

# TREM2 Promotes Natural Killer Cell Development in CD3-CD122<sup>+</sup>NK1.1<sup>+</sup> pNK Cells

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## Research Article

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# Abstract

**Background:** Triggering receptor expressed on myeloid cells 2 (TREM2) signaling is considered to regulate anti-inflammatory responses in macrophages, dendritic cell maturation, osteoclast development, induction of obesity, and Alzheimer's disease pathogenesis. However, little is known regarding the effect of TREM2 on natural killer (NK) cells.

**Results:** Here, we demonstrated for the first time that CD3<sup>-</sup>CD122<sup>+</sup>NK1.1<sup>+</sup> precursor NK (pNK) cells expressed TREM2 and their population increased in TREM2-overexpressing transgenic (TREM2-TG) mice compared with that in female C57BL/6J wild type (WT) mice. Both NK cell-activating receptors and NK cell-associated genes were expressed at higher levels in various tissues of TREM2-TG mice than in WT mice. In addition, bone marrow-derived hematopoietic stem cells (HSCs) of TREM2-TG mice (TG-HSCs) successfully differentiated into NK cells *in vitro*, with a higher yield from TG-HSCs than from WT-HSCs. In contrast, TREM2 signaling inhibition by TREM2-Ig or a phosphatidylinositol 3-kinase (PI3K) inhibitor affected the expression of the NK cell receptor repertoire and decreased the expression levels of NK cell-associated genes, resulting in significant impairment of NK cell differentiation. Moreover, in melanoma-bearing WT mice, injection of bone marrow cells from TREM2-TG mice exerted greater antitumor effects than that with cells from WT control mice.

**Conclusions:** Collectively, our data clearly showed that TREM2 promoted NK cell development and tumor regression, suggesting TREM2 as a new candidate for cancer immunotherapy.

## Background

Natural killer (NK) cells play roles in both innate and adaptive immunity [1], which kill target cells using various mechanisms, including exocytosis of perforin and granzyme molecules, promotion of death receptor-mediated apoptosis, and secretion of interferon (IFN)- $\gamma$  [2, 3]. The function of NK cells is regulated by balancing signals from activating and inhibitory receptors that interact with their respective ligands on target cells, including virus-infected, tumor, and allogeneic cells [4]. The ligand profile of the target cell allows for differentiation between self and non-self, as well as between normal and abnormal cells. Signals from NK cell-activating receptors, such as NKG2C, NKG2D, NKG2E, Ly49C, Ly49D, Ly49H, and Ly49E (in mice), lead to cytotoxicity and cytokine production via signaling through DAP10 or DAP12 [5, 6]. In particular, the NKG2D/DAP10 receptor complex conveys activating signals by recruiting the p85 subunit of phosphatidylinositol 3-kinase (PI3K), leading to NK cell-mediated cytotoxicity [7, 8]. In contrast, engagement of Ly49A, Ly49C, Ly49G2, Ly49I, and NKG2A inhibitory receptors by major histocompatibility complex (MHC) class I hinders cytotoxicity and cytokine release [7, 8]. Interestingly, triggering receptor expressed on myeloid cells 2 (TREM2) is associated with DAP12 protein, which contains the immunoreceptor tyrosine-based activation motif (ITAM) that is structurally similar to NK cell receptors.

NK cells are derived from hematopoietic stem cells (HSCs) in the bone marrow (BM), fetal thymus, fetal liver, and umbilical cord blood [9–12]. *In vitro* culture of HSCs in medium supplemented with the stem cell

factors FLT3L and interleukin (IL)-7 (defined as c-kit<sup>+</sup> lineage<sup>-</sup> HSCs) leads to their differentiation into precursor NK (pNK) cells that express the CD122 receptor (IL-2R $\beta^+$ /CD15R $\beta^+$ ) [12, 13]. These *in vitro*-differentiated CD122<sup>+</sup> pNK cells further differentiate into immature NK cells (NK1.1<sup>+</sup>, CD49<sup>-</sup>NKG2<sup>+</sup>, and Ly49<sup>-</sup>) and mature NK (mNK) cells (NK1.1<sup>+</sup>, CD49/NKG2<sup>+</sup>, and Ly49<sup>+</sup>) when co-cultured with OP9 stromal cells in the presence of IL-2 or IL-15[12–16]. Moreover, OP9 stromal cells secrete growth factors that support NK cell differentiation *in vitro*. We and other groups have previously reported gene expression profiles specific to NK cell differentiation *in vitro*. Among these, inhibitor of DNA binding 2 (Id2) is an important transcription factor for NK cell differentiation[17]. In addition, E4bp4, which acts downstream of IL-15 receptor signaling, is an essential transcription factor for NK cell development and function [18]. Engagement of the IL-15 receptor with its ligand mediates NK cell activation [19] and differentiation [20] through the PI3K-protein kinase B (AKT) pathway. Interestingly, we also identified *TREM2* in the CD122<sup>+</sup> pNK cell gene expression profile [10].

TREM2 is associated with DAP12, which contains the ITAM that serves as a docking site for Src kinases in dendritic cells (DCs) [7], osteoclasts [8], monocytes[21, 22], macrophages[23, 24], and microglia [21]. TREM2/DAP12 signaling induces PI3K and extracellular signal-regulated kinases [25], promotes the upregulation of CC chemokine receptor 7 in DCs [7], and increases phagocytosis in DCs [26, 27]. In addition, TREM2/DAP12 signaling negatively regulates the inflammatory response in microglia [27] and macrophages [28]. TREM2 undergoes intramembranous proteolysis by  $\gamma$ -secretase, while its extracellular domain is cleaved and removed by sheddase, disintegrin, and metalloproteinase domain-containing protein 10 [29, 30]. However, it is unclear whether the soluble and C-terminal fragments produced by this proteolytic event function as scavenger receptors or play biological roles [31, 32]. Moreover, TREM2/DAP12 has been shown to induce obesity by promoting adipogenesis and upregulating the expression of adipogenic regulators within adipocytes via WNT10b/ $\beta$ -catenin signaling [33]. Recently, TREM2 expression has also been identified as a risk factor for late-onset dementia and Alzheimer's disease [31],[34–39]. Furthermore, TREM2 acts as a tumor suppressor in colorectal carcinoma and hepatocellular carcinoma through WNT1/ $\beta$ -catenin and extracellular signal-regulated kinase signaling or PI3K/AKT/ $\beta$ -catenin signaling [40, 41]. Nonetheless, although the structure of TREM2 is similar to that of NK cell receptors, its effect on NK cells remains unknown.

Here, we demonstrated that overexpression of TREM2 promoted NK cell differentiation and enhanced their cytotoxicity toward tumor cells *in vivo* and *in vitro*. Conversely, treatment with TREM2-Ig or a PI3K inhibitor inhibited NK cell differentiation, suggesting that activation of the PI3K pathway by TREM2/DAP12 signaling plays a crucial role in both the differentiation and effector function of NK cells.

## Results

# TREM2-overexpressing transgenic (TREM2-TG) mice show a higher NK cell population than wild type (WT) mice

To investigate the effect of TREM2 on NK cell development, we analyzed NK populations in previously generated TREM2-overexpressing transgenic (TREM2-TG) and wild type (WT) mice using flow cytometry [33]. The expression of NK receptor repertoires, NK1.1<sup>+</sup> population percentage, and their absolute numbers in the spleen, BM, and liver were higher in TREM2-TG mice than in WT mice (Fig. 1A and B). Furthermore, the absolute number of NK cells expressing the NKG2A/NKG2C/NKG2E receptor was higher in the spleen (Additional file 1, Fig. S1, left panel) and liver (right panel) of TREM2-TG mice than that in those of WT mice. A slight increase in the BM of TREM2-TG mice (middle panel) was also observed. Similarly, the percentage and absolute numbers of Ly49C/F/H/I<sup>+</sup> and Ly49D<sup>+</sup> NK cells in the spleen and BM of TREM2-TG mice were significantly higher than in those of WT mice (Fig. 1B and Additional file 1, Fig. S1).

To investigate whether NK cells express TREM2, we isolated BM cells from WT and TREM2-TG mice, stained them with NK-specific markers, and performed flow cytometry analysis. The t-SNE density plots of the BM cells of WT and TREM2-TG mice are shown in Fig. 1C–E left and middle panels, respectively. The cell population showed both NK1.1<sup>+</sup> (Cluster 2, indigo) and TREM2 surface protein (red) expression (Fig. 1C, third panel). Furthermore, the NK1.1<sup>+</sup>TREM2<sup>+</sup> (indigo and red) cell population was increased in the BM of TREM2-TG mice (Fig. 1C, red arrow) compared with that of WT mice. Likewise, Fig. 1D shows the CD122<sup>+</sup> cells (Cluster 6, indigo) and TREM2<sup>+</sup> cell population (red, third panel), demonstrating an increase in CD122<sup>+</sup>TREM2<sup>+</sup> double-positive (indigo and red) cells in TREM2-TG mice (red circle). We further analyzed the TREM2 expression in the CD3<sup>-</sup>CD122<sup>+</sup>NK1.1<sup>+</sup> pNK cells. As shown in Fig. 1E, TREM2-expressing pNK cells (CD3<sup>-</sup>CD122<sup>+</sup>NK1.1<sup>+</sup>) were increased in TREM2-TG mice compared with that in WT mice. In addition, the TREM2<sup>+</sup> pNK cell population (TREM2<sup>+</sup>CD122<sup>+</sup>NK1.1<sup>+</sup>) was significantly increased in the BM of TG mice (4.68%) compared with that in the BM of WT mice (1.63%; Fig. 1F). These data showed that TREM2 was expressed in CD3<sup>-</sup>CD122<sup>+</sup>NK1.1<sup>+</sup> pNK cells

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to identify whether TREM2 regulates NK cell function-associated genes using splenic NK1.1<sup>+</sup> cells from WT and TREM2-TG mice. The expression levels of IFN- $\gamma$  ( $3.29 \pm 1.158$ -fold increase), perforin ( $18.25 \pm 5.3$ -fold increase), and granzyme B ( $57 \pm 16.97$ -fold increase) were significantly higher in the spleens of TREM2-TG mice than in the spleens of WT mice (Additional file 1, Fig. S2A). Consequently, TREM2-TG splenic NK cells showed significantly higher NK cell-mediated cytotoxicity than those of WT mice (Additional file 1, Fig. S2B). Interestingly, no differences were observed in CD4<sup>+</sup> T, CD8<sup>+</sup> T, and B220<sup>+</sup> cell populations and their absolute numbers in the spleen between WT and TREM2-TG mice (Additional file 1, Fig. S3).

### **Inhibition of TREM2 signaling reduces NK cell populations *in vivo***

To verify the effect of TREM2 on NK cell development *in vivo*, we inhibited TREM2 signaling in WT mice via intraperitoneal injection of a TREM2-Ig fusion protein or a humanized (hu)-Ig control. Three days after injection, the spleen, BM, and liver cells were isolated, and NK cell populations and the expression of NK-specific receptors were analyzed via flow cytometry. The NK1.1<sup>+</sup> cell population was reduced in the

spleens of TREM2-Ig-injected mice, with respect to both the percentage of the splenocyte population and absolute number of NK1.1<sup>+</sup> cells, compared with that in the spleens of hu-Ig-injected control mice (3.525% ± 0.32% vs. 5% ± 0.5%; Fig. 2A and Additional file 1, Fig. S4). Similarly, the NK1.1<sup>+</sup> population percentage and the absolute number of NK1.1<sup>+</sup> cells were lower in the BM and liver of TREM2-Ig-injected mice compared with that in the BM and liver of hu-Ig-injected control mice (Fig. 2A). Furthermore, the NK1.1<sup>+</sup>/NKG2A/C/E<sup>+</sup> cell population percentage was lower in the spleens of TREM2-Ig-injected mice (1.9%) than that in the spleens of hu-Ig-injected control mice (2.4%; Additional file 1, Fig. S4, left panel). Both the NK1.1<sup>+</sup>/NKG2A/C/E<sup>+</sup> (0.6% vs. 1.1%) and NK1.1<sup>+</sup>/Ly49D<sup>+</sup> (0.5% vs. 1%) populations were reduced in the BM of TREM2-Ig-injected mice than in the BM of control mice, and the absolute number of NK1.1<sup>+</sup> cells was also decreased by TREM2 signal inhibition (TREM2-Ig treatment; Fig. 2B and Additional file 1, Fig. S4, middle panel). In addition, both the NK1.1<sup>+</sup>/NKG2A/C/E<sup>+</sup> (4.9% vs. 6.3%) and NK1.1<sup>+</sup>/Ly49D<sup>+</sup> (2% vs. 2.4%) populations, as well as the absolute number of NK1.1<sup>+</sup> cells were decreased in the livers of TREM2-Ig-injected mice compared with those in the livers of control mice (Fig. 2B and Additional file 1, Fig. S4, right panel). However, the absolute numbers of NK1.1<sup>+</sup>/NKG2A/C/E<sup>+</sup>, Ly49C/F/H/I<sup>+</sup>, and Ly49D<sup>+</sup> cells were significantly decreased in the spleen (Fig. 2B, left panel) and BM (Fig. 2B, middle panel), but not in the liver (Fig. 2B, right panel) of TREM2-Ig-injected mice compared with those in hu-Ig-injected mice (Fig. 2B, right panel). Together, these data indicated that inhibition of TREM2 signaling by TREM2-Ig resulted in a decrease in the number of NK cells and the expression of their signature surface receptors *in vivo*.

### **TREM2 promotes NK cell differentiation** *in vitro*

Our results showed that TREM2 signaling increased the number of NK cells *in vivo*. However, this is not sufficient to conclude that TREM2 enhances commitment to the NK cell fate and differentiation of the NK cell lineage. Therefore, to determine the effects of TREM2 on the differentiation of NK cells, we isolated c-kit<sup>+</sup> Lin<sup>-</sup>HSCs from the BM of WT and TREM2-TG mice and differentiated them into pNK and mNK cells *in vitro*. During NK cell differentiation, pNK cells were treated with TREM2-Ig or hu-Ig to inhibit TREM2 signaling. As a result, the percentage and absolute numbers of NK1.1<sup>+</sup>/NKG2A/C/E<sup>+</sup> cells were approximately 2-fold higher in mNK cells derived from hu-Ig-treated pNK cells of TREM2-TG mice (25%) than in their counterparts derived from hu-Ig-treated pNK cells of WT mice (14%; Fig. 3A). However, this NK1.1<sup>+</sup> cell population dramatically decreased when both WT-pNK (46.8% ± 0.8–10.6% ± 2.7%) and TREM2-TG-pNK (53.8% ± 2.8–13.3% ± 0.88%) cells were treated with TREM2-Ig during differentiation (Fig. 3A and B, upper panel). The absolute number of NK1.1<sup>+</sup> cells decreased by 3.97- (WT) and 4.77-fold (TREM2-TG) after treatment with TREM2-Ig (Fig. 3C). Additionally, the percentage and absolute number of NK cells expressing Ly49C/F/H/I or Ly49D were reduced in both NK cells derived from WT-pNK (6.7–1.8% or 3.9–2.2%, respectively) and TREM2-pNK (7.3–4.7% or 7.1–3.1%, respectively) after TREM2-Ig treatment during differentiation (Fig. 3A and C). In contrast, the total number of cells differentiated from TREM2-TG-pNK or WT-pNK cells did not differ significantly, regardless of TREM2-Ig treatment (Fig. 3B, lower panel). Subsequently, we performed RT-PCR analyses to identify the expression of NK cell-

associated genes regulated by TREM2 (Fig. 3D and Additional file 1, Fig. S5). NK cells that differentiated from TREM2-TG-pNK cells treated with hu-Ig showed increased IFN- $\gamma$  ( $3.97 \pm 0.63$ -fold) and Fas ligand ( $4.5 \pm 0.3$ -fold) expression compared with that in NK cells derived from WT-pNK cells treated with hu-Ig. We also observed increased expression levels of granzyme B ( $1.8 \pm 0.36$ -fold), perforin ( $1.25 \pm 0.05$ -fold), and TNF-related apoptosis-inducing ligand (TRAIL;  $1.9 \pm 0.22$ -fold) in NK cells derived from TREM2-TG-pNK cells treated with hu-Ig compared with those in NK cells derived from WT-pNK cells treated with hu-Ig. In contrast, the expression levels of these genes were reduced in NK cells derived from both WT-pNK and TG-pNK cells when TREM2 signaling was inhibited by TREM2-Ig (Fig. 3D). Moreover, the expression levels of E4bp4, Id2, CD122, and CD123 increased in TREM2-TG-pNK-derived NK cells compared with those in WT-pNK-derived NK cells treated with hu-Ig, whereas the expression levels of these genes in differentiated NK cells decreased significantly after TREM2-Ig treatment (Additional file 1, Fig. S5).

## TREM2 signaling inhibits tumor progression

As described above, inhibition of the TREM2 signaling pathway by TREM2-Ig reduced NK cell receptor and NK cell-associated gene expression, along with the absolute number of NK cells *in vitro* (Fig. 3). To confirm whether TREM2 affects tumor progression *in vivo*, we injected TREM2-TG or WT mice with B16F10 melanoma cells after intraperitoneal injection of hu-Ig or TREM2-Ig. As shown in Fig. 4A, on day 25, the tumor volume in WT mice treated with TREM2-Ig (WT + TREM2-Ig) was significantly higher than that in WT mice treated with hu-Ig (WT + hu-Ig). Differences in tumor volume between the two groups became even more prominent after day 25. Additionally, on day 25, the tumor volume in TREM2-TG mice treated with TREM2-Ig (TG + TREM2-Ig;  $1988.14 \pm 426.2$  mm<sup>3</sup>) was higher than that in TREM2-TG mice treated with hu-Ig (TG + hu-Ig;  $970.3 \pm 257.11$  mm<sup>3</sup>). Furthermore, on day 31, the tumor volume was significantly lower in TG + hu-Ig mice ( $1700.82 \pm 171.142$  mm<sup>3</sup>) than in WT + hu-Ig mice ( $3088.09 \pm 808.67$  mm<sup>3</sup>). On average, the tumor volume in TREM2-TG mice ( $3718.48 \pm 1095.74$  mm<sup>3</sup>) was approximately 2-fold lower than that in WT mice ( $7915.32 \pm 839.09$  mm<sup>3</sup>). These data showed that tumor progression in TREM2-TG mice was significantly reduced compared with that in WT mice, and tumor progression in both TREM2-TG and WT mice increased upon TREM2-Ig treatment. In addition, differences in survival rate were observed upon inhibition of TREM2 signaling. The survival rate of tumor-bearing WT mice injected with TREM2-Ig was 14.2%, which was significantly lower than that of tumor-bearing WT mice injected with hu-Ig (42.8%), tumor-bearing TREM2-TG mice injected with TREM-Ig (71.4%), and tumor-bearing TREM2-TG mice injected with hu-Ig (100%) on day 38 (Fig. 4B). Furthermore, the number of metastatic melanomas in the lungs of TREM2-Ig-injected WT mice ( $24 \pm 4$ , B16F10 cell spots) was higher than that in the lungs of hu-Ig-injected WT mice ( $3 \pm 1$ , B16F12 cell spots; Fig. 4C). Surprisingly, B16F10 melanoma cells were rarely observed in the lungs of hu-Ig-injected TREM2-TG mice, whereas melanoma cells were apparent in the lungs of TREM2-Ig-injected TREM2-TG mice ( $19 \pm 1$ , B16F10 cell spots).

## Adoptive transfer of TREM2-TG BM cells promotes tumor regression

As mentioned above, TREM2-TG mice showed a significantly lower tumor volume and rare metastatic tumor spots compared with WT mice when they were injected with B16F10 melanoma cells. This may be related to the effects of TREM2-overexpressing monocytes/macrophages or DCs, which secrete cytokines and indirectly activate T cells and NK cells. To investigate whether TREM2-overexpressing NK cells cause tumor regression, we transplanted CD45.2 TG-BMs (TG to WT) or CD45.2 WT-BMs (WT to WT) into sublethally irradiated WT recipients (CD45.1). Four weeks after adoptive transplantation, we analyzed the NK cell population in various organs in each group using flow cytometry. Higher proportions of NK cells (NK1.1<sup>+</sup>/CD3<sup>-</sup>/CD45.2<sup>+</sup>) were detected in the spleen (3.51% vs. 0.73%), BM (1.0% vs. 0.36%), and lungs (10% vs. 7%) of recipient mice (CD45.1 WT) engrafted with CD45.2 TG-BMs than in those engrafted with CD45.2 WT-BMs (Fig. 5A). These data indicated that TREM2-TG mice had a larger NK cell population than WT mice.

We then used an *in vivo* tumor model to determine whether TREM2 signaling affects the antitumor effect of NK cells. To this end, we subcutaneously transplanted B16-F10 melanoma cells into WT mice (CD45.1) engrafted with BMs (CD45.2) from WT or TREM2-TG mice and measured the tumor volume every other day. The tumor volume measured 21 days post-inoculation in mice transplanted with TREM2-TG-BMs (TG to WT, 1079 ± 221.5 mm<sup>3</sup>) was lower than that in mice that received WT-BMs (WT to WT controls, 3122.7 ± 1269 mm<sup>3</sup>; Fig. 5B). Furthermore, 27 days post-inoculation, the survival rate (75%) of tumor-bearing mice transplanted with TREM2-TG-BMs was significantly higher than that of tumor-bearing mice that had received WT-BMs (0%; Fig. 5C). To observe lung metastatic melanoma, we sacrificed mice from each group on day 14. Several melanomas (large black spots, 14 ± 1) were observed in the lung tissues of WT BM recipients (open bars, Fig. 5D); however, few melanomas were detectable (2 ± 1) in the counterparts transplanted with TREM2-TG cells (solid bars, Fig. 5D).

## TREM2 regulates NK cell differentiation via PI3K signaling

TREM2-DAP12 signaling, triggered by TREM2 ligand binding, may promote or inhibit proinflammatory responses, induce obesity [33], and mediate neurodegeneration [34, 38]. DAP12, an adaptor protein of TREM2, mediates downstream signaling via the cytoplasmic ITAM domain, which recruits SYK and activates PI3K, phospholipase C, and Vav signaling cascades [42]. To investigate how TREM2 signaling regulates NK cell differentiation, we treated pNK cells differentiated from WT-HSCs or TG-HSCs with the PI3K inhibitor Ly294002 during their differentiation into mNK cells. After 14 days, differentiated mNK cells were stained with NK-specific markers and analyzed via flow cytometry (Additional file 1, Fig. S6A). In the absence of the PI3K inhibitor, the population of NK1.1<sup>+</sup>/NKG2ACE<sup>+</sup> cells differentiated from TREM2-TG-pNK cells (dimethyl sulfoxide (DMSO) control; Additional file 1, Fig. S6A, lower panel) was 2-fold higher than that of NK cells differentiated from WT-pNK cells (DMSO control; Fig. S6A, upper panel).

Interestingly, NK1.1<sup>+</sup>/NKG2ACE<sup>+</sup> cell populations derived from both WT- and TREM2-TG-pNK cells decreased (10-fold) after Ly294002 treatment during NK cell maturation.

We also analyzed the expression of NK cell-associated genes in mNK cells differentiating in the presence or absence of the PI3K inhibitor. The expression levels of IFN-γ, perforin, and granzyme B were increased

by 4- to 5-fold in mNK cells differentiated from TREM2-TG-pNK cells than that in mNK cells differentiated from WT-HSCs. Similarly, the expression levels of Fas ligand, TRAIL, and IL-15R $\alpha$  were higher in mNK cells differentiated from TREM2-TG-pNK cells than those in cells differentiated from WT-pNK cells. With the exception of E4bp4 and IL-15R $\alpha$ , NK cell-related gene expression levels significantly decreased in mNK cells treated with Ly49294002 during NK cell differentiation (Additional file 1, Fig S6). In particular, the expression level of Id2 decreased by more than 2-fold in mNK cells after treatment with Ly294002.

## Discussion

TREMs have emerged as critical immune regulators that modulate inflammatory responses in macrophages, glial cells, and DCs [41, 43–45]. Recently, several groups reported TREM2 as a novel tumor suppressor in colorectal and hepatocellular carcinoma [40, 46]. However, the function of TREM2 in NK cells has not yet been elucidated. NK cells mediate innate as well as adaptive immune responses, including cytotoxic activity against tumors. However, autologous NK therapy for patients with cancer has several limitations [47]. *Ex vivo* NK cell expansion is difficult, and IL-2 treatment for NK cell activation causes severe side effects [40]. Therefore, it is necessary to find new ways to alter NK cell function, which is modulated by activating receptors that have binding motifs for the associated adaptor protein DAP12. Interestingly, the structure of TREM2 is very similar to that of other NK cell receptors that transmit intracellular signals via DAP12, although its function in NK cells remains unclear.

In this study, we found that TREM2 is expressed in CD3<sup>-</sup>CD122<sup>+</sup>NK1.1<sup>+</sup> pNK cells, while the NK cell population increased in the BM of TREM2-TG mice compared with that in the BM of WT mice. Most recently, it was reported that liver lymphocytes express DAP12 as well as low levels of TREM2, which is clear evidence supporting our findings [48].

The late NK cell maturation stage (CD3<sup>-</sup>NK1.1<sup>+</sup>) is subdivided into four distinct subsets: CD27<sup>lo</sup>Mac-1<sup>lo</sup>, CD27<sup>hi</sup>Mac-1<sup>lo</sup>, CD27<sup>hi</sup>Mac-1<sup>hi</sup>, and CD27<sup>lo</sup>Mac-1<sup>hi</sup> [49–51]. The intermediate CD27<sup>hi</sup>Mac-1<sup>hi</sup> population shows the strongest cytotoxicity and secretes cytokines [52], and also has higher proliferation potential and an enhanced ability to interact with DCs [51]. Conversely, CD27<sup>lo</sup>Mac-1<sup>hi</sup> NK cells, the most abundant mNK cells, are effective killer cells *in vivo*, and are particularly effective on MHC class I-negative tumor cells [49]. According to our data, TREM2 overexpression affected the CD27<sup>hi</sup>Mac-1<sup>lo</sup>, CD27<sup>hi</sup>Mac-1<sup>hi</sup>, and CD27<sup>lo</sup>Mac-1<sup>hi</sup> populations in the BM, suggesting that TREM2 promoted NK cell maturation in the BM with strong expression of TREM2 ligands (Additional file 1, Fig. S7). In contrast, in the peripheral blood, the number of NK1.1<sup>+</sup>/CD3<sup>-</sup> cells was higher in TREM2-TG mice (8.2%) than in WT mice (5.7%), whereas the NK1.1<sup>+</sup>/CD3<sup>-</sup>/CD27<sup>hi</sup>/Mac-1<sup>hi</sup> cell population was comparable in the two types of mice (Additional file 1, Fig. S7B). These data suggested that TREM2 did not affect NK cell maturation in the peripheral blood although more NK cells were released into the blood in TREM2-TG mice, as NK cell maturation was promoted in the BM of TREM2-TG mice.

In addition, we established a tumor-bearing mouse model to demonstrate that the NK cell population increased by TREM2 overexpression, which reduced tumor progression. The tumor volume was lower in

tumor-bearing WT mice transplanted with BM cells of TREM2-TG mice than that in tumor-bearing WT mice transplanted with BM cells of WT mice. Metastasis of tumor cells to the lung tissue was reduced, and the survival rate of tumor-bearing WT mice transplanted with BM cells of TREM2-TG mice was higher than that of mice transplanted with BM cells of WT mice. NK cells promote the maturation of DCs via IFN- $\gamma$ , an important proinflammatory cytokine [53], and DCs stimulate cytotoxicity and cytokine secretion by NK cells via IL-12 [54]. This bidirectional crosstalk between NK cells and DCs is an important mechanism in innate and adaptive immune responses [55, 56]. Therefore, TREM2 reduces tumor progression *in vivo* by directly improving NK cell cytotoxicity, and TREM2-overexpressing DCs and macrophages can develop and stimulate NK cell function *in vivo*.

We co-cultured pNK cells derived from WT-HSCs and TG-HSCs with OP9 stromal cells, which support hematopoiesis by secreting growth factors, to induce further differentiation into mNK cells. Interestingly, Bartosz et al. [57] reported that NK cells may be derived from myeloid progenitors. Accordingly, we hypothesized that TREM2 in pNK cells or myeloid progenitors enhanced both the differentiation of NK cells *in vitro* and their cytotoxicity in the presence of OP9 cells. Recently, apolipoprotein E has been reported as a ligand of TREM2, although this is now considered controversial [58]. Consequently, the definitive identification of TREM2 ligands remains necessary for the development of NK cell therapies.

Previous studies have demonstrated that PI3K, and not PLC- $\gamma$ , plays a critical role in the development of mNK cells [59]. The absence of PLC- $\gamma$  does not disrupt NK cell development but causes defects in NK cell cytotoxicity [60, 61]. However, Tassi et al. [62] demonstrated that PLC- $\gamma$ 2 is crucial for the development of the NK cell receptor repertoire. Interestingly, expression of the NKG2A/NKG2C/NKG2E receptor in mNK cells, differentiated from WT-pNK or TREM2-TG-pNK cells, decreased significantly from 40.68–8.96% after PLC- $\gamma$  inhibitor treatment (Additional file 1, Fig. S8A). Expression of Id2 was upregulated by TREM2 in mNK cells differentiated from WT-pNK or TREM2-TG-pNK cells, and was downregulated upon PI3K inhibitor treatment. Moreover, the expression of E4bp4, an essential factor for NK cell development [18], was upregulated by TREM2 in NK cells differentiated from pNK cells and was downregulated following PLC- $\gamma$  inhibitor treatment (Additional file 1, Fig. S8B), indicating that TREM2 influenced NK cell differentiation not only via the PI3K signaling pathway but also via PLC- $\gamma$  signaling.

## Conclusions

In conclusion, we demonstrated that TREM2 played an important role not only in myeloid cells, but also in CD3<sup>-</sup>CD122<sup>+</sup>NK1.1<sup>+</sup> pNK cells. Furthermore, TREM2 promoted NK cell differentiation, as well as the expression of NK cell receptor repertoires and cytokines, suggesting that TREM2 might be an effective candidate for new NK cell therapies.

## Methods

### Mice

Five to seven-week-old female C57BL/6J WT and TREM2-TG mice were used in this study [33]. All animal experiments were carried out following the guidelines of the Institutional Animal Care Committee of Chonnam National University (CNU IACUC-YB-2017-19).

## Differentiation of HSCs into mNK cells

Murine HSCs were sorted from BM cell populations by negative or positive selection using a magnetic-activated cell sorter (MACS), as described previously [10]. Briefly, total BM cell samples were prepared by flushing the femurs from C57BL/6 mice, followed by filtration through a 70- $\mu$ m cell strainer (Falcon, San Jose, CA, USA). Total BM samples were cleared of erythrocytes via erythrocyte lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) treatment. The suspensions of single BM cells were labeled using a cocktail of biotinylated antibodies against lineage (Lin<sup>+</sup>) markers (CD11b, Gr-1, B220, NK1.1, CD2, and TER-119), which were then incubated with streptavidin-magnetic beads. The samples were depleted of magnetically labeled Lin<sup>+</sup> cells by retention on CS column beads in the magnetic field of a VarioMACS Separator (Miltenyi Biotec, Sunnyvale, CA, USA). c-Kit<sup>+</sup> cells among the Lin<sup>-</sup> cells were positively selected with magnetic bead-conjugated antibodies against c-kit, and then the cell suspension was run through an MS magnetic column.

HSCs were stimulated to differentiate into NK cells as described previously [11]. In brief, purified Lin<sup>-</sup>, c-kit<sup>+</sup> HSCs were plated on a 24-well plate (Corning, ME, USA) at  $1 \times 10^6$  cells/well and cultured in RPMI medium supplemented with a mixture of IL-7 (0.5 ng/mL), stem cell factor (30 ng/mL), Flt3-L (50 ng/mL), indomethacin (20  $\mu$ g/mL), and gentamycin (20  $\mu$ g/mL) at 37 °C and 5% CO<sub>2</sub>. Three days later, half of the culture supernatant was removed and replaced with fresh medium containing the same cytokines. After 7 days, the cells were co-cultured with or without OP9 stromal cells (American Type Culture Collection, Manassas, VA, USA) in the presence of mouse IL-15 (20 ng/mL). Three days later, half of the culture medium was changed with fresh medium containing the same cytokines, and the cells were cultured for an additional 7 days. The purity of the cultured cells was determined on days 0, 7, and 14 by staining with stage-specific antibodies during the differentiation of NK cells, and their purity was analyzed using a flow cytometer.

## Isolation of NK1.1<sup>+</sup> cells from the spleen

Splenocytes were isolated from the spleens of the mice, and the cell suspension was filtered through a 20- $\mu$ m cell strainer. After removal of erythrocytes via treatment with erythrocyte lysis buffer, single cells in suspension were first incubated with a biotinylated antibody against NK1.1 (BD Pharmingen, San Diego, CA, USA), followed by incubation with streptavidin-magnetic beads. NK1.1<sup>+</sup> cells were then purified using MACS (Miltenyi Biotec) according to the manufacturer's instructions.

## Flow cytometry analysis

To determine the developmental status of NK cells differentiated from HSCs of WT and TREM2-TG mice, we performed flow cytometry analysis of HSCs, pNKs, and mNKs co-cultured with OP9 cells using antibodies against the markers with stage-specific expression during the differentiation of NK cells. In

brief, HSCs were stained with 1  $\mu$ L of fluorescein isothiocyanate (FITC)-conjugated c-kit, phycoerythrin (PE)-conjugated Sca-1, and biotin/streptavidin/cytochrome-conjugated IL-7Ra. pNKs were stained with 1  $\mu$ L of FITC-conjugated anti-CD122 and 0.5  $\mu$ L of PE-conjugated anti-NK1.1. mNKs were stained with FITC-conjugated anti-NKG2A/C/E, anti-Ly49C/F/H/I, anti-Ly49D, and PE-conjugated anti-NK1.1. The cells were incubated with the antibodies for 30 min on ice, and then washed twice with staining buffer (phosphate-buffered saline containing 3% fetal bovine serum and 0.1% NaN<sub>3</sub>). The cells were analyzed using a FACS Calibur flow cytometer (BD Bioscience, San Jose, CA, USA) and Cell Quest software. The data shown in histograms or dot plots are representative of replicates.

## BM adoptive transfer

WT (CD45.1) recipients were irradiated with 6.5 Gy, followed by injection with WT (CD45.2) or TREM2-TG (CD45.2) BM cells ( $1 \times 10^6$ ) intravenously. Four to eight weeks after the cells were transplanted, the BM and spleen were harvested, and single-cell suspensions were prepared as described above. Erythrocytes were lysed, lymphoid cell populations were counted, and the number of NK cells was assessed using flow cytometry with antibodies against NK1.1, CD45.1, or CD45.2.

## Tumor models

To determine the tumor volume and survival rate, we subcutaneously injected B16F10 melanoma cells ( $5 \times 10^5$  cells/mouse) (ATCC, VA, USA) into the left flank of WT mice, TREM2-TG mice, BM-transplanted WT mice, and mice intraperitoneally injected with TREM2-Ig or hu-Ig (as a control). After B16F10 cell injection, the tumor volume was measured every 2 days.

## RT-PCR

Total cellular RNA was extracted using Trizol B reagent (Tel-Test, Friendswood, TX, USA) according to the manufacturer's instructions. Aliquots of total RNA were transcribed into cDNA at 37 °C for 1 h in a total reaction volume of 20  $\mu$ L with 2.5 U of Moloney murine leukemia virus reverse transcriptase (Roche, Mannheim, Germany). Reverse-transcribed cDNA was added to a PCR mixture consisting of 10  $\times$  PCR buffer, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Bioneer, Daejeon, Korea), and 10 pmol of primers for each gene. For  $\beta$ -actin amplification, 27 cycles were performed, and for all other genes, 30 or 35 cycles were performed. The amplification profile included denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and extension at 72 °C for 10 min. PCR products were electrophoresed and visualized via ethidium bromide staining.

## Cytotoxicity assay

A standard lactate dehydrogenase-release assay kit (Promega, WI, USA) was used to measure the cytotoxicity of NK cells, according to the manufacturer's instructions. In brief, NK cells were stimulated with 20 ng/mL of recombinant murine IL-2 for 48 h, washed twice with phosphate-buffered saline, and seeded into 96-well round-bottom microtiter tissue culture plates at various effector: target cell ratios. Target cell samples ( $1 \times 10^4$  cells per well) were tested in triplicate. The cells were incubated for 4 h at

37 °C in a 5% CO<sub>2</sub> humidified incubator. Culture supernatants (50 µL) were then collected and combined with 50 µL of the substrate. The plates were covered with aluminum foil for protection against light and incubated at room temperature for 30 min, after which 50 µL of stop solution was added to each well. Absorbance at 490 nm was measured within 1 h of adding the stop solution. The results are expressed as the percentage of specific release based on the following formula: percent specific release =  $[(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$ .

## Statistical analysis

All values are expressed as mean  $\pm$  standard error of the mean. All experiments were repeated at least three times independently. Student's *t*-test and analysis of variance were employed for statistical analysis using GraphPad Prism 5 (San Diego, CA, USA). Differences were considered statistically significant at  $P < 0.05$ .

## Abbreviations

**TREM2:** Triggering receptor expressed on myeloid cells 2

**NK:** Natural killer

**pNK:** precursor natural killer

**mNK:** mature natural killer

**TREM2-TG:** TREM2-overexpressing transgenic

**HSC:** Hematopoietic stem cell

**TG-HSC:** Bone marrow-derived HSC of TREM2-TG mice

**PI3K:** Phosphatidylinositol 3-kinase

**MHC:** Major histocompatibility complex

**ITAM:** Immunoreceptor tyrosine-based activation motif

**Id2:** Inhibitor of DNA binding 2

**RT-PCR:** Reverse transcription-polymerase chain reaction

**TRAIL:** TNF-related apoptosis-inducing ligand

**WT+TREM2-Ig:** WT mice treated with TREM2-Ig

**Hu:** Humanized

**WT+hu-Ig:** WT mice treated with hu-Ig

**TG+TREM2-Ig:** TREM2-TG mice treated with TREM2-Ig

**TG+hu-Ig:** TREM2-TG mice treated with hu-Ig

**DMSO:** Dimethyl sulfoxide

**MACS:** Magnetic-activated cell sorter

## Declarations

### Ethics approval and consent to participate

This study includes data with laboratory animals. All animal experimental protocols were approved by the Ethics Committee of the Institutional Animal Care and Use Committee (IACUC) of Chonnam National University (CNU IACUC-YB-2017-19). The study was carried out in compliance with the ARRIVE guidelines.

### Consent for publication

Not applicable

### Availability of data and materials

Not applicable

### Competing interests

The authors declare that they have no competing interests.

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### Author's contributions

HSK and EMK designed the experiments. HYL, JWY, and EHL performed most of the experiments, including NK cell differentiation, flow cytometry analysis, and NK cytotoxicity assay. KYJ, HRC, SMK, and JWY performed the RT-PCR analysis and animal experiments. HYL, HSK, and EMK wrote the manuscript. All authors read and approved the final manuscript.

### Acknowledgements

Not applicable

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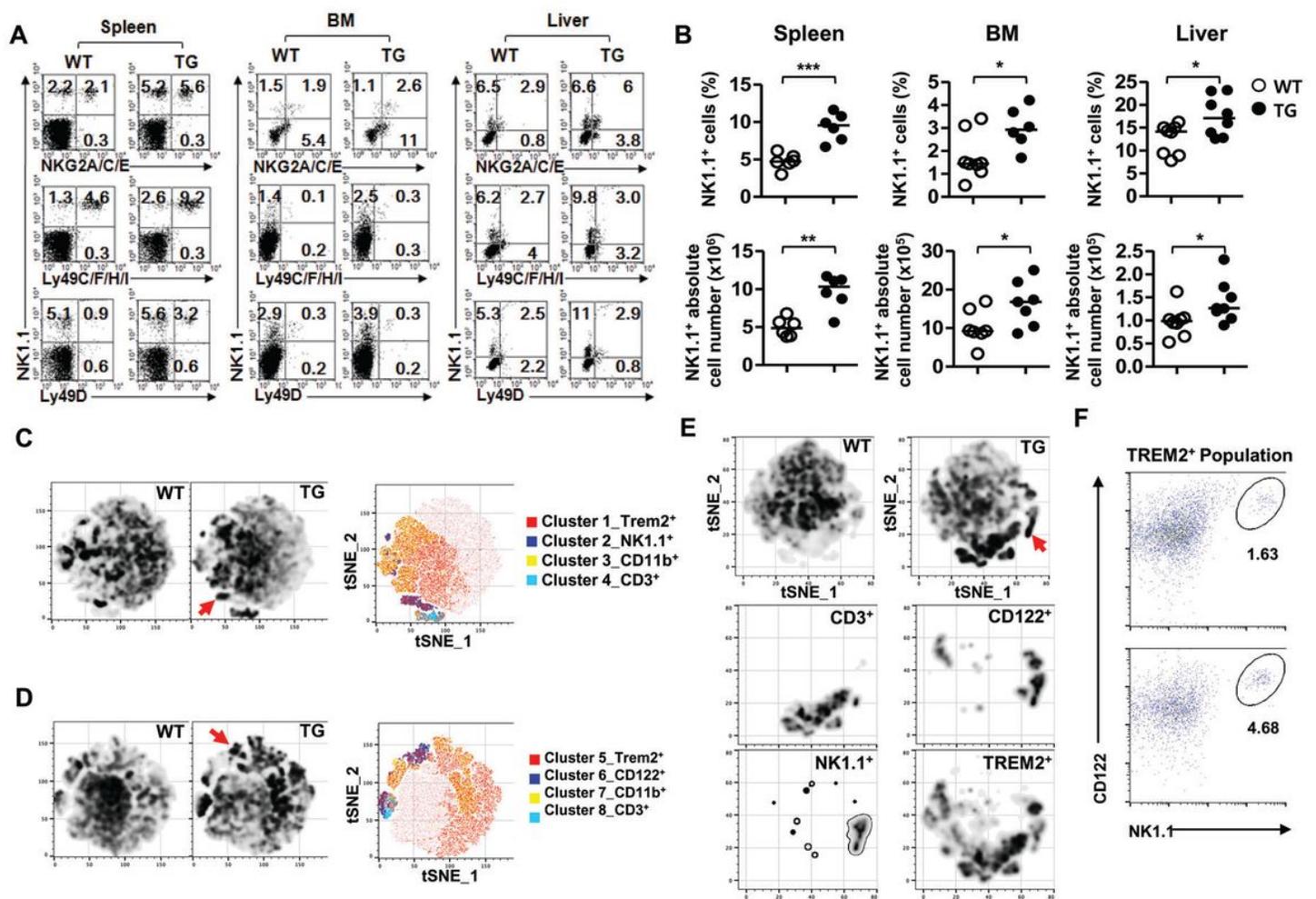
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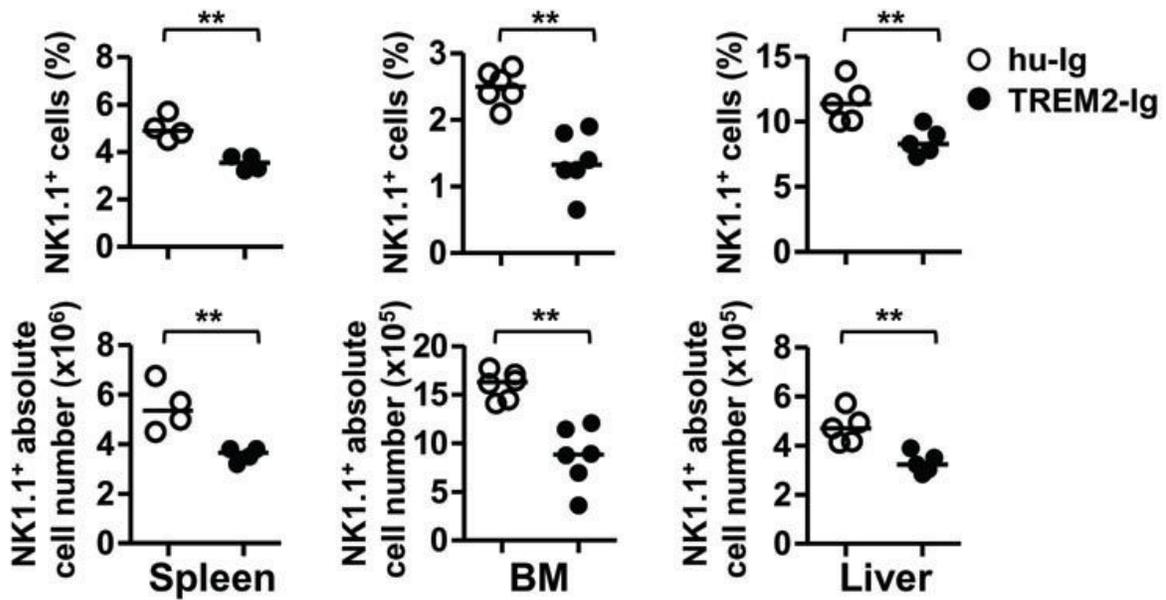
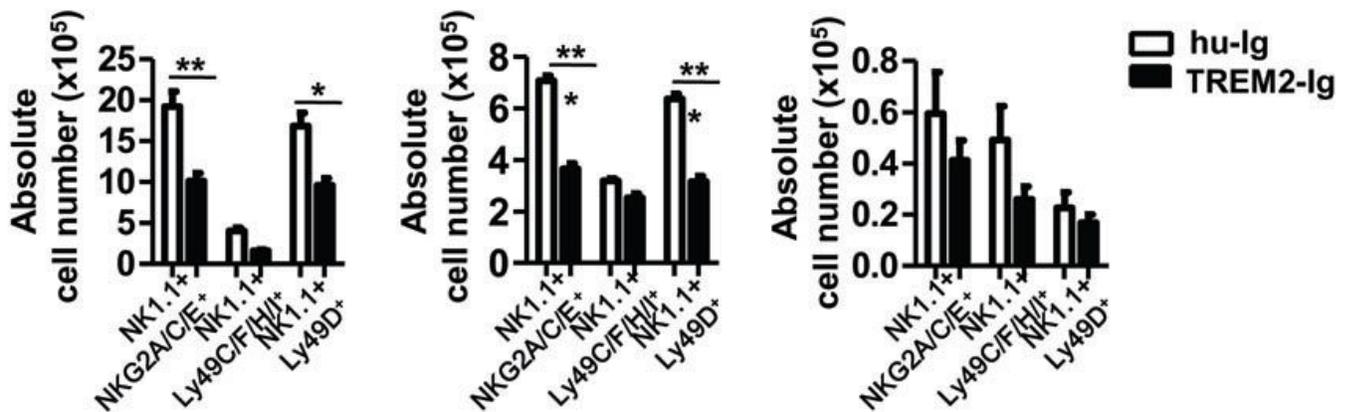
## Figures



**Figure 1**

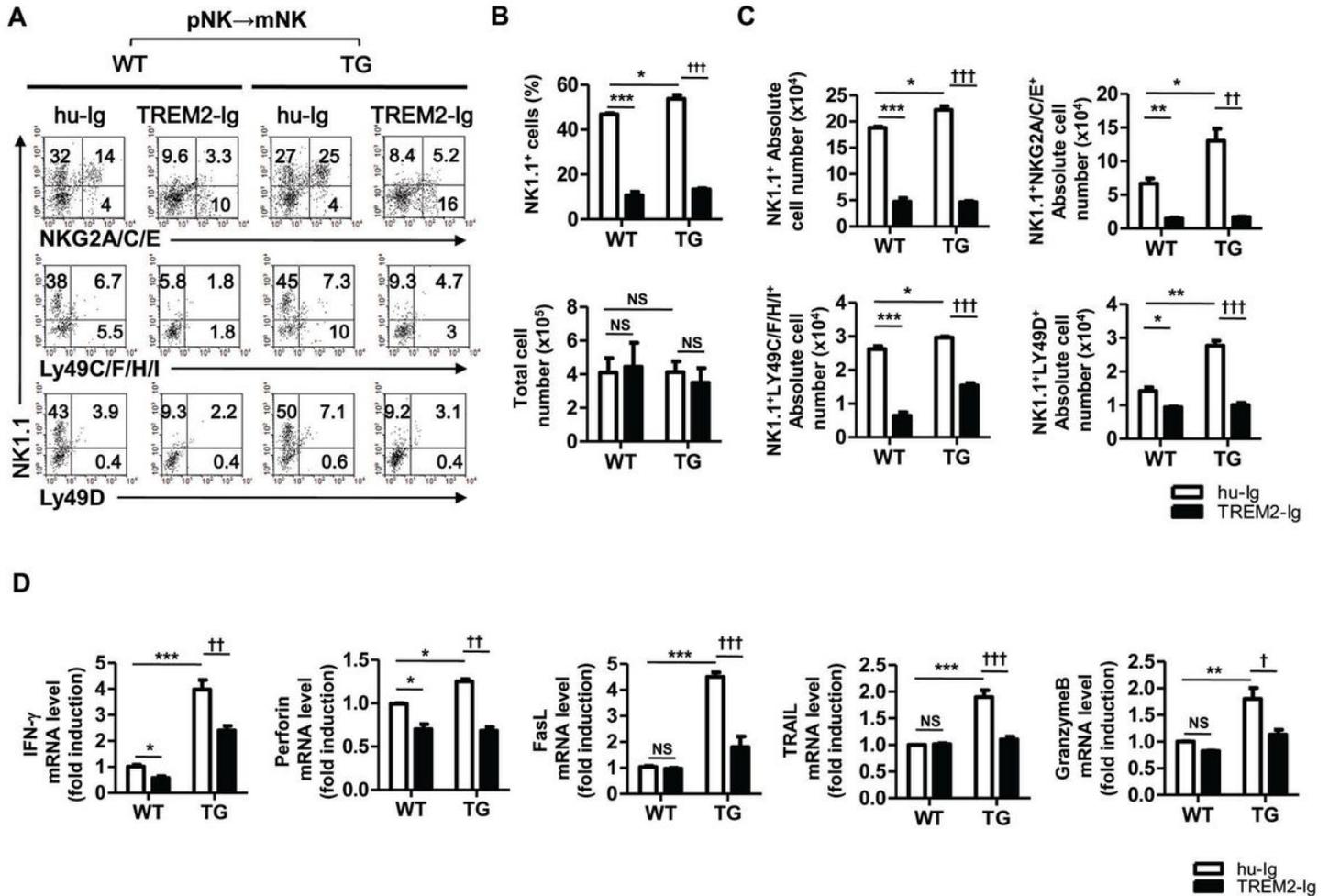
NK cell population increase in TREM2-TG mice. (A) Flow cytometry analysis data represent the surface expression of NK-specific receptors (NGK2A/C/E, Ly49C/F/H/I, Ly49D) in the spleen, BM, and liver of WT and TREM2-TG mice. (B) The percentage (top panel) and absolute number (bottom panel) of NK1.1<sup>+</sup> cells in the spleen (N=6), BM (N=7), and liver (N=8) of WT and TREM2-TG mice. Each symbol represents

an individual mouse; horizontal lines indicate the mean. \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 by Student's t-test. (C-E) Representative tSNE plots show BM cell population of WT (left panel) and TG mice (middle panel) stained with TREM2/NK1.1/CD11b/CD3/TREM2 (C) or TREM2/CD122+/CD11b/CD3 specific antibodies (D). Each cluster represents a group of TREM2+ (Cluster1, 5), CD11b+ (Cluster 3,7), CD3+ (Cluster4, 8), NK1.1+ (Cluster2), and CD122+ (Cluster 6) cell population, respectively (C and D right panel). (C, right panel) Cluster2 (indigo) represents NK1.1+ cells and cluster1 (red) represents TREM2+ cells (D, right panel). Red circles indicate the TREM2+ population on the NK1.1+ cells (C) or CD122+ cells (D). (E) These tSNE plots showed CD3+, CD122+, NK1.1+, and TREM2+ cell population in the BMs of WT (left panel) and TG mice (right panel). Red circles indicate the CD3-CD122+NK1.1+TREM2+ population in BMs of TG mice. Each tSNE plot represents CD3+, CD122+, NK1.1+, TREM2+ cell population separately. (F) Dot plots represent that BM of TG mice (lower) and WT mice (upper), which were stained with TREM2, CD122+, and NK1.1+ antibodies. Black circles indicate CD122+NK1.1+ population in the TREM2+ population. tSNE plots and dot plots were analyzed using Flow jo software.

**A****B****Figure 2**

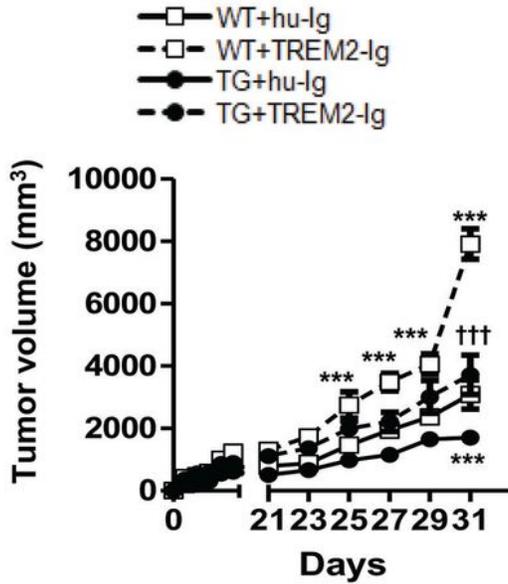
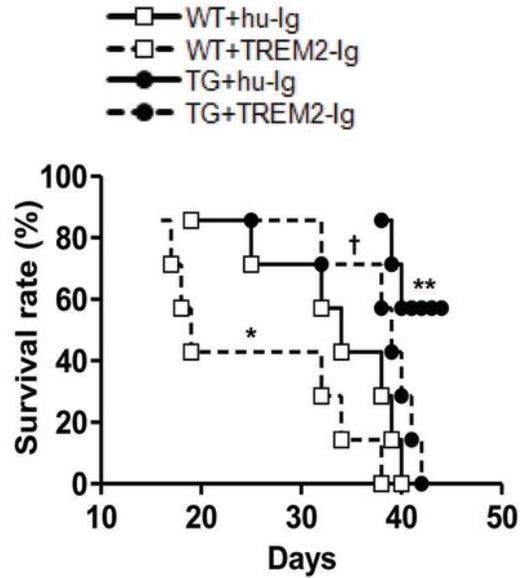
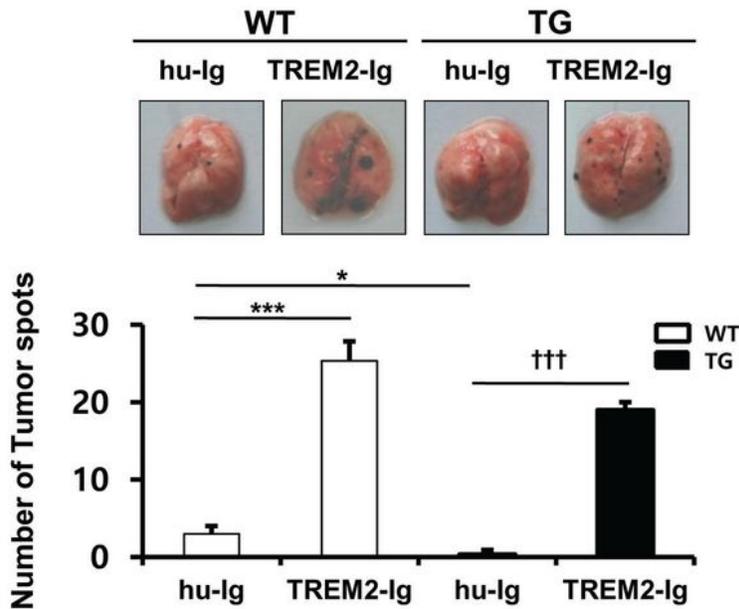
Blockade of TREM2 signaling reduces the NK cell population in vivo. (A) WT mice were injected with 100  $\mu$ g of TREM2-Ig or hu-Ig (control) twice per week for 4 weeks intraperitoneally, then the expression of NK cell-specific receptors in the spleen, BM, and liver cells of WT mice was analyzed using flow cytometry. (A) The percentage (top panel) and absolute number (bottom panel) of NK1.1+ cells from the spleen (N=4), BM (N=6), and liver (N=5) of mice as shown in Fig. 1A. Each symbol represents an individual mouse; horizontal lines indicate the mean. (B) Graphs of absolute cell number of NK cells expressing each NK cell receptor in the spleen (left panel), BM (middle panel), and liver (right panel) of mice treated with hu-Ig (open bar) or TREM2-Ig (solid bar). For each population, the absolute number determined by

calculation from flow cytometry profiles. Data are shown as mean  $\pm$  SEM from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  by based on two-way ANOVA analysis with Bonferroni posthoc test.



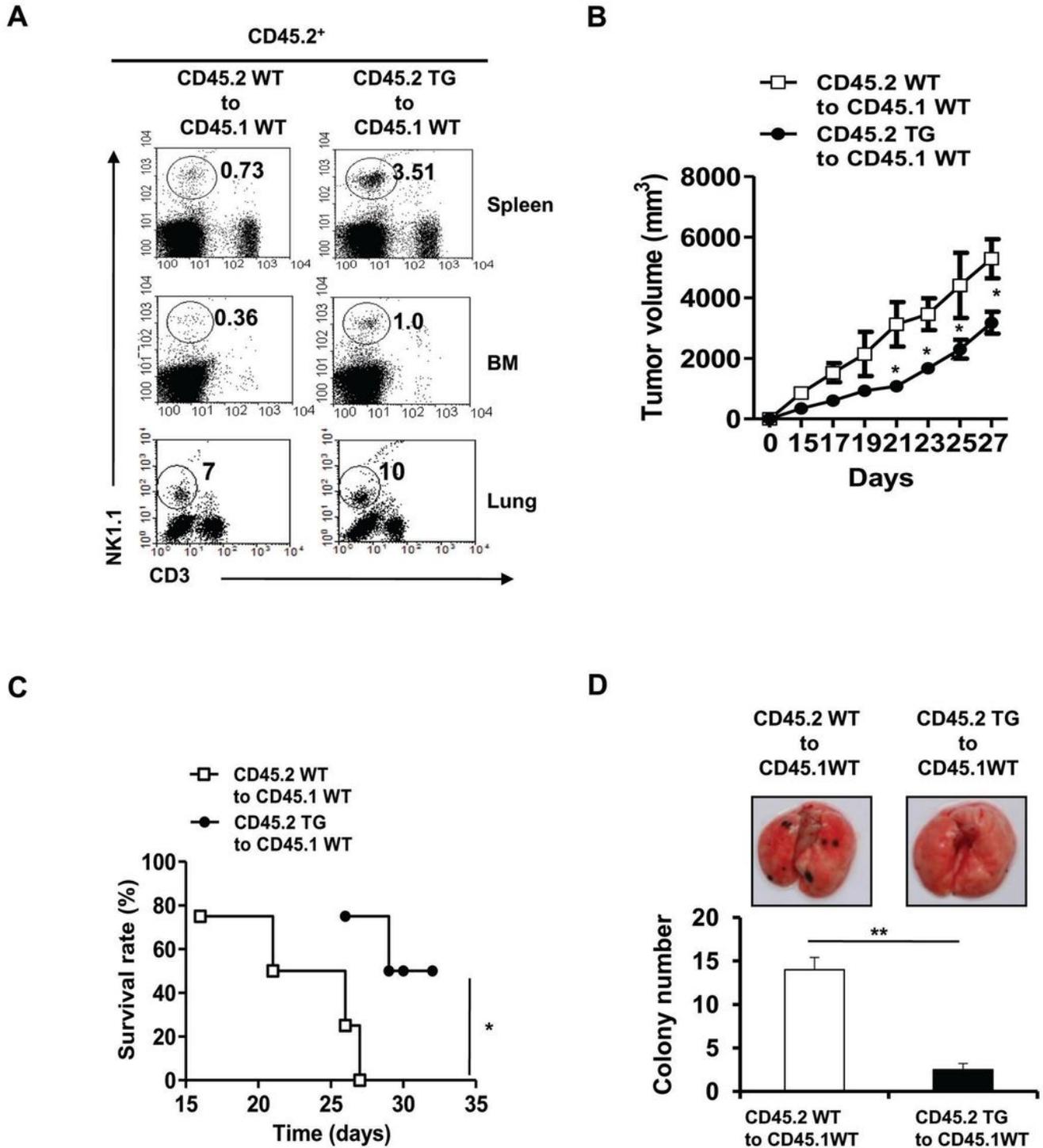
**Figure 3**

Effects of TREM2 on the differentiation of NK cells from HSCs in vitro. (A) Representative flow cytometry plots of NK cell receptor expression on the differentiated NK cells from WT-HSCs or TREM2-TG-HSCs. Cells treated with hu-Ig or TREM2-Ig during the pNK to mNK cell differentiation simultaneously. (B) Graphs indicate the total number of cells (upper panel) and percentage of NK1.1+ cells (lower panel) differentiated from WT or TG -HSCs, where were treated with hu-Ig (open bar) or TREM2-Ig (solid bar). (C) Graphs show the absolute cell number of the NK1.1+ cell population, NK receptor-expressing NK cells that were differentiated from WT-HSCs treated with hu-Ig (open bar) or TREM2-Ig (solid bar). (D) Real-time-qPCR analysis of IFN- $\gamma$ , Perforin, Granzyme B, FasL, and TRAIL mRNAs in mNK cells derived from WT-HSCs or TG-HSCs. Values are presented as the mean  $\pm$  standard error of the mean of three independent experiments (B-D). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. WT-hu-Ig and † $P < 0.05$ , †† $P < 0.01$ , and ††† $P < 0.001$  vs. TG-hu-Ig by two-way ANOVA analysis with Bonferroni posthoc test. Three independent experiments were performed.

**A****B****C****Figure 4**

TREM2 signaling prevents tumor progression in vivo. (A) Tumor volumes of WT and TREM2-TG mice. WT and TREM2-TG Mice were injected with B16-F10 cells subcutaneously after intraperitoneal injection of hu-Ig or TREM2-Ig. Tumor volumes were measured every two days. (B) The survival rate of tumor-bearing WT and TREM2-TG mice. Seven mice per group were used. Survival probabilities were analyzed using the Kaplan–Meier method. The significance of differences between groups assessed using the log-rank test.

All statistical tests were two-sided, with  $*P < 0.05$  taken to indicate significance. Significance of difference between samples was determined using two-way ANOVA analysis with Bonferroni posthoc test. WT+hu-Ig vs. TG+hu-Ig ( $**P < 0.01$ ), WT+hu-Ig vs. WT+TREM2-Ig ( $*P < 0.05$ ), and TG+hu-Ig vs. TG+TREM2-Ig ( $\dagger P < 0.05$ ,  $\dagger\dagger\dagger P < 0.001$ ) were compared. (C) Representative photographs of lungs with metastatic colonies from mice of each group (top panel). Graph quantitates the total number of metastatic colonies in the lungs of each group treated with hu-Ig or TREM2-Ig (bottom panel). WT+hu-Ig vs. TG+hu-Ig ( $*P < 0.01$ ), WT+hu-Ig vs. WT+TREM2-Ig ( $***P < 0.05$ ), and TG+hu-Ig vs. TG+TREM2-Ig ( $\dagger\dagger\dagger P < 0.001$ ) were compared. Significance of difference between samples was determined using Student's t-test.



**Figure 5**

Adoptive bone marrow transplantation of TREM2 TG mice increase NK cell population in WT mice. (A) Representative flow cytometry plots of CD3-NK1.1+ cells from BM, spleen, and lung of lethally irradiated WT CD45.1 recipient 4 weeks after tail vein injection of total BM from WT or TREM2-TG (CD45.2) donors. (B) Graph depicting tumor volumes in tumor-bearing CD45.1 WT mice after intravenous injection with BM cells of CD45.2 WT (open square, N=4) or CD45.2 TREM2-TG mice (solid circle, N=4). Tumor volume was

measured every 2 days, after 15 days of injection. Significance of difference was analyzed by two-way ANOVA analysis with Bonferroni posthoc test (\*P < 0.05). Three independent experiments repeated. (C) The survival rate of tumor-bearing CD45.1 WT mice after intravenous injection with BM cells from CD45.2 WT (open square, N=4) or CD45.2 TREM2-TG mice (solid circle, N=4). \*P < 0.05. (D) Representative photographs of lungs with metastatic colonies (top panel) of tumor-bearing CD45.1 WT mice after intravenous injection with BM cells of CD45.2 WT or CD45.2 TREM2-TG mice. Graph quantifies the total number of metastatic colonies in the lung (bottom panel). \*\*P < 0.01 using Student's t-test.

## Supplementary Files

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