

Expression of Gpnmb in NK Cell Development from Hematopoietic Stem Cells

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Background: Molecular mechanisms of natural killer (NK) cell development from hematopoietic stem cells (HSCs) have not been clearly elucidated, although the roles of some genes in NK cell development have been reported previously. Thus, searching for molecules and genes related NK cell developmental stage is important to understand the molecular events of NK cell development. **Methods:** From our previous SAGE data-base, Gpnmb (Glycoprotein non-metastatic melanoma protein B) was selected for further analysis. We confirmed the level of mRNA and protein of Gpnmb through RT-PCR, quantitative PCR, and FACS analysis. Then we performed cell-based ELISA and FACS analysis, to know whether there are some molecules which can bind to Gpnmb. Using neutralizing antibody, we blocked the interaction between NK cells and OP9 cells, and checked IFN- γ production by ELISA kit. **Results:** Gpnmb expression was elevated during *in vitro* developmental stage and bound to OP9 cells, but not to NK precursor cells. In addition, we confirmed that the levels of Gpnmb were increased at NK precursor stage *in vivo*. We confirmed syndecan4 as a candidate of Gpnmb's binding molecule. When the interaction between NK cells and OP9 cells were inhibited *in vitro*, IFN- γ production from NK cells were reduced. **Conclusion:** Based on these observations, it is concluded that Gpnmb has a potential role in NK cell development from HSCs.

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INTRODUCTION

Natural killer (NK) cells are derived from hematopoietic stem cells (HSCs) which can be differentiated into several hema-

topoietic lineages. It has been reported that NK cells are mainly matured in bone marrow as they express several receptors (1). These receptors endow NK cells to advance into the late stages of maturation. Although the outline of NK cell development from hematopoietic stem cells was reported previously (2,3), it is far from full understanding of NK cell development. Thus, the identification of other receptors related differentiation of NK cells is a critical issue to understand NK cell development.

Based on our published SAGE database (4), we selected some genes that may have potential to affect the differentiation of NK cells. Among these candidates, Gpnmb (glycoprotein non-metastatic melanoma protein B) showed a pattern of elevated expression in precursor NK (pNK) stage, compared to HSCs. Gpnmb is a typical trans-membrane glycoprotein composed with 574 amino acids. It is found on the plasma membrane and membranes of the endosome compartments. Gpnmb contains heparin binding motif and integrin recognition RGD domain in its extracellular region, while in the cytoplasmic region Gpnmb contains ITAM (Immunoreceptor tyrosine-based activation motif) and lysosomal targeting motif. It was previously reported that it is involved in osteoblast differentiation and participates in melanoma subsistence (5,6). In breast cancer, Gpnmb has tumor suppressor functions or metastatic functions (7). Gpnmb is also known as osteoactivin, dendritic cell-associated heparin sulfate proteoglycan-integrin ligand, and hematopoietic growth factor inducible neurokinin-1 type (7).

In this report, we evaluated the potential role of Gpnmb in NK cell developmental from HSCs. Expression of Gpnmb was increased during developmental stage of NK cells and

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maintained until they got full maturation. During this stage, pNK cells have chance to interact with stromal cells for the full maturation (8). Thus, we checked the roles of Gpnmb in interacting between these cells. Finally we identified that Gpnmb induces NK cells differentiation in aspect of IFN- γ production through *in vitro* co-culture system of NK cells and stromal cells.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from DaeHan Bio-Link (Korea). Whenever we have the necessity of fostering mice, we housed mice under specific pathogen-free (SPF) conditions as recommended by institutional regulation, and used between 6 and 10 weeks of age.

RT PCR and Quantitative real time PCR

To verify the expression of NK cell associated genes from each developmental stage, we conducted isolation of mRNA classically using TRIzol (Invitrogen). After quantitative normalization for each gene using β -actin expression, for Gpnmb detection, we accomplished RT-PCR using 3' primer-TCCCATCTCGAAGGTGAAAG and 5' primer-CAATTGTGATG-GTGGCTCTG. To detection of SD1, SD2, SD3, and SD4, we designed 3' primer- GAAGTGCTGGGAGGTGTCAT and 5' primer- GCTTGGTGGGTTTCTGGTAG, 3' primer- CCTCAGCAA-ACCAAAGATG and 5' primer- TAACACCAGTCCGCAACAAG, 3' primer- CTGGCTCCTCTAACCATCCA, and 5' primer- ACTGTGTGGGCTTGTGACTG, and 3' primer- ATCCTCTTTG-CCGTTTTCT and 5' primer- CACAACCCTCCACTCTCTC, respectively. Levels of mRNA expression were normalized relative to β -actin gene expression levels.

Antibodies and Flow cytometric Analysis

All antibodies for flow cytometric analysis were purchased from Becton Dickinson (BD) and PharMingen (SanDiego, CA). Mouse IgG Fc conjugated recombinant Gpnmb, and anti-Gpnmb antibody were purchased from R&D systems and used as manufacturer's guide line and reported (9). Cells were stained with the indicated antibodies in a staining buffer for 15min in 4°C, washed away, and analyzed by flow cytometry (FACScalibur: BD Biosciences) using CellQuest Pro (BD Biosciences) program.

in vitro differentiation of NK cells from HSCs

NK cell differentiation from HSCs was performed as described previously (3). In brief, after removing erythrocytes in extract from mouse bone marrow by ACK solution, c-Kit⁺, Lin⁻ (B cells, T/NK cells, granulocytes, monocytes, NK/NKT cells, and erythrocytes were depleted by B220, CD2, Gr-1, CD11b, NK1.1, and TER-119, respectively) were purified by MACS Cell Separation kit (Miltenyi Biotec) according to the manufacturer's protocol. Antibodies used for MACS purification were purchased from Becton-Dickinson (BD) and PharMingen (San Diego, CA).

Co-culture with OP9

To perform a co-culture with OP9 stromal cells, OP9 cells were plated at a density of 5×10^3 cells/ml in a 24-well plate with alpha-MEM (Invitrogen) supplemented with 20% fetal bovine serum. Attached OP9 cells were irradiated with 2000 rads for 24 h prior to the procedure. After removing alpha-MEM medium, pNK cells which were cultured for 7 days from HSC stage were plate onto OP9 cells at a density of 2×10^6 cells/ml in the presence IL-15 (50 ng/ml), and incubated for another 5 days.

Cell-based ELISA

OP9 cells were seeded at near 70% confluence in a 96-well plate containing alpha-MEM supplemented with 20% fetal bovine serum on 1 day prior to cell fixation. After removing residual liquid, cells were fixed by 3.7% formaldehyde diluted in PBS for 10 min at room temperature, washed 3 times with PBST, and blocked with 10% FBS diluted PBS for 1 h at room temperature. After washing, recombinant Gpnmb and control IgG were added into 96-well for 2 h at room temperature. Cells were washed 3 times, added with anti-mouse HRP as a secondary antibody at room temperature for 1h, washed 3 times again, added with substrate and stop solution, and then scanned at 450 nm.

Mouse IFN- γ ELISA

To detect IFN- γ , we collected the supernatants of NK cell culture on 12 day after, diluted, and used mouse IFN- γ ELISA kit from R&D systems. Then we followed manufacturer's guideline.

RESULTS

Gpnmb expression was increased during *in vitro* NK cell differentiation

To confirm the expression pattern of Gpnmb during NK cell differentiation, we checked mRNA and protein level of Gpnmb in *in vitro* culture system (Fig. 1). HSCs isolated from wild type mice were cultured in RPMI media containing SCF, Flt3L, and IL-7 for 7 days (pNK). After cultivation, we changed RPMI media containing IL-15 and cultured for another 5 days (mature NK, mNK). As shown in Fig. 1A, the expression of Gpnmb was not detected at HSC, but it was increased from 7 days of cultivation. To know relative quantity of Gpnmb mRNA, we performed real-time PCR (Fig. 1B). The dramatic change of Gpnmb mRNA suggests that Gpnmb may have some roles in NK cell differentiation. To know the expression level of Gpnmb protein *in vitro*, we checked its expression in the cells at each *in vitro* stage. On the 7 day, when pNK cells express CD122 (10), Gpnmb expression was increased in CD122 positive cells (Fig. 1C, top). On the 12 day when mNK cells express both CD122 and NK1.1, more Gpnmb expression was observed in CD122 and NK1.1 double positive populations (Fig. 1C, bottom). Overall, these results demonstrate that Gpnmb expression was increased dur-

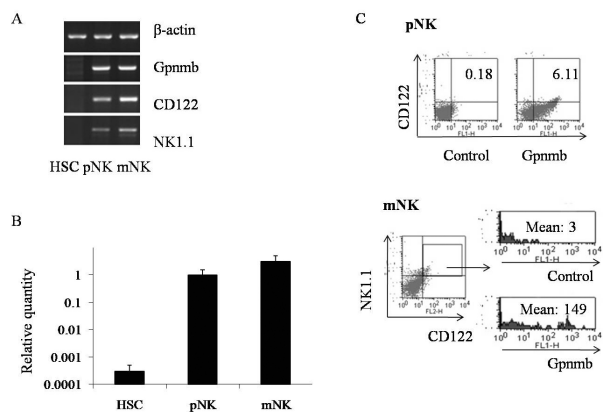


Figure 1. Gpnmb expression was increased during NK differentiation. (A) HSCs, pNK, and mNK cells were collected during *in vitro* NK differentiation, and isolated total RNAs. The expression of Gpnmb, CD122, and NK1.1 were analyzed by RT-PCR. The results shown are representative of at least three individual experiments with similar results. (B) Using cDNA from Fig. 1A, the expressions of Gpnmb were examined by real time quantitative PCR. A vertical axis shows the relative quantity. Data are representative of three independent experiments. (C) During *in vitro* culture, pNK and mNK cells were collected, stained with anti-Gpnmb antibody, and analyzed via flow cytometry.

ing *in vitro* NK cell developmental stage.

Level of Gpnmb expression was elevated at pNK stage *in vivo*

To confirm whether each *in vivo* developmental stage of NK cells has the same expression pattern with *in vitro* developmental stage, we checked the expression of Gpnmb in bone marrow cells. After removing erythrocytes, lymphocytes from bone marrow were stained with several markers indicating each developmental status. HSC represented by c-kit positive and lineage negative showed that mean value of Gpnmb was 12.6 (Fig. 2, G1). In contrast to HSC, pNK cells stained as CD122 positive and lineage/NK1.1 negative displayed higher mean value of Gpnmb (Fig. 2, G2 and G3). CD122 and NK1.1 double positive mNK cells from bone marrow showed the reduced expression of Gpnmb (Fig. 2, G4), similar to human mNK cells, which CD56 positive or CD94 positive cells showed the low expression level of Gpnmb *in vivo* (data not shown). It seems that Gpnmb may function mainly at pNK stage rather than mNK stage at least *in vivo* system. Meanwhile, comparing Gpnmb expression *in vitro* or *in vivo*, mNK cells have different expression pattern of Gpnmb. This

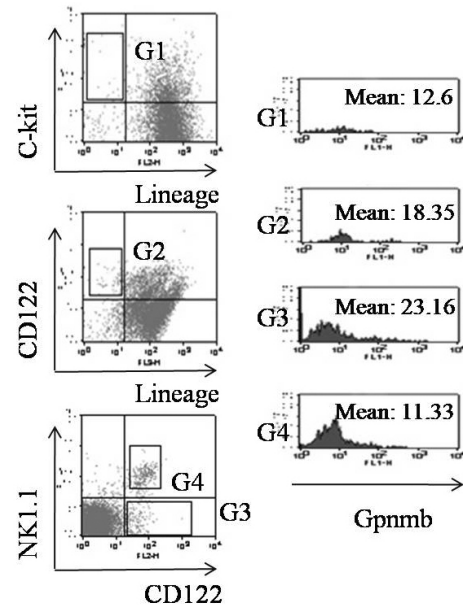


Figure 2. Gpnmb expression was elevated at pNK stage *in vivo*. Single cell suspensions of bone marrow were stained with several markers. G1, G2, G3, and G4 represented lineage-/c-kit+, lineage-/CD122+, CD122+/NK1.1-, and CD122+/NK1.1+, respectively. The mean values are determined from the FITC intensity in gated population.

may be due to the different microenvironment conditions of *in vitro* and *in vivo* systems or uneven maturation of NK cells in *in vitro* culture system. Collectively, Gpnmb may be related to CD122 expression at pNK stage where NK cell development is accelerated by expressing CD122 molecule.

Gpnmb binds ligand of stromal cells

During differentiation, it is already reported that bone marrow environment is an essential factor for NK cell maturation (11). To know the effect of Gpnmb expression on NK cell differentiation, we tested whether Gpnmb can bind to bone marrow stromal cells. For this purpose, we checked interaction of Gpnmb with OP9 stromal cells using recombinant Gpnmb conjugated Fc region. Recombinant Gpnmb conjugated Fc region or control IgG were added on plated OP9 cells, and their optical density value were analyzed by cell-based ELISA using mouse HRP (Fig. 3A). Compared to control IgG or without Gpnmb, recombinant Gpnmb showed high level of optical density value, indicating that Gpnmb can bind to unidentified component of OP9 cells. Meanwhile, we also checked the interaction of pNK cells by themselves, because it is possible that pNK cell might be a binding partner each other. However, pNK cells showed unchanged levels of anti-mouse IgG in the presence of recombinant Gpnmb or control IgG

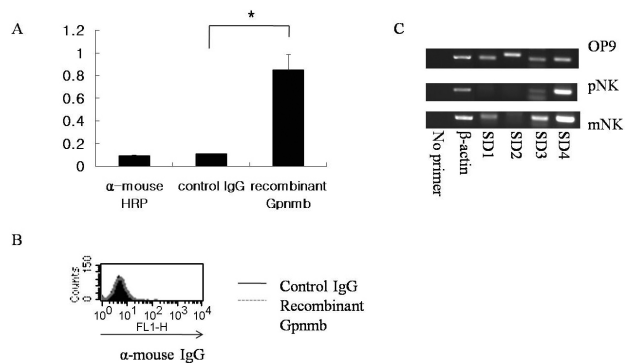


Figure 3. Gpnmb binds to the ligand of stromal cells. (A) OP9 cells were plated at about 70% confluence. After one day cultivation, cell-based ELISA was performed. For conjugation, control IgG and recombinant Gpnmb protein were used. A vertical axis shows optical density value. The values represent the means of 3 or 4 individual experiments. * $p < 0.05$. (B) pNK cells were collected and stained with control IgG and recombinant Gpnmb to know whether Gpnmb is involved in binding of NK cells by themselves. As a secondary antibody, anti-mouse IgG conjugated FITC was used. The results represent at least three individual experiments. (C) OP9, pNK cells, and mNK cells were collected, and total RNAs were isolated. As a negative control, no primer was used, and the expressions of SD family were examined.

(Fig. 3B). These results show that pNK cell interacts with stromal cell, but not with pNK cell itself.

It has been reported that Gpnmb binds to heparin heparan sulfate proteoglycan (12). In addition, one of the binding partner of Gpnmb is syndecan4 (SD4) on activated T cell which functions as a negative regulator on T cell activation, although SD4 on B cell did not bind to Gpnmb (12,13). Thus, we determined the expression of SD4 and other SD family in OP9 cells to determine a candidate which enables to bind Gpnmb on cell surface (Fig. 3C). As shown in Fig. 3C, OP9 cells showed SD1, SD2, SD3, and SD4 mRNA expression. In case of pNK cells, they expressed SD4 mRNA, but not SD1, SD2, and SD3 mRNA. However, mNK cells showed elevated SD family expressions. A previous report suggested that there is discordance between expression and binding activity, because SD4 can form diverse heparan sulfate structures (13). Considering all of these results, it seems that Gpnmb on NK cells binds to SD4 on OP9 cells directly, but not to SD4 on NK cells which might have different heparan sulfate structure at pNK stage. More study will be required to resolve this issue.

Gpnmb is involved in IFN- γ production

To know the meaning of Gpnmb binding to stromal cells dur-

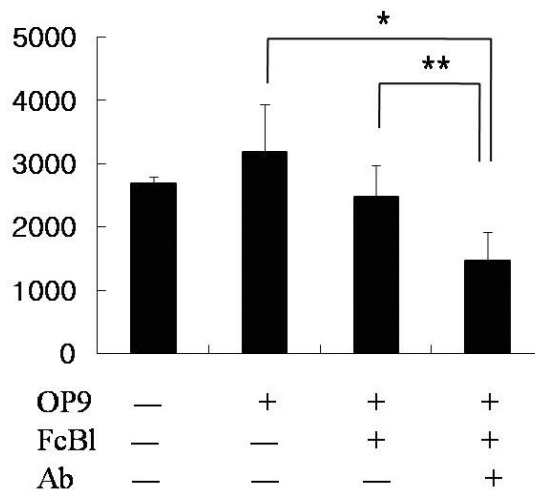


Figure 4. Neutralizing anti-Gpnmb antibody induced the reduction of IFN- γ production. After irradiation of OP9 cells, pNK cells were co-cultured with OP9 cells or OP9 cells with Fc Blocker (FcBI) (1ug/ml) alone or both FcBI and anti-Gpnmb antibody (1.5 ug/ml). On 12 day, supernatants were collected and assayed for mouse IFN- γ ELISA. A vertical axis shows IFN- γ concentration (pg/ml). The results shown are mean values which are representative of at least three individual experiments. * $p < 0.01$, ** $p < 0.1$.

ing NK cell differentiation, we test the inhibition effects of anti- Gpnmb antibody on IFN- γ production which is a key molecule for NK cell function and development (14). Using neutralizing anti-Gpnmb antibody, we found that inhibition of interaction between pNK cells and OP9 cells reduced IFN- γ production (Fig. 4). Compared with IFN- γ production of other control groups, NK cells treated with anti-Gpnmb antibody (lane 4) produced low level of IFN- γ , about 50% reduction. These results indicate that Gpnmb has an important role in IFN- γ production and NK cell development.

DISCUSSION

Our results demonstrate that Gpnmb elevated at pNK stage has a positive role in NK cell differentiation. Its expression was increased during developmental stage, bound to OP9 cells through unfound mechanism, and blocking interaction with OP9 cells by anti- Gpnmb antibody reduced IFN- γ production.

Gpnmb has a heparin binding motif (15), and SD family and glypican family are major candidates of cell surface-heparan sulfate (16). Thus, Gpnmb has a possibility to bind to SD family and glypican family. As it was reported that SD4 is a binding molecule of Gpnmb (13), thus we examined SD family as binding candidates including SD4. Because SD4 is expressed constructively in endothelial cells and B cells (16,17), other cells might able to bind to Gpnmb. Based on expression pattern of SD4 and binding pattern, it seems that SD4 is a candidate for Gpnmb's binding partner in this case. In blocking experiments between OP9 cells and Gpnmb of NK cells, we scrutinized several developmental markers such as CD122, NK1.1, DX5, NKG2D, and IFN- γ . Although there were no consistent changes in all markers, IFN- γ production was remarkably reduced. IFN- γ production has been considered as an essential marker in NK cell development (14). Furthermore, IFN- γ production has been used as a key molecule to monitoring NK cell activity (18,19). It is very interesting that Gpnmb can modulate IFN- γ production during NK cell development. It seems that Gpnmb regulates NK cell development by controlling NK cell and stromal cell interaction via IFN- γ production. The direct effects of Gpnmb on NK cell development in connection with stromal cell will be required.

In conclusion, the expression of Gpnmb was increased as NK cells were matured. It bound to stromal cells directly probably via SD4. This interaction was involved in IFN- γ

production. Taken together, Gpnmb has a potential value as a positive regulator in NK cell development.

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