CD99-Dependent Expansion of Myeloid-Derived Suppressor Cells and Attenuation of Graft-Versus-Host Disease

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CD99 is involved in many cellular events, such as the generation of Hodgkin and Reed-Sternberg cells, T cell co-stimulation, and leukocyte transendothelial migration. However, these studies have been limited to *in vitro* or *in vivo* experiments using CD99-deficient cell lines or anti-CD99 antibodies. In the present study, using CD99-deficient mice established by the exchangeable gene trap method, we investigated the physiologic function of murine CD99. In a B6 splenocytes → bm12 graft-versus-host disease model, wild-type cells were minimally lethal, whereas all mice that received CD99-deficient donor cells developed an early and more severe pathology. Graft-versus-host disease in these mice was associated with insufficient expansion of myeloid-derived suppressor cells. This was confirmed by experiments illustrating that the injection of wild-type donor cells depleted of Mac-1^{+} cells led to an almost identical disease course as the CD99-deficient donor system. Therefore, these results suggest that CD99 plays a crucial role in the attenuation of graft-versus-host disease by regulating the expansion of myeloid-derived suppressor cells.

INTRODUCTION

Human CD99, encoded by the *MIC2* gene in pseudoautosomal region 1 (PAR1) of the X chromosome, is a ubiquitous 32 kDa transmembrane protein with a highly O-glycosylated extracellular region (Hahn et al., 1997; Park et al., 2005). It is expressed in all leukocyte lineages and involved in many cellular events. Engagement of CD99 on human thymocytes with agonistic antibodies induces homotypic aggregation (Hahn et al., 1997), apoptosis (Bernard et al., 1997), and upregulation of TCR and MHC class molecules on the surface of thymocytes (Choi et al., 1998). In mature T cells, CD99 delivers effective co-stimulatory signals (Oh et al., 2007). In B cells, the downregulation of CD99 by EBV-encoded latent membrane protein-1 (LMP-1) leads to the generation of Hodgkin and Reed-Sternberg cells related to Hodgkin's disease (Kim et al., 1998; 2000; Lee et al., 2011). Moreover, transendothelial migration of monocytes is also regulated by human CD99 (Schenkel et al., 2002). In addition to these observations in humans, a mouse homologue of human CD99 (also designated D4) was identified as a ligand of the paired Ig-like type 2 receptor (PILR) (Park et al., 2005; Shiratori et al., 2004). Its functional analogy with human CD99 is supported by reports that it also participates in the transendothelial migration of leukocytes and recruitment into inflamed tissue (Bixel et al., 2004; Dufour et al., 2008). Until recently, however, these studies have been limited to *in vitro* or *in vivo* experiments using CD99-deficient cell lines or anti-CD99 antibody due to the unavailability of CD99-deficient mice.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of activated immature myeloid cells with morphology similar to granulocytes or monocytes (Movahedi et al., 2008) that accumulate under numerous pathologic conditions including cancer, infection, inflammatory disease, and stress (Gabrilovich and Nagaraj, 2009). MDSCs are characterized by the co-expression of myeloid-cell lineage differentiation antigen Gr-1 and CD11b (Mac-1) in mice (Gabrilovich and Nagaraj, 2009). MDSC expansion and activation are influenced by several factors, representative of which are cyclooxygenase 2 (also known as PTGS2) and vascular endothelial growth factor (VEGF), which are produced by tumor cells, tumor stromal cells, and activated T cells (Gabrilovich and Nagaraj, 2009). These factors are mainly involved in the upregulation of immune suppressive factors in MDSCs and their expansion. MDSCs were recently reported to play a potentially important role in determining the severity of graft-versus-host disease (GVHD) (Rao et al., 2003) by suppressing alloreactivity (Highfill et al., 2010; Morecki et al., 2008).

In the present study, we found that there was significant aggravation of GVHD when splenocytes of CD99-deficient mice

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were used as donor cells. In subsequent experiments to identify the mechanism by which CD99 is related to the disease course of GVHD, we found that expansion of MDSCs is severely compromised in the absence of CD99.

MATERIALS AND METHODS

Mice
A heterozygote mutant C56BL/6 mouse clone (CD99$^{+/GT}$) with an insertional mutation between exons 2 and 3 of the CD99 gene (21-B6T44 clone in Exchangeable Gene Trap Clone database; http://egtc.jp/action/access/clone_detail?id=21-B6T44) was produced using the pU-21T exchangeable gene trap vector and deposited in the Center for Animal Resources and Development (CARD) R-BASE of the Kumamoto University of Japan (http://cardb.cc.kumamoto-u.ac.jp/transgenic/index.jsp). Wild-type C57BL/6J (B6, H-2^b), B6.C-H2bm12/KhEg (bm12), and CD45.1 congenic B6 mice were purchased from the Jackson Laboratory (USA). CD99$^{GT/GT}$ mice were backcrossed to CD45.1$^+$ congenic B6 mice to generate CD45.1$^+$ CD99$^{GT/GT}$ mice. All mice were maintained at the Center for Animal Resource and Development at Seoul National University. The animal studies were performed after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (IACUC approval No. SNU-091125-5).

Genotyping of CD99$^{GT/GT}$ mice
DNA samples for CD99$^{GT/GT}$ genotyping were extracted from the tails of mice and PCR was performed using tail genomic DNA as a template. For wild-type alleles, a 5′ primer (5′-CGA GTGACGACTTCAACCTGGGC-3′) located in exon 2 and a 3′ primer (5′-TGAGTCTCCGTGTGGCCTTG-3′) located in exon 5 were used to generate a 917 bp wild-type fragment. PCR was performed for 35 cycles (60 s at 94°C; 60 s at 60°C; 60 s at 72°C). To detect the trap allele, a 5′Z-1 primer (5′-GCGTTACC CAACTTAATCG-3′) and a 3′ Z-2 primer (5′-TGTGAGCGAGT AACAACCCG-3′) located in the pU21 were used to generate a 320 bp fragment. For amplification of the 3′ flanking region of the trap vector, a 5′ primer (5′-AAGGCCCCAACGCCCAAGA AGCC-3′) located in exon 3 and a 3′ primer (5′-AGGGCGTCC TCCAGG TCGAA-3′) located in exon 4 were used.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis
Total cellular RNA was prepared from spleens using the RNeasy Mini kit (Qiagen, USA) according to the manufacturer's instructions. cDNA was synthesized using oligo-dT primers, and used as a template for PCR with the following primers: mouse CD99, 5′ primer (5′-CCAGTGACGACTTCAACCTG-3′) and 3′ primer (5′-GATAGGCCACGAAGCTCGACAC-3′); mouse β-actin, 5′ primer (5′-GACGGCCAGGTCATCACTAT-3′) and 3′ primer (5′-GTACTTGCGCTCAGGAGGAG-3′).

Antibodies and flow cytometric analysis
FITC-, PE-, and APC-anti-mouse CD4 (RM4.5), CD8 (53-6.7), PE-anti-mouse CD11b (M1/70), APC-anti-mouse CD11c (HL3), PerCy5.5-anti-mouse CD45.1 (A20), FITC-anti-mouse B220 (RA3-6B2), APC-anti-mouse NK1.1 (PK136), FITC-anti-mouse TCRβ (H57-597), APC-anti-mouse Gr-1 (RB6-8C5) antibodies, and APC-streptavidin were purchased from eBioscience (USA) or BD Biosciences (USA). PE- and biotin-conjugated anti-mouse CD99 (EJ2) monoclonal antibody was obtained from Dinona (Korea). Fresh cell suspensions of thymocytes, splenocytes, and lymph nodes were stained with antibodies and then

analyzed using a flow cytometer (FACSCalibur; BD, USA) and CellQuest Pro software (BD, USA).

Induction of GVHD
Recipient bm12 mice received 600 cGy of irradiation from a ^{137}Cs source split into two doses and separated by 4 h. One day later, 1.5×10^7 unfractionated spleen cells from B6 mice were intravenously injected into irradiated recipients. For adoptive transfer of fractionated cells, splenocytes were subjected to magnetic cell sorting (Miltenyi Biotec, USA), according to the manufacturer's instructions, and purity was usually greater than 95%. To compare the development of GVHD between recipients of fractionated and unfractionated populations, the number of CD4$^+$ T cells injected into each mouse was equalized. At the indicated times, the bone marrow and spleen cells were harvested for flow cytometric analysis, and the cytokine concentration in serum was measured by cytometric bead array (BD).

Suppressor assay
To investigate the *in vitro* suppressor activity of MDSCs, a mixed lymphocyte reaction was performed. CD4$^+$ T cells were purified from CD45.1 B6 mice via magnetic sorting and labeled with CFSE as described previously (Lo et al., 2011). CD45.1 B6-derived Mac-1$^+$Gr-1$^+$ MDSCs were purified from the spleens of bm12 mice, who had received CD45.1 B6 splenocytes, via flow cytometric sorting. T cell-depleted splenocytes were purified from B6 mice via magnetic sorting. CFSE-labeled CD4$^+$ T cells (3×10^5) and 2,000 rad-irradiated bm12 splenocytes (3×10^5) were cultured in the presence of either sorted MDSCs or T cell-depleted B6 splenocytes (3×10^5). After 5 days' culture, cells were stained with anti-CD4 and anti-CD45.1 antibodies and the percentage of CFSE^{lo} cells in the gated CD45.1$^+$CD4$^+$ population was measured via flow cytometric analysis.

EL4 tumor model
Mice were subcutaneously injected with 5×10^6 EL4 cells and monitored every 2 to 3 days to evaluate tumor growth. Subcutaneous tumors were measured with calipers along the perpendicular axis of the tumor and size was expressed as tumor volume based on the following formula: tumor volume (mm^3) = (major axis) $\times$ (minor axis)$^2 \times$ 0.52 (Li et al., 2004).

Statistical analysis
All data were analyzed using GraphPad Prism software (Graph-Pad Software, USA). Time curves for progression to death were prepared by the Kaplan-Meier method. Significance between animal groups was computed using a *t*-test. A value of *p* < 0.05 was considered statistically significant.

RESULTS

Systemic deletion of CD99 does not affect the cellularity of spleen leukocyte subpopulations
To investigate the physiological role of mouse CD99 in the development and function of immune cells, heterozygote 21-B6T44 clone mice, in which pU-21T exchangeable gene trap vectors were inserted into the CD99 (pilr-l) gene, were intercrossed, and the offspring were screened via PCR analysis of genomic DNA. When primer sets specific for the inserted gene trap vector or targeted to exon 2 and exon 5 of the mouse CD99 gene were used, insertion of the gene trap vector between exons 2 and 3 of the CD99 gene caused the wild-type allele to be unamplified and thus the genotype was easily identified (Fig. 1A, upper and middle). We also excluded the possibility that a large deletion in the 3′ flanking region of the trap

A

B

C

D
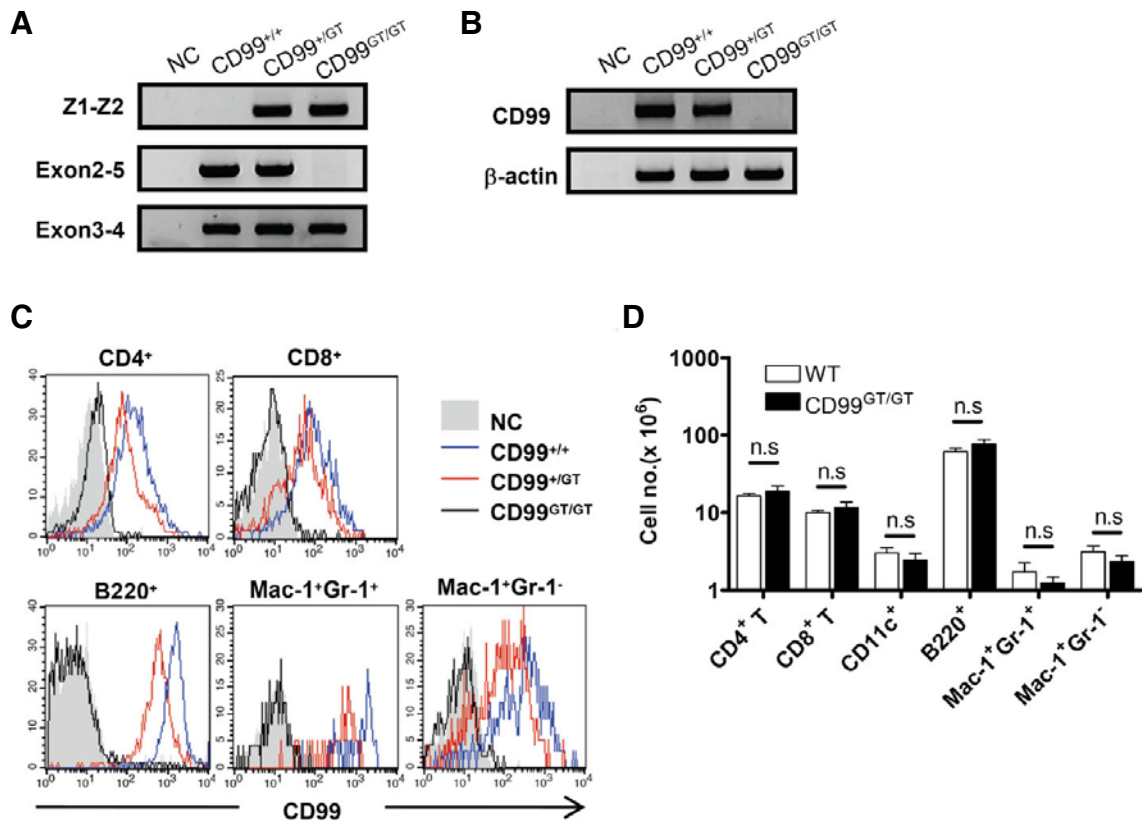
Fig. 1. Genotypic and phenotypic analysis of wild-type mice and insertional mutants in CD99 via gene trap. (A) Representative results of PCR genotyping. To detect wild-type and trap alleles, genomic DNA was prepared from the tails of wild-type (CD99$^{+/+}$), heterozygous (CD99$^{+/GT}$), and homozygous insertional mutant (CD99$^{GT/GT}$) mice, amplified using a primer pair that detected the pU-21T gene trap vector, murine CD99 exons 2 and 5, or murine CD99 exons 3 and 4, and analyzed in parallel with a negative control (NC). (B) Absence of CD99 gene transcripts in CD99$^{GT/GT}$ splenocytes. RT-PCR analysis of splenocytes obtained from wild-type, CD99$^{+/GT}$, and CD99$^{GT/GT}$ mice demonstrated the expression of CD99 transcripts only in wild-type and CD99$^{+/GT}$ mice. Transcription of β-actin was used as a positive control. (C) No expression of CD99 protein on the surface of CD99$^{GT/GT}$ splenocytes. Flow cytometric analysis of splenocytes gated on each population revealed higher CD99 expression on the surface of B cells (B220$^+$) and Mac-1$^+$Gr-1$^+$ cells than that of T cells and macrophages (Mac-1$^+$Gr-1$^-$). CD99 expression level in CD99$^{+/GT}$ mice was about two-fold lower than that of wild-type littermates. (D) Cellularity of each leukocyte subpopulation in spleen is not affected by CD99 deletion. The absolute number of splenic cell subsets of wild-type and CD99$^{GT/GT}$ mice was calculated based on the relative proportion determined by flow cytometry. The data represent five mice per group. n.s, not significant.

vector was present, via amplification of the region encompassing the exons 3 and 4 of the CD99 gene (Fig. 1A, lower). The absence of CD99 gene transcripts in the homozygote mutant was confirmed via RT-PCR analysis of RNA extracted from the spleen (Fig. 1B), whereas the expression of CD99 in wild-type spleens has been documented (Park et al., 2005).

Next, we compared CD99 expression in spleen cell subpopulations using flow cytometry (Fig. 1C). Consistent with our RT-PCR results, CD99 was not expressed in splenocytes of homozygous mutant B6 mice. In wild-type B6 mice, the expression level of CD99 in B cells and Mac-1$^+$Gr-1$^+$ cells was higher than that in T cells. In the splenocytes of heterozygous mice (CD99$^{+/GT}$), the CD99 expression level was half of that of the wild-type. Systemic deletion of CD99 did not affect the cellularity of each leukocyte subpopulation in spleen (Fig. 1D) and both heterozygote and homozygote mice appeared normal and were fertile.

Development of lethal GVHD and insufficient expansion of MDSCs in irradiated bm12 mice that received CD99$^{GT/GT}$ splenocytes

To compare the immune function of CD99-deficient mice to that

of wild-type mice, we used a GVHD model, which was generated by B6 splenocyte transfer into irradiated MHC class II-different bm12 hosts (B6 splenocytes → bm12) (Rao et al., 2003). In accordance with previous descriptions (Rao et al., 2003; Sprent et al., 1990; 1995), when unfractionated splenocytes from B6 mice were transferred into sublethally irradiated bm12 mice, host mice recovered their body weight after transient weight loss during the first week post-transplant and showed minimal lethality during the 10-week follow-up period (Fig. 2A). In contrast, inoculation with CD99-deficient splenocytes caused continuous weight loss in recipients resulting in early death of all CD99$^{GT/GT}$ splenocytes → bm12 mice (Fig. 2A). In agreement with the clinical course of the disease, CD99 deficiency in donor cells caused a more severe drop in cellularity of host cells in spleen and bone marrow (Fig. 2B, left).

In the B6 splenocytes → bm12 GVHD model, the donor non-T cell compartment represses CD4$^+$ T cell-mediated acute GVHD (Rao et al., 2003). The clinical course in bm12 mice transplanted with purified B6 CD4$^+$ T cells in that study was similar to that of bm12 mice that received CD99$^{GT/GT}$ splenocytes in the present study. Based on these findings, we assessed

whether the lack of CD99 expression in donor splenocytes affected the non-T cell compartment. Seven days after donor cell transfer, there was no statistically significant difference in the number of donor cells in spleens of bm12 mice that received wild-type and CD99-deficient splenocytes (Fig. 2B, right, and Table 1). During the next 7 days, there was a three-fold increase in the total number of donor cells in the spleens of wild-type cell recipients (Fig. 2B, right, and Table 1). Specifically, a marked increase in the number of Mac-1$^+$Gr-1$^+$ MDSCs (about 22-fold) contributed to the major change in donor cell number, and thus donor cells were composed of 40% MDSCs, which were more abundant than CD4$^+$ T cells 14 days after transplant (Table 1 and Fig. 2C). This was in sharp contrast to the three-fold increase in the number of CD99-deficient MDSCs during the same period (Table 1). In contrast, there was less difference in the cell numbers of other donor-derived cell populations between the two groups of mice (Table 1). Therefore, taken together, these results suggest that CD99 may modulate GVHD progression by promoting the expansion of MDSCs.

Table 1. Summary of donor cell numbers in recipient spleens

Donor cell type	Days	Cell no. (mean $\pm$ SE $\times 10^5$)*			Fold change	
		CD99$^{+/+}$	CD99$^{GT/GT}$	p	CD99$^{+/+}$	CD99$^{GT/GT}$
Total	7	59 $\pm$ 10	49 $\pm$ 11	0.53	3.4	1.6
	14	197 $\pm$ 30	76 $\pm$ 11	0.002		
CD4$^+$ T	7	31 $\pm$ 5	23 $\pm$ 5	0.32	1.2	0.9
	14	36 $\pm$ 7	20 $\pm$ 3	0.06		
CD8$^+$ T	7	12 $\pm$ 2	10 $\pm$ 2	0.43	2.0	1.4
	14	25 $\pm$ 6	14 $\pm$ 2	0.07		
B cell	7	8 $\pm$ 2	5 $\pm$ 1	0.15	1.5	1.0
	14	13 $\pm$ 4	5 $\pm$ 1	0.04		
Mac-I$^+$Gr-1$^+$	7	4 $\pm$ 1	6 $\pm$ 1	0.22	21.6	2.7
	14	84 $\pm$ 13	16 $\pm$5	0.0005		

*Number of mice in each group: CD99$^{+/+}$ day 7 ($n = 4$), CD99$^{GT/GT}$ day 7 ($n = 5$), CD99$^{+/+}$ day 14 ($n = 4$), CD99$^{GT/GT}$ day 14 ($n = 6$).
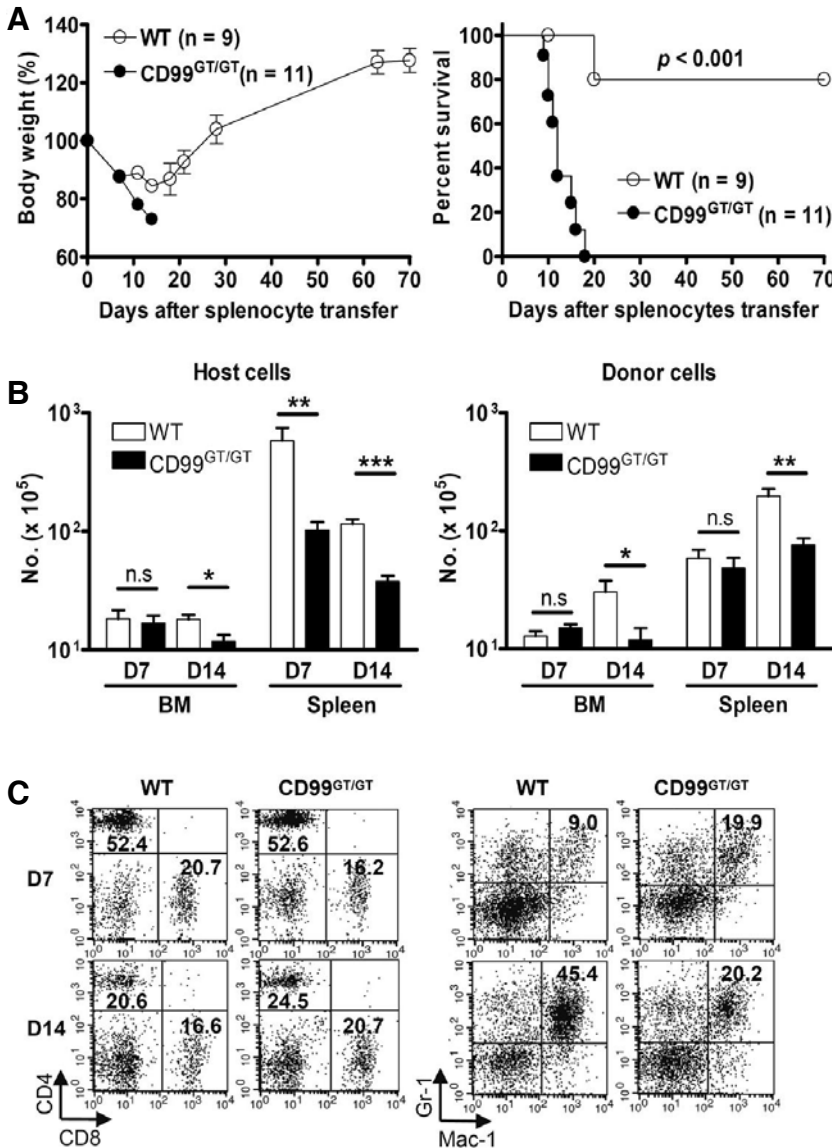
Fig. 2. The absence of CD99 on donor cells aggravates acute GVHD in MHC class II-only disparate recipients. (A) Early death of bm12 mice that received CD99$^{GT/GT}$ splenocytes compared to recipients of wild-type cells. Sublethally irradiated bm12 mice were infused with 1.5 $\times$ 10^7 splenocytes obtained from wild-type (WT) or CD99$^{GT/GT}$ donor mice on day 0. Mice were monitored for body weight (left) and survival (right). Data are pooled from two independent experiments and numbers in parentheses are the total number of recipient mice in each group. (B) Decreased expansion of CD99-deficient donor cells, compared to wild-type cells in bm12 mice. To assess the severity of GVHD, cells in bone marrow (BM) and spleen were analyzed by flow cytometry on days 7 and 14 after adoptive transfer, and the absolute number of host (left) and donor (right) cells were calculated by multiplying the percentage of CD45.1$^-$ (host) and CD45.1$^+$ (donor) by the cellularity. Data are mean values $\pm$ SEM from four to six animals in each group and representative data from two independent experiments are shown. (C) Defect in the expansion of donor-derived Mac-1$^+$Gr-1$^+$ MDSCs in bm12 mice that received CD99$^{GT/GT}$ splenocytes compared to recipients of wild-type cells. To evaluate the fraction of donor CD4$^+$ T cells, CD8$^+$ T cells, and Mac-1$^+$Gr-1$^+$ MDSCs, cells from the spleens of recipient bm12 mice were stained with the indicated antibodies on days 7 and 14 after adoptive transfer of 1.5 $\times$ 10^7 wild-type or CD99-deficient splenocytes and were analyzed by flow cytometry. Representative data from three independent experiments are shown. n.s, not significant; *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$.
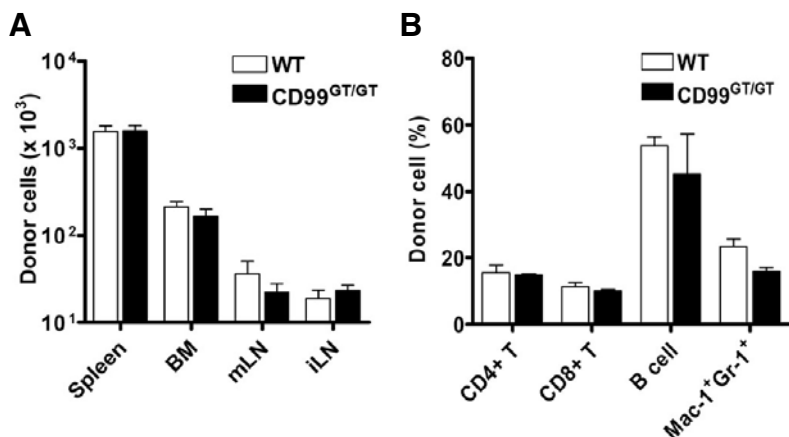
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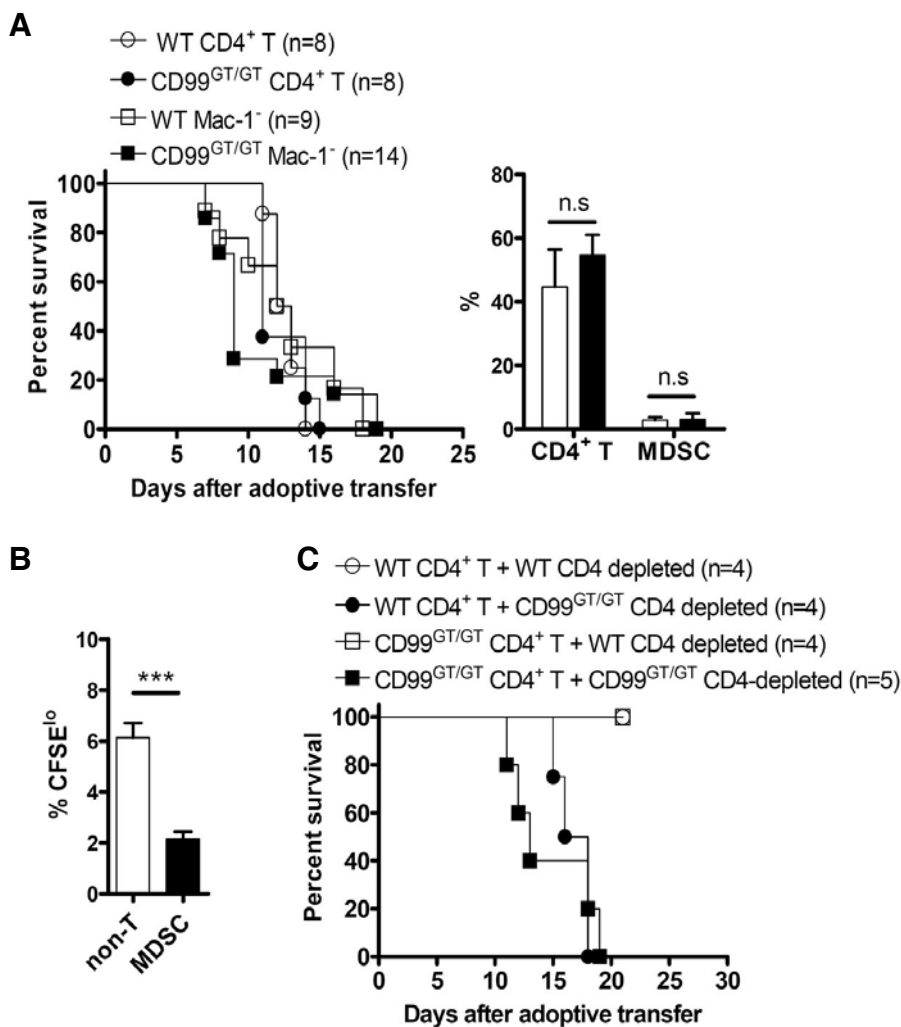
Fig. 3. Essential role of CD99 in the non-lymphoid population for suppression of GVHD. (A) Accelerated mortality of bm12 mice received Mac-1-depleted splenocytes from wild-type or CD99$^{GT/GT}$ mice. Groups of bm12 mice received purified CD4$^+$ T cells (2.5×10^6) or Mac-1-depleted cells (Mac-1$^-$; 1.5×10^7) from wild-type (WT) or CD99$^{GT/GT}$ mice on day 0. The survival rate pooled from two independent experiments is shown, and numbers in parentheses indicate the total number of recipient mice in each group (left). The percentages of CD4$^+$ T cells and Mac-1$^+$Gr-1$^+$ MDSCs among the donor cells in the spleens of recipients were also measured via flow cytometric analysis on day 10 after adoptive transfer, and data are mean values $\pm$ SEM from three animals in each group (right). (B) MDSCs suppress T cell allo-response *in vitro*. CFSE-labeled CD45.1 B6 CD4$^+$ T cells were stimulated with irradiated bm12 splenocytes in the presence of either T cell-depleted B6 splenocytes (non-T) or Mac-1$^+$Gr-1$^+$ MDSCs derived from donor cells in bm12 mice received B6 splenocytes. After 5 days' culture, the percentage of CFSE^{lo} cells in the gated CD4$^+$ T population was compared via flow cytometric analysis. (C) The suppression of GVHD progress depends on the CD99 in the non-T cell population. Purified CD4$^+$ T cells (2.5×10^6) were co-transferred with CD4-depleted cells (1.25×10^7) into sublethally irradiated bm12 mice as indicated; survival rate is shown. Numbers in parentheses are the total number of recipient mice in each group.

Defect in MDSC expansion from CD99-deficient donor cells accelerates lethal GVHD

To determine whether the defect in MDSC expansion culminates in the accelerated mortality of CD99$^{GT/GT}$ splenocytes → bm12 mice, we transferred purified CD4$^+$ T cells or Mac-1-depleted splenocytes from B6 mice into the sublethally irradiated bm12 mice. Consistent with a previous report (Rao et al., 2003), injection of CD4$^+$ T cells caused rapid disease and the survival rate was not affected by the lack of CD99 in donor cells (Fig. 3A, left), indicating that CD99 expressed on CD4$^+$ T cells had no influence on their alloreactivity. Moreover, groups of mice that received Mac-1-depleted splenocytes from wild-type or CD99$^{GT/GT}$ mice also followed a similar disease course (Fig. 3A, left). In the spleens of these recipients, the fraction of donor-derived MDSCs was markedly reduced by Mac-1-depletion prior to adoptive transfer (Fig. 3A, right). To further investigate whether MDSCs could suppress the alloreactivity of CD4$^+$ T cells, donor-derived Mac-1$^+$Gr-1$^+$ MDSCs, which expanded after transfer of wild-type splenocytes to irradiated bm12 mice, were purified, and CFSE-labeled CD45.1 B6 CD4$^+$ T cells were stimulated with irradiated bm12 splenocytes in the presence of MDSCs or T cell-depleted B6 splenocytes as a negative control. After 5 days' culture, about 6% of CD4$^+$ T cells in control wells
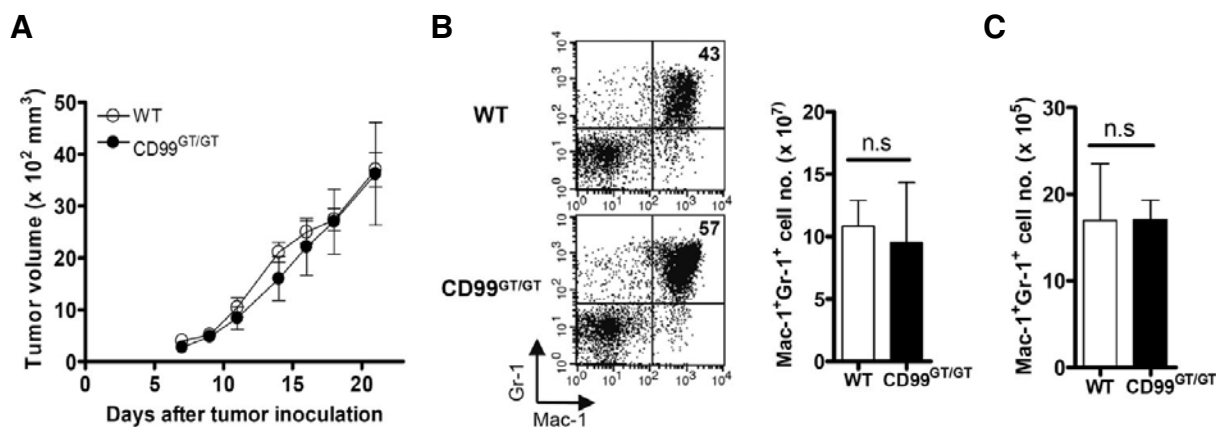
showed diluted CFSE (Fig. 3B). In contrast, MDSCs inhibited the proliferation of CD4$^+$ T cells. Thus, these data support the suggestion that the compromised expansion of donor-derived MDSCs might cause acute lethal GVHD in recipients of CD99-deficient splenocytes.

In the B6 splenocytes → bm12 GVHD model, a difference at a single MHC class II allele between donor and recipient leads the donor CD4$^+$ T cells to act as the main alloreactive cells (Rao et al., 2003; Sprent et al., 1990). Consequently, to investigate whether CD99 in CD4$^+$ T cells or other cell populations are relevant for sufficient MDSC expansion, CD4-depleted splenocytes were infused into irradiated bm12 mice along with CD4$^+$ T cells. Notably, wild-type CD4-negative fraction co-transferred with wild-type or CD99-deficient CD4$^+$ T cells protected the recipient mice from acute GVHD (Fig. 3C). In contrast, the absence of CD99 in the CD4-negative donor cell fraction did not ameliorate the symptoms of acute GVHD, independent of CD99 expression by the donor CD4$^+$ T cells. Therefore, these data suggest that MDSC expansion in the B6 splenocytes → bm12 model depends on CD99 molecules in the donor cell population other than alloreactive T cells.

A

B

Fig. 4. Comparable migration properties of wild-type and CD99-deficient MDSCs. (A) The absolute number of CD99$^{GT/GT}$ and wild-type donor cells in the spleen, bone marrow, and lymph nodes of recipient mice was similar. Splenocytes (1.5×10^7) from CD45.1$^+$ wild-type (WT) or CD99$^{GT/GT}$ mice were injected into sublethally irradiated bm12 mice, and 24 h after cell transfer the number of donor cells was calculated by multiplying the percentage of CD45.1$^+$ cells by the total cell number in the spleen, bone marrow (BM), mesenteric lymph node (mLN), and inguinal lymph node (iLN). (B) Comparable homing activities of T cells, B cells, or MDSCs between CD99$^{GT/GT}$ and wild-type donors. Cells in the spleen were stained with antibodies against CD45.1, CD4, CD8, B220, Mac-1, and Gr-1, and the donor cell fraction in each subset was analyzed by flow cytometry. The data are mean values ± SEM from four recipients in each group and representative data from two independent experiments are shown.

A   **B**   **C**

Fig. 5. CD99-independent expansion of MDSCs in tumor-bearing mice. (A) Similar tumor growth rate in CD99$^{GT/GT}$ and wild-type mice. Wild-type (WT) and CD99$^{GT/GT}$ mice were injected subcutaneously with 5×10^6 EL4 cells. Tumor growth was measured using calipers and average tumor volume was obtained from the width and length of the tumor. (B, C) MDSC expansion in tumor-bearing mice is independent on the CD99 expression status of host mice. The Mac-1$^+$Gr-1$^+$ MDSC fraction in the spleen (B) and tumor (C) of EL-4 tumor-bearing wild-type and CD99$^{GT/GT}$ mice was assessed by flow cytometry. A respresentative flow cytometric prolife is presented (B, left), and the total number of MDSCs in the spleen (B, right) and tumor (C) in wild-type (n = 4) or CD99-deficient (n = 3) mice 3 weeks after tumor challenge was calculated. Representative data from three independent experiments are shown. n.s, not significant.

### Comparable migration properties of CD99-deficient and wild-type MDSCs

Murine CD99 participates in transendothelial migration of T cells, neutrophils, and monocytes (Bixel et al., 2004; 2007; Dufour et al., 2008). Therefore, we tested the possibility that accelerated GVHD in CD99$^{GT/GT}$ splenocytes → bm12 mice is secondary to altered migratory properties of CD99$^{GT/GT}$ splenocytes compared to wild-type cells. Splenocytes from CD45.1$^+$ congenic wild-type or CD99$^{GT/GT}$ mice were injected into sublethally irradiated bm12 hosts. Twenty-four hours later, the absolute number of CD45.1$^+$ CD99$^{GT/GT}$ and wild-type cells in the spleen, bone marrow, and lymph nodes of the recipient mice were similar (Fig. 4A). Further analysis of donor cell subpopulations in the spleen showed no significant difference in homing activities of T cells, B cells, or MDSCs between CD99$^{GT/GT}$ and wild-type donors (Fig. 4B), suggesting that the difference in the migratory properties of the donor cells was unlikely to account for the contrasting GVHD phenotypes induced by CD99$^{GT/GT}$ and wild-type donors.

CD99-independent expansion of MDSCs in tumor-bearing mice

MDSCs accumulate in cancer patients and animal models (Gabrilovich and Nagaraj, 2009). To evaluate the role of CD99 in the cancer-induced expansion of MDSCs, mice were subcutaneously injected with 5×10^6 EL4 cells and tumor growth was monitored every 2 or 3 days. There was no difference between wild-type and CD99$^{GT/GT}$ mice (Fig. 5A). After 3 weeks, tumor-bearing mice were sacrificed to measure MDSCs in the spleen and tumor by flow cytometry. In both types of mice, Mac-1$^+$Gr-1$^+$ MDSCs markedly expanded (Fig. 5B left) and their number in the spleens or tumors of both groups did not differ (Fig. 5B right, and Fig. 5C). Thus, tumor-induced expansion of MDSCs does not depend on CD99 molecules.

Comparable serum soluble factor concentrations between CD99-deficient and wild-type groups

The expansion and activation of MDSCs is influenced by several factors, such as IL-1β, IL-4, IL-6, IL-10, IL-13, GM-CFS, and IFN-γ (Gabrilovich and Nagaraj, 2009). Some of these
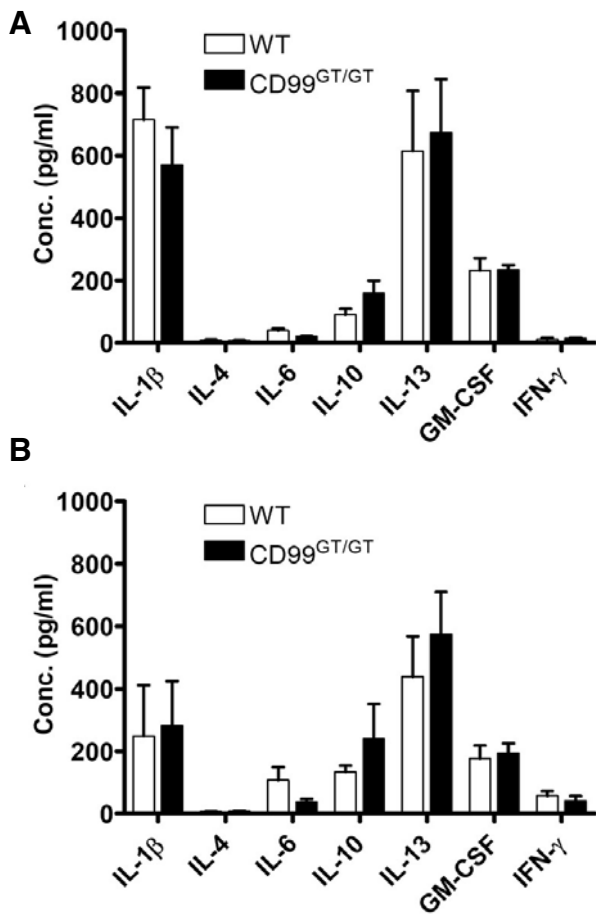
Fig. 6. Comparable serum concentration of soluble factors that induce the expansion and activation of MDSCs between CD99-deficient and wild-type groups. (A) No difference in serum IL-1β, IL-4, IL-6, IL-10, IL-13, GM-CFS, and IFN-γ concentrations between bm12 mice that received wild-type (WT) and CD99-deficient (CD99$^{GT/GT}$) splenocytes. Serum cytokine levels were analyzed by cytometric bead array on day 7 after adoptive transfer. Data are mean values ± SEM from five animals in each group. (B) No difference in serum cytokine concentration between tumor-bearing wild-type and CD99-deficient (CD99$^{GT/GT}$) mice. Data are mean values ± SEM from five animals in each group 3 weeks after tumor challenge.

cytokines are produced primarily by tumor cells and others are produced by activated T cells and tumor stromal cells. This raises the possibility that CD99 expression in donor cells might affect the serum concentration of these soluble factors and thereby modulate the expansion of MDSCs in the GVHD model. To test this possibility, sera were collected from bm12 mice on day 7 after adoptive transfer of wild-type or CD99-deficient splenocytes, and serum cytokine concentrations were compared. As shown in Fig. 6A, there was no difference in serum IL-1β, IL-4, IL-6, IL-10, IL-13, GM-CFS, and IFN-γ concentrations between the two groups. This was also the case in the tumor-bearing mouse model (Fig. 6B).

DISCUSSION

In the present work, we investigated the physiologic function of CD99 using CD99-deficient mice. Systemic deletion of CD99 by the exchangeable gene trap method did not affect the cellu-

larity of leukocyte subpopulations in the spleen. Unlike wild-type donor cells, however, CD99-deficient splenocytes caused lethal GVHD when adoptively transferred into irradiated bm12 hosts. In this GVHD model, we found that expansion of donor-derived MDSCs and the resulting attenuation of acute GVHD were dependent on CD99. On the contrary, tumor-induced expansion of MDSCs in CD99-deficient mice was comparable to that of wild-type mice.

To investigate the physiologic function of CD99, we used a CD99 mutant mouse clone established by the exchangeable gene trap method (EGTC 21-B6T44). The trap vector was confirmed to be inserted between exons 2 and 3 of the CD99 gene in the 21-B6T44 mouse clone via 5′ RACE. The absence of a CD99 transcript and protein expression in the homozygous mutants of this clone was confirmed by RT-PCR and flow cytometry. Then we investigated the *in vivo* impact of CD99 deficiency using a well-defined GVHD model in mutant MHC class II-different mice (Rao et al., 2003; Sprent et al., 1990). The most remarkable finding in bm12 mice that received CD99-deficient splenocytes was that they succumbed to lethal acute GVHD. The early lethality in these recipients was in sharp contrast to the clinical course in recipients of wild-type B6 splenocytes where there was minimal lethality (Sprent et al., 1990; 1995). This type of acute GVHD also developed in bm12 mice that received either purified B6 CD4$^+$ T cells alone or B6 ccr2$^{-/-}$ total splenocytes (Rao et al., 2003). Previous results obtained from ccr2$^{-/-}$ mice led to the hypothesis that cross-talk between the T cell and non-T cell compartments is important for amelioration of acute GVHD and that this interaction is dysregulated in B6 ccr2$^{-/-}$ splenocytes → bm12 mice (Rao et al., 2003). Here, we analyzed the Mac-1$^+$Gr-1$^+$ population in recipient mice during the course of GVHD, as MDSCs increase in numerous pathologic conditions and have suppressive effects on the adaptive immune response (Gabrilovich and Nagaraj, 2009). As expected, the donor MDSC population increased markedly during the second week post-transplantation in B6 splenocytes → bm12 mice but not in recipients of CD99-deficient splenocytes. Moreover, depletion of the Mac-1$^+$ fraction from donor cells prior to transplantation caused identical disease course regardless of CD99 expression in donor cells. Therefore, these findings suggest that MDSCs might be the major player responsible for repression of alloreactive CD4$^+$ T cells, and that MDSC expansion requires CD99 expression in donor cells.

While CD99 operated in the expansion of MDSCs in GVHD, this was not the case in the tumor model. The expansion and activation of MDSCs in a tumor system requires several soluble factors, which are spontaneously produced from tumor or stromal cells rather than as a result of cross-talk via cell-cell interaction (Gabrilovich and Nagaraj, 2009). These factors include prostaglandins (Sinha et al., 2007), VEGF (Gabrilovich et al., 1998), stem cell factor (SCF) (Pan et al., 2008), GM-CSF (Serafini et al., 2004), M-CSF (Menetrier-Caux et al., 1998), and inflammatory cytokines such as IL-6 and IL-1β (Bunt et al., 2007; Song et al., 2005). In contrast, during the development of GVHD, suppression of this pathology seems to primarily need diverse cross-talk between immune cells. In particular, in the B6 splenocytes → bm12 GVHD model, the development of GVHD is dominantly dependent on the CD4$^+$ T cells, as the only haplotype difference in donor and recipient mice is located on a single MHC class allele, I-A^b. Thus, under these circumstances, it seems that the interaction between alloreactive T cells and non-T cell compartments might contribute to the expansion of MDSCs in a CD99-dependent manner.

We next addressed whether cell migration was impaired by the lack of CD99 molecules, given that CD99 may play an im-

portant role in the *in vitro* transendothelial migration of human monocytes (Schenkel et al., 2002), and *in vivo* blocking studies using anti-CD99 antibodies support the idea that murine CD99 plays a significant role in the migration of T cells, neutrophils, and monocytes to a site of inflammation (Bixel et al., 2004; 2007; Dufour et al., 2008). However, we found contradictory results; there was no difference in migratory activities between wild-type and CD99-deficient leukocytes. Although the reason for this apparent discrepancy is not clear, two possibilities may be considered. In our system, cell migration was designed to be directed to the lymphoid organs, whereas in other model systems, cells were directed to the inflammatory foci. In addition, other researchers used blocking antibodies for simple physical inhibition of CD99 molecules. In contrast, our model was a CD99$^{GT/GT}$ mouse system where cells can modulate other redundant molecules involved in transmigration, such as PECAM-1, ICAM-2, junctional adhesion molecules (JAMs), and endothelial cell-selective adhesion molecul*e* (ESAM) (Vaporciyan et al., 1993; Vestweber, 2007; Woodfin et al., 2010).

In summary, we found that massive expansion of donor MDSCs after leukocyte transplantation ameliorates acute GVHD and that CD99 plays a major role in the efficient expansion of these cells. Considering that the only known ligand of murine CD99 is PILR, which is expressed on the surface of leukocytes (Shiratori et al., 2004), ligation of CD99 in MDSCs by PILR in CD4$^+$ T cells is a candidate cellular mechanism for promotion of MDSC expansion. Conversely, ligation of PILR in CD4$^+$ T cells by CD99 in MDSCs or other non-T cells might affect T cell function, such as the secretion of cytokines that regulate MDSC expansion. In the present study, no difference in serum cytokine concentration was found between bm12 mice that received CD99-deficient and wild-type splenocytes, supporting the former possibility. However, it remains to be clarified whether direct contact between effector T cells and non-T cell compartments through PILR-CD99 interaction is involved in MDSC expansion.

ACKNOWLEDGMENTS

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