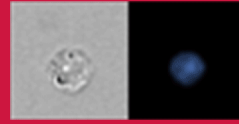


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CUTTING EDGE

Cutting Edge: Lectin-Like Transcript 1 Is a Ligand for the CD161 Receptor¹

Hatice Aldemir,^{2,3*} Virginie Prod'homme,^{2,4*} Marie-Jeanne Dumaurier,^{*} Christelle Retiere,[†] Gwenola Poupon,^{*} Julie Cazareth,^{*} Franck Bihl,^{*} and Veronique M. Braud^{5*}

Human NK cells and subsets of T cells or NKT cells express the orphan C-type lectin receptor CD161 (NKR-P1A) of unknown function. In contrast to rodents that possess several NKR-P1 genes coding for either activating or inhibitory receptors, the nature of signals delivered by the single human NKR-P1A receptor is still to be clarified. In this article, we show that the lectin-like transcript 1 (LLT1) molecule is a ligand for the CD161 receptor. Engagement of CD161 on NK cells with LLT1 expressed on target cells inhibited NK cell-mediated cytotoxicity and IFN- γ secretion. Conversely, LLT1/CD161 interaction in the presence of a TCR signal enhanced IFN- γ production by T cells. These findings identify a novel ligand/receptor pair that differentially regulate NK and T cell functions. The Journal of Immunology, 2005, 176: 7791–7795.

Natural killer cells are innate immune lymphocytes that can also participate in the initiation and development of adaptative immune responses. They mediate their role through production of cytokines and direct killing of transformed or infected cells. Their function is tightly regulated by a fine balance of inhibitory and activating signals that are delivered by a diverse array of cell surface receptors (1, 2). A prerequisite for a NK cell attack is the presence on target cells of ligands for activating receptors and low level or absence of ligands for inhibitory receptors. It was believed that NK self-tolerance was achieved by expression on each NK cell of at least one self-MHC specific inhibitory receptor (3). However, this dogma has been challenged recently by the identification of a NK cell population in normal mice that lack inhibitory receptors specific for self-MHC class I molecules (4, 5). Therefore, additional surface receptors contribute to NK self-tolerance and to the modulation of NK cell responses. Their characterization and the identification of their physiological ligands are required to allow a comprehensive understanding of NK cell function.

Human CD161 (NKR-P1A) is a C-type lectin receptor whose ligand and function still remain to be elucidated. It is likely to play a crucial role because it is expressed by most human NK cells and also by CD1d-restricted NKT cells and subsets of circulating and tissue-infiltrating T cells (6, 7). A single human gene *NKR-P1A* has been cloned while rodents possess several *NKR-P1* genes (6). Anti-CD161 Abs either had no effect, stimulated, or inhibited human NK cells and costimulated NKT cells (2, 6, 8, 9). These effects could not be correlated with any known signaling motifs, absent from the CD161 sequence. Therefore, how CD161 signals remains elusive.

To understand the physiological role of CD161, we set out experiments to identify its ligand. It was described recently that some members of the mouse NKR-P1 family of receptors interact with C-type lectin-related (Clr)⁶ molecules. The inhibitory NKR-P1B and NKR-P1D recognize Clr-b and the activating NKR-P1F associates with Clr-g (10, 11). We speculated that the ligand of the CD161 receptor would be homologous to the mouse Clr molecules. The human lectin-like transcript 1 (LLT1) was found to be the best candidate with 43–48% of homology at the amino acid level. LLT1 seems to be primarily expressed by monocytes and B cells in peripheral blood (12). However, LLT1 is induced rapidly in PMA-stimulated PBMCs (13) and in IL-2-activated NK cells or T cells (14). Cross-linking of LLT1 with an Ab induced production of IFN- γ by NK cells (12). Using LLT1-multimer, we now show that LLT1 binds specifically to CD161. Interestingly, this interaction inhibits NK cell-mediated cytotoxicity and IFN- γ production while enhancing CD3-triggered IFN- γ production by T cells.

Materials and Methods

Generation of LLT1 multimers

Full-length LLT1 cDNA was amplified by RT-PCR from total RNA extracted from human polyclonal NK cells. The coding sequence for the extracellular domains of LLT1 (residues aa 60–191) was PCR amplified and cloned into pcDNA3 vector (Invitrogen Life Technologies) in frame with the signal sequence of human CD5 and the Fc portion of human IgG (hlgG)1. LLT1-Fc

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⁶ Abbreviations used in this paper: Clr, C-type lectin related; LLT1, lectin-like transcript 1; hlgG, human IgG; EGFP, enhanced GFP.

dimers were produced in 293T cells. Proteins of ~55 kDa under reducing conditions and ~110 kDa under nonreducing conditions were detected on a SDS-PAGE gel. Consistent with the presence of two *N*-glycosylation sites in the coding sequence of LLT1 extracellular domains, treatment with the endoglycosidase-F (PNGase-F) produced a protein of the predicted ~48 kDa size. A multimeric complex was generated by incubating the dimers with protein A-biotin (Pierce) at a molar ratio of 1:1. Saturating amounts of purified hIgG were added to block the remaining free protein A sites. A multimer control was generated using protein A-biotin saturated with purified hIgG. These complexes were conjugated to streptavidin-allophycocyanin (BD Biosciences). Ten micrograms of LLT1- and Ctrl-hIgG-multimers was incubated for 45 min at 4°C with 2×10^5 cells for each flow cytometry stain.

Generation of stable transfectants

Full-length CD161 cDNA amplified by RT-PCR from RNA extracted from human PBMCs and LLT1 cDNA was cloned into pIRES2-enhanced GFP (EGFP) vector (BD Clontech). Transfected 293T, C1R, HeLa, and P815 cells were selected in neomycin-containing medium, and EGFP-positive cells were cell sorted. CD161 expression was confirmed by flow cytometry using anti-CD161 Abs, and LLT1 expression was confirmed by RT-PCR.

Culture of polyclonal NK and T cell populations

PBMCs were separated by Ficoll-Paque Plus density gradient centrifugation (Amersham Biosciences) from blood purchased from the Etablissement Français du Sang. Polyclonal NK cells were isolated by positive magnetic selection with anti-CD56 MicroBeads (Miltenyi Biotec). Purified NK cells were cocultured subsequently with irradiated allogeneic PBMCs and B-EBV feeder cells in X-VIVO 15 medium (Cambrex) supplemented with 500 U/ml rIL-2 (Chiron). NK cells were maintained in rIL-2 or treated for 3 days with 5 ng/ml rIL-2 (R&D Systems) before functional assays. All the NK cells used contained <5% CD3⁺CD56⁺ T cells. Polyclonal T cells were cultured from the flow-through of the NK cell purification using anti-CD56 MicroBeads (Miltenyi Biotec). T cells were cultured in RPMI 1640 medium (Cambrex) supplemented with 10% FCS (Perbio) and 30 U/ml rIL-2.

Monoclonal Abs and flow cytometric analysis

NK cells were immunostained with anti-CD56 (N901, NKH1), anti-CD3 (UCHT1), and anti-CD161 191B8 (mouse IgG2a) or DX12 (mouse IgG1) (BD Biosciences and Beckman Coulter). DX12 and 191B8 mAbs recognize two different epitopes on CD161. Anti-TNP (mIgG2a and mIgG1) mAbs were used as isotype IgG controls. Fluorescence was analyzed on a FACSCalibur cytometer with the CellQuest Pro Software (BD Biosciences).

NK cell-mediated cytotoxicity assays

Four-hour ⁵¹Cr release assays were performed using standard techniques. Polyclonal NK cells were preincubated for 15 min with blocking anti-CD161 or isotype IgG control mAbs (10 μg/ml) and anti-CD16 mAb (0.5 μg/ml) before addition of the labeled target cells. Chromium release was measured in a multi detector gamma counter (United Technologies Packard). The percentage of specific lysis was calculated as ((experimental release) - (spontaneous release))/((maximum release) - (spontaneous release)) × 100.

Measurement of CD107a surface expression

Polyclonal NK cells were stimulated for 5 h with C1R or C1R-LLT1 at an E:T ratio of 1:5. Anti-CD107a (H4A3) Ab (40 μl/ml) (BD Biosciences) was added throughout the assay, and brefeldin A (10 μg/ml) (Sigma Aldrich) was added for the last 4 h.

Measurement of IFN-γ production

Polyclonal NK cells were incubated for 4 h in RPMI 1640 medium supplemented with 10% FCS (Perbio), rIL-2 (200 U/ml), and brefeldin A (10 μg/ml), either alone or with target cells C1R or C1R-LLT1 at an E:T ratio of 1:5. Intracellular IFN-γ was detected using the anti-IFN-γ (B-B1) mAb (Diaclone).

Results and Discussion

LLT1 interacts with the CD161 receptor

Because of LLT1 homology with the mouse Clr molecules, we tested the binding of LLT1 to CD161 using a multimerized soluble form of LLT1. Binding was tested onto 293T cells stably transfected with CD161 and compared with the parental cell line or 293T cells stably transfected with LLT1. The transfectants were selected on the basis of EGFP expression as the

full-length cDNAs of CD161 and LLT1 were cloned into pIRES2-EGFP vector. LLT1 multimers bound to 293T-CD161 cells but not to 293T or 293T-LLT1 cells while control hIgG-multimers did not stain any of the cells (Fig. 1A). This indicated that LLT1 interacts with CD161. The specificity of the binding was confirmed by blocking LLT1-multimer staining with increasing concentrations of anti-CD161 mAbs DX12 (Fig. 1B and 1C) and 191B8 (data not shown). When LLT1 multimers were incubated with fresh PBMCs or NK cell lines, we did not detect binding, which is likely to be correlated with the lower level of CD161 surface expression and/or low-affinity interaction as previously reported for other MHC class I- and mouse NKR-P1-multimers (10, 15). Alternatively, the binding of LLT1-multimers was impaired by the presence of LLT1 in *cis*- (14) similarly to the lack of binding of H-2D^k-multimers to Ly49A because of the presence of H-2D^d in the same cell (16). Our data demonstrate that LLT1 is the human ortholog of mouse Clr molecules (10, 11). Interestingly, CD161 and LLT1 both belong to the same family of C-type lectin receptors and were characterized independently as homodimers on the cell surface (6, 12).

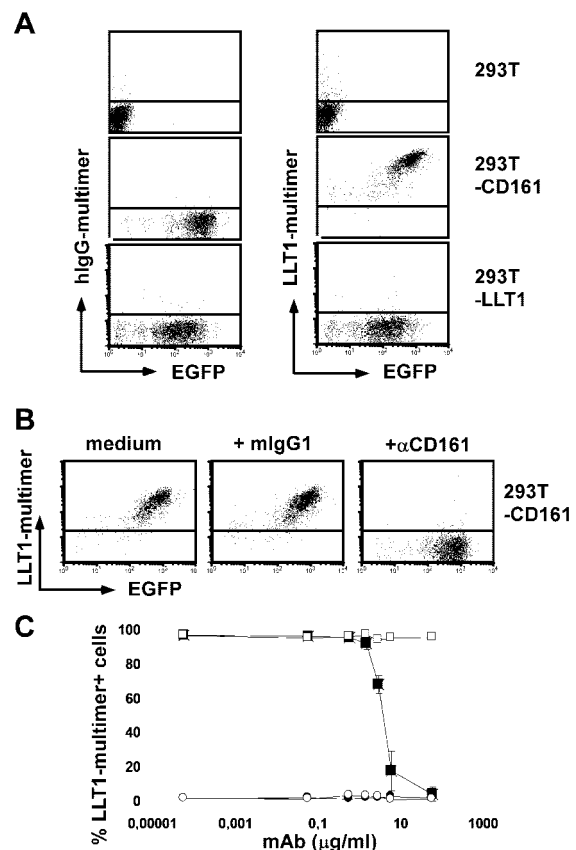


FIGURE 1. LLT1 is a ligand for the human CD161 receptor. *A*, 293T cells either untransfected or stably transfected with pIRES2-EGFP-CD161 or -LLT1 were stained with control hIgG- and LLT1-multimers. *B*, The binding of LLT1-multimer to 293T-CD161 transfectants was blocked specifically by anti-CD161 mAb DX12 (10 μg/ml). *C*, 293T-CD161 (squares) or 293T-LLT1 (circles) transfectants were stained with LLT1-multimer in the presence of increasing concentrations of anti-CD161 DX12 (filled symbols) mAb or anti-CD94 (open symbols) mAb as a control. Results are shown as mean ± SEM of three experiments.

LLT1 binding to CD161 inhibits NK cell-mediated cytotoxicity and IFN- γ production

To determine the outcome of LLT1 interaction with CD161 on NK cell effector functions, we stably expressed LLT1 in various NK cell targets. In the absence of specific Ab to LLT1, we confirmed the presence of LLT1 transcript by RT-PCR. None of the NK cell targets possessed the LLT1 transcript while it was detected in the stable transfectants (data not shown). Interestingly, we could also indirectly assess LLT1 surface expression because incubation of CD161⁺ NK cells or T cells with LLT1 expressing target cells consistently resulted in a reduction of CD161 level of expression, similarly to CD161 cross-linking using mAb (Figs. 2–5). This decrease was rapid, detected after a 15-min incubation of NK cells with C1R-LLT1 transfectants (Fig. 2A). The use of Transwells (0.4 μ m) prevented this effect (Fig. 2B), therefore excluding LLT1 shedding, which could block anti-CD161 mAb staining. Fixation of NK cells also prevented decrease of CD161 expression on NK cells exposed to C1R-LLT1 (Fig. 2A), suggesting that CD161 was internalized following binding to LLT1. Therefore, LLT1 interaction with CD161 affects cell surface expression of CD161 in a similar fashion to MIC-induced down-regulation of NKG2D (17). It remains to establish whether this mechanism is used to modulate LLT1/CD161 signaling events.

To test the effect of LLT1 binding to CD161 on NK cell killing, polyclonal NK cell populations were selected that were >99% CD161⁺. They were assessed for cytolytic activity against C1R, 293T, and Hela cell lines either untransfected or transfected with LLT1. We first used a novel assay measuring expression of the lysosomal-associated membrane protein-1 (or CD107a) at the NK cell surface after degranulation (18). A significant inhibition of CD107a cell surface expression was measured when NK cells were incubated with C1R-LLT1 com-

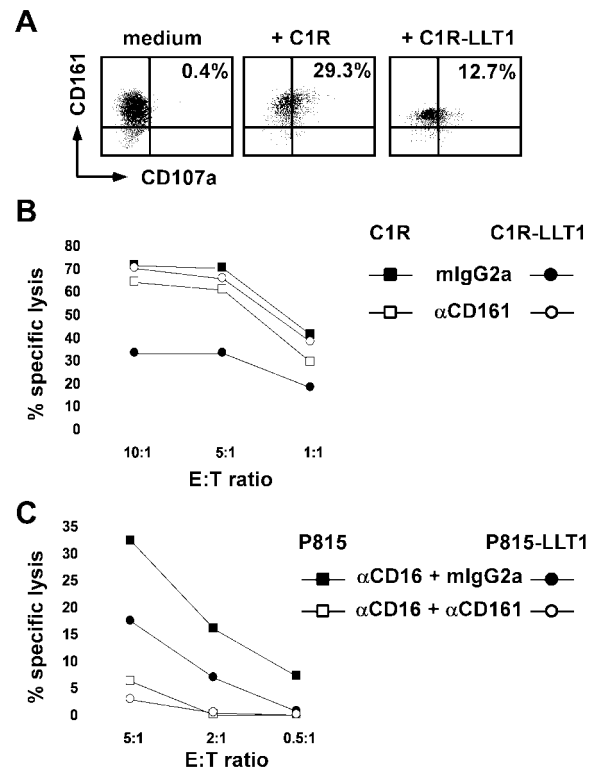


FIGURE 3. LLT1 interaction with CD161 inhibits NK cell-mediated cytotoxicity. *A*, Monitoring of CD107a expression on NK cells unstimulated or stimulated with C1R and C1R-LLT1 for 5 h. The percentage of NK cells expressing CD107a is indicated. *B*, C1R and C1R-LLT1 were used as targets in a 4-h ⁵¹Cr release assay with polyclonal CD161⁺ NK cells. Blocking mAb and isotype control mAb were added at 10 μ g/ml. *C*, Redirected killing assay using polyclonal NK cells and P815 cells transfected or not with LLT1. Suboptimal concentration of anti-CD16 mAb (0.5 μ g/ml) was added to anti-CD161 (191B8) or isotype control (10 μ g/ml) mAbs. Data are representative of four to eight experiments.

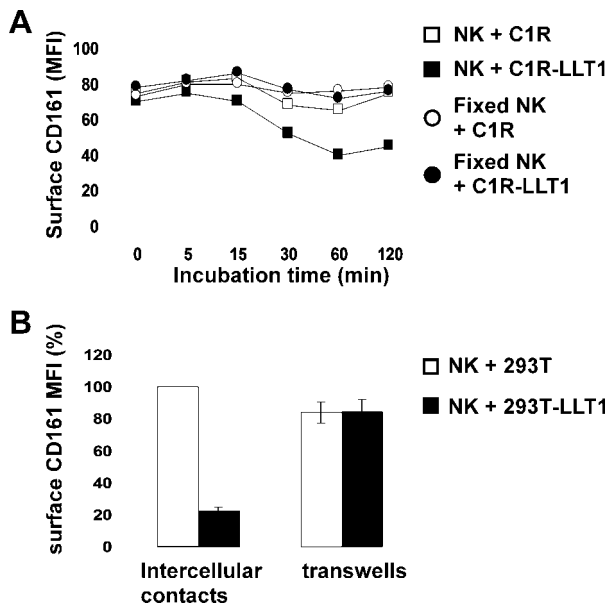


FIGURE 2. LLT1 interaction with CD161 results in a down-regulation of CD161. *A*, Polyclonal NK cells fixed or not before incubation with C1R and C1R-LLT1 cells were monitored for cell surface expression of CD161. *B*, Polyclonal NK cells were incubated for 4 h with 293T or 293T-LLT1 cells and CD161 cell surface expression was monitored. The mean fluorescence intensities obtained after exposure to control 293T cells were arbitrarily set as 100%. Results are shown as mean \pm SEM of three experiments.

pared with C1R (Fig. 3A). Degranulation was correlated with cytolytic activity. Polyclonal CD161⁺ NK cells killed the untransfected C1R cells while LLT1 expression significantly protected C1R target cells from lysis (Fig. 3B). Similar inhibition was obtained when 293T and Hela cells expressing LLT1 were incubated with NK cells (data not shown). Protection from lysis was specific as killing was restored by addition of blocking anti-CD161 but not isotype IgG control mAb (Fig. 3B). A contribution of EGFP to this effect was excluded as mock-transfected target cells behaved identically to untransfected targets (data not shown). The inhibition was enhanced when IL-12-activated polyclonal NK cells were used, which is consistent with IL-12 up-regulating CD161 surface expression (8). To further confirm these results, we performed a redirected killing assay using the FcR-bearing cell line P815. Both anti-CD161 mAb and expression of LLT1 inhibited the direct (data not shown) or anti-CD16-triggered killing of P815 (Fig. 3C). Altogether, these results demonstrate that LLT1 interaction with CD161 results in inhibition of NK cell-mediated cytotoxicity.

We next sought to assess the effect of LLT1 interaction with CD161 on the production of IFN- γ by NK cells. Polyclonal CD161⁺ NK cells were incubated for 4 h with target cells in the presence of brefeldin A, and IFN- γ production was measured by flow cytometry after intracellular staining. Incubation with C1R activated NK cells to produce IFN- γ but exposure to

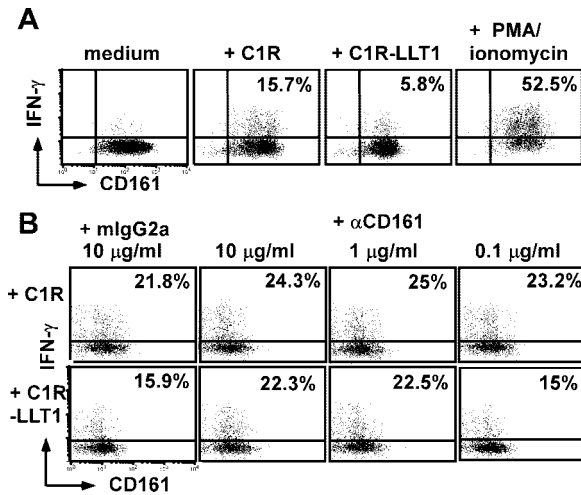


FIGURE 4. LLT1 binding to CD161 inhibits IFN- γ production by NK cells. Polyclonal NK cells were stimulated for 4 h with C1R and C1R-LLT1 in the presence of brefeldin A (10 μ g/ml) and rIL-2 (200 U/ml). The percentage of NK cells secreting IFN- γ is indicated. *A*, Incubation with target cells only or with PMA (5 ng/ml) and ionomycin (0.5 μ g/ml). *B*, Incubation in the presence of increasing concentrations of blocking anti-CD161 (191B8) or isotype control mAbs. Data are representative of eight independent experiments.

C1R-LLT1 significantly reduced the cytokine secretion (Fig. 4*A*). This inhibition resulted from LLT1 binding to CD161 as an anti-CD161 blocking mAb restored the production of IFN- γ by NK cells incubated with C1R-LLT1 to a level similar to NK cells stimulated with C1R (Fig. 4*B*). Addition of anti-CD161 mAb to NK cells cocultured with C1R had no effect, which is consistent with the lack of LLT1 transcription in C1R. Inhibition of IFN- γ production by NK cells following LLT1 interaction with CD161 was observed consistently independently of the state of activation of the polyclonal NK cells and was enhanced by pretreatment of NK cells with rIL-12 (Fig. 4 and data not shown). Production of IFN- γ induced by plate-bound anti-CD16 mAb was also significantly inhibited by addition of anti-CD161 mAb (data not shown).

Previous studies using Abs to cross-link CD161 had given conflicting results with either no effect, inhibition, or activation of NK cell functions (2, 6, 8). We now demonstrate that binding of CD161 on NK cells to LLT1 on target cells decreases NK cell-mediated cytotoxicity and IFN- γ production. This inhibition was measured using polyclonal NK cell populations expressing various other inhibitory receptors such as killer Ig-related receptors, CD94/NKG2A, and LIR-1/ILT-2. Therefore, LLT1 binding to CD161 seems to represent another pair of ligand/receptor with significant inhibitory capacity in NK cells.

CD161 engagement with LLT1 enhances IFN- γ production triggered by CD3 ligation on T cells

CD161 is expressed by subpopulations of circulating and tissue-infiltrating T cells and by CD1d-restricted NKT cells (6, 7). Anti-CD161 mAb costimulated TCR-mediated cytokine secretion and proliferation of NKT cells (9). With the knowledge of the ligand, it was therefore important to further investigate the role of CD161 on T cell function. Polyclonal T cells were stimulated with plate-bound anti-CD3 and/or anti-CD161 mAbs or with P815 cells expressing LLT1 in the presence of anti-CD3 mAb. By contrast to CD3 cross-linking, CD161 ligation did not trigger IFN- γ production by T cells

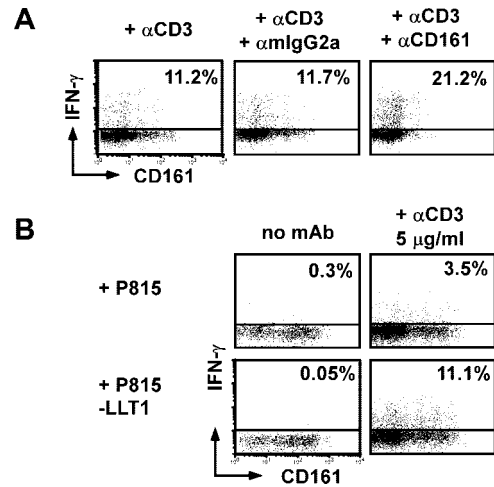


FIGURE 5. Simultaneous ligation of CD161 and CD3 enhances IFN- γ production by T cells. *A*, Polyclonal T cells were incubated for 4 h with plate-bound anti-CD3 (500 ng/ml) alone or together with isotype control or anti-CD161 (191B8) (5 μ g/ml) mAbs. *B*, Polyclonal T cells were stimulated with P815 transfected or not with LLT1 in the presence of anti-CD3 mAb (5 μ g/ml). Dot plots represent the percentage of T cells secreting IFN- γ . Data are representative of two to four independent experiments.

(data not shown). However, simultaneous engagement of CD3 and CD161 enhanced IFN- γ secretion compared with CD3 ligation alone (Fig. 5, *A* and *B*). A similar increase in IFN- γ production was seen using a CD161⁺ T cell clone TALL-104 (data not shown). These results reveal an interesting feature of CD161 in that it is capable of triggering opposing signals in NK and T cells, despite transcripts and proteins being identical in both cell types (data not shown). Although several genes encode for NKR-P1 receptors with either activating or inhibitory function in rodents, a single human *NKR-P1* gene has been cloned to date, which therefore signals differently depending on the cell type in which it is expressed. CD161 is not the sole receptor exerting opposing effects on NK and T cell functions. CD81 interacting with hepatitis C virus envelope protein E2 shows similar behavior (19, 20). However, CD81 and CD161 do not seem to associate in our preliminary coimmunoprecipitation experiments (data not shown). An additional intriguing observation is that CD161 does not possess any of the known signaling motifs. Therefore, further studies are needed to understand how CD161 exerts its different effects on NK and T cells.

In conclusion, these data clearly demonstrate that LLT1 is a ligand for the CD161 receptor, and their interaction differentially regulates NK and T cell functions. LLT1 is a host-encoded non-MHC ligand. Together with MHC-specific inhibitory receptors, this novel interaction may contribute to NK self-tolerance. LLT1/CD161 interaction may also be used to spare MHC class I-deficient cells from NK cell attack. In addition, LLT1 engagement of CD161 augmented TCR-dependent IFN- γ production by T cells, which is likely to play a significant role in immune responses. Interestingly, CD161 is more frequently expressed on T and NKT cells than any other NK receptors (2). Its relevance in diseases needs to be assessed. Finally, a recent study reported that LLT1 cross-linking with a mAb induced production of IFN- γ by NK cells (12), suggesting that LLT1/CD161 signaling may be bi-directional. Expression of both LLT1 and CD161 on NK or T cells predicts a "cis-" interaction. The balance between *cis* vs *trans* interactions may

modulate NK and T cell activation thresholds similarly to Ly49A binding to H-2D^d (16). It remains to also establish whether down-regulation of CD161 following binding to LLT1 participates in the regulation of cellular activation.

The identification of LLT1 interaction with CD161 opens up new avenues to understand the physiological role of these two cell surface molecules in immune responses and to assess their potential as new targets for the prevention and treatment of diseases.

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Disclosures

The authors have no financial conflict of interest.

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