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GPR56 as a novel marker identifying the CD56^{dull} CD16^{+} NK cell subset both in blood stream and in inflamed peripheral tissues

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Abstract

To define novel human NK cell markers, we generated two mAbs specific for G-protein-coupled receptor 56 (GPR56), a surface glycoprotein that appears to be involved in cell-to-cell and cell-to-matrix interactions. GPR56 has been described in selected normal tissues, and in certain tumors, while, as yet, its expression on leukocytes is unknown. In this study, we show that anti-GPR56 mAbs, among leukocytes, prevalently recognize NK cells. In particular, these mAbs brightly stain CD56^{dull} CD16^{+} NK cells while react poorly with CD56^{bright} CD16$^{+/-}$ NK cells. Consistently, we found that GPR56 was expressed on NK cells populating inflamed peripheral tissues while it was absent in lymph node-derived NK cells. We also show that activating stimuli, such as cytokines or exposure to monocyte-derived dendritic cell, down-regulate NK cell expression of GPR56 both at the protein and at the transcriptional level. Interestingly, IL-18, known to induce *de novo* expression of CCR7 on CD56^{dull} CD16^{+} NK cells, displayed the highest capability of modulating GPR56. Thus, together with the identification of GPR56 as a novel marker capable of discriminating different NK cells subsets, our data suggest that GPR56 may take part to the mechanisms regulating NK cell migration through the blood stream, peripheral tissues and lymph nodes.

Keywords: cell activation, cell surface molecules, human, NK cells

Introduction

In recent years, NK cells have been progressively assigned to additional novel roles within the immune system (1–3). Indeed, together with the classical well-known functions, i.e. cytolytic activity and cytokine production, NK cells by interacting with dendritic cell (DC), would directly influence the priming of naive T cells toward T_h1 polarization thus acting as regulators of adaptive immune response (4–8). This newly identified functional capability is the result of the combined action of different NK cell subsets unique in their ability to fulfill defined functions. These include: the CD56^{bright} CD16$^{+/-}$ NK cells (5–15% of circulating NK cells), which are poorly cytolytic, while producing large amounts of T_h1 cytokines and the CD56^{dull} CD16^{+} NK cells (80–90% of circulating NK cells), which conversely are highly cytotoxic and produce lower levels of T_h1 cytokines (9). In addition, a third NK cell subset, characterized by the CD56^{-} CD16^{+} phenotype, displays poor cytolytic potential and appear to include cells capable of producing T_h2-type cytokines (10). The CD56^{-} CD16^{+} cell population is frequently expanded in HIV-infected individuals but can be detected also in the peripheral blood of most healthy donors (1–10% of circulating NK cells) (11). Regarding the ability to interact with DC,

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the CD56^{dull} CD16^{+} NK cells induce DC maturation both by secreting cytokines (tumor necrosis factor-α and IFN-γ) and by killing those DC that do not undergo a productive maturation (a process called 'DC editing'), whereas the CD56^{bright} CD16$^{+/-}$ NK cells easily respond to maturing DC both by proliferating and by secreting IFN-γ (12–16).

The different functional/phenotypic features of the two major NK cell subsets suggest that they may serve at different functions in different body compartments. During inflammatory events in peripheral tissues, CD56^{dull} CD16^{+} NK cells can be recruited from peripheral blood by inflammatory chemokines, including IL-8, fractalkine and chemerin (5, 17–20). Once at these sites, recruited NK cells can be activated by different cytokines, pathogen-derived TLR-ligands as well as by the interaction with susceptible targets such as virus-infected or tumor-transformed cells (1, 5, 20–24). As a consequence, CD56^{dull} CD16^{+} NK cells acquire the capability to recognize and kill target cells, secrete cytokines and contribute to the induction of appropriate DC maturation. Following antigen capture and contact with NK cells, maturing DC leave inflamed tissues, reach draining lymph node, where they present antigen to T cells and drive an appropriate adaptive immune response. At these sites, in particular in the T-cell-rich area, maturing DC would encounter IFNγ-producing NK cells and this interaction would be crucial for skewing T-cell priming toward T_h1 polarization (6, 7, 25–27).

This model also takes into account the chemokine receptors and selectin profile of the two main circulating NK cell subsets. CD56^{bright} CD16$^{+/-}$ NK cells are CD62L^{+} CCR7^{+} CXCR1^{-} CX3CR1^{-}, therefore are endowed with receptors driving their migration in secondary lymphoid organs. On the contrary, CD56^{dull} CD16^{+} NK cells that are CD62L$^{+/-}$ CCR7^{-} CXCR1^{+} CX3CR1^{+} ChemR23^{+} would be mainly recruited into inflamed peripheral tissues (1, 5, 9, 16, 17, 28, 29).

However, while the presence of CD56^{bright} CD16$^{+/-}$ NK cells in secondary lymphoid organs has been clearly documented (25–27), limited information only is available on the location of NK cells outside the blood stream and on the mechanisms that sustain their homing in tissues.

In this study, we show that GPR56, a surface molecule possibly involved in the cellular interaction with extracellular matrix (ECM) and in the process of melanoma cell migration (30), is differentially expressed on the two major human NK cell subsets. Although, on NK cells, a defined functional role for GPR56 could not be assigned, this novel NK cell marker may allow a further and better characterization of NK cells in different body compartments, including peripheral tissues, lymph node and peripheral blood.

Methods

Monoclonal antibodies

The following mAbs, produced in our lab, were used in this study: JT3A (anti-CD3, IgG2a), c218 (anti-CD56, IgG1), A13 (anti-Vδ1 IgG1), BB3 (anti-Vδ2 IgGa), c127 (anti-CD16 IgG1), PP35 (anti-CD244, IgG1), E59/126 (anti-CD300a IgG1), c227 (anti-CD69 IgG1), KS38 (anti-NKp44 IgM), FM95 (anti-CD86, IgG1) and FM184 (anti-CD1a, IgM). D1.12 (IgG2a, anti-HLA-DR) mAb was provided by Dr R. S. Accolla (University of Insubria, Varese, Italy). Anti-CD3-FITC/anti-CD56PC5, anti-CD14-FITC (IgG2A), ZM3.8 (anti-ILT3-PC5) mAbs (Immunotech, Marseille, France); anti-CD123 (IgG1) (BD-Pharmingen, San Diego, CA, USA); anti-BDCA-2, anti-BDCA-3 and anti-BDCA-4 mAbs (Miltenyi, Bergisch Gladbach, Germany) were commercially available.

Cell isolation and culture

PBMCs were derived from healthy donors by Ficoll-Hipaque gradients. To obtain purified NK cells, the RosetteSep^{TM} NK cell Enrichment Kit (StemCell Technologies, Vancouver, Canada) was used: whole blood was incubated with the RosetteSep antibodies cocktail that target and cross-link unwanted cells to RBCs, forming rosette. After centrifugation on Ficoll-Hipaque gradients to separate rosetted cells, the recovered NK cells were assessed for purity by cytofluorimetry. Only those populations displaying >95% of CD56^{+} CD3^{-} CD14^{-} NK cells were selected. To obtain polyclonal NK cell lines, CD56^{+} CD3^{-} CD14^{-} cells were cultured on irradiated feeder cells in the presence of 300 U ml^{-1} rhIL-2 (Proleukin; Chiron, Emeryville, CA, USA) and 5 μg ml^{-1} PHA (Gibco, Paisley, Scotland).

Freshly isolated NK cells were cultured in the presence of one or another of the following cytokines: rhIL-2 (300 U ml^{-1}), rhIL-15 (20 ng ml^{-1}), rhIL-12 (2.5 ng ml^{-1}) (Peprotech, London, UK) and rhIL-18 (1 ng ml^{-1}) (MBL, Naka-Ku Nagoya, Japan). At the day indicated in the text, cells were harvested and analyzed by FACS for the expression of CD69, NKp44 (31) and GPR56.

Lymphocytes from lymph node were kindly provided by Guido Ferlazzo (Messina, Italy). Lymph node were from patients who underwent surgery for cancer resection.

To generate DC, PBMCs were derived from healthy donors and plastic adherent cells were cultured in the presence of IL-4 and granulocyte macrophage colony-stimulating factor (Peprotech) at the final concentration of 20 and 25 ng ml^{-1}, respectively (15). After 6 days of culture, cells were characterized by the CD14^{-}CD1a^{+}CD86^{-} phenotype corresponding to immature DC (iDC). In co-culture experiments, the NK/DC ratio was 5:1.

Flow cytofluorimetric analysis

For cytofluorimetric analysis (FACS-Calibur; BD, Mountain View, CA, USA), cells were stained with FITC- or PC5-labeled specific mAbs or with unlabeled mAbs followed by PE- or FITC-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associated, Birmingham, AL, USA or BD). In the analysis of NK cells/DC co-cultures, NK cells were gated on the basis of FSC/SSC parameters and confirmed by the assessment of CD56 expression.

Biochemical characterization and identification of the molecule recognized by DF206 and SEM279 mAbs

In total, 20×10^6 cells were lysed in 1% NP-40 and immunoprecipitated with Sepharose-CnBr (Pharmacia Biotech Inc., Piscataway, NJ, USA)-coupled mAbs. Samples were analyzed by discontinuous SDS–PAGE and transferred to Immobilon P (Millipore Corp., Bedford, MA, USA). Membranes

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were probed with Biotin (Pierce, Rockford, IL, USA)-labeled purified mAbs. After staining with Neutravidin (Pierce), the Renaissance Chemiluminescence Kit (NEN, Boston, MA, USA) was used for detection.

mAb-reactive molecules were purified from membrane cell lysates. Briefly, membrane lysates from 5×10^9 FO-1 cells were incubated with Sepharose-CnBr-coupled SEM279 mAb (Over/Night, 4°C). After extensive washes, the affinity-purified proteins were eluted with 0.1 M glycine and (without SDS–PAGE analysis) digested with trypsin (Promega, Madison, WI, USA). The resulting peptides were analyzed using an automated LCQ-DECA MS/MS ion trap mass spectrometer coupled to a HPLC Surveyor (Thermo Finnigan). Protein identification was performed using SEQUEST software, from Thermo Electron, operating on a 10 processor computer cluster (AETHIA, Turin, Italy) and searched against an IPI human protein database. Peptide MS/MS assignments were filtered following the HUPO criteria: Xcorr $\geqslant$ 1.9 for the singly charged ions, Xcorr $\geqslant$ 2.2 for doubly charged ions and Xcorr $\geqslant$ 3.7 for triply charged ions, peptide probability $\leqslant$ 0.01, Delta Cn $\geqslant$ 0.1 and Rsp $\leqslant$ 4.26. To identify the largest panel of peptides, the option no enzyme was used for the in silica digestion of human databases.

Reverse transcription–PCR analysis, DNA sequencing and cell transfection

Total RNA was extracted from resting and IL-2 activated polyclonal NK cell populations using Micro or Mini RNeasy Kit (Qiagen) following the manufacturer's instruction. Complementary DNA (cDNA) synthesis was obtained using hexameric primers. Four different sets of primers were used in this study. The primer sequences used for GPR56 ORF amplification (2245 bp) were GPR56 ORF up: 5′-CAGCGGCATCTCCCTTG-3′/GPR56 ORF down: 5′-TTTCAAAGCTCCTCACTATG-3′, while those utilized for CD2 ORF (1122 bp) were CD2 ORF up: 5′-AACCAACCCCTAAGATGAG-3′/CD2 ORF down 5′-AGGGCAGAAATCCACAGTG-3′. The set of primers β-actin-up: 5′-ACTCCATCATGAAGTGTGACG-3′/β-actin-down: 5′-CATACTCCTGCTTGCTGATCC-3′ allowed to amplify a 250-bp fragment of human beta-actin cDNA and the primers pair GPR56 sense 5′-CCATCTTTCTGGTGACGC-3′/GPR56 anti-sense 5′-GAGCTGATGGGGAGCCTG-3′ amplified a 476-bp segment of GPR56 transcript. The amplification profile was 30 cycles: 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, except for GPR56 ORF PCR that required 35 cycles, an annealing temperature of 55°C and an extension time of 45 s. The PCR products were resolved into a 1% agarose gel, subcloned into pcDNA3.1/V5/His TOPO^{TM} vector using the Eukaryotic TOPO TA Cloning Kit (Invitrogen) and sequenced. DNA sequencing was performed using d-Rhodamine Terminator Cycle Sequencing Kit and a 3100 ABI automatic sequencer (PerkinElmer/Applied Biosystems, Foster City, CA, USA).

Quantitative PCR (Q-PCR) amplification was performed using TaqMan assay (Applied Biosystems 7700 Sequence Detector). A two-step PCR procedure of 15 s at 95°C and 1 min at 60°C was applied for 40 cycles. GPR56 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts were analyzed using Hs00173754_m1 kit and human GAPDH Endogenous control kit, respectively (Applied Biosystems). The GAPDH expression was used to normalize the GPR56 messenger RNA (mRNA) amount. The normalized GPR56 mRNA transcript, present in IL-2 activated NK cells, was calculated as time-fold mRNA detected in the corresponding resting NK cells (indicated as 1 in this study). The samples were analyzed in three independent experiments and each reaction was performed in triplicate.

HEK-293T cells were transfected with GPR56 pcDNA3.1 construct using FuGene-6 reagent following the manufacturer's instruction. After 48 h, transfected cells were assessed by FACS for reactivity with DF206 or SEM279 mAbs.

Immunocytochemical and immunohistochemical stainings

Cytospin preparations were fixed in ethanol, immunostained with SEM279 mAb (mouse IgG2a, dilution 1:5) followed by NovoLink Polymer Detection System (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) and visualized with DAB.

Tissue samples from skin biopsies obtained from five patients with lichen planus were snap frozen in isopentane precooled to liquid nitrogen and stored at −80°C. Thin (5-μm) cryostat sections were air-dried overnight at room temperature and fixed in acetone for 10 min before staining. Expression of GPR56 was evaluated by immunohistochemistry. Tissue sections were incubated with SEM279 mAb (dilution 1:5) for 1 h at room temperature followed by NovoLink Polymer Detection System and visualized by applying 3-amino-9-ethylcarbazole. Nuclei were counterstained with hematoxylin. Double immunofluorescence was performed using SEM279 mAb combined with a set of primary antibodies, including anti-CD56 mAb (123C3.D5, IgG1, dilution 1:20), anti-CD94 mAb (39 BIO-CL1, IgG1, dilution 1:10, kindly provided by M. Colonna, Washington University, St Louis, MO, USA), anti-CD3 mAb (SK7, IgG1, dilution 1:20; BD Bioscience, San Josè, CA, USA), MART-1/Melan-A (clone A103, IgG1, dilution 1:10; DakoCytomation, Glostrup, Denmark) followed by appropriate Texas red- or FITC-conjugated isotype-specific secondary antibodies (Southern Biotech, Birmingham, AL, USA). Immunostained sections were photographed using Olympus BX60 fluorescence microscope, equipped with the Olympus DP-70 digital camera, and images were analyzed by Analysis 3.2 soft imaging system GMBH.

Results

Identification of two novel mAbs preferentially reacting with human CD56^{dull} CD16^{+} peripheral blood NK cells

Mice were immunized with purified human NK cells freshly isolated from healthy individuals. After cell fusion (31), mAbs were selected by triple immunofluorescence staining and cytofluorimetric analysis on the basis of their reactivity with different PBMC populations. Two mAbs, termed DF206 (IgG1) and SEM279 (IgG2a), were identified that, within the PBMC, stained prevalently the NK cell population. In particular, among lymphocytes, both mAbs selectively stained the CD56^{+} CD3^{-} NK cells and a variable fraction of CD3^{+} T cells (ranging from 2 to 28% of the whole circulating T cells, mean 14%, $n = 10$) (Fig. 1A and data not shown for SEM279). In some donors, they also reacted with a small fraction of CD56^{-} CD3^{-} cells while in no instances they stained

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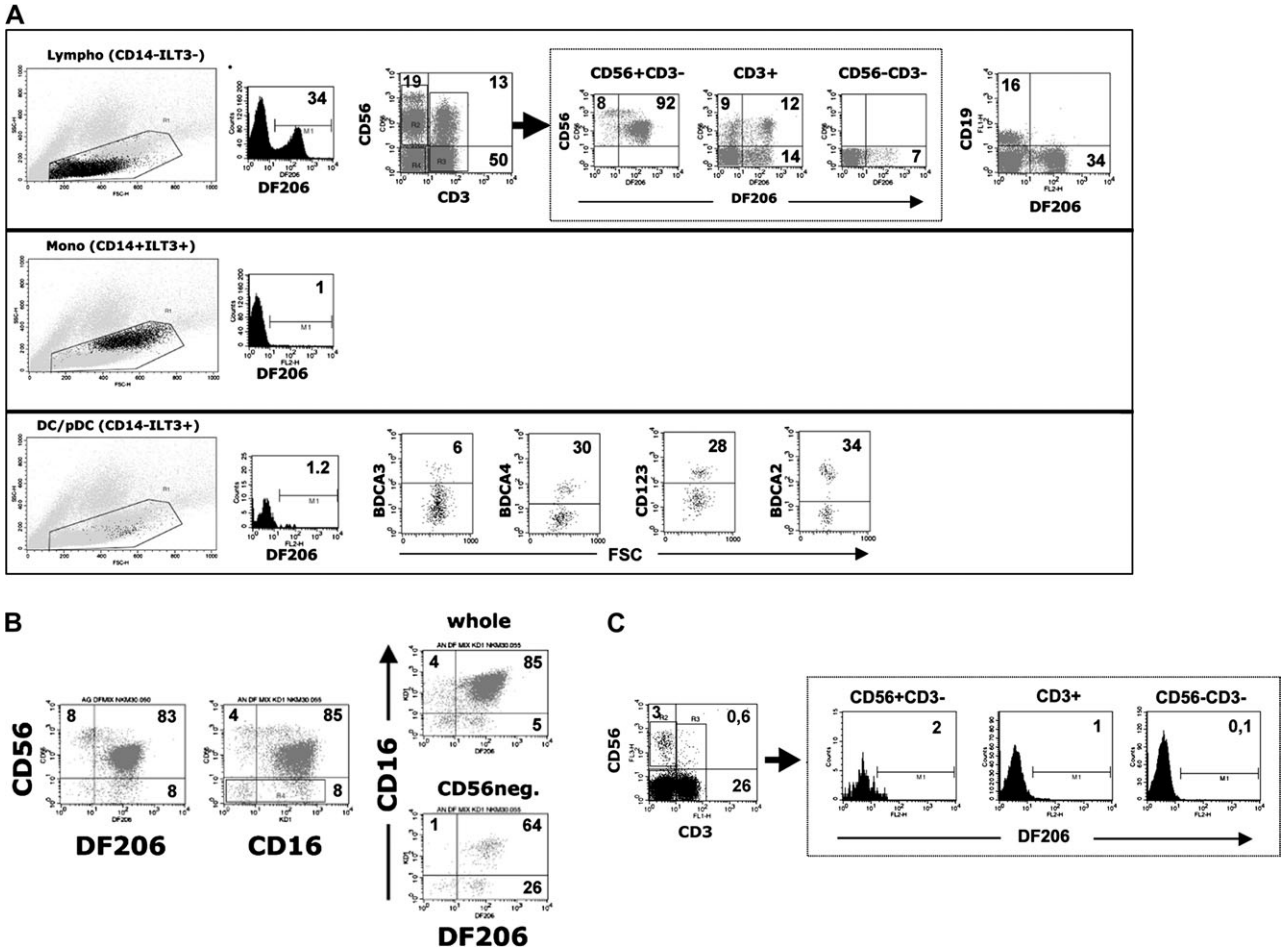

Fig. 1. DF206 mAb prevalently reacts with CD56^{dull} CD16^{+} NK cells. (A) Cytofluorimetric analysis of DF206 mAb reactivity on different PBMC sub-populations gated basing on physical parameters (FSC versus SSC) and immune phenotype (dark gray dots left panels): CD14^{-}ILT3^{-} (lymphocytes), CD14^{+}ILT3^{+} (monocytes) and CD14^{-}ILT3^{+} (DC/pDC). In the upper line is shown the DF206 mAb reactivity on the whole lymphocyte population (single staining), on T-cell and NK cell subsets (triple staining) or on CD19^{+} B cells (double staining). Data in the central line are referred to monocytes. Data in the lower line are referred to the DC/pDC population that includes: the BDCA3^{dull} myeloid DC1, the BDCA3^{bright} myeloid DC2 and the CD123^{bright} BDCA4^{+} BDCA2^{+} pDC cell subsets. (B) DF206 mAb-reactive molecule, CD56 and CD16 expression was comparatively analyzed by triple staining and FACS analysis on purified NK cells freshly isolated from blood. (C) Cytofluorimetric analysis of DF206 mAb reactivity on lymphocytes derived from lymph node. The different gated sub-populations are shown. In each experiment, the percentages of single-positive or double-positive cells are indicated. SEM279 mAb gave similar FACS profiles (data not shown).

CD56^{-} CD3^{-} CD19^{+} B cells (Fig. 1A and data not shown for SEM279).

Interestingly, these mAbs stained brightly CD56^{dull} NK cells, while stained weakly the CD56^{bright} NK cell subset (Fig. 1A and data not shown for SEM279). Thus, the molecule recognized by these mAbs, similar to CD16, was differentially expressed by the two major NK cell sub-populations. Regarding the fraction of T cells reacting with DF206 and SEM279 mAbs, these were highly heterogenous since they included both CD56^{+} and CD56^{-} cells, expressing the TCR γ/δ^{+}, the Vα24^{+} or the CD8^{+} CD244^{+} CD300a^{+} TCR α/β^{+} phenotype (data not shown).

Finally, both mAbs did not react with monocytes, circulating myeloid DC, plasmocytoid DC (Fig. 1A) or granulocytes (data not shown).

To examine in more detail the NK cell subsets recognized by DF206 and SEM279 mAbs, their reactivity was compared with that of both anti-CD56 and anti-CD16 mAbs on purified fresh NK cells. In line with the results obtained in PBMC, the two novel mAbs strongly reacted with the CD56^{dull} CD16^{+} NK cells while poorly stained the CD56^{bright} CD16$^{+/-}$ NK subset. Finally, both mAbs also stained most CD56^{-} CD16^{+} NK cells (Fig. 1B and data not shown for SEM279).

We also analyzed DF206 and SEM279 mAbs reactivity in lymphocytes derived from lymph node. In this context, recent studies demonstrated that these secondary lymphoid compartments are mainly populated by NK cells belonging to CD56^{bright} CD16$^{+/-}$ subset (25, 26). As shown by the representative staining profiles in Fig. 1(C), neither the CD3^{-} CD56^{+} NK cells nor the CD3^{+} T cells were significantly stained by these mAbs.

Finally, we analyzed the reactivity of the mAbs on a panel of cell including *in vitro*-activated T or NK cells, cultured DCs and tumor cell lines (Table 1). In all instances the

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Table 1. DF206 and SEM279 mAbs reactivity

Cells	Histotype	mAbs reactivity
PHA blasts[a]		_[b]
IL-2 NK cells[c]		+/–
Mo-DC[d]		–
Fibroblasts		–
NK-92	NK cell line	–
YT	NK cell line	–
NK-L	NK cell line	–
NK-33	NK cell line	–
H9	T cell line	–
JA3	T cell line	–
721-221	B cell line	–
DAUDI	B cell line	–
RAJI	Burkitt lymphoma cell line	–
K562	Erythroleukemia cell line	–
HL-60	Promyelocytic leukemia cell line	–
FO-1	Melanoma cell line	+
M14	Melanoma cell line	++
M15	Melanoma cell line	+/–
Mel1106	Melanoma cell line	+
Mel1386	Melanoma cell line	–
ACN	Neuroblastoma cell line	+
A172	Glioblastoma cell line	–
H460	Lung carcinoma cell line	+
A549	Lung carcinoma cell line	–
SKOV3	Ovarian carcinoma cell line	+/–
OVCAR3	Ovarian carcinoma cell line	–

[a]T cells activated *in vitro* with PHA.
[b]Cells were analyzed by cytofluorimetric analysis, the MFI is indicated as follows: ++ = MFI > 100, + = 40 < MFI < 100 and +/– = 10 < MFI < 40.
[c]Polyclonal NK cell population cultured in IL-2 for 20 days.
[d]DC derived from monocytes that have been cultured in the presence of granulocyte macrophage colony-stimulating factor + IL-4.

pattern of reactivity of the two mAbs resulted identical in all cell types analyzed.

Identification of the molecule recognized by DF206 and SEM279 mAbs

As shown in Table 1, the mAbs stained weakly activated NK cell populations while strongly reacted with certain melanoma cell lines. Thus, to characterize the molecules recognized by the mAbs, cell lysates derived from M14 and FO-1 melanoma cell lines and, as negative control, a Burkitt lymphoma cell line, were immunoprecipitated with DF206 and SEM279 mAbs. Samples were analyzed in SDS–PAGE and, after blotting, membranes were probed with the two mAbs (Fig. 2A). Under non-reducing conditions, both mAbs identified two molecules of ~60 to 65 and 90 to 100 kDa, respectively.

To ascertain the identity of mAb-reactive molecules, these were purified from FO-1 cell membranes by affinity chromatography (see Methods). Tryptic-digested molecules were analyzed by liquid chromatography and tandem mass spectrometry. Seven different peptides allowed the identification of the mAb-reactive molecule (Table 2). It corresponded to the G-protein-coupled receptor 56 (GPR56) also termed 7-transmembrane protein with no EGF-like N-terminal domains 1 (TM7XN1) (NP_958933).

GPR56 is a cell surface glycoprotein composed of an extracellular domain (containing seven N-glycosylation consensus sequences) that is non-covalently bound to a highly hydrophobic seven spanning transmembrane (7TM) portion (32, 33). All the peptides identified by mass spectra analysis were localized in the extracellular portion (Table 2). In this context, both DF206 and SEM279 mAbs recognized in western blot two bands corresponding to the glycosylated extracellular portion of GPR56 either alone (±65 kDa) or associated with the 7TM portion (±100 kDa). Thus, biochemical data together with cytofluorimetric analysis show

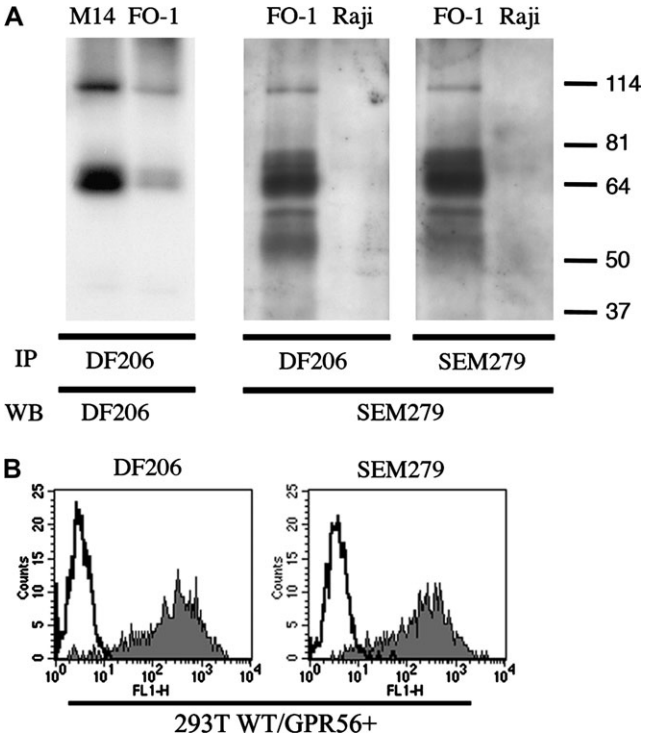

Fig. 2. DF206 and SEM279 mAbs specifically recognize GPR56. (A) M14 and FO-1 melanoma cell lines were immunoprecipitated with DF206 and SEM279 mAbs. Samples were analyzed in an 8% SDS–PAGE under non-reducing conditions and probed with biotin-labeled mAbs. Molecular weight markers (kilodaltons) are indicated. The Burkitt lymphoma cell line Raji was used as negative control. (B) Staining of GPR56 pcDNA3.1-transfected cells: HEK-293T cells untransfected (white) or transiently transfected with GPR56 pcDNA3.1 construct (gray) were stained with the indicated mAbs and analyzed by FACS.

Table 2. Identification of the molecule recognized by DF206 and SEM279 mAbs

Sequence	*m*/*z*	Position
SSLHYKPTPDLR	1413.7	44–55
ISIENSEEALTVHAPFPAAH PASR	2544.3	56–79
DLQLLSQFLK	1204.7	182–191
RPSAAPASQQLQSLESK	1797.9	199–215
LQPTAGLQDLHIHSR	1686.8	240–254
LLLVDFSSQALFQDK	1723.9	288–302
VLGIVVQNTK	1070.7	312–321

Amino acid sequence, mass-to-charge ratio (*m*/*z*) and position in the GPR56 protein sequence of the peptides identified by mass spectra analysis.

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that both mAbs react with epitopes located in the extracellular portion of the molecule.

The identification of GPR56 as the DF206 and SEM279 mAb-reactive molecule was confirmed by transfection experiments. To this end, total RNA extracted from the FO1 cell line was retrotranscribed and amplified, using a set of primers specific for the GRP56 ORF. The obtained PCR products were cloned into an expression vector and sequenced, and the GPR56-pcDNA3.1 construct was transiently transfected in HEK-293T cell line. As shown in Fig. 2(B), both DF206 and SEM279 mAbs brightly stained the GPR56-pcDNA3.1-transfected cells.

The expression of GPR56 is down-regulated upon NK cell activation

At variance with freshly drawn NK cells, long-term IL-2 cultured NK cell populations displayed poor reactivity with the DF206 and SEM279 mAbs (Table 1). This poor reactivity was not ameliorated after cell permeabilization (data not shown). These findings suggested that the process of NK cell activation could affect the expression of the GPR56 molecule. To analyze this possibility, freshly purified NK cells were cultured in the presence of IL-2 and analyzed at various time intervals for the expression of both GPR56 and NKp44 (31) and CD69 cell activation markers. As shown in Fig. 3(A), IL-2 treatment induced a progressive reduction of GPR56 expression that was paralleled by the progressive acquisition of the NKp44 molecule at the NK cell surface. Maximal down-regulation of GPR56 occurred after ~20 days of culture (Fig. 3A). On the other hand, no direct correlation could be established with the expression of CD69 since this molecule was already expressed at early time upon NK cell activation (data not shown). The inverse correlation between GPR56 and NKp44 expression was observed also with

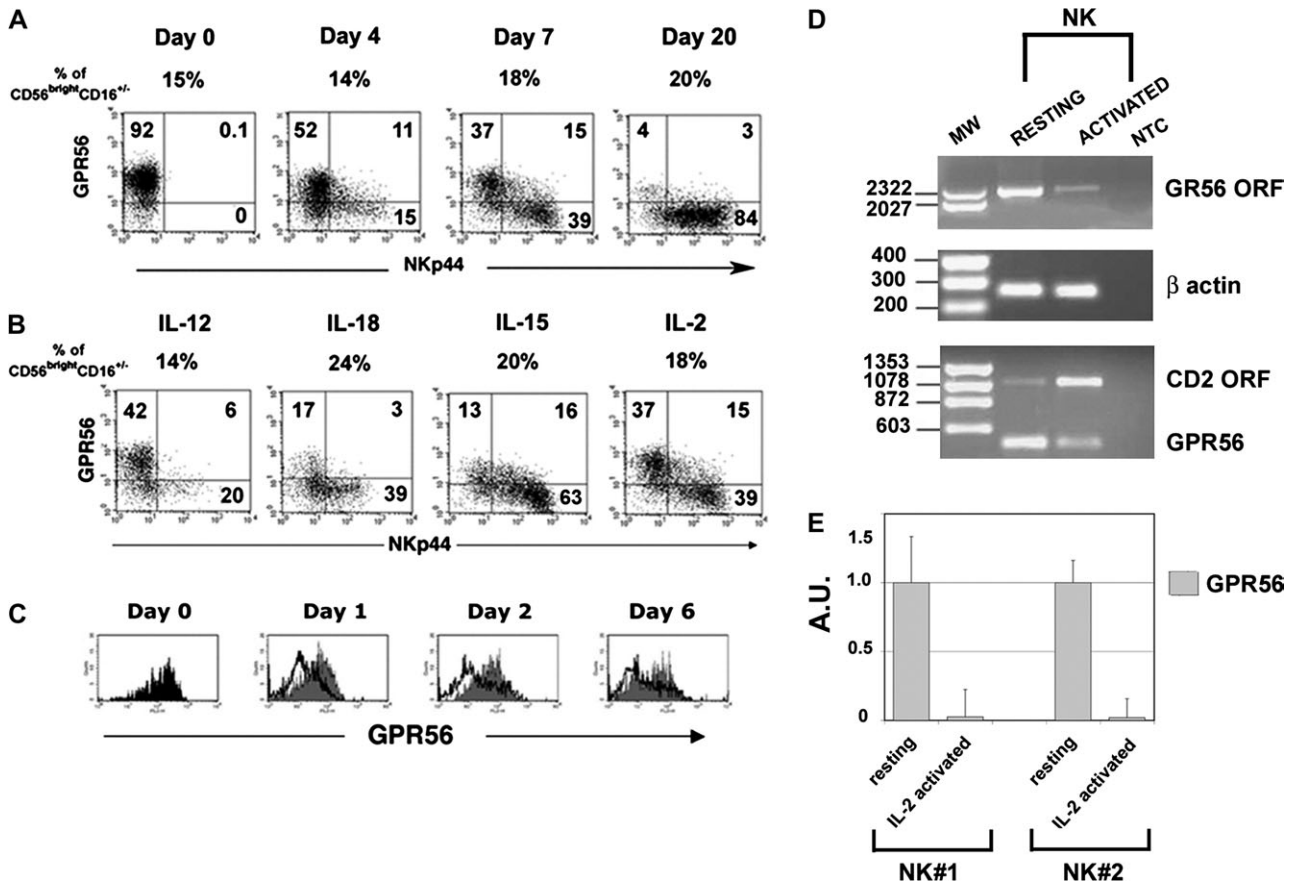

Fig. 3. Cytokines or DC induce GPR56 down-regulation in NK cells. Freshly isolated NK cells have been cultured in different conditions and then analyzed by FACS or by reverse transcription–PCR analysis for the expression of GPR56 and NKp44 or CD2. (A) NK cells were cultured in the presence of rhIL-2 and analyzed at the indicated time intervals. The percentage of CD56^{bright} CD16$^{+/-}$ NK cells at each time point is also indicated. (B) NK cells were exposed to the indicated cytokines and analyzed after 7 days of culture. The percentage of CD56^{bright} CD16$^{+/-}$ NK cells is also indicated. In (A) and (B), the percentage of cells included in each quadrant is indicated. (C) NK cells were cultured in the presence of monocyte-derived DC + LPS (1 μg ml^{-1}) (white profiles) or rhIL-2 (gray profiles) and evaluated at different time points. Black profile indicates the expression of GPR56 on NK cells at day 0. LPS alone did not induce down-regulation of GPR56 (data not shown). (D) Reverse transcription–PCR analysis of GPR56, beta-actin and CD2 transcripts was performed on total RNA extracted from resting or IL-2-activated polyclonal NK cell populations. The PCR products obtained using sets of primers specific for GPR56 ORF and a segment of beta-actin cDNA, or CD2 ORF and a segment of GPR56 transcript, were resolved into a 1% agarose gel. Molecular weights are indicated on the left. (E) Analysis of GPR56 transcript by Q-PCR: GPR56 mRNA present in resting and IL-2-activated NK cells, derived from two different donors, was analyzed by Q-PCR. All values were normalized to GAPDH transcript and the data are represented in time-fold the normalized GPR56 transcript detected in resting NK cells (used as reference). Each bar is the mean of three independent experiments of Q-PCR performed in triplicate (standard deviation is indicated).

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IL-15 and an even stronger effect was detected by culturing NK cells with IL-18. On the other hand, treatment with IL-12 resulted in poor effects in terms of both GPR56 down-regulation and in NKp44 up-regulation (Fig. 3B). As shown in Fig. 3(A and B), the percentage of CD56^{bright} CD16$^{+/-}$ NK cells did not change significantly, thus ruling out the occurrence of a large expansion of CD16$^{+/-}$ GPR56$^{+/-}$ NK cells during culture with different cytokines.

Down-regulation of the GPR56 molecule at the NK cell surface occurred also when NK cells were cultured together with monocyte-derived maturing DC (Fig. 3C). In this case, however, the kinetic of down-regulation was faster than that observed with IL-2 or IL-15 while it was similar to that induced by IL-18 (Fig. 3C). Although not shown, iDC induced only little GPR56 down-regulation on NK cells (with a mean fluorescence intensity decrease of 20% as compared with 70% induced by maturing DC). These results are in line with the concept that DC can contribute to the process of the NK cell activation and that IL-18 may be involved in this process (7, 21, 34).

To investigate whether the decrease of GPR56 is regulated at transcriptional level, we analyzed GPR56 coding mRNA by reverse transcription–PCR. The intensity of the GPR56-specific PCR product, amplified using a set of primers specific for GPR56 ORF, was higher in freshly isolated as compared with IL-2-activated NK cell populations (Fig. 3D). Similar results were obtained also using a semi-quantitative PCR analysis. Indeed, using a combination of two different sets of primers, one that amplify a 476-bp segment of GPR56 transcript and another specific for the CD2 ORF (used as internal control), we observed that the level of GPR56 transcript was lower in activated as compared with freshly isolated NK cells (Fig. 3D). Finally, to quantify the observed decrease, we performed a Q-PCR analysis. To this end, we compared the GPR56 amount in resting versus IL-2-activated NK cells derived from two different donors. In this analysis, GAPDH mRNA was used to normalize the GPR56 mRNA amount and the normalized GPR56 transcript present in IL-2-activated cell population was reported as time-fold mRNA detected in the corresponding resting NK cells. As shown in Fig. 3(E), upon 10 days of culture in the presence of IL-2, the amount of GPR56 transcript decreased dramatically (~40 times).

These data demonstrate that GPR56 expression on NK cells strictly paralleled the results on the quantitative evaluation of the transcripts coding for this receptor in either resting or activated NK cells.

Expression of GPR56 on NK cells from inflamed peripheral tissues

Next tumor cell lines and human NK cells were tested by immunocytochemistry for the expression of GPR56 using the SEM279 mAb. According to the expression of GPR56 in melanomas (see above), a strong reactivity of SEM279 mAb was observed on cytospin preparations of the human melanoma cell line FO1. The positivity was diffuse in the cytoplasm with the presence of a membrane rim (Fig. 4A); no reactivity was observed on the control murine cell line P815 (Fig. 4B). To define the distribution and cellular localization of GPR56 in NK cells, its expression was tested on freshly isolated and IL-2 activated human NK cells. As expected, a strong reactivity was observed in most freshly isolated NK cells with an obvious membrane localization while no reactivity was detected in the majority of activated NK cells (Fig. 4C and D).

We recently documented the presence of NK cells in mucosal biopsies obtained from patients with oral lichen planus (OLP), a mucocutaneous inflammatory disease (17). Therefore, we tested the expression of GPR56 by immunohistochemistry on tissue sections obtained from five OLP patients. SEM279 mAb stained consistent amounts of

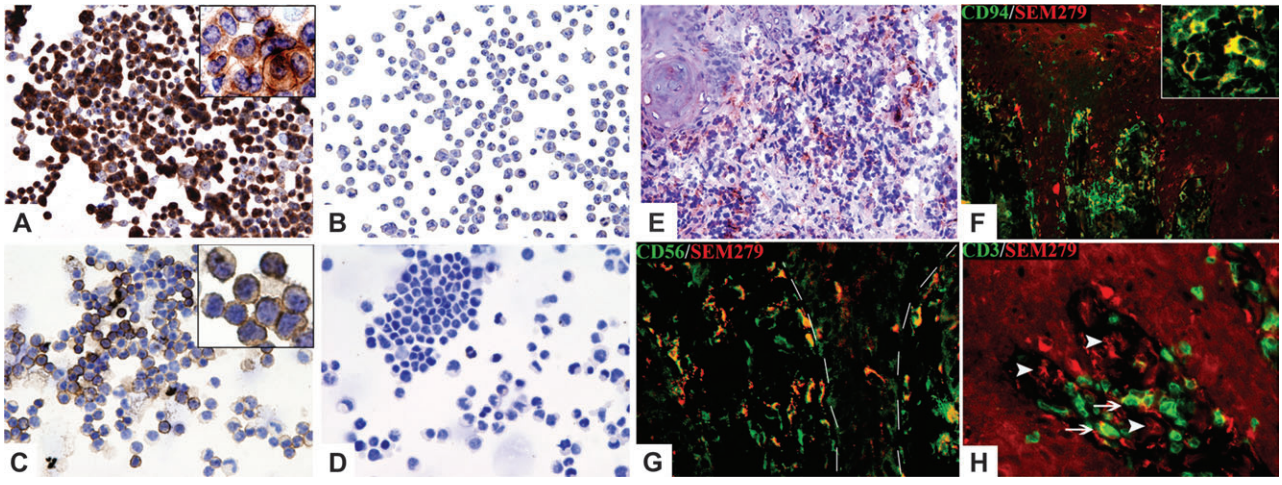

Fig. 4. Immunocytochemical and immunohistologic analysis of GPR56 expression in melanoma cells, NK cells and lichen planus biopsies. SEM279 mAb reactivity on cytospin preparations of the melanoma cell line FO-1 (A), the murine cell line P815 (B), freshly isolated (C) or IL-2-cultured (D) NK cells. GPR56 expression in NK cells infiltrating lichen planus biopsies (E–H). In lichen planus, numerous GPR56^{+} cells are observed in the stromal inflammatory infiltrate (E). The GPR56^{+} cell population is composed of mononuclear cells with round to oval morphology, co-expressing the NK cell markers CD94 (yellow cells, F) or CD56 (yellow cells, G; the dotted line indicates the epithelial–stromal junction) and mostly lacking the T-cell marker CD3 (H, arrows: GPR56^{+}CD3^{+} cells; arrowheads: GPR56^{+}CD3^{-} cells). In (E), nuclei are counterstained with hematoxylin. Original magnification ×200 (A, B and E–G), ×400 (C, D and H), ×800 (inset in F) and ×1000 (insets in A and C).

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mononuclear cells mainly located in the inflammatory stromal infiltrate and at the stromal/epithelial interface (Fig. 4E). Double immunofluorescence staining showed that most GPR56^{+} cells co-expressed CD94 (Fig. 4F) and CD56 (Fig. 4G) but only rarely CD3 (Fig. 4H arrows and Supplementary Figure 1, available at *International Immunology* Online), suggesting their NK cell identity. Thus, this molecule is expressed in cells recruited at inflammatory sites confirming that this event involves CD56^{dull} NK cells. The finding that GPR56 is still expressed (i.e. is not down-modulated) on a substantial NK cell fraction after extravasation suggests that NK cells do not undergo immediate and indiscriminate activation once recruited at sites of inflammation. These data are in line with previous findings indicating that within inflammatory peripheral tissues only a fraction (30–40%) of recruited NK cells becomes activated as judged on the basis of their CD69 expression (17). Interestingly, a strong reactivity of GPR56-specific mAbs was also observed on MART-1^{+} melanocytes located in the basal layer of the epithelium (Supplementary Figure 2, available at *International Immunology* Online).

Discussion

In the present study, we identify the GPR56 molecule as a novel NK cell marker allowing the discrimination between different NK cell subsets in humans. By using newly generated specific mAbs, we could analyze the surface expression and the cellular distribution of GPR56 on leukocytes derived from blood stream, lymph node and inflamed peripheral tissues. The specificity of the anti-GPR56 mAbs was assessed by the mass spectrometer analysis of the peptides derived from the affinity-purified molecules and was confirmed by staining experiments on cells transfected with the GPR56 construct. Our study revealed that anti-GPR56 mAbs strongly reacted with CD56^{dull} CD16^{+} NK cells while only a weak staining was detected on CD56^{bright} CD16$^{+/-}$ NK cells. GPR56 expression on CD56^{dull} CD16^{+} NK cells was not stable rather it appeared to be affected by the status of activation of the cells. Indeed, exposure to different activating stimuli, including exogenous cytokines such as IL-2, IL-15 and IL-18 or monocyte-derived DCs, induced at the NK cell surface a progressive GPR56 down-regulation that correlated with the parallel acquisition of the NKp44 activation marker. This GPR56 down-regulation occurred at the transcriptional level while it did not involve mechanisms of protein retention in the cytoplasm since, on activated NK cells, GPR56 was poorly detected even in mAb-specific intra-cytoplasmic staining. This also suggests that, although splice variants coding for intra-cytoplasmic forms of GPR56 have been described (35), these do not appear to be involved in GPR56 down-modulation.

GPR56 is a member of the increasing family of the human G-protein-coupled receptors (GPCRs) with long N-termini termed LN-7TM (36, 37). Although they share a common signaling mechanism (involving interaction with G-proteins) (38, 39) and certain structural features, including the presence of 7TM domains, a GPCR proteolytic site (GPS) (40) and Ser/Thr-rich regions forming a mucin-like stalk, each member display an N-terminal motif that is unique within the LN-7TM family (36, 37). The GPS motif is crucial for the proteolytic cleavage of several members of this family, including GPR56 (30, 40, 41). In particular, it has been shown that a precursor protein is subjected to an intracellular proteolytic processing that results in a mature protein composed of two associated subunits (extracellular and 7TM), which is expressed at the cell surface (30, 40, 41).

Although the functional role of most GPCR is still unknown, their structure and tissue distribution suggest that they may be involved in cell-to-cell or cell-to-ECM interactions (32, 33, 36, 37). In particular, a role for GPR56 in the mechanisms of cell adhesion has been suggested in different studies dealing with GPR56^{+} melanoma or glioblastoma tumor cells (33, 42). In addition, GPR56 has been suggested to be essential for a correct migration of neuronal progenitors during brain cortical development (43). More recently it has been shown that GPR56 expressed on melanoma cells could bind to the widespread tissue transglutaminase (TG)2 (30). This enzyme has been shown to participate to the organization of ECM by directly interacting with different ECM proteins and stabilizing their three-dimensional structure (44). In addition, TG2 would also interact with cellular integrins thus mediating cell-to-ECM interactions and affecting cell spreading and migration (45).

Basing on these observations, the expression of GPR56 on CD56^{dull} CD16^{+} NK cells could enable this NK cell subset to interact with ECM within peripheral tissues. Therefore, the differential GPR56 expression on CD56^{dull} CD16^{+} and CD56^{bright} CD16$^{+/-}$ NK cells would be in line with the current hypothesis on the different homing capabilities of the two main NK cell subsets. Indeed, the CD56^{dull} CD16^{+} subset is characterized by a set of chemokine receptors (CXCR1, ChemR23) that allow the extravasation into inflamed peripheral tissues. Accordingly, GPR56 was detected in most NK cells from inflamed peripheral tissues (lichen planus—Fig. 4F and G). On the other hand, the CD56^{bright} CD16$^{+/-}$ cells, by means of CCR7, can reach and home into lymph node in response to CCL19/21 secondary lymphoid organ chemokines. Along this line lymph node NK cells appeared essentially GPR56^{-} (Fig. 1C).

The 7TM-GPCR family members display, within their structure, multiple sites with different binding specificities. Therefore, it cannot be ruled out that, also in the case of GPR56, beside TG2 additional ligands may be identified with consequent implications in the function of CD56^{dull} CD16^{+} NK cells.

In our study we also show that, upon different activating stimuli, NK cells profoundly down-regulate GPR56 expression. Although the meaning of such phenomenon is yet unknown, one could speculate that GPR56 down-regulation may allow activated NK cells to move more efficiently within injured tissues. Alternatively, this may represent a negative feedback mechanism aimed either to avoid excessive accumulation of activated NK cells in inflamed tissues or to end the local immune reaction. In line with this hypothesis, both the kinetics of GPR56 down-regulation (Fig. 3) and the presence of GPR56^{+} NK cells in inflamed peripheral tissues (Fig. 4) indicate that GPR56 modulation may represent a late event in the process of NK cell activation. A further possibility is that, within inflamed peripheral

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tissues, microenvironmental stimuli may play a crucial role in down-regulating the GPR56 expression thus favoring migration of NK cells into lymph node. An example is represented by IL-18 that was shown to induce *de novo* expression of CCR7 on CD56^{dull} CD16^{+} NK cells (7, 21). Notably, we observed that IL-18 is the cytokine displaying the highest capability of modulating GPR56 from the NK cell surface. Thus, IL-18-exposed NK cells down-regulate GPR56, express CCR7 and migrate to lymph node where GPR56^{+} cells are not detectable. This may suggest that expression of GPR56 may represent a signal of retention for NK cells recruited in inflamed peripheral tissues.

In this context, it has been recently shown that the GPR56 expression on melanoma cells inversely correlates with their capability to leave primary tumor and generate metastasis (30, 33). In addition, it has also been suggested that TG2-mediated engagement of GPR56 would inhibit melanoma cell proliferation (30). Although not shown, in experiments of mAb-mediated cross-linking we failed to modulate NK cell cytolytic activity, cytokine production and proliferation. Nevertheless, we cannot rule out a possible role of GPR56 in these NK cell functions, either because our mAbs may not recognize a 'functional' GPR56 epitope or because GPR56 signaling may require the co-engagement of co-receptors (such as CD81) (38).

GPR56 expression was previously demonstrated in selected normal tissues (including uterus, pancreas and kidney) (42) and in certain tumors (including melanomas, gliomas and esophageal squamous cell carcinoma) (30, 33, 42, 46). Moreover, GPR56 has been postulated to represent a marker of normal hematopoietic and neuronal stem cells (47). Although the presence of GPR56 transcripts in NK cells is reported in a human microarray data bank (http://biogps.gnf.org), no data were available on its protein expression and distribution on these cells. The GPR56-specific mAbs generated in our lab allowed us to evaluate for the first time the expression of such a molecule on mature leukocytes and will represent a potent tool to confirm and/or evaluate the expression of GPR56 protein in different cell types and in a variety of physiological and pathological conditions.

Supplementary data

Supplementary Figures 1 and 2 are available at *International Immunology* Online.

Disclosure

The authors declare no competing financial interests. The European Commission is not liable for any use that may be made of the information contained.

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