Amelioration of acute graft-versus-host disease by NKG2A engagement on donor T cells

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Acute graft-versus-host disease (aGVHD) remains a major complication of allogeneic bone marrow transplantation, which is caused by donor T cells specific for host alloantigens. In a murine model, we found that donor T cells expressed a natural killer cell inhibitory receptor, CD94/NKG2A, during the course of aGVHD. Administration of an anti-NKG2A mAb markedly inhibited the expansion of donor T cells and ameliorated the aGVHD pathologies. These results suggested that the CD94/NKG2A inhibitory receptor expressed on host-reactive donor T cells can be a novel target for the amelioration of aGVHD.

Introduction

Allogeneic bone marrow transplantation (BMT) has been a clinical treatment modality for hematopoietic disorders and hematological malignancies. Graft-versus-host disease (GVHD) remains a major complication after allogeneic BMT, which is caused by donor T cells specific for host alloantigens. An acute form of GVHD (aGVHD) affects the skin, liver, gut, and lymphoid organs where inflammatory reactions characterized by mononuclear cell (MNC) infiltration and histopathological damage take place, which lead to erythroderma, hepatitis, diarrhea, wasting, immunodeficiency and, finally, death. A murine model of aGVHD, which is caused by transfusion of C57BL/6 (B6) splenic T cells into (DBA/2 x B6)F1 or (BALB/c x B6)F1 (CBF1), has been frequently used to investigate the pathogenesis of aGVHD [1, 2].

Recent studies have revealed two types of natural killer (NK) cell surface receptors that activate or inhibit NK cell functions [3–5]. Among them, CD94/NKG2 heterodimers are unique in their recognition of a non-classical major histocompatibility complex (MHC) class I molecule (HLA-E in humans and Qa-1 in mice) [6–9] and the presence of both activating (NKG2C and NKG2E) and inhibitory (NKG2A) isoforms [8, 9]. HLA-E and Qa-1 present transporter associated with antigen processing (TAP)-dependent peptides derived from MHC class I signal sequences and thus indirectly represent the integrity of classical MHC class I expression and TAP function in normal cells [10, 11]. NKG2A contains immunoreceptor tyrosine-based inhibitory motifs (ITIM) recruiting tyrosine phosphatases and thus inhibits NK cell activation [12, 13]. NKG2C and NKG2E do not contain ITIM, but associate with DAP12 recruiting tyrosine kinases and thus initiate NK cell activation [9, 14–16]. Among these isoforms, CD94/NKG2A is the predominant one expressed by both

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Abbreviations: aGVHD: acute graft-versus-host disease · ALT: alanine aminotransferase · B6: C57BL/6 · BMT: bone marrow transplantation · CBF1: (BALB/c x B6)F1 · G3PDH: glyceraldehyde 3-phosphate dehydrogenase · MNC: mononuclear cell ·
human and murine NK cells, and thus the major function of CD94/NKG2 is thought to protect normal cells from NK cell-mediated damage [3, 4].

It has recently been reported that CD94/NKG2A can be also expressed by T cells and inhibit T cell activation *in vitro* [17–22]. Moreover, increased expression of CD94 on human peripheral blood CD8+ T cells has been noted after allogeneic BMT [23]. However, the functional role of CD94/NKG2A in the pathogenesis of GVHD remains unclear. In this study, by using the murine aGVHD model, we demonstrated that CD94/NKG2A is expressed on donor T cells during the course of aGVHD and that its engagement by anti-NKG2A mAb could ameliorate aGVHD.

**Results**

**Expression of CD94/NKG2A by donor T cells**

We first examined cell surface expression of CD94/NKG2A on donor and recipient T cells in a murine aGVHD model, which was caused by i.v. injection of B6 splenocytes into CBF1 mice, by flow cytometry using an anti-CD94 mAb (18d3) [9] and an anti-NKG2A/C/E mAb (20d5) [9]. We used B6-Ly5.1 congenic mice as the donor to discriminate donor (Ly5.1+) and recipient (Ly5.1-) T cells. As represented in Fig. 1A and B, while neither CD94 nor NKG2A/C/E were expressed on B6 donor T cells before i.v. injection, approximately 10% of donor CD4+ T cells and 60% of donor CD8+ T cells in the spleen and liver expressed CD94 and NKG2A/C/E at day 14 and 21 after i.v. injection into CBF1 recipients. Neither NKT cells nor NK cells were included in these donor T cell populations, as estimated by the expression of NK1.1 (data not shown). By contrast, expression of either CD94 or NKG2A/C/E was not observed on recipient T cells at any time point (data not shown).

To determine the NKG2 isof orm expressed by donor T cells, we performed RT-PCR using specific primers for NKG2A, C, or E. As shown in Fig. 1C, expression of NKG2A, but not NKG2C or E, mRNA was found in donor CD4+ and CD8+ T cells from the spleen and liver at day 14 after transfer. These results indicated that donor T cells, especially CD8+, expressed the CD94/NKG2A inhibitory receptor during the course of aGVHD.

**Cytotoxic T lymphocytes against host alloantigen mostly reside in the NKG2A/C/E+ population of donor T cells**

We next examined whether CD94/NKG2A was expressed on donor T cells reactive with host alloantigen. To address this, we isolated whole Ly5.1+, Ly5.1+NKG2A/C/E+, and Ly5.1+NKG2A/C/E+ donor T cell popu-
lations from the spleens of aGVHD mice on day 14 and then tested their cytotoxic activity against P815 and A20.2J target cells expressing the host alloantigen (H-2\(^d\)). As shown in Fig. 2, cytotoxic activities against both P815 and A20.2J mostly resided in the NKG2A/C/E\(^+\) population of donor T cells. These results indicated that host-reactive donor cytotoxic T lymphocytes (CTL) expressed CD94/NKG2A during aGVHD.

**Effect of anti-NKG2A/C/E mAb treatment on the expression of CD94/NKG2A on donor T cells**

To explore the function of CD94/NKG2A expressed on donor T cells during aGVHD, we i.p. administrated 250 \(\mu\)g of the anti-NKG2A/C/E mAb (20d5) or control rat IgG every 2 days from day 0 to day 14 after i.v. injection of B6 splenocytes into CBF\(_1\) mice. As shown in Fig. 3A, slightly reduced levels of CD94 expression were observed on donor T cells from the liver of 20d5-treated recipients as compared to those from the control IgG-treated recipients. In contrast to donor T cells from the control IgG-treated recipients, those from the 20d5-treated recipients were not stained with biotinylated 20d5 (Fig. 3A), suggesting that the NKG2A molecules on donor T cells were saturated by the administered 20d5. This notion was further substantiated by a direct staining with anti-rat IgG secondary Ab (Fig. 3B). Similar results were obtained with donor T cells from the spleen (data not shown). These results indicated that the in vivo treatment with 20d5 induced a weak down-modulation of CD94/NKG2A on donor T cells, but not a depletion of CD94/NKG2A-expressing donor T cells. The administration of 20d5 also did not deplete NK1.1\(^+\) NK cells that expressed CD94/NKG2A at a higher level than the donor T cells (data not shown).

**Administration of anti-NKG2A/C/E mAb inhibits donor T cell expansion**

We next examined the effect of anti-NKG2A/C/E mAb (20d5) treatment on the expansion of donor T cells in the spleen and liver. As shown in Fig. 4A, both CD4\(^+\) and CD8\(^+\) donor T cells markedly expanded in the spleen of the control IgG-treated recipients at day 14 and 21 after transfer, but this expansion was significantly inhibited by the 20d5 treatment. A similar inhibition by 20d5 was observed against the donor T cell expansion in the liver (Fig. 4B).
Administration of anti-NKG2A/C/E mAb ameliorates aGVHD pathologies

We next examined the effect of anti-NKG2A/C/E mAb (20d5) treatment on aGVHD-associated pathologies. As shown in Fig. 5A, the serum alanine aminotransferase (ALT) level, an indicator of liver damage, was markedly increased at day 14 after transfer of B6 splenocytes into control IgG-treated CBF1 mice, but this increase was significantly inhibited by the 20d5 treatment. We also examined the histopathological changes in the liver, small intestine, and spleen at day 14 after transfer. As shown in Fig. 5B, typical histological features of aGVHD, including a massive infiltration of MNC in the periportal area in the liver, MNC infiltrates in the submucosal area and villous degeneration in the small intestine, and emergence of megakaryocytic blasts and lymphoid hypoplasia represented by loss of germinal centers in the spleen, were found in the control IgG-treated recipients, but all these pathological changes were ameliorated in the 20d5-treated recipients. A longer monitoring of the recipients showed that the 20d5 treatment significantly prolonged survival (45.0 ± 8.2 days) as compared to the control IgG treatment (32.2 ± 6.4 days) (p = 0.0333 by Mann-Whitney U-test), but all the recipients eventually died by 60 days (data not shown).

Protective effect of anti-NKG2A/C/E mAb on aGVHD is donor cell dose-dependent

We next examined the effect of anti-NKG2A/C/E mAb (20d5) treatment on aGVHD induced by twofold less (3.5 × 10⁷ cells) or twofold more (14 × 10⁷ cells) donor splenocytes. As shown in Fig. 6A, the 20d5 treatment significantly inhibited the expansion of donor T cells in the spleen when 3.5 × 10⁷ or 7 × 10⁷ donor splenocytes were infused. However, such an inhibitory effect of 20d5 was not statistically significant when 14 × 10⁷ donor splenocytes were infused. Similar
results were obtained for donor T cell expansion in the liver, although the expansion of CD4+ T cells was still significantly inhibited by 20d5 even when 14 \times 10^7 donor splenocytes were infused (Fig. 6B). Consistently, the increase in serum ALT levels was significantly inhibited by the 20d5 treatment when 3.5 \times 10^7 or 7 \times 10^7 donor splenocytes were infused, but not when 14 \times 10^7 donor splenocytes were infused (Fig. 6C). These results indicated that the protective effect of 20d5 was limited at a higher donor cell dose.

**Administration of anti-NKG2A/C/E mAb inhibits proliferative and cytotoxic responses of donor T cells against host alloantigen**

We finally examined the effect of anti-NKG2A/C/E mAb (20d5) treatment on donor T cell responses to host alloantigen (H2d). At day 14 after i.v. injection of B6-Ly5.1 donor splenocytes into control IgG- or anti-NKG2A/C/E mAb (20d5)-treated CBF1 recipients, we isolated Ly5.1+ donor T cells from the spleen and tested their proliferative response to irradiated BALB/c (H2d) splenocytes and their cytotoxic activity against H2d target cells (P815 and A20.2J). As shown in Fig. 7A, the proliferative response of B6 donor
T cells to BALB/c splenocytes was significantly inhibited by the 20d5 treatment as compared to the control IgG treatment. Moreover, cytotoxic activity of donor T cells against H-2d target cells was also significantly reduced by the 20d5 treatment as compared to the control IgG treatment (Fig. 7B). These results indicated that the 20d5 treatment inhibited the proliferative response and the CTL development of donor T cells against host alloantigen.

Discussion

In this study, we demonstrated that the CD94/NKG2A inhibitory receptor was expressed preferentially on donor T cells in a murine GVHD model. Administration of anti-NKG2A mAb inhibited donor T cell expansion, the proliferative response of donor T cells to host alloantigen, and CTL activity against host alloantigen, resulting in improvement of aGVHD pathologies. These results suggested that the CD94/NKG2A inhibitory

Fig. 6. Protective effect of anti-NKG2A/C/E mAb on aGVHD is donor cell dose dependent. For induction of aGVHD, CBF1 mice received 3.5 × 10⁷, 7 × 10⁷, or 14 × 10⁷ donor splenocytes from B6-Ly5.1 mice by i.v. injection. Splenic (A) and hepatic (B) MNC were isolated from control IgG- or anti-NKG2A/C/E mAb (20d5)-treated aGVHD mice at 14 days after transfer, and stained with PE-labeled anti-NK1.1 mAb, FITC-labeled anti-CD4 or CD8 mAb, and biotinylated anti-Ly5.1 mAb, followed by Tri-Color-labeled streptavidin. Donor and recipient T cells were identified as Ly5.1+ and Ly5.1−, respectively. (C) Serum ALT levels in control IgG- or anti-NKG2A/C/E mAb (20d5)-treated aGVHD mice were determined at 14 days after transfer. Data are represented as the means ± SD of five mice in each group. *, p < 0.05; **, p < 0.01. Similar results were obtained in two independent experiments.

Fig. 7. Administration of anti-NKG2A/C/E mAb inhibits proliferative and CTL responses of donor T cells against host alloantigen. aGVHD was induced by transfer of 7 × 10⁷ donor splenocytes from B6-Ly5.1 mice into CBF1 mice. Ly5.1+ donor T cells were isolated from the spleens of control IgG- or anti-NKG2A/C/E mAb (20d5)-treated normal B6 or aGVHD mice at 14 days after transfer. (A) The proliferative response against irradiated B6 (H-2b) or BALB/c (H-2d) target cells was estimated by [³H]thymidine (Tdr) uptake for the last 18 h of a 3-day culture. Data are indicated as the means ± SD of triplicate samples. *, p < 0.05. Similar results were obtained in three independent experiments. (B) Cytotoxic activity was tested against P815 and A20.2J (both H-2d) target cells by a 4-h ⁵¹Cr-release assay at the indicated E/T ratios. Data are indicated as the means ± SD of triplicate samples. *, p < 0.05 compared to control IgG-treated aGVHD mice. Similar results were obtained in three independent experiments.
receptor on donor T cells could be a novel target for amelioration of aGVHD.

It has been demonstrated that in vitro stimulation of human T cells with superantigens or allogeneic cells in the presence of IL-15 [24] or TGF-β [21] induced CD94/NKG2A expression predominantly on CD8^+^ cells. CD94/NKG2A was also detected on memory CD8^+^ cells in human peripheral blood [25], which might be induced by IL-15, since IL-15 plays a critical role in the development and maintenance of memory CD8^+^ T cells [26, 27]. Moreover, CD8^+^ T cells that infiltrated primary and metastatic melanomas expressed CD94/NKG2A [28], which might be induced by tumor-derived TGF-β. Also in the murine system, it has recently been reported that antigen-specific CD8^+^ T cells expressed CD94/NKG2A during viral and bacterial infections [17, 29, 30] in an IL-15-independent manner [29]. We now demonstrated the expression of CD94/NKG2A on a minor population (10–15%) of donor CD4^+^ T cells and most (60–70%) of donor CD8^+^ T cells during murine aGVHD, which might be also induced by IL-15 or TGF-β. In this respect, it may be noteworthy that TGF-β has been suggested to act protectively against aGVHD [31–33], which might be mediated by the CD94/NKG2A induction on donor T cells. As far as we tested, CD94/NKG2A expression was not induced on either CD8^+^ or CD4^+^ T cells in allogeneic MLR in vitro, suggesting that some environment in vivo, possibly rich in IL-15 and/or TGF-β, may be required for the CD94/NKG2A expression on T cells. Further studies are needed to address these possibilities.

It has been demonstrated that the IL-15-induced CD94/NKG2A expression on alloreactive CD8^+^ T cells led to an impairment of allospecific CTL activity, which could be restored by blockade with anti-CD94 mAb [24]. It has also been reported that the in vitro expanded CD94/NKG2A^+^ subpopulation of melanoma-specific CD8^+^ T cells exhibited impaired cytotoxic activity, which could be restored by blockade with anti-CD94 mAb [28]. More importantly, the CD94/NKG2A expressed on virus-specific CD8^+^ T cells during acute polyoma virus infection was responsible for the impaired CTL activity, which could be restored by blockade of CD94/NKG2A or Qa-1 [17]. Since Qa-1 has a broad tissue distribution [34], the CD94/NKG2A and Qa-1 interaction has been implicated in protection of normal cells from self NK cells [3, 4]. Analogously, the CD94/NKG2A expression on virus-specific CTL has been proposed to play a physiological role to restrain an excessive CTL activation, to prevent immunopathological tissue damage [17]. A similar scenario may be applicable to the CD94/NKG2A expressed on donor T cells during aGVHD, which may represent an intrinsic mechanism that limits aGVHD pathology. Consistent with this notion, donor T cells expanded progressively in the spleen and liver during days 7–14 after transfer, but this expansion was rather suppressed during days 14–21 in the present aGVHD model (Fig. 4). Moreover, the liver damage represented by serum ALT levels peaked at day 14 and then was improved (Fig. 5). This spontaneous suppression of the donor T cell expansion and the liver damage might be associated with the expression of CD94/NKG2A on donor T cells (Fig. 1). However, it should be also noted that CD94/NKG2A expression was not necessarily correlated with inhibition of T cell functions in some viral infections [29, 30]. Further studies are now under way to determine the physiological role of CD94/NKG2A in regulating aGVHD.

It has been reported that donor NK cells were involved in the development of aGVHD [35–37]. Thus, the ameliorating effect of 20d5 on aGVHD might be also mediated by inhibition of donor NK cell activation by 20d5. However, in our preliminary experiments, the 20d5 treatment could inhibit donor T cell expansion and aGVHD pathologies even when the donor splenocytes were depleted of NK cells. Therefore, it is more likely that the 20d5 treatment directly inhibited the CD94/NKG2A-expressing donor T cells, as shown in Figs. 4 and 7.

It has been demonstrated that the cross-linking of CD94 or NKG2A by mAb and Fc receptors on target cells inhibited cytotoxic activity and cytokine production of NK cells and CD8^+^ T cells in vitro [13, 38], which seems to result from transmission of an inhibitory signal via CD94/NKG2A. However, in our preliminary experiments, addition of 20d5 to primary or secondary MLR in vitro did not significantly inhibit the proliferative response or the alloreactive CTL induction. Moreover, addition of 20d5 did not significantly affect the proliferative response or the CTL activity of donor T cells from control IgG-treated aGVHD mice in vitro (data not shown). These results suggest that chronic ligation of NKG2A by 20d5 or higher cross-linking by Fc receptors and complement/complement receptors might be required for the 20d5 effect in vivo. Nevertheless, it is likely that the administration of anti-NKG2A mAb ameliorated aGVHD (Fig. 5) by transducing an inhibitory signal upon cross-linking in vivo, which functionally inactivated the host-reactive donor T cells expressing CD94/NKG2A in the present model. Consistently, the expansion of donor T cells in the spleen and liver, the proliferative response of donor T cells to host alloantigen, and the CTL activity against host alloantigen were markedly impaired by the anti-NKG2A treatment (Figs. 4, 6, 7). Since a similar expression of CD94/NKG2A on donor T cells has been observed for human peripheral blood T cells after allogeneic BMT [23], the engagement of CD94/NKG2A on donor T cells by an agonistic mAb may be a novel strategy for amelioration of aGVHD also in human cases.
Materials and methods

Mice

Female C57BL/6-Ly5.1 (B6-Ly5.1, H-2b) and (BALB/c × B6)F1 (CBF1, H-2k/b) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and CLEA Japan, Inc. (Tokyo, Japan), respectively, and used at 8–10 wk of age. All mice were maintained under specific pathogen-free conditions and treated according to the protocols approved by the University Committee on Use and Care of Animals at Niigata University.

Induction of aGVHD and mAb treatment

B6-Ly5.1 splenocytes (3.5 × 10^7, 7 × 10^7, or 14 × 10^7 cells) were i.v. injected into CBF1 mice on day 0. An anti-mouse NKG2A/C/E mAb (20d5, rat IgG2a; 250 μg) [9] or control rat IgG (Sigma, St. Louis, MO; 250 μg) was i.p. administrated every 2 days from day 0 to day 14.

Cell preparation

Splenic and liver MNC were prepared as described [39]. Ly5.1^+, Ly5.1^+NKG2A/C/E^–, Ly5.1^+NKG2A/C/E^+, CD4^+Ly5.1^+, CD8^+Ly5.1^+, CD4^+Ly5.1^+, and CD8^+Ly5.1^+ populations were isolated by sorting on a FACSvantage (Becton Dickinson, San Jose, CA).

Flow cytometric analysis

An anti-mouse NKG2A/C/E mAb (20d5) and an anti-mouse CD94 mAb (18d3) [9] were biotinylated by a standard method. Total RNA was isolated using RNA easy Mini kit (Qiagen, Hilden, Germany) to block Fc receptor, and then incubated with anti-CD16/CD32 mAb (2.4G2; BD Biosciences, San Diego, CA). Tri-Color-conjugated streptavidin and Tri-Color-conjugated goat anti-rat IgG (R35-95) (BD Biosciences, San Diego, CA). Tri-Color-conjugated mAb to mouse CD3 (145-2C11), NK1.1 (PK136), and IFN-γ (XMG1.2) (BD Biosciences, San Diego, CA) were purchased from Caltag (San Francisco, CA). Cells were pre-incubated with anti-CD16/CD32 mAb (2.4G2; BD Biosciences) to block Fcγ receptor, and then incubated with the relevant mAb for 30 min at 4°C, washed and analyzed on a FACScan (Becton Dickinson).

RT-PCR

Total RNA was isolated using RNA easy Mini kit (Qiagen, Hilden, Germany). RT-PCR was performed from 5 μg of total RNA by using an RT-PCR kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer’s instructions. The following primers were used for NKG2A: sense 5'-CAT TCA TCG AGC AGG AAA TC-3', antisense 5'-GCT GAC CTC TGC CCT'TCC GA-3'; NKG2C: sense 5'-GAC TTC AAC ATG CTT CCG AC-3', antisense 5'-GCC TGA CCT CTT CCC TTC CG-3'; NKG2E: sense 5'-GAG ATC GCC TGC TGG AGT TT-3', antisense 5'-GCT GAC CTC TGC CCT'TCC GA-3'; glyceraldehyde 3-phosphate dehydrogenase (G3PDH): sense 5'-ACC ACA GTC CAT GAA ATC AC-3', antisense 5'-TCC ACC ACC CTG TGT CAG TA-3'. PCR was performed on a GeneAmp PCR System 9700 thermal cycler (PerkinElmer, Foster City, CA) with first heating to 94°C for 5 min and then 35 cycles at 94°C for 45 s for denaturation, 58°C for 45 s for annealing, and 72°C for 90 s for extension, followed by a final incubation at 72°C for 7 min. The PCR products were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining under UV illumination.

Pathological examination

Serum ALT levels were measured spectrophotometrically by a standard enzymatic method using a commercial kit (Wako Chemical Inc., Osaka, Japan). Tissues were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections (4-μm) were stained with hematoxylin and eosin.

Proliferation assay

Ly5.1^+ B6 donor T cells (2 × 10^7/well) were co-cultured with irradiated (30 Gy) B6 or BALB/c splenocytes (5 × 10^7/well) in a round-bottom 96-well plate for 3 days. [3H]thymidine (0.5 μCi/well) uptake was assessed for the last 18 h as described [40].

Cytotoxicity assay

Cytotoxic activity of Ly5.1^+ B6 donor T cells was tested against 51Cr-labeled P815 (DBA/2-derived mastocytoma, H-2b) and A20.2J (BALB/c-derived B lymphoma, H-2d) target cells (1 × 10^5/well) at the indicated effector-to-target (E/T) ratios by a standard 4-h 51Cr-release assay. Percent cytotoxicity was calculated as described [39].

Statistical analysis

Significant differences between two groups were determined by two-tailed Student’s t-test; p values <0.05 were considered statistically significant.

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