CD160-activating NK cell effector functions depend on the phosphatidylinositol 3-kinase recruitment

Magali Rabot¹, Hicham El Costa¹, Beata Polgar¹, Anne Marie-Cardine², Maryse Aguerre-Girr¹, Aliz Barakonyi¹, Salvatore Valitutti¹, Armand Bensussan²* and Philippe Le Bouteiller¹*

¹Institut National de la Santé et de la Recherche Médicale Unité 563 and Université Paul Sabatier de Toulouse, Hôpital Purpan, 31024 Toulouse Cedex 3, France
²Institut National de la Santé et de la Recherche Médicale Unité 841, Faculté de Médecine de Créteil, 94010 Créteil Cedex, France

Keywords: cytokine, innate immunity, signaling pathway

Abstract

CD160 NK cell-activating receptor is a glycosyl-phosphatidylinositol-anchored molecule that, upon specific engagement, triggers both cytotoxicity and a unique cytokine production [IFN-γ, tumor necrosis factor-α (TNF-α) and IL-6] through an undefined signaling pathway. In the current study, we have identified several signaling molecules recruited after mAb-specific CD160 engagement in freshly isolated human circulating NK cells. Using confocal microscopy, we found that CD160 engagement induces the recruitment and co-localization of phosphorylated molecules with redistributed, capped CD160 at the cell surface. We then demonstrated that phosphatidylinositol 3-kinase (PI3K) signaling molecule is required for CD160-mediated cytotoxicity and cytokine release. First, we observed by confocal microscopy that engagement of CD160 induces its polarization and co-localization with PI3K. Second, we showed that pharmacological inhibitors of PI3K abrogate both CD160-mediated cytotoxicity and IFN-γ, TNF-α and IL-6 cytokine release. We further found that CD160 engagement induced marked phosphorylation of Akt, as evidenced by western blotting. We identified additional CD160-mediated signaling molecules recruited downstream and upstream of PI3K. Both induction of phosphorylated ERK molecules after CD160-specific engagement and prevention of CD160-induced cytokine release by MEK pharmacological inhibitor indicate that ERK downstream pathway is implicated. Similarly, we identified that Syk molecule upstream of PI3K is involved in the signaling cascade mediated by CD160 engagement. Two different Syk-specific inhibitors blocked CD160-mediated cytokine release, and CD160-specific engagement induced the enhancement of phosphorylated Syk proteins. These data demonstrate that PI3K is a crucial signaling element for both effector functions of the CD160 NK cell-activating receptor.

Introduction

NK cells use various combinations of activating and inhibitory cell-surface receptors to mediate target cell killing and cytokine secretion upon interaction with specific ligands (1–3). Among the various human NK cell-activating receptors described to date, the MHC class I-dependent CD160 receptor containing a single Ig domain exhibits unique properties. First, in contrast to other human NK cell receptor genes located on chromosomes 12 and 19 (4), CD160 gene is located on chromosome 1 (5). Second, CD160 is a glycosyl-phosphatidylinositol (GPI)-anchored receptor (6). Third, CD160 is expressed on the CD56dim CD16+ peripheral blood (PB)-NK cell major subset that is highly cytotoxic (6). Fourth, CD160 cell-surface expression is rapidly down-modulated after PB-NK cell activation (7). We have previously demonstrated that CD160 is an activating NK cell receptor: engagement of the 80-kDa CD160 membrane receptor by anti-CD160 mAb cross-linking or by its specific physiological ligand, HLA-C, not only triggers strong cytotoxicity (8) but also potent IFN-γ, tumor necrosis factor-α (TNF-α), IL-6 (9) and IL-8 cytokine production (unpublished results). Moreover, it has been found that CD160 gene and gene products are present in both mice (10, 11) and rabbits (unpublished data), strongly suggesting that this receptor, recognized by the CL1-R2 mAb in these species, exerts conserved effector...
functions. CD160 receptor lacks tyrosine-based cytoplasmic signaling motifs. Contrasting with CD160, the other activating NK cell receptors have a short cytoplasmic tail and their charged transmembrane domain can associate with adaptor signaling proteins of two types, namely, DAP10 and immunoreceptor tyrosine-based activatory motif (ITAM)-bearing molecules (3, 12). Human activating NK cell receptors can be grouped in the following categories according to their intracellular signaling pathways: (i) NK2G2D receptor that is coupled to the transmembrane adaptor DAP10 which recruits the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and Grb2 upon phosphorylation, leading to a full cytotoxic response when triggered (13); (ii) the receptors that signal through the ITAM present on different adaptors including DAP12, CD3ζ, and FcRγ, and which recruit and activate Syk and/or ZAP70 protein kinases after phosphorylation by Src family kinases (14); CD16 (FcγRIII), natural cytotoxicity receptor Nkp44, killer cell Ig-like receptors (KIRs) with short cytoplasmic domain, C-type lectin CD94/NKG2C/E receptors and (iii) other receptors that signal by different pathways not using ITAM adaptors or DAP10 (2B4, DNAM-1), some of them being as yet largely unknown (CD2, NKp80). Yet, the signal events that drive CD160-mediated effector functions have not been defined. We set out experiments to define the signaling pathway that is activated by CD160-specific engagement. Here we demonstrate that mAb cross-linking of CD160 NK cell receptor induces the recruitment of PI3K, a critical signaling event leading to both cytotoxicity and cytokine secretion. We further identified the implication of Syk, Akt and ERK signaling elements.

Methods

Cells and pharmacological inhibitors

Fresh human PB-NK cells were isolated from normal volunteer blood donors (Etablissement Français du Sang, Toulouse, France) by magnetic negative selection using a MACS column (Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer’s protocol. Using double-color flow cytometry analysis, we found that PB-NK cell purity (CD3+ /CD56+) was consistently >95%. More than 85% of these purified PB-NK cells were CD160+. Such freshly isolated PB-NK cells were used immediately. The pharmacological inhibitors of PI3K, Wortmannin and LY294002, were purchased, respectively, from Sigma (Saint-Quentin Fallavier, France) and Calbiochem (San Diego, CA, USA). MEK1/2-specific inhibitor U0126, Piceatannol (Syk and ZAP70 inhibitor), ‘Syk inhibitor’ and ‘Jak inhibitor I’ were all purchased from Calbiochem. All the inhibitors were diluted in dimethyl sulfoxide (DMSO), used as negative control. NK cell viability (checked by trypan blue counting and flow cytometry annexin V-propidium iodide double staining) was intact in all groups, excluding the possibility that non-specific toxicity of these pharmacological reagents might result in loss of NK cell function.

Western blotting of phosphotyrosines

PB-NK cells (1 × 10⁶) were cross-linked during 5 min with CL1-R2 anti-CD160 mAb or IgG1 isotype control and then lysed immediately in 1% Triton X-100 lysis buffer [20 mM Tris–HCl, pH 7.5/150 mM NaCl/10 mM NaF/1% Triton X-100/1 mM phenylmethylsulphonyl fluoride (PMSF)/1 mM sodium vanadate/1 μg/ml 1 leupeptin/1 μg/ml aprotime]. After centrifugation at 13 000 r.p.m. at 4°C, equal amounts of proteins were fractionated by 10% SDS–PAGE and transferred onto nitrocellulose membrane. Immunobots were probed with an anti-phosphotyrosine (PTyr) mAb (4G10, Upstate Biotechnology Inc., Bucks, UK) followed by goat antimouse Ig coupled to HRP (Amersham Biosciences, Orsay, France). The detection was realized by autoradiography with an enhanced chemiluminescence detection system (Amersham).

Confocal microscopy

For capping experiments, human PB-NK cells were washed in PBS/1% FCS and incubated on ice for 20 min with anti-CD160 mAb (BY55, IgM, 10 μg ml⁻¹, produced locally) or anti-NKG2D mAb (1D11, IgG1, 10 μg ml⁻¹, BD Biosciences, San Diego, CA, USA). Cells were washed three times with PBS/1% FCS and incubated either at 37 or 4°C with an isotype-specific goat anti-mouse secondary antibody conjugated to FITC (CliniSciences, Montrouge, France, 10 μM) in PBS/1% FCS for 30 min. The cells were laid on poly-L-lysine-coated slides for 5 min at 37°C, fixed with 3% PFA, permeabilized with 0.1% Triton X-100 and stained with the following antibodies in PBS/3% BSA/10 mM HEPES: anti-PTyr (PY99, IgG2b, Santa Cruz, CA, USA), anti-PI3K p85α subunit (IgG1, Santa Cruz), anti-PI3K p110 subunit (IgG2a, Santa Cruz) or anti-CD45 (9.4, IgG2a, American Type Tissue Collection-LGC Promochem, Molsheim, France). Primary antibodies were revealed using Ig isotype-matched goat anti-mouse secondary antibodies conjugated to Cy5 or Alexa 546 (Molecular Probes, Cergy-Pontoise, France). The samples were mounted in 90% glycerol–PBS containing 2.5% 1-4-diazabicyclo(2.2.2)octane and were examined using a Carl Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany). To rule out any putative cross-reactivity, PB-NK cells were incubated with one of the primary antibodies (anti-CD160, anti-NKG2D, anti-PTyr, anti-PI3K or anti-CD45), followed by a mix of all secondary antibodies (goat anti-mouse anti-IgM–FITC, anti-IgG2b–Cy5 and anti-IgG1– or anti-IgG2a–Alexa 546). To determine the percentage of each co-capping, 200 cells were randomly scored from three independent experiments.

Redirected cytotoxicity assay

Redirected PB-NK cell cytotoxicity assays used the FcγRIII/IIl B815 murine mastocytoma cell line as targets in a 4-h ⁵¹Cr release assay, in the presence of 10 μg ml⁻¹ of anti-CD160 mAb (CL1-R2, IgG1, produced locally), anti-CD16 (3G8 mAb, IgG1, Beckman-Coulter, Villepinte, France) or a control mouse IgG1 (BD Biosciences), as previously described (8). PB-NK cells were pre-treated or not with the inhibitors of PI3K Wortmannin (0.01, 0.1 or 1 μM) or LY294002 (1, 10 or 25 μM) (Sigma) or DMSO solvent control for 30 min at 37°C, washed and used for the ⁵¹Cr release assay (Packard γ-counter).
Cytokine assay

Purified PB-NK cells were pre-treated or not with various concentrations of indicated inhibitors or DMSO solvent control for 30 min at 37°C. Cells were then briefly washed and used for mAb-specific cross-linking. mAb-specific engagement of CD160 (CL1-R2 mAb) or CD16 (3G8 mAb) was performed at the final concentration of 10 μg ml⁻¹ for 16 h at 37°C in 5% CO₂. IgG1 isotype controls were also used at the same conditions. Supernatants were collected for the measurements of cytokine release and stored at −80°C until further analysis. In some experiments, IL-2 (400 IU ml⁻¹) was added during the incubation time. The Tn1/Tn2 CBA kit (BD Biosciences) was used for simultaneous measurement of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ, according to the manufacturer’s instructions and as previously reported (9). Analysis was made on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences). The mean fluorescence was compared with standard curves and cytokine concentrations (pg ml⁻¹) calculated by using the CBA software provided (BD Biosciences). IL-2 measurements were excluded from the analysis because, in some experiments, NK cells were activated by this cytokine. IL-4 and IL-10 data were not shown because they were always negative.

Western blot

PB-NK cells (2.5 × 10⁶) were incubated for 30 min on ice with 1 μg of purified anti-CD160 (BY55) or anti-2B4/CD244 mAb (C1.7, IgG1, Beckman-Coulter). Goat anti-mouse IgM or IgG was then added, and cells were placed at 37°C for the indicated time. Following washes in cold PBS, cells were lysed in 1% Triton X-100 lysis buffer (20 mM Tris–HCl, pH 7.5/150 mM NaCl/10 mM NaF/1% Triton X-100/1 mM PMSF/1 mM sodium vanadate/1 μg ml⁻¹ leupeptin/1 μg ml⁻¹ aprotinin) for 1 h at 4°C. After a centrifugation step at 13 000 r.p.m. and 4°C, lysates were supplemented with SDS sample buffer, and proteins were fractionated by 10% SDS–PAGE and transferred onto nitrocellulose membrane. Immunoblots were first probed with an anti-phospho-Akt mAb (Cell Signaling Technologies/Ozyme, St Quentin en Yvelines, France) followed by goat anti-mouse Ig coupled to HRP. Membranes were then stripped and revealed using an anti-Akt mAb (Cell Signaling Technologies/Ozyme). The detection of the proteins was realized by autoradiography with an enhanced chemiluminescence detection system (Amersham Biosciences).

Phosphoprotein level analysis

Human purified PB-NK cells (10⁶) (or total PBMC) were stimulated by CL1-R2 anti-CD160 mAb, anti-CD16 mAb (3G8) or anti-CD3 mAb (UCHT1, IgG1, BD Biosciences) or IgG1 isotype control at the final concentration of 10 μg ml⁻¹ for 5 min at 37°C. Cells were then lysed in lysis buffer from BD™ Phospho Flex Set (BD Biosciences) and used for phospho-ERK and phospho-Syk measurements, according to manufacturer’s instructions (15). Analysis was made on a FACSCalibur flow cytometer. Mean fluorescence was compared with standard curves and phosphoprotein concentrations (units ml⁻¹) calculated by using the CBA software provided.

Results

Specific engagement of CD160 NK cell receptor induces the recruitment of phosphorylated tyrosines

We have previously shown that engagement of CD160 by specific mAbs induced both cytotoxicity and IFN-γ, TNF-α and IL-6 production (8, 9). Protein tyrosine phosphorylation is a crucial mechanism used by receptors to transmit their intracellular signals (16). In order to analyze the tyrosine phosphorylation of proteins induced by CD160 engagement, PB-NK cells were stimulated with anti-CD160 mAb and subjected to western blotting with anti-PTyr mAb 4G10. The blot shown in Fig. 1(A) indicated that CD160-specific engagement increased tyrosine phosphorylation of a number of proteins, as compared with untreated cells or IgG1 isotype control-treated cells. To determine whether CD160 engagement recruited these phosphorylated proteins, we cross-linked CD160 on PB-NK cells with anti-CD160 mAb. Following cell fixation, permeabilization and incubation with anti-PTyr antibody, the results were evaluated by confocal microscopy. In control experiments performed at 4°C, CD160 staining is detectable on the whole cell surface of PB-NK cells, whereas the PTyr staining is mostly membrane associated but can also be observed diffused in the cytosol (Fig. 1B, upper panel). By contrast, in experiments performed at 37°C, specific engagement of CD160 induced its redistribution (capping) at the cell surface of PB-NK cells (Fig. 1B, lower panel). Staining with anti-PTyr antibody revealed a similar redistribution of PTyr staining and its co-localization with CD160. Similar results were obtained with the CD160-expressing NK-92 cell line (data not shown). These results demonstrate that specific mAb-mediated engagement of CD160 induces the recruitment of phosphorylated tyrosine that co-localize with this receptor.

PI3K is required for CD160-induced cytotoxicity and cytokine production

After determining that CD160 engagement caused marked co-localized tyrosine phosphorylation, we set out to identify the signaling pathways transduced by this receptor. We first analyzed whether the pivotal PI3K, known to be implicated in the NK lytic process (17), was recruited after CD160 activation. Using confocal microscopy, we first found that specific CD160 mAb engagement induced the capping of CD160 and its co-localization with PI3K when the experiments were performed at 37°C (Fig. 2A, lower panel). By contrast, when experiments were performed at 4°C, CD160 staining was distributed all over the surface of PB-NK cells, and PI3K staining was also not polarized (Fig. 2A, upper panel). As expected, we found that a specific mAb engagement of NKG2D, another activating NK receptor previously known to recruit PI3K (17), results in a similar co-capping between NKG2D and PI3K when the experiments were performed at 37°C but not at 4°C (Fig. 2B, lower and upper panels, respectively). In a control experiment, we found an absence of co-capping between CD160 and CD45 (Fig. 2C). To further determine the role of PI3K in the CD160-mediated pathway, we tested the effects of pharmacological inhibitors of PI3K on both effector functions. We found that...
the PI3K inhibitors Wortmannin and LY294002 abrogated CD160-induced redirected cell lysis against P815 target cells in a dose-dependent manner (Fig. 3). A similar dose-dependent blocking effect of anti-CD16 mAb-mediated cytotoxicity was obtained with the same PI3K inhibitors, confirming previous observations (14). We then tested whether these PI3K inhibitors had inhibitory effects on the CD160-mediated cytokine release. We demonstrated that both Wortmannin and LY294002 caused a large reduction in CD160-induced IFN-γ, TNF-α and IL-6 production (Fig. 4) as compared with control samples of either media or DMSO (used as solvent for the pharmacological inhibitors).

We then investigated whether CD160 engagement activated the Akt serine/threonine kinase downstream of PI3K (14). After mAb BY55-specific engagement of CD160, Akt activation was assessed by immunoblotting PB-NK cell lysates with an antibody specific for the phosphorylated activated form of Akt. We found that CD160-specific engagement induced marked phosphorylation of Akt within 1–5 min (Fig. 5, left-hand side panel), as observed after 2B4/CD244-activating NK cell receptor engagement (Fig. 5, right-hand side panel). These findings further demonstrate the activation of PI3K after specific engagement of CD160.

Fig. 1. CD160-specific engagement induces the recruitment of phosphorylated tyrosines. (A) Human PB-NK cells were untreated (no antibody), treated with IgG1 (IgG1 control) or cross-linked with CL1R2 anti-CD160 mAb (CD160) and then lysed. Phosphorylated tyrosine molecules were detected by western blotting using the 4G10 anti-pTyr mAb. Molecular weights (in kDa) are indicated on the left. (B) Human PB-NK cells were incubated for 20 min at 4°C with BY55 anti-CD160 mAb followed by FITC-conjugated goat anti-mouse Ig (green) for 30 min at 4 or 37°C. After fixation and permeabilization, cells were stained with anti-pTyr mAb followed by a CY5-conjugated goat anti-mouse Ig (blue) and analyzed by confocal microscopy. Data are from one representative experiment out of three, using different donor bloods. Two hundred cells were randomly scored for pTyr co-localization with CD160. The mean percentage of co-capping from three independent experiments was 94%.

Fig. 2. CD160-specific engagement induces PI3K recruitment. PB-NK cells were incubated for 20 min at 4°C with BY55 anti-CD160 mAb (A and C) or anti-NKG2D mAb (B), followed by incubation with FITC-conjugated goat anti-mouse Ig for 30 min at 4 or 37°C (green). After fixation and permeabilization, PB-NK cells were then stained with anti-PI3K (A and B) or anti-CD45 (C) mAbs followed by Alexa 546-conjugated goat anti-mouse antibody (red). For each antibody, 200 cells were randomly scored from three independent experiments performed with three different donors for PI3K enrichment in CD160 or NKG2D caps. The percentage of co-capping is 92% for CD160/PI3K and 88% for NKG2D/PI3K.
Taken together, all these results demonstrate that PI3K is a major signaling mediator of CD160-induced cytotoxicity and cytokine production in PB-NK cells.

CD160 engagement recruits MEK and ERK downstream mediators of PI3K

To determine which signaling pathway downstream of PI3K was activated after CD160 engagement, we tested the effect of the MEK1/2-specific pharmacological inhibitor U0126 on CD160-induced cytokine production by CL1-R2 mAb. We found that this inhibitor blocked CD160-induced IFN-γ, TNF-α and IL-6 production in a dose-dependent pattern (Fig. 6A), thus demonstrating that the mitogen-activated protein kinase pathway was activated following CD160-specific engagement. We then analyzed whether CD160 engagement recruited ERK downstream signaling molecule. PB-NK cells were stimulated by anti-CD160 mAb, anti-CD16 mAb or control IgG and phospho-ERK induction was then analyzed on cell lysates by flow cytometry. CD160 mAb cross-linking increased phospho-ERK after 5 min (Fig. 6B, left-hand side panel). A similar phospho-ERK enhancement was detected after CD16 engagement, whereas IgG control treatment had no effect. As positive control, we found that an anti-CD3 mAb treatment of PBMC also triggers ERK phosphorylation (Fig. 6B, right-hand side panel), as already reported following TCR engagement (18). Together, these data demonstrate the involvement of both Akt and ERK downstream elements of PI3K after CD160 engagement and the direct involvement of MEK in the CD160-mediated specific cytokine release.

CD160 engagement recruits Syk upstream element of PI3K

We finally searched for PI3K upstream signaling events which could be activated by CD160-specific engagement. We focused on Syk, a major tyrosine kinase signaling molecule used by several activating NK cell receptors (19). We first found that the Syk pharmacological inhibitors Piceatannol (Syk family inhibitor) and Syk inhibitor blocked mAb CD160-induced IFN-γ release in a dose-dependent manner (Fig. 7A, left-hand side panel) as well as TNF-α and IL-6 production (data not shown). Similarly, the blocking of IFN-γ production by the same Syk inhibitors was observed following CD16 mAb-specific engagement (Fig. 7A, middle panel). Syk inhibitor had no effect on the production of IFN-γ by PB-NK cells activated by IL-2 (Fig. 7A, right-hand side panel). As negative control, a Jak pharmacological inhibitor did not affect production of IFN-γ by PB-NK cells after either CD160 mAb- or CD16 mAb-specific engagement, whereas it blocked IFN-γ release specifically mediated by a strong (400 IU ml⁻¹) IL-2 activation of PB-NK cells (Fig. 7A, right-hand side panel). These results indicate that Syk activation.
controls the CD160-mediated cytokine release. To demonstrate that Syk is recruited following CD160 engagement, we analyzed the level of phosphorylated Syk molecules triggered by CD160-specific engagement. PB-NK cells were stimulated by anti-CD160 mAb, anti-CD16 mAb or control IgG and the levels of phospho-Syk were subsequently analyzed by flow cytometry. CD160 mAb cross-linking increased phospho-Syk after 5 min (Fig. 7B). As expected, a similar phospho-Syk enhancement was obtained after CD16 mAb engagement, whereas IgG control treatment had no effect as compared with untreated PB-NK cells. Together, these data demonstrate the activation of Syk after CD160 engagement and that such involvement was necessary for the CD160-mediated cytokine release.

Discussion

We show here, using functional, biochemical, flow cytometry and confocal microscopy methods, that PI3K plays a pivotal role in the CD160-mediated signaling pathway, resulting in the activation of cytotoxicity and cytokine release in fresh PB-NK cells. This functional effect is inferred from the efficiency of the PI3K pharmacological inhibitors to block both CD160-mediated effector functions. We provided further demonstration that CD160 engagement recruits PI3K by showing that such engagement induces marked phosphorylation of Akt and ERK downstream signaling elements within a very short period of time. PI3K was described as key upstream regulator of MEK1/2–ERK signaling molecules in the cytolytic process (17). Whether PI3K also controls the cytokine production mediated by ligand engagement of the different activating NK cell receptors is a subject of debate (14). Here we provide evidence that CD160-mediated cytokine production is blocked by PI3K- and MEK-specific inhibitors, thereby demonstrating that PI3K pathway can also control cytokine production. Such dual activating effector function using the PI3K pathway is also characteristic of CD16. This is distinct from NKG2D/DAP10-mediated activation which only triggers cytotoxicity but is insufficient to induce cytokine secretion (20).

We found that CD160 engagement also involves the PI3K upstream element Syk, since the CD160-mediated cytokine release is prevented by the use of Syk-specific inhibitors, and that Syk becomes phosphorylated after CD160 engagement. These data show that CD160-mediated signaling pathway is Syk dependent which is a second difference...
when compared with the NKG2D–DAP10-mediated pathway which is mostly Syk independent (19, 20). The pattern of CD160-recruited molecules rather resembles one of the ITAM-dependent activating receptors described to date (14). Whether CD160 engagement only activates the PI3K–ERK signaling pathway and/or other downstream pathways is unknown. All the results described here have been obtained after BY55 (IgM) or CL1-R2 (IgG1) mAb-mediated CD160 cross-linking. The signaling events mediated by engagement of CD160 with its physiological ligand, HLA-C, are very likely to be the same as both types of engagement were previously shown to trigger exactly the same activating effector functions, i.e. cytotoxicity and IFN-γ, TNF-α and IL-6 secretion (8, 9).

As NKG2D signaling pathway is distinct from that of KIR and other C-lectin like receptors, its specific engagement is likely less susceptible to signals mediated by inhibitory KIRs (21). In contrast to NKG2D, involvement of Syk in the CD160 signaling pathway may explain why inhibitory KIRs down-modulate CD160-mediated effector functions (8, 9). How CD160-mediated signaling could be negatively controlled by these KIRs in the absence of pathogens or tumors...
Fig. 7. Syk pharmacological inhibitors abrogate CD160-mediated IFN-γ production and CD16 mAb cross-linking induces Syk phosphorylation. (A) After pre-treatment with different concentrations of Syk-specific inhibitors piceatannol (Pic) and Syk inhibitor (SI) or Jak inhibitor I (JI) control, PB-NK cells were activated by CL1-R2 anti-CD160 mAb, anti-CD16 mAb or IL-2 00 IU ml⁻¹ for 16 h. Sample supernatants were analyzed by CBA for IFN-γ production. Cytokine concentrations in the samples were calculated relative to the appropriate calibration curves with standard dilutions for each cytokine. Results are expressed as mean ± SD of triplicates. Data are representative of four independent experiments performed on different donors. (B) PB-NK cells were stimulated by anti-CD160 mAb, anti-CD16 mAb or IgG1 isotype control and phospho-Syk measurements were analyzed by flow cytometry on denatured cell lysate samples. Data are representative of three different donors.
remains to be determined. Similarly, our current observation that the levels of CD160-mediated signaling molecules activation and the subsequent levels of CD160-mediated effector functions vary from one donor to another (some donors are even unresponsive) needs to be explained.

The identity of putative CD160-associated proteins—if any—that may transduce activating signals is not yet known. CD160 being a GPI-anchored molecule, is unable to associate with DAP10 or DAP12 signaling adaptors. One hypothesis is that engagement of CD160 induces rafts aggregation and thus massive recruitment and subsequent activation of early signaling molecules such as Src kinases, without assistance of any associated proteins. Another possibility is that CD160 may associate with intermediate unidentified signaling molecules localized in the lipid raft domains (22). CD160 NK cell receptor might activate the p56<sup>ck</sup> protein tyrosine kinase, since it has been shown to associate with CD160 in cytotoxic CD8<sup>+</sup> T cells (23). Our observation that Src inhibitors abrogate the cytokine release mediated by specific CD160 mAb engagement (data not shown) favors these hypotheses. CD160 might possibly associate with other activating receptors present in the lipid raft as shown for NKG2D receptor (supplementary data Fig. S1 is available at International Immunology Online). Furthermore, we have recently found that engagement of CD160 leads to its colocalization with the co-receptor CD2 on PB-NK cells (24). Further experiments are in progress to investigate how the GPI anchor of CD160 can confer signaling capacity.

In summary, our results outline a CD160-mediated signaling pathway that triggers cytotoxicity and cytokine production. Overall, our data establish a pivotal role for PI3K in the CD160-mediated effector functions of the PB-NK cells, and identify some of the upstream and downstream signaling molecules of PI3K. Understanding how this CD160-mediated signaling pathway is integrated in vivo with the other activating ITAM- and DAP10-dependent pathways to target viral infection or tumor cells warrants further analysis.

Supplementary data
Supplementary Fig. S1 is available at International Immunology Online.

Acknowledgements
We thank Sabina Müller, Aurélie Wiedemann and Nathalie Pizzato for their helpful advice. This work was supported by institutional grants from Institut National de la Santé et de la Recherche Médicale (P.L.B., A.B. and S.V.), Paul Sabatier University (P.L.B., S.V. and M.R.), Paris XII University (A.B. and A.M.C.), Association pour la Recherche sur le Cancer (A.B. and B.P.) and Libanese CNRS and Tabbib Nafsak magazine (H.E.C.). P.L.B’s team is member of the ‘EMBIC’ European Network of Excellence.

Abbreviations

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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>DMSO</td>
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<tr>
<td>GPI</td>
<td>glycosyl-phosphatidylinositol</td>
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