-expressing pDCs have the capacity to produce IFN-α due to the lack of FcγRIIA/TLR-7 activation of the latter population. Thus, our observation may indicate that induction of IFN-α production and increased SLAM molecule expression by RNA-IC stimulation of pDCs are linked processes in the activation of pDCs, although this needs to be formally proven.
expression of CD229 and CD319 on pDCs may support the pDC–NK cell cross-talk. 

Finally, we investigated the expression of the SLAM molecules on pDCs and NK cells from patients with SLE. Patients with SLE had decreased expression of CD84 and CD319 on circulating pDCs, which is at variance with the in vitro stimulation of PBMCs from healthy individuals with RNA-ICs. However, RNA-IC stimulation of SLE PBMCs increased the expression of CD319 and CD229 on pDCs, demonstrating that SLE pDCs retain their capacity to upregulate these molecules. One explanation for the decreased SLAM expression on circulating pDCs in SLE could be that SLE IC-activated cells migrate from the circulation to inflamed tissues. Such redistribution from blood to tissues of pDCs that produce IFN-α was also described in SLE (42, 43) and might explain our observation. On SLE NK cells, decreased expression of CD229 on CD56dim NK cells and increased expression of CD319 and CD48 on CD56bright NK cells were observed. The exact consequence of the altered expression of SLAM molecules in SLE NK cells is unknown, but it might reflect several previous observations. For instance, the lower expression of CD229 on CD56dim NK cells may contribute to the decreased cytokotopy of SLE NK cells (44, 45). In contrast, the increased expression of SLAM molecules on CD56bright NK cells may play a role in the increased proinflammatory cytokine production in SLE (46). Conceivably supporting the latter interpretation is the recent observation that patients with SLE have increased expression of CD229 and NTB-A on T cells and that activation of CD229 or NTB-A, together with CD3, promotes IL-17 production (30).

In summary, our study shows that RNA-ICs regulate a subset of SLAM molecules on pDCs and NK cells and that lupus patients have an altered expression of CD319 and CD229 on these cell types. Our observations are important in light of emerging data demonstrating that the SLAM family of receptors is implicated in autoimmune disease processes. Because SLAM molecules have been suggested as both possible biomarkers and therapeutic targets in SLE (30), further studies of the precise function of SLAM receptors on pDCs and their role in the pathogenesis of SLE seem highly warranted.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


producing cells (plasmacytoid dendritic cells) and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG. J. Immunol. 171: 3296–3302.


**SUPPLEMENTARY TABLE I.** Receptors and ligands analyzed on pDCs and NK cells.

<table>
<thead>
<tr>
<th><strong>Activating NK receptors</strong></th>
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<tr>
<td>NKG2C - HLA-E</td>
<td></td>
</tr>
<tr>
<td>NKp30 -</td>
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<tr>
<td>NKp80 -</td>
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<tr>
<th><strong>Inhibitory NK receptors</strong></th>
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<tr>
<td>NKG2A - HLA-E</td>
<td></td>
</tr>
<tr>
<td>HLA-ABC</td>
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<tr>
<td>HLA-G</td>
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<tr>
<th><strong>Adhesion molecules</strong></th>
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<td>CD11a - ICAM-1</td>
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<td>ICAM-2</td>
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<td>ICAM-3</td>
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<td>CD31 - CD38</td>
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<td>ULBP2</td>
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<td>ULBP3</td>
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<th><strong>Other molecules</strong></th>
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<tr>
<td>CD69</td>
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<td>CD7</td>
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Receptor and ligands analyzed on pDCs, CD56\textsuperscript{dim} NK and CD56\textsuperscript{bright} NK cells by flow cytometry following 6 h culture of PBMC with or without addition of RNA-IC.
Supplementary Figure 1

A

CD69

CD319

B

CD150
CD48
CD229
CD244
CD84
NTB-A
CD319

Fold increased MFI
**SUPPLEMENTARY FIGURE 1.** Expression of CD69 and SLAM family receptors on B cells and T cells. PBMCs were cultured for 6 h in medium only or stimulated with RNA-IC, and surface expression of CD69 and SLAM family receptors was determined by flow cytometry on B cells, CD8+ and CD8− T cells, as specified. (A) Histograms for culture in medium only are depicted by dashed lines, RNA-IC by solid lines and isotype staining by shaded gray. (B) Fold increase in surface expression (median fluorescence intensity (MFI)) of SLAM family members in 6 h RNA-IC-stimulated PBMC cultures relative to culturing in medium only. Data (mean±SD) are from four healthy donors. B cells were gated as CD11c−CD14−CD3−CD19+ and T cells as CD11c−CD14−CD19−CD3+ cells.