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CD161 (Human NKR-P1A) Signaling in NK Cells Involves the Activation of Acid Sphingomyelinase

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NK and NKT cells play a major role in both innate immunity and in influencing the development of adaptive immune responses. CD161 (human NKR-P1A), a protein encoded in the NK gene complex, is a major phenotypic marker of both these cell types and is thought to be involved in the regulation of NK and NKT cell function. However, the mechanisms of action and signaling pathways of CD161 are poorly understood. To identify molecules able to interact with the cytoplasmic tail of human CD161 (NKR-P1A), we have conducted a yeast two-hybrid screen and identified acid sphingomyelinase as a novel intracellular signaling pathway linked to CD161. mAb-mediated cross-linking of CD161, in both transfectants and primary human NK cells, triggers the activation of acid, but not neutral sphingomyelinase. The sphingomyelinases represent the catabolic pathway for N-acyl-sphingosine (ceramide) generation, an emerging second messenger with key roles in the induction of apoptosis, proliferation, and differentiation. These data therefore define a novel signal transduction pathway for the CD161 (NKR-P1A) receptor and provide fresh insights into NK and NKT cell biology. The Journal of Immunology, 2006, 176: 2397–2406.

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Materials and Methods

Antibodies
mAbs specific for human CD161 (clone DX12), rat NKR-P1 (clone 10/78), human CD3, human CD56, and human CD25 were obtained from BD Pharmingen. mAb to TCR V\beta24 (clone 15) was purchased from Beckman Coulter. mAbs HP-3G10 (specific for human CD1 and HP-3B1 (specific for CD94) were a gift of Professor M. López-Botet (Universitat Pompeu Fabra, Barcelona, Spain). The CD59-specific mAb (MEM-43) was purchased from Abcam. Polyclonal Abs were obtained from Upstate Biotechnology (protein kinase B (PKB)/aAkt and Rsk/MAPK-activated protein (MAPKAP)-kinase 1a) and from New England Biolabs (PKB\aAkt phosphorylated on Ser473). Rabbit IgG specific for murine Ig and FITC-conjugated F(ab')2 of rabbit anti-murine Ig were obtained from DakoCytomation. Mouse IgG1 (MOPC21) isotype control Ab was purchased from Sigma-Aldrich. Mouse IgG1 (MOPC21) isotype control Ab was purchased from Sigma-Aldrich.

Cell lines and NK primary cell lines
YT, an FcR-negative, CD161-negative NK cell tumor line (18) was provided by Z. Eshhar (Weizmann Institute, Rehovot, Israel). 293T cells were originally obtained from American Type Culture Collection and were maintained in RPMI 1640 medium or DMEM supplemented with 10% heat-inactivated FCS (Harlan Sera Laboratory). 10\muM glutamine, 50 \muM 2-ME, 1 \muM sodium pyruvate, 1 \muM nonessential amino acids, and 50 \muM each streptomycin and penicillin. All cell culture reagents were from Invitrogen Life Technologies. Primary human NK cells (>95% CD3-negative, >95% CD56-positive) were obtained from healthy adult donors as described (19) and experiments were conducted at least 5 days after recombination human IL-2 stimulation. To generate CD161-expressing T cell lines, freshly isolated PBMC were stained with CD3-FITC and CD161-PE (BD Pharmingen) and bulk sorted using a DakoCytomation Mo-Flo sorter. Sorted cells (depending on donor, between 3 and 18% of initial CD3\+ PBMC) were expanded in culture under polyclonal activation conditions in RPMI 1640 supplemented with human serum (10%), purified PHA (0.5 kg/ml), recombinant human IL-2 (100 IU/ml), and 30 Gy-irradiated feeder cells (allogeneic PBMC \times 10\+ cells/ml; allogeneic B lymphoblastoid cells: 5 \times 10\+ cells/ml). Cells were checked periodically for expression of CD161. These lines were enriched for V\beta24-expressing T cells, but did not exclusively express this TCR (phenotypic data on one of the two lines used in these experiments are included in Fig. 6a). mAb stimulation experiments were performed 2–3 wk after restimulation.

Plasmids and transfection
The full-length CD161 was generated by RT-PCR from cDNA of an NK cell line using the oligonucleotides 5’-CGGAAATTCGCCACCATGGACCAACAAAAGCTATATGG3’ and 5’-GGGATCCCTACCAAGGCAGTACACATTCACC-3’ and cloned into the vector pBABE-CMV (21). The construct was confirmed by sequencing. Stable transfectants of CD161 were prepared in 293T cells by lipofectin-based transfection (SuperFect transfection reagent; Qiagen) and in YT cells by retrovirus gene-mediated transfer as previously described (21). CD161-positive cells were selected in puromycin and bulk sorted by FACS.

Yeast two-hybrid interactions
The CytoTrap yeast two-hybrid system was from Stratagene. Briefly, yeast temperature-sensitive cdc25H cells were cotransformed with constructs prepared in pSOS and pMyr vectors according to manufacturer’s conditions. The cytoplasmic tail fragment of CD161 was amplified from cDNA prepared from an NK cell line using the primers 5’-CGGAAATTCGCCACCATGGACCAACAAAAGCTATATGG3’ and 5’-GGGATCCCTACCAAGGCAGTACACATTCACC-3’. This fragment was cloned into the pSOS vector to be used as bait. A human spleen cDNA library in the pMyr vector (BD Pharmingen) and bulk sorted using a DakoCytomation Mo-Flo sorter. Sorted cells (depending on donor, between 3 and 18% of initial CD3\+ PBMC) were expanded in culture under polyclonal activation conditions in RPMI 1640 supplemented with human serum (10%), purified PHA (0.5 kg/ml), recombinant human IL-2 (100 IU/ml), and 30 Gy-irradiated feeder cells (allogeneic PBMC \times 10\+ cells/ml; allogeneic B lymphoblastoid cells: 5 \times 10\+ cells/ml). Cells were checked periodically for expression of CD161. These lines were enriched for V\beta24-expressing T cells, but did not exclusively express this TCR (phenotypic data on one of the two lines used in these experiments are included in Fig. 6a). mAb stimulation experiments were performed 2–3 wk after restimulation.

Sphingomyelinase assays and determination of ceramide levels
Both Asmase and nSMase activities were assayed as previously described with some modifications (22). Briefly, cells were grown starved (DMEM/10\% FCS) for 16 h before the experiments. Cells were incubated with Abs against either CD161 or IgG1 isotype control at 4°C for 15 min followed by incubation at 37°C with rabbit anti-mouse IgG (4 \mu g/ml) for the indicated time. Stimulation was stopped by immersion of samples in methanol/dry ice (−70°C) for 5 s followed by centrifugation at 1300 \times g for 3 min at 4°C. Stimulus with TNF-α, a known inducer of Asmase activity, was included in some experiments. For the Asmase assays, cells were preincubated with 100 \mu M imipramine or 10 \mu M SR33557, inhibitors of Asmase but not nSMase (23, 24). Cell pellets were resuspended in ice-cold Asmase or nSMase lysis buffers and further incubated for 15 min at 4°C. Cellular lysates were centrifuged at 500 \times g at 4°C, and supernatants used for enzymatic activity measurements. Protein content was measured (Coomassie Plus protein assay reagent; Pierce) and equal amounts of proteins (50–80 \mu g) were added to a final volume of 180 \mu l containing 454 pmol of [N-methyl-\(^{14}\)C]sphingomyelin (specific activity 55 mCi/mmol; Amersham Biosciences). Radioactive phosphocholine produced from \(^{14}\)C-labeled sphingomyelin was extracted and quantitated as indicated below.

Detergent extraction and isolation of lipid rafts
YT-CD161 cells were stimulated, as previously described, with either isotype control or anti-CD161 mAbs for 3 min at 37°C. To isolate membrane rafts, cells (3 \times 10\+ YT cells) were homogenized using eight strokes of a Dounce homogenizer and lysed for 30 min in ice-cold lysis buffer. Nuclei and cell debris were removed by centrifugation at 100,000 \times g for 20 min. For the discontinuous sucrose gradient, the preparation was made 40% with respect to sucrose. Then 0.8 ml of the lysate-sucrose mixture was overlaid sequentially with 6 ml of 30% sucrose and 4.5 ml of 4% (w/v) sucrose
prepared in TNE (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA). The mixture was centrifuged at 40,000 rpm for 16–20 h in a SW40Ti rotor (Beckman Instruments). From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 11 fractions. Efficiency of fractionation was monitored by Western blot of fractions using CD59-specific mAbs to define the lipid raft containing fractions. Immunoprecipitation was performed after addition of anti-CD161 mAb DX12 and rabbit anti-mouse Ig Ab to lipid raft and membrane fractions. Immunoprecipitated CD161 molecules were visualized by immunoblotting using the CD161-specific mAb HP-3G10. aSMase activity in the fractions was assayed as previously described.

Kinase assays
PKB/Akt and Rsk1 were determined as described by Alessi et al. (26) with some modifications. YT-CD161 cells were serum starved overnight before the experiments. For treatment with inhibitors cells were further preincubated with 100 nM wortmannin (Calbiochem) for 15 min, 10 μM SR33557 for 15 min, or 100 μM imipramine (Sigma-Aldrich) for 1 h and then stimulated with CD161 or control mAbs. After cross-linking, the cells were washed once in cold PBS and lysed on ice with 1 ml of lysis buffer containing protease and phosphatase inhibitors (20 mM Tris-HCl (pH 7.6), 0.1% (w/v) Triton X-100, 10% (v/v) glycerol, 137 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 25 mM sodium fluoride, 0.5 μM microcystin-LR, 1 μg/ml leupeptin, and pepstatin). Lysates were centrifuged for 5 min at 13,000 × g and kinases were immunoprecipitated from ≈300 μg of postnuclear cell-lysate protein with Abs against PKB/Akt1 and Rsk1/MAPKAP-kinase 1α (sheep and rabbit polyclonal IgG Abs, respectively) overnight at 4°C. Immunocomplexes were recovered with either protein G- or protein A-Sepharose and washed extensively with lysis buffer and then with kinase buffer (50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.1% (v/v) 2-ME, 0.03% Brij-35). Assay of PKB/Akt1 and Rsk1/MAPKAP-kinase 1α activity was performed in kinase buffer containing 50 μM substrate peptide GRPRTSSFAEG (Crosstide; Upstate Biotechnology), 15 μM PKA inhibitor peptide (Upstate Biotechnology), 15 mM magnesium acetate, and 1 μCi/assay of [γ-32P]ATP (Amersham Biosciences). After 30 min at 25°C, samples were spun, aliquots blotted onto paper, and reactions were stopped after three 0.75% (v/v) phosphoric acid washes, followed by one acetone wash. Radioactivity was measured with 3 ml of scintillation liquid.

Flow cytometry
All flow cytometric analyses were performed on a FACScan instrument (BD Biosciences).

Functional analysis of CD3+ and CD161+ T cells
These experiments were based on the assays for costimulation by CD161 described by Exley et al. (5). For activation of T cells (105/well), anti-CD3 mAb T3D (1 μg/ml), anti-CD161 mAb DX1 (10 μg/ml) or both mAbs were added to plates. In some experiments, T cells were recovered after 6 h of stimulation with plate-bound Ab in the presence of Golgi-Stop (BD Biosciences), and intracellular staining for IFN-γ performed. Intracellular staining for IFN-γ was done with the Cytofix/Cytoperm with kit and R-PE-conjugated 45B3 mAb (BD Biosciences) according to the manufacturer’s instructions. IFN-γ production was determined by flow cytometry.

Statistical analysis
Unpaired two-tailed Student’s t test was used for data analysis, calculated using PRISM software (GraphPad). Graphs show mean values of triplicate experiments, and error bars represent the SD. The p values obtained are shown for each experiment.

Results
Interaction of aSMase with the cytoplasmic domain of CD161
To identify novel proteins interacting with CD161, we used the yeast two-hybrid system to screen for proteins able to bind to the cytosolic domain of human CD161 (Met1–Gly4). A library of human spleen cDNA in the pMyr vector was screened for possible interactions with the cytosolic tail of CD161 as bait. After screening 4.6 × 109 clones and checking the specificity of the

FIGURE 1. aSMase associates with CD161. A, A yeast two-hybrid screen identified CD161 and aSMase as interacting partners. The position of the three independent clones compared with the full-length cDNA of human aSMase and a schematic of the predicted domain structure of the sphingomyelinase enzyme: M, Metallophos-Calciun-kinin-like phosphoesterase domain; S, saposin-like type B domain. B, Flow cytometric analysis to show the levels of CD161 expression on YT parent and YT-CD161 cells. C, aSMase coprecipitates with CD161 after cross-linking with CD161, but not control, Abs (see Materials and Methods). D, aSMase enzymatic activity can be detected in CD161, but not control, immunoprecipitates from YT-CD161 cells. YT-CD161 cells were stimulated by cross-linking with mAb against CD161 for 3 min and then lysed. mAbs to the indicated molecules were added to 100 μg of lysate for 1 h. The immune complexes were then recovered using protein A-Sepharose, and aSMase activity measured. Representative data from one of three experiments are shown.
interaction, the human aSMase gene was identified in three distinct clones (Fig. 1A). The nucleotide sequence of the three clones finished in different 3' positions as it would be expected from independent clones, but all of them started at the same 5' position, perhaps because of a stop during the retrotranscription while the cDNA was being made. In fact, we were not able to rescue the full-length aSMase cDNA from the library by PCR. One other cDNA, corresponding to an Expressed Sequence Tag database of unknown function, was identified in the yeast two-hybrid screen as a candidate molecule to interact with CD161, but we could not demonstrate any specific association of this cDNA with aSMase in assays of protein-protein interactions and so did not study this molecule further.

To confirm the association of aSMase with CD161 in a different experimental system, coprecipitation experiments were conducted. YT cells transfected with CD161 were incubated on ice with an isotype control CD56-specific mAb or CD161-specific mAb, washed, and then moved to 37°C with the addition of a cross-linking Ab. After the indicated time, the cells were lysed in digitonin, immune complexes recovered with protein A-Sepharose, and the presence of aSMase tested either by Western blot or by specific enzymatic assays. The Western blot (Fig. 1B) revealed an ~75-kDa band corresponding to aSMase in immunoprecipitates with CD161, but not isotype control or CD56 mAbs. No association of aSMase could be detected with CD161 in unstimulated cells (data not shown). Enzymatic assays confirmed the presence of aSMase activity in the CD161 immunoprecipitates from YT transfectants compared with control immunoprecipitates with anti-CD56 mAbs and IgG1 isotype control (Fig. 1C). In contrast, no significant differences between control and CD161 immunoprecipitates were observed when nSMase activities were assayed (data not shown). Taken together, these experiments confirm the interaction of aSMase with CD161. Comparison of the amount of aSMase activity associated with immunoprecipitated CD161 with aSMase activity in whole cell lysates (data not shown) indicates that 1–2% of total cellular aSMase activity is recruited to CD161.
on stimulation, an estimate consistent with densitometric analysis of the immunoprecipitation and Western blot data.

**CD161 is linked to aSMase activation**

To test whether CD161 stimulation can cause aSMase activation in intact cells, experiments measuring aSMase activity after mAb-mediated cross-linking of CD161 were conducted. Ligation of CD161 results in a statistically significant specific increase in aSMase, but not nSMase activity (Fig. 2A). This increased enzymatic activity was accompanied by a marked elevation of intracellular ceramide levels (Fig. 2A, inset). No increased aSMase activity was seen after stimulation of untransfected YT cells with anti-CD161 mAb (data not shown). Fig. 2B shows that the increased aSMase activity seen after CD161 cross-linking occurred rapidly and was transient, declining to basal levels after 10–20 min. Both the rapid rate of induction of aSMase activity and the amount of enzyme activity induced by CD161 stimulation is similar to that observed after stimulation with other molecules such as TNF-α or CD28 (27). The stimulation of sphingomyelinase activity seen after ligation of CD161 was substantially reduced by the presence of 10 µM aSMase inhibitor SR33557 (data not shown).

**CD161 cross-linking stimulates aSMase in human NK primary cell lines**

To determine whether CD161-triggering leads to activation of aSMase in a more physiological context, we repeated the previous experiments in a panel of NK primary cell lines instead of transfectants or tumor cells. Although the absolute levels of aSMase activity were lower in the primary NK cells than in the YT tumor cell line, these data show that CD161 activation results in specific aSMase stimulation (Fig. 3A), comparable, in terms of fold-induction, to that seen in YT-CD161 transfectant cells, despite the lower levels of CD161 surface expression (Fig. 3B) and the heterogeneity of the cells used.

**CD161 stimulation results in activation of the PI3K/PKB/Akt and ERK pathways**

The previous experiments demonstrate that ligation of CD161 triggers a pulse of aSMase activation and ceramide production that could affect diverse cellular processes, including apoptosis, proliferation, and differentiation (28). Ceramide generation is known to affect the activation of both protein kinases such as ceramide-activated protein kinase/kinase suppressor of Ras and protein kinase C (PKC)ζ and protein phosphatases PP1 and PP2A (29–31) and

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**FIGURE 4.** CD161 stimulation results in activation of the PI3K/PKB/Akt and ERK pathways. YT-CD161 cells were stimulated by control or CD161 cross-linking at 37°C for 5 min, and then lysed. Where indicated, YT cells were pretreated with 100 nM wortmannin or 10 µM SR33557 for 15 min, or 100 µM imipramine for 1 h before stimulation. Statistical significance (⁎) is indicated. A, PKB was immunoprecipitated from equal amounts of lysate and the enzyme activity assayed (see Materials and Methods). Results are expressed as cpm, and the data presented represent one of multiple experiments performed in triplicate. ⁎, p < 0.01. B, Rsk1 (MAPKAP kinase-1α) was immunoprecipitated from equal amounts of lysate and the enzyme activity assayed (see Materials and Methods). Results are expressed as cpm, and the data presented represent one of multiple experiments performed in triplicate. ⁎, p < 0.01. C, The 50 µg of lysate were run on 10% SDS-PAGE, transferred to PVDF, and PKB phosphorylated (p-PKB) on Ser473 and total PKB visualized by Western blotting with specific antisera. D, Aliquots of YT cells were pretreated with imipramine before stimulation with anti-CD161 and lysis. Rsk1 (MAPKAP kinase-1α) was immunoprecipitated from equal amounts of lysate and the enzyme activity assayed (see Materials and Methods). Results are expressed as cpm, and the data presented represent one of multiple experiments performed in triplicate. ⁎, p < 0.01.
activation of these enzymes can subsequently lead to altered phosphorylation, and activity, of a variety of molecules including Akt/PKB and proline-directed kinases such as MAPK. We therefore studied the effect of CD161 cross-linking on firstly, PKB/Akt activation, as a readout of possible effects of ceramide on PI3K and PKCζ (32–36), and secondly Rsk1/MAPKAP-kinase 1α activation as a readout of ceramide-activated protein kinase/kinase suppressor of Ras activation. Rsk are phosphorylated by ERK1/ERK2 (37) and have been suggested to be a substantial output of the MAPK cascade (38), which can in turn be regulated by kinase suppressor of Ras, both directly and indirectly via effects on Raf-1 (reviewed in Refs. 39 and 40) and perhaps by also PKCζ (41). Interestingly, at least in vitro, Rsk1 can also be dephosphorylated by both protein phosphatases PP1 and PP2A (37). Representative data for the effects of CD161 ligation on the activation of PKB/Akt and Rsk1 kinases are shown in Fig. 4, A and B. Ab-mediated ligation of CD161 induces a roughly 3-fold activation of both these enzymes, whereas inclusion of wortmannin, an inhibitor of PI3K, largely blocks PKB and Rsk activation. Similarly, pretreatment with SR33557, an inhibitor of aSMase, also reduces kinase activation.

PKB/Akt is activated by, and dependent upon, multitesite phosphorylation. Full activation of PKB/Akt requires phosphorylation on Ser473 (26); thus to confirm specific activation of PKB/Akt. Western blots with phospho-Ser473-specific Abs and loading controls were conducted. Fig. 4C confirms that CD161 cross-linking leads to activation of PKB/Akt and shows that the inhibition of PKB activation by SR33557 is titratable. Pretreatment of cells with an unrelated inhibitor of aSMase, imipramine, also blocks PKB activation. Fig. 4D demonstrates that imipramine treatment also blocks CD161-mediated Rsk1 activation.

Low doses of C₆-ceramide can trigger activation of PKB

That a pulse of ceramide, produced after CD161 ligation, leads to PI3K-dependent activation of PKB is surprising because a number of studies have reported that ceramide production leads to inhibition of PKB activation. However, in general, these studies have been based on analyses of effects produced by treatment of cells with high concentrations (around 100 μM) of cell permeable ceramide analogs. The amount of ceramide likely to be produced after treatment with SR33557, but not completely, perhaps reflecting the differing requirements of expression of activation markers and entry into cell cycle (data not shown).

Experiments of Western blotting with phospho-Ser473-specific antisera confirmed that CD161 cross-linking enhanced CD3-triggered PKB/Akt activation in primary T cells and that this enhancement depended on aSMase activity (Fig. 6D).

CD161 and aSMase associate in detergent-resistant membrane fractions

Although the vast majority of cellular aSMase localizes to an endosomal/lysosomal compartment, 1–2% of this enzyme can also be found in detergent-resistant membrane raft fractions (42, 43), often in association with caveolin. These membrane rafts are enriched in glycosphingolipids and sphingomyelin (44, 45) and have been suggested to be sites for ceramide generation in response to various stimuli (reviewed in Refs. 46 and 47). We therefore investigated whether CD161 was located in membrane rafts, either constitutively or recruited there after cross-linking, and whether the CD161 molecules associated with aSMase in detergent-resistant or detergent-soluble membrane fractions. For these experiments YT-CD161 cells were stimulated by CD161 cross-linking, lysed, and fractionated by discontinuous sucrose gradient centrifugation according to standard methods (48). Fig. 7A shows that the membrane raft-associated CD59 (GPI-linked) protein was recovered in the expected fractions for both cells treated with anti-CD161 or isotype control IgG1 mAb, confirming efficient fractionation of the cells. Western blot analysis of the raft and detergent-soluble fractions (Fig. 7B) shows that ligation of CD161 on the intact cell
promotes the recruitment of a sizable proportion of CD161 molecules into the membrane raft fractions. To study the possible association of the receptor and aSMase CD161, immunoprecipitates from the detergent-resistant (membrane raft) and detergent-soluble fractions of control and CD161 stimulated cells were analyzed for aSMase activity. These data (Fig. 7C) show clearly that although aSMase activity is found in both the fractions corresponding to lipid microdomains and in the detergent-soluble material at the bottom of the gradient, CD161-associated aSMase activity is found only in the membrane raft fractions.

Discussion

In the present work, yeast two hybrid studies led to the hypothesis that CD161 signaling involved activation of aSMase. This suggestion was then confirmed in biochemical and functional studies using CD161 transfectants (Figs. 1 and 2), primary human NK and NKT cell lines expressing CD161 (Figs. 3 and 6) and CD161-positive rat NK tumor cell lines (data not shown). mAb-mediated ligation of CD161 results in association of the receptor with an aSMase in detergent-resistant membrane rafts and enzyme activation that leads to ceramide production, downstream signaling events such as activation of PKB/Akt and Rsk1/MAPKAP-kinase 1 and proliferation.

The observation that CD161 ligation triggers aSMase activation in detergent-resistant membrane raft fractions is consistent with a number of previous reports showing that membrane rafts are sites of aSMase and nSMase activation and ceramide generation in response to various stimuli including IL-1β, CD95, and TNF (42, 43, 50, 51). CD161 is clearly recruited to these membrane rafts (Fig. 7), and confocal microscopy analysis has not provided any evidence of CD161 internalization after ligation of the receptor, (D. Pozo and M. Valés-Gómez, unpublished observations), supporting the suggestion that the interaction with aSMase is occurring at the cell surface. Quantitation of aSMase activity and protein associated with CD161 immunoprecipitated from whole cell lysates indicates that 1–2% of total cellular aSMase activity associates with CD161 on stimulation. Given that only 1–2% of cellular aSMase is located in membrane rafts (42, 43), the vast majority of aSMase resides in lysosomes. These data suggest that virtually all the membrane aSMase is recruited to CD161 after cross-linking with specific mAbs.

Inspection of an alignment of the cytoplasmic tails of rodent and human CD161 molecules reveals that they are quite distinct; for bound CD3 mAb (1 μg/ml) and/or plate-bound CD161 mAb (10 μg/ml). Some aliquots of the T cell lines were cultured with either imipramine (100 μM), SR33557 (10 μM) or DMSO for 30 min before plating with mAbs. T cell proliferation, measured by [3H]thymidine incorporation (cpm), was determined in triplicate at 72 h (SEM shown). One representative experiment (of six) is shown.

FIGURE 6. The costimulatory function of CD161 depends on aSMase function. A, Representative flow cytometry data showing the expression of CD3, CD161, Vα24, and CD94, compared with IgG1 isotype control, on the cell surface of one of the in vitro cultured CD3/CD161 sorted T cell lines. B, CD161-expressing T cell lines from two donors (10^5 cells/well, 3 wk after restimulation) were stimulated with limiting quantities of plate-bound mAb (10 μg/ml), or anti-CD3 mAb (1 μg/ml), or a mixture of CD3 (1 μg/ml) and CD161-specific mAb (10 μg/ml) in the presence or absence of the aSMase inhibitor imipramine (100 μM) or DMSO. After 6 h of stimulation with plate-bound Ab in the presence of Golgi-Stop (BD Biosciences), intracellular staining for IFN-γ was performed and assessed by flow cytometric analysis. B, Sorted CD3⁺ or CD161⁺ T cells were stimulated by treatment with either IgG1 control mAb (10 μg/ml), anti-CD3 mAb (1 μg/ml), or a mixture of CD3 (1 μg/ml) and CD161-specific mAb (10 μg/ml) on ice for 15 min, followed by rabbit anti-mouse cross-linking Ab at 37°C for 3 min. Where indicated, cells were pretreated with DMSO or 10 μM SR33557 for 15 min before stimulation. Cells were then lysed and 50 μg of lysate run on 10% SDS-PAGE, transferred to PVDF, and PKB phosphorylated (p-PKBα) on Ser473, and total PKB visualized by Western blotting with specific antisera.
example, human CD161 (4) does not contain the cytoplasmic tail p56Lck binding motif found in CD4 and CD8 and in all of the murine NKR-P1 molecules (12, 14). Only one patch of sequence was conserved between the rat and human receptors. This region contains various serine residues and interestingly, the cytoplasmic tail of human CD161 becomes phosphorylated on serine, but not tyrosine, residues after mAb-mediated cross-linking of the receptor (data not shown). It is possible that this region may be the site of association between the receptor and the enzyme, but definitive proof of this conjecture is lacking because to date it has not proved possible to achieve efficient cell surface expression of CD161 molecules with deletions in the intracytoplasmic portion of the receptor (D. Pozo and H. Reyburn, unpublished observations). The yeast-two hybrid studies indicate that the cytoplasmic tail of CD161 interacts with the C-terminal half of the aSMase enzyme. The regions of the aSMase molecule present in these partial cDNA clones are not known to be involved in protein-protein interactions, but are highly conserved in aSMase enzymes from various species. Little is known about the domain structure of aSMase; the diagram shown (Fig. 1A) is based on sequence homologies and is derived from the Pfam database (www.sanger.ac.uk/Software/Pfam/). It should be pointed out that aSMase is a member of a multigene family and shares motifs with a large family of metallophosphoesterases, thus although the enzyme identified in the yeast two-hybrid studies corresponds to aSMase (NM_000543), we cannot rule out the possibility that in the biochemical and functional studies (Figs. 1–3) in which only aSMase activity was measured, other related enzymes may also be activated after CD161 ligation. A number of points have to be borne in mind when considering the downstream signaling effects induced by CD161-stimulated aSMase activation. Sphingomyelinases are enzymes that catalyze the hydrolysis of sphingomyelin (ceramide phosphorylcholine) into ceramide and phosphorylcholine. Ceramide appears to be a lipid second messenger in programmed cell death (apoptosis), cell differentiation, and cell proliferation and has been reported to regulate directly or indirectly the activity of a number of enzymes and signaling components (29–31). However, confident interpretation of the published data is hampered by the fact that most observations come from studies using high concentrations of synthetic short chain ceramide analogs for treating cells over long periods of time, which may not reflect the transient physiologic generation of small amounts of ceramide in response to exogenous stimuli (reviewed in Refs. 28 and 52). For example, it has been shown that short-term exposure to low dose ceramide resulted in enhanced T cell proliferation (27, 53) whereas long term ceramide treatment resulted in inhibition of T cell proliferation (54).

In our experiments (Fig. 2) CD161-induced aSMase activation followed the same short-term kinetic as that seen for other receptor-mediated stimulation of aSMase activity and ceramide production (55). This observation is therefore at least consistent with ceramide having a growth promoting effect in this system, a hypothesis supported by the observation that activation of both PKB/Akt and Rsk1/MAPKAP-kinase 1α is seen after stimulation via CD161 (Figs. 4 and 6D). PKB/Akt and Rsk1/MAPKAP-kinase 1α activation is associated with enhanced cell survival, via phosphorylation and inhibition of the proapoptotic protein Bad (56–58).
These observations are thus entirely consistent with the idea that CD161 acts as a stimulatory molecule for human CD34+ immature thymocytes (11) and a costimulatory molecule for human NKT cells (5). Indeed, costimulation of T cell proliferation on cross-linking of CD161 was confirmed in these experiments (Fig. 6B) and it was further shown that inhibition of aSMase activity can block this effect. Strikingly, aSMase activation is also a feature of signaling via the better characterized T cell costimulatory molecules CD28 and LFA-1 (27, 59). Ceramide production after signaling via the better characterized T cell costimulatory molecule CD161 seems to be entirely dependent on PI3K stimulation. Although perhaps unexpected that wortmannin, a potent and highly specific inhibitor of PI3K, is able to block activation of elements of the MAPK pathway is consistent with previous observations (60, 61). Treatment with SR33557 or imipramine, inhibitors of aSMase, partially blocked activation of PKB and Rsk, an observation consistent with previous work in which SR33557 treatment consistently achieved a 50–70% inhibition of aSMase activity (24, 62).

It is also important to remember that a pulse of ceramide production will also affect the dynamic balance between cellular levels of ceramide and other sphingolipids that regulate opposing signaling pathways (30), e.g., ceramide can be rapidly metabolized to sphingolipids with properties different from, or even opposite to, ceramide: simple sphingolipid metabolites have been found to be important mediators of many signaling pathways related to cell proliferation, cell differentiation, cell cycle arrest, or apoptotic death. In this context, a possible explanation for the different reported effects of NKR-P1 receptors on human lymphocytes (4, 5, 11) could be differences in the extent and duration of ceramide generation.

It is also worth noting that in addition to its potential role as an intracellular second messenger molecule, ceramide may also have an indirect impact on cellular signaling due to its role as a membrane structural component (46, 52, 63). The conversion of sphingomyelin to ceramide would be expected to have marked alterations on both membrane transport and intracellular vesicle transport. For example, detergent resistant microdomains, “lipid rafts” contain large amounts of sphingomyelin (44, 45), and it has been reported (64), although this point is controversial (65), that T cells from mice deficient in aSMase appear to be deficient in lipid rafts, a phenotype accompanied by reduced signaling via protein tyrosine kinases. Consistent with these data, it has recently been shown that in human monocyte cell lines aSMase is rapidly activated on FcγRII cross-linking with consequent ceramide generation that controls FcγRII clustering and phosphorylation in lipid rafts (66). Determination of the extent of the contribution of mechanisms such as these to the observed signal transduction pathways triggered by CD161 ligation will be a major object of future research.

Another logical next step will be to test whether any murine CD161 molecules associate with aSMase, in addition to src family kinases such as p56Lck (14), and then to study whether any alteration of CD161 function can be detected in aSMase knockout mice. Initial studies of one strain of amsase−/− mice have not detected any gross disturbance in NK or T cell development or function (67), but NKR-P1 function was not tested directly in these experiments and this testing is not simple because mice, unlike humans, express multiple distinct NKR-P1 proteins (3) that can interact with src family tyrosine kinases such as p56Lck as well as, putatively, aSMase. In this situation of potential redundancy in function it is not clear that removing one element for signal transduction would necessarily inactivate NKR-P1 function. Interpretation of experiments with aSMase-deficient mice is further complicated by the observation that the two, independently generated, strains of aSMase knockout mice described to date have markedly different phenotypes (64, 65).

In any event and taking into consideration the major role played by NK and NKT cells in immune responses to pathogens and cancer and in the regulation of autoimmunity, that signaling via CD161, a major cell surface marker of these cell types, involves alterations in sphingomyelin metabolism could lead to new approaches toward intelligent manipulation of the immune system in these conditions (68).

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Disclosures
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