A novel subset of NK cells expressing high levels of inhibitory FcγRIIB modulating antibody-dependent function

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Abstract: NK cells can kill antibody-coated target cells following engagement of FcγRIIA, the major activating FcγR expressed by these cells. The presence of FcγRIIC (CD32C) has also been reported, but its contribution to the FcγR-dependent effector functions of NK cells remains debated. We demonstrate here that inhibitory FcγRIIB is also expressed by a small subset of CD56+ /NKp46+ NK cells and can efficiently down-modulate their FcγR-dependent effector function. Immunofluorescence analyses of NK cells from 52 healthy donors showed the presence of CD56bright/FcγRII− (5.2%±3.4), CD56dim/FcγRIIlo− (94.1%±3.4), and CD56dim/FcγRIIbright (0.64%±0.72) cells. QRT-PCR and protein analyses performed on isolated FcγRIIibright NK cells indicated that FcγRIIB is strongly expressed by these cells but not by FcγRIIilow cells. In addition, FcγRIIibright cells showed a weaker antibody-dependent degranulation when incubated with IgG-coated target cells compared with FcγRIIilow NK cells, although a strong FcγRIIIA expression was detected in both cells. Furthermore, the addition of anti-FcγRIIFab paralleled a higher degranulation of FcγRIIibright NK cells, indicating a direct role for FcγRIIB in this down-modulating effect. Thus, it is proposed that FcγRIIBbright NK cells represent a new NK cell compartment able to down-modulate NK cell functions triggered by the engagement of activating FcγR. J. Leukoc. Biol. 84: 1511–1520; 2008.

Key Words: degranulation · Fcgamma receptor · FcgammaRIIB · natural killer cells · natural killer receptors

INTRODUCTION

Human natural killer (NK) cells can kill immunoglobulin G (IgG)-coated targets through antibody-dependent cellular cytoxicity (ADCC) following engagement of IgG Fc receptors (FcγR) expressed at their membrane [1, 2]. The receptor responsible for mediating ADCC in NK cells, FcγRIIA (CD16), is an intermediate-affinity FcγR that triggers NK cell cytotoxicity and cytokine production following its engagement by IgG bound to cell surface antigens [3]. Although FcγRIIIA is commonly thought to be the only FcγR expressed on NK cells, studies have demonstrated an heterogeneous expression of FcγRIIC (CD32C) on human NK cells from ~40% healthy subjects using RT-PCR and flow cytometry analyses [4–6]. FcγRIIB belongs to the FcγRII family that consists of different structurally related 42-kDa molecules with low affinity for IgG [3, 7–10], including also the activating FcγRIIA and the inhibitory FcγRIIB [9–12]. The FCGR2C gene results from an unequal crossover event between FCGR2A and FCGR2B genes and encodes a Fcγ receptor exhibiting more than 99% homology with FcγRIIB in the extracellular domain [9, 11, 13]. FcγRIIB family members differ in their cellular expression, function, and ligand binding specificities [14]. Cross-linking of FcγRIIIA results in up-regulation of intracellular Ca2+ concentration, phagocytosis of opsonized particles, as well as internalization of immune complexes [15–17]. In contrast, engagement of FcγRIIB results in the inhibition of cell activation triggered via cell surface activating receptors [18, 19]. FcγRIIB is mostly present on B cells, basophils, mast cells, and cells from the monocytic lineage [3]. However, it has been suggested that NK cells from a number of rheumatoid arthritis patients and from one healthy individual might also express FcγRIIB rather than FcγRIIC [20, 21]. The role of FcγRIIC remains debatable, as the FCGR2C gene is often found to contain an in-frame termination codon, and this receptor has been considered as evolving into a pseudogene [11, 22, 23]. However, it has been demonstrated that some FcγRIIC splice-variants act as activating FcγR [24].

Both FcγRIIIA and FcγRIIB play a role in the antitumor activity of therapeutic monoclonal antibodies (mAbs). An improved clinical response has been observed after rituximab treatment in lymphoma patients harboring the high IgG1 binder FcγRIIIA-158V, suggesting an important role of FcγRIIA-positive effector cells in the clinical outcome of the patients [25, 26]. In contrast, FcγRIIB has been shown to

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negatively impact on the antitumor efficacy of trastuzumab in mice xenografted with an HER-2-positive human breast carcinoma [27]. Moreover, more recent studies highlighted the in vivo implication of FcγRIIA + NK cells in the clinical response to this antibody, in patients with HER-2-positive primary breast cancer [28, 29].

Altogether, these data raise the possibility that an expression of the inhibitory FcγRIIB by NK cells could be responsible for a reduced NK cell antitumor activity in patients treated with cytotoxic therapeutic mAbs. The present study was therefore performed as a first step to analyze whether FcγRIIB could be detected on NK cells from healthy donors and could act as a negative regulator of activating FcγR-dependent functions in these cells. We demonstrate herein that peripheral blood NKP46+/CD56+/CD3- NK cells from healthy donors contain a subset of CD56dim cells that strongly express FcγRIIB. These CD56dim/FcγRII+ bright NK cells exhibit a pattern of NK-cell receptors (NKR) expression different from that of NK cells that express a low level, if any, of FcγRII (CD56dim/FcγRII- NK cells). Finally, the in vitro degranulation capacity of FcγRIIIB+ bright NK cells was lower than that of the FcγRII- cells following incubation with anti-CD20-coated CD20+ Raji cells. The use of anti-FcγRII Fab fragments demonstrated that FcγRIIB is responsible for this reduced degranulation, indicating an active role of this receptor in the control of FcγRIIA-dependent functions in NK cells.

MATERIALS AND METHODS

Cell lines

All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C, in an atmosphere containing 5% CO2. The human FcγRII+ Raji lymphoblastoid cell line (CCL-86, ATCC) was maintained in RPMI 1640 + Glutamax ( Gibco-Invitrogen, Carlsbad, CA, USA) with 10% heat-inactivated fetal cell serum (FCS) (PAA Laboratories, Pasching, Germany), 100 U/ml penicillin/100 μg/ml streptomycin (Gibco), 5% sodium pyruvate (Gibco), 0.1% β-mercapto-ethanol (Gibco) (CLICK medium). The human FcγRIIA+ erythroleukemic cell line K562 (CCL-243, ATCC) was maintained in RPMI 1640 + Glutamax with 10% FCS, 100 U/ml penicillin/100 μg/ml streptomycin (complete medium).

NK cell purification and flow cytometry cell sorting

Peripheral blood mononuclear cells (PBMCs) from anonymous healthy volunteers (Etablissement Français du Sang, Hôtel Dieu Hospital, Paris, France) were isolated from peripheral blood by centrifugation on a Ficoll/Hypaque gradient (PAA Laboratories). NK cells were purified using the negative selection NK cell isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany). Purified cells were at least 95% CD56+. Less than 3% of cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 (clone RM052 from Immunotech, Marseille, France), anti-CD3 (clone SK7 from Becton Dickinson, San Jose, CA, USA), or anti-CD19 (clone HIB19 from Becton Dickinson) mAbs. For sorting experiments, freshly purified NK cells were stained with FITC-conjugated anti-FcγRII, clone AT10 from AbD Serotec (Cergy Saint-Christophe, France), phycoerythrin (PE)-conjugated anti-CD3 (clone UCHT1) and allophycocyanin (APC)-conjugated anti-CD56 (clone NCAM16.2) from Becton Dickinson, prior to flow cytometry sorting of CD56+/CD3+/FcγRII+ and CD56dim/CD3+/FcγRII+ NK cells using a BD FACSAria Cell-Sorting System (Becton Dickinson).

Evaluation of FcγR expression by flow cytometry

To analyze FcγR expression on CD56+ NK cells, cells were stained with APC-conjugated anti-CD56 (clone NCAM16.2) and PE-conjugated anti-CD3 (clone UCHT1), or with anti-NKp46 (clone BAR283) mAbs (Becton Dickinson), in combination with one of the FITC-conjugated anti-FcγR mAbs [anti-FcγRI, clone AT10 from Serotec or clone 3D3 from Becton Dickinson, or anti-FcγRIII, clone 3G8 from Becton Dickinson]. Alternatively, unlabeled anti-FcγRII KB61 [30] was labeled using the AlexaFluor488-Zenon Mouse IgG1 Labeling kit (Molecular Probes-Invitrogen) following manufacturer's instructions. In some experiments, Fab fragments of anti-FcγRIIA/BC (clone KB61) or anti-FcγRII (clone IV.3) [31] mAbs were labeled using the Lynx Rapid RPE Antibody Conjugation kit (Abh Serotec). For triple labeling experiments with anti-NKp46 and anti-CD3 antibodies, KB61 mAb was coupled with AlexaFluor488 following manufacturer's instructions (Molecular Probes-Invitrogen). In some experiments, freshly purified NK cells were preincubated for 30 min on ice with the unlabeled anti-FcγRII mAb 3G8 that binds to the FcγRII IgG binding site [32] before being directly labeled with the anti-FcγRII mAb (AT10-FITC) in combination with APC-conjugated anti-CD56 (clone NCAM16.2) and PE-conjugated anti-CD3 (clone UCHT1).

Four-color flow cytometry

Immunophenotypic analysis of freshly purified NK cells was carried out by 4-color analysis on a FACS Calibur (Becton Dickinson) with CELLQuest Pro software (Becton Dickinson). Cells were stained with the following mAbs: FITC-conjugated anti-FcγRI (clone AT10) (Serotec); APC-conjugated anti-CD56 (clone NCAM16.2), Phycoerythrin-cyanin 5 (PE-Cy5)-conjugated anti-CD3 (clone UCHT1), both from Becton Dickinson and one of the following PE-conjugated mAbs: anti-FcγRII (clone B73.1) from Becton Dickinson; anti-NK2G2 (clone 134/391) and anti-NKp60 (clone 239/127) from R&D Systems; anti-NKp2A (clone Z199), anti-NKp2D (clone ON72), and anti-CD94 (clone NP-3B1), anti-CD161 (clone 191B9), anti-NKp30 (clone Z23), anti-NKp44 (clone Z231), anti-NKp46 (clone BAR283), anti-ILT-2 (clone HP-F1), anti-CD138a/β (clone EB6.B), anti-CD158b/1 (clone GL183), anti-CD158e/1 (clone Z27), and anti-CD158f (clone FES127), all from Beckman Coulter (Fullerton, CA, USA). Perforin expression by NK cells was assessed by intracellular staining with an AlexaFluor647-conjugated anti-perforin mAb (clone dG9) (BioLegend), in combination with PE-conjugated anti-CD56 (clone B159) from Becton Dickinson, and the PE-Cy5 anti-CD3 and FITC-anti-FcγRII. 4-color staining was carried out and CD56+/CD3- events falling in the lymphocyte population were analyzed for FcγRIII and each tested for receptor expression.

Confocal microscopy

Freshly purified NK cells (5×10⁶) were stained with 2 μg/ml primary antibodies (mouse IgG1, APC-conjugated anti-CD56 mAb (clone NCAM16.2) in combination with mouse IgG1 anti-FcγR mAbs (anti-FcγRI, clone KB61, or anti-FcγRIII, clone 3G8) for 30 min on ice. All incubations were done using PBS containing 5% human AB serum. Cells were then washed twice in PBS and incubated on ice for 30 min with biotin-conjugated goat anti-mouse (GAM)-IgG1, (1/100 dilution, The Binding Site, Birmingham, UK) and, after two washes in PBS, Cy3-conjugated streptavidin (1/1000 dilution; Jackson Immunoresearch Laboratories, West Grove, PA, USA) was added for an additional 20 min on ice. Cells were then washed two more times in PBS, fixed with 0.5% formaldehyde containing PBS and resuspended with 15 μl Mowiol mounting medium prior to spreading on a 75-μm capillary gap microscope glass slide (Dako ChemMate; Dako, Glostrup, Denmark). Immunofluorescence analysis was performed with a confocal laser scanner microscope Zeiss LSM 510 (Zeiss, Oberkochen, Germany) and the Zeiss LSM Image Browser V3.1.0.99 software (Zeiss). The wavelength of the HeNe laser was set at 543 nm for Cy3 excitation and at 633 nm for APC excitation. Fluorescence emission was revealed by BP 560-610 band pass filter for Cy3 and by LP 560 long pass filter for APC. Double-staining immunofluorescence images were acquired sequentially in the red and blue channels at a resolution of 512×512 pixels and analyzed using the Image J freeware (version 1.37v).

Quantification of FcγRIIb transcripts by real-time PCR

Total cellular RNA was isolated from sorted CD56dim/CD3+/FcγRIIbright and CD56+/CD3-/FcγRII- NK cells using the RNeasy mini kit or RNeasy micro kit (Qiagen). The integrity and the quantity of RNA were evaluated on a bioanalyzer-2100 (Agilent Technologies, Santa Clara, CA, USA). cDNA were then
generated by reverse transcription (RT) of 10 ng total cellular RNA and linearly amplified using the Quantitect whole Transcriptome kit (Qiagen). Each cDNA sample was then used as a template for quantitative PCR reactions performed using the universal PCR master Mix (Applied-Biosystems, Foster City, CA, USA). FcRRII-specific primers and probe for real-time RT-PCR (Assay ID: Hs00269610_m1; Applied-Biosystems) on a ABI Prism 7900HT Sequence detection system (Taqman; Applied-Biosystems). Human β-actin (ACTB) Endogenous Control (part no. 4333762T; Applied-Biosystems) was used as internal control. The RQ manager software V3.2 (Applied-Biosystems) was used to establish the PCR cycle at which the fluorescence exceeded a set threshold, C_{T}, for each sample. Data were then analyzed according to the 2-ΔΔCT method, where ΔCT represents C_{T} FcRRII− −/− NK cells (target) − ΔC_{T} FcRRII+ +/− NK cells (calibrator).

The n-fold difference between the amounts of FcRRII transcripts of the target (FcRRII+ +/− NK cells) relative to the calibrator (FcRRII− −/− NK cells) was then calculated as 2-ΔΔCT [33].

**Protein extraction, immunoprecipitation, and Western blot analysis**

Hydroporphic proteins from CD56dim/CD3/FcRRIIright and CD56high/CD3/FcRRIIleft flow cytometry-sorted freshly purified NK cells, and from FcRRII+ Raji and FcRRII+ K562 cells were isolated using the MEM-PER eukaryotic membrane protein extraction reagent kit (Pierce, Rockford, IL, USA) following manufacturer's instructions. The hydrophobic protein extracts were immunoprecipitated with anti-FcRRII KB61-coated Protein G-Sepharose beads (Protein G-4-fast flow, Amersham Biosciences-GE HealthCare, Piscataway, NJ, USA). After multiple washing, eluates from the immunosorbent were boiled, fractionated by SDS-PAGE in reducing conditions and transferred onto Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were saturated with Tris buffered saline (TBS) containing 0.1% Tween 20 (Merck, Nottingham, UK) and 5% BSA (Sigma Chemical, Saint Louis, MO, USA), pH 7.4, and incubated with previously described rabbit polyclonal Abs raised in our laboratory (2 μg/ml) reacting specifically with the cytoplasmic tail of human FcRRII (rabbit anti-human FcRRII) (sc-2004, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a final concentration of 0.25 μg/ml. Rabbit 7522 antibodies bind both FcRRIIA and FcRRIIC, as these receptors exhibit highly homologous intracellular domains. Antigen/antibody complexes were then detected using an enhanced chemoluminescence (ECL) kit (Amersham Biosciences-GE HealthCare).

**CD107a mobilization assay**

Degranulation was assessed with a CD107a mobilization assay [36, 37] using NK cells and MHC class I human erythroleukemic K562 target cells at different effector/target (E/T) cell ratios, or CD20+ human lymphoblastoid Raji target cells (E/T=15/1) coated with 5 or 500 ng/ml of a chimeric anti-human CD20 mAb (clone EMAB-6, Laboratoire Français de Fractionnement et des Biologicals, Lille, France) [38]. In some experiments, Fab fragments of the anti-FcRRII KB61 mAb (that inhibits the IgG binding to FcRRII) were added at various concentrations (0.1, 0.5 and 1.0 μg/ml), in order to inhibit the engagement of FcRRII by the anti-CD20 mAb. Fab fragments of the anti-FcRRII Fab mAb [31] were used as negative control. PE-Cy5-conjugated anti-CD107a (clone H4A3, Becton Dickinson) mAb and monensin (5 μM, Sigma) were added to the effector and target cell mixtures that were then incubated for 4 h. Monensin is added to prevent the acidicification of the endosomal compartments, which could alter the fluorescence of internalized CD107a and CD107a mAb complexes [36]. Cells were then washed in PBS containing 2 mM EDTA and stained for extracellular markers [APC-conjugated anti-CD56, PE-conjugated anti-CD3 and FITC-conjugated anti-FcRRII (clone 3D3 from Becton Dickinson mAbs)], to evaluate CD107a expression level by different NK cell subpopulations defined on their FcRRII expression level. PE-Cy5−− cells were scored by flow cytometry, revealing cells that have undergone degranulation. Results are presented as means ± SD of positive-stained cells.

**Statistical analysis**

Statistical analyses were performed using the Statview 5.0 software package for Windows (SAS Institute, Cary, NC, USA) for NKR phenotypic characterization and analysis of CD107a expression. Statistically significant differences were calculated using the Student paired t test. P values are indicated in the figures.

**RESULTS**

FcRRII expression defines a novel NK cell subset in healthy donors

Freshly purified NK cells isolated by negative selection from PBMCs of healthy donors were analyzed for FcRRII expression by flow cytometry using the anti-FcRRII KB61 mAb [30]. Three subpopulations of CD56+/CD3− NK cells could be defined based on FcRRII expression levels. Fig. 1A shows representatative flow cytometry data obtained from two of the tested donors (n=52). First, CD56bright NK cells (~5% of total NK cells) were either FcRRII-negative or exhibited only marginal, as also seen for FcRRIIA [39] (Fig. 1A, middle panels, solid arrowheads). Second, as previously reported by Metes et al. [5] and Stewart-Akers et al. [21], a large subpopulation of CD56dim NK cells (>90% of total NK cells) that expressed either low levels (left middle panel) of FcRRII or not (right middle panel), hence termed CD56dim/FcRRII−− NK cell subpopulation, was observed (Fig. 1A, middle panels, gray-filled arrowheads). This cell subpopulation was detected in all the individuals tested, ranging from 0.13% to 2.28% (n=52, median value: 0.64%). Similarly to FcRRIIA (CD16), FcRRII was detected only on CD56dim/CD3− NK cells. Coexpression of the two receptors was always observed by quadruple CD56/CD3/FcRRII/FcRRI staining (see Fig. 5A, upper panel).

To confirm that the CD56dim/FcRRIIright+ cells observed are, indeed, NK cells, double Nkp46/FcRRII staining was also performed, since Nkp46 is considered to be a specific NK cell receptor [40]. Fig. 1A (lower panels) shows the data obtained with the same two donors. As already observed when NK cells were defined as CD56+/CD3− cells, Nkp46+/− NK cells expressing high levels of FcRRII (Nkp46+/− FcRRIIright+−) were detected. Similar results were obtained with 10 donors.

Confocal microscopy experiments using purified NK cells also showed the expression of FcRRII by CD56+ cells (Fig. 1B). As observed in flow cytometry analyses, NK cells that strongly expressed CD56 (CD56right+− blue staining) were not labeled with the anti-FcRRII KB61 mAb, NK cells with a faint expression of CD56 were either moderately or strongly labeled with KB61 mAb (red staining), a situation most likely reflecting CD56dim/FcRRIIright− and CD56dim/FcRRIIright+ NK cell subpopulations, respectively.

The expression of FcRRII on CD56dim/CD3− NK cells was further confirmed using two other anti-FcRRII mAbs (3D3 and AT10). All three antibodies allowed the detection of the CD56dim/FcRRIIright− subpopulation (Fig. 2A). Of note, the staining of CD56dim/FcRRIIright− cells by the AT10 mAb was weaker when compared with the other two mAbs (3D3 and...
Evidence that the detection of the FcγRIIbright NK cell subset was not due to a nonspecific binding of the Fc region of the anti-FcγRII mAbs to FcγRIIA was obtained from the following results. First, triple labeling of freshly purified NK cells with APC-anti-CD56, FITC-anti-CD3, and PE-labeled KB61 Fab fragments allowed the detection of the FcγRIIbright NK cell subset (Fig. 2B). Interestingly, the FcγRIIbright NK cells were not detected using PE-labeled Fab fragments of the IV.3 mAb, which is preferentially directed against FcγRIIA (Fig. 2B) [31]. Second, preincubation of freshly purified NK cells with an anti-FcγRIII binding site mAb (3G8) prior to triple CD56/CD3/FcγRII staining did not hinder the detection of FcγRIIbright NK cells (Fig. 2C).

**Phenotypic characterization of the FcγRIIbright NK cell subset**

To assess whether FcγRIIbright NK cells show a characteristic expression pattern of activating and/or inhibitory NKRs, NKR expression by these cells was compared with that of FcγRIIlo− cells using 4-color flow cytometry analyses. Quadruple stainings (anti-CD3, anti-CD56, anti-FcγRII, and one of the anti-NKR mAbs) were carried out on freshly purified NK cells. NKG2D, CD161, Nkp44, Nkp46, Nkp80, and CD158i were expressed at similar levels between the two subpopulations (data not shown). NKG2C, an activating receptor homologue to the inhibitory NKG2A receptor, was similarly expressed by the two NK cell subpopulations (Fig. 3). In contrast, CD94, the activating Nkp30 and the inhibitory NKG2A receptors were expressed at significantly lower levels by FcγRIIbright NK cells, while the inhibitory ILT-2 and the KIRs CD158a/h, CD158b1/b2j, and CD158c1/e2 showed a higher expression (Fig. 3).

**FcγRIIbright NK cells predominantly express high levels of the inhibitory FcγRIIB**

To characterize the type of FcγRII expressed by FcγRIIbright NK cells, NK cells were first enriched by negative selection (>70% CD56+CD3− cells) from more than 1×10^6 PBMCs from a single healthy donor. Five independent experiments using different donors were performed. Isolated cells were triple-labeled with anti-CD3, anti-CD56 and anti-FcγRII mAbs to sort FcγRII−, FcγRIIbright and FcγRIIbright CD56dim/CD3− NK cells. Hydrophobic proteins were then extracted from the cell lysates of 5×10^6 FcγRII− and 1 to 2.5×10^5 FcγRIIbright sorted NK cells depending on the experiment. FcγRII immunoprecipitation was then performed using the KB61 mAb, and Western blot analysis using specific rabbit polyclonal antibodies raised against FcγRIIB or FcγRIIA/C intracellular domains [34, 35] were then carried out. Figure 4A shows the results obtained with two of the five donors. It indicates that FcγRIIB was strongly detected in the immunoprecipitates of the FcγRIIbright NK cell lysates but not in those of FcγRIIlo−.
NK cells (Fig. 4A, upper panels). The same observation was made with the three other donors tested (data not shown). By contrast, only trace amount of activating FcγRIIIA/C could be detected in FcγRIIIBright NK cells of only one of the donors tested (Fig. 4A, lower panels). A low level of activating FcγRIIIA/C could be detected in FcγRIIIdim- NK cell lysate, or not (Fig. 4A, lower panels).

To further assess the presence of FcγRIIB in FcγRIIIBright NK cells, FcγRIIIIdim and FcγRIIIIBright CD56dim/CD3− NK cells from four other healthy donors were also FACS-sorted and FcγRIIB transcripts were quantified by real-time PCR. Data were analyzed according to the comparative C_{T} method (see Materials and Methods) [33]. FcγRIIB transcripts were expressed by FcγRIIIIBright NK cells at levels between 82 and 7×10^{5} times more than FcγRIIIIdim NK cells for the four tested donors (Fig. 4B), thus confirming that FcγRIIIIBright cells predominantly express FcγRIIB.

FcγRIIIIBright NK cells exhibit reduced degranulation in FcγR-dependent in vitro assay

The functional activity of CD56dim/FcγRIIIIBright NK cells was then analyzed using the CD107a mobilization assay and was compared with that of CD56dim/FcγRIIIIdim NK cells. CD107a is a marker of intracytoplasmic cytotoxic granules that can be detected by flow cytometry using a fluorochrome-conjugated anti-CD107a mAb during granule-mediated exocytosis as it is transiently expressed at the degranulating cell membrane.

CD107a (LAMP-1) mobilization assay therefore permits the evaluation of NK cell degranulation at the single cell level by flow cytometry [36, 37].

First, we evaluated FcγRIIIA and perforin expression by FcγRIIIIBright NK cells. As shown in Fig. 5A, CD56dim/CD3−/FcγRIIIIBright NK cells strongly express FcγRIIIA on their cell surface, and intracellular staining also showed that these cells expressed perforin.

Second, freshly purified human NK cells from healthy donors were cultured for 4 h in various conditions in the presence of PE-Cy5-conjugated anti-CD107a mAb and monensin. Cells were then harvested and stained with FITC-conjugated anti-FcγRII, PE-conjugated anti-CD3, and APC-conjugated anti-CD56 mAbs prior to flow cytometry analyses. Cells in the CD56dim/CD3− lymphocyte gate were analyzed for CD107a expression. Two gates were defined, delineating the two CD56dim FcγRII NK cell subpopulations (R1: FcγRIIIIdim-cells, R2: FcγRIIIIBright-cells).

In these experiments, freshly purified NK cells were incubated with either the MHC Class I NK-sensitive K562 cells to evaluate degranulation induced by NK cell natural activity, or with CD20+ Raji cells and an anti-CD20 mAb (5 or 500 ng/ml) to evaluate their FcγR-mediated degranulation. Figure 5B shows the results obtained in one representative experiment. CD107a was barely detected at the surface of nonstimulated NK cells after 4 h incubation (% CD107a+: R1/R2 = 0.7%) (Fig. 5B, upper left panel). However, when mixed with the
MHC Class I NK-sensitive K562 cells, CD107a was expressed at a similar level on FcγRII^{bright} cells (R2: 6.1% CD107a⁰) and FcγRII^{lo/-} cells (R1: 6.2% CD107a⁰) (Fig. 5B, upper right panel), showing that this FcγRII^{bright} NK cell subpopulation exhibits a potent natural killing activity. When incubated with CD20⁺ Raji cells and 500 ng/ml anti-CD20 mAb, CD107a was detected on both FcγRII^{lo/-} and FcγRII^{bright} NK cells. Interestingly, the FcγRII^{bright} cells showed a reduced degranulation (R2: 19.3% CD107a⁰) compared with FcγRII^{lo/-} NK cells (R1: 29.7% CD107a⁰) (Fig. 5B, lower left panel). Six independent experiments were performed using different healthy donors and led to similar results. Figure 5C summarizes the results obtained in these experiments, represented as mean percentages ± SD of CD107a⁺ cells amongst the two NK cell populations in presence of CD20⁺ Raji cells and anti-CD20 mAb. A significant decrease of degranulation by CD56^{dim} FcγRII^{bright} NK cells compared with FcγRII^{lo/-} cells was observed in presence of both 5 and 500 ng/ml anti-CD20 mAb.

Furthermore, to assess the role of FcγRII in the FcγR-dependent degranulation assay, NK cells were also incubated in presence of CD20⁺ Raji cells, the anti-CD20 mAb (500 ng/ml), and different concentrations of anti-FcγRII KB61 Fab fragments (0.1, 0.5, or 1.0 μg/ml). KB61 Fab fragments were added at the beginning of the incubation in order to inhibit FcγRII engagement by the anti-CD20 mAb. Four independent experiments were performed. Figure 5B (lower right panel) shows the results obtained in one experiment. Degranulation of FcγRII^{lo/-} NK cells (R1) was not affected by the addition of KB61 Fab fragments [29.7% (Fig. 5B, lower left panel) vs. 30.9% (Fig. 5B, lower right panel) CD107a⁺ cells in the absence or in the presence of KB61 Fab fragments, respectively], indicating that FcγRII expressed by FcγRII^{lo/-} NK cells do not play a significant role in the FcγR-dependent degranulation of these cells. By contrast, the degranulation of FcγRII^{bright} NK cells (R2) was significantly increased when KB61 Fab fragments was added [19.3% (Fig. 5B, lower left panel) vs. 29.8% (Fig. 5B, lower right panel) CD107a⁺ cells in the absence or in the presence of KB61 Fab fragments, respectively], leading to a percentage of CD107a⁺ cells comparable to that of FcγRII^{lo/-} NK cells. By contrast to KB61 Fab
degranulation was observed, while Fc
CD3- subset of NK cells (termed herein Fc
present study the low level of Fc
was reported more than 10 years ago [4, 5]. We confirm in the

count in NK cell studies, despite the fact that this observation

individuals express Fc
The observation that human NK cells from certain healthy

discussed above, with the medium

mAbs. Of note, the use of the AT10 mAb reproducibly led to
a much lower staining of these cells. This particular binding
pattern likely reflects the previously described predominant
expression of the FcyRIIC by these NK cells, since AT10 mAb
has been proposed as binding weakly the FcyRIIC but not the
other FcyRII (IIA and IIB) [20]. In contrast, the KB61 mAb,

strongly binds the three FcyRII (IIA, IIB, and IIC) [20]. RT-PCR analyses confirmed the presence of FcyRIIc1 and
FcyRIIc3 transcripts in healthy donors exhibiting a KB61
staining of their FcyRIIlo NK cell subset (data not shown).

Interestingly, a small subset of NK cells that strongly
express FcyRIIb (termed FcyRIIbNK NK cells) differing from
the FcyRIIlo NK cell subset, and comprising less than 2%

median value±SD: 0.64%±0.72) of total CD56+/CD3− NK

NK cells were also found expressed on these FcyRIIbNK

NK cells. Furthermore, the strong expression of FcyRIIb NK

NK cells (C–F) were purified, reverse transcribed, and
linearly amplified. cDNA were then analyzed
for FcyRIIb transcripts by real-time PCR using the comparative 2−ΔΔCT method (see Methods).

Results represent the n-fold difference between the amount of FcyRIIb transcripts of the FcyRIIb NK cells (target) and that of the FcyRIIlo NK cells (calibrator). All results were calibrated with an internal control (β-actin) to normalize for differences in total cDNA.

**DISCUSSION**

The observation that human NK cells from certain healthy
individuals express FcyRII (CD32) is barely taken into account in NK cell studies, despite the fact that this observation was reported more than 10 years ago [4, 5]. We confirm in the present study the low level of FcyRII expression by a CD56dim/CD3− subset of NK cells (termed herein FcyRIIlo NK cells) from ~40% healthy donors. FcyRII could be detected at the surface of FcyRIIlo NK cells using anti-FcyRII KB61 or 3D3 mAbs. Of note, the use of the AT10 mAb reproducibly led to a much lower staining of these cells. This particular binding pattern likely reflects the previously described predominant expression of the FcyRIIC by these NK cells, since AT10 mAb
by the FcγRII^lo/- subpopulation. The detection of the FcγRII^bright NK cells by AT10 mAb to a level comparable to that observed with the KB61 mAb suggested that FcγRII^bright NK cells do not predominantly express the activating FcγRIIC. Furthermore, the weak binding of Fab fragments of the IV.3 mAb [which binds preferentially to FcγRIIA and marginally to FcγRIIB] to the FcγRII^bright NK cell subset also indicated a weak or absent FcγRIIA expression by this NK cell subset.

Figure 5. FcγRIIB-dependent reduced degranulation of CD56^dim/FcγRII^bright NK cells. (A) FcγRII, FcγRIII, and perforin expression by FcγRII^bright and FcγRII^lo/- NK cells. NK cells were stained with fluorochrome-conjugated anti-CD56, anti-CD3, anti-FcγRII (clone AT10) mAbs and either with the anti-FcγRII 3G8 mAb (left panel) or with an anti-perforin mAb (intracellular staining) (right panel). CD56^+/CD3^- cells from the lymphocyte population were analyzed. (B–D) FcγRIIB-dependent reduced degranulation of CD56^dim/FcγRII^bright NK. Freshly purified NK cells were incubated for 4 h in various conditions, in the presence of monensin and PE-Cy5-conjugated anti-CD107a mAb. Cells were then harvested and stained (CD56/CD3/FcγRII) prior to FACS analyses. CD56^+/CD3^- cells from the lymphocyte population were analyzed. (B) Representative results obtained from one donor are shown. Upper left panel shows results obtained with nonstimulated NK cells; upper right panel shows results obtained with NK cells incubated with the MHC-NK-sensitive K562 cells (E/T: 5/1); lower panels show results obtained with NK cells incubated with CD20^+ Raji cells (E/T: 15/1) in the presence of anti-CD20 mAb (500 ng/ml) alone (lower left panel) or with anti-FcγRII KB61 Fab fragments (1 µg/ml) (lower right panel). Histograms representing the CD107a expression in gates R1 (CD56 dim/FcγRII^lo/- cells) and R2 (CD56^dim/FcγRII^bright cells) are shown, and percentages of CD107a^+ cells are given in each histogram. (C) Mean % of CD107a^+ cells (±SD) obtained in the antibody-dependent degranulation assay performed with six donors. NK cells were mixed with CD20^+ Raji cells (E/T: 15/1) and 500 or 5 ng/ml anti-CD20 mAb, or not. (D) Mean % CD107a^+ cells (±SD) obtained in the antibody-dependent degranulation assay performed with four of the six donors tested (C) in the presence of KB61 Fab fragments. NK cells were incubated with CD20^+ Raji cells (E/T: 15/1), 500 ng/ml anti-CD20 mAb, and KB61 Fab fragments (0.1, 0.5, and 1.0 µg/ml) or not. Statistically significant differences were calculated using the Student paired t test.
ments suggested an expression of the inhibitory FcγRIIB by FcγRIIB^bright^ cells. It was confirmed by immunoprecipitation/Western-blot and real-time semiquantitative PCR experiments carried out on FACS-sorted NK cells showing that FcγRIIB^bright^ NK cells, but not FcγRIIB^{dim/lo} NK cells, strongly express the inhibitory FcγRIIB. A marginal expression of the activating FcγRIIA/C at the protein level was detected in only one of the five donors tested.

Finally, the FcγR-dependent CD107a mobilization assay showed a reduced degranulation of the FcγRIIB^bright^ subpopulation compared with that detected in the FcγRIIB^{dim/lo} subpopulation. This occurred despite the fact that FcγRIIB^bright^ NK cells possess an antibody-dependent cytotoxic arsenal (strong expression of FcγRIIHA and positive intracellular staining of perforin). Moreover, the possibility of a reduced activation potential of these FcγRIIB^bright^ NK cells can be excluded, since these cells showed a good degranulation ability when incubated with the MHC Class I NK-sensitive K562 cells. When a saturating quantity of Fab fragments of anti-FcγRII mAb (clone KB61, directed against the FcγRII IgG binding site) was added in the FcγR-dependent degranulation assay, FcγRIIB^bright^ NK cells degranulation was significantly increased and reached a level comparable to FcγRIIB^{lo/-} NK cells. These results demonstrate that the FcγRIIB inhibitory function is responsible for the reduced degranulation of the FcγRIIB^bright^ NK cells. Of note, the fact that FcγRIIB^{dim/lo} NK cells showed the same level of degranulation in the presence of KB61 Fab or not illustrates the marginal contribution of FcγRIIC in FcγR-dependent degranulation by FcγRIIB^{lo/-} NK cells in these experimental conditions.

In summary, we have shown that FcγRIIB expression levels by human circulating CD56^{+}/CD3^- NK cells from healthy donors define three functionally different subsets, the previously described CD56^{bright}/FCγRII and CD56^{dim}/FCγRII^{lo/-} NK cells [5, 21] and a newly defined CD56^{dim}/FCγRII^{bright} small subpopulation of NK cells expressing high levels of a functionally active inhibitory FcγRIIB. In addition, this subset also shows an increase in the expression of the inhibitory ILT-2 and all KIR tested, suggesting an inhibitory phenotype of these cells. The low number of these FcγRIIB^bright^ NK cells in the peripheral blood of healthy donors raises the question of whether these cells play a significant role in controlling NK cell functions. A local increase of the numbers of FcγRIIB at sites of inflammation, infections or tumors might have important consequences on the whole NK cell compartment capacity, through the down-modulation of the different functions triggered by the engagement of activating FcγRIIIA, from ADCC to the release of proinflammatory molecules. Evaluation of this CD56^{dim}/FCγRII^bright^ NK cell subset in various pathologies might help to better understand its physiological role.

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