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Brief Conclusive Report

Specific expression of GPR56 by human cytotoxic lymphocytes

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ABSTRACT

We here report the existence of a new cluster of adhesion-GPCRs in human immune cells. Analysis of a comprehensive immune cell transcriptome dataset indicated that expression of the closely related receptors, GPR56, GPR97, and GPR114, is associated with single lymphocyte and granulocyte subsets. Applying flow cytometric analysis with newly generated mAb, we show that expression of GPR56 is restricted to cytotoxic NK and T lymphocytes, including $CD8^+$, $CD4^+$, and $\gamma\delta$ T cells. Primary infection with human CMV, which generates a vast population of $CD8^+$ T cells with an effector phenotype, induced a strong increase in GPR56 expression in virus-specific $CD8^+$ T cells that remained detectable during latency. In NK-92 cells, ectopic expression of GPR56 inhibited spontaneous and SDF-1-stimulated cell migration. Our data suggest that GPR56 expression is a common trait of human cytotoxic lymphocytes and might affect the migratory properties of these cells. *J. Leukoc. Biol.* 90: 735–740; 2011.

Introduction

The adhesion class of GPCRs comprises 33 members in humans with a broad cellular distribution in the developing embryo, the reproductive tracts, and the nervous and the immune system [1, 2]. Adhesion-GPCRs differ from classical GPCRs by their unusual molecular structure. Intramolecular processing at a GPCR-proteolytic site proximal to the first transmembrane helix gives rise to a membrane-spanning and an extracellular subunit, which reassociates noncovalently, resulting in expression of a heterodimeric receptor at the cell surface. The extracellular subunits of adhesion-GPCRs are exceptionally long and contain a variety of structural domains that can facilitate cell-cell and cell-matrix interactions. Ligand profiles and in vitro studies have indicated a role of adhesion-GPCRs in cell adhesion and migration. More recent evidence, coming forth from genetic models, has confined this concept by suggesting that the primary function of adhesion-GPCRs may relate to the proper positioning of cells in a variety of organs [3]. However, despite much research interest, neither the mechanisms of activation nor the downstream signaling pathways of these nonclassical GPCRs are understood yet. Another shortcoming is the poor, basic characterization of many adhesion-GPCRs; approximately one-half of them has not been studied at the protein level.

Immune cells express a group of adhesion-GPCRs with N-terminal EGF-like domains, called EGF-TM7 receptors [4]. Whereas the EGF-TM7 family member CD97 is widely present on almost all leukocytes, expression of its relatives EMR1–4 (EMR1 is the human homologue of mouse F4/80) is restricted to myeloid cells, including monocytes, macrophages, DCs, and granulocytes. Whether leukocytes also express other adhesion-GPCRs has not been studied yet systematically. In a recent report, Della Chiesa and coworkers [5] described GPR56 as a novel marker for $CD56^{dull}$ cytotoxic NK cells. We here report that a cluster of adhesion-GPCRs, comprising GPR56, GPR97, and GPR114, is expressed in immune cells. With the use of newly generated mAb, we show that GPR56 expression identifies cytotoxic NK and T cells. We demonstrate that virus-specific $CD8^+$ T cells stably express GPR56 during human CMV infection. Finally, we provide evidence that GPR56 might regulate lymphocyte migration.

Abbreviations: APC=allophycocyanin, *B3GAT1*=β-1, 3 glucuronosyltransferase 1, EMR=EGF-like modules containing mucin-like receptor protein, KLRG1=killer cell lectin-like receptor subfamily G member 1, mFc=mouse Fc, SDF-1=stromal cell-derived factor-1, TM7= seven-transmembrane

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MATERIALS AND METHODS

Microarray analysis

The transcriptome of purified human leukocyte subsets was analyzed using Affymetrix HG-U133 A and B arrays (Affymetrix, Santa Clara, CA, USA) [6]. Gene expression profiling in the $CD8^+$ T cell during primary human CMV infection was done on Whole Human Genome 44K microarrays (Agilent Technology, Santa Clara, CA, USA) [7]. The complete datasets are available from the Gene Expression Omnibus database (Accession Numbers GSE3982 and GSE12589).

Generation and purification of GPR56-mFc fusion protein

A GPR56-mFc expression construct was generated by subcloning the entire extracellular domain of GPR56 into pSecTag2A-mFc (Invitrogen, San Diego, CA, USA). Generation of the construct, expression in human embryonic kidney-293T cells, and purification of the GPR56-mFc fusion protein were done essentially as reported [8].

Generation of GPR56-specific mAb

Purified GPR56-mFc protein was injected s.c. into Balb/c mice to generate mAb using standard protocols. In brief, for the first antigen challenge, 100 μg GPR56-mFc with complete adjuvant was injected into mice. At Days 21, 35, and 51, mice were boosted with 100 μg GPR56-mFc with incomplete adjuvant. A final boost of 50 μg GPR56-mFc without adjuvant was given at Day 65. Following preliminary screening, splenocytes were purified from sensitized mice and fused with the NS-1 cell. Hybridoma cells were selected in hypoxanthine, aminopterin, thymidine medium and subcloned subsequently to identify GPR56-specific clones by ELISA. A total of five monoclonal hybridoma lines (CG1, CG2, CG3, CG4, and CG5) was successfully established.

GPR56 overexpression

CHO-K1 cells were transfected with pcDNA3.1-based expression constructs encoding GPR56 or EMR2 as described previously [9]. NK-92 cells were infected with a pFB-Neo retroviral expression construct (Stratagene, La Jolla, CA, USA) encoding GPR56, using protocols described previously [10]. Stable NK-92 cells were selected in culture medium containing IL-2 (100 U/ml) and G418 (0.5 mg/ml) for 14 days.

Western blot analysis

Total cell lysates of tranfected CHO-K1 cells were separated in 10% SDS/PAGE gel and subjected to standard Western blot analysis. CG1-5 or 2A1 (anti-EMR2) mAb (5 μg/ml) were used as the primary antibody, followed by HRP-conjugated anti-mouse IgG (1:1000) as the secondary antibody.

Flow cytometry

Mononuclear cells were isolated from heparinized peripheral blood from healthy individuals using standard density gradient centrifugation techniques and Lymphoprep (Axis-Shield, Oslo, Norway). NK-92 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). For flow cytometry analysis, on average, 3×10^5 cells were incubated with fluorescent label-conjugated mAb as described [11]. Antibodies with the following specificities were used: CD3 (clone SK7, labeled with APC, PerCP-Cy5.5, or APC-H7), CD4 (clone SK3, labeled with PerCP-Cy5.5 or PE-Cy7), CD8 (clone SK1, labeled with PerCp-Cy5.5), CD19 (clone SJ25C1, labeled with PerCP-Cy5.5), CD20 (clone L27, labeled with APC), CD28 (clone CD28.2, labeled with APC), CD56 (clone B159, labeled with APC), CD94 (clone HP-3D9, labeled with APC), and TCR$\gamma\delta$ (clone B1, labeled with FITC; all BD Biosciences, San Diego, CA, USA); CD16 (clone 3G8, labeled with APC; Caltag Laboratories, Burlingame, CA, USA); CD27 (clone CLB-CD27/1-9F4, labeled with FITC) and granzyme B (clone GB11, labeled with PE; both Sanquin, Amsterdam, The Netherlands); CD45RA (clone 2H4-B, labeled with PE), TCRVα24 (clone C15, labeled with FITC), and TCRVβ11 (clone C21, labeled with PE; Beckman-Coulter, Fullerton, CA, USA); and CD57 (clone NC1, labeled with FITC; Immunotools, Friesoythe, Germany). PE- and APC-conjugated goat anti-mouse-Ig (Beckman-Coulter and BD Biosciences, respectively) was used as a second-step reagent. Staining with APC-conjugated HLA-A0201 tetramer, loaded with CMV immediate early protein 1-derived peptide VLEETSVML, was performed as described [7]. Flow cytometry analysis was performed using FACSCalibur and FACSCanto machines (BD Biosciences) and the FlowJo software package (Tree Star, Ashland, OR, USA).

Transwell migration

Retrovirally transduced NK-92 cells (2×10^5), starved in serum-free RPMI-1640 medium containing 0.3% BSA and 100 U/ml IL-2 at 37°C for 6 h, were loaded in a volume of 100 μl on transwell filters with a pore size of 5 μm (Corning, Corning, NY, USA). SDF-1 (CXCL12) was added as chemoattractant to the lower compartment at various concentrations. After 2.5 h incubation at 37°C, cells in the lower compartment were harvested and quantified by flow cytometry at a fixed, high speed for 105 s. A two-tailed unpaired t test was used to compare the levels of migration.

RESULTS AND DISCUSSION

Transcription of *GPR56*, *GPR97*, and *GPR114* in leukocytes

By using Affymetrix microarrays, we recently developed transcript profiles for all major human leukocyte populations, including neutrophils, eosinophils, basophils, mast cells, macrophages, DCs, NK cells, and B and T cell subsets [6]. This dataset, which allows the identification of genes expressed in immune cells, was used to analyze the expression pattern of adhesion-GPCRs. Next to the members of the EGF-TM7 family, CD97, EMR1, EMR2, and EMR3, we identified three novel receptors with distinct gene expression profiles in human leukocytes (**Fig. 1A**). These receptors, called GPR56, GPR97, and GPR114, are structurally similar molecules with an extracellular region of 250–400 aa (Fig. 1B). The genes encoding *GPR56*, *GPR97*, and *GPR114* cluster on the long arm of chromosome 16, suggesting a common evolutionary origin (Fig. 1C). High levels of the *GPR56* transcript were detected in NK cells, effector/memory T cells, and $\gamma\delta$ T cells. Transcripts of *GPR97* and *GPR114* were primarily found in granulocytes, and GPR*114* was present in eosinophils and *GPR97* in neutrophils and eosinophils. Other adhesion-GPCRs, present on the Affymetrix arrays [*BAI1-3*, *CELSR1-3*, *ELTD1* (ETL), *LPHN1-3*, *GPR64* (HE6), *GPR98* (VLGR1), *GPR110*, *GPR115*, *GPR116*, *GPR123*, *GPR124*, *GPR125*, *GPR126*, *GPR133*, and *GPR144*], were not expressed in leukocytes (Supplemental Fig. 1).

Generation of GPR56-specific mAb

To start with the characterization of the newly identified adhesion-GPCRs on immune cells, we generated mAb against human GPR56, using soluble GPR56-mFc protein as the antigen (Supplemental Fig. 2A). A total of five hybridoma clones, CG1–5, producing mouse anti-human GPR56 mAb, was successfully established. The specificity and utility of these mAb were evaluated by Western blot analysis and flow cytometry.

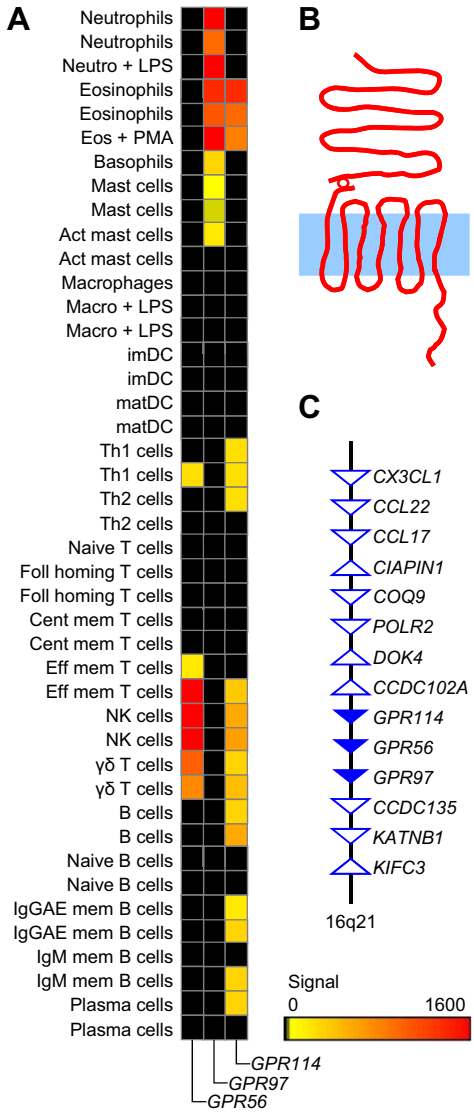

Figure 1. Transcription of the *GPR56/GPR97/GPR114* cluster in leukocytes. (A) Leukocyte transcription profile of *GPR56*, *GPR97*, and *GPR114* revealed by hybridization of Affymetrix microarrays with cRNAs derived from isolated peripheral blood leukocyte subsets of two individual healthy volunteers. The color scale indicates a transcript expression signal, where black = absent, yellow = moderately expressed, and red = highly expressed. imDC, immature DC; matDC, mature DC. (B) Cartoon representation of GPR56. At the cell surface, GPR56 is expressed as a heterodimer, consisting of an extracellular α-chain noncovalently bound to a membrane-spanning β-chain. (C) Organization of the *GPR56/GPR97/GPR114* locus on the long arm of human chromosome 16. Genes are depicted (not to scale) as triangles with the transcriptional orientation indicated.

Western blot analysis showed that all mAb recognize the purified GPR56-mFc protein (Supplemental Fig. 2B). Moreover, CG2, CG3, and CG4 detected a specific 60- to 75-kDa fragment in the cell lysate of GPR56- but not mock- and EMR2-transfected CHO-K1 cells. By flow cytometry of transfected CHO-K1 cells, a clear surface staining of GPR56 was obtained with CG2 and CG4 (Supplemental Fig. 2C).

Expression of GPR56 by cytotoxic lymphocytes

With the use of mAb CG4, we characterized the expression of GPR56 on circulating human leukocytes. In line with Della Chiesa et al. [5], we found abundant GPR56 expression on cytotoxic $CD56^{dull}$ NK cells but not on the developmentally and functionally less-mature $CD56^{bright}$ cells (**Fig. 2A**). Subdivision of the $CD56^{dull}$ population, based on CD94 surface density [12], revealed a small but consistent increase in GPR56 expression during the transition from intermediary $CD94^{high}$ to fully mature $CD94^{low}$ cytotoxic NK cells.

We next studied different T cell lineages. NKT cells, a separate population of T cells that coexpresses invariant TCR and NK-lineage molecules and executes effector functions by the production of large amounts of IFN-γ [13], did not express GPR56 (data not shown). $CD8^+$ T cells can be subdivided, based on the expression of CD27 and CD45RA, into naive, memory-type, and effector-type cells [14], Although $CD27^-$ $CD45RA^+$ effector-type cells strongly expressed GPR56, staining was very weak on memory cells and absent on naive cells (Fig. 2B). Similarly, naive and memory $CD4^+$ T cells did not express GPR56 (Fig. 2C). Cytotoxic $CD4^+$ T cells that lack expression of CD27 and CD28 are rare in normal subjects but emerge as a consequence of chronic viral infection [15]. In a CMV-positive donor with large numbers of cytotoxic $CD4^+$ T cells, we found expression levels of GPR56 on $CD4^+CD27^-$ cells comparable with that on cytotoxic NK cells and $CD8^+$ T cells (Fig. 2C, right panel). Expression of GPR56 correlated even better with lack of CD28, which strictly defines cytotoxic $CD4^+$ T cells (data not shown). Finally, we analyzed $\gamma\delta$ T cells, a small population of functionally variable T lymphocytes [16]. Again, expression of GPR56 was restricted to $CD27^-CD45RA^+$ effector-type cells, which all were $GPR56^+$ (Fig. 2D). In conclusion, we found abundant and highly specific expression of GPR56 in all cytotoxic subsets of NK and T lymphocytes. Expression of GPR56 completely overlapped with the presence of the cytolytic enzymes perforin, granzyme A, and granzyme B in these cells (Fig. 2E). Noncytotoxic NK and T cells, B cells, and myeloid cells, including granulocytes, monocytes, and DCs, did not express GPR56 (data not shown).

Persistent infection with CMV results in a strong increase in virus-specific, effector-type $CD8^+$ T cells with key features such as cytolytic potential, IFN-γ production, and migratory capacity [7]. With the use of expression profiling of CMV-specific $CD8^+$ T cells of renal transplant recipients experiencing primary CMV infection after receiving a CMV-seropositive donor organ, we observed a strong up-regulation of *GPR56* gene expression during the peak of the response, followed by a further increase until 1 year after viral infection (**Fig. 3A**). Kinetics of *GPR56* expression closely correlated with the expression of *B3GAT1*, the glucuronyltransferase that generates CD57, and *KLRG1*, both markers of replicative senescence [17–19]. Analysis of protein expression revealed that $CD8^+$ T cells, specific for the CMV immediate early protein 1, expressed similar levels of surface GPR56 during all phases of the response, even at 5 years after viral infection (Fig. 3B). A comparable staining was obtained on CMV pp65-specific $CD8^+$ T cells (data not shown). Thus, like key features of CMV-specific $CD8^+$ T cells,

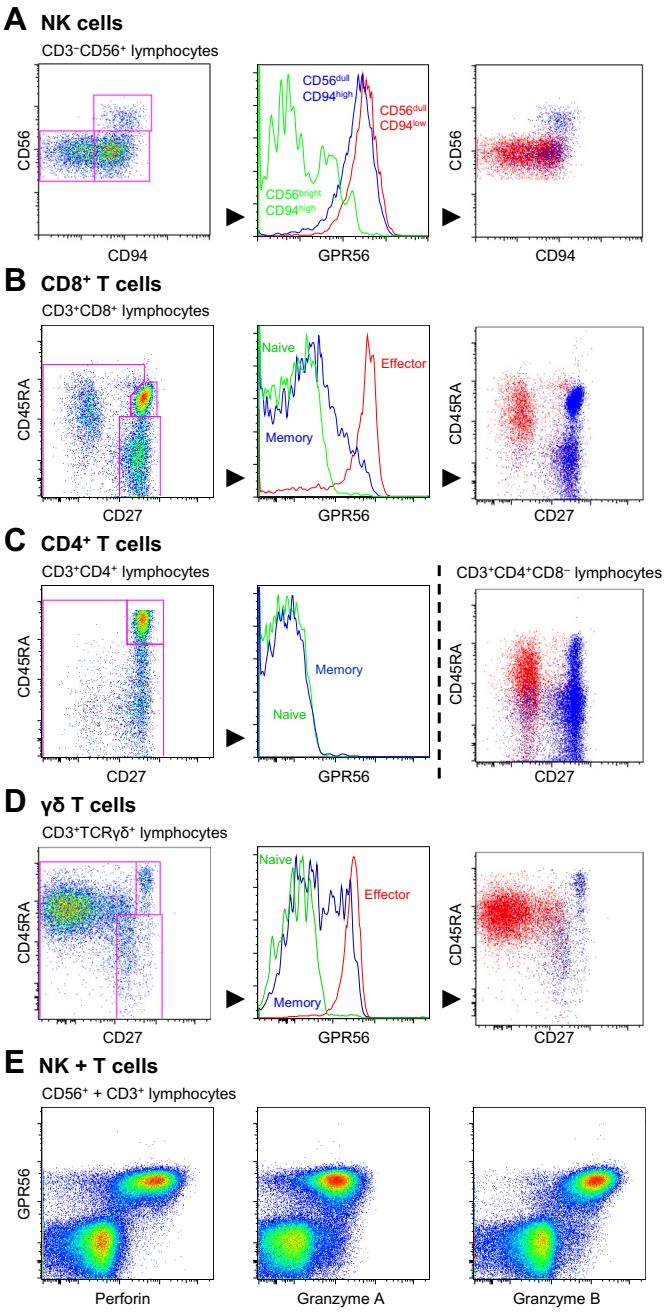

Figure 2. Cytotoxic lymphocytes express GPR56. (A) $CD3^{-}CD56^{+}$ NK cells were subdivided based on expression of CD56 and CD94 into $CD56^{bright}CD94^{high}$, $CD56^{dull}CD94^{high}$, and $CD56^{dull}CD94^{low}$ cells (left panel) and analyzed for expression of GPR56 (middle panel). $GPR56^{+}$ cells (in red) were cytotoxic cells with a dull expression of CD56 (right panel). (B) $CD8^{+}$ T cells were subdivided based on the expression of CD27 and CD45RA into $CD27^{+}CD45RA^{+}$ naive, $CD27^{+}CD45RA^{-}$ memory-type, and $CD27^{-}CD45RA^{+}$ effector-type cells (left panel). GPR56 expression in these subsets was determined (middle panel). $GPR56^{+}$ cells (in red) had an effector cell phenotype (right panel). (C) $CD4^{+}$ T cells were subdivided based on the expression of CD27 and CD45RA into $CD27^{+}CD45RA^{+}$ naive and $CD27^{+}CD45RA^{-}$ memory cells (left panel). GPR56 expression in these subsets was determined (middle panel). The right panel shows $GPR56^{+}$ cells (in red, geometric mean fluorescence intensity=8200) with a $CD27^{-}CD45RA^{+}$ cytotoxic phenotype from a donor with a large fraction of these normally rare cells. (D) γδ T cells, defined by the expression of a γδ TCR, were subdivided based on the expression of CD27 and CD45RA, into $CD27^{+}CD45RA^{+}$ naive, $CD27^{+}CD45RA^{-}$ memory-type, and $CD27^{-}CD45RA^{+}$ effector-type cells (left panel). GPR56 expression in these subsets was determined (middle panel). $GPR56^{+}$ cells (in red) had an effector cell phenotype (right panel). (E) NK and T cells were analyzed for coexpression of GPR56 with the cytolytic enzymes perforin, granzyme A, and granzyme B. Flow cytometry plots in this figure are representative of three to seven independently analyzed donors.

expression of GPR56 was installed during primary infection and stably persisted throughout the latency stage.

Ectopic expression of GPR56 inhibits lymphocyte migration

Functional maturation affects the migratory properties of lymphocytes [7]. Iguchi and coworkers [20] previously demonstrated that ectopic expression of GPR56 in neural progenitor cells negatively regulates migrating activity. With the use of retroviral transduction, we expressed GPR56 in NK-92, a cytotoxic NK cell line that lacks GPR56 expression, independent of its reliance on IL-2 (Fig. 4A, and data not shown). In line with the effect of GPR56 overexpression in neural progenitor cells, ectopic expression of GPR56 reduced spontaneous transwell migration of NK-92 cells by ~70% (Fig. 4B). Upon stimulation with SDF-1 (CXCL12), transmigration of GPR56-expressing cells was reduced by 70–80% as compared with control NK-92 cells. Hence, expression of GPR56 might regulate the migratory capacity of cytotoxic lymphocytes.

Concluding remarks

GPR56 was discovered independently in human heart tissue and melanoma cells by two studies in 1999 [21, 22]. Later work demonstrated a role of GPR56 in brain development. Mutations in various parts of the receptor were found to cause a human brain cortical malformation, called bilateral frontoparietal polymicrogyria [23, 24], which is mirrored in mice lacking a functional *Gpr56* gene [25]. A critical role for GPR56 in the adhesion of developing neurons to basal lamina molecules and consequently, the proper positioning of these cells were demonstrated [26]. Other studies found GPR56 in hematopoietic and neuronal stem cells [27, 28] and in a variety of tumors, including melanomas, gliomas, and different carcinomas [29–31]. Binding of mouse GPR56 to tissue transglutaminase 2 inhibited melanoma growth and spreading [30]. Biochemical studies revealed association of GPR56 with the tetraspanin CD81 in the cell membrane and engagement of different G_{α} proteins [32]; however, as for other adhesion-GPCRs, a link between receptor ligation and downstream signaling has not been established.

We here describe the expression of GPR56 in immune cells, thereby confirming a recent report that described GPR56 as novel marker for $CD56^{dull}$ lymphocytes. With the use of protein purification and sequencing, Della Chiesa and colleagues [5] identified the antigen recognized by two mAb with specificity for circulating and sessile NK cells as GPR56. Our study aimed to find novel adhesion-GPCRs on immune cells. We

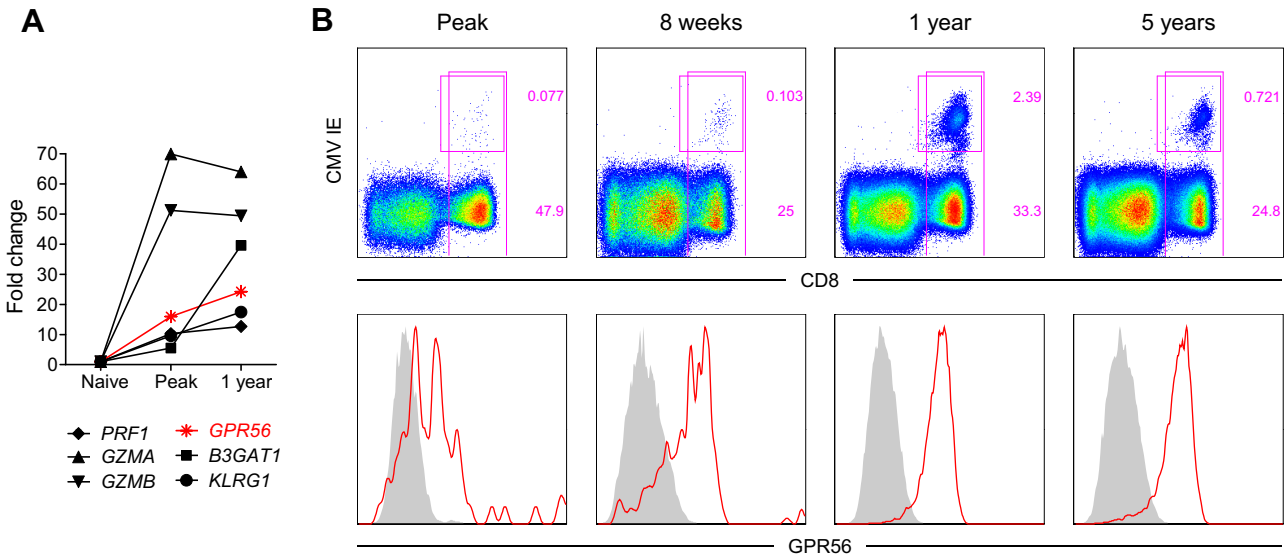

Figure 3. Alterations in GPR56 expression of CD8$^+$ T cells during primary CMV infection. (A) Gene expression of *GPR56*, *PRF1* (perforin), *GZMA* (granzyme A), *GZMB* (granzyme B), *B3GAT1* (the glucuronyltransferase that generates the CD57 epitope), and *KLRG1* in naïve cells in CMV-specific cells from renal transplant recipients at peak of and 1 year after infection was analyzed by hybridizing Agilent microarrays with cRNA from sorted peripheral blood leukocytes of three individual donors [7]. Expression is depicted as fold-change compared with naïve CD8$^+$ T cells. (B, upper panels) Staining of CMV-specific cells using a tetramer against an immediate early protein 1 (IE) epitope during a primary response in the same donor. Numbers depict the percentage of CD8$^+$ or tetramer$^+$ cells within the CD8$^+$ population. (Lower panels) GPR56 expression on tetramer$^+$ cells (red lines) compared with naive CD8$^+$ T cells (gray histograms). Flow cytometric analysis is shown for one representative patient of two.

found a gene cluster, encoding the related receptors GPR56, GPR97, and GPR114, to be expressed in leukocytes. Based on newly generated antibodies, we show that GPR56 is not restricted to cytotoxic NK cells but is also found on all populations of cytotoxic T cells. Expression of GPR56 closely correlated with a cytotoxic cell signature defined by the expression of cytolytic enzymes and cell surface markers, such as CD57 and KLRG1, and regulated cell migration. We conclude that GPR56 is a novel pan-cytotoxic lymphocyte marker in humans.

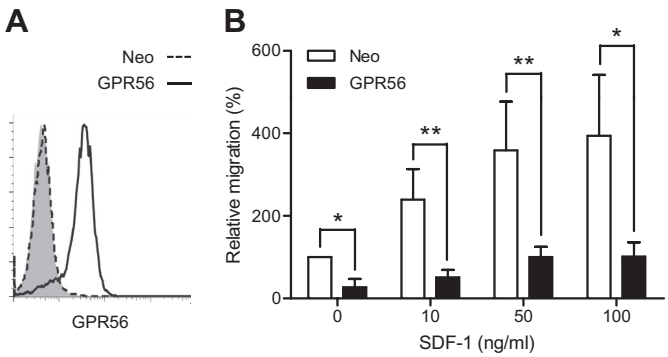

Figure 4. Ectopic expression of GPR56 inhibits lymphocyte migration. (A) GPR56 expression in retrovirally transduced NK-92 cells depicted by flow cytometry. (B) GPR56- and neo-transduced NK-92 were allowed to transmigrate for 2.5 h through 5-μm transwell filters in the absence or presence of 10–100 ng/ml SDF-1 in the lower compartment. Cells that migrated through the filter were quantified by flow cytometry. Indicated is the relative mean ± SD (untreated, neo-transduced cells=100%) of three independent experiments. *$P < 0.05$; **$P < 0.005$.

AUTHORSHIP

Y-M.P., M.D.B.G., K-F.C., and E.B.M.R. performed experiments and interpreted results; P.A.B., R.A.W.L., and C.R.M. designed research and interpreted results; H-H.L. and J.H. designed research, interpreted results, and wrote the paper.

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