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The Role of Regulatory T Cells Expanded by Anti-DR3 Antibody for Alleviation of Acute Graft-versus-Host Disease

급성 이식편대숙주질환 완화를 위한 항 DR3 항체에 의해 증가된 조절 T 세포의 역할

2015 년 2 월

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by

Kim, Byung-Su

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Microbiology and Immunology) at Seoul National University College of Medicine

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Approved by Thesis Committee:

Professor SUNG-SOO YOON Chairman

Professor CHUNG-GYU PARK Vice chairman

Professor Eung Soo Hwang

Professor Dong-Kyu Ye

Professor Young Yiel Lee

Abstract

The Role of Regulatory T Cells Expanded by Anti-DR3 Antibody for Alleviation of Acute Graft-versus-Host Disease

Kim, Byung-Su (2007-30966)
Department of Microbiology and Immunology
Seoul National University College of Medicine

Introduction: Regulatory T cells (Treg) alleviate acute graft-versus-host disease (aGVHD) while preserving graft-versus-tumor (GVT) effects. However, their low frequency hinders clinical translation. Although a lot of protocols to expand Treg have been reported, they still have limitations. DR3 belongs to the TNF receptor superfamily and is expressed primarily on T cells. Its agonistic antibody (Ab) was reported to expand Treg *in vivo* and prevent the rejection of a cardiac allograft.

Methods: C57BL/6 donor mice were intraperitoneally injected with anti-DR3 Ab or hamster IgG isotype control and conventional T cells (Tcon) were isolated from them four days later. Tcon and T cell-depleted (TCD) bone marrow (BM) cells were transplanted to lethally irradiated Balb/c recipients. Clinical signs of aGVHD and bioluminescence imaging (BLI) were regularly monitored. Recipient mice were sacrificed for flow cytometric analyses and serum cytokine assay at predefined time points.

Results: Donor mice treated with anti-DR3 Ab yielded Tcon containing higher proportions of Treg that suppressed Tcon proliferation with lower numbers. Mixed lymphocyte reaction (MLR) showed that these Tcon maintained higher Treg proportions, were less proliferative, had reduced Th1 differentiation and more PD-1 expression on Treg in response to allogeneic stimuli. *In vivo* studies confirmed that Tcon from anti-DR3-treated mice expanded to a lesser extent and caused the re-

duced aGVHD. Treg from anti-DR3-treated donors expanded robustly and their proportions within donor $CD4^+$ T cells were maintained higher. CD25 expression and BrdU incorporation of non-Treg $CD4^+$ and $CD8^+$ T cells derived from anti-DR3-treated donors were reduced *in vivo*. Recipients of Tcon from anti-DR3-treated donors had lower serum IFNγ, IL-1β, and TNFα levels. Tcon from anti-DR3-treated donors also preserved their GVT effects.

Conclusions: Together these data suggest that treating donors with anti-DR3 Ab can effectively expand donor Treg, alleviate aGVHD and preserve GVT effects. Conclusively, anti-DR3 Ab could be utilized as one of the strategies for *in situ* expansion of regulatory T cells.

Key words: acute graft-versus-host disease, bone marrow transplantation, regulatory T cells, DR3

Contents

Abbreviations

Ab	antibody
aGVHD	acute graft-versus-host disease
APC	antigen-presenting cells
ATG	anti-thymocyte globulin
ATRA	all-trans retinoic acid
BLI	bioluminescence imaging
BM	bone marrow
BMT	bone marrow transplantation
CCD	charge-coupled device
CNI	calcineurin inhibitor
CTL	cytotoxic T lymphocytes
DLI	donor lymphocyte infusion
EAE	experimental autoimmune encephalomyelitis
FCS	fetal calf serum
G-CSF	granulocyte colony stimulating factor
GMP	good manufacturing practice
GVT	graft-versus-tumor
GZB	granzyme B
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
iTreg	induced regulatory T cells
LN	lymph nodes
LPS	lipopolysaccharides
LTi	lymphoid tissue inducer cells
MACS	magnetic activated cell sorter
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
mTOR	mammalian target of rapamycin
PBSC	peripheral blood stem cell
PI3K	phosphatidylinositol 3-kinase

RBC	red blood cell
STAT	signal transducer and activator of transcription
TCD	T cell-depleted
Tcon	conventional T cells
TCR	T cell receptor
TNBS	2,4,6 trinitrobenzenesulfonic acid
Tr1	T regulatory type 1 cells
Treg	regulatory T cells
UCB	umbilical cord blood
WT	wild-type

Introduction

What is aGVHD

Allogeneic bone marrow transplantation (BMT) is a potentially curative treatment for a variety of hematologic malignancies (1,2) as well as autoimmune disorders (3,4). Although BMT has been increasingly performed in recent years, its overall success is often limited by the aGVHD. aGVHD is immunologic destruction of host tissues by donor lymphocytes predominantly affecting the liver, intestinal mucosa and skin (5). Conditioning regimens of BMT create lymphopenic environments and result in severe inflammation including the storm of proinflammatory cytokines and increased expression of adhesion molecules on host tissues. Donor T cells recognize host antigen-presenting cells (APC), proliferate and are activated in response to such conditions (6). Activated T cells produce large amounts of Th1 cytokines such as IFNγ, IL-2 and TNFα (5,6). These reactions finally differentiate cytotoxic T lymphocytes (CTL) that can damage host cells (7). TNFα is another important mediator of host tissue destruction and gut tissues are especially susceptible to TNFα (8). Microbial lipopolysaccharides (LPS) can translocate into the bloodstream through gastrointestinal mucosa damaged by a conditioning regimen and amplify severe inflammation (9). Therefore, it is very difficult to treat aGVHD once it has been established.

How to control aGVHD

Pharmacologic agents are the mainstream of contemporary armamentarium for prophylaxis and treatment of aGVHD. The combination therapy of short-course methotrexate and a calcineurin inhibitor (CNI) such as cyclosporine or tacrolimus has been a gold standard of aGVHD prophylaxis for the last 30 years (10). CNI interferes with calcium-dependent IL-2 gene activation and inhibits activation and proliferation of not only Tcon but also Treg (11). Anti-thymocyte globulin (ATG) is

another popular agent for GVHD prophylaxis. However, it cannot improve survival because of the increased risk of opportunistic infection and tumor relapse associated with it (12). Rapamycin down-regulates IL-2/phosphatidylino-sitol 3-kinase (PI3K) signaling pathway of Tcon through inhibiting mammalian target of rapamycin (mTOR) (13). Treg preferentially use IL-2 receptor β/signal transducer and activator of transcription (STAT)5 pathway and are not interfered with rapamycin (14). Standard GVHD prophylaxis regimens fail in about 40% of patients who have received human leukocyte antigen (HLA)-matched BMT (15). Treatment outcome of diagnosed aGVHD is even worse and 50~60% of patients do not respond. Therefore, the current status of aGVHD control is not satisfactory and more effective therapeutic strategies are required.

Control of aGVHD by natural Treg

Natural Treg are the $Foxp3^+$ subset of $CD4^+$ T cells and develop in the thymus. They suppress the activation not only of self-reactive but also of allo-reactive T cells (17). One of the most seminal findings in the field of aGVHD is that donor natural Treg prevent development of aGVHD in a murine model (18). This finding has been repeatedly demonstrated in numerous clinical and preclinical studies (19). At the first clinical trial, natural Treg were purified from peripheral blood using the magnetic activated cell sorter (MACS) under good manufacturing practice (GMP) conditions and adoptively transferred to recipients without further manipulation. Approximately 50% of the purified cells had $CD4^+CD25^{high}$ phenotype and showed the suppressive development of aGVHD (20). Another study reported that natural $CD4^+CD25^+$ Treg were isolated using the MACS technique and infused into recipients before the injection of donor Tcon. Approximately 70% of Treg were $Foxp3^+$ subset and prevented aGVHD in the absence of any post-transplantation immunosuppression without interfering GVT effects (21). However, adoptive cell therapy using natural Treg cannot be

used widely since its low frequency makes it difficult to obtain enough yields of Treg from donors (22).

Expansion of natural Treg and its limitations

To overcome this, a lot of protocols of *ex vivo* expansion of Treg were explored (23). *Ex vivo* expansion of Treg was accomplished by high-dose IL-2 and combined stimulation of CD3/CD28 (24,25). This method required specialized facilities for human T cell culture and ~10 days delay in the time to transplantation (23). Overgrowth of contaminating effector T cells was often observed and rapamycin was tried to prevent it (26). Umbilical cord blood (UCB) is another source for natural Treg expansion since MACS-purified Treg from UCB contains very few $CD25^{dim}$ $CD4^{+}$ effector/memory T cells (27). Contaminating $CD25^{-}$ naïve T cells from UCB are anergic and hypoproliferative, thereby, they do not affect the suppressive function of expanded UCB Treg (28). However, expansion yields of UCB Treg are very variable and often unpredictable so that routine use of this technique is impractical (23). *In vivo* expansion of Treg after BMT has been also attempted. The combined administration of rapamycin and IL-2 to recipients expanded donor-type Treg and reduced lethal aGVHD in a murine major histocompatibility complex (MHC) I/II-mismatch murine model (29). However, systemic side effects associated with IL-2 administration limit clinical translation of this strategy.

Use of induced Treg (iTreg) and T regulatory type 1 (Tr1) cells

iTreg cells exit from the thymus as $Foxp3^{-}$ naïve $CD4^{+}$ T cells and Foxp3 expression is induced in the periphery (30). iTreg cells can be generated and expanded *in vitro* from human naïve T cells by stimulating them in presence of TGFβ and all-trans retinoic acid (ATRA) or rapamycin (31). While outcomes of TGFβ/ATRA protocol showed variable success rates, iTreg generated by rapamycin/TGFβ protocol sup-

pressed aGVHD in a xenogenic model (32). Tr1 cells are $CD25^-$ $Foxp3^-$ $IL\text{-}10^+$ subpopulation of T cells and they were reported to have a role in the long-term tolerance of BMT (33). Alloantigen-specific Tr1 cells can be generated *in vitro* from naïve T cells and show theoretical advantages over adoptive cell therapy of polyclonal natural Treg (34). However, it also requires prolonged culture of donor T cells in the presence of host mononuclear cells and IL-10 (34). Hypomethylating agents (azacitidine and decitabine) and histone deacetylase inhibitors (vorinostat) are mainly used for treating myelodysplastic syndrome or other hematologic malignancies. These drugs were recently shown to induce Foxp3 expression from $CD4^+CD25^-$ Tcon *in vivo* and suppress aGVHD in a murine model (35,36). However, these agents touch broad aspects of an immune system and can affect engraftment of hematopoietic stem cells adversely.

Death receptor 3 (DR3, TNFRSF25)

Death receptor 3 (DR3, TNFRSF25) belongs to a TNF receptor superfamily and is primarily expressed on regulatory T cells, lymphoid tissue inducer cells (LTi) and NKT cells. Its natural ligand, TL1a is expressed on endothelial cells and APC (37). Early studies reported that they induced pathogenic inflammation in various disease models (38). TL1a co-stimulated proliferation and cytokine production in $CD4^+$ T cells through DR3. $DR3^{-/-}$ T cells showed reduced proliferation and altered cytokine production *in vitro*. $DR3^{-/-}$ T cells also caused decreased inflammation in the experimental autoimmune encephalomyelitis (EAE) model (39). Interestingly, TL1a preferentially stimulated memory T cells rather than naïve T cells (40). TL1a has been linked to human inflammatory bowel disease (IBD, 41). Constitutively TL1a-expressing transgenic mice spontaneously develop IBD and treatment of TL1a-neutralizing Ab abrogated 2,4,6 trinitrobenzenesulfonic acid (TNBS)-induced colitis (42). Interestingly, $Foxp3^+$ Treg were present in increased numbers (42).

Treg expansion by DR3 stimulation

Schreiber et al. recently showed that agonistic anti-DR3 Ab expanded Treg *in vivo* through the T cell receptor (TCR) engagement and IL-2 receptor-dependent mechanism (42). This Ab prevented the development of allergic lung inflammation by increasing the Treg proportion of $CD4^+$ T cells in the lung and suppressing Th2 cytokine production (42). Anti-DR3 Ab also promoted a cardiac allograft survival by increasing the Treg proportion of T cells that resided in a cardiac graft as well as a spleen (43). To confirm whether a physiologic ligand TL1a could also expand Treg, TL1a-Ig fusion protein was generated (44). TL1a-Ig mediated rapid proliferation of $Foxp3^+$ Treg as well as $CD4^+Foxp^-$ Tcon *in vitro*. TL1a-Ig also blocked de novo biogenesis of iTreg. DR3 stimulation by TL1a-Ig *in vivo* induced expansion of Treg, which was dependent on TCR engagement with MHC class II. TL1a-Ig–mediated Treg expansion *in vivo* was protective against allergic lung inflammation, a mouse model for asthma (44). To improve the outcome of BMT, it is necessary to develop a novel protocol of Treg expansion, which should expand Treg more rapidly but not require specialized facilities. Anti-DR3 Ab was reported to expand Treg *in vivo* in just 5 days by a single injection. Furthermore, this Ab affect only limited cell populations such as T cells and NKT cells. In this study, Tcon were isolated from anti-DR3-treated donor mice and transplanted to MHC I/II-mismatched allogeneic recipients to show increased Treg, reduced aGVHD and preserved GVT effects.

Materials and Methods

Mice and cell line

C57BL/6 (H-2k^b CD45.2$^+$) and BALB/c (H-2k^d CD45.2$^+$) mice were purchased from the Jackson Laboratory and luciferase-expressing (*luc*$^+$) C57BL/6 (H-2k^b Thy-1.1$^+$ CD45.1$^+$) mice were bred by backcrossing FVB/N L2G85 transgenic mice into Thy-1.1$^+$ CD45.1$^+$ C57BL/6 strain for >14 generations and were obtained from Dr. Negrin, Stanford University (45). Mice were used at age 8-16 weeks and only sex-matched combinations were used for transplant experiments. BCL_1 is the murine B cell lymphoma cell line that arose from the Balb/c strain and closely resembles human chronic lymphocytic leukemia (46). Generation of *luc*$^+$/*gfp*$^+$-BCL_1 cell line was previously described and it is also a gift from Dr. Negrin, Stanford University (47).

Antibodies and reagents

The FcR blocking reagent, anti-CD4 microbead, anti-CD8 microbead, LS columns, and a manual MidiMACS separator were purchased from Miltenyi Biotec (Auburn, CA) and used to isolate Tcon from donor mice. Anti-APC microbead (Miltenyi Biotec) was used to enrich CD25$^+$ cells. Anti-DR3 Ab (clone: 4C12) and hamster IgG isotype control Ab (clone: HTK888) were purchased from Biolegend (San Diego, CA). Monoclonal Ab to the following molecules were used for flow cytometric analysis: H-2K^b (AF6-88.5), CD45.1 (A20), CD3ε (145-2C11), CD4 (GK1.5), CD8a (53-6.7), Foxp3 (FJK-16s), CD25 (PC61), T-bet (4B10), granzyme B (GB11), PD-1 (RMP1-30), IFNγ (XMG1.2), TNFα (MP6-XT22), and BrdU (BU20A) from Biolegend and eBioscience (San Diego, CA). Fixable Viability Dye eFluor® 506 (eBioscience) was used to exclude dead cells. To fix and permeabilize cells, Foxp3/Transcription Factor Fixation/Permeabilization buffer set (eBioscience) was used. BrdU and DNase I were purchased from Sigma (St. Louis, MO). [^{3}H]thymidine was obtained from Perkin-Elmer (Shelton, CT).

Cell isolation/sorting after Ab administration

Wild-type (WT) or *luc*$^+$ CD45.1$^+$ C57BL/6 donor mice were treated according to the purpose of each experiment. They were administered with anti-DR3 or isotype control Ab 0.5 mg/kg at 4 days before transplantation (day -4) and sacrificed at day 0. Lymph nodes (LN; cervical, axillary, inguinal, and mesenteric) and a spleen were harvested and processed in PBS supplemented with 2% fetal calf serum (FCS) to single cell suspension. Cells were blocked with FcR blocking reagent after red blood cell (RBC) lysis. To isolate Tcon, they were bound with anti-CD4/anti-CD8 microbeads and run through a LS column and a MidiMACS separator. Tcon were eluted manually from the column. For CD25$^+$ Treg sorting, cells were stained with anti-CD25 APC and anti-CD4 eF450, bound with anti-APC microbead and positively selected by a LS column and a MidiMACS separator (Miltenyi Biotec). CD25$^+$-enriched cells were then loaded on a BD FACS Aria II cell sorter (BD Biosciences; San Jose, CA) and CD4$^+$CD25$^+$ cells were sorted. This purification protocol yielded >95% pure CD4$^+$CD25$^+$Foxp3$^+$ cells. To deplete T cells from BM, cells were flushed out from femurs and tibias of naïve WT C57BL/6 mice at day 0. They were bound with anti-CD4/anti-CD8 microbeads and run through a LS column and a MidiMACS separator. Flow-through was taken as TCD-BM.

Mouse models: MHC I/II-mismatch aGVHD and BCL_1 tumor models

aGvHD was induced as described previously (11). Briefly, BALB/c recipient mice were lethally irradiated with 850cGy, given in 2 split doses of 425cGy at least 4 hours apart, on day 0. TCD-BM cells were isolated from WT C57BL/6 donor mice that received no treatment while Tcon were from Ab-treated C57BL6 donor mice. 5x10^6 TCD-BM and 1x10^6 Tcon were intravenously injected into recipient mice within 4 hours of irradiation (Figure 1). For a few experiments that were fo-

cused on the later period of aGVHD, $5x10^5$ Tcon and $5x10^6$ TCD-BM were injected. Mice were kept in disposable cages from Innovive (San Diego, CA) and provided with Uniprim® Teklad diet from Harlan Laboratories (Indianapolis, IN) for a minimum of 30 days after transplantation. Recipient mice were monitored and weighed 2-3 times per a week. Clinical evidence of aGVHD was evaluated and scored as described in Table 1 (48). To make a BCL_1 tumor model, 20,000 *luc*$^+$/*gfp*$^+$-BCL_1 cells were intravenously injected to Balb/c mice via a tail vein at day -8 and their engraftment was confirmed by BLI at day -1. These mice were lethally irradiated and transplanted with $5x10^6$ TCD-BM and $1x10^6$ Tcon at day 0 as described above.

Mixed lymphocyte reaction (MLR)

For conventional MLR experiments, Tcon were isolated from anti-DR3 or isotype-treated B6 donor mice by the manual MACS selection as described above. A spleen was harvested from a Balb/c mouse and processed to single cell suspension. After RBC lysis, cells were γ-irradiated (3000rad). B6 Tcon ($2x10^5$/well) and irradiated Balb/c splenocytes ($2x10^5$/well) were cultured together using a RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (GIBCO; Grand Island, NY), 5 µg/mL 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 30 IU/mL of IL-2 (Chiron; Emeryville, CA) in a 37°C humidified, 5% CO_2 incubator. Cultures were set up in triplicates in 96-well flat-bottom plates and the volume of each well was 200 µL. After 96 hours culture, cells were pulsed with 0.037 MBq/well (1 µCi/well) [^{3}H]thymidine for 16 hours. Cells were harvested onto filter membranes using a Wallac harvester and the amount of incorporated [^{3}H]thymidine was measured with a Wallac Betaplate counter (all from Perkin-Elmer; Shelton, CT). For suppressive MLR experiments, Tcon were isolated from naïve B6 mice by the manual MACS selection while CD25$^+$ Treg were MACS-enriched and sorted from Ab-treated B6 donor mice as described above. Naïve B6

Tcon ($2x10^5$/well), irradiated Balb/c splenocytes ($4x10^5$/well), and $CD25^+$ Treg from Ab-treated donors (at various ratios to naïve B6 Tcon) were cultured for 96 hours and then pulsed with [^{3}H]thymidine in the same way as for conventional MLR experiments.

Flow cytometric analysis: Foxp3, cytokines and BrdU staining

Cells were harvested from MLR plates after 96 hours culture or re-isolated from BM transplant recipients. Briefly, LN (cervical, axillary, inguinal, and mesenteric) and a spleen were harvested from recipient mice at days 3, 7, 14, and 28 according to the purpose of each experiment and then processed to single cell suspension. For intracellular cytokine staining, cells were stimulated with Cell Stimulation Cocktail (eBioscience). Briefly, 1 µL of cocktail was diluted to 500 µL of cell culture medium and cells were incubated in a 37°C humidified, 5% CO_2 incubator for 5 hours. After that, cells were washed with staining buffer for use in Ab staining protocols. Cells were blocked with FcR blocking reagent after RBC lysis then stained with Ab for surface markers and viability dye. They were fixed with Foxp3/Transcription Factor Fixation/Permeabilization buffer set (eBioscience) and then stained with Ab to Foxp3, T-bet, and cytokines. For BrdU staining, recipient mice were intraperitoneally injected with 1mg of BrdU 1 and 2 days before LN/spleen harvest. Re-isolated cells were surface stained, fixed using the Foxp3/Transcription Factor Fixation/Permeabilization buffer set (eBioscience), treated with 30 µg of DNase I for 1 hour at 37°C, and then stained with Ab to BrdU. All assays were performed according to the manufacturer's instructions and analytical flow cytometry was done on a 4-laser LSR II cytometer (BD Biosciences). Collected data were analyzed using FlowJo software (TreeStar; Ashland, OR).

In vivo bioluminescence imaging (BLI)

In vivo BLI was performed as described previously (47). Briefly, mice

were injected intraperitoneally with luciferin (10 μg/g of body weight). Ten minutes later, the mice were anesthetized and imaged using an IVIS Spectrum charge-coupled device (CCD) imaging system (Xenogen; Alameda, CA) for 5 minutes. Imaging data were analyzed and quantified with the Living Image Software (Caliper Life Sciences; Hopkinton, MA).

Multiplex cytokine assays

Serums were collected from BM transplant recipients by a heart puncture at days 3, 7, 14, and 28 according to the purpose of each experiment and immediately stored at -80°C in a freezer until the assay day. 20 different cytokines were analyzed in a multiplex assay system using fluorescently labeled microsphere beads (Cytokine Mouse 20-plex Panel for the Luminex® platform, LMC0006, Invitrogen; Carlsbad, CA). The assay was performed in duplicate according to the manufacturer's instructions. Cytokine levels were quantitated using the Luminex 200 system (Luminex; Austin, TX)

Statistical analysis

Post-transplantation survival curves were plotted using Kaplan-Meier method and compared by a log-rank test. Serial cytokine levels, changes in donor Treg and aGVHD scores were compared using the multiple *t* tests with Holm-Sidak correction for dealing with multiple comparisons across all time points. This statistical method was also used when the one variable was compared in similar culture conditions. Otherwise, data were analyzed using the 2-tailed Student *t* test. A *P* value <0.05 was considered statistically significant. Prism 6 (GraphPad Software; La Jolla, CA) was used for all statistical analysis.

Results

Tcon isolated from anti-DR3-treated mice contain higher proportion of Treg that are functional with lower numbers

Although agonistic anti-DR3 Ab (clone: 4C12) had been shown to induce rapid proliferation of Treg in peripheral blood (42), it was not certain that this Ab could expand Treg in lymphoid tissues to increase Treg yields from donor mice. To investigate this, Tcon donor mice were treated as described in the Materials and Methods section and sacrificed 4 days later to harvest LN and a spleen. Proportions of Treg were significantly increased in Tcon graft which were isolated from anti-DR3-treated donor mice (Figure 2A; $P<0.0001$). Absolute Treg numbers obtained from a single donor were also increased with anti-DR3 treatment (Figure 2B; $P<0.0001$). More than 20 Tcon isolations were performed for this study and in every case, Treg proportions of Tcon graft as well as absolute Treg numbers from anti-DR3-treated donors were increased.

To determine the suppressive capability of Treg from anti-DR3-treated donors, B6 mice were treated with anti-DR3 Ab or isotype control and Treg ($CD4^+CD25^+$ subpopulation) were sorted out. Other B6 mice received no treatment and naïve Tcon were isolated from them using the MACS technique. Fixed numbers of naïve B6 Tcon and irradiated Balb/c splenocytes were cultured together and sorted $CD4^+CD25^+$ Treg were added at a 1:1 or lower ratios to naïve B6 Tcon. They were incubated for 96 hours and pulsed with [^{3}H]thymidine. Figure 2C shows that Treg from anti-DR3-treated donors suppressed Tcon proliferation more efficiently than Treg from isotype-treated donors at lower Treg:Tcon ratios ($P<0.05$). At higher Treg:Tcon ratios, both Treg types suppressed Tcon proliferation almost completely. These results suggest that treating donor mice with anti-DR3 Ab increases the number as well as the functional activity of Treg that play a crucial role in suppressing aGVHD.

Tcon from anti-DR3-treated donors are less proliferative, less activated and maintain higher Treg level in MLR

To investigate how Tcon from anti-DR3-treated donors respond to allogeneic stimuli, MLR experiments were performed. Tcon from anti-DR3-treated donors showed significantly decreased thymidine uptake (Figure 3A; $P<0.001$). They were also stained for Foxp3 expression before and after MLR and analyzed by flow cytometry. Tcon from anti-DR3-treated donors had higher Treg proportion after MLR compared to Tcon from isotype-treated donors (Figure 3B; $P<0.001$). When comparing before and after allogeneic MLR, the proportion of Treg was significantly decreased in Tcon from isotype-treated donors (Figure 3B; $P<0.01$) but not changed in Tcon from anti-DR3-treated donors (Figure 3B; not significant). These results suggest that Tcon from anti-DR3-treated donors had reduced proliferation capacity and their Treg proportion can be sustained in an allogeneic environment.

Since Th1 differentiation of donor Tcon is the main pathway of aGVHD, further allogeneic MLR experiments were performed and T-bet, IFNγ, TNFα and granzyme B (GZB) expressions analyzed by flow cytometry. TNFα production was significantly reduced in non-Treg CD4 (Figure 3C; $P<0.05$) and CD8 T cells (Figure 3C; $P<0.001$) from anti-DR3-treated donors compared to the same subpopulation from isotype-treated donors. T-bet expression was significantly decreased in CD8 T cells from anti-DR3-treated donors compared to those from isotype-treated donors (Figure 3D; $P<0.05$). T-bet expression was equally very low in non-Treg CD4 T cells from both groups. GZB production was also significantly decreased in CD8 T cells from anti-DR3-treated donors (Figure 3E; $P<0.001$). These results show that Th1 differentiation of Tcon from anti-DR3-treated donors was suppressed even in the allogeneic environment.

PD-1 and GZB expressions of Treg in allogeneic environment were also analyzed by flow cytometry since it has been suggested that Treg

use these molecules to exert regulatory functions (49). Figure 3F and 3G show that Treg from anti-DR3-treated donors produced more PD-1 (P<0.05) and GZB (P<0.01) than Treg from isotype-treated donors. These results suggest that Treg from anti-DR3-treated donors possessed augmented suppressive functions.

Tcon from anti-DR3-treated donors expand poorly *in vivo* and cause reduced aGVHD mortality and morbidity

To investigate whether treating donors with anti-DR3 Ab can reduce the mortality and morbidity rates associated with aGVHD, a major mismatch aGVHD model was used. Briefly, Tcon were isolated from anti-DR3 or isotype-treated donors and $1x10^6$ of this Tcon transplanted to lethally irradiated recipient mice together with $5x10^6$ TCD-BM from naïve donor mice as described in the Materials and Methods section. Mice were examined more than once a week to confirm death or to measure aGVHD score. Most recipient mice that received Tcon from anti-DR3-treated donors survived and they showed significantly improved survival compared to other mice receiving Tcon from isotype-treated donors (Figure 4A; P<0.0001). Both groups of recipients had similar aGVHD scores for the first two weeks after transplantation when the cytokine storm induced by lethal irradiation reached its peak. After that, recipients of Tcon from isotype-treated donors had their aGVHD becoming worse again and most of them died between day 30 and 60. In contrast, recipients of Tcon from anti-DR3-treated donors only experienced mild weight loss (10~20% of baseline weight) and skin GVHD. They showed stable aGVHD scores that were significantly lower at multiple time points (Figure 4B; P<0.0001). To track *in vivo* expansion of Tcon, Tcon were isolated from *luc*$^+$ B6 donor mice which were treated with Anti-DR3 Ab or isotype control and $1x10^6$ of Tcon were transplanted to lethally irradiated Balb/c recipients together with $5x10^6$ TCD-BM from naïve wild-type B6 mice for serial BLI. While Tcon from anti-DR3-treated donors showed limited expansion across a

whole period, expansion of Tcon from isotype-treated donors showed sharp peaks and became significantly different on day 6 and 30 after transplantation (Figure 4C; $P<0.0001$). Distribution patterns were not different between the two groups of Tcon (Figure 4D).

Treg from anti-DR3-treated donors expand robustly after BMT and suppress Tcon proliferation *in vivo*

Since it has been suggested that higher Treg proportion of donor CD4 T cells is associated with reduced aGVHD risk (50-52), the Treg proportion of CD4 T cells that were derived from anti-DR3-treated donors was measured. Tcon from CD45.1$^+$ B6 donor mice that were treated with Anti-DR3 Ab or isotype control were isolated and $1x10^6$ Tcon were transplanted to lethally irradiated Balb/c recipients together with $5x10^6$ TCD-BM from naïve wild-type B6 mice. LN and a spleen were harvested on days 3, 7, and 14 from recipients and processed into single-cell suspensions. Re-isolated cells were stained for CD45.1 and other markers, fixed, and the Foxp3 expression was examined by flow cytometric analysis. CD45.1 congenic marker was used to gate T cells derived from anti-DR3 or isotype-treated donors. Treg derived from anti-DR3-treated donors increased in number over time after transplantation but Treg from isotype-treated donors did not (Figure 5A LN, not significant at day 3 and 7, $P<0.0001$ at day 14; Spleen, not significant at day 3, $P<0.01$ at day 7, $P<0.0001$ at day 14). Treg proportion of CD4 T cells derived from isotype-treated donors rapidly decreased early after transplantation. Recipients of Tcon from anti-DR3-treated donors maintained higher Treg proportions of donor CD4 T cells at least for the first two weeks after transplantation (Figure 5B LN, $P<0.001$ at day 3, $P<0.0001$ at day 7, $P<0.05$ at day 14; Spleen, $P<0.0001$ at day 3, $P<0.05$ at day 7, $P<0.05$ at day 14). To investigate the proliferation of T cells derived from anti-DR3-treated donors, recipient mice were intraperitoneally injected with BrdU 1mg at day 5 and 6 then sacrificed at day 7. Re-isolated cells were fixed and stained with

anti-BrdU Ab as described in the Materials and Methods section. Proliferating fraction of non-Treg CD4 T cells that were derived from anti-DR3-treated donors was significantly reduced (Figure 5C, $P<0.01$ in LN, $P<0.001$ in spleen) compared to those from isotype-treated donors. CD8 T cells showed the same results (Figure 5D, $P<0.05$ in LN, $P<0.001$ in spleen).

To investigate Treg proportion of donor CD4 T cells for the later period, it was necessary to avoid death of recipient mice and selection bias so that $5x10^5$ Tcon were transplanted to recipient mice and peripheral blood was collected every 20 days. Figure 7A shows that recipients of Tcon from anti-DR3-treated donors maintained higher donor Treg proportion until day 60 compared to recipients of Tcon from isotype-treated donors ($P<0.0001$ at day 20, $P<0.001$ at day 40 and 60). Proliferation of T cells derived from anti-DR3-treated donors was investigated once again for the later phase of aGVHD. Recipient mice were transplanted with $5x10^5$ Tcon that were isolated from anti-DR3 or isotype-treated donors, injected with BrdU 1mg at day 26 and 27, then sacrificed at day 28. Proliferating fraction of non-Treg CD4 T cells (Figure 7B) and CD8 T cells (Figure 7C) derived from anti-DR3-treated donors significantly decreased compared to those from isotype-treated donors (Figure 7B $P<0.001$ in LN, $P<0.01$ in spleen; Figure 7C $P<0.01$ in LN, $P<0.05$ in spleen). These results suggest that higher Treg proportion of Tcon was maintained and contributed to the sustained suppression of non-Treg T cells.

Tcon from anti-DR3-treated donors down-regulate CD25 and cause lower serum inflammatory cytokines

CD25 is the common marker of activated T cells and has been used frequently to measure the T cell activation status in mouse aGVHD studies (53). Figure 6A shows that non-Treg CD4 T cells derived from anti-DR3-treated donors had lower CD25 expression compared to those from isotype-treated donors (not significant in LN, $P<0.01$ in

spleen) at day 3 after transplantation. CD8 T cells from anti-DR3-treated donors also showed lower CD25 expression at day 3 (Figure 6B, $P<0.01$ in LN, $P<0.001$ in spleen). In addition, serum levels of IFNγ, IL-1β and TNFα were measured since they are known to play an important role in aGVHD pathogenesis. It was particularly interesting to track their serial changes since a certain cytokine is preferentially involved with the specific stage of aGVHD (7). Serums were collected from recipient mice through a heart puncture at days 3, 7, and 14 after BMT and used for the magnetic beads-based multiplex assay. IFNγ (Figure 6C) and IL-1β (Figure 6D) levels increased early after transplantation and then rapidly decreased in recipients of Tcon from isotype-treated donors but did not change much in recipients of Tcon from anti-DR3-treated donors ($P<0.0001$ at day 3). TNFα levels were stable and equivalent between the two groups until day 7. However, TNFα level in recipients of Tcon from isotype-treated donors increased and became significantly higher at day 14 (Figure 6E, $P<0.0001$ at day 14). Serum IFNγ and TNFα levels were measured again at day 28 after transplantation. Recipient mice were transplanted with $5x10^5$ Tcon that were isolated from anti-DR3 or isotype-treated donors. Recipients of Tcon from anti-DR3-treated donors had lower serum IFNγ and TNFα levels that were insignificant for IFNγ (Figure 7D, not significant) but significant for TNFα (Figure 7E, $P<0.01$). Intracellular TNFα staining showed that this difference was significant only in CD8 T cells (Figure 7F, ns, not significant; ***P 0.0001 to 0.001). These data suggest that Tcon from anti-DR3-treated donors caused less T cell activation and lower inflammatory cytokine levels in the major-mismatch aGVHD model.

Tcon from anti-DR3-treated donors retain GVT effects

In the clinic, measures to prevent or treat aGVHD frequently impair GVT effects of BMT and fail to improve patients' outcome. Therefore, it is very crucial to alleviate aGVHD while preserving GVT effects at the

same time. It was previously reported that natural Treg did not impair GVT effects when they were sorted from naïve mice of the same strain and transplanted together with Tcon (18). Attempts were made to confirm whether Tcon from anti-DR3-treated donors could retain their GVT effects since not only could this Ab upregulate Treg activity through PD-1 upregulation but they also maintain higher Treg proportions for a long period after transplantation. BCL_1-*gfp*$^+$/*luc*$^+$ cell line was intravenously injected to Balb/c recipients 8 days before BMT to track tumor cell proliferation *in vivo*. WT B6 mice were treated with anti-DR3 or isotype control 4 days before transplantation for Tcon isolation. $1x10^6$ Tcon were injected into recipients and BMT was performed as described in the Materials and Methods section. BLI was repeated to track the tumor burden *in vivo*. Every mouse injected with BCL_1-*gfp*$^+$/*luc*$^+$ cell line was confirmed to have this cell line engrafted at one day before transplantation. Figure 8A and 8B show that BCL_1-*gfp*$^+$/*luc*$^+$ cell line was initially cleared early after transplantation and started to proliferate in spleen. Soon tumor cells spread to the whole body of recipient mice that received BCL_1-*gfp*$^+$/*luc*$^+$ cell line with no Tcon. In contrast, recipient mice that received BCL_1-*gfp*$^+$/*luc*$^+$ cell line and Tcon isolated either from anti-DR3 or isotype-treated donor mice showed no signs of tumor engraftment until day 80. Almost all recipient mice that received BCL_1 and Tcon from isotype-treated donors died of aGVHD. Recipient mice that received BCL_1 and Tcon from anti-DR3-treated donors showed significantly longer survival (Figure 8C, $P<0.0001$). Recipient mice that received BCL_1 only had a similar survival as the recipient mice of Tcon from anti-DR3-treated donors. These results confirm that Tcon from anti-DR3-treated donors retain their GVT activity.

Discussion

DR3 stimulation results in sustainably higher donor Treg frequency, which is important for aGVHD control

It was decided to test anti-DR3 Ab for alleviation of aGVHD based on prior studies that showed Treg expansion and increased survival of heart grafts by this Ab. However, it was not certain that Tcon from anti-DR3-treated mice could maintain higher Treg proportion after BMT since increased Treg frequency started to decline at 5~6 days after anti-DR3 Ab treatment for steady-state mice. Furthermore, severe aGVHD was associated with decreased Treg frequency (50-52). Donor Tcon from anti-DR3-treated mice might be able to escape from suppression by Treg since they faced storms of inflammatory cytokines and severe lymphopenic environment, which were quite different conditions from solid organ transplantations.

To answer the first question, allogeneic MLR experiments were performed and it was found that the Treg proportion of Tcon from anti-DR3-treated mice did not change significantly but the Treg proportion of Tcon from isotype-treated mice decreased significantly after 96 hours culture with allogeneic splenocytes (Figure 3B). Figure 5A confirms that Treg from anti-DR3-treated donors can expand over time while Treg from isotype-treated donors cannot. The higher Treg proportions of Tcon from anti-DR3-treated donors were maintained at least for the early phase of aGVHD induced by $1x10^6$ Tcon and virtually for the whole period of aGVHD induced by $5x10^5$ Tcon (Figure 5B, Figure 7A). The superior suppressive activity of DR3-stimulated Treg limited the expansion of $Foxp3^-$ Tcon and helped to maintain relative higher Treg proportions. It is another important finding of this study that recipient mice of the Tcon from anti-DR3-treated donors showed significantly better survival and decreased aGVHD morbidity than recipients of Tcon from isotype-treated donors. This difference became more prominent as days passed and aGVHD score curves diverged signifi-

cantly at day 30 after transplantation (Figure 4A & 4B). Curves of total photon flux from BLI looked very similar with aGVHD score curves, suggesting that aGVHD symptoms were driven by donor Tcon (Figure 4C).

DR3 stimulation causes prolonged reduction of serum TNFα, the important effector cytokine of aGVHD

TNFα is the most important cytokine for the effector phase of aGVHD and gastrointestinal tissues are especially susceptible to TNFα to provide an amplification loop of the cytokine storm (54). Interestingly, serum TNFα levels also showed the compatible pattern with aGVHD score and BLI data. Serum TNFα levels neither increased nor showed differences between the recipients of anti-DR3- vs. isotype-stimulated Tcon for the first week but at day 14 they increased and showed a significant difference (Figure 6E, $1x10^6$ Tcon). The serum TNFα data from different experiments ($5x10^5$ Tcon) were significantly different again at day 28 (Figure 7E). Intracellular cytokine staining performed at day 28 suggested that donor CD8 CTL might be responsible for this difference (Fig 7F). Shin et al. reported that combined treatment of IL-2 and rapamycin to recipient mice expanded donor Treg and decreased intracellular production of IFNγ and TNFα. However, neither IL-2 nor rapamycin alone was enough for donor Treg expansion and decreased cytokine productions (30). This finding suggests that donor Treg expansion is associated with reduced production of IFNγ and TNFα from T cells.

Recipients of Tcon from anti-DR3-treated donors had quite stable serum IFNγ levels while recipients of Tcon from isotype-treated donors had very high level of serum IFNγ at day 3, which decreased rapidly thereafter. Serial trends of serum IFNγ and TNFα, the two most well known cytokines of aGVHD pathophysiology, suggested that IFNγ was more important for the activation phase and TNFα for the effector phase. Proliferating fraction of each Tcon subset (non-Treg CD4 and/o

CD8) was measured by *in vivo* BrdU labeling. Figures 5C~D showed that the Tcon derived from anti-DR3-treated donors had reduced proliferating fractions of non-Treg CD4 and CD8 T cells at day 7 after BMT. From different experiments using $5x10^5$ Tcon, similar findings were observed at day 28 (Figure 7B~C). All of these data suggested that non-Treg T cells could not escape from the suppression by Treg.

DR3-stimulated Treg do not interfere with GVT effects

Suppressive MLR experiments were performed to evaluate the regulatory capabilities of Treg that were sorted from anti-DR3-treated mice (Figure 2C). The results showed that these Treg could suppress Tcon proliferation even with lower numbers. However, the exact mechanisms of superior suppression by DR3-stimulated Treg were largely unknown. Up to now, it has been suggested that Treg use surface molecules (CTLA4, LAG3, and PD-1/PD-L1) and cytokines (TGFβ, IL-10, IL-35, granzyme B) to inhibit dendritic cells and effector T cells (49, 55). Furthermore, Kitazawa et al. reported that blockade of PD-1/PD-L1 pathway abrogated Treg-mediated suppression of aGVHD (56). Saha et al. also reported similar findings (53). Therefore allogeneic MLR experiments were performed in this study to measure the PD-1 expression by Treg cells. Figure 3F showed that Treg from anti-DR3-treated mice expressed more PD-1 than Treg from isotype-treated mice in allogeneic environment. GZB was also upregulated in these Treg (Figure 3G).

It is well documented that Treg is responsible for decreasing anti-tumor immunity in some tumor models although it was reported that natural Treg did not interfere with the GVT effects of allogeneic Tcon in BMT settings (18). Since Treg from anti-DR3-treated mice increased the expression of PD-1 in my data and blockade of PD-1/PD-L1 pathway was reported to increase proliferation and function of adoptively transferred CTL (57), it is possible that DR3-stimulated Treg may interfere with GVT effects. In order to investigate that possibility, the BCL_1 cell line

was used. This cell line has Balb/c background and is a murine counterpart of human chronic lymphocytic leukemia. Figures 8A~B show that Tcon from anti-DR3-treated donors had the same GVT effects as Tcon from isotype-treated donors. Recipients of the BCL_1 cell line and Tcon from anti-DR3-treated donors showed significantly longer survival than those of the BCL_1 cell line and Tcon from isotype-treated donors.

Treg could facilitate donor lymphoid reconstitution and generate diverse TCR repertoire by protecting thymic and peripheral LN architecture in the murine BMT experiments (58). The resultant enhanced lymphoid reconstitution by Treg provided long-term protective immunity against the lethal viral infection. Mice with severe aGVHD had disrupted lymphoid organs from GVHD and remained lymphopenic with a restricted TCR repertoire. Immune reconstitution takes at least several months after the human allogeneic hematopoietic stem cell transplantation and patients are at increased risk of severe infections and the relapse of malignant diseases (59). This study has found that not only Treg but also Tcon from anti-DR3-treated donors expanded robustly over time after BMT, which suggests the enhanced immune reconstitution in recipients. This phenomenon can explain how GVT effects were retained regardless of increased PD-1 expression.

Conclusion: administration of anti-DR3 Ab can be easily translated to clinical trials

Tcon isolated from anti-DR3-treated mice could maintain higher Treg proportions for a long period after transplantation to allogeneic recipients. This was translated to better survival and lower aGVHD morbidity. Their GVT effects were preserved. Recipients of these Tcon had lower inflammatory cytokines, decreased T cell infiltration into target tissues and reduced proliferation of donor Tcon. This is the first study to demonstrate the benefits of Treg expansion by anti-DR3 Ab for aGVHD prevention. The current practice is to infuse donor Treg prior to the donor lymphocyte infusion (DLI) to prevent aGVHD associated

with it. Donors should come to a hospital separately for the Treg collection and for the DLI collection. *Ex vivo* Treg expansion takes about 2 weeks and it fails occasionally. In contrast, Treg expansion by anti-DR3 Ab is simple and rapid. This Ab is administered only once to donors and the collection for DLI can be performed within 5 days. Treg collection and its *ex vivo* expansion are not required. This is the similar process as the peripheral blood stem cell (PBSC) mobilization by granulocyte colony stimulating factor (G-CSF). This study demonstrates that treating donors with anti-DR3 Ab can prevent aGVHD while retaining GVT effects. Furthermore, the convenience of this Ab will help to remove the current limitations of Treg cell therapy and facilitate their clinical applications.

Table 1

Assessment of clinical GVHD in transplanted animals

Criteria	Score 0	Score 1	Score 2
Weight loss	<10%	>10% to 25%	>25%
Posture	Normal	Hunching noted only at rest	Severe hunching impairs movement
Activity	Normal	Mild to moderately decreased	Staionary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling/poor grooming
Skin integrity	Normal	Scaling or paws/tail	Obvious areas of denuded skin

Figure Legends

Figure 1: Scheme of BM transplantation using anti-DR3-treated B6 donor mice

WT or CD45.1$^+$ *luc*$^+$ donor B6 mice were intraperitoneally injected with 0.5 mg/kg of anti-DR3 Ab (4C12) or isotype control (Hamster IgG) on Day -4. WT Balb/c recipients were lethally irradiated (850cGy) and injected with TCD-BM (5x10^6) from WT B6 donors on Day 0. LN/Sp were harvested from Ab-treated B6 mice, Tcon were isolated using CD4 & CD8 MACS beads, and 1x10^6 Tcon were given on Day 0. Recipient mice were monitored for serial Foxp3 level, GVHD score, BLI, and survival.

Figure 2: Expanded Treg isolated from anti-DR3-treated mice and their suppressive activity

Tcon were isolated from WT or CD45.1$^+$ B6 donors treated either with anti-DR3 or with isotype control Ab as described. (A~B) They were surface stained, fixed, and stained for Foxp3 to show Treg (%) of CD4 T cells. Number of Treg obtained from one donor = (%Foxp3$^+$ of live cells) x (count of live cells) / (number of donor mice). The boxes extend from the 25th to 75th percentile and the line in the middle is the median. Whiskers are plotted by the Tukey method and dots represent outliers. Data from the two groups were compared by the unpaired *t* test (*****P*<0.0001). These are combined results of 24 Tcon isolations. (C) CD25$^+$ Treg were sorted from anti-DR3- or isotype-treated mice (purity >95%) and Tcon were isolated from naïve B6 mice as described. CD25$^+$ Treg were plated at various ratios to naïve B6 Tcon (2x10^5/well) in the presence of γ-irradiated (3000cGy) Balb/c splenocytes (4x10^5/well). Cells were cultured for 96 hours and then pulsed with [^{3}H]thymidine (1 mCi/well) for 16 hours. The thymidine uptakes (CPM) of the two groups (CD25$^+$ Treg from anti-DR3- vs. isotype Ab-treated donors) were measured and compared by multiple *t* tests using Holm-

Sidak correction. Only significant comparisons are marked with asterisks (*P 0.01 to 0.05; **P 0.001 to 0.01). These are representative results of more than two experiments.

Figure 3: Tcon from anti-DR3-treated mice are less proliferative, less activated and maintain higher Treg level

Tcon were isolated from WT or CD45.1$^+$ B6 donors treated either with anti-DR3 or with isotype control Ab. They were plated at 1:1 ratio with γ-irradiated (3000cGy) Balb/c splenocytes (2x10^5/well), cultured for 96 hours as described. (A) They were pulsed with [^{3}H]thymidine (1 mCi/well) for 16 hours. The thymidine uptakes (CPM) of the two groups (Tcon from anti-DR3- vs. isotype Ab-treated donors) were measured and compared by the unpaired *t* test (***P 0.0001 to 0.001). (B) Isolated Tcon were surface stained, fixed and stained for Foxp3 before and after allogeneic MLR. Treg proportions of CD4 T cells from the two groups (Tcon from anti-DR3- vs. isotype Ab-treated donors) were compared by unpaired *t* test while the data from before vs. after allogeneic MLR were compared by paired *t* test (**P 0.001 to 0.01; ***P 0.0001 to 0.001; ns, not significant). (C) Cells were harvested from the MLR plate after treatment with the Cell Stimulation Cocktail (eBioscience) for 5 hours. (D~G) Cells were harvested from the MLR plate without stimulation. They were surface stained, fixed, and stained for Foxp3 and other intracellular antigens. (C) TNFα, (D) T-bet, (E) granzyme B (GZB) for CD8 T cells. (F) PD-1 and (G) GZB for Treg. (C~G) Data from the two groups (Tcon from anti-DR3- vs. isotype Ab-treated donors) were compared by unpaired *t* test (ns, not significant; *P 0.01 to 0.05; **P 0.001 to 0.01; ***P 0.0001 to 0.001). These are representative results of more than two experiments.

Figure 4: Recipients of Tcon from anti-DR3-treated donors have reduced Tcon expansion and longer survival

Tcon were isolated from CD45.1$^+$ *luc*$^+$ B6 donors that were treated with anti-DR3 or isotype control Ab. WT Balb/c recipients were lethally irradiated and transplanted with 1x10^6 Tcon and 5x10^6 TCD-BM as described. Survival (A) and aGVHD score (B) of recipient mice were measured at least twice a week until day 30 and then every 7~10 days. These are the combined results of three independent experiments. Survival curves were compared by the log rank test (****P<0.0001) and aGVHD scores were compared by multiple *t* tests with Holm-Sidak correction. Only significant comparisons are marked with asterisks (****P<0.0001). (C~D) BLI was repeated at days 3, 6, 12, 20, 30, and 44 after transplantation. (C) Curves of total photon flux from the two groups (anti-DR3- vs. isotype Ab-treated donors) are shown and compared by multiple *t* tests with Holm-Sidak correction. Only significant comparisons are marked with asterisks (****P<0.0001). (D) Representative images of recipient mice at day 6 and 30 are displayed. These are the representative data of 3 independent experiments.

Figure 5: Increased Treg expansion but decreased proliferation of Tcon derived from anti-DR3-treated donors

Tcon were isolated from CD45.1$^+$ B6 donors treated either with anti-DR3 or with isotype control Ab. WT Balb/c recipients were lethally irradiated and transplanted with 1x10^6 Tcon and 5x10^6 TCD-BM as described. (A~B) LN and a spleen were harvested from recipients at days 3, 7 and 14 for flow cytometric analysis of Foxp3. (A) the absolute number of donor Treg and (B) Treg proportion of donor Tcon are shown. Data from the two groups (Tcon from anti-DR3- vs. isotype Ab-treated donors) were compared by multiple *t* tests using Holm-Sidak correction (*P 0.01 to 0.05; **P 0.001 to 0.01; ***P 0.0001 to 0.001; ****P<0.0001). These are the combined results of three independent transplantations. (C~D) Recipient mice were intraperitoneally injected with BrdU 1mg at days 5 & 6 and then LN/Spleen were harvested at day 7. BrdU staining was done as described. BrdU uptake of donor (C)

Foxp3$^-$ CD4 T cells, (D) CD8 T cells are shown. Data from the two groups (Tcon from anti-DR3- vs. isotype Ab-treated donors) were compared by unpaired *t* test (**P* 0.01 to 0.05; ***P* 0.001 to 0.01; ****P* 0.0001 to 0.001). These are the representative data of three independent transplantations.

Figure 6: Surface CD25 expression of non-Treg T cells and serum levels of inflammatory cytokines

Tcon were isolated from CD45.1$^+$ B6 donors treated either with anti-DR3 or with isotype control Ab. WT Balb/c recipients were lethally irradiated and transplanted with 1x10^6 Tcon and 5x10^6 TCD-BM as described. (A, B) LN/Spleen were harvested from recipients at day 3. Re-isolated cells were surface stained for CD25, fixed and stained for Foxp3. CD25 expression of donor (A) Foxp3$^-$ CD4 and (B) CD8 T cells are shown. Data from the two groups were compared by unpaired *t* test (***P* 0.001 to 0.01; ****P* 0.0001 to 0.001). These are representative data of two transplantations. (C~E) Serums were collected from recipient mice at days 3, 7, and 14. Serum cytokine levels were measured by the multiplex assay. (C) IFN-γ, (D) IL-1β, and (E) TNFα. Data from the two groups were compared by multiple *t* tests with Holm-Sidak correction (*****P*<0.0001). These are the combined results of more than two transplantations.

Figure 7: Treg proportions, proliferation of donor-derived Tcon and serum cytokines for the later phase of aGVHD

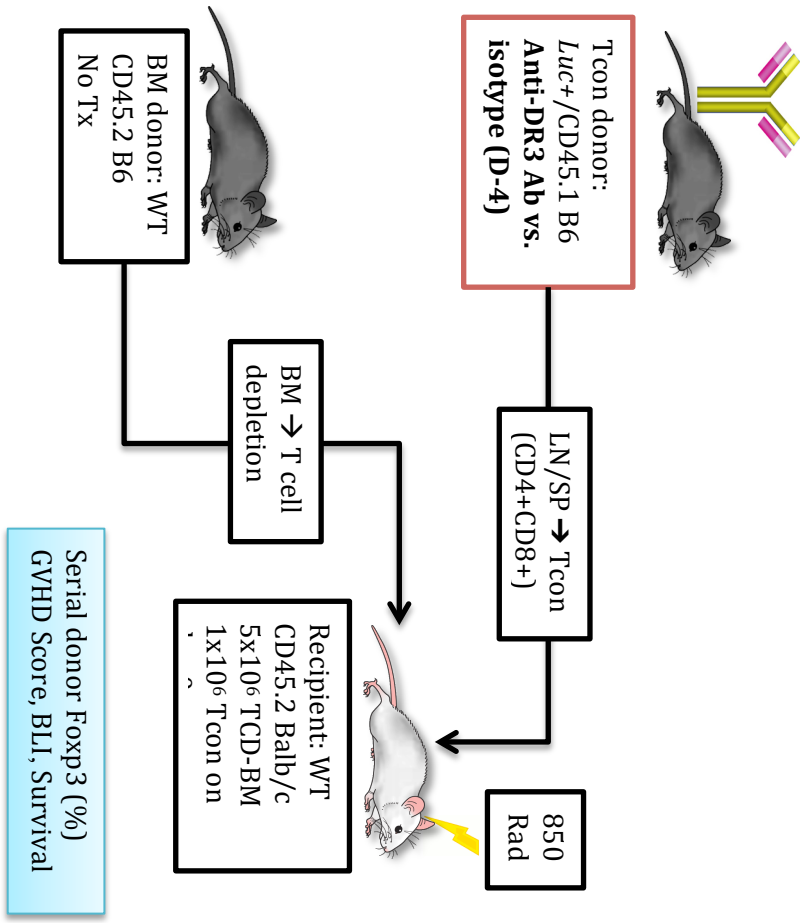
Tcon were isolated from CD45.1$^+$ B6 donors treated either with anti-DR3 or with isotype control Ab. WT Balb/c recipients were lethally irradiated and transplanted with 5x10^5 Tcon and 5x10^6 TCD-BM as described. (A) Recipient mice were bled at days 20, 40 and 60 after transplantation. Re-isolated cells were surface stained, fixed and stained for Foxp3. (B~C) Recipients were intraperitoneally injected with BrdU 1mg at day 26 & 27 and then LN/Spleen were harvested at day

28. Re-isolated cells were surface stained, fixed and stained for Foxp3 & BrdU. Donor (B) $Foxp3^-$ CD4 T cells and (C) CD8 T cells. (A~C) Flow cytometric data from the two groups (anti-DR3- vs. isotype Ab-treated donors) were compared by multiple *t* tests with Holm-Sidak correction. Only significant comparisons are marked with asterisks (**P* 0.01 to 0.05; ***P* 0.001 to 0.01; ****P* 0.0001 to 0.001; *****P*<0.0001). (D, E) Serums were collected from recipient mice at day 28 and stored in -80°C. Cytokine levels were measured by the multiplex assay. (D) IFNγ and (E) TNFα. Cytokine data of two groups (anti-DR3- vs. isotype Ab-treated donors) were compared by unpaired *t* tests (**P* 0.01 to 0.05; ***P* 0.001 to 0.01). These are the combined results of two transplantations (F) Intracellular production of TNFα was compared by unpaired *t* test (ns, not significant; ****P* 0.0001 to 0.001).

Figure 8: Tcon from anti-DR3-treated donors retain GVT effects

WT Balb/c recipient mice were intravenously injected with 20,000 luc^+/gfp^+-BCL_1 cells at day -8. Tumor engraftment was confirmed by BLI at day -1. Tcon were isolated from WT B6 donors treated either with anti-DR3 or with isotype control Ab. Recipients were lethally irradiated and transplanted with $1x10^6$ Tcon and $5x10^6$ TCD-BM as described. (A, B) Tumor burden was monitored by BLI at days 7, 14, 22, 30, 37, 44, 48, and 52. These are the representative results of two independent transplantations. (C) Survival data from two independent transplantations were combined and plotted. Survival curves were compared by the log rank test (*****P*<0.0001; ns, not significant).

Figure 1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(CD4+CD8+)
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BM donor: WT
CD45.2 B6
No Tx
BM → T cell
depletion
Recipient: WT
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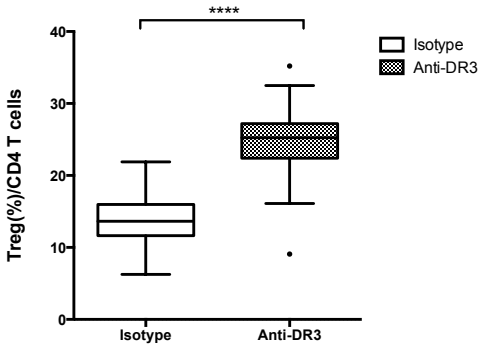
1x10⁶ Tcon on
Serial donor Foxp3 (%)
GVHD Score, BLI, Survival

Figure 2

A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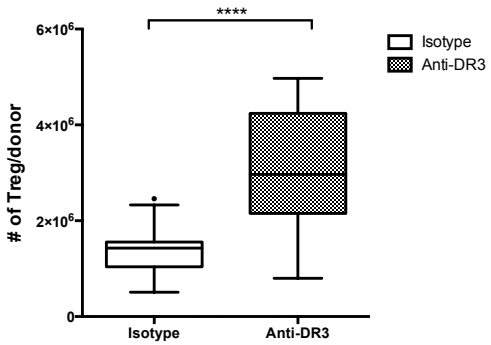
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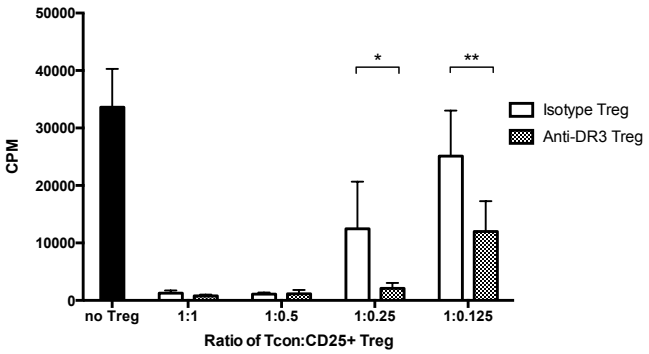
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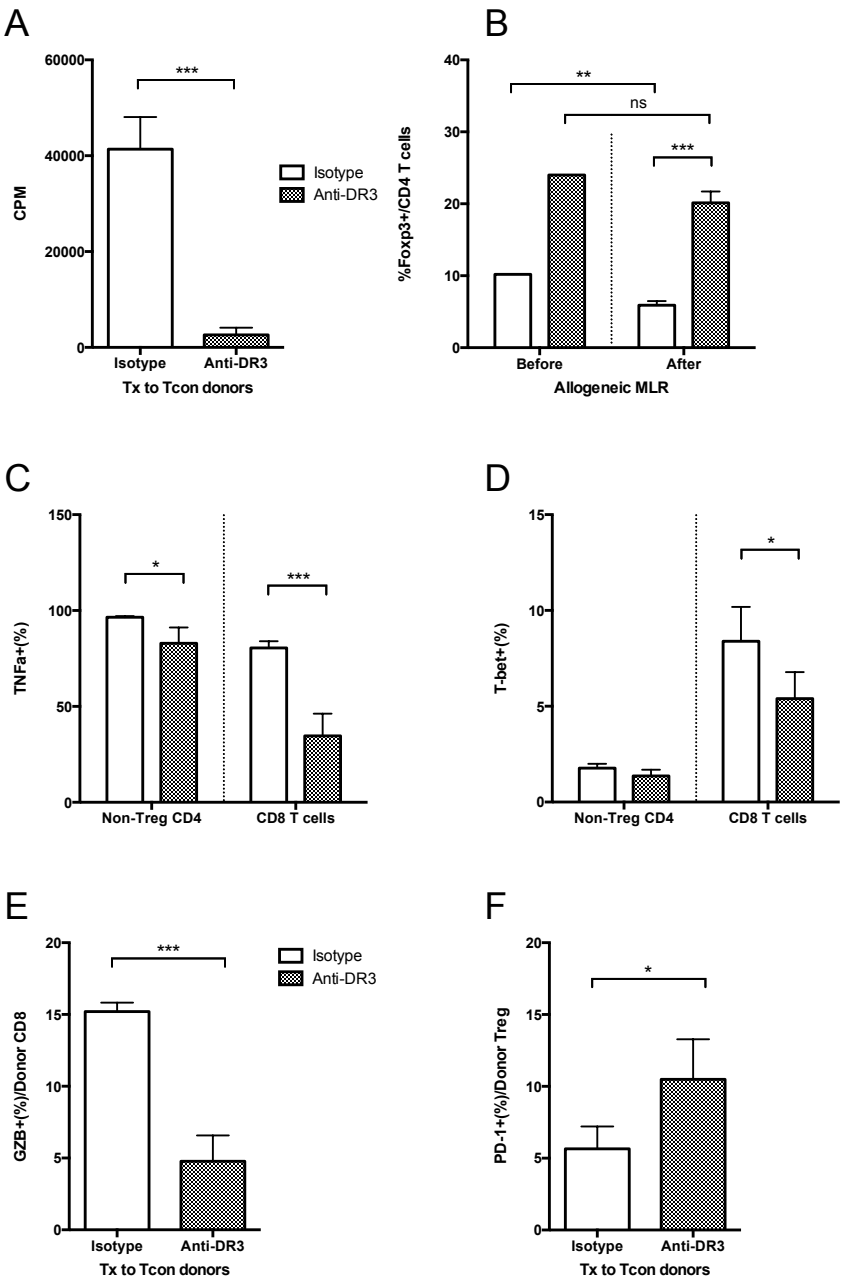
1:0.125
Ratio of Tcon:CD25+ Treg

Figure 3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Allogeneic MLR
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TNFa+(%)
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Non-Treg CD4
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T-bet+(%)
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Non-Treg CD4
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Tx to Tcon donors

G

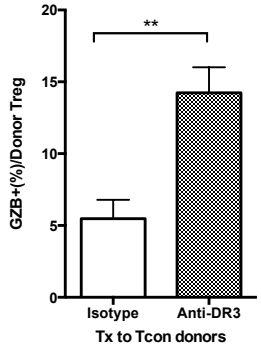
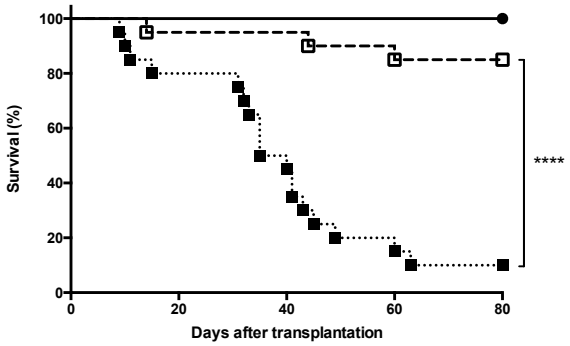
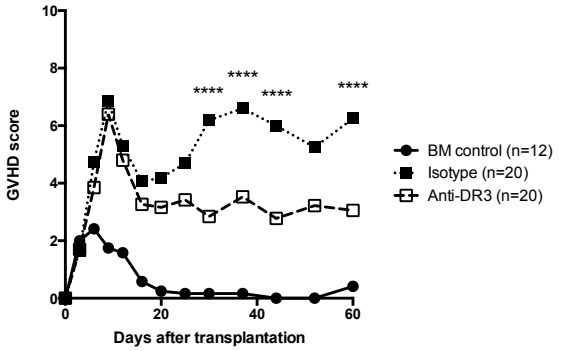


Figure 4

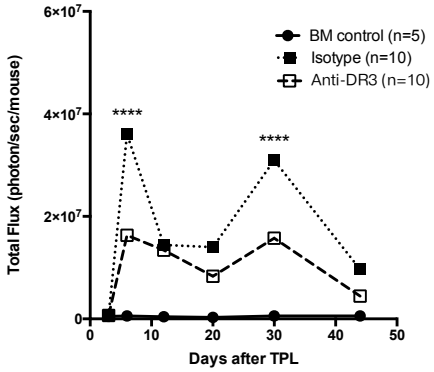
A


Survival (%)
100
80
60
40
20
0
0
20
40
60
80
Days after transplantation
****

B


GVHD score
10
8
6
4
2
0
0
20
40
60
Days after transplantation
****
****
****
****
BM control (n=12)
Isotype (n=20)
Anti-DR3 (n=20)

C


Total Flux (photon/sec/mouse)
6×10^7
4×10^7
2×10^7
0
0
10
20
30
40
50
Days after TPL
****
****
BM control (n=5)
Isotype (n=10)
Anti-DR3 (n=10)

D

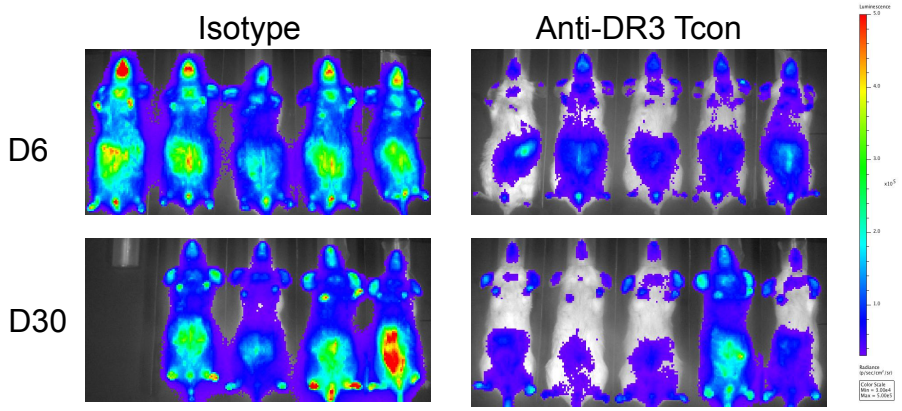
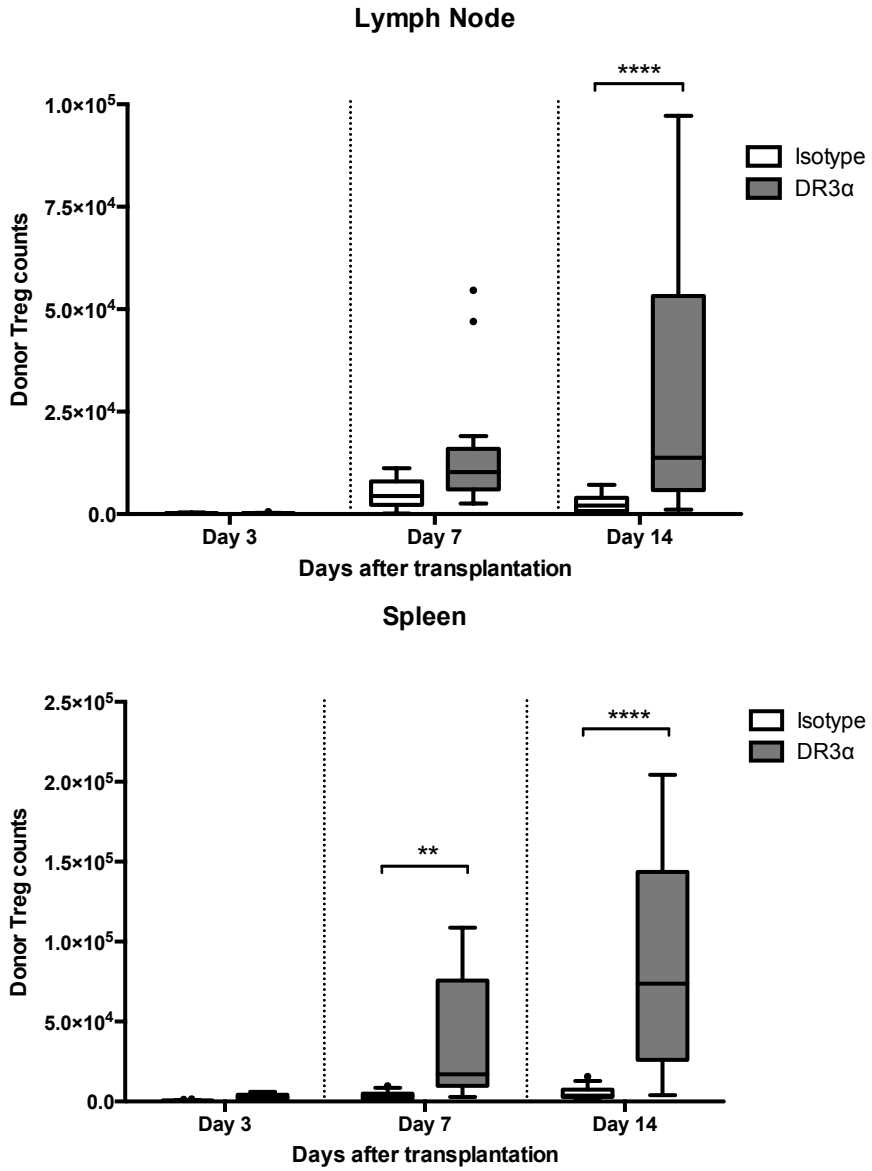


Figure 5

A


Lymph Node
****
1.0×10^5
7.5×10^4
5.0×10^4
2.5×10^4
0.0
Donor Treg counts
Isotype
DR3α
Day 3
Day 7
Day 14
Days after transplantation
Spleen
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
**
****
Donor Treg counts
Isotype
DR3α
Day 3
Day 7
Day 14
Days after transplantation

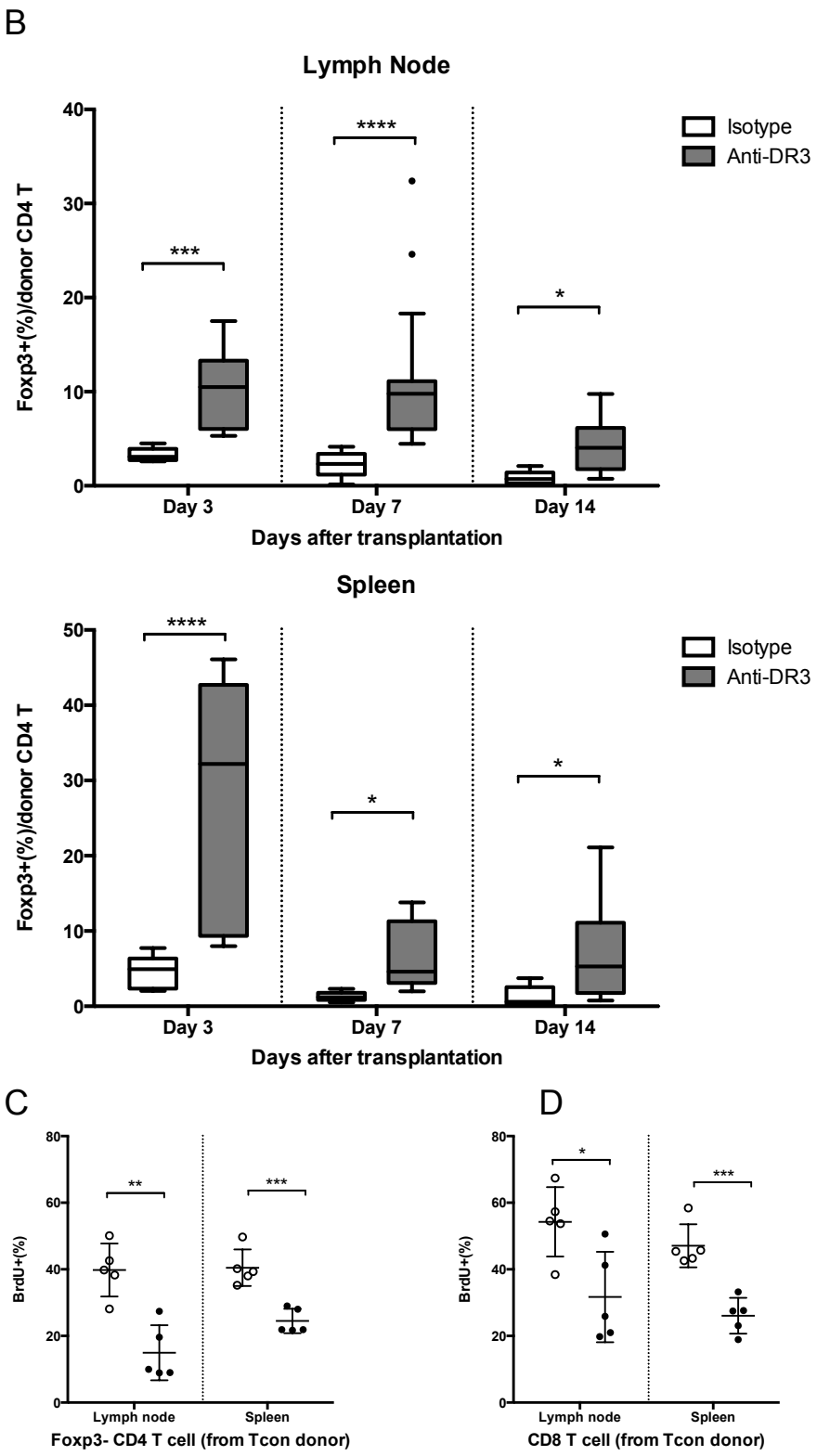
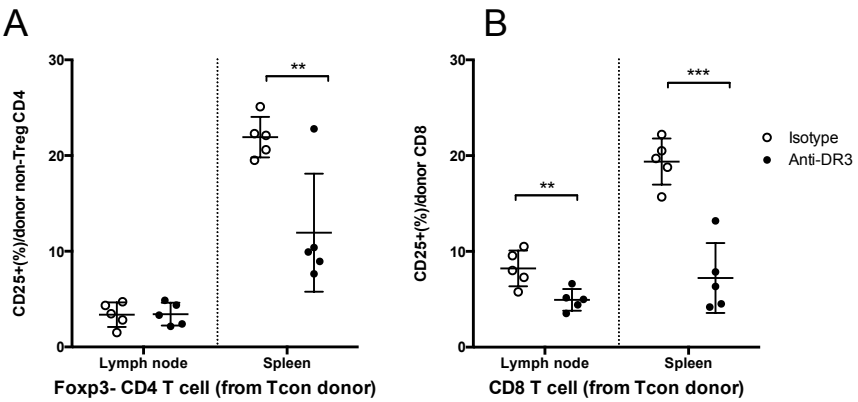
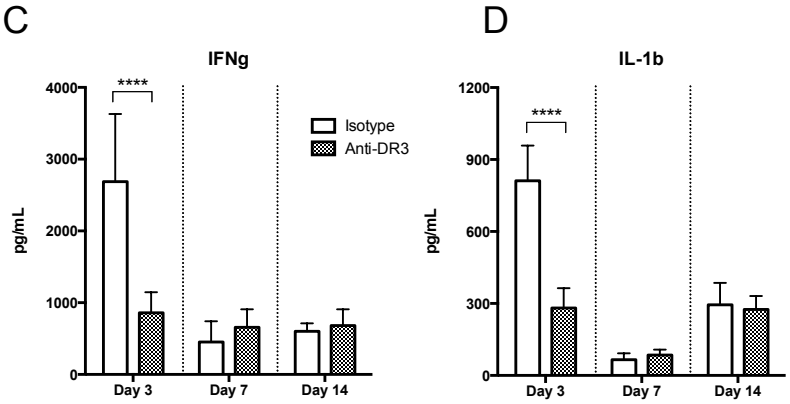

B
Lymph Node
Foxp3+(%)/donor CD4 T
Isotype
Anti-DR3
***
****
*
Day 3
Day 7
Day 14
Days after transplantation
Spleen
Foxp3+(%)/donor CD4 T
Isotype
Anti-DR3
****
*
*
Day 3
Day 7
Day 14
Days after transplantation
C
BrdU+(%)
**
***
Lymph node
Spleen
Foxp3- CD4 T cell (from Tcon donor)
D
BrdU+(%)
*
***
Lymph node
Spleen
CD8 T cell (from Tcon donor)

Figure 6


A
CD25+(%)/donor non-Treg CD4
30
20
10
0
**
Lymph node
Spleen
Foxp3- CD4 T cell (from Tcon donor)
B
CD25+(%)/donor CD8
30
20
10
0
**
***
Lymph node
Spleen
CD8 T cell (from Tcon donor)
Isotype
Anti-DR3


C
IFNg
pg/mL
4000
3000
2000
1000
0
****
Isotype
Anti-DR3
Day 3
Day 7
Day 14
D
IL-1b
pg/mL
1200
900
600
300
0
****
Day 3
Day 7
Day 14

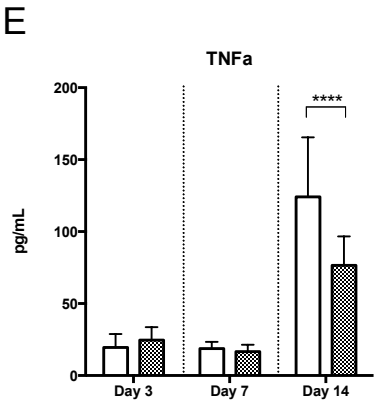
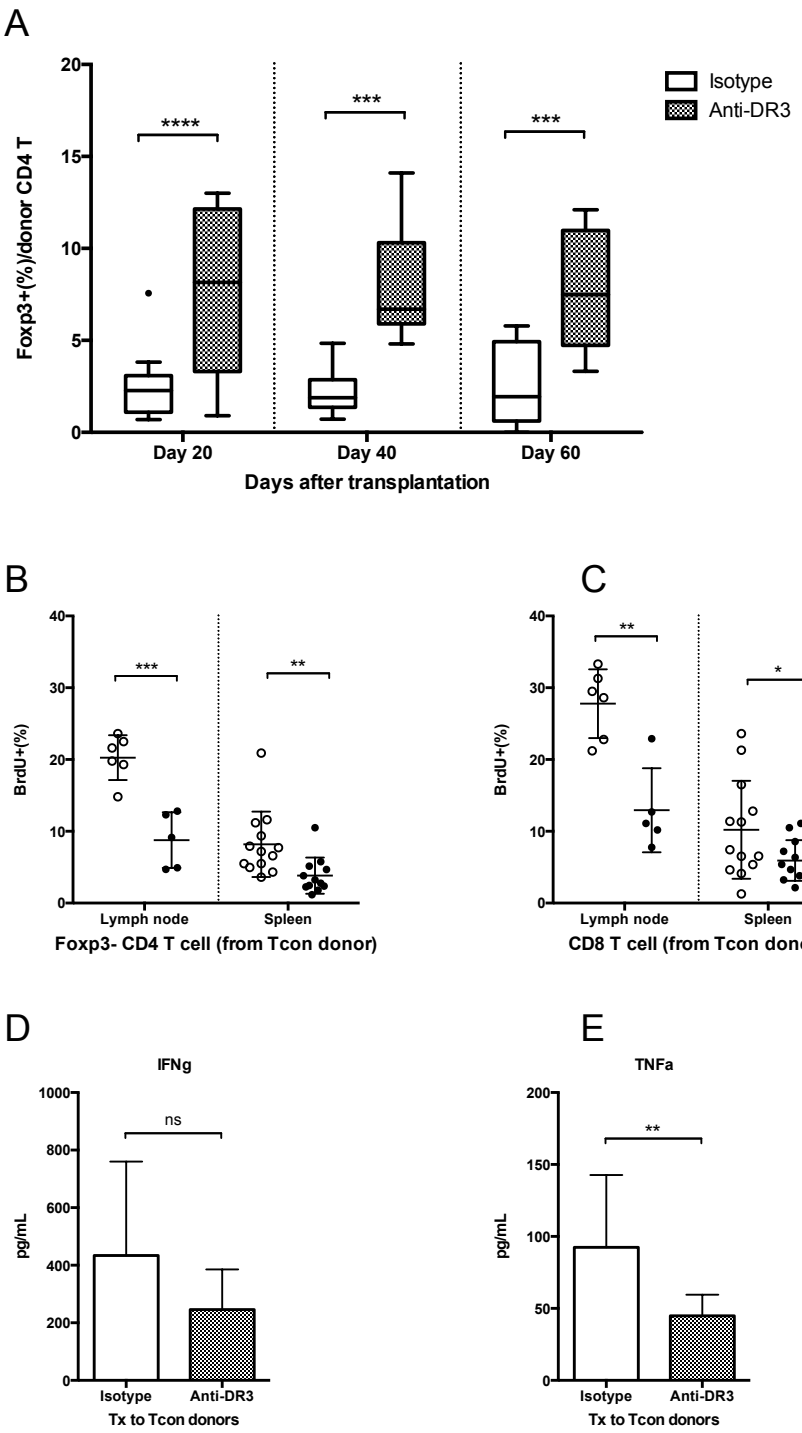

E
TNFa
pg/mL
200
150
100
50
0
****
Day 3
Day 7
Day 14

Figure 7


A
Foxp3+(%)/donor CD4 T
Isotype
Anti-DR3
****
***
***
Day 20
Day 40
Day 60
Days after transplantation
B
BrdU+(%)
***
**
Lymph node
Spleen
Foxp3- CD4 T cell (from Tcon donor)
C
BrdU+(%)
**
*
Lymph node
Spleen
CD8 T cell (from Tcon donor)
D
IFNg
pg/mL
ns
Isotype
Anti-DR3
Tx to Tcon donors
E
TNFa
pg/mL
**
Isotype
Anti-DR3
Tx to Tcon donors

F

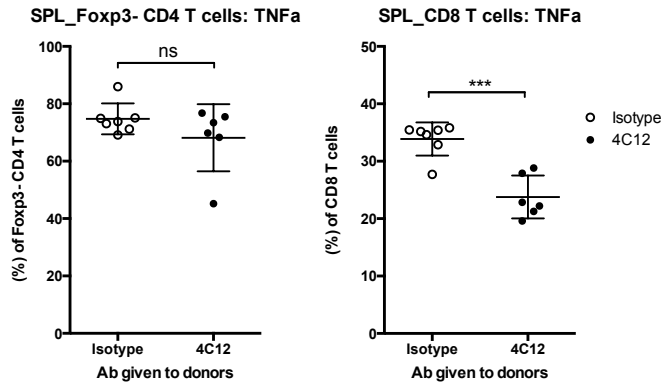
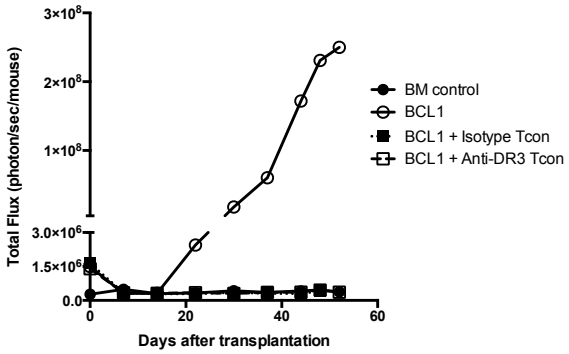
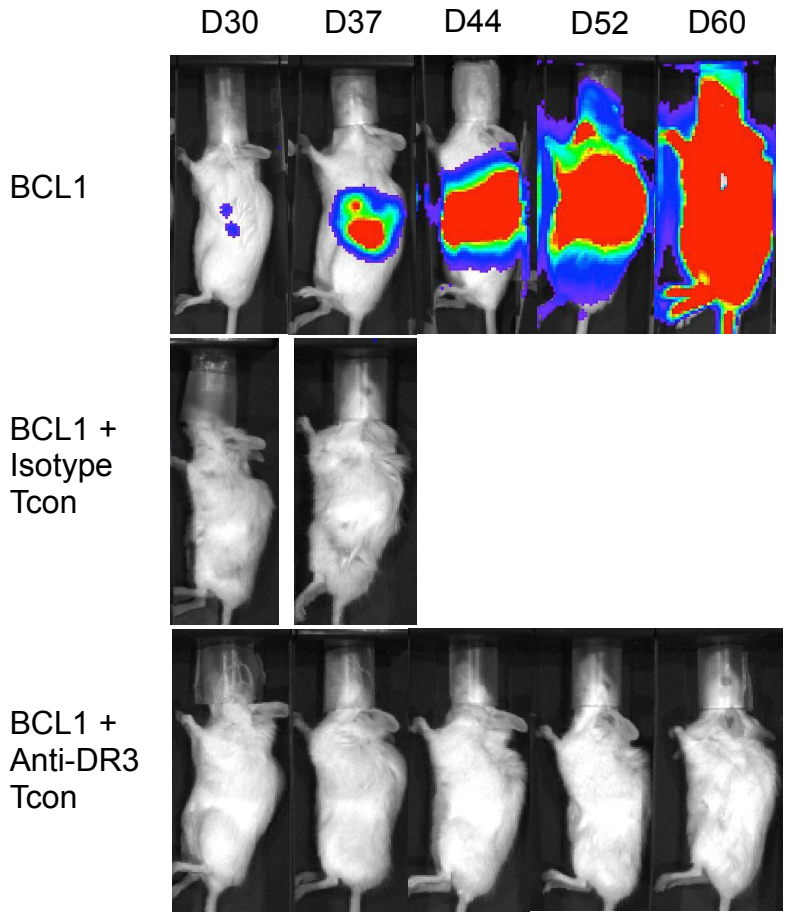

SPL_Foxp3- CD4 T cells: TNFa
ns
(%) of Foxp3- CD4 T cells
100
80
60
40
20
0
Isotype
4C12
Ab given to donors
SPL_CD8 T cells: TNFa
***
(%) of CD8 T cells
50
40
30
20
10
0
Isotype
4C12
Ab given to donors
Isotype
4C12

Figure 8

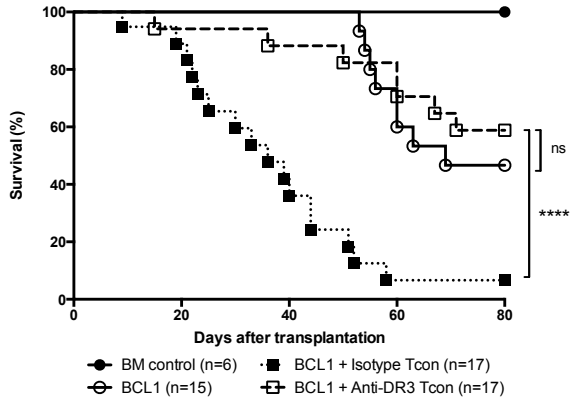
A


3×10^8
2×10^8
1×10^8
3.0×10^6
1.5×10^6
0.0
Total Flux (photon/sec/mouse)
0
20
40
60
Days after transplantation
BM control
BCL1
BCL1 + Isotype Tcon
BCL1 + Anti-DR3 Tcon

B


D30
D37
D44
D52
D60
BCL1
BCL1 + Isotype Tcon
BCL1 + Anti-DR3 Tcon

C


100
80
60
40
20
0
Survival (%)
0
20
40
60
80
Days after transplantation
ns
****
BM control (n=6)
BCL1 + Isotype Tcon (n=17)
BCL1 (n=15)
BCL1 + Anti-DR3 Tcon (n=17)

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국 문 초 록

급성 이식편대숙주질환 완화를 위한 항 DR3 항체에 의해 증가된 조절 T 세포의 역할

서울대학교 의과대학
의학과 미생물학교실
김 병 수 (2007-30966)

도입: 조절 T 세포는 급성 이식편대숙주질환을 완화하면서 이식편대종양효과를 유지한다. 그러나, 이들의 낮은 빈도로 인해 임상에 적용하는데 있어 어려움이 있다. 조절 T 세포를 증가시키기 위한 방법들이 다수 보고되어 있으나, 아직은 제한점이 많다. DR3 는 TNF 수용체집단에 속하며 T 세포 등에 주로 발현된다. 이에 대한 항진성 한체는 생체내 조절 T 세포를 증가시키며 동종이식된 심장이식편의 거부반응을 예방할 수 있음이 보고되었다.

방법: 항 DR3 항체 또는 햄스터 IgG 동형대조항체를 C57BL/6 공여자 쥐에게 복강내 주사로 투여하고 4 일 후 통상적 T 세포를 분리하였다. 통상적 T 세포와 T 세포를 제거한 골수세포를 치사량의 방사선을 조사한 Balb/c 수용자 쥐에게 이식하였다. 급성 이식편대숙주반응의 임상 징후와 생체발광 영상을 정기적으로 추적 시행하였다. 일부 수용자 쥐는 유세포분석 또는 혈청 싸이토카인 측정을 위해 사전에 정한 날자에 희생되었다.

결과: 항 DR3 항체를 투여한 공여자 쥐로부터 얻어진 통상적 T 세포는 더 높은 비율의 조절 T 세포를 가지고 있었으며 이 조절 T 세포는 더 적은 수로도 통상적 T 세포의 증식을 억제할 수 있었다. 임파구혼합반응 실험은 항 DR3 항체를 투여한 공여자에서 분리한 통상적 T 세포가 동종항원에 반응 후 증식력이 낮으며, 높은 조절 T 세포의 비율이 유지되고, Th1 분화가 감소되며, 조절 T 세포의 PD-1 발현이 감소되었음을 보였다. 쥐를 이용한 생체내 실험에서 항 DR3 항체를 투여한 공여자 쥐에서 얻은 통상적 T 세포는 이식 후 증식이 적었으며 감소된 급성이식편대숙주질환을 유발하였다. 항 DR3 항체를 투여한 쥐에서 유래된 조절 T 세포는 이식 후 활발하게 수가 증가되었으며

공여자 T 세포내의 비율도 더 높게 유지되었다. 항 DR3 항체를 투여한 쥐에서 기원한 비조절 CD4 T 세포와 CD8 T 세포는 이식 후 CD25 의 발현과 BrdU 섭취가 더 적었다. 항 DR3 항체를 투여한 공여자 쥐에서 얻은 통상적 T 세포를 이식한 숙주 쥐에서 IFNγ, IL-1β, TNFα 의 혈청내 농도도 더 낮았다. 항 DR3 항체를 투여한 쥐에서 얻은 통상적 T 세포는 온전한 이식편대종양효과를 보였다.

결론: 이 결과들은 모두 공여자에게 항 DR3 항체를 투여함으로써 공여자 조절 T 세포를 효과적으로 증가시키고, 급성 이식편대숙주질환을 예방하며, 이식편대 종양효과를 유지할 수 있음을 시사한다. 결론적으로 항 DR3 항체는 조절 T 세포의 생체내 증가를 위한 효과적인 방법의 하나로 이용될 수 있다.

핵심단어: 급성 이식편대숙주질환, 골수이식, 조절 T 세포, DR3