Soluble HLA-G dampens CD94/NKG2A expression and function and differentially modulates chemotaxis and cytokine/chemokine secretion in CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells

**Short title:** Multiple effects of soluble HLA-G on NK cells

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ABSTRACT

Soluble HLA-G (sHLA-G) inhibits NK cell functions. Here we investigated sHLA-G-mediated modulation of i) chemokine receptor and NK receptor expression and function and ii) cytokine/chemokine secretion in CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells. sHLA-G-treated or untreated peripheral blood (PB) and tonsil NK cells were analyzed for chemokine receptor and NK receptor expression by flow cytometry. sHLA-G downmodulated i) CXCR3 on PB and tonsil CD56\textsuperscript{bright} and CD56\textsuperscript{dim}, ii) CCR2 on PB and tonsil CD56\textsuperscript{bright}, iii) CX3CR1 on PB CD56\textsuperscript{dim}, iv) CXCR5 on tonsil CD56\textsuperscript{dim} and v) CD94/NKG2A on PB and tonsil CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells. Such sHLA-G-mediated downmodulations were reverted by adding anti-HLA-G or anti-ILT2 mAbs. sHLA-G inhibited chemotaxis of i) PB NK cells towards CXCL10, CXCL11 and CX3CL1 and ii) PB CD56\textsuperscript{bright} NK cells towards CCL2 and CXCL10. Interferon-\(\gamma\) secretion induced by NKp46 engagement was inhibited by NKG2A engagement in untreated but not in sHLA-G-treated NK cells. sHLA-G upregulated secretion of i) CCL22 in CD56\textsuperscript{bright} and CD56\textsuperscript{dim} and ii) CCL2, CCL8 and CXCL2-CXCL3 in CD56\textsuperscript{dim} PB NK cells. Signal transduction experiments showed sHLA-G-mediated downmodulation of Stat5 phosphorylation in PB NK cells. In conclusion, our data delineated novel mechanisms of sHLA-G-mediated inhibition of NK cell functions.
INTRODUCTION

Natural killer (NK) cells are a lymphocyte population that can spontaneously kill tumor cells and virus-infected cells. Recently, it has been shown that NK cells are also major producers of pro-inflammatory and immunosuppressive cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α) and IL-10, in physiological and pathological conditions\(^1\). Moreover, NK cells secrete hematopoietic growth factors, i.e. granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor and IL-3, and chemokines, including CCL2 (MCP-1), CCL3 (MIP1-α), CCL4 (MIP1-β), CCL5 (RANTES), and CXCL8 (IL-8)\(^1\). Importantly, NK cell major functions can be modulated by their interaction with other immune cell types, such as dendritic cells, T and B lymphocytes, macrophages and neutrophils\(^2\). Altogether, these features delineate a central role of NK cells in innate immune response against tumors and pathogens\(^3\), and in innate and adaptive immunity regulation.

Multiple activating or inhibitory receptors regulate NK cell functions, and the balance between activating/inhibitory signals is crucial to determine NK-cell activation and target cells lysis, allowing NK cells to acquire self-tolerance and avoiding autoreactivity. In particular, HLA-class I molecules play a key role in self-tolerance by interacting with NK cell inhibitory receptors\(^4\). These receptors include CD94/NKG2A heterodimer, which recognizes HLA-E molecule, and Killer-Ig-like Receptor (KIR, CD158) family, which is composed of clonally distributed receptors specific for determinants shared by classical HLA class Ia alleles\(^4\). NK cell activation may be induced by proinflammatory cytokines such as IL-2, IL-15 and IL-18 and by the interaction between activating NK receptors and their ligands on target cells. In humans, activating receptors include NKp46, NKp30, NKp44 (natural cytotoxicity receptors), DNAM-1, NKG2D, and co-receptors 2B4 and NTBA\(^4\).

Different chemokine receptors control NK cells migration and recirculation through peripheral blood, inflamed tissues and secondary lymphoid organs\(^5\). In particular, CCR7 and CXCR3, through
a gradient of CCL19/CCL21 and CXCL9/CXCL10/CXCL11, respectively, drive NK cell migration to lymph nodes, where they promote Th1-polarization, while CXCR1, CX3CR1 and chem23R, specific receptors for CXCL8 (IL-8), CXC3CL1 (fractalkine) and Chemerin, respectively, are involved in NK cell binding to endothelial cells and migration to injured tissues5-7.

Two major subsets of peripheral blood NK cells have been identified on the basis of surface density of CD56 molecule, an isoform of human neural cell adhesion molecule, that may be involved in NK cells/target cells interactions. CD56bright/CD16dim- NK cells express high levels of CD56, are virtually negative for CD16 and exhibit low cytotoxicity, while secreting large amounts of Th-1 cytokines in response to cytokines (i.e. IL-12 and IL-18). These cells express CCR7 and CXCR3 and predominate in secondary lymphoid organs8,9. On the other hand, the main NK cell subset in peripheral blood is represented by CD56dim/CD16+ NK cells, which exert potent natural and antibody-dependent cytotoxicity (ADCC), express CXCR1, CX3CR1 and chem23R, and display higher expression of IL-2R common γ chain compared to CD56bright counterparts. Both NK cell subsets express CD94/NKG2A, while KIRs expression is mainly detected in CD56dim/CD16+ subset10.

Several studies have demonstrated HLA-G inhibitory effects on NK cells. HLA-G belongs to non-classical HLA Ib molecules, that also include HLA-E, -F and –H. These molecules, at variance with classical HLA-Ia molecules (HLA-A, -B and –C), display potent immunoregulatory properties targeting NK cells, T and B lymphocytes, monocytes and dendritic cells11.

HLA-G shows a complex splicing pattern of primary transcript, that yields four membrane-bound (HLA-G1, -G2, -G3 and –G4) and three soluble (HLA-G5, -G6 and –G7) isoforms12. Soluble HLA-G (sHLA-G) can be also generated by shedding of membrane-bound isoforms by metalloproteases. Both soluble and membrane-bound HLA-G molecules display similar immunoregulatory properties mediated by the interaction with different receptors. In particular, HLA-G is the unique known ligand of KIR2DL4 (CD158d), whose expression is mainly detected in CD56bright/CD16dim- NK
cells. Additional HLA-G receptors are represented by immunoglobulin-like transcript (ILT)2 (CD85j, LILRB1) and ILT4 (CD85d, LILRB2). Both lymphoid and myeloid cells express ILT2, while ILT4 is present in myeloid cells only. ILT2 and ILT4 are inhibitory receptors, whereas KIR2DL4 seems capable to transduce both inhibitory and activating signals. HLA-G also binds CD160 on NK cells, endothelial cells and T lymphocytes.

HLA-G molecules inhibit the cytolytic function of NK cells and T lymphocytes. During pregnancy, decidual NK cell expression of ILT2 and KIR2DL4 and their interaction with HLA-G expressed and secreted by extravillous cytotrophoblast play a crucial role in protecting semiallogeneic fetal tissues from maternal uterine NK cytolysis. In tumors and viral infection, HLA-G overexpression and release by tumor cells and virus-infected cells represent a further mechanism of escape from immune system recognition.

HLA-G molecules inhibit NK cell-mediated cytotoxicity through downregulation of perforin and Stat3, and may also induce fratricide killing of NK cells. More recently, it has been demonstrated that sHLA-G inhibits NK cell mediated lysis impairing actin reorganization and perforin granule polarization toward target cells. Moreover, the impaired cytoskeletal reorganization affects CD2 localization in late NK-to-target cell immune synapse.

Finally, secretion of different cytokines and chemokines by peripheral blood and decidual NK cells can be modulated by sHLA-G towards pro-inflammatory or immunosuppressive cytokine secretion profile, depending on KIR2DL4 or ILT2 engagement by HLA-G molecules.

In the present study, we have explored the potential role of sHLA-G in the modulation of surface phenotype and function of CD56bright and CD56dim NK cell subsets. In particular, sHLA-G treated NK cells have been analyzed for i) modulation of chemokine receptors and chemotaxis, ii) phenotypic and functional modulation of activating or inhibitory NK cell receptors and iii) secretion of cytokines and chemokines. Finally, sHLA-G signaling pathways in these NK cell subsets have been investigated.
MATERIALS AND METHODS

Soluble HLA-G production. Soluble HLA-G was produced in 721.221.G1 human lymphoblastoid cell line (kindly provided by Dr. Francesco Puppo, DIMI, Genoa). The purity of sHLA-G preparation was assessed by mass spectrometry (see Supplemental data).

Purified sHLA-G was tested at 100 ng/ml. Dose-response experiments have been performed using serial dilutions from 100 ng/ml to 6.25 ng/ml. In some experiments, HLA-G1 molecules were aggregated on nanoparticles, previously employed to enhance HLA-G bioactivity. This experimental model also facilitates anti-HLA-G mAb blocking experiments. Notably, control beads did not affect the expression of receptors here investigated. Results obtained with HLA-G beads and sHLA-G were comparable.

Goat-anti mouse IgG Adembeads (Ademtech, Paris, France) were incubated for 1 h at RT with anti-HLA-G mAb MEM-G/9. After washes, coated beads were incubated overnight at 4°C with HLA-G containing supernatants (HLA-G beads) or medium (control beads). Nanoparticles were then washed and resuspended in culture medium before being used (3x10⁴ beads/cell).

Cell isolation and culture.

The study was approved by the Ethical Committee of G. Gaslini Institute, Genoa, Italy. Surgically removed tonsils and normal peripheral blood (PB) samples were obtained following written informed consent. Mononuclear cells (MNC) were isolated by Ficoll-Hypaque (Sigma) density gradient.

Total NK cells, CD56bright NK cells and CD56dim NK cells were isolated from PB samples using NK isolation kit, CD56bright NK cell isolation kit and CD16+/CD56+ NK cell isolation kit, respectively (Myltenyi Biotec, Bergisch Gladbach, Germany), following manufacturer’s protocol.

NK cells were treated in vitro for 24-36h in RPMI 10% FBS at 37°C and 5% CO₂ with i) sHLA-G (100 ng/ml) or medium alone, or with ii) HLA-G beads or control beads (3x10⁴ nanoparticles per cell) before being analyzed. In some experiments, NK cells were cultured 24 h with rhIL-2.
(Proleukin, Chiron Italia S.r.l., Milan, Italy) at the concentration of 500 I.U./ml before being treated with sHLA-G as detailed above.

**Antibodies and flow cytometry.**

Cells were stained with fluorochrome-conjugated mAbs or with isotype and fluorochrome-matched control antibodies, or with unconjugated mAbs followed by fluorochrome-conjugated isotype-specific goat anti-mouse second reagent (see Supplemental data), and then were run on a Gallios cytometer (Beckman Coulter, Miami, FL, USA). $10^4$ events were acquired and analyzed using Kaluza software (Beckman Coulter).

**Blocking experiments**

Blocking experiments with anti-HLA-G mAb have been performed by pre-treatment of HLA-G beads with anti-HLA-G blocking mAb 87G (Exbio). Beads were incubated with 50 μg/ml of 87G mAb 30’ at 37°C, 5% CO₂, before being added to cells.

Blocking experiments of ILT2 receptor have been performed by adding 20 μg/ml of anti-ILT2/CD85j blocking antibody (clone #292319, R&D systems) or isotype-matched control (Invitrogen, CA, USA) 30’ before stimulating cells as described above.

**Chemotaxis**

Chemotaxis was investigated as described in Supplemental Data. Migration index was calculated as follows: ($n°$ of migrated cells / $n°$ of dispensed cells*100). Migration index obtained with medium alone was subtracted from each corresponding migration index value.

**IFN-γ release by NK cells**

Crosslinking experiments were performed on total PB NK cells. Plates were coated overnight at 4°C with 100 μl of different monoclonal antibodies: i) anti-NKp46 activating receptor (2.5 μg/ml), ii) anti-NKG2A inhibitory receptor (2.5 μg/ml), iii) anti-NKp46 + anti-NKG2A, iv) PBS. Plates were then washed three times in PBS. NK cells (previously treated with sHLA-G or medium) were
plated at 100,000 cells/well in RPMI 10% FBS + IL12p70 (0.1 ng/ml) and incubated at 37°C 5% CO₂ for 24h. Supernatants were then collected and centrifuged at 3000 g for 10’. IFN-γ was measured using IFN-γ ELISA set (Immunotools, Friesoythe, Germany), following manufacturer’s protocol. Samples were tested in triplicate diluted 1:10 in dilution buffer (PBS 1% FBS, 0.05% Tween-20). Fold increase of IFN-γ secretion was calculated for each experimental set as following: (ng/ml IFN-γ secreted with specific mAbs / ng/ml IFN-γ secreted with irrelevant isotype-matched mAb).

**Antibody array**

Cytokine production was investigated on supernatants collected from purified PB CD56<sup>bright</sup> and CD16<sup>+</sup>/CD56<sup>dim</sup> NK cells either untreated or treated with sHLA-G, using RayBio Human Cytokine Antibody Array 3 (Table S2, RayBiotech, Inc., Norcross, GA, USA), following manufacturer’s protocol. Samples were tested after 1:4 dilution in culture medium. Protein levels were quantified by scanning densitometry of the radiography films using VersaDoc 3000 Gel Imaging System (BioRad, CA, USA). Results are expressed as relative density, obtained as follows: density of specific spot/mean density of housekeeping proteins spots.

**Signaling pathway**

sHLA-G signaling pathways were investigated by flow cytometry on total PB NK cells, either untreated or treated with sHLA-G (see Supplemental data).

**Statistics**

Statistical analysis was performed using Prism software 5.03 (GraphPad Software Inc., La Jolla, CA USA). t test or Mann-Whitney test have been used, depending on data distribution. The significance range is the following: * p value < 0.05 (significant), ** p value < 0.005, *** p value < 0.0005.
RESULTS

sHLA-G dampens the expression of different chemokine receptors on NK cells

We evaluated by flow cytometry the expression of chemokine receptors on NK cells from peripheral blood (PB), gating on CD56^{bright} or CD56^{dim} cell fractions (Fig.1, panel A), after culture in the presence or absence of sHLA-G. Representative histograms of flow cytometric analysis of chemokine receptors expression on CD56^{bright} and CD56^{dim} NK cells are shown in Fig.S1 and S2, respectively.

As shown in Fig.1, panel B, sHLA-G significantly downregulated CCR2 expression on CD56^{bright} NK cells (MRFI median 2,51±0,08 vs 1,31±0,04, p=0,004), whereas no CC chemokine receptor was modulated on CD56^{dim} NK cells (Fig.1, panel C).

sHLA-G significantly downregulated CXCR3 expression, both in CD56^{bright} (MRFI median 21,1±1,51 vs 1,26±0,04, p=0,004) and CD56^{dim} (MRFI median 3,77±0,96 vs 1,31±0,02, p=0,004) NK cells (Fig.1, panel D and E, respectively), while that of other CXC receptors was unaffected by the same treatment.

As shown in Fig. 1, panel G, CX3CR1, whose expression was detected at high levels on PB CD56^{dim} NK cells, was significantly downmodulated by sHLA-G treatment (MRFI median 41,23±3,83 vs 14,54±2,29, p=0,05). Conversely, CX3CR1 expression was low to absent in PB CD56^{bright} NK cells, and was not modulated by sHLA-G treatment (Fig.1, panel F).

CCR2 and CXCR3 expression was also tested on purified PB CD56^{bright} NK cells (Fig.1, panel H). Again, sHLA-G significantly downmodulated the expression of both CCR2 (MRFI median 2,79±0,22 vs 1,65±0,25, p=0,02) and CXCR3 (MRFI median 8,18±0,88 vs 1,46±0,08, p=0,004) (Fig.1, panel I).

Finally, the expression of chemokine receptors was evaluated on NK cells from tonsil as prototype of a secondary lymphoid organ, gating on CD56^{bright} or CD56^{dim} cells (Fig.2, panel A). sHLA-G significantly downregulated the expression of CXCR3 both in CD56^{bright} (MRFI median 14,58±1,62
vs 6.31±0.85, p=0.05) and CD56^{dim} (MRFI median 14.5±0.83 vs 1.96±0.2, p=0.05) NK cells (Fig. 2, panel B and C respectively). Moreover, sHLA-G downregulated expression of i) CCR2 in CD56^{bright} (MRFI median 2 ±0.92 vs 1.61±0.18, p=0.05) and ii) CXCR5 in CD56^{dim} NK cells (MRFI median 12.93±0.8 vs 7.06±0.52, p=0.05).

**sHLA-G downregulates the expression of CD94/NKG2A on NK cells**

We next evaluated the effect of sHLA-G treatment on the expression of a panel of NK receptors. These included activating receptors and co-receptors (NKG2D, DNAM-1, NKp30, NKp44, NKp46, NTBA, 2B4) as well as KIR2DL1/S1, KIR2DL2/L3/S2, KIR2DL4, CD94/NKG2A and ILT2 inhibitory receptors.

This analysis was performed by flow cytometry, gating on CD56^{bright} and CD56^{dim} NK cells isolated from PB (Fig.3 panel A and B respectively) or human tonsil (Fig.3 panel C and D respectively). As shown in Fig. 3, sHLA-G treatment downregulated the expression of CD94/NKG2A heterodimer, both in CD56^{bright} (Panel A, NKG2A MRFI median : 7.37±1.57 vs 3.69±0.4, p=0.04; CD94 MRFI median : 11.97±2.05 vs 6.08±0.86, p=0.02) and CD56^{dim} (Panel B, NKG2A MRFI median : 8.56±1.84 vs 4.42±0.57, p=0.04; CD94 MRFI median : 17.79±3.21 vs 7.15±1.21, p=0.007) PB NK cells. Representative histograms of flow cytometric analysis of NK cell receptors expression on PB CD56^{bright} and CD56^{dim} NK cells are shown in Fig.S3 and S4, respectively.

Expression of CD94/NKG2A was also significantly downregulated by sHLA-G in CD56^{bright} (Fig.3, Panel C, NKG2A MRFI median : 78.04±18.30 vs 40.44±10.53, p=0.05; CD94 MRFI median : 73.16±26.59 vs 37.48±7.80, p=0.05) and CD56^{dim} (Fig.3, Panel D, NKG2A MRFI median : 13.04±1.13 vs 6.72±0.77, p=0.05; CD94 MRFI median : 14.76±2.05 vs 6.58±0.94, p=0.05) tonsil NK cells.

On the contrary, the expression of all the other NK receptors analyzed was not significantly modified by sHLA-G treatment, either in PB or tonsil NK cell populations.
sHLA-G mediated downregulation of chemokine receptors and NK receptors is dose-dependent

We have tested whether chemokine receptor and NK receptor downmodulation induced by sHLA-G was dose-dependent. Total PB NK cells were cultured with medium alone or in the presence of serial dilutions (1:2) of sHLA-G, starting from 100 ng/ml to 6.25 ng/ml. Next, the expression of CXCR3, CX3CR1, CD94 and NKG2A was analyzed by flow cytometry.

As shown in Fig.4, CXCR3 (panel A), CX3CR1 (panel B) and NKG2A (panel C) expression on NK cells (MRFI 2.38±0.27, 17.52±0.24 and 3.16±0.09, respectively) was significantly downregulated by sHLA-G at 100 ng/ml (MRFI 1.11±0.02, 7.68±0.68 and 2.2±0.11, respectively, p=0.02), 50 ng/ml (MRFI 1.13±0.01, 2.26±0.34 and 2.54±0.13, respectively, p=0.02), 25 ng/ml (MRFI 1.21±0.01, 9.61±1.16 and 2.65±0.08, respectively p=0.02) and 12.5 ng/ml (MRFI 1.35±0.08, 12.06±2.5 and 2.81±0.06, respectively, p=0.02), but not at 6.25 ng/ml (MRFI 1.8±0.06, 17.05±0.51 and 3.11±0.06, respectively, p=0.02).

CD94 expression (Fig.4, panel C, MRFI 4.59±0.83) was dampened by sHLA-G at 100 ng/ml (MRFI 2.17±0.32, p=0.02) and 50 ng/ml (MRFI 2.26±0.34, p=0.02), but not at 25 ng/ml (MRFI 2.70±0.45), 12.5 ng/ml (MRFI 3.53±0.64) and 6.25 ng/ml(MRFI 3.62±0.67).

Blocking experiments

To demonstrate unambiguously the role of sHLA-G in the downmodulation of chemokine receptor and NK receptors expression on NK cells, sHLA-G treatment was performed in the presence of an anti-HLA-G (87G) mAb, that blocks the interaction between HLA-G and its receptors.

Expression of CXCR3 and NKG2A was evaluated on total PB NK cells that had been treated with i) control beads, ii) HLA-G beads and iii) HLA-G beads pre-incubated with 87G mAb or irrelevant isotype-matched mAb.

As shown in Fig.5, panel A, HLA-G beads dowregulated the expression of CXCR3 (upper panel) on NK cells, as compared with cells treated with control beads (MRFI median : 4.24±0.62 vs
1.49±0.06, p=0.002). Preincubation of HLA-G beads with the anti-HLA-G mAb, but not with irrelevant isotype matched mAb, significantly restored the expression of CXCR3 (MRFI median: 2.11±0.03 vs 1.49±0.06, p=0.002), thus demonstrating that modulation of CXCR3 expression was mediated by sHLA-G. Similar results were obtained analyzing NKG2A expression (Fig.5, panel A, lower panel). Indeed, sHLA-G-induced downregulation of NKG2A expression (MRFI median: 17.08±1.75 vs 11.61±1.11, p=0.01) was significantly reverted by pre-incubation of HLA-G beads with anti-HLA-G mAb (MRFI median: 17.01±1.90 vs 11.61±1.11, p=0.01), whereas such effect was not obtained using irrelevant isotype matched mAb (Fig.5, panel A, lower panel).

Finally, in order to investigate whether ILT2, a receptor for HLA-G, was involved in downmodulation of CXCR3 (as previously reported) and NKG2A, additional experiments were performed using an anti-ILT2/CD85j blocking mAb. As shown in Fig 5, panel B, sHLA-G mediated inhibition of CXCR3 (upper panel) and NKG2A (lower panel) expression on NK cells was partly but significantly reverted by pre-incubating these cells with anti-ILT2/CD85j mAb (MRFI median: CTR 30.65±10.47 and 14.67±0.44 respectively; sHLA-G 5.48±0.53 and 7.45±0.2 respectively; sHLA-G + anti-ILT2 mAb 14.2±4.64 and 8.61±0.29 respectively, p=0.01), but not with irrelevant isotype-matched mAb, thus demonstrating that sHLA-G modulated CXCR3 and NKG2A expression partly through the interaction with ILT2 receptor.

**sHLA-G inhibits chemotaxis of NK cells towards CXCL10, CXCL11, CX3CL1 and CCL2**

We performed *in vitro* chemotaxis experiments on total PB NK cells and PB CD56<sup>bright</sup> NK cells, either untreated or treated with sHLA-G. We used as chemoattractants i) CXCL10 and CXCL11, the ligands of CXCR3 (which was downregulated by sHLA-G on both CD56<sup>dim</sup> and CD56<sup>bright</sup> PB NK cells), ii) CX3CL1, ligand of CX3CR1, which was downmodulated by sHLA-G on CD56<sup>dim</sup> NK cells and iii) CCL2, the ligand of CCR2, which was downregulated by sHLA-G on CD56<sup>bright</sup> PB NK cells. CCL21, a ligand of CCR7, which was not modulated by sHLA-G treatment, was also tested as control.
As shown in Fig.5, panel C, sHLA-G significantly inhibited chemotaxis of total PB NK cells towards CXCL10 (migration index median 1.87±0.48 vs 0±0.37, p=0.02), CXCL11 (migration index median 3.2±0.59 vs 1.07±0.25, p=0.007) and CX3CL1 (migration index median 2.8±0.67 vs 0.27±0.21, p=0.05).

Chemotaxis of CD56<sup>bright</sup> PB NK cells towards CCL2 (migration index median 1.33±0.31 vs 0.64±0.14, p=0.02) and CXCL10 (migration index median 4.12±1.46 vs 1.18±0.46, p=0.05) was also inhibited by sHLA-G treatment (Fig.5, panel D).

Finally, chemotaxis of either total PB NK cells or CD56<sup>bright</sup> PB NK cells towards CCL21 was unaffected by sHLA-G.

**sHLA-G-mediated downregulation of CD94/NKG2A receptor results in impaired inhibitory function**

We tested whether sHLA-G-mediated downmodulation of the inhibitory CD94/NKG2A receptor on NK cells was associated with an impairment of its inhibitory function. To this end, in the presence of suboptimal doses of rIL-12, total PB NK cells (either untreated or treated with sHLA-G) were cultured in wells pre-coated with an agonistic mAb against the NKp46 activating receptor, alone or in combination with an agonistic mAb against NKG2A (Fig.5, panel E). Culture supernatants were recovered and analyzed for the amount of IFN-γ by ELISA.

As previously described, in untreated NK cells mAb-mediated crosslinking of NKp46 induced the release of IFN-γ (IFN-γ fold increase 3.35±0.37), which was significantly reduced by the simultaneous engagement of NKG2A (IFN-γ fold increase 2.5±0.24, p=0.05). Engagement of NKp46 induced IFN-γ secretion also in sHLA-G treated NK cells (IFN-γ fold increase 3.13±0.3). In this case however, engagement of NKG2A failed to downmodulate the NKp46-mediated release of IFN-γ (IFN-γ fold increase 3.15±0.33), thus indicating that inhibitory activity of NKG2A was dampened by sHLA-G treatment.
sHLA-G modulated chemokine secretion by CD56\textsuperscript{dim} and CD56\textsuperscript{bright} PB NK cells

We investigated the chemokine secretion pattern of PB purified CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells, either untreated or treated with sHLA-G. Results are expressed as relative density.

As shown in Fig.6, sHLA-G significantly increased the secretion of CCL22/MDC by CD56\textsuperscript{dim} NK cells (panel A, 0,66±0,04 vs 0,97±0,05, p=0,02) and CD56\textsuperscript{bright} NK cells (panel B, 0,71±0,02 vs 0,92±0,02, p=0,02). Moreover, in CD56\textsuperscript{dim} NK cells sHLA-G significantly augmented the secretion of CCL2/MCP-1 (0,76±0,03 vs 1,05±0,02, p=0,02), CXCL2-CXCL3/GRO (0,71±0,005 vs 0,93±0,001, p=0,02) and CCL8/MCP-2 (0,62±0,02 vs 0,83±0,07, p=0,02).

sHLA-G affects phosphorylation of Stat5

Finally, we investigated the expression of proteins involved in signal transduction in NK cells, either untreated or treated with sHLA-G, gating on CD56\textsuperscript{bright} or CD56\textsuperscript{dim} cell fractions as described above. In particular, we evaluated the phosphorylation of i) Akt, that is crucial for cell cycle progression, and ii) Stat1, Stat3 and Stat5, which regulate the transcription of several genes\textsuperscript{29}.

As shown in Fig. 6, sHLA-G significantly downregulated the expression of p-Stat5, both in CD56\textsuperscript{bright} (panel C, MRFI median 1,57±0,01 vs 1,48±0,03, p=0,05) and in CD56\textsuperscript{dim} NK cells (panel D, MRFI median 1,75±0,01 vs 1,47±0,06, p=0,05) NK cells. p-Akt (as well as Akt), p-Stat1 and p-Stat3 were not affected by sHLA-G treatment.
DISCUSSION

In this study we have investigated for the first time sHLA-G-mediated modulation of expression and function of different molecules related to NK cell activity.

We have previously demonstrated that sHLA-G inhibited chemotaxis of different T cell subsets through the downregulation of the expression of different chemokine receptors\(^2\). Here, we report that sHLA-G modulated expression and function of different chemokine receptors on CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) resting NK cells. Similar results were obtained using NK cells from peripheral blood and tonsil, tested as prototype of secondary lymphoid organ. Likewise, the effects of sHLA-G on IL-2 activated CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) NK cells were similar to those detected with the corresponding resting cell fractions (see Supplemental Data).

sHLA-G significantly downregulated CXCR3 expression both in CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) NK cells and impaired their migration towards CXCL10 and CXCL11, that together with CXCL9 are ligands of this receptor\(^3\). CXCR3 drives NK cells migration to inflamed tissues, in particular during viral infections, thus promoting NK cell mediated lysis of virus-infected cells and leading to the resolution of viral infection. Indeed, dysregulation of CXCR3 signaling impairs antiviral responses \textit{in vivo}\(^4\). Moreover, CXCR3 is essential, in association with CXCR4, for CD56\(^{\text{bright}}\) NK cells recruitment to the endometrium during the menstrual cycle and in the decidua during pregnancy\(^5\). Furthermore, CD56\(^{\text{bright}}\) NK cells recruited through CXCR3 engagement in psoriatic skin are key players in the pathogenesis of psoriasis\(^6\). Similarly, in allergic contact dermatitis CD56\(^{\text{bright}}\) NK cells are recruited, mainly through CXCR3, to the skin, where they release type-1 cytokines that induced apoptosis of keratinocytes\(^7\).

Our data suggest that high levels of serum sHLA-G, that have been detected in patients with solid or hematological tumors and viral infections\(^8\), might impair NK cells migration to inflamed tissues through downmodulation of CXCR3 expression and chemotaxis to CXCL9, CXCL10 and CXCL11. Reduced recruitment of NK cells would translate into lower NK activity against virus-
infected or tumor cells. On the other hand, decreased levels of serum sHLA-G observed in patients with different autoimmune and inflammatory diseases might facilitate migration of NK cells to inflamed tissues, leading to disease exacerbations and tissue damage\cite{4,35,36}.

We have also demonstrated that sHLA-G downregulated CCR2 expression in PB and tonsil CD56\textsuperscript{bright} NK cells, and impaired their migration towards CCL2. CCR2 plays an important role in the recruitment of NK cells to inflamed tissues during viral infection and tuberculosis\cite{37,38}. Moreover, infiltration of NK cells in a mouse model of established metastatic tumors was mediated by CCL2/CCR2 interaction\cite{39}. Again, we can speculate that high levels of serum sHLA-G might reduce the migration of CD56\textsuperscript{bright} NK cells to the site of inflammation, thus leading to impaired anti-viral or anti-tumor immune responses.

CX\textsubscript{3}CR1, the receptor for CX\textsubscript{3}CL1 (fractalkine) is highly expressed on CD56\textsuperscript{dim}, but not on CD56\textsuperscript{bright} NK cells. It has been demonstrated that CX\textsubscript{3}CL1 is preferentially expressed and released in Th1-mediated disorders, such as psoriasis and granulomatous lymphadenitis induced by Mycobacterium tuberculosis. NK cells are recruited through CX\textsubscript{3}CR1/CX\textsubscript{3}CL1 interaction to the site of inflammation, together with Th1 cells that also express CX\textsubscript{3}CR1 at high levels. Co-recruitment of NK cells and Th1 T cells results in the amplification of Th1 responses\cite{40}. Moreover, CX\textsubscript{3}CR1 drives NK cell migration towards inflamed tissues during multiple sclerosis\cite{41} and rheumatoid arthritis\cite{42}. Finally, CX\textsubscript{3}CR1 has a pivotal role in the recruitment of activated cytotoxic NK cells to the tumor. Indeed, defective anti-tumor responses detected in CX\textsubscript{3}CR1\textsuperscript{-/-} mice have been correlated with reduced NK cell migration to the tumor microenvironment\cite{43}.

Our data suggested that high serum levels of sHLA-G, through downmodulation of CX\textsubscript{3}CR1 expression and inhibition of NK cell migration towards CX\textsubscript{3}CL1, might affect NK cell recruitment in different pathological conditions. This effect may translate into an amelioration of Th-1 mediated inflammatory diseases and autoimmune diseases, but, on the other hand, may impair NK-cell mediated antitumor responses.
Surprisingly, we found that CXCR5 expression was downregulated by sHLA-G on CD56\textsuperscript{dim} NK cells from tonsil but not from peripheral blood. Although the reason(s) for this discrepancy is unknown, it is conceivable that the pro-inflammatory environment typical of surgically removed tonsils may synergize with sHLA-G in downregulation of CXCR5 expression.

Inhibitory activities of HLA-G molecules (both membrane-bound and soluble isoforms) on NK-cell mediated lysis have been reported in the last years\textsuperscript{15,20-25}. However, the potential modulation of activating and inhibitory NK receptors induced by HLA-G has never been investigated.

In this study, we demonstrate that sHLA-G did not affect the expression of activating NK receptors and co-receptors (NKp30, NKp44, NKp46, NKG2D, NTBA, 2B4 and DNAM-1) and KIR2DL1/S1, KIR2DL2, L3/S2, KIR2DL4 and ILT2/CD85j inhibitory receptors on CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells from peripheral blood and tonsils. In another experimental model, sHLA-G was found to boost expression of its own receptor ILT2/CD85j\textsuperscript{44}, a finding that was not confirmed by our study and by another published paper\textsuperscript{28}. This is likely due to different cell types used as targets for sHLA-G and/or to different experimental settings.

Surprisingly, sHLA-G induced a phenotypical and functional downregulation of CD94 and NKG2A molecules, that associate to form the heterodimeric receptor CD94/NKG2A, which binds HLA-E molecule\textsuperscript{45}. HLA-E, like HLA-G, belongs to HLA-Ib subfamily. The physiological role of HLA-E is to bind peptides derived from the leader sequence of other HLA-class I molecules, and to present them to NK cells through interaction with CD94/NKG2A receptor, thus allowing NK cells to monitor the expression of HLA-class I molecules on target cells. In fact, the interaction between HLA-E and CD94/NKG2A inhibits NK-cell mediated lysis of HLA-E\textsuperscript{+} target cells\textsuperscript{45}. It is tempting to speculate that sHLA-G performed a “negative feedback” on NK cells, rendering NK cells, that have been previously sensitized to sHLA-G, less prone to be subsequently inhibited by HLA-E molecule. It has been demonstrated that nonapeptides derived from HLA-G leader peptide are loaded on HLA-E molecules\textsuperscript{46}. When HLA-G is overexpressed, i.e. in tumor cells, the augmented
generation of HLA-G-derived nonapeptides leads to surface HLA-E up-regulation. In tumor cells, HLA-G can inhibit NK cell functions directly or indirectly through the up-regulation of HLA-E, that inhibits NK cell mediated lysis by interacting with CD94/NKG2A\textsuperscript{47}. Although this is true for surface HLA-G on target cells that express both HLA-G and HLA-E, it is conceivable that sHLA-G behaves differently from the surface molecule. On the other hand, in physiological conditions, HLA-E molecules loaded with HLA-G-derived nonapeptides are preferentially recognized by NK cells through CD94/NKG2C activating receptor\textsuperscript{48}. In this view, our finding that sHLA-G can downmodulate CD94/NKG2A expression on NK cells may be relevant in the process of tolerance breaking that occurs during cytotrophoblast implantation\textsuperscript{48}.

We have demonstrated that downregulation of chemokine receptors and NK receptors induced by sHLA-G is dose-dependent. Notably, such effect was achieved at high concentration of sHLA-G, similar to sHLA-G serum levels detected in pathological conditions, but not at concentration similar to physiological HLA-G serum levels.

Results about HLA-G-induced modulation of cytokine and chemokine secretion by NK cells are still contradictory. Li et al. have demonstrated that sHLA-G induced proinflammatory and proangiogenic factor release in decidual CD56\textsuperscript{+} NK cells through KIR2DL4 ligation\textsuperscript{22}. Similar results have been obtained by van der Meer et al., that have demonstrated an increased secretion of the pro-inflammatory cytokines TNF-\(\alpha\) and IFN-\(\gamma\) by peripheral blood and uterine NK cells induced by sHLA-G\textsuperscript{24}. Moreover, Rajagopalan et al. have demonstrated that sHLA-G induced release of proinflammatory cytokines and chemokines in resting NK cells\textsuperscript{25}. In contrast, Morel et al. have demonstrated that HLA-I molecules (including HLA-G) downmodulate IFN-\(\gamma\) secretion by NK cells through ILT2/CD85j ligation\textsuperscript{23}.

We have here analyzed for the first time differential effects of sHLA-G on cytokine and chemokine secretion in purified CD56\textsuperscript{bright} and CD56\textsuperscript{dim} peripheral blood NK cells. CCL22/MDC secretion was increased by sHLA-G treatment both in CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells. These data may be
relevant, since it has been previously demonstrated that up-regulation of CCL22 secretion by NK cells may augment the recruitment of regulatory T cells in tumor microenvironment of Lewis lung carcinoma, thus leading to the establishment of an immune-suppressive microenvironment\textsuperscript{19}. Chemokine secretion seems to be more affected by sHLA-G treatment in CD56\textsuperscript{dim} than in CD56\textsuperscript{bright} NK cells. In fact, sHLA-G also increased the secretion of MCP-1 (CCL2), MCP-2 (CCL8), and GRO chemokines (CXCL2 and CXCL3) by CD56\textsuperscript{dim} NK cells. Finally, we evaluated the expression of proteins involved in signal transduction. We have demonstrated that sHLA-G induced p-Stat5 downregulation both in CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells, thus leading to de-regulation of the transcription of several genes. These data are in line with our previous study on T lymphocytes, in which we have demonstrated that sHLA-G downregulated Stat5 phosphorylation through an increased phosphorylation of the protein phosphatase SHP-2\textsuperscript{28}, and another study showing that sHLA-G downregulated JAK2, Stat-3 and Stat-5 phosphorylation in erythroid cells\textsuperscript{50}. The reasons why, at variance with a previous report\textsuperscript{18}, we did not detect any difference in p-Stat3 expression in CD56\textsuperscript{dim} or CD56\textsuperscript{bright} NK cells upon treatment with sHLA-G or medium are unknown, but may be related to the different sources of NK cells tested. Signal transduction initiated by sHLA-G in CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells likely involves additional pathways beside that related to Stat-5 phosphorylation. Furthermore, post-transcriptional mechanisms may control the expression in NK cells of the molecules here investigated. Such mechanisms may be especially relevant in the case of CCR2, whose expression was differentially regulated by sHLA-G in CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells.

In conclusion, our data delineate novel aspects of the control of NK cell function operated by sHLA-G molecules that may have important implications in disease pathogenesis. In particular, sHLA-G i) downmodulated expression of CXCR3, CX\textsubscript{3}CR1 and CCR2 and migration towards their specific ligands, that might impair NK cell recirculation and function; ii) modulated the expression and function of the CD94/NKG2A receptor, possibly preventing HLA-E mediated inhibition in
sHLA-G sensitized NK cells through a negative feedback, and iii) modulated cytokine and chemokine secretion by NK cells, perhaps impacting on the recruitment of different cell population, such as regulatory T cells\textsuperscript{59}.

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Authorship and Conflict of Interest Statements

FM designed the research, performed most of the experiments, analyzed data and wrote the paper; EF and AP performed some experiments; RC, CB and AD provided some reagents, performed some experiments, analyzed data and wrote the manuscript; VP designed the study, analyzed data and wrote the manuscript. None of the authors declare any conflict of interest.
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FIGURE LEGENDS

Figure 1. Modulation of chemokine receptors expression by sHLA-G on peripheral blood NK cells. Panels A-G Cytofluorimetric analysis of chemokine receptor expression on purified total peripheral blood (PB) NK cells, gating on CD56\textsuperscript{bright} or CD56\textsuperscript{dim} NK cells. Panel A shows a representative staining with anti-CD56 mAb. CC chemokine receptor expression on CD56\textsuperscript{bright} (Panel B) and CD56\textsuperscript{dim} (Panel C) NK cells, CXC chemokine receptor expression on CD56\textsuperscript{bright} (Panel D) and CD56\textsuperscript{dim} (Panel E) NK cells and CX\textsubscript{3}CR1 expression on CD56\textsuperscript{bright} (Panel F) and CD56\textsuperscript{dim} (Panel G) NK cells, either untreated (black bars) or treated with sHLA-G (white bars). Results are expressed as mean relative fluorescence intensity (MRFI). Means of five different experiments ± SD are shown. Asterisks indicate significant differences. The insets in panels B, D, E and G show representative stainings of NK cells, untreated (ctr) or treated with sHLA-G, with anti-CCR2, anti-CXCR3 and anti-CX\textsubscript{3}CR1 mAbs, respectively. Black profiles indicate staining with specific mAbs, whereas grey profiles indicate staining with irrelevant isotype-matched mAb.

Panels H and I Cytofluorimetric analysis of chemokine receptor expression on purified PB CD56\textsuperscript{bright} NK cells. Panel H shows a representative staining with anti-CD56 mAb. Panel I shows CCR2 and CXCR3 expression on CD56\textsuperscript{bright} NK cells untreated (black bars) or treated with sHLA-G (white bars). Results are expressed as MRFI. Means of five different experiments ± SD are shown. Asterisks indicate significant differences. The inset shows representative stainings of CD56\textsuperscript{bright} NK cells, untreated (ctr) or treated with sHLA-G, with anti-CCR2 and anti-CXCR3 mAbs, respectively. Black profiles indicate staining with specific mAbs, whereas grey profiles indicate staining with irrelevant isotype-matched mAb.

Figure 2. Modulation of chemokine receptor expression by sHLA-G on NK cells from tonsil. Cytofluorimetric analysis of chemokine receptor expression on purified total NK cells from tonsil,
gating on CD56$^{\text{bright}}$ or CD56$^{\text{dim}}$ NK cells on the basis of CD56 expression. **Panel A** shows a representative staining with anti-CD56 mAb. The expression of chemokine receptors was evaluated on CD56$^{\text{bright}}$ (**Panel B**) and CD56$^{\text{dim}}$ (**Panel C**) NK cells, either untreated (black bars) or treated with sHLA-G (white bars). Results are expressed as MRFI. Means of five different experiments ± SD are shown. Asterisks indicate significant differences. The insets in panels B and C show representative stainings of NK cells, untreated (ctr) or treated with sHLA-G, with anti-CXCR3 and anti-CXCR5 mAbs. Black profiles indicate staining with specific mAbs, whereas grey profiles indicate staining with irrelevant isotype-matched mAb.

**Figure 3. Expression of activating and inhibitory receptors on sHLA-G-treated NK cells.** Cytofluorimetric analysis of the expression of activatory and inhibitory receptors on purified total NK cells, gating on CD56$^{\text{bright}}$ or CD56$^{\text{dim}}$ NK cells on the basis of CD56 expression. Expression of NK cell receptors was evaluated on PB CD56$^{\text{bright}}$ (**Panel A**) and CD56$^{\text{dim}}$ NK cells (**Panel B**) or tonsil CD56$^{\text{bright}}$ (**Panel C**) and CD56$^{\text{dim}}$ NK cells (**Panel D**), either untreated (black bars) or treated with sHLA-G (white bars).

Results are expressed as MRFI. Means of five different experiments ± SD are shown. Asterisks indicate significant differences. The insets in panels A,B,C and D show representative stainings of NK cells, untreated (ctr) or treated with sHLA-G, with anti-NKG2A and anti-CD94 mAbs, respectively. Black profiles indicate staining with specific mAbs, whereas grey profiles indicate staining with irrelevant isotype-matched mAb.

**Figure 4. Dose-response experiments.** Total PB NK cells were treated with serial dilutions (1:2) of sHLA-G, (range: 100 ng/ml - 6,25 ng/ml). Cytofluorimetric analysis of CXCR3 (**Panel A**), CX3CR1 (**Panel B**), CD94 (**Panel C**) and NKG2A (**Panel B**) expression was performed. Results are expressed as MRFI. Means of three different experiments ± SD are shown. Asterisks indicate significant differences.

**Figure 5. Blocking experiments and functional assays.** **Panel A.** Total PB NK cells were treated
with beads i) uncoated (ctr, black bars), ii) coated with sHLA-G (beads HLA-G, white bars), iii) coated with sHLA-G and treated with 87G anti-HLA-G blocking mAb (striped bars) or iv) coated with sHLA-G and treated with isotype-matched irrelevant ctr mAb (grey bars) before being admixed with NK cells. Cytofluorimetric analysis of CXCR3 or NKG2A expression has been performed. Panel B shows blocking experiments performed on total PB NK cells untreated (ctr, black bar) or treated with sHLA-G (white bar), or pre-incubated with anti-ILT2/CD85j mAb (striped bar) or isotype-matched mAb (grey bar) and then treated with sHLA-G. Cytofluorimetric analysis of CXCR3 or NKG2A expression is shown. Results are expressed as MRFI. Means of three different experiments ± SD are shown. Asterisks indicate significant differences.

Chemotaxis was performed on total PB NK cells (panel C) or PB CD56\textsuperscript{bright} NK cells (panel D), using as chemoattractants i) CXCL10, CXCL11, CX3CL1 and CCL21 or ii) CCL2, CXCL10 and CCL21, respectively. Results are expressed as migration index (\(\text{n° of migrated cells}/\text{n° of total cells}*100\)). Means of five different experiments ± SD are shown. Asterisks indicate significant differences. Panel E shows IFN-\(\gamma\) secretion by total PB NK cells treated with anti-NKp46 mAb alone or in combination with anti-NKG2A mAb. NK cells were either untreated (black bars) or treated with sHLA-G (white bars) before mAb-mediated crosslinking of NKp46 and/or NKG2A molecules. Results are expressed as IFN-\(\gamma\) fold increase (\(\text{ng/ml IFN-\(\gamma\) secreted by specific mAb-treated cells divided by ng/ml IFN-\(\gamma\) secreted by irrelevant isotype matched treated cells}\)). Means of three different experiments ± SD are shown. Asterisks indicate significant differences.

**Figure 6. Modulation of cytokine/chemokine secretion and signal transduction by sHLA-G**

Cytokine and chemokine secretion was assessed on supernatants from purified PB CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells by antibody array. Means of three different experiments ± SD for modulated cytokines/chemokines are shown in panel A (CD56\textsuperscript{dim} NK cells) and panel B (CD56\textsuperscript{bright} NK cells). Black bars indicate untreated NK cells, whereas white bars indicate NK cells treated with sHLA-G. Results are expressed as relative density (\(\text{density of specific spot/ mean of density of housekeeping}\)).
proteins spot). Asterisks indicate significant differences.

Analysis of signal transduction was performed on total PB NK cells either untreated (black bars) or treated with sHLA-G (white bars), gating on CD56\textsuperscript{bright} (panel C) and CD56\textsuperscript{dim} (panel D) NK cells. Results are expressed as MRFI. Means of three different experiments ± SD are shown. Asterisks indicate significant differences.
Figure 2

(A) Flow cytometry dot plot showing CD56 and SSC fluorescence. Bright and dimm gates are indicated.

(B) MRFI plot showing expression of various chemokine receptors (CCR2, CCR7, CXCR1, CXCR3, CXCR4, CXCR5, CXCR7) under control (ctr) and sHLA-G conditions. Significance at *p=0.05.

(C) MRFI plot showing CXCR3 and CXCR5 expression under control (ctr) and sHLA-G conditions. Significance at *p=0.05.
Figure 5

A

CXCR3 expression (MRFI)

** p=0.002

NKG2A expression (MRFI)

* p=0.01

B

CXCR3 expression (MRFI)

CTR

sHLA-G

sHLA-G + anti ILT2 mAb

sHLA-G + ctrl mAb

NKG2A expression (MRFI)

CTR

sHLA-G

sHLA-G + anti ILT2 mAb

sHLA-G + ctrl mAb

C

D

E

CXCL10  CXCL11  CX3CL1  CCL21

migration index

** p=0.007

* p=0.02

* p=0.05

migration index

* p=0.05

* p=0.02

* p=0.02

IFN-γ (fold increase)

anti Nkp46

anti Nkp46 + anti NKG2A

anti Nkp46 + anti Nkp46

anti Nkp46 + anti Nkp46

anti Nkp46 + anti Nkp46

ctr  sHLA-G

ctr  sHLA-G
Figure 6

A

- CCL8/MCP-2
- * p=0.02
- CCL2/3/GRO
- * p=0.02
- CCL2/MDC
- * p=0.02
- CCL2/MCP-1
- * p=0.02

B

- CCL15/MIP-1α
- * p=0.02
- CCL22/MDC
- * p=0.02
- CXCL8/IL-8

C

- MFI
- * p=0.05

D

- MRFI
- * p=0.05
Soluble HLA-G dampens CD94/NKG2A expression and function and differentially modulates chemotaxis and cytokine/chemokine secretion in CD56brght and CD56dim NK cells

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