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# A Soluble Form of the MHC Class I-Specific CD160 Receptor Is Released from Human Activated NK Lymphocytes and Inhibits Cell-Mediated Cytotoxicity<sup>1</sup>

Jérôme Giustiniani, Anne Marie-Cardine, and Armand Bensussan<sup>2</sup>

CD160 is a GPI-anchored lymphocyte surface receptor in which expression is mostly restricted to the highly cytotoxic CD56<sup>dim</sup>CD16<sup>+</sup> peripheral blood NK subset. We previously reported that MHC class I (MHC-I) molecules bind to CD160 receptors on circulating NK lymphocytes and that this interaction triggers their cytotoxic activity and cytokine production. We also observed that CD160 surface expression on NK cells is down-modulated upon activation with PMA or IL-2. In this study, we further report that short-time incubation of NK lymphocytes with IL-15 converts the membrane-bound CD160 to a soluble form through a proteolytic cleavage involving a metalloprotease. Thus, CD160 is no longer detected at the cell surface, but can be immunoprecipitated from the NK cell culture medium. Interestingly, CD160 transcript remains highly expressed during the process of protein shedding. In addition, we demonstrate that CD160 mRNA synthesis can be induced in CD56<sup>bright</sup> separated lymphocytes following exposure to IL-15. By producing a Flag-tagged soluble CD160 protein, we establish that its binding to MHC-I molecules results in the inhibition of the cytotoxic CD8<sup>+</sup> T lymphocyte activity and of the CD160-mediated NK cell cytotoxicity. Thus, we show that activated NK lymphocytes release a soluble form of CD160 that functionally impairs the MHC-I-specific cytotoxic CD8<sup>+</sup> T lymphocyte responsiveness. *The Journal of Immunology*, 2007, 178: 1293–1300.

Natural killer cells are a class of lymphocytes involved in innate immunity that provide an important first line of immune surveillance to limit virus propagation and tumor burden before the onset of adaptive T and B lymphocyte immunity (1–3). In humans, NK lymphocytes are phenotypically characterized by the expression of CD56, an isoform of the neural cell adhesion molecule, and by the absence of CD3 (1, 4, 5). In peripheral blood (PB),<sup>3</sup> two subsets of NK cells were identified, namely, a CD56<sup>dim</sup> and a CD56<sup>bright</sup> population corresponding to ~95 and 5% of the total circulating NK pool, respectively. The predominant CD56<sup>dim</sup> subset, which mainly consists of killer cells, is CD16<sup>+</sup> and perforin<sup>+</sup>. In contrast, the CD56<sup>bright</sup> subset exerts immunoregulatory functions and poor cytotoxic activity and lacks or expresses few CD16 molecules (6–8).

Immunoregulatory, as well as cytotoxic effector, NK lymphocyte functions are regulated by signals deriving from the engagement of activating and inhibitory receptors (9). Most inhibitory NK receptors (NKR) engage MHC class I (MHC-I) molecules on target cells (10). In humans, these receptors can be classified into two

groups, recognizing either common allelic determinants of MHC-I molecules, or surveying MHC-I molecules in general. The NKRs belonging to the latter group structurally correspond to C-type lectin- or Ig-like receptors, as the HLA-E-specific CD94/NKG2A heterodimer (11) or the leukocyte Ig-like transcript 2 (ILT-2), that show a broad specificity for several MHC-Ia or Ib molecules (12). In contrast, the receptors of the first group only belong to the Ig superfamily (also referred to as killer-cell Ig-like receptors (KIRs)) and include: the receptors KIR2DL1 and KIR2DL2/DL3, specific for distinct alleles of HLA-Cw; KIR3DL1 that interacts with HLA molecules exhibiting the Bw\*04 specificity; KIR3DL2 which may recognize peculiar HLA-A alleles; and KIR2DL4 which binds to the MHC-Ib molecule HLA-G (9, 12). The inhibitory NKRs encompass an intracellular tail, where the characteristic ITIM involved in the generation of inhibitory signals once the receptor is engaged is located. In contrast, the activating NKRs lack such a cytoplasmic tail and mediate their signaling through the association with adaptor molecules bearing an ITAM motif. In most cases, an activating NKR corresponds to each MHC-I molecule-specific inhibitory NKR. However, while both receptors originate from distinct genes located on chromosome 19 in humans (13), their amino acid sequences present enough homology so that they can be recognized by the same mAb. Importantly, most of the activating receptors group includes a set of unique receptors that are not MHC-I specific. These receptors correspond to the natural cytotoxic receptors NKp46/CD335, NKp44/CD336, and NKp30/CD337 (14). Although NKp46/CD335 has been reported to recognize the viral hemagglutinin in a sialic acid-dependent way (15), NKp30/CD337 was found to interact with the pp65 CMV protein (16). These receptors are uniquely expressed on NK cells. Another activating receptor is the NKG2D homodimer, in which expression is not restricted to NK lymphocytes. It was shown to be specific for several ligands including the stress-inducible surface glycoproteins MHC-I chain-related gene (MIC) A and B, and the UL16-binding proteins 1–4 (17).

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<sup>3</sup> Abbreviations used in this paper: PB, peripheral blood; NKR, NK receptor; MHC-I, MHC class I; ILT, Ig-like transcript; KIR, killer-cell Ig-like receptor; sCD160, soluble CD160; 1,10 PNT, 1,10 phenanthroline; MIC, MHC-I chain-related gene; PLD, phospholipase D.

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Besides these activating receptors nonrestricted to MHC-I molecules, we reported the identification of the MHC-Ia/Ib-specific Ig-like type activating receptor BY55/CD160. CD160 expression is restricted to the cytotoxic CD56<sup>dim</sup> NK lymphocyte subset (18–20) and to circulating CD8<sup>+</sup> T cells expressing granzyme B and perforin (21). We previously established that the engagement of CD160 receptors at the cell surface leads to the production of cytokines and the induction of cytotoxic functions (22, 23). These CD160-mediated processes are restricted to freshly isolated PB-NK lymphocytes and were not observed on cytokine-activated NK lymphocytes or on in vitro-expanded NK lymphocyte clones (18, 23). In addition, CD160 receptor appears to be unique, as it is encoded by a gene located on human chromosome 1 and corresponds to a GPI-anchored Ig-like molecule in which surface expression is rapidly down-modulated upon NK cell activation (24).

Previous studies established a critical role for IL-15 in the mechanisms regulating NK cell differentiation and functions. Thus, mice lacking the IL-15R  $\alpha$ -subunit presented a complete defect in NK cell production (25). Similarly, mice genetically deficient in IL-15 lacked NK cells, indicating an obligate role for IL-15 in the development and functional maturation of these cells (26). More recently, IL-15 has been implicated in the up-regulation of NK cell receptor expression and function, as demonstrated for NKG2D (27) and NKp44 (28).

The release of several membrane-bound molecules under a soluble form has been demonstrated, as for the TNF- $\alpha$  molecules (29) or the glycoprotein MICA (30). It has been shown that the shedding of these molecules distally affects the target cells or modulates the cell-cell interaction by reducing the ligand densities. We therefore investigated CD160 processing upon IL-15-mediated activation of NK cells. We describe a novel immunoregulatory function for a soluble form of CD160, which can be proteolytically generated under the control of a metalloprotease following NK cell activation.

## Materials and Methods

### Cells

PBMC were isolated from heparinized venous blood obtained from healthy donors, by density gradient centrifugation over lymphocytes separation medium (PAA Laboratories). Fresh PB-NK cells were isolated using a MACS and a NK cell isolation kit according to the manufacturers' recommendations (Miltenyi Biotec). PB-NK cell purity was shown to be >90%. The selection of in vitro allogeneic-MHC-I-restricted effector T lymphocytes was performed as previously reported (31). Briefly, PBMC were cocultured for 6 days with irradiated EBV-transformed B cells in RPMI 1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100  $\mu$ g/ml), L-glutamine (2 mM), and 10% heat-inactivated human serum (Jacques Boy Institute, Lyon, France). At day 6 of the mixed lymphocyte culture, the CD8<sup>+</sup> population was isolated using CD8<sup>+</sup> microbeads according to the manufacturer's instructions (Miltenyi Biotec). Cells were then extensively washed and cultured overnight at 37°C and further tested as effector cells against the specific allogeneic EBV-B target cell line in lymphocyte-mediated cytotoxicity assays. CD8<sup>+</sup> cells represented >90% of the isolated lymphocyte population. To separate the CD56<sup>dim</sup> and CD56<sup>bright</sup> PB-NK cell populations, PB-NK cells were stained with an anti-CD56 PE-conjugated mAb, and sorted using an ELITE cell sorter (Beckman Coulter).

All cell lines used in this study were cultured in standard culture medium containing 10% FCS (Perbio Science). The 721.221-HLACw3 stable transfectants (221-Cw3; provided by Dr. P. Le Bouteiller, Institut National de la Santé et de la Recherche Médicale, Unité 563, Toulouse, France) were obtained by transfection of 721.221 cells (221) with a HLA-Cw3 coding vector.

### Abs and flow cytometry

The Abs used in this study were the following: anti-Flag M2 mAb (Sigma-Aldrich), rabbit anti-GPI-phospholipase D (Caltag Laboratories), anti-CD56 mAb (Beckman Coulter), and anti-CD160 mAb (BY55 (IgM) and CL1-R2 (IgG1), produced locally). Irrelevant isotype-matched Abs were

used as negative controls. FITC- or PE-conjugated goat anti-mouse IgG or IgM (Beckman Coulter) or goat anti-rabbit IgG (Caltag Laboratories) were used as secondary reagents. Cells were phenotyped by indirect immunofluorescence. Briefly, the cells were incubated with the specific mAb for 30 min at 4°C, washed twice in PBS, and further incubated with the appropriate FITC- or PE-labeled secondary Abs. After washing, cells were analyzed by flow cytometry on an EPICS XL apparatus (Beckman Coulter). For intracellular staining, the cells were permeabilized in saponin buffer (PBS/0.1% and BSA/0.1% saponin; Sigma-Aldrich) before staining, and all subsequent steps were performed in saponin buffer, as described above.

For soluble CD160 (sCD160) binding assays, COS7 cells were transfected with an expression vector coding for a Flag-tagged soluble CD160 protein. Following cell recovery, cell culture medium was collected and concentrated in a Centricon Plus-20 centrifugal filter (cut-off: 30 kDa; Millipore). Several dilutions of COS7 cell culture medium, corresponding to concentrations of sCD160 ranging from 0.5 to 10  $\mu$ g/ml were tested for their ability to label HLA-Cw3-expressing cells. Typically, 10<sup>5</sup> 221-Cw3 cells were incubated with 50  $\mu$ l of sCD160-Flag containing supernatant in a 96-well round-bottom plate. Culture medium obtained from COS7 cells transfected with an empty vector was used as negative control. After 1 h at 37°C, cells were washed and fixed in PBS containing 2% paraformaldehyde for 20 min at 4°C. Cells were then washed twice, incubated with the anti-Flag M2 mAb for 20 min at 4°C, and stained with FITC-conjugated goat anti-mouse Abs. After washes, the cells were analyzed using an XL flow cytometer (Beckman Coulter).

### Inhibition of the GPI-anchored CD160 cleavage

The phospholipase inhibitors U73122, U73343, and 1,10 phenanthroline (1,10 PNT) were purchased from Sigma-Aldrich. A total of 3  $\times$  10<sup>5</sup>/ml PB-NK cells were cultured for 72 h in RPMI 1640 supplemented with 10% heat-inactivated human serum, penicillin/streptomycin, L-glutamine, and IL-15 (10 ng/ml; PeproTech). The cells were then washed and incubated for 8–12 h at 37°C in IL-15-containing medium alone, or supplemented with 1,10 PNT (10  $\mu$ M), U73122 (2  $\mu$ M), or U73343 (2  $\mu$ M). U73343, an inactive analog of U73122, was used as negative control. After two washes in PBS, cells were processed for CD160 immunostaining and flow cytometry analysis.

### RNA extraction, reverse transcription, and cDNA amplification (RT-PCR)

Total RNA was isolated using the TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies). For each reverse transcription, 5  $\mu$ g of RNA was used. Reverse transcription was performed using 500 ng of an oligo(dT) primer (Invitrogen Life Technologies) and the Powerscript reverse transcriptase (BD Clontech) in a total volume of 20  $\mu$ l. Specific primers for the amplification of CD160 and GPI-phospholipase D1 (PLD1) variant 1 and 2 cDNA were designed on the basis of published sequences (20, 32). The CD160 primers were as follows: 5'-TGCAGGAT GCTGTTGGAACCC-3' (forward) and 5'-CCTGTGCCTGTTGATTCT TG-3' (reverse). The primer sequences for the cDNA amplification of GPI-PLD1 variant 1 were 5'-ATGGATGGCGTGCCTGACCTGGCC-3 (forward) and 5'-CAGCGGTGGCTGCAGGTCGGATGT-3' (reverse), and 5'-GTGTTGGACTTTAACGTGGACGGC-3' (forward) and 5'-CAGC AGAGGCTGCGCGTCAGATAT-3' (reverse) for the GPI-PLD1 variant 2.  $\beta$ -actin cDNA amplification was performed in parallel as internal control. The synthesis of specific cDNA fragments was achieved by using 1  $\mu$ l of the reverse-transcribed product according to a standard procedure (Invitrogen Life Technologies), in a total volume of 20  $\mu$ l. Each sample was subjected to denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 90 s) steps for 35 cycles. The amplified products were separated on a 1% agarose gel.

### Production and quantification of soluble CD160-Flag

A cDNA encoding a C-terminal Flag (DYKDDDK)-tagged sCD160 (sCD160-Flag) was generated by PCR amplification of the sequence corresponding to aa 1–160 of CD160 with the following primers: 5'-TGCAGGAT GCTGTTGGAACCC-3' (forward) and 5'-TCACTGTGCATCGTCGCTCT TGTAGTCGCTGAAGTGAAGAGTGCCTTC-3' (Flag-reverse). After purification, the resulting PCR product was ligated into the pcDNA3 expression vector (Invitrogen Life Technologies) and the construct double-strand was sequenced.

COS7 cells were transiently transfected with the pcDNA3 vector, or the sCD160-Flag expression vector, using the DEAE-dextran method, and subsequently cultured for 72 h in serum-free RPMI 1640 medium supplemented with L-glutamine and antibiotics. An ELISA was developed to



detect the produced sCD160-Flag protein in the cell culture medium, as previously performed for the quantification of soluble CD100 (33). Briefly, the anti-Flag M2 mAb (5  $\mu$ g/well) was coated in a 96-well plate (MaxiSorp; Nunc, ClineScience) overnight at 4°C. All subsequent steps were performed at 4°C. Following saturation with PBS/1% BSA for 4 h, the sCD160-Flag containing medium of transfected COS7 cells was added for 2 h. After extensive washes with PBS/1% BSA, the anti-CD160 (CL1-R2)-biotinylated mAb (diluted in PBS/1% BSA) was added. After washes and incubation with streptavidin-alkaline phosphatase, the revelation step was performed using the pNpp Liquid Substrate System for ELISA (Sigma-Aldrich). After a 1-h incubation in the dark, at room temperature, the absorbance was measured at 405 nm using a plate reader spectrophotometer (Packard Instrument). A standard curve was realized using purified sCD160-Flag protein. To this aim, sCD160-Flag was immunoprecipitated from transfected COS7 culture medium using CL1-R2 mAb coupled to protein G-Sepharose beads (Amersham Biosciences) and eluted in 2 mM glycine-HCl (pH 2.8). After neutralization, a second immunoprecipitation step was performed with agarose-coupled anti-Flag mAb (Sigma-Aldrich). sCD160-Flag was finally eluted in 2 mM glycine-HCl (pH 2.8). The eluate was neutralized, submitted to dialysis in PBS, and concentrated (Centricon; Millipore). The protein concentration was then estimated on a silver-stained gel by comparison with known quantities of BSA.

### Immunoprecipitation and immunoblotting

Culture medium (10 ml) from transfected COS7 cells was incubated with 5  $\mu$ g of anti-Flag M2 mAb for 90 min at 4°C, and immune complexes were collected with 20  $\mu$ l of protein G-Sepharose beads. Alternatively,  $2 \times 10^7$  control or IL-15-activated PB-NK lymphocytes were cultured for 24–48 h in 10 ml of RPMI 1640 medium without serum. Culture supernatants were collected and incubated with CL1-R2 mAb (10  $\mu$ g/test) followed by protein G-Sepharose beads. After washes, the precipitated proteins were separated by SDS-8% PAGE. The proteins were then transferred onto a nitrocellulose membrane and subjected to Western blot analysis using the anti-CD160 (CL1-R2, 5  $\mu$ g/ml) or anti-Flag M2 (5  $\mu$ g/ml) mAb. HRP-conjugated goat anti-mouse Abs (Jackson ImmunoResearch Laboratories) were used as secondary Abs, and the immunoreactive proteins were visualized using an ECL kit (Amersham Biosciences).

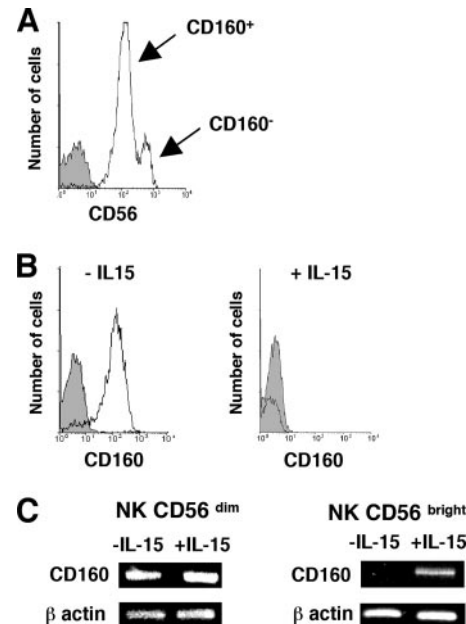
### Lymphocyte-mediated cytotoxicity

The lymphocyte cytotoxicity was tested in a  $^{51}\text{Cr}$ -release assay. Target cells were labeled with 100  $\mu\text{Ci}$  of  $\text{Na}^{51}\text{CrO}_4$  for 90 min at 37°C, and washed three times in RPMI 1640 medium containing 10% FCS. The target cells were then plated in 96-well V-bottom microtiter plates (Greiner) for 1 h at 37°C. When necessary, the cells were subjected to a preincubation step with 50  $\mu$ l of culture medium from COS7 cells transfected with pcDNA3 or sCD160-Flag expression vector. The effector cells were then added in a final volume of 150  $\mu$ l/well. Assays at various E:T cell ratios on  $10^3$  target cells were performed in triplicate. After 4 h of culture at 37°C, the plates were spun down and 100  $\mu$ l of the cell supernatant were collected from each well. The determination of  $^{51}\text{Cr}$  release was done using a gamma-counter (Packard Instrument). The percentage of specific lysis was determined as previously reported (18). The lysis was considered as significant when representing >10% of the maximum level of cell lysis.

## Results

### CD160 membrane expression is decreased in IL-15-cultured PB-NK lymphocytes

Initial findings revealed a loss of BY55/CD160 cell surface expression on NK cells treated with PMA (18). More recently, we reported that NK lymphocytes cultured in the presence of IL-2 exhibit a decreased cell surface reactivity toward an anti-CD160 mAb, when compared with untreated cells (23). Similar CD160 immunolabelings were performed on highly purified PB-NK cells consisting in  $\text{CD56}^{\text{dim}}\text{CD160}^+$  and  $\text{CD56}^{\text{bright}}\text{CD160}^-$  lymphocyte subsets at a 9:1 ratio (Fig. 1A). We observed that a 72-h incubation time of these cells with IL-15 results in a strong decrease in anti-CD160 mAb recognition at their cell surface, as revealed by flow cytometry analysis (Fig. 1B). Both anti-CD160 Abs, namely CL1-R2 and BY55, that are directed against distinct epitopes of the molecule, lost their reactivity toward IL-15-activated NK lymphocytes. Interestingly we found that, while CD160 molecules become undetectable at the cell surface of IL-15-acti-

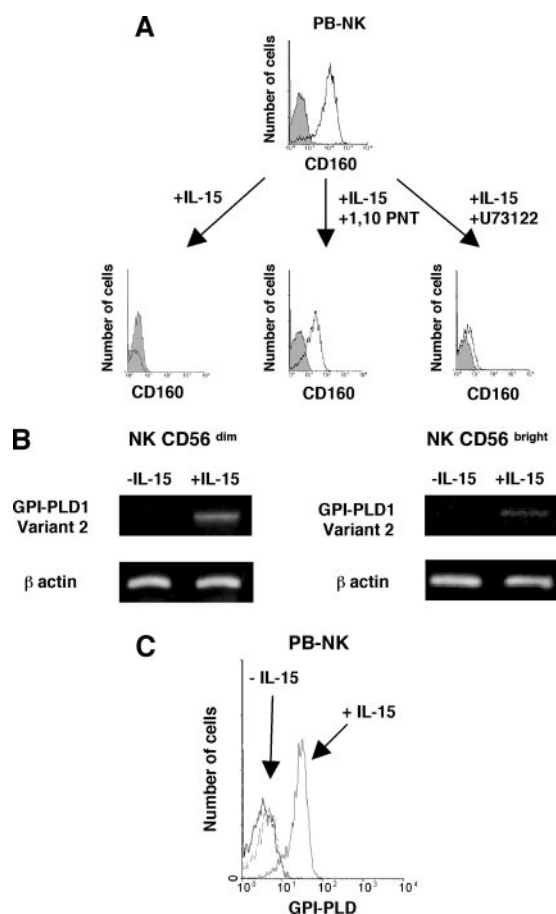


**FIGURE 1.** Down-expression of CD160 at the cell surface of IL-15-activated PB-NK lymphocytes. *A*, CD56 expression level delineates two subpopulations of NK cells. PB-NK cells were isolated from the PBMC of a healthy donor and immunolabeled using a PE-conjugated control IgG or anti-CD56 mAb. Flow cytometry cell analysis was conducted and allowed the detection of the  $\text{CD56}^{\text{dim}}\text{CD160}^+$  ( $\geq 90\%$ ) and  $\text{CD56}^{\text{bright}}\text{CD160}^-$  ( $\leq 10\%$ ) subpopulations, as indicated. *B*, Flow cytometry analysis of membrane-associated CD160 on resting and IL-15-treated PB-NK lymphocytes. PB-NK cells were cultured in medium alone (*left panel*) or supplemented with IL-15 (10 ng/ml; *right panel*) for 72 h. Cells were labeled with either the BY55 and anti-IgM FITC-coupled secondary Abs or the CL1-R2 and anti-IgG FITC-coupled secondary Abs, and further analyzed by flow cytometry. *C*, Analysis of CD160 mRNA synthesis in PB-NK cell subpopulations.  $\text{CD56}^{\text{dim}}$  and  $\text{CD56}^{\text{bright}}$  NK cell subsets were obtained from purified PB-NK lymphocytes by immunostaining with an anti-CD56 PE-conjugated mAb followed by a cell sorting procedure. Each subpopulation was then maintained in culture in the absence ( $-IL-15$ ) or presence ( $+IL-15$ ) of cytokine for 72 h. Total mRNA was extracted from each cell type and processed for reverse-transcription and CD160 cDNA-specific amplification. Amplification of the same reverse-transcribed product with  $\beta$ -actin primers was used as internal control.

vated  $\text{CD56}^{\text{dim}}$  NK lymphocytes, the level of CD160 transcripts is not modified in these cells and remains identical with the one detected in nontreated cells (Fig. 1C, *left panel*). In contrast, and in agreement with their  $\text{CD160}^-$  phenotype, CD160 mRNA synthesis is not detected in resting  $\text{CD56}^{\text{bright}}$  NK cells, but is induced upon their incubation with IL-15 (Fig. 1C, *right panel*). Thus, the nondetection of membrane-bound CD160 on IL-15-treated NK cells did not result from an activation-induced down-regulation of CD160 mRNA transcription.

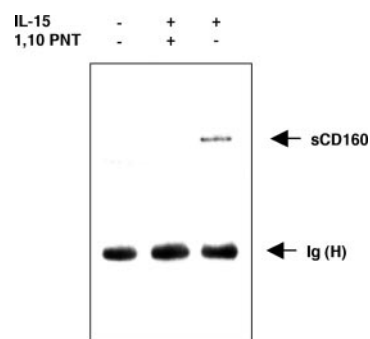
### Membrane-bound CD160 receptor is cleaved through a metalloprotease-dependent process

The release of membrane-bound proteins under a soluble form, mediated through a proteolytic cleavage, has been reported for various molecules (29, 30). We therefore investigated whether a similar mechanism can be responsible for the decreased detection of the GPI-anchored CD160 at the surface of activated  $\text{CD56}^{\text{dim}}$  NK lymphocytes. PB-NK lymphocytes were stimulated with IL-15 and further incubated in the presence of the phospholipase C-type inhibitor U73122, or the GPI-specific PLD (GPI-PLD) inhibitor 1,10 PNT. Cells were subsequently subjected to flow cytometry



**FIGURE 2.** The down-modulation of CD160 cell surface expression on IL-15-activated PB-NK lymphocytes involves a  $Zn^{2+}$ -dependent protease and correlates with the neosynthesis of the GPI-PLD enzyme. **A**, Inhibition of the IL-15-induced CD160 down-expression in the presence of 1,10 PNT. Freshly isolated PB-NK cells were cultured in IL-15-containing medium alone, or supplemented with the phospholipase inhibitor 1,10 PNT (10  $\mu$ M) or U73122 (2  $\mu$ M). Membrane-bound CD160 was detected by flow cytometry analysis with the anti-CD160 mAb BY55 plus anti-IgM-FITC Abs. A control IgM was used as negative control. **B**, Expression of CD160 mRNA following IL-15 activation of NK cells. Sorted CD56<sup>dim</sup> or CD56<sup>bright</sup> NK cells were grown in culture medium  $\pm$  IL-15 for 72 h before total mRNA extraction. After reverse-transcription, cDNA amplification was performed using primer pairs leading to the synthesis of products corresponding to the specific coding sequence of the GPI-PLD1 variant 2 and  $\beta$ -actin. **C**, The IL-15 treatment induces the synthesis of GPI-PLD by PB-NK cells. Resting or IL-15-activated PB-NK lymphocytes were subjected to a permeabilization step in saponin-containing buffer before immunolabeling. Cells were then incubated with specific anti-GPI-PLD or control Abs and FITC-coupled secondary reagent. The intracellular protein immunostaining was further detected by flow cytometry analysis.

analysis to visualize membrane-bound CD160 receptors. The results shown in Fig. 2A demonstrated that the IL-15-induced down-modulation of CD160 cell surface expression is not affected by the addition of U73122 inhibitor. In contrast, it is partially impaired when 1,10 PNT is added to the cell culture medium. This first observation, together with previous studies reporting the involvement of the GPI-PLD1 protease in the release process of GPI-anchored membrane receptors (34, 35), prompted us to examine the expression of this enzyme in PB-NK lymphocytes. The presence of mRNA transcripts corresponding to the GPI-PLD1 known variant 1 and 2 (32) was first assessed by RT-PCR. The results from a representative experiment performed on NK cells separated



**FIGURE 3.** Detection of soluble CD160 molecules within the extracellular environment of IL-15-stimulated PB-NK cells. PB-NK cells were left untreated or activated for 72 h with IL-15, then washed and incubated with IL-15 alone or supplemented with 1,10 PNT protease inhibitor. Cell culture supernatants were collected and subjected to anti-CD160 immunoprecipitation using CL1-R2 mAb. Following protein separation by SDS-8% PAGE under reducing conditions, the precipitated proteins were transferred onto a nitrocellulose membrane and subjected to anti-CD160 immunoblotting. The immunoreactive proteins were visualized using HRP-conjugated secondary Abs and an ECL detection system. Positions of sCD160 and of the Ig H chains (H) are indicated.

from the PBMC of a healthy individual indicate that neither the CD56<sup>bright</sup> nor the CD56<sup>dim</sup> PB-NK subsets express the transcripts for the GPI-PLD1 variant 2 (Fig. 2B). Note that the circulating PB-NK lymphocytes also show no synthesis of the GPI-PLD1 variant 1 transcript, while both variant 1 and 2 mRNAs are detected in PBMC and purified T lymphocytes (data not shown). Importantly, we established that the GPI-PLD1 variant 2 transcript synthesis is induced in both CD56<sup>bright</sup> and CD56<sup>dim</sup> NK pools when cultured in the presence of IL-15 (Fig. 2B). These data were further confirmed at the protein level by realizing immunostaining experiment with specific anti-PLD Abs. Indeed, we observed an induction of the GPI-PLD protein expression in permeabilized circulating NK lymphocytes upon IL-15 treatment (Fig. 2C). Altogether, these results demonstrate that the disappearance of CD160 from the NK cell membrane correlates with the neosynthesis of the GPI-PLD enzyme, indicating that this  $Zn^{2+}$ -dependent protease may be responsible for the cleavage of membrane-bound CD160 and its release under a soluble form.

#### *Characterization of sCD160 molecules released in IL-15-stimulated PB-NK cell extracellular environment*

To definitely demonstrate the IL-15-mediated release of soluble CD160 by activated NK lymphocytes, and to better characterize this soluble form at the molecular level, anti-CD160 immunoprecipitates were prepared from resting or IL-15-treated PB-NK cell culture medium. The analysis of the immunoprecipitated proteins by Western blot using the anti-CD160 mAb CL1-R2 leads to the detection of a unique protein band with an apparent molecular mass of 80 kDa (Fig. 3). A similar recognition pattern was obtained when the anti-CD160 mAb BY55 was used for immunoprecipitation on YT and NK lymphocytes total cell lysates (data not shown), thus indicating that the membrane-bound and soluble forms of CD160 exhibit the same multimeric structure, which is resistant to reducing agents (20). Importantly, no protein band is detected in immunoprecipitates obtained from the culture supernatant of nonactivated PB-NK lymphocytes, or of IL-15-stimulated cells cultured in the presence of 1,10-PNT inhibitor (Fig. 3). This latter observation indicates that sCD160 is not constitutively produced by circulating NK lymphocytes and further confirms the phospholipase dependence of CD160 proteolytic cleavage.

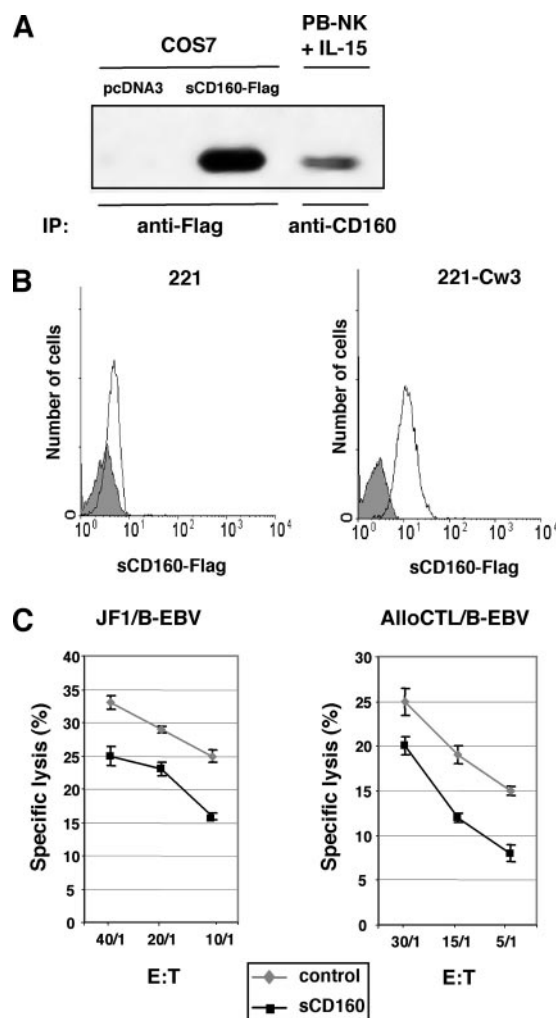
*An sCD160-Flag fusion protein binds to MHC-I molecules and inhibits the activity of cytotoxic lymphocytes*

To investigate the functional role of sCD160, we generated an expression vector coding for a C-terminal Flag-tagged soluble CD160 protein (sCD160-Flag). This fusion protein, when expressed by transiently transfected COS7 cells, exhibits the same multimeric structure that sCD160 molecules precipitated from an IL-15-treated PB-NK culture supernatant, as demonstrated by its detection as a 80-kDa polypeptide upon anti-CD160 mAb immunoblotting (Fig. 4A). By performing sCD160-Flag binding assays on HLA-Cw3-expressing 721.221 cells, we establish that sCD160-Flag protein efficiently interacts with the MHC-I molecules, a maximum binding being observed at a concentration of 5  $\mu$ g/ml recombinant protein (Fig. 4B). Importantly, the use of a similar, or higher, concentration of sCD160-Flag fails to significantly label the parental cell line 221, inferring the specificity of the detected interaction.

The ability of sCD160 molecules to interact with MHC-I molecules led us to determine whether this association could functionally affect the MHC-I-restricted CTL activities. Therefore, the cytolytic activity of the HLA-A11-restricted human cytotoxic CD8<sup>+</sup> T cell clone JF1 (36) was tested against the specific HLA-A11 EBV-transformed B cell line. The target cells were preincubated with a culture supernatant obtained from COS7 cells transfected with either the empty expression vector (control) or sCD160-Flag coding construct. A representative experiment, shown in Fig. 4C (left panel), reveals that sCD160 partially inhibits the specific CTL activity exerted by the JF1 clone. The level of inhibition observed never exceeded 25–30% for all E:T ratios tested. Furthermore, the incubation of the target cells with higher concentration of sCD160-Flag (>5  $\mu$ g/ml) did not result in the detection of higher inhibition levels and almost no inhibition was obtained when <1  $\mu$ g/ml sCD160-Flag was used (data not shown). Similarly, the JF1 clone cytotoxic activity was not altered upon preincubation of the target cells with a Flag-tagged soluble CD100 protein, thus inferring the specificity of the inhibition observed with sCD160-Flag protein (data not shown).

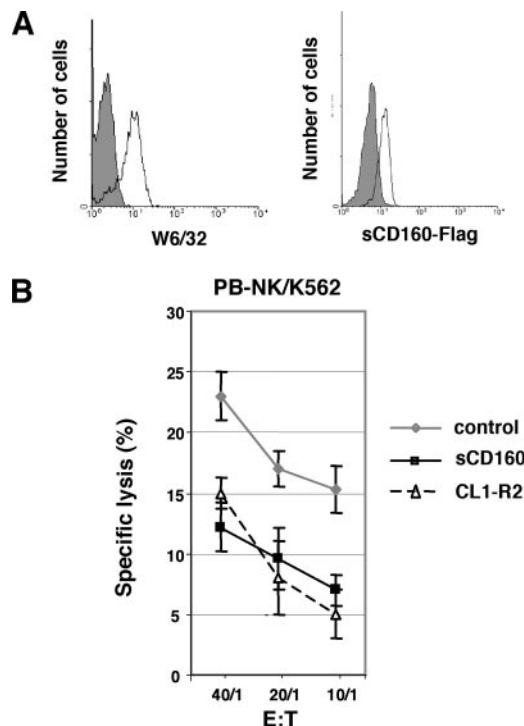
Importantly, an sCD160-induced inhibition of cytotoxicity is also observed when CD8<sup>+</sup> CTL isolated from 6-day allogeneic mixed lymphocyte cultures are used as effector cells (Fig. 4C, right panel). This suggests that the sCD160-mediated down-modulation of cytolytic activity is not restricted to cytotoxic T cell clones, but could also be effective on primary allogeneic stimulated T lymphocytes.

We previously reported that the lysis of K562 cells by freshly isolated PB-NK lymphocytes (that expressed significant amount of CD160, see Fig. 1B) is partly dependent on CD160/HLA-C interaction (23). In addition, the K562 NK lymphocyte-sensitive target cells expressed low amounts of MHC-I molecules, as demonstrated by flow cytometry analysis using the anti-MHC-I mAb W6/32 (Fig. 5A, left panel). Note that we recently identified the MHC-I molecules expressed by these NK cell targets as HLA-Cw3 (23). We further establish that sCD160-Flag protein binds to the HLA-C molecules expressed by K562 cells (Fig. 5A, right panel). Consequently, a significant inhibition of PB-NK lymphocyte cytotoxicity toward K562 cells is observed (Fig. 5B). As reported elsewhere, the addition of the anti-CD160 mAb CL1-R2 (Fig. 5B), or of the anti-MHC-I mAb W6/32 (data not shown), similarly impairs the PB-NK cell-mediated cytotoxicity against K562 cells. These data indicate that the binding of sCD160 to MHC-I molecules impairs the recognition of the target cells by the cytotoxic effector cells, thus decreasing their cytolytic activity.



**FIGURE 4.** sCD160 inhibits the cytolytic activity of NK and allogeneic CD8<sup>+</sup> CTL toward an EBV-B cell line. **A**, The in vitro-produced sCD160-Flag fusion protein structurally corresponds to sCD160 molecules released by activated PB-NK cells. COS7 cells were transiently transfected with the pcDNA3 control vector or with the expression construct encoding a Flag-tagged soluble CD160 protein (sCD160-Flag). Following a 2-day culture, the cell supernatants were collected and anti-Flag immunoprecipitates were prepared. An anti-CD160 immunoprecipitation was performed in parallel on the culture medium from IL-15-treated PB-NK lymphocytes. Immunoprecipitates were resolved by reducing SDS-8% PAGE, and subjected to Western blot analysis using the anti-CD160 mAb CL1-R2. Proteins were finally detected by autoradiography as described in the legend of Fig. 3. **B**, sCD160-Flag fusion protein efficiently binds to HLA-C molecules. The parental cell line 221 or the HLA-Cw3 expressing transfectants (221-Cw3) were incubated with culture medium obtained from COS7 cells transfected with pcDNA3 (control) or sCD160-Flag coding vector. A 5  $\mu$ g/ml concentration of sCD160-Flag-containing medium was used in the shown experiment. The binding of sCD160-Flag to the cells was assessed by immunostaining using the anti-Flag mAb and anti-IgG FITC-coupled Abs, and subsequent flow cytometry analysis. **C**, sCD160 inhibits the cytotoxic activity of CTLs. Cytotoxic assays were conducted using <sup>51</sup>Cr-labeled HLA-A11-expressing EBV-transformed B cells as target cells. The target cells were incubated with control or sCD160-Flag-containing culture medium from transfected COS7 cells before contact with the effector cells. The HLA-A11-specific human cytotoxic T cell clone JF1 (left panel) or sorted allogeneic CD8<sup>+</sup> CTL (right panel), were selected as effector cells. Each experimental condition was performed in triplicate and included three different E:T ratios. <sup>51</sup>Cr release was measured in the coculture supernatants, and results were expressed as the percent of specific lysis  $\pm$  SD.





**FIGURE 5.** The interaction of sCD160 with MHC-I molecules on K562 cells down-regulates the PB-NK cell cytotoxic activity. **A**, Binding of sCD160-Flag to K562 cells. K562 cells were either labeled with the anti-MHC-I molecules mAb W6/32 (left panel) or subjected to sCD160-Flag binding assay as described in the legend of Fig. 4B. **B**, K562 cells were preincubated with control or sCD160-Flag-containing medium before contact with the effector PB-NK lymphocytes. Alternatively, the anti-CD160 mAb CL1-R2 was added to the effector cells before starting the coculture. K562 cell lysis was quantified as explained in *Materials and Methods*.

## Discussion

In the present study, we establish that the GPI-anchored CD160 receptor expressed by human NK lymphocytes is processed under a soluble form following the exposure of the cells to IL-15. The generation of sCD160 occurs through a proteolytic cleavage of the membrane-bound protein and involves a metalloprotease. A metalloprotease-dependent shedding, leading to the release of soluble factors, has been reported for numerous transmembrane proteins such as MICA (30), the p60TNFR (37), FasL (38), or FcγRIII/CD16 (39). We similarly reported that sTA/CD106, initially identified at the cell surface of TCRγδ T cell clones, can be cleaved and released in the extracellular environment via a protein kinase C-dependent process (40). More recently, we found that the transmembrane immune semaphorin CD100/SEMA4D is released through a proteolytic cleavage upon T lymphocyte activation (33). Interestingly, functional studies revealed that the soluble form of CD100 inhibits the migration ability of human monocytes and immature dendritic cells (41).

The endogenous GPI-PLD has been identified as the enzyme responsible for the cleavage and release of various GPI-anchored membrane receptors (34). Thus, it has been shown that the GPI-membrane anchored carcinoembryonic Ag is cleaved from the human colorectal tumor cell line LS180 by a mechanism involving the GPI-PLD protein (35). Two human GPI-specific PLD cDNA have been cloned, corresponding to two protein variants (32). We demonstrate for the first time that resting PB-NK cells do not express both transcripts (Fig. 2B), while their synthesis is detected in purified T lymphocytes. However, the expression of GPI-PLD is efficiently induced upon activation of the PB-NK cells with IL-15

(Fig. 2C). Concomitantly, sCD160 molecules are detected within the activated-cell culture medium (Fig. 3). It should be mentioned that high amounts of sCD160 molecules can also be immunoprecipitated from the culture supernatant of the cytokine-independent NK cell line YT, which constitutively expressed the GPI-PLD enzyme (data not shown). By using specific phospholipase inhibitors, we further demonstrate that the Zn<sup>2+</sup> chelator 1,10 PNT blocks the generation of sCD160 and therefore the cleavage of the membrane-bound receptor. Similar results have been reported for the GPI-anchored membrane receptor CD55, which proteolytic cleavage depends on a cell-associated GPI-specific PLD and is strongly impaired in the presence of 1,10 PNT (37). Altogether, our data suggest that the neosynthesized GPI-PLD could be responsible for the activation-dependent shedding of CD160 on PB-NK cells.

Interestingly, we observe that CD160 transcripts are not detectable in sorted CD56<sup>bright</sup> PB-NK lymphocytes that fail to exhibit cytotoxic functions. This confirms our initial findings obtained by flow cytometry using the anti-CD160 mAb BY55, which demonstrated that BY55-reactive lymphocytes corresponded to NK cells and to cytotoxic CD8<sup>+</sup> T lymphocytes in bone marrow, cord, and PB (18, 42). Furthermore, we reported that the intestinal intraepithelial lymphocytes, which spontaneously exhibit a cytotoxic activity, are reactive with BY55 mAb (20). CD160 mRNA synthesis is induced when CD56<sup>bright</sup> NK lymphocytes are exposed to IL-15. Interestingly, this cytokine was also reported to be crucial for driving CD34<sup>+</sup> progenitors toward cytotoxic NK lymphocytes (43). However, CD160 could not be detected at the cell surface of these IL-15-activated NK lymphocytes, probably due to its cleavage following the induced-synthesis of the GPI-PLD.

To determine whether similarly to the GPI-anchored receptor, sCD160 is capable to bind to MHC-I molecules, an sCD160-Flag fusion protein was produced and tested for its ability to interact with 221-HLA-Cw3 transfectants. We report that sCD160-Flag, which presents the same characteristics that the cellularly cleaved sCD160 in terms of molecular structure, efficiently labels the HLA-Cw3-expressing cells (Fig. 4B). The establishment of sCD160/HLA-C interactions was found to have functional consequences. Indeed, we demonstrate that the preincubation of the target cells with sCD160 partially inhibits the CTL activity mediated by an MHC-I-restricted cytotoxic T cell clone, or by allogeneic stimulated CD8<sup>+</sup> T lymphocytes (Fig. 4C). sCD160-mediated CTL inhibition is directly related to the MHC-I molecule recognition, as no inhibition of cytotoxic activity was detected in the presence of sCD160 in an anti-CD3 mAb-redirected cytotoxicity assay using the murine mastocytoma cell line P815 as target cells (data not shown). Recently, the ILT-2 (CD85j) and ILT-4 (CD85d) receptors, which recognize a broad range of classical and nonclassical human MHC-I molecules, as murine (44) and human (19) CD160, were found to compete with CD8 for the binding to the α3 domain of MHC-I molecules (45). It is therefore possible that sCD160, as membrane-bound ILT-2/CD85j, could function as an inhibitory molecule through its association with the MHC-I molecules. Indeed, the MHC-I molecules complexed to sCD160 might not be recognized by the CD8 coreceptors expressed by cytotoxic lymphocytes, as a result of binding-induced conformational changes or steric hindrance.

We previously reported that freshly isolated PB-NK cells, expressing significant amount of membrane-bound CD160, mediate MHC-I-restricted cytotoxicity against the low-bearing HLA-C molecule K562 target cell line (23). We established that in this case, PB-NK lymphocytes cytotoxicity triggering is due to CD160/MHC-I interaction. Here, we show that sCD160, when ligated to MHC-I molecules on K562 target cells, is as efficient as an anti-CD160 mAb to inhibit PB-NK cell cytotoxicity (Fig. 5B).

In addition, and as expected from a previous observation, a similar inhibition of cytotoxicity is obtained when K562 cells are preincubated with the anti-HLA-B, -C mAb B1.23.2 (data not shown). These results further confirm that the recruitment of CD160 by low amount of MHC-I molecules triggers the PB-NK cell cytotoxic activity. We additionally observed that an increased expression of MHC-I molecules on the target cells resulted in a total inhibition of CD160-mediated PB-NK cell cytotoxic activity (47). This probably occurs as a consequence of the simultaneous recruitment of MHC-I-dependent inhibitory receptors that overcome CD160-induced activating signals (23). Experiments were undertaken to inhibit the negative effects resulting from the engagement of these inhibitory receptors. Thus, a target EBV-B cell line shown to be resistant to NK cell lysis, was preincubated with sCD160. No PB-NK cell cytotoxicity was obtained under these experimental conditions, despite an effective binding of the soluble protein to the target cells (data not shown). These observations tend toward the conclusion that sCD160 does not interfere in the establishment of interactions between the MHC-I molecules and the inhibitory receptors. One explanation would be that sCD160, and the inhibitory NK receptors, recognize distinct binding sites on MHC-I molecules. Nevertheless, we clearly demonstrate that activated NK lymphocytes release a cleaved molecule, which inhibits the MHC-I-specific CD8<sup>+</sup> CTL effector function.

An alternative mechanism leading to the inhibition of the CTL effector function triggered by NKG2D/MICA interactions has been recently reported (30, 46). In this newly identified process, the proteolytically shed MICA protein produced by the tumor cells binds to the NKG2D receptors expressed at the cell surface of effector lymphocytes, thus modulating the NKG2D-mediated tumor immune surveillance. This might correspond to a possible mechanism used by tumor cells to escape to the immune system. In contrast, sCD160-induced CTL inhibition seems to rather correspond to a physiological mechanism involved in the control of the CD8<sup>+</sup> cytotoxic effector T lymphocyte activity. In light of our results, a benefit role for sCD160 molecule could be proposed in the context of immune cell regulation. Indeed, this molecule may induce tolerance in some pathological situations, such as the rejection of allogeneic grafted organs. However, sCD160 may also provide inopportune signals, leading for example to the inhibition of CD8<sup>+</sup> CTL activity during viral infections. Moreover, the identification of a cytokine-dependent process, leading to the production of sCD160 as a functional soluble factor, opens new perspectives regarding the tight regulation of CD8<sup>+</sup> cytotoxic effector T lymphocyte activity.

## Disclosures

The authors have no financial conflict of interest.

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