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Induction of Lectin-like Transcript 1 (LLT1) Protein Cell Surface Expression by Pathogens and Interferon-γ Contributes to Modulate Immune Responses*

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Background: CD161 expressed by NK cells and T cells interacts with LLT1.
Results: LLT1 expression profile reveals LLT1 is induced by pathogens and IFN-γ and LLT1/CD161 interaction inhibits NK cell functions whereas it costimulates T cells.
Conclusion: The link between LLT1 expression and pathogen stimulation points toward a role in modulating immune responses to pathogens
Significance: LLT1/CD161 interaction is relevant in immunity to infection.

CD161 is a C-type lectin-like receptor expressed on human natural killer (NK) cells and subsets of T cells. CD161 has been described as an inhibitory receptor that regulates NK cell-mediated cytotoxicity and IFN-γ production. Its role on T cells has remained unclear. Studies have shown that triggering of CD161 enhances NK T cell proliferation and T cell-IFN-γ production while inhibiting TNF-α production by $CD8^+$ T cells. Lectin-like transcript 1 (LLT1), the ligand of CD161, was found to be expressed on Toll-like receptor (TLR)-activated plasmacytoid and monocyte-derived dendritic cells (DC) and on activated B cells. Using newly developed anti-LLT1 mAbs, we show that LLT1 is not expressed on the surface of circulating B and T lymphocytes, NK cells, monocytes, and dendritic cells but that LLT1 is up-regulated upon activation. Not only TLR-stimulated dendritic cells and B cells but also T cell receptor-activated T cells and activated NK cells up-regulate LLT1. Interestingly, IFN-γ increases LLT1 expression level on antigen-presenting cells. LLT1 is also induced on B cells upon viral infection such as Epstein-Barr virus or HIV infection and in inflamed tonsils. Finally, expression of LLT1 on B cells inhibits NK cell function but costimulates T cell proliferation or IFN-γ production, and coengagement of CD161 with CD3 increases IL-17 secretion. Altogether, our results point toward a role for LLT1/CD161 in modulating immune responses to pathogens.

The immune response is a complex network of interactions and regulations. It involves an innate immune system that uses a restricted set of invariant pattern-recognition receptors (PRRs)[2] that recognize pathogen-associated molecular patterns and an adaptive immune system generating a diverse repertoire of antigen receptors expressed by T and B lymphocytes leading to efficient effector responses and immunological memory (1). PRRs facilitate uptake of microbes and induce chemokines, proinflammatory cytokines, anti-microbial peptides, and complement activation. PRRs also up-regulate costimulatory molecules and induce cytokines that are crucial for the generation of adaptive immune responses. One of the best studied family of PRRs is the Toll-like receptor (TLR) family. TLR-activated antigen-presenting cells (APCs) display all of the necessary signals to induce efficient T cell responses. Nonetheless, the rules governing T cell activation remain poorly understood. An increasing number of costimulatory molecules have been described, but their role respective to PRRs and T cell activation still awaits investigation.

CD161, also known as NKR-P1A, is a C-type lectin-like receptor belonging to the C-type lectin family of receptors whose encoding genes are clustered in the natural killer (NK) cell complex on human chromosome 12. The *KLRB1* gene encodes CD161, a type II transmembrane glycoprotein expressed as a disulfide-linked homodimer on the cell surface of most human NK cells (2) and diverse subsets of T lymphocytes including invariant NK T cells (3), mucosa-associated invariant T cells (4), γδ T cells (5), and αβ T cells (2). CD161 is expressed mainly by effector and central memory T cells but is

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[2] The abbreviations used are: PRR, pattern-recognition receptor; APC, antigen-presenting cell; BCR, B cell receptor; CFSE, carboxyfluorescein succinimidyl ester; Clr, C-type lectin-related; DC, dendritic cell; LLT1, lectin-like transcript 1; NK, natural killer; PBMC, peripheral blood mononuclear cell; PGE2, prostaglandin E2; PHA, phytohemagglutinin; sCD40L, soluble CD40 ligand; SEB, staphylococcal enterotoxin B; TLR, Toll-like receptor.

not found on naïve T cells (6, 7). Although CD161 has been associated with IFN-γ and IL-17 secretion by $CD4^+$ T cells (8, 9), its function on human cells still remains unclear.

We and others identified lectin-like transcript 1 (LLT1) as the ligand of CD161 (10, 11). LLT1 is encoded by one of the alternatively spliced transcript variants of the *CLEC2D* gene also located in the NK cell complex (12). A recent study detected LLT1 expression on TLR-activated plasmacytoid and monocyte-derived dendritic cells (DCs) as well as on TLR or B cell receptor (BCR)-activated B cells (11). However, extensive study of LLT1 expression profile is still lacking. It has clearly been established that the LLT1/CD161 interaction inhibits NK cell-mediated cytotoxicity and IFN-γ secretion (10, 13), but its role on T cells remains controversial. The interaction either costimulated the proliferation and cytokine secretion of T cells and NKT cells (3, 10, 14), decreased TNF-α production by $CD8^+$ T cells (13), or had no effect (8, 11). Earlier studies have also suggested that CD161 could be involved in $CD4^+$ and γδ T cell trans-endothelial migration (5, 15).

To clarify the role of the LLT1/CD161 interaction, we used newly developed anti-LLT1 mAbs to define precisely the expression profile of LLT1 (12). LLT1 is expressed by hematopoietic cells, and its expression was found to be tightly correlated with activation on T cells, NK cells, B cells, and DCs. In agreement with a previous study (11), we found that activated B cells and TLR-activated DCs expressed LLT1 on the cell surface. In addition, we identified IFN-γ as a key signal amplifying LLT1 induction by TLR on APCs. Accordingly, we found LLT1 induction upon viral infections and in inflamed tonsils. Functional studies with LLT1-expressing B cells demonstrated that the LLT1/CD161 interaction inhibited NK cell function and costimulated proliferation and IFN-γ secretion by $CD161^+$ T cells. In addition, we showed that coengagement of CD161 and CD3 increased IL-17 secretion by Th17 cells. LLT1/CD161 therefore represents an additional ligand/receptor pair that regulates both innate and adaptive immune responses.

EXPERIMENTAL PROCEDURES

Cell Lines—C1R and C1R-LLT1 B cell lines, Raji Burkitt lymphoma, 293T and 293T-CD161 human embryonic kidney, COS African green monkey kidney, THP-1 acute monocytic leukemia, and P815 mouse mastocytoma cells were maintained in complete RPMI 1640 medium or Iscove's modified Dulbecco's medium (Lonza) supplemented with 10% FCS (Pierce), penicillin (100 units/ml), and streptomycin (100 μg/ml).

Primary Cells—Peripheral Blood Mononuclear Cells (PBMCs) were separated by Ficoll-Paque Plus density gradient centrifugation (Amersham Biosciences) from blood purchased from the Etablissement Francais du Sang. Monocytes; B cells and NK cells were isolated by positive magnetic selection with anti-CD14, anti-CD19, or anti-CD56 Microbeads (Miltenyi Biotec), respectively, and further sorted by flow cytometry on a FACSVantage (Becton Dickinson) using anti-CD3, -CD56, -CD19, and -CD14 mAbs (BD Biosciences). T cells were sorted directly by flow cytometry as $CD3^+$, $CD3^+CD4^+$, or $CD3^+CD8^+$ cells using anti-CD3, -CD4, and -CD8 mAbs. Purity was typically >92–99%. Monocyte-derived DCs were generated from positively selected $CD14^+$ monocytes cultured for 5 days in the presence of 200 units/ml IL-4 (BD Biosciences) and 50 ng/ml GM-CSF (PeproTech). For NK cell assays purified $CD56^+$ cells were subsequently cocultured with irradiated allogeneic PBMCs and B-EBV feeder cells in X-VIVO 15 medium (Cambrex) supplemented with 10% FCS and 500 units/ml IL-2 (Chiron). All NK cells used contained <5% $CD3^+CD56^+$ T cells. Th17 clonal cells were generated as follows. PBMCs were cultured in RPMI 1640 medium supplemented with 10% FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM β-mercaptoethanol, 1 μM PGE2 (BD Biosciences), 10 ng/ml IL-23 (eBioscience), 10 ng/ml IL-1β (BD Biosciences) and 0.1 μg/ml anti-CD3 OKT3 for 12 days. On day 2, culture medium was supplemented with 20 units/ml IL-2. On day 12, $CD161^+CD4^+CD3^+$ cells were sorted by flow cytometry using anti-CD161, -CD4, and -CD3 mAbs and subsequently cocultured with irradiated allogeneic PBMCs in complete medium. After 3 weeks, clones were screened by flow cytometry for IL-17 production after phorbol 12-myristate 13-acetate/ionomycin stimulation using Alexa Fluor 647-conjugated anti-human IL-17A (eBioscience). The selected IL-17-producing clones were amplified further in the presence of allogeneic irradiated PBMCs in complete medium. B cells from discarded tonsils were obtained after informed consent from patients undergoing routine tonsillectomies at the Lenval Hospital, Nice. Cells were mechanically collected and the suspension passed through 70-μm nylon cell strainers (BD Biosciences).

Antibodies—In house-generated anti-LLT1 mAbs (clones 2F1 and 4F68) have been described previously (12). 4F68 was cloned and the variable region fused to human IgG4 Fc domain (4F68-hIgG4). The following antibodies were used in functional assays: anti-LLT1 (mAb3480) (R&D Systems), anti-CLEC2D/A (4C7) (Abnova), anti-CD161 (HP3G10) (kindly provided by M. Lopez-Botet, Spain), anti-TNP (mIgG1), and human IgG4 (Sigma-Aldrich). $F(ab')_2$ fragments of 4F68, HP3G10, and mIgG1 isotype control were prepared by pepsin digestion (Sigma) at a 3:100 (w/w) ratio of pepsin to IgG in 0.2 mol/liter sodium acetate buffer (pH 4.0) at 37 °C, for 4 h. LLT1-Fc multimer was generated as described previously (10).

Real-time RT-PCR and Western Blotting—Real-time RT-PCR amplification of *CLEC2D* variant 1 was performed as described previously (12). Transcript levels were expressed relative to β-actin. For Western blot analysis, whole cell lysates were prepared as described previously (12), loaded on 12% SDS-PAGE, and transferred to nitrocellulose membranes. LLT1 was detected using 2F1, and β-actin was used as control.

Flow Cytometry—Cells were incubated in PBS, 0.1% BSA, 10% normal mouse serum, and 100 μg/ml human Ig for 30 min on ice, to block Fc receptors efficiently prior to incubation with biotinylated anti-LLT1 (4F68) or isotype mIgG1 control, followed by APC-conjugated streptavidin (BD Biosciences). The various cell populations were phenotyped using labeled antibodies (BD Biosciences) to CD3, CD4, CD8, CD19, CD14, CD83, CD56, CD161, CD69, and CD38. Cytokine production was monitored by intracellular staining using phycoerythrin-conjugated anti-IFN-γ or anti-IL-4 mAbs and Alexa Fluor 647-conjugated anti-IL-17 or anti-IL-10 mAbs. NK cell degranulation was detected using phycoerythrin-conjugated anti-CD107a mAb.

Cell Stimulations and Viral Infections—Polyclonal T cells were cultured for 72 h in the presence of 0.5 μg/ml PHA (Remel), or for 24 h on plate-bound anti-CD3 UCHT1 or mIgG1 isotype control MOPC-21 (1 μg/ml) (Sigma). Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen)-labeled $CD4^+$ T cells were cultured for 5 days in wells coated with anti-CD3 OKT3 (3 μg/ml). B cells were cultured for 60 h in the presence of 2.5 μg/ml soluble CD40 ligand (sCD40L) (PeproTech) and 5 μg/ml $F(ab')_2$ anti-IgM (Beckman Coulter), or of 5 μg/ml TLR7/8 ligand CL097, TLR7 ligand imiquimod, TLR8 ligand ssRNA40, or TLR9 ligand CpG ODN2006 (Invivogen) supplemented when indicated with 0.5 μg/ml IFN-γ or IL-4. NK cells were cultured for 18 h in the presence of 293T cells, COS cells, THP1 cells, or C1R cells, or P815 cells coated with anti-CD16 3G8 or a mIgG2A isotype control (50 μg/ml), at an E:T ratio of 1:3. DCs were cultured for 48 h in the presence of a mixture of cytokines, including IL-1β (10 ng/ml), IL-6 (10 ng/ml), TNF-α (10 ng/ml), and PGE2 (1000 units/ml), or of 25 μg/ml TLR3 ligand poly(I:C), 1 μg/ml TLR4 ligand LPS, 5 μg/ml TLR7/8 ligand CL097, 5 μg/ml TLR7 ligand imiquimod, or 5 μg/ml TLR8 ligand ssRNA40. LPS-stimulated DCs were stimulated with 2.5 μg/ml sCD40L, or 0.3 μg/ml IL-12, IL-4, IFN-γ or IL-17A. PBMCs were incubated with EBV supernatant (from B95.8 cells, kindly provided by C. Willem, EFS, Nantes, France) or HIV-1 IIIB supernatant for 66 h or 72 h, respectively.

NK and T Cell Assays—Polyclonal NK cells were stimulated for 4–5 h with C1R or C1R-LLT1 cells at an E:T ratio of 1:3 in the presence or not of the indicated mAbs either as mIgG1, $F(ab')_2$ fragments, or hIgG4 Fc-fusion proteins (10 μg/ml). CD161 down-regulation, NK cell degranulation, and IFN-γ production were monitored as described previously (10). Autologous B and T cells from PBMCs were first stimulated for 24 h with 5 μg/ml CpG ODN2006 to up-regulate LLT1 on B cells. Anti-LLT1 or isotype controls (20 μg/ml) were then added to the culture medium, followed 30 min later by the addition of staphylococcal enterotoxin B (SEB) (10 ng/ml) (Sigma). After 5 days of culture, cells were stimulated for 4 h with 5 ng/ml phorbol 12-myristate 13-acetate and 500 ng/ml ionomycin (Sigma) in the presence of 10 μg/ml brefeldin A, followed by intracellular staining using phycoerythrin-conjugated anti-human IFN-γ (BD Biosciences). Autologous B and T cells from tonsils were labeled with 2.5 μM CFSE and cultured in the presence of SEB (250 pg/ml) and anti-LLT1 or isotype controls (20 μg/ml). After 5 days, the dilution of CFSE was monitored by flow cytometry. Th17 clones were incubated for 4 h on plate-bound anti-CD3 UCHT1 at increasing concentrations, in combination with plate-bound anti-CD161 (HP3G10) or mIgG1 isotype control (5 μg/ml) and 10 μg/ml brefeldin A. IL-17 secretion was detected by intracellular staining with Alexa Fluor 647-conjugated anti-human IL-17A. The IL-17 secretion index was calculated as follows: (percentage $IL\text{-}17^+$ cells with anti-CD161 − percentage $IL\text{-}17^+$ cells with isotype control mIgG1)/(percentage $IL\text{-}17^+$ cells with isotype control mIgG1) * 100.

RESULTS

LLT1 Is Induced upon Activation on Hematopoietic Cells—The LLT1 expression profile was studied both at the transcriptional level using specific real-time RT-PCR and at the protein level using two novel anti-LLT1 mAbs that we have generated (12). We showed previously that *CLEC2D* transcript variant 1 (LLT1) was detected only in a few cell lines of the B cell lineage such as Raji, used here as an internal control (12). Low levels of LLT1 mRNA were detected in PBMCs from healthy donors compared with Raji, and transcripts were found primarily in T and B lymphocytes, at lower levels in NK cells, and were absent in monocytes (Fig. 1*A*). Despite the presence of transcripts, however, no LLT1 protein was detected either at the cell surface or intracellularly, indicating that LLT1 translation is tightly regulated and that LLT1 is not expressed by hematopoietic cells under resting conditions (Fig. 1, *B* and *C*).

We thus searched for signals that could induce LLT1 expression on PBMCs. Stimulation of polyclonal T cells with PHA for 72 h resulted in a 2-fold increase of LLT1 transcripts and the detection of LLT1 protein by Western blotting (Fig. 2, *A* and *B*). LLT1 is heavily glycosylated and runs as a diffuse band with higher molecular mass corresponding to cell surface-expressed LLT1 and lower molecular mass corresponding to intracellular LLT1 (Figs. 2*B*, 3*B*, and 4*B*). In correlation with the detection of LLT1 protein in whole cell lysates, we detected LLT1 at the cell surface of T cells, both on $CD4^+$ and $CD8^+$ cells by flow cytometry using 4F68 mAb (Fig. 2*C*, *upper*). Although less efficient (12), anti-LLT1 mAbs clone 2F1 and mAb3480 also detected LLT1 at the cell surface of PHA-stimulated T cells (data not shown). Importantly, PHA-mediated induction of LLT1 at the cell surface was only detectable after 24 h of stimulation and was maximal between 48 and 72 h (data not shown). LLT1 was also induced upon cross-linking for 24 h of the T cell receptors using plate-bound anti-CD3 (Fig. 2*C*, *lower*). Interestingly, LLT1 expression was closely associated with activation because its expression was detected exclusively on T cells expressing the early activation marker CD69 and on proliferating T cells (Fig. 2*C*, *lower*). LLT1 could be detected indifferently on both $CD161^+$ and $CD161^-$ T cells (data not shown). The induction of LLT1 cell surface expression was also detected on activated invariant NKT cells, as well as on IFN-γ-, IL-4-, IL-17-, and IL-10-secreting $CD4^+$ T cells (data not shown). In addition to activated T cells, we also detected LLT1 on activated B cells after engagement of the BCR and CD40 for 60 h (Fig. 2*D*). These results on B cells are in agreement with a previous report (11). Finally, we could detect intracellular LLT1 protein expression in IL-2-activated polyclonal NK cell lines generated in the presence of allogeneic PBMCs and B cells by Western blotting (Fig. 2*E*), but cell surface expression was detected only on NK cells incubated for 18 h with some but not all NK-sensitive targets (Fig. 2*F*, *upper*). Because NK cell activation results from a balance between the engagement of activating and inhibitory receptors, whose ligands can be differentially expressed on target cells, we tested whether LLT1 expression required the engagement of specific NK receptors. We thus performed redirected killing assays using the FcR-bearing P815 cells coated with anti-NKR mAbs either alone or in combination. LLT1 was

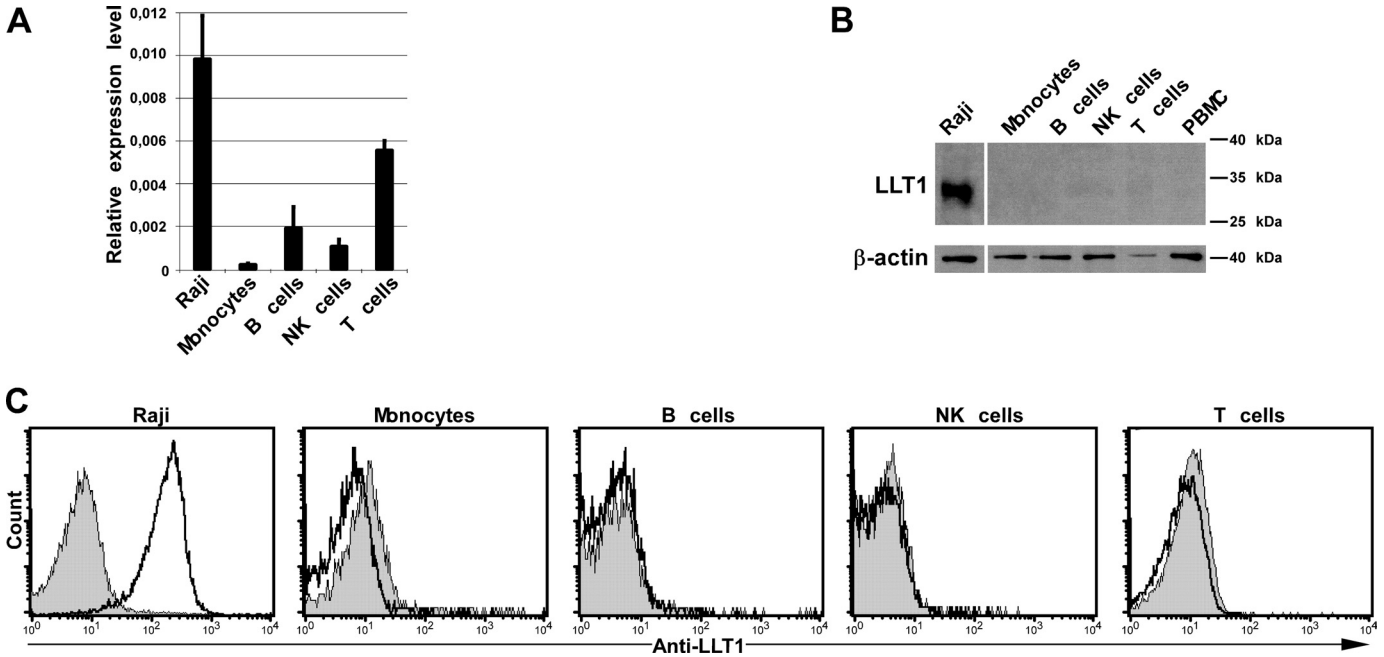
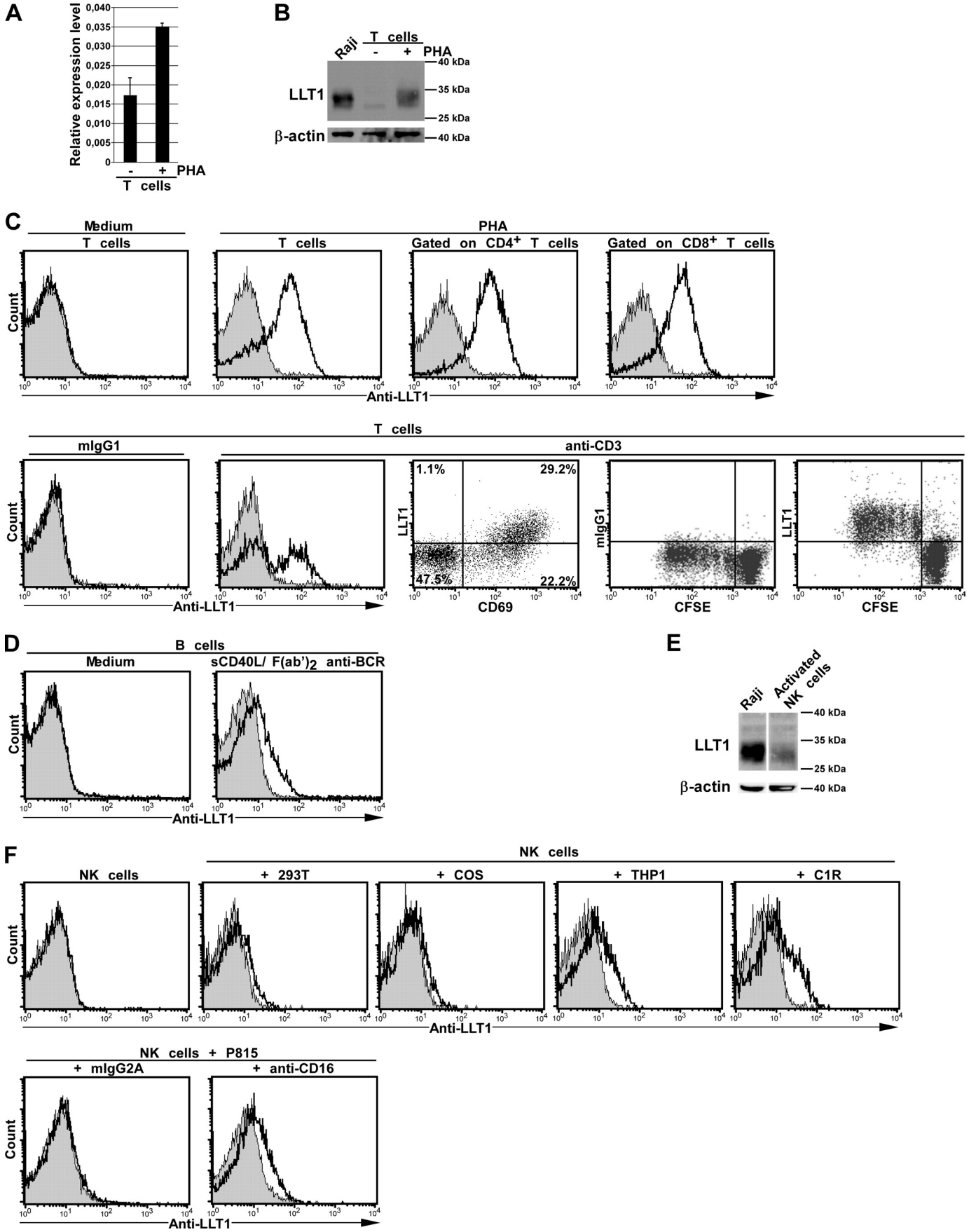


FIGURE 1. **LLT1 expression in resting PBMCs.** *A*, *CLEC2D* transcript variant 1 (LLT1) quantification by real-time RT-PCR in Raji or the indicated cell populations isolated from peripheral blood of a representative healthy donor. *B*, LLT1 protein detection by Western blot analysis of whole cell lysates of the indicated cell populations using 2F1 mAb. β-Actin was used as internal control. *C*, cell surface expression of LLT1 analyzed by flow cytometry using 4F68 mAb (*solid line*) compared with mIgG1 isotype control (*filled histogram*). Data are representative of three independent experiments (*A* and *B*) and >20 independent experiments (*C*).

induced only upon CD16 cross-linking but not upon NKG2D, CD94/NKG2C, NKp30, NKp44, or NKp46 engagement (Fig. 2*F*, *lower*, and data not shown). Cross-linking of CD16 simultaneously with the above activating NKRs did not enhance LLT1 induction, and the level of expression of LLT1 upon cross-linking of CD16 remained consistently low (data not shown). CD16 is not a natural cytotoxicity receptor but mediates antibody-dependent cellular cytotoxicity and has been identified as the dominant receptor in the hierarchy of receptors that trigger NK cells (16). Our results therefore suggest that LLT1 expression on NK cells is induced when a threshold of activation is reached as obtained with CD16 engagement or in the presence of NK-sensitive targets.

LLT1 Expression on APCs Is Regulated by Pathogen-associated Stimuli—CD161, the receptor of LLT1, is expressed on NK cells and subsets of T cells and is thought to interact with LLT1 on APCs (10, 11, 13). We therefore examined the regulation of LLT1 expression on DCs and B cells. Monocyte-derived DCs were generated from the culture of blood purified $CD14^+$ monocytes in the presence of GM-CSF and IL-4. On day 5, immature DCs were stimulated with a panel of TLR ligands or a type 2 polarized DC (DC2)-inducing mixture of cytokines (17) and subsequently analyzed for the presence of LLT1 mRNA and protein. Similar to monocytes, immature DCs did not express LLT1 (Fig. 3, *A–D*). In contrast, LLT1 expression was induced upon specific TLR stimulations on mature DCs (Fig. 3). The most potent stimulator of LLT1 expression was LPS, which triggers TLR4. Indeed, we detected a 6-fold increase in LLT1 mRNA and significant amount of LLT1 protein, although a large part displayed the lower molecular mass, suggesting an intracellular localization. Stimulation of TLR3 with poly(I:C) induced transcription of LLT1 but to a lesser extent than TLR4 stimulation, and very low levels of LLT1 protein could be detected. In contrast, TLR8 stimulation with CL097, TLR2/6 stimulation with Pam2CSK4, NOD2 with MDP, and DC maturation with a DC2-inducing mixture of cytokines (IL-1β, TNF-α, IL-6, and PGE2) did not lead to LLT1 induction (Fig. 3, *A*, *B*, and *D*, and data not shown). Flagellin and CpG were used because negative controls as human monocyte-derived DCs do not express TLR5 and TLR9. Interestingly, the combined stimulation of TLR4 (LPS) with TLR8 (CL097 and ssRNA) further enhanced cell surface expression of LLT1, suggesting that TLRs can have a synergistic effect (Fig. 3*E*, *upper*). Importantly, we found that IFN-γ reproducibly increased LLT1 expression intracellularly and on the surface of LPS-matured DCs whereas IL-12, IL-4, IL-17, IL-10, and TNF-α did not have any effect and sCD40L decreased it, suggesting that type 1 polarized DC (DC1)-inducing stimulus (LPS + IFN-γ) is the most potent inducer of cell surface LLT1 (Fig. 3, *C* and *E*, *lower*).

We next performed similar TLR stimulations of B cells purified from blood. We found that TLR7 stimulation with CL097 or imiquimod and TLR-9 stimulation with CpG ODN induced LLT1 expression both intracellularly and on the cell surface (Fig. 4, *B* and *C*) whereas the increase of transcripts was only marginal (Fig. 4*A*). These results were also obtained with unpurified B cells, upon TLR stimulation of PBMCs (data not shown). Addition of IFN-γ during the stimulation of B cells with CL097 increased cell surface expression of LLT1 whereas addition of IL-4 tended to decrease LLT1 up-regulation (Fig. 4*D*). Addition of IL-17 or sCD40L had no effect (data not shown). Based on our findings, we next tested whether acute viral infections could induce LLT1 expression. As shown in Fig. 4*E*, LLT1 cell surface expression was detected on peripheral B cells following *in vitro* infection of PBMCs with EBV and


A
Relative expression level
0,040
0,035
0,030
0,025
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- + PHA
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- + PHA
LLT1
β-actin
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T cells
Gated on CD4+ T cells
Gated on CD8+ T cells
Count
Anti-LLT1
T cells
mIgG1
anti-CD3
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Anti-LLT1
1.1%
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CD69
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+ THP1
+ C1R
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Anti-LLT1
NK cells + P815
+ mIgG2A
+ anti-CD16
Count
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HIV-1. Interestingly, we could not detect LLT1 expression on peripheral B cells from patients with high HIV-1 viral loads, suggesting that LLT1 induction seen on B cells upon acute HIV infection is transient or that the HIV-1 virus regulates LLT1 expression (data not shown). Similarly, we detected LLT1 cell surface expression *in vivo* on a significant proportion of tonsillar B cells isolated from human inflamed tonsils collected from children suffering from chronic and recurrent tonsillitis triggered by viral and bacterial infections (Fig. 4*F*). LLT1 expression was correlated with high levels of CD38 found in germinal center B cells and on plasma blasts. These observations underline the association between LLT1 induction and antigenic stimulation.

Physiological Roles of LLT1/CD161 Interaction—To assess the role of the LLT1/CD161 interaction, we developed a blocking anti-LLT1 mAb. Clone 4F68 blocked the binding of LLT1-Fc multimer to 293T-CD161 transfectants in a dose-dependent manner (Fig. 5*A*) and was the sole among all anti-LLT1 mAbs described so far which was able to block LLT1-induced down-regulation of CD161 cell surface expression (Fig. 5*B*). For use in functional assays, we generated $F(ab')_2$ fragments of 4F68 mIgG1 and cloned the mAb to fuse the variable region to the constant domain of human IgG4 which has a lower affinity for the Fcγ receptors (4F68-hIgG4).

CD161 is expressed by the majority of NK cells, and LLT1 expression on NK-sensitive target cells was previously found to inhibit both NK cell-mediated cytotoxicity and IFN-γ production (10, 11, 13). Using the blocking anti-LLT1 4F68 either as mIgG1, $F(ab')_2$ fragments, or as a chimeric molecule with hIgG4, we confirmed that the LLT1/CD161 interaction inhibits NK cell functions. Similar to anti-CD161 mAb, anti-LLT1 4F68 mAb restored NK cell degranulation and IFN-γ secretion in the presence of C1R-LLT1 target cells (Fig. 5, *C* and *D*).

CD161 is also expressed by a significant proportion of T cells including invariant NK T cells, mucosa-associated invariant T cells, $\gamma\delta$ T cells, and $\alpha\beta$ T cells, but the role of the LLT1/CD161 interaction in modulating the function of these T cell populations remains controversial. We therefore studied the effect of the blocking anti-LLT1 mAb 4F68 in functional assays *in vitro*. When we compared the different subsets of $CD4^+$ T cells generated *in vitro* and secreting IFN-γ (Th1), IL-4 (Th2), IL-17 (Th17), or IL-10 (Treg), we found that CD161 was expressed more frequently on IFN-γ- and IL-17-secreting T cells. 80% of the cells with a Th1 or Th17 phenotype expressed CD161 whereas only 40% were $CD161^+$ among the IL-4- or IL-10-secreting T cells (data not shown). We therefore focused on the role of the LLT1/CD161 interaction in the regulation of IFN-γ- and IL-17-secreting T cells. Previous studies including our own suggested that CD161 may function as a costimulatory receptor (3, 10, 14). To elucidate further the role of LLT1/CD161 on T cells, we set up a cellular assay in which peripheral B cells prestimulated with CpG ODN to induce LLT1 cell surface expression were incubated with autologous polyclonal T cells in the presence of SEB. These polyclonal T cells included $CD161^{bright}$ $CD8^+$ mucosa-associated invariant T cells (4, 18) and $CD161^+CD4^+$ T cells. Fig. 6*A* shows that blocking the LLT1/CD161 interaction by addition of anti-LLT1 mAb 4F68 decreased the percentage of IFN-γ-secreting $CD161^+$ T cells. The reduction was more significant when the percentage of IFN-γ^{bright} $CD161^+$ T cells was considered. In a different experimental setting, we mixed tonsillar B cells and T cells in the presence of SEB and looked at T cell proliferation. As described above, tonsillar B cells expressed a significant level of cell surface LLT1 (Fig. 4*F*). Incubation with blocking anti-LLT1 mAb 4F68 decreased $CD161^+$ T cell proliferation (Fig. 6*B*). Finally, we investigated the function of CD161 receptor on Th17 clones generated *in vitro*. Cross-linking of CD161 together with CD3 increased IL-17 secretion, up to 30% at 0.5 μg/ml anti-CD3 (Fig. 6*C*). Altogether, these results indicate that CD161 may represent an additional costimulatory molecule involved in the regulation of T cell activation.

DISCUSSION

The immune system is set to defend the host against a diversity of microbial pathogens. Triggering of immune responses results from the activation of several signals, including signals provided by T cell receptor or BCR cross-linking, signals delivered through costimulatory molecules, and signals from TLRs or cytokine receptors. Using newly developed antibodies that are highly specific for LLT1, we have shown that LLT1 is induced upon activation through a combination of these signals and that the LLT1/CD161 interaction on one hand inhibits NK cell activation and on the other hand costimulates T cells. The LLT1/CD161 interaction therefore has a dual role in that it contributes to mount an efficient T cell response against microbes but at the same time is involved in the control of NK cell responses and tolerance. This is reminiscent of costimulatory molecules such as CD28/CTLA-4 that have been shown to deliver both stimulatory and inhibitory signals for T cell responses (19). Also, LLT1 shares characteristics of several costimulatory molecules that have broad immunoregulatory functions and are induced by PRRs such as TLRs (1, 20). Similarly to costimulatory molecules, LLT1 is inducibly expressed on T cells but also on APCs such as DCs and B cells. Such pattern of expression necessitates undertaking cell type-specific analyses of their functions.

In this study, we focused on the role of LLT1 expressed by APCs. We confirmed and extended previous findings that

FIGURE 2. **LLT1 expression is induced on activated lymphocytes.** *A–C*, polyclonal T cells cultured for 72 h in the absence or presence of PHA or 24 h on plate-bound anti-CD3. CFSE-labeled $CD4^+$ T cells were cultured for 5 days on plate-bound anti-CD3. *A*, *CLEC2D* transcript variant 1 (LLT1) quantification by real-time RT-PCR. *B* and *E*, LLT1 protein detection by Western blot analysis of whole cell lysates using 2F1 mAb. Raji was used as control, and β-actin was used as internal control. NK cells were generated from allogeneic stimulation in the presence of IL-2. *C*, *D*, and *F*, cell surface expression of LLT1 analyzed by flow cytometry using 4F68 mAb (*solid line*) compared with mIgG1 isotype control (*filled histogram*). *C*, on T cells following PHA (*upper*) or plate-bound anti-CD3 (*lower*) stimulation. The proportions of plate-bound anti-CD3 activated T cells expressing LLT1 and CD69 are indicated. LLT1 expression is measured on CFSE-labeled $CD4^+$ T cells. *D*, on polyclonal B cells cultured for 60 h in the absence or presence of sCD40L and anti-BCR $F(ab')_2$ fragments. *F*, on polyclonal NK cells cultured for 18 h in the absence or presence of the indicated target cells (*upper*) and in the presence of P815 cells coated with mIgG2a isotype control or anti-CD16 mAb (*lower*). Data are representative of at least three independent experiments.

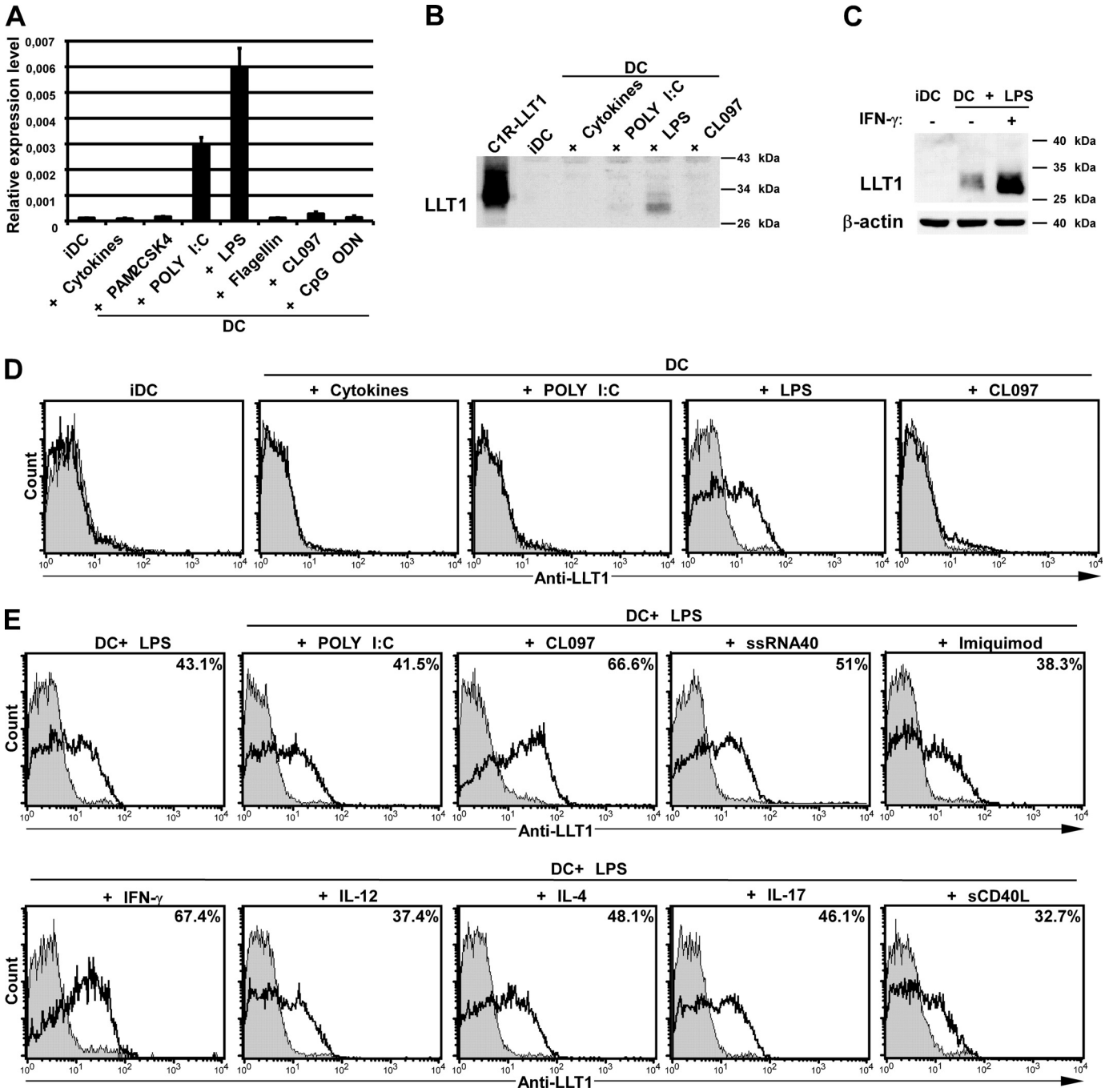

FIGURE 3. **LLT1 is induced on LPS-matured DCs.** *A*, *CLEC2D* transcript variant 1 (LLT1) quantification by real-time RT-PCR in immature DCs or DCs matured with the indicated stimuli. *B* and *C*, LLT1 protein detection by Western blot analysis of whole cell lysates using 2F1 mAb. C1R-LLT1 was used as control,. and β-actin was used as internal control. *D* and *E*, cell surface expression of LLT1 analyzed by flow cytometry using 4F68 mAb (*solid line*) compared with mIgG1 isotype control (*filled histogram*). The proportion of LLT1-expressing DCs among total DCs is indicated. Data are representative of at least three independent experiments (*A*, *D*, and *E*) and two independent experiments (*B* and *C*).

LLT1 is induced upon specific TLR stimulations on DCs and B cells (11). We found a predominant expression of LLT1 on LPS-matured DCs which was significantly increased by IFN-γ. Interestingly, although factors such as LPS or poly(I:C) can induce IL-12 production by DCs, the ability to generate stable DC1 which have an elevated ability to produce IL-12 and promote Th1 responses seems to be predominantly controlled by IFN-γ stimulation (21). LLT1 induction seems therefore associated with a Th1-polarizing environment. In contrast, DC2 generated by stimulation with a mixture of cytokines (IL-1β, IL-6, TNF-α, and PGE2) (17) failed to up-regulate LLT1. We also detected significant expression of LLT1 on activated B cells, either following BCR and CD40 cross-linking or following TLR7 and TLR9 stimulation. Similarly to DCs, the presence of IFN-γ increased LLT1 cell surface expression on B cells. In contrast, IL-4 decreased LLT1 surface expression. Cytokines play a key role in the commitment of naïve B cells into effector B cell subsets (22). IFN-γ contributes to the development of

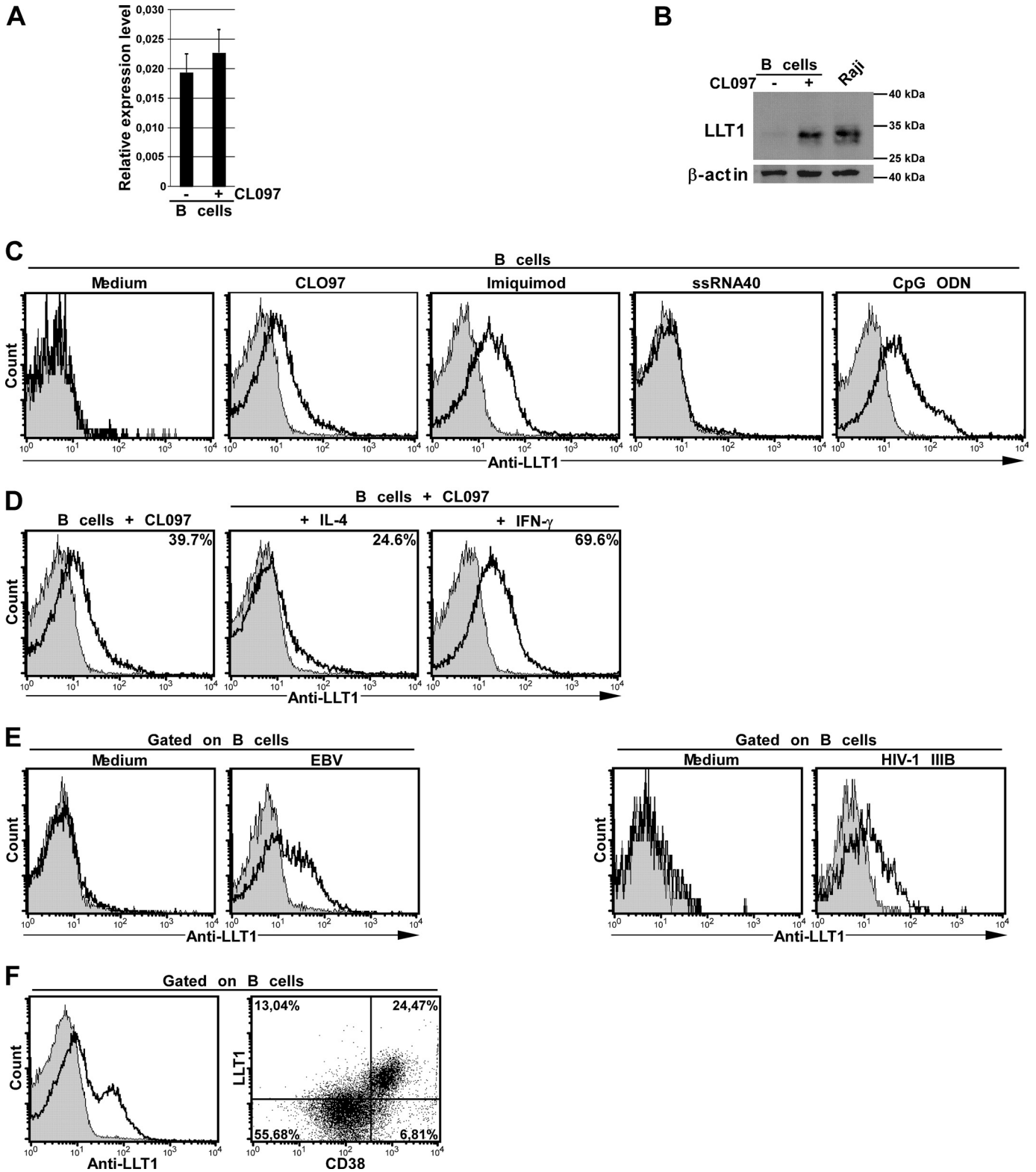

FIGURE 4. **LLT1 expression is induced on activated B cells.** *A*, *CLEC2D* transcript variant 1 (LLT1) quantification by real-time RT-PCR in purified peripheral B cells stimulated or not for 38 h with the TLR7/8 ligand CL097. *B*, LLT1 protein detection by Western blot analysis of whole cell lysates using 2F1 mAb following 60 h of stimulation. Raji was used as positive control and β-actin as internal control. *C* and *D*, cell surface expression of LLT1 analyzed by flow cytometry using 4F68 mAb (*solid line*) compared with mIgG1 isotype control (*filled histogram*) following 60 h of stimulation. *D*, proportion of LLT1-expressing B cells among total B cells. *E* and *F*, cell surface expression of LLT1 on $CD19^{+}CD3^{-}$ gated B cells, analyzed by flow cytometry using 4F68 mAb (*solid line*) compared with mIgG1 isotype control (*filled histogram*) *E*, following *in vitro* infection of PBMCs with EBV or HIV. *F*, in human inflamed tonsils in combination with CD38. Data are representative of at least three independent experiments (*A*, *C*, and *F*) and two independent experiments (*B*, *D*, and *E*).

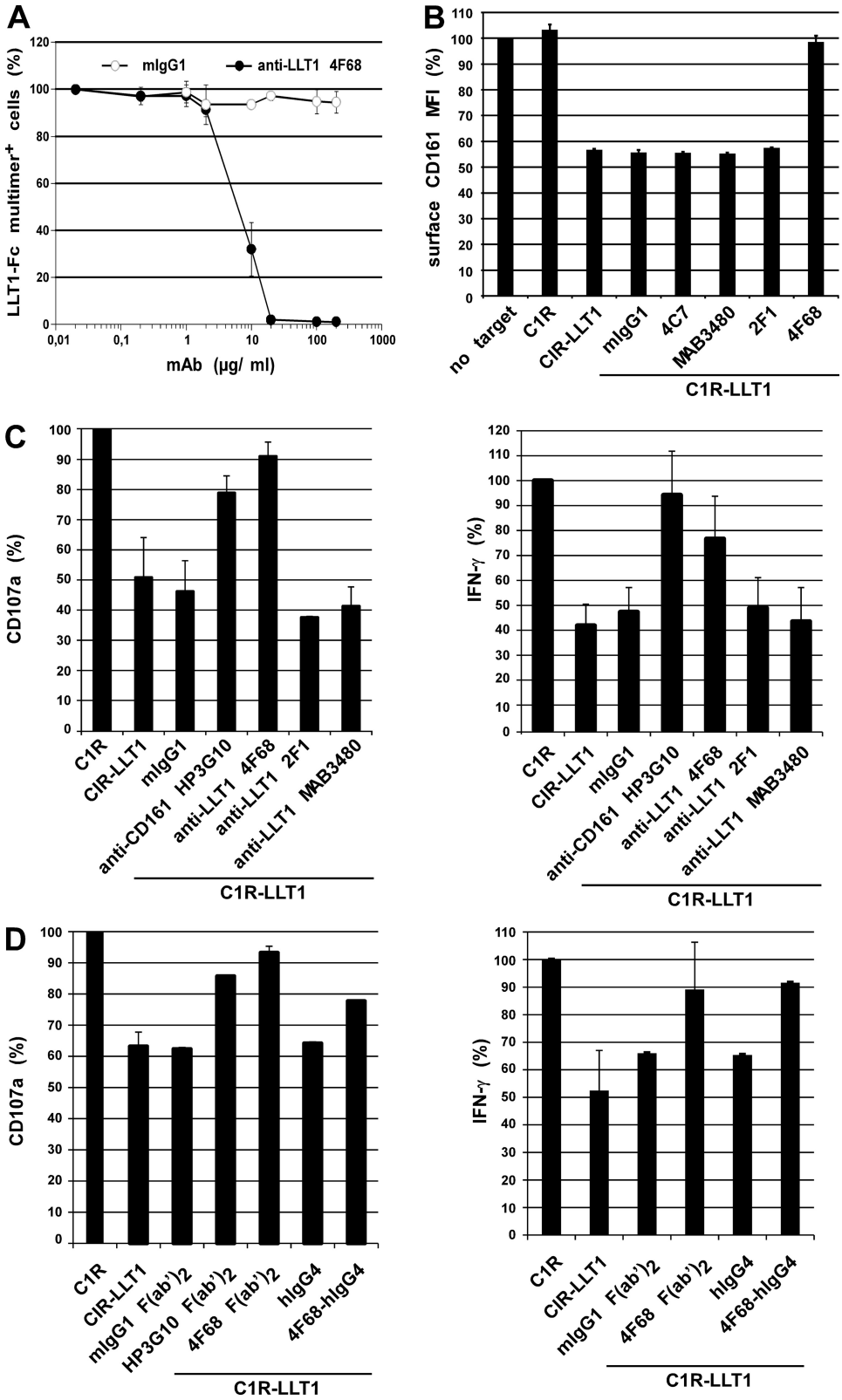

FIGURE 5. **Anti-LLT1 mAb 4F68 blocks LLT1/CD161 interaction and restores NK cell functions.** *A*, 293T-CD161 cells were stained with LLT1-Fc multimer in the presence of increasing concentrations of anti-LLT1 4F68 mAb (*filled circles*) or mIgG1 isotype control (*open circles*). Results are shown as mean ± S.E. (*error bars*) of four experiments. *B*, cell surface expression of CD161 analyzed by flow cytometry using 191B8 mAb on NK cells incubated alone or with C1R or C1R-LLT1 in the presence or absence of the indicated anti-LLT1 mAbs and isotype control mIgG1. The mean fluorescence intensity obtained with NK cells alone was arbitrarily set as 100%. Data are mean ± S.D. of triplicates and are representative of five experiments. *C* and *D*, NK cell degranulation (*left*) and NK cell secretion of IFN-γ (*right*) following incubation with C1R or C1R-LLT1 targets in the presence of the indicated mIgG1 (*C*), or the indicated $F(ab')_2$ fragments and hIgG4-Fc chimeric molecules (*D*). The percentage of $CD107a^+$ and IFN-γ^+ NK cells in the presence of C1R cells was arbitrarily set as 100%. Average and S.D. of three (*C*) and two (*D*) independent experiments with triplicates are indicated.

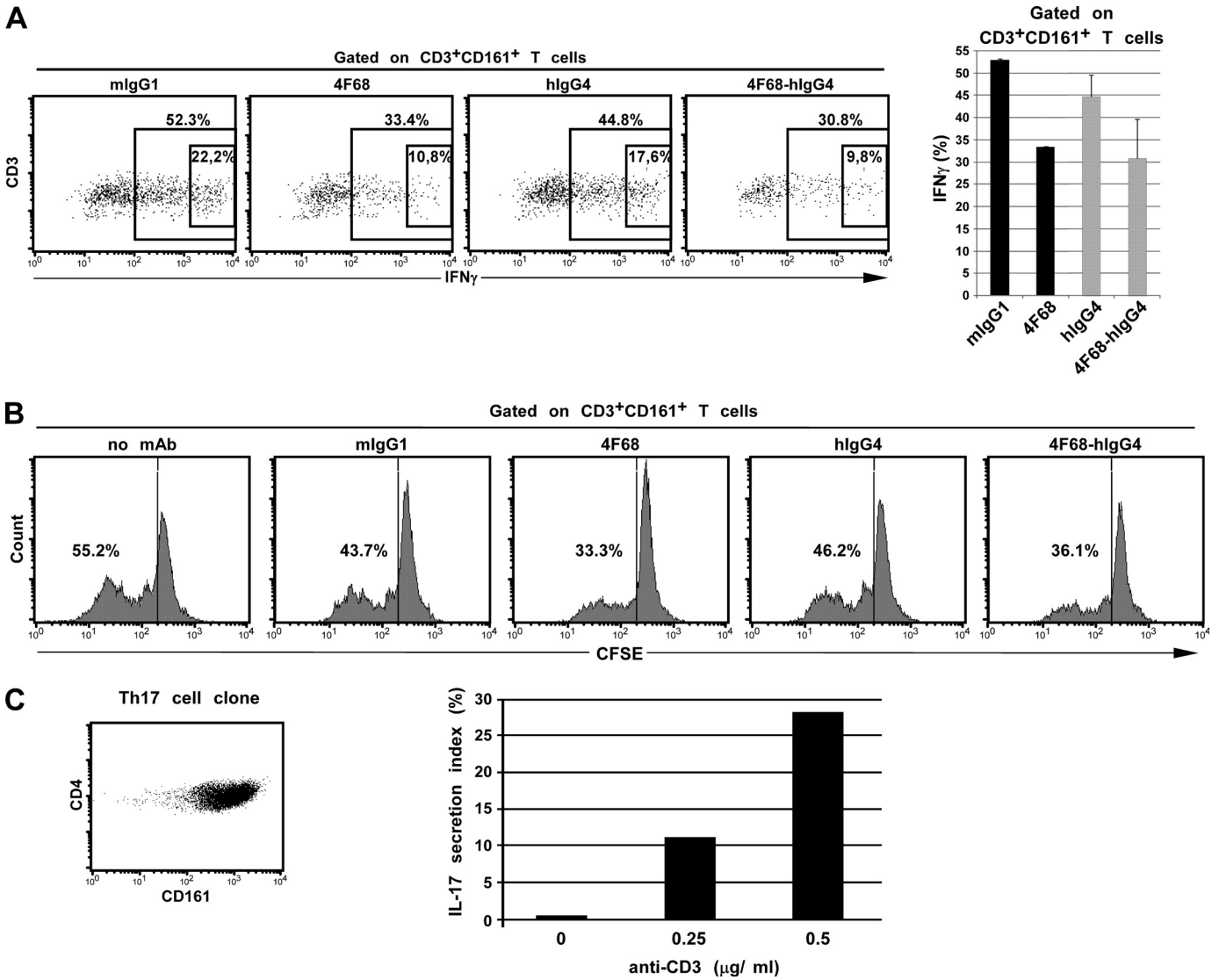

FIGURE 6. **LLT1 expressed on B cells costimulates T cells.** *A*, IFN-γ secretion by T cells stimulated with SEB and autologous B cells expressing LLT1 following CpG stimulation, in the presence of the indicated isotype controls or anti-LLT1 mAbs. The percentage of IFN-γ^+ and IFN-γ^{high} CD161$^+$ T cells is indicated on the *dot-plots* and graphically. *B*, CD56-depleted human tonsillar cells labeled with CFSE incubated with SEB in the presence of the indicated mAbs or hIgG4-Fc chimeric molecules. The percentage of proliferative CD3$^+$CD161$^+$ T cells is indicated. *C*, IL-17 secretion by a representative CD4$^+$CD161$^+$ Th17 clone stimulated by increasing concentrations of plate-bound anti-CD3, in the presence of a saturating concentration of plate-bound isotype control or anti-CD161 mAb. Results are expressed as a percentage of IL-17 secretion index calculated as follows: (percentage IL-17$^+$ cells with anti-CD161 − percentage IL-17$^+$ cells with isotype control mIgG1)/(percentage IL-17$^+$ cells with isotype control mIgG1) * 100. Data are representative of two independent experiments.

Be-1 cells that secrete IFN-γ, TNF-α, and IL-12. Interestingly, these Be-1 cells also promote Th1 responses. Our results therefore indicate that LLT1 up-regulation is preferentially triggered by a Th1-polarizing environment. This will favor its interaction with CD161-expressing T cells and NK cells because (i) IL-12 was reported to up-regulate CD161 (23), (ii) we observed a high frequency of CD161-expressing cells among IFN-γ-secreting T cells, and (iii) the majority of NK cells that are high producers of IFN-γ express CD161. CD161 is also expressed on a majority of Th17 cells, many of which secrete both IFN-γ and IL-17. Interestingly, we found that the LLT1/CD161 interaction costimulated the secretion of these two cytokines. The finding that LLT1 is induced by pathogens provides a link with the preferential expression of its receptor CD161 on Th1 and Th17 cells. The induction of LLT1 by TLRs could also represent one mechanism coupling microbial recognition with the initiation of adaptive immune responses (24). Indeed, the LLT1/CD161 interaction may participate in the switch from innate to adaptive immunity. Clusters of DCs, T cells getting primed, and activated NK cells are found in secondary lymphoid organs such as lymph nodes draining infection sites (25–27). An initial expansion and peak of activation of NK cells which is controlled by both CD4$^+$ T cells and DCs is followed by a contraction phase and expansion of activated T cells secreting cytokines such as IFN-γ (26, 28). In this context, the homeostatic control of the NK cell response is crucial to avoid immune pathologies, and we propose that induction of LLT1 on APCs upon stimulation with appropriate pathogens may participate in this control. How the interaction between LLT1 and CD161 can differentially inhibit and costimulate NK and T cells needs further investigation, but the implication of acid sphingomyelinase has been proposed (14). Importantly, CD161 expression was

reported on effector and memory T cells but not on naïve T cells (6). The role of the LLT1/CD161 interaction seems therefore to be restricted to the regulation of effector and memory T cell responses rather than primary activation of naïve T cells.

We found that the inhibitory and costimulatory signals delivered by CD161 are low (Figs. 5 and 6) and necessitate sufficient levels of LLT1 and CD161 on the cell surface to be detected. In particular, we show that a certain threshold of activation needs to be reached to get induction of LLT1 on the cell surface, which only occurs after 24 h of stimulation and is optimal at 48 h (Figs. 2–4). These findings correlate with the low affinity interaction between CD161 and LLT1, which shows a $K_D \sim 48$ μM (29). Also, CD161-mediated inhibition in NK cells has been linked to the presence in the intracytoplasmic domain of CD161, of a sequence homologous to immunoreceptor tyrosine-based inhibitory motifs but displaying an alanine at position −2 which decreases its inhibitory potential (30). Our data suggest that signals delivered by CD161 are tightly controlled, consistent with a role of fine tuning NK and T cell responses alongside other cell surface molecules. Alternatively, LLT1/CD161 may also play a role in the homing of NK and T cells to tissues, but this needs to be investigated further (15).

Because LLT1 was found to be expressed on T and NK cells in addition to APCs (Figs. 2–4), the LLT1/CD161 interaction may occur in *cis*. However, although we detected high levels of LLT1 on activated T cells, the addition of blocking anti-LLT1 mAb in the absence of APCs did not modulate the T cell activation threshold and T cell effector functions (data not shown). Further work is needed to decipher the physiological role of LLT1 on activated T and NK cells.

The induction of LLT1 by TLRs indicates that the LLT1/CD161 interaction regulates immune responses during infection. This was confirmed by the detection of LLT1 cell surface expression on B cells upon EBV or HIV infection and in inflamed tonsils, suggesting that the LLT1/CD161 interaction has important functions in infectious diseases, particularly viral and bacterial infections. However, the costimulatory role of CD161 in T cells may also be particularly relevant in diseases such as multiple sclerosis in which an expansion of $CD161^+CD8^+$ T cells secreting proinflammatory cytokines has been observed (31). Interestingly, an association between EBV infection and multiple sclerosis has been reported. We show that EBV induces LLT1 expression on the cell surface of B cells. Although correlative, these findings add another weight to the hypothesis that some of these $CD161^+CD8^+$ T cells in multiple sclerosis would recognize EBV epitopes.

Whereas CD161 is the unique member of the NKR-P1 family in humans, several NKR-P1 molecules have been described in rodents sharing between 45 and 48% homology with human CD161 (32–37). Rat and mouse *klrb1* genes encodes both inhibitory receptors (rNKR-P1B and -G, mNKR-P1B/D) with a conserved immunoreceptor tyrosine-based inhibitory motif in their cytoplasmic domain and activating receptors (rNKR-P1A and -F, mNKR-P1A, -C, and -F) that lack consensus immunoreceptor tyrosine-based inhibitory motifs but display a charged residue in their transmembrane region. Some C-type lectin-related (Clr) molecules encoded by *clec2d* genes in the NK cell complex are ligands for several mNKR-P1 and all rNKR-P1 receptors. Whereas partial conservation of specificity for Clr ligands is described among rNKR-P1 (38), Clr/mNKR-P1 interactions seem to be more specific with Clr-b described as the ligand of mNKR-P1B/D (39, 40) and Clr-g and Clr-x as ligands of mNKR-P1F (40, 41). The multiplicity of NKR-P1 molecules in rodents renders comparison with human system difficult, in particular when functional role is concerned. Further work will be needed to identify potential functional orthologs for LLT1.

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