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Prevention of chronic graft-versus-host disease by stimulation with glucocorticoid-induced TNF receptor

Juyang Kim[1], Woon Sun Choi[1], Hye Jeong Kim[1] and Byungsuk Kwon[1,2]

[1]The Immunomodulation Research Center and
Department of Biological Science
University of Ulsan
Ulsan 680-749, Korea
[2]Corresponding author: Tel, 82-52-259-2860;
Fax, 82-52-259-2740; E-mail, bkwon@mail.ulsan.ac.kr

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Abbreviations: AICD, activation-induced cell death; BDF_1, (C57BL/6 × DBA/2)F_1; cGVHD, chronic graft-versus-host disease; GITR, glucocorticoid-induced TNF receptor

Abstract

GITR (glucocorticoid-induced TNF receptor) is a recently identified member of the TNF receptor superfamily. The receptor is preferentially expressed on $CD4^+CD25^+$ regulatory T cells and GITR signals break the suppressive activity of the subset. In this study, we wanted to reveal the *in vivo* function of GITR in chronic graft-versus-host disease (cGVHD), a lupus-like autoimmune disease. A single injection of anti-GITR monoclonal antibody (DTA-1) was effective in blocking the progression of cGVHD in the parent-into-F_1 model. Treatment of DTA-1 significantly decreased levels of IgG_1 anti-DNA autoantibody, inhibited glomerulonephritis, and increased survival. The DTA-1-mediated inhibition of autoantibody production correlated with deletion of B cells and could occur independently of $CD4^+CD25^+$ regulatory T cells. Our results indicate that anti-GITR monoclonal antibody may be used as a potential immunotherapeutic agent for preventing cGVHD.

Keywords: autoantibodies; B-lymphocytes; glomerulonephritis; graft vs host disease; immunotherapy; TNFRSF18 protein, mouse

Introduction

Graft-versus-host disease (GVHD) commonly occurs in patients who receive allogeneic stem cell transplants and remains a significant cause of morbidity (Murphy, 2000). In GVHD, immunocompetent donor cells attack a genetically disparate host. GVHD can exist as two distinct clinical entities: acute GVHD (aGVHD) and chronic GVHD (cGVHD). Donor $CD8^+$ T cells and T helper type 1 (Th1) $CD4^+$ T cells cause acute GVHD by attacking host tissues (Murphy and Blazar, 1999), whereas donor Th2 $CD4^+$ T cells play a key role in causing cGVHD by hyper-activating recipient B cells and thus breaking B-cell tolerance to self-antigens (Gleichmann *et al*., 1984; Via and Shearer, 1988).

Numerous model systems have been developed for examining GVHD. In a well-known cGVHD model, cGVHD is induced by transferring parental DBA/2 T cells into normal (C57BL/6 × DBA/2)F_1 (BDF_1) mice. An immune complex glomerulonephritis that progresses to global glomerulosclersosis and renal failure is prominent in recipient F_1 mice (Bergijk *et al*., 1992), among other multisystem disorders comprising a spectrum of abnormalities such as vasculitis, polyarthritis, and mononuclear cell infiltration of multiple organs (Van Rappard-Van Der Veen *et al*., 1983; Rolink *et al*., 1983).

GITR (glucocorticoid-induced TNF receptor) was originally identified by comparing untreated and dexamethasone-treated murine T-cell hybridoma cells (Nocentini *et al*., 1999). Later, human GITR and its ligand were identified by searching an expressed sequence tag (EST) database (Kwon *et al*., 1999; 2003; Gurney *et al*., 1999). GITR expression is up-regulated on T cells after activation, and a high level of GITR is constitutively expressed on $CD4^+CD25^+$ regulatory T cells (McHugh *et al*., 2002; Shimizu *et al*., 2002). GITR signals abrogate their suppressive function (McHugh *et al*., 2002; Shimizu *et al*., 2002). However, recent studies have demonstrated that GITR functions as a co-stimulatory molecule for conventional T cells *in vitro* (Ronchetti *et al*., 2004; Kohm *et al*., 2004; Ji *et al*., 2004; Kanamaru *et al*., 2004) and that GITR-deficient mouse $CD4^+CD25^+$ regulatory T cells were able to exert the suppressive activity on conventional $CD25^-CD4^+$ T cells as equally as wild-type $CD4^+CD25^+$ regulatory T cells (Ronchetti *et al*., 2004).

The function of GITR remains largely to be defined. In this study, we wanted to reveal the *in vivo* function of GITR in the parental-into-F_1 cGVHD model.

Materials and Methods

Disease induction

Female DBA/2 (H-2^d) and BDF_1 (H-$2^{b/d}$) mice, 6-8 weeks of age, were purchased from Orient (Seoul, Korea) and were used to induce cGVHD. Single-cell suspensions in PBS were prepared from the spleens and lymph nodes of normal DBA/2 parental donors, filtered through a sterile mesh (BD Falcon, San Diego, CA) and washed. After the erythrocytes were lysed in hemolysis buffer (144 mM NH_4Cl and 17 mM Tris-HCl, pH 7.2), the remaining cells were resuspended at 8×10^7 cells/0.2 ml in PBS. cGVHD was induced by transfer of 8×10^7 of DBA/2 parental cells into the tail vein of normal, unirradiated BDF_1 mice. Immediately thereafter, 200 μg of DTA-1 or control immunoglobulin (Ig) was administered intraperitoneally. In some experiments, $CD4^+CD25^+$ regulatory T cells were removed by anti-CD25-conjugated magnetic beads (Miltenyi Biotech, Aurburn, CA) from DBA/2 spleen/lymph node cells. The remaining cells (8×10^7) were transferred into BDF_1 mice to induce cGVHD.

ELISA, flow cytometry, and histology

ELISA was used to measure serum titers of anti-DNA IgG_1 as described previously (Kim *et al*., 2005). Flow cytometry was also described previously (Kim *et al*., 2005). For histopathology, kidneys were collected and immediately immersed in 10% neutral-buffered formalin. The formalin-fixed tissue was embedded in paraffin, and 4-μm sections were stained with hematoxylin and eosin (H&E) or periodic acid schiff (PAS) and evaluated by microscopy. For immunohistochemistry, kidneys were embedded in optimal cutting temperature (OCT) compound (Sakura Fineteck, Torrence, CA) and snap-frozen in liquid nitrogen. Sections (8 μm) were air-dried, fixed with acetone, and stained with fluorescein isothiocyanate (FITC)-conjugated anti- mouse IgG (BD Biosciences Pharmingen, San Diego, CA). Fluorescence was examined by confocal microscopy (Olympus, Tokyo, Japan).

Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences between experimental groups.

Results

Agonistic anti-GITR antibody (DTA-1) has been shown to abolish the immunosuppressive activity of $CD4^+CD25^+$ regulatory T cells (Shimizu *et al*., 2002). Since $CD4^+CD25^+$ regulatory T cells can inhibit both humoral and cell-mediated branches of the immune response (Wood and Sakaguchi *et al*., 2003), we expected that DTA-1 would exacerbate cGVHD. Unexpectedly, a one-time administration of DTA-1 (200 μg) at the phase of disease induction significantly blocked the production of autoantibody. As seen previously (Kim *et al*., 2005), high levels of

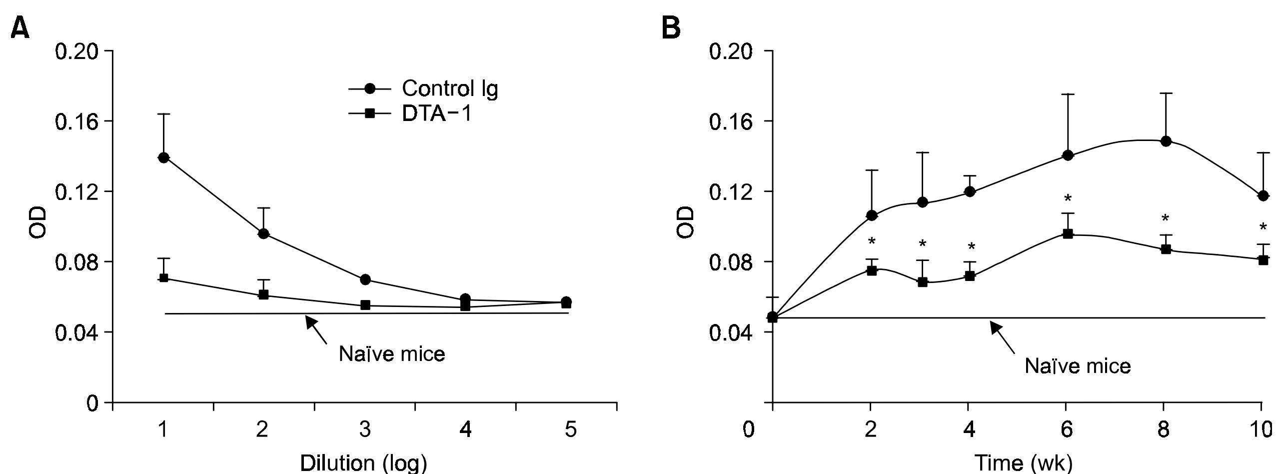
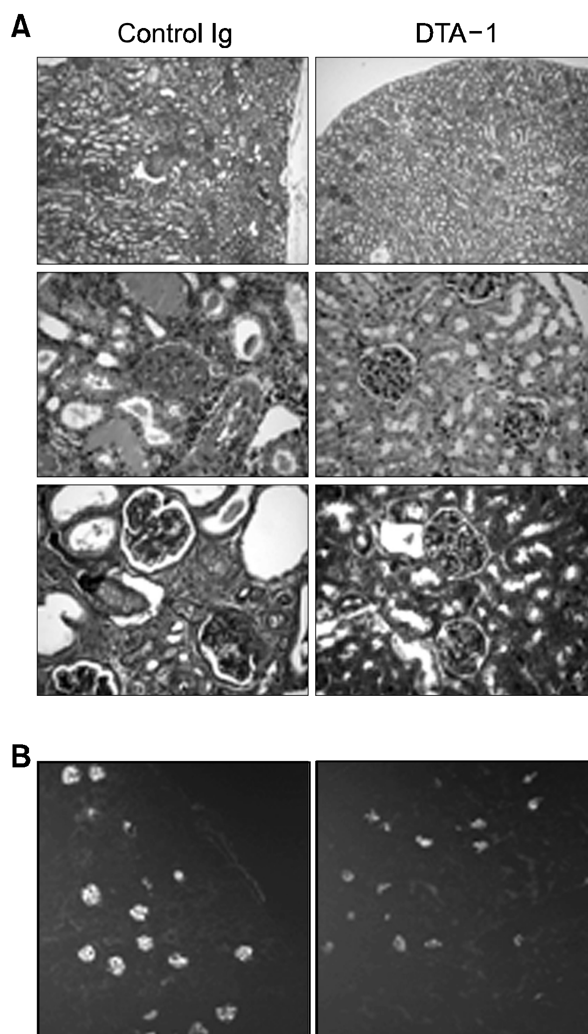


Figure 1. DTA-1 inhibits the production of IgG_1 anti-DNA autoantibody in cGVHD. cGVHD was induced by transferring 8×10^7 DBA/2 spleen/ lymph node cells into BDF_1 mice. Immediately thereafter, 200 μg of DTA-1 or control Ig was injected. Serum samples were collected every 1 or 2 weeks, and assayed in duplicate by ELISA for IgG_1 anti-DNA autoantibody. The optical density (OD) of duplicate samples for each mouse was measured at 450 nm, using serially diluted serum samples. Titration curves for anti-DNA IgG_1 are shown in the panel A at week 2. Similar titration curves were obtained at other time points. OD values are means ± SD (n = 10 per group) of 10-fold dilution of samples and are representative of more than 3 independent experiments. $^*P < 0.05$, between the 2 groups at the indicated time points.



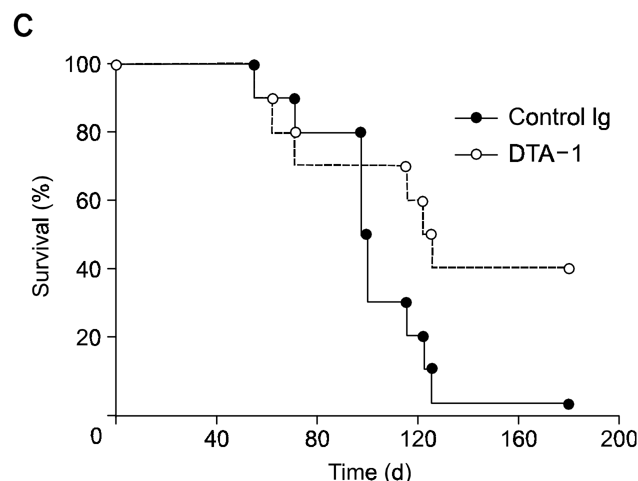


Figure 2. DTA-1 inhibits immune complex formation and glomerulonephritis. (A) Kidneys were collected from cGVHD mice at week 12 after disease induction and were fixed in formalin. Sections from control Ig- or DTA-1-treated mice were stained with H&E or PAS. *Upper panel*, H&E staining (lower magnification); *middle panel*, H&E staining (higher magnification); *lower panel*, PAS staining. (B) Kidneys from 12-week cGVHD mice were collected and snap-frozen, and sections were stained with FITC-labeled rat anti-mouse IgG. (C) DTA-1 treatment increased survival (n = 10 per group). Kidneys of n = 5-10 per group were analyzed for histology and histochemistry. Data shown are representative of more than 3 independent experiments.

anti-DNA IgG_1 began to be detected in the serum of control cGVHD mice at week 2 after disease induction (Figure 1). In contrast, administration of DTA-1 resulted in a marked reduction in levels of anti-DNA IgG_1. The decrease was observed as early as week 2 and maintained thereafter (Figure 1).

Kidney disease is considered to be the primary cause of mortality in mice afflicted with cGVHD. In particular, autoantibody-immune complex deposits directly result in glomerular damage. As DTA-1 significantly inhibited autoantibody production, we investigated whether it could prevent renal disease. The kidneys from control cGVHD mice demonstrated severe glomerulonephritis. Virtually all of the glomeruli exhibited global or segmental glomerulosclerosis (Figure 2A). Histological sections from control cGVHD mice also displayed severe tubular damage, prominent perivascular inflammatory cell infiltrates and fibrosis (Figure 2A). In contrast, the general kidney pathology was greatly reduced in the DTA-1-treated cGVHD mice (Figure 2A). Consistent with the histological examination, IgG deposition in the kidney was significantly decreased in the DTA-1-treated cGVHD mice (Figure 2B). Finally, we confirmed that DTA-1-treated cGVHD mice had increased survival (Figure 2C). Thus, DTA-1 treatment has an effect on cGVHD mortality as well as morbidity. Taken together, our findings suggest that DTA-1 treatment prevents cGVHD by inhibiting the production of autoantibodies, and so inhibiting the induction and development of renal disease.

To address the mechanism underlying the inhibition of cGVHD by DTA-1 treatment, we investigated the lymphocyte populations in cGVHD mouse spleens. We consistently found a marked decrease in B-cell numbers 7 days after disease induction and B-cell lymphopenia after 14 days (Figure 3). Since B cells involve the production of autoantibody in cGVHD, our results indicate that elimination of B cells may be responsible for the inhibition of

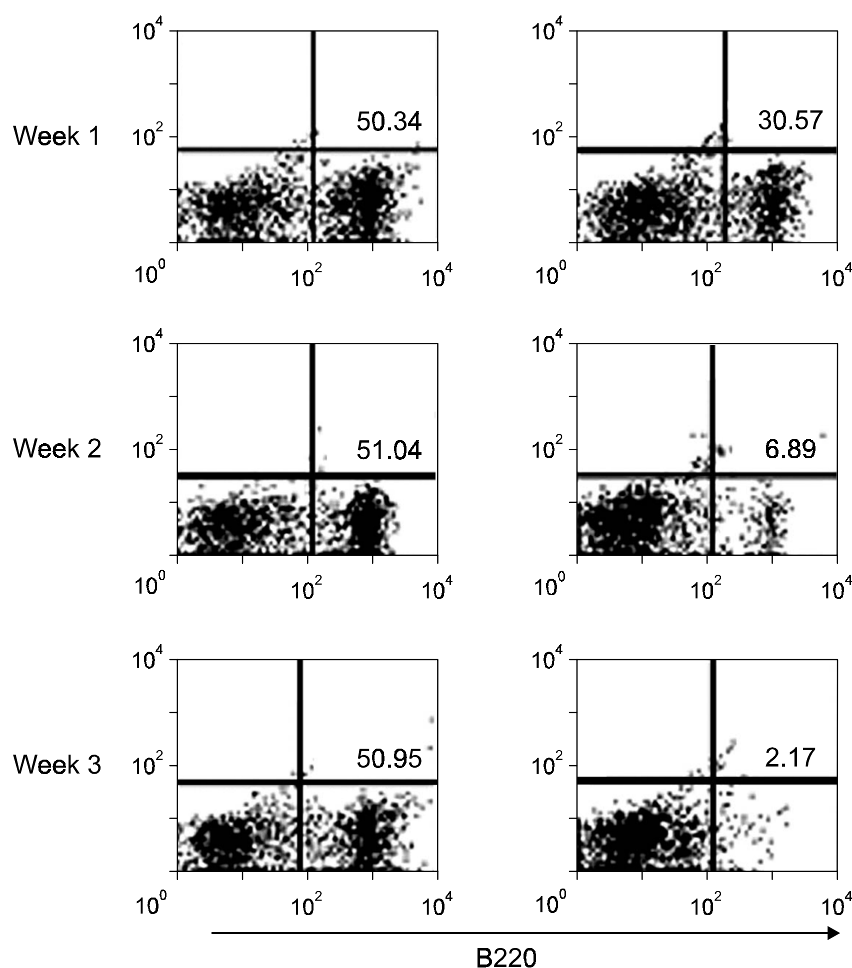


Figure 3. B cells are deleted after GITR stimulation. Splenocytes prepared at weeks 1, 2, and 3 after disease induction were analyzed for B220 staining. FACS plots shown are representative of more than 3 independent experiments (n = 3-5 per group).

autoantibody production by DTA-1 treatment.

The effect of DTA-1 could not be explained by the breakdown of immunosuppressive activity of $CD4^+CD25^+$ regulatory T cells. To determine whether or not the DTA-1-mediated inhibition of cGVHD is indeed independent of $CD4^+CD25^+$ regulatory T cells, we induced cGVHD by transferring $CD4^+CD25^+$ T cell-depleted DBA/2 parental cells into BDF_1 recipients. We previously demonstrated that donor $CD4^+CD25^+$ regulatory T cells were able to inhibit cGVHD (Kim *et al.*, 2005). Our results showed that deletion of $CD4^+CD25^+$ regulatory T cells did not influence the effect of DTA-1 on levels of anti-DNA IgG_1 and survival (Figure 4). These results clearly demonstrate that there exists an anti-GITR antibody's mechanism of action that is independent of $CD4^+CD25^+$ regulatory T cells.

Discussion

The main finding presented in this report is to reveal a GITR co-stimulatory pathway independent of $CD4^+CD25^+$ regulatory T cells *in vivo*. To be equally important, we provide a possibility of GITR stimulation as an immunotherapeutic tool for cGVHD and presumably other autoimmune diseases. There are reports showing *in vitro* that GITR signals co-stimulate $CD4^+CD25^-$ conventional T cells (Ji *et al.*, 2004; Kanamaru *et al.*, 2004; Kohm *et al.*, 2004; Ronchetti *et al.*, 2004) and $CD8^+$ T cells (Ronchetti *et al.*, 2004; Muriglan *et al.*, 2004; La *et al.*, 2005). In our cGVHD model, *in vivo* ligation of GITR prevents donor $CD8^+$ T-cell anergy and subsequently induces a sustained activation of alloreactive donor $CD8^+$ T cells (Kim *et al.,* 2006). Since the inhibition of cGVHD by GITR stimulation occurred in the absence of $CD4^+CD25^+$ regulatory T cells, our results also indicate that GITR signals directly co-stimulate conventional T cells *in vivo*.

There could be several possible explanations for the mechanism underlying our observations. It has been shown that GITR co-stimulation induces the activation-induced cell death (AICD) of alloreactive

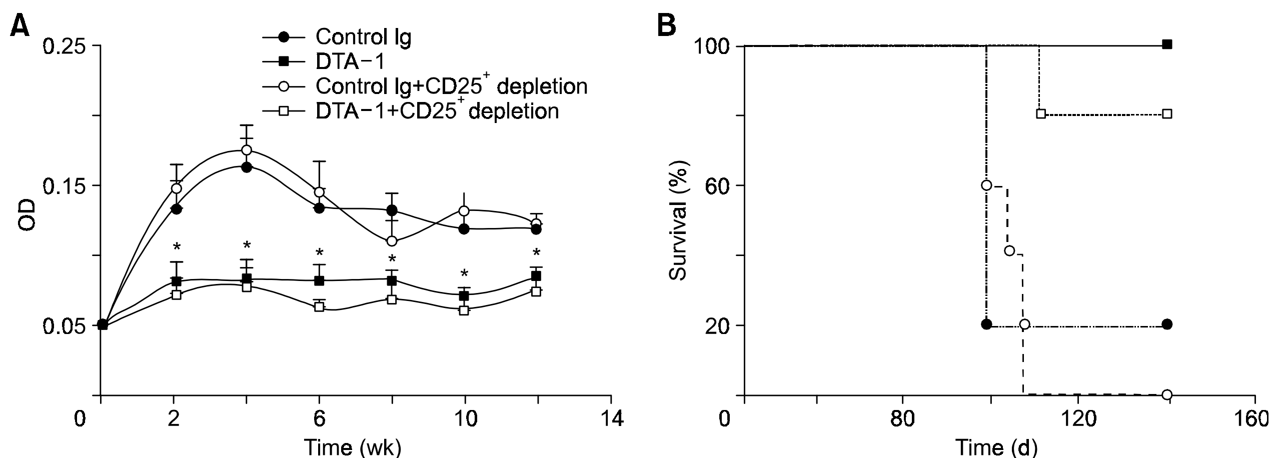


Figure 4. CD4⁺CD25⁺ regulatory T cells are not required for DTA-1-mediated inhibition of cGVHD. Donor CD4⁺CD25⁺ regulatory T cells were depleted before transfer using anti-CD25-conjugated microbeads. (A) OD values are mean ± SD of *n* = 10 per group at 10-fold dilution of samples. $^{*}P < 0.05$, between control Ig-treated groups and DTA-1-treated groups. (B) Survival curves (*n* = 10 per group). Data shown are representative of more than 3 independent experiments.

$CD4^+$ T cells, whereas it enforces the activity of alloreactive $CD8^+$ T cells (Muriglan *et al*., 2004). Therefore, one explanation is that GITR stimulation might prevent cGVHD by eliminating donor $CD4^+$ T cells required for the development of cGVHD (Morris *et al*., 1999). Stimulation of 4-1BB, a similar member of the TNF receptor superfamily, has been shown to be highly efficient in inducing the AICD of donor $CD4^+$ T cells (Kim *et al*., 2005), and so completely preventing cGVHD. However, we could not obtain any evidence supporting the hypothesis that GITR stimulation with a lower lose of DTA-1 (200 μg) preferentially induces the AICD of donor $CD4^+$ T cells (not shown). Another possibility is that GITR stimulation might block cGVHD through enhancing the cytotoxic activity of alloreactive donor $CD8^+$ T cells that can kill host splenocytes, including autoreactive B cells. This explanation is supported by our observations that DTA-1 was capable of eliminating B cells (Figure 3). Indeed, our study indicates that treatment of a higher dose of anti-GITR antibody can strongly activate alloreactive donor $CD8^+$ T cells in cGVHD and shift cGVHD towards an acute form (Kim *et al.,* 2006). The activation of donor $CD8^+$ T cells subsequently may lead to B-cell killing through two mechanisms: direct killing by their cytotoxic activity and indirect killing via secreting IFN-γ (Kim *et al.,* 2006; Sun *et al*., 2002).

Traditionally, immunotherapy has been targeted for blockade of co-stimulatory pathways to inhibit autoimmune diseases and graft rejection. Paradoxically, recent reports have provided evidence that stimulation of co-stimulatory pathways is highly potent in blocking the progression of autoimmune diseases (Maur *et al*., 2002; Sun *et al*., 2002; Foell *et al*., 2003; Seo *et al*., 2004) or GVHD (Yu *et al*., 2004; Kim *et al*., 2005). The therapeutic effect of strong co-stimulation *in vivo* is due to the deletion of autoreactive or alloreactive $CD4^+$ T cells. Therefore, administration of agonistic monoclonal antibodies against co-stimulatory molecules offers a promising new therapeutic approach for autoimmune diseases and GVHD.

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