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Defects in Regulatory T Cells Due to CD28 Deficiency Induce a Qualitative Change of Allogeneic Immune Response in Chronic Graft-versus-Host Disease

Yuki Akieda,† Ei Wakamatsu,† Tomoe Nakamura,† Yasuo Ishida,† Shuhei Ogawa,* and Ryo Abe*†

In patients receiving allogeneic hematopoietic cell transplantation, chronic graft-versus-host disease (cGVHD) remains a frequent complication and resembles autoimmune diseases such as systemic lupus erythematosus and systemic sclerosis. Our previous work demonstrated the critical role of CD28 costimulation of donor T cells for GVHD induction. In this study, we investigate the role of CD28 costimulation of host T cells in cGVHD. CD28-intact mice as hosts showed systemic lupus erythematosus–type cGVHD, whereas CD28-deficient mice developed a distinct phenotype of cGVHD, with fibrotic damage in skin and internal organs, resembling systemic sclerosis. This phenotype was due to a lack of signaling through the C-terminal proline-rich motif within host CD28's cytoplasmic tail, a motif previously shown to be required for development of regulatory T cells (Tregs) and function of conventional T cells. Adoptive transfer experiments demonstrated that a defect in host CD4+CD25+ Tregs, but not in conventional T cells, was responsible for disease phenotype. Host Treg deficiency altered the cytokine pattern of donor CD4+ T cells and the Ag specific expression of autoantibodies, and these might lead to phenotypic change. Thus, host CD28 signaling controlled the pathogenesis of cGVHD through effects on host Tregs, whose status impacts qualitatively on the allogeneic immune responses. The Journal of Immunology, 2015, 194: 4162–4174.

Abbreviations used in this article: aGVHD, acute GVHD; B6, C57BL/6; cGVHD, chronic GVHD; GvHD, graft-versus-host disease; HCT, hematopoietic cell transplantation; Itk, IL-2 inducible T cell kinase; Lck, lymphocyte-specific protein tyrosine kinase; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; Tconv, conventional T cell; Tg, transgenic; TM, transmembrane domain; Treg, regulatory T cell; WT, wild-type.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/$25.00 (5), but the risk for cGVHD has stayed the same or even increased (6, 7). However, the pathogenesis of cGVHD is poorly understood, in part because its phenotype is so variable. Further study is thus needed to elucidate mechanisms of cGVHD pathogenesis and to find ways to control it.

The costimulatory receptor CD28 regulates a wide range of functions. It is constitutively expressed in naive T cells and interacts with its ligands B7-1/2 (CD80/86) on APCs. TCR signaling with CD28 costimulation is essential for T cell activation, IL-2 production, proliferation, survival, and Th cell differentiation (8, 9). Furthermore, CD28 costimulation regulates the thymic development, peripheral homeostasis, and suppressive function of Foxp3+ regulatory T cells (Tregs) in a cell-intrinsic manner (10–12). The CD28 receptor contains a short cytoplasmic tail composed of a YMNMT motif and two proline-rich (PxxP) motifs. These are involved in CD28-mediated costimulatory signal transduction (13). Upon CD28 engagement, protein tyrosine kinases are recruited to the cytoplasmic tail of CD28 where they phosphorylate CD28 (14–16). Next, CD28 recruits several adaptor proteins, including the p85 regulatory subunit of PI3K, growth factor receptor–bound protein 2, and growth factor receptor–bound protein 2–related adaptor protein, to the phosphorylated YMNMT motif (16–19), which then activate MAPK, Akt, NFAT, and NF-κB. The two proline-rich domains of CD28, N-terminal PxxP and C-terminal PxxP (P^{306}RRP and P^{206}YAP, respectively) require phosphorylation by tyrosine kinases to recruit the signal molecules (20, 21). The C-terminal PxxP motif binds the src-family lymphocyte-specific protein tyrosine kinase (Lck) (22) and activates protein kinase C 0 and NF-κB (23–25). The N-terminal PxxP motif binds the SH3 domains of IL-2 inducible T cell kinase (Itk) and Tec (16, 26). Our previous works demonstrated that, in GVHD murine models, CD28-deficient donor T cells could not induce GVHD (27, 28), and the treatment with CTLA4-Ig (CTLA4 fusion protein) prevented the induction of GVHD (29). Thus, CD28 costimulation in donor T cells played an important role for the induction of GVHD.

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Abbreviations used in this article: aGVHD, acute GVHD; B6, C57BL/6; cGVHD, chronic GVHD; GvHD, graft-versus-host disease; HCT, hematopoietic cell transplantation; Itk, IL-2 inducible T cell kinase; Lck, lymphocyte-specific protein tyrosine kinase; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; Tconv, conventional T cell; Tg, transgenic; TM, transmembrane domain; Treg, regulatory T cell; WT, wild-type.
CTLA4-Ig also potentially inhibits the CD28 costimulation in host T cells, but the role of CD28 costimulation in host T cells remains to be elucidated. A comprehensive understanding of CD28 costimulation in host T cells, in addition to donor T cells, is thought to be required for preventing GVHD.

In this study, to reveal the role of CD28 costimulation in host T cells in the pathogenesis of cGVHD, we used CD28-deficient mice as hosts. We found that lack of CD28 signaling in host T cells converted SLE-type cGVHD to SSC-type cGVHD. As previously reported, the transplant of allogeneic donor cells into wild-type (WT) hosts resulted in SLE-type cGVHD, with the production of anti-dsDNA autoantibody and the development of glomerulonephritis. In this study, CD28-deficient hosts showed SSC-type cGVHD with fibrotic damage to the skin and internal organs. These results highlighted that CD28 signaling in host T cells played an important role in the pathogenesis of cGVHD. Moreover, host CD28 signaling, particularly the pathway that drives Treg development, played a protective role in the development of SSC-type cGVHD. Indeed, SSC-like phenotype was due mainly to the defect in host CD4+CD25+ Tregs, but not in CD4+CD25-, CD8+, and NK1.1+ T cells. Finally, the defect in host Tregs altered the cytokine production of donor CD4+ T cells and target Ags of autoantibodies. These results suggest that CD28 signaling controls the pathogenesis of cGVHD through effects on Tregs, whose status influences qualitatively the allogeneic immune responses.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from Sankyo Labo Service (Tokyo, Japan). CD28−/− mice were generated as previously described (30) and were kindly provided by Dr. C. June (University of Pennsylvania Perelman School of Medicine, Philadelphia, PA). CD28−/− mice, CD28−/− transgenic (Tg) strain (CD28 transmembrane domain [TM], YF) were generated in our laboratory as described previously (26). CD28−/− Tg strains (nPA and nPA) were provided by Dr. A. Singer (National Cancer Institute, Bethesda, MD) (11). All CD28 transgenes (WT, YF, nPA, nPA, and CD28TM) were expressed under the control of human CD2 promotor-enhancer elements on Gdf2−/− background. B6.C-H2bm12 (bm12) mice (31), congenic B6 (CD45.1) mice, and NK1.1+ T cells. Final CD28 signaling played a protective role in the development of SSC-type cGVHD. Indeed, SSC-like phenotype was due mainly to the defect in host CD4+CD25+ Tregs, but not in CD4+CD25−, CD8+, and NK1.1− T cells. Finally, the defect in host Tregs altered the cytokine production of donor CD4+ T cells and target Ags of autoantibodies. These results suggest that CD28 signaling controls the pathogenesis of cGVHD through effects on Tregs, whose status influences qualitatively the allogeneic immune responses.

Histology and immunohistochemistry

Tissues were fixed with 20% neutral-buffered formalin, embedded in paraffin, and sectioned. Those sections were stained with H&E, or Mason’s trichrome to visualize collagen.

For immunohistofluorescence analysis, skin and kidney tissues were embedded in optimal cutting temperature medium and were frozen in liquid nitrogen. Frozen sections (5 μm) were dried and fixed in cold acetone for 5 min. For the detection of immune complex depositions, sections were incubated with AlexaFluor488–conjugated anti-mouse IgG (H+L) F(ab′)2 (Cell Signaling Technology, Boston, MA), FITC–conjugated anti-mouse IgM, anti-mouse IgG2c, IgG2b, IgG3 (Bethyl Laboratories), and complement C3 (MP Biomedicals, Solon, OH) for 2 h at room temperature and were extensively washed with PBS. For type I collagen staining, cryosections were first incubated with rabbit anti-mouse type I collagen (Millipore, Billerica, MA) and then with Alexa Fluor488–conjugated goat anti-rabbit IgG (H+L) (Biotrend). The stained sections were counterstained with Hoechst 33342 (Sigma-Aldrich). For the detection of infiltrating cells, cryosections were fixed in 4% paraformaldehyde and then were incubated with Cy5-conjugated anti-mouse CD4 (GK1.5, laboratory prepared), biotin-conjugated anti-mouse CD45.1 (A20; BD Pharmingen), and finally with streptavidin–PE (Biologend).

For indirect immunofluorescence, 5-μm acetone or cryosections were fixed with 20% neutral-buffered formalin and then were incubated with Cy5-conjugated anti-mouse IgG (H+L) F(ab′)2 (Cell Signaling Technology). Stained sections were visualized with fluorescence microscopy B29000 (KEYENCE, Osaka, Japan) or confocal laser-scanning microscope Fluoview FV10i (Olympus, Tokyo, Japan). Fluorescent images were depicted with pseudocolors.

Measurement of serum cytokines level

Luminex Bead Array Assay Kit, Multiplex for cytokines was used according to the manufacturer’s instructions.

Real-time PCR

Total RNA was extracted from snap-frozen tissues, homogenized in TRIzol reagent (Sigma-Aldrich), and then treated with DNase. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) with priming by oligo-(deoxythymidine) (Invitrogen). Quantitative real-time PCR was performed in a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA) with a SYBR Green PCR Mix and specific primers as follows: Ifng; 5′-GGA TGC ATT CAT GAG TAT TGC-3′ (forward), 5′-CCT TTT CCG CCT CTT GAG G-3′ (reverse), Il4; 5′-AGC TCC TCA CAG CAA CGA AAG A-3′ (forward), 5′-TAT GCA CAA CAC TCT GTA AGC AGC-3′ (reverse), Gp96d; 5′-TGA AGC AGC CAT CTG AGG G-3′ (forward), 5′-CGA AGG TGG AAG AGT GGG AGG-3′ (reverse).

Preparation of T cells for adoptive transfer

T cells from spleen and lymph nodes were enriched by negative selection on a plate coated with anti-MHC class II (M5/114) mAb. For the depletion of CD4+, CD8+ or NK1.1− T cells, enriched T cells were then incubated with anti-CD4 (RL 172/4), anti-CD8 (83-12-5), or anti-NK1.1 (PK136) mAb, plus rabbit complement. Unfractionated CD4+ T cells or CD25−/− depleted

Measurement of Igs in serum

Serum Igs were measured with ELISA as described previously (29).

Serum Igs were measured with ELISA as described previously (29).
CD4 T cells were purified using anti-CD8 (83-12-5) and anti-NK1.1 (PK136) and/or anti-CD25 (7D4) mAb cocktails, plus complement. The purity of enriched T cells was >94%. The residual target cells were ~2% of total mononuclear cells after depletion.

**In vitro Treg suppression assay**

CD4 T cells from splenocytes were enriched by negative selection on a plate coated with anti-MHC class II (M5/114) mAb and anti-CD8 (3.155) mAb. CD4^+CD25^-CD44^int/low naive T cells and CD4^+CD25^- T cells (Treg) were sorted from enriched CD4^+ T cells. Naive CD4^+ T cells were cultured with 8 × 10^4 APCs, titrated number of Tregs, and 0.3 μg/ml anti-CD3 (2C11) mAb for 3 d in 96-well round-bottom plates. Proliferation of naive T cells was assessed as dilution of CFSE dye by flow cytometry.

**Isolation of lymphocytes from tissues**

Shaved back skin was harvested, s.c. fat was removed, and skin was minced with scissors. Skin samples were digested in PBS with 2.5% FCS, 1 mg/ml collagenase type D (Roche), 0.15 mg/ml DNase I (Sigma-Aldrich), and 0.3 mg/ml Dispase (Life Technologies) at 37˚C for 15 min. Digested samples were passed through 70-μm mesh, and the remaining skin tissues were repeatedly digested. Kidney was collected, decapsulated, and then minced with scissors. Kidney samples were digested in PBS with 2.5% FCS, 1 mg/ml collagenase type D, and 0.3 mg/ml DNase I at 37˚C for 20 min. After washes and centrifuge, cells of skin or kidney samples were suspended in 40% Percoll (GE Healthcare), gently layered onto the 80% Percoll, and centrifuged at 780 g for 20 min at room temperature. Lymphocytes were isolated from the Percoll interface.

**Statistical analysis**

Differences between groups were examined by a two-tailed unpaired Student t test, or log-rank test in Prism6 (GraphPad Software, San Diego, CA). The p values <0.05 were considered significant.

**Results**

**WT hosts and CD28-deficient hosts develop different phenotypes of cGVHD**

We previously showed that the CTLA4-Ig fusion protein prevents cGVHD (27), and that CD28-deficient donor T cells cannot induce cGVHD (27). Thus, CD28 costimulation is essential for the alloreactivity of donor T cells and the induction of cGVHD. When we induced cGVHD, we observed that, like donor T cells, host T cells also showed an increase in early activation markers (data not shown). However, the role of CD28 costimulation in host T cells remains unclear. We therefore investigated the role of CD28 signaling in host T cells by using Cd28^-/- mice as hosts.

Two mouse strains, B6 and B6.C-H2^bm12 ("bm12"), differ only by three amino acids in the β-chain of the I-A molecule, yet this difference is sufficient to induce alloreactivity in CD4^+ T cells. The transfer of bm12 splenocytes into B6 mice results in SLE-type cGVHD, which is characterized by anti-dsDNA autoantibody production and by glomerulonephritis with immune complex deposition (34). When splenocytes from donor bm12 mice were transferred into B6 mice or into Cd28^-/- mice, skin lesions, hair loss, and deposition gradually developed in Cd28^-/- hosts. In contrast, none of the B6 hosts manifested such symptoms (Fig. 1A and 1B). This indicated that CD28 signaling in host T cells protects against skin cGVHD. However, the Cd28^-/- group was divided into mice affected with skin lesions and unaffected mice (Fig. 1A). We postulated that unaffected Cd28^-/- hosts had produced Abs against the CD28 on donor T cells. Cd28^-/- mice would lack immunological tolerance to CD28, because when their immune systems were developing, CD28 was never expressed. As predicted, anti-Cd28 Abs were detected in Cd28^-/- hosts. Indeed, Cd28^-/- hosts that produced a high amount of anti-CD28 Abs showed no sign of skin disease, but Cd28^-/- hosts that produced a low amount of anti-CD28 Abs developed skin disease (Fig. 1C). Furthermore, even in Cd28^-/- hosts with high anti-CD28 Abs, donor CD4 T cells were decreased but not absent (data not shown). This indicated that anti-CD28 Abs might block CD28 costimulation in donor T cells, preventing activation and proliferation.

To avoid the production of anti-CD28 Abs, we used as hosts CD28TM-Tg mice, which express a CD28 transgene lacking cytoplasmic domain (TM) under a human CD2 promoter/enhancer in a Cd28^-/- background (28). As expected, they did not produce Abs to CD28 (Fig. 1C). CD28WT-Tg mice express an intact WT CD28 transgene on the same background, and we used these as controls. When using CD28TM-Tg mice as hosts, skin lesions developed in all mice (Fig. 1A). Symptoms appeared after day 14 and progressed over the long term (Fig. 1A and 1B). None of CD28-intact hosts (B6 and CD28WT-Tg) manifested such symptoms (Fig. 1A, 1B, 1D, and 1F). The widespread skin lesions in CD28TM-Tg hosts included thickened and tight tail, edema, and shiny skin at the extremities (Fig. 1E and 1G). Weight changes of B6 hosts were similar to those in control mice (B6 and CD28TM-Tg without injection of donor cells), whereas CD28TM-Tg hosts had slight weight loss (Fig. 1H). These results suggested that deficiency of CD28 signaling in host T cells caused skin disease.

**CD28 deficiency in host T cells converts cGVHD from SLE-type to SSC-type**

WT and CD28TM-Tg hosts at 14 wk, after the transfer of donor cells, were histologically analyzed. In WT hosts, which develop SLE-type cGVHD, the skin appeared normal (Fig. 2A, left panels). In CD28TM-Tg hosts, skin had dermal thickening and fibrosis, follicular dropout, subdermal fat loss, lymphocyte infiltration, and an increase of histiocytes and fibroblasts (Fig. 2A, right panels, and 2B). Because these features are often observed in patients and in mouse models of SSC (35, 36), it was considered that the CD28TM-Tg hosts had developed SSC-type cGVHD. In humans, SSC and SSC-type cGVHD are also characterized by excess collagen deposition in skin (37). To examine whether this also occurred in the CD28TM-Tg hosts, skin tissues were stained by anti-type I collagen Ab. Collagen deposition was increased in the dermis of CD28TM-Tg hosts, but not in that of WT hosts (Fig. 2A, bottom panels). We then examined the internal organs, because in humans with SSC-type cGVHD, progressive fibrotic damage leads to organ dysfunction and death (3). In CD28TM-Tg hosts, the fibrotic change extended to the glomeruli and tubulo-interstitium of kidney. In both hosts, perivascular inflammatory infiltration was observed (Fig. 2C). In CD28TM-Tg hosts, fibrosis with inflammatory infiltration occurred in the tongue, salivary glands, liver, and pancreas (Fig. 2C, lower panels). The tongue showed subepithelial lymphocytic infiltration, the salivary glands showed perivascular lymphocytic infiltration and inflammation, the liver showed perivascular lymphocytic infiltration around the portal area, and the pancreas displayed perivascular and periductal infiltration. In WT hosts, lymphocytes infiltrated the portal area of the liver and salivary glands, but not the tongue or pancreas (Fig. 2C, upper panels). These results indicated that the CD28TM-Tg hosts showed the characteristics of SSC-type cGVHD in organs.

B6 hosts given bm12 donor cells were reported to show high serum levels of anti-dsDNA autoantibodies, a characteristic of SLE (38). To evaluate whether CD28TM-Tg hosts, which showed an SSC-like phenotype, might have developed this SLE-type manifestation, we measured serum levels of Igs with ELISA. WT hosts showed a gradual elevation of anti-dsDNA IgG, but CD28TM-Tg hosts had no elevation of anti-dsDNA IgG and significantly lower
levels of total IgG subclasses overall (Fig. 3A–E). WT hosts showed a transient increase in IgE, but CD28TM-Tg hosts showed a sustained increase (Fig. 3F).

In WT hosts, immune complex is deposited in glomeruli, as seen in patients and in murine models of SLE (39). To evaluate whether this also occurred in CD28TM-Tg hosts, we analyzed kidney tissues 14 wk after transplant. In WT hosts, deposition of immune complex containing complement component C3 was observed in both glomerular capillary loops and mesangium. In contrast, in CD28TM-Tg hosts, only IgG was deposited at the mesangium (Fig. 3G). Weight changes (H) of B6 hosts and TM-Tg hosts are shown. Data are means ± SEM. Data represent two or more independent experiments; five mice per group. Statistical comparisons are as follow: *p < 0.05 for TM-Tg hosts versus B6 hosts, †p < 0.05 for Cd28−/− hosts versus TM-Tg hosts, ‡p < 0.05 for Cd28−/− hosts versus B6 hosts.

In CD28-deficient hosts, disease phenotype is associated with the cytoplasmic C-terminal proline-rich motif of CD28

The cytoplasmic tail of CD28 has several binding sites for signal molecules: the Y189MNM motif, the N-terminal proline-rich motif (P194RRP), and the C-terminal proline-rich motif (P206YAP). These motifs are thought to be associated with PI3K, Itk, and Lck, respectively, and to initiate signaling cascade (8). To understand their role in costimulation, three types of Tg mice were generated with mutations in the cytoplasmic domain of CD28: Y189 to F (hereafter YF), P194RRP to A194RRA (nPA), and P206YAP to A206YAA (cPA) whose CD28 cannot associate with the respective protein. Our previous work demonstrated that after CD28-ligation, both YF and nPA mutants were impaired for the tyrosine phosphorylation of CD28, the activation of Erk and Akt, proliferation, and IL-2 production. In contrast, cPA mutants showed intact tyrosine phosphorylation of CD28, activation of Erk, but impaired CD28-dependent T cell proliferation and IL-2 production (21). Furthermore, none of the mutant donor T cells was able to induce aGVHD in vivo (Ref. 28 and S. Ogawa and R. Abe, unpublished observations). Among the mutant strains, in cPA, intrathymic Foxp3+ Treg generation is impaired in a cell-intrinsic manner, suggesting that only signaling through P206YAP motif is necessary for thymic Treg development (11).

To identify which CD28 signaling pathway was responsible for phenotypic change of cGVHD in host T cells, we used these CD28 mutant Tg mice as hosts. When donor bm12 splenocytes were transferred into CD28 mutant Tg mice, cPA hosts showed more skin GVHD, but YF and nPA hosts showed no skin GVHD (Fig. 4A and 4B). This suggested that the cGVHD phenotypic change caused by CD28 signal deficiency in host T cells was primarily...
due to the lack of signaling through the C-terminal proline-rich motif, which presumably altered the Lck signaling pathway. Also, the change of cGVHD phenotype in CD28TM-Tg hosts might be due to reduction of Tregs. Because cPA hosts had less severe skin lesions than CD28TM-Tg hosts (Fig. 4B), we next counted Foxp3+ Tregs in the hosts during cGVHD. We found that cPA hosts had fewer Tregs than WT, YF, and nPA hosts, but more than CD28TM-Tg hosts (Fig. 4C), suggesting that in CD28-deficient hosts, the onset of SSc-type symptoms and the severity of skin lesions might be correlated with the number of host Tregs.

A defect in host CD4+CD25+ Tregs changes cGVHD’s phenotype

Because the cPA mutation impaired both CD28-dependent conventional T cell (Tconv) proliferation and IL-2 production and Treg development (11), we could not rule out the possibility that functional defects of Tconvs and NKT cells caused the cGVHD phenotypic change. To identify whether the defect in Tconvs (CD4+ and CD8+), or NK1.1+ T cells or CD4+ Tregs caused the cGVHD phenotypic change, we adoptively transferred fractionated T cells from CD28-intact mice into CD28TM-Tg mice before GVHD induction.

First, to test whether CD28-intact T cells could suppress cGVHD phenotype in CD28TM-Tg hosts, we added back unfractionated WT or CD28TM-Tg T cells into hosts. As expected, hosts that were added back with WT cells developed fewer skin lesions than unadded back CD28TM-Tg hosts. Add-back with CD28TM-Tg cells had no effect (Fig. 5A). Next, we compared CD28TM-Tg hosts that were added back with WT T cells, either unfractionated, CD4+-depleted, CD8+-depleted, or NK1.1+-depleted cells prevented skin lesions (Fig. 5A, 5B, and 5E). Thus, defects in host CD4+ T cells changed the disease phenotype.

To determine whether defects of CD4+ Tconvs or CD4+CD25+ Tregs controlled cGVHD phenotype, we compared CD28TM-Tg hosts that were added back either with unfractionated CD4+ T cells or with CD25+-depleted CD4+ T cells from CD28-intact mice. Unfractionated CD4+ T cells prevented the SSc phenotype, but CD25+-depleted cells did not (Fig. 5C–E). In addition, in hosts that were added back with WT T cells, the serum level of anti-dsDNA IgG was increased when unfractionated CD4+ T cells were used but was unchanged when CD25+-depleted CD4+ T cells were used (Fig. 5F).

These results showed that add-back with unfractionated CD4+ T cells (containing CD4+CD25+ Tregs) reconverted cGVHD from SSc-type to SLE-type, but did not prevent all cGVHD, suggesting that a defect in host CD4+CD25+ Tregs changed the phenotype.

In addition to fewer Tregs, a defect of Treg function might also contribute to this phenotypic change. To assess the suppressive capability of CD4+CD25+ Tregs in CD28TM-Tg mice, we performed an in vitro Treg suppression assay. As shown in Fig. 6A and 6B, compared with WT Tregs, three times more CD28TM-Tg Tregs were needed to produce the same effect. This suggests that CD28TM-Tg Tregs had less suppressive capability than did WT Tregs. Furthermore, the homing ability of Tregs is important for suppression of the immune response in peripheral tissues. A previous report demonstrated that skin-homing receptor CCR4-deficient Tregs are unable to prevent skin inflammation in Foxp3-deficient scurfy mice (40). We examined whether CD28-deficient Tregs had a defect in chemokine receptor expression and found that they had less CCR4 expression than did WT Tregs (Fig. 6C, top, and 6D, left). Although the frequency of CCR6-expressing cells in Tregs was equivalent between WT and CD28TM-Tg Tregs, CCR6 expression on the CD28TM-Tg Treg surface was lower than that of WT Tregs (Fig. 6C, bottom, and 6D, right). Thus, in CD28TM-Tg hosts, there were fewer Tregs, and the cells were defective. Both defects caused the phenotypic change of cGVHD.

FIGURE 2. CD28TM-Tg hosts receiving transplants show pathological features similar to SSc-type cGVHD. Tissue sections from transplant recipients, after 14 wk. bm12 spleen cells were transplanted into WT mice or into TM-Tg mice. (A) Skin: (top panels) H&E staining; (bottom panels) type I collagen (green) and nucleus (blue) staining. Scale bars, 50 μm. (B) Data represent pathological GVHD scores for skin tissues obtained from WT and TM-Tg hosts in (A). Each dot represents one mouse. Means are indicated by a horizontal bar. **p < 0.01. (C) Masson’s trichrome stain to visualize collagen (blue). Scale bars, 100 μm. Data are representative of two or more experiments with five mice per group.
Defects in host Tregs enhance donor Th2 responses

We investigated mechanisms whereby the defects in host Tregs changed the phenotype of cGVHD. In CD28TM-Tg hosts, preferential deposition of IgG1 subclass and higher serum IgE level were observed (Fig. 3H and 3F). Because class switch recombination is regulated by cytokines (41), these results suggest that the cytokine environment might be different between WT hosts and CD28TM-Tg hosts. In this [bm12 → B6] cGVHD murine model, donor CD4+ T cells play a central and sufficient role for the initiation and formation of cGVHD (39), and similarly in CD28TM-Tg hosts, with purified CD4+ T cells as donor cells, cGVHD phenotype was similar to that with unfractionated donor cells (data not shown). Therefore, to ask how donor CD4+ T cell properties differed in WT hosts and CD28TM-Tg hosts, we analyzed their cytokine production profile at 5 wk after donor cells transfer, when skin disease in CD28TM-Tg hosts begins to progress (Fig. 1B). Intracellular cytokine staining of splenic CD4+ T cells showed that IFN-γ–producing cells were equally common. IL-17–producing cells were few and equivalent in both hosts. In contrast, there were more IL-4–producing cells in CD28TM-Tg hosts than in WT hosts (Fig. 7A and 7B). The yield of total CD4+ T cells was the same in the two hosts (data not shown). This suggested that in CD28TM-Tg hosts the defect in host Tregs augmented Th2 differentiation in donor CD4+ T cells. The serum levels of Th2 cytokines such as IL-5, IL-6, IL-10, and IL-13 were also higher (Fig. 7C). We next examined cytokine expression in skin and kidney. In skin, IFN-γ mRNA expression was higher in CD28TM-Tg hosts than WT hosts, and IL-4 mRNA was expressed only in CD28TM-Tg hosts (Fig. 7D, upper panels). In kidney, IFN-γ mRNA was highly expressed in both hosts, whereas IL-4 mRNA expression was only increased in CD28TM-Tg hosts (Fig. 7D, lower panels). To investigate whether cytokine expression correlated with an infiltration of donor CD4+ T cells, skin and kidney were immunostained. In CD28TM-Tg hosts, but not WT hosts, donor CD4+ T cells diffusely infiltrated skin, especially the dermal area. In WT hosts, almost all CD4+ T cells in skin were derived from the host (Fig. 7E, upper panels). In contrast, in both hosts, perivascular infiltration by donor CD4+ T cells was detected in kidney (Fig. 7E, lower panels). These results were quantitatively analyzed by flow cytometry of lymphocytes from skin and kidney. In WT hosts, no donor (CD45.2+) CD4+ T cells infiltrated into skin, compared with abundant infiltration in CD28TM-Tg hosts (0% for WT hosts versus 21 ± 5.4% [mean ± SEM] for CD28TM-Tg hosts; Fig. 7F).
In both hosts, donor CD4+ T cells infiltrated into kidney (7.4 ± 1.4% for WT hosts versus 16 ± 2.9% for CD28TM-Tg hosts; Fig. 7F). These results suggested that the increased IFN-γ and IL-4 mRNA expression in the skin of CD28TM-Tg hosts was due to infiltration by donor CD4+ T cells, and the high expression of IL-4 mRNA in kidney of CD28TM-Tg hosts was due to the augmentation of the Th2 subset in donor CD4+ T cells. Taken together, the defect in host Tregs altered donor CD4+ T cell cytokine production and infiltration, leading to phenotypic conversion of cGVHD.

Host Treg deficiency alters Ag specificities of autoantibody

Tissues such as skin, tongue, and pancreas, which were unaffected in WT hosts, became targeted in CD28TM-Tg hosts (Fig. 2). Besides, in these hosts, substantial IgG was deposited in glomeruli despite very low serum level of anti-dsDNA IgG (Fig. 3A and 3G). These observations indicate that Abs in CD28TM-Tg hosts could bind Ags other than dsDNA, generating Ag–Ab complexes that are then deposited on glomeruli. We hypothesized that Ag specificities of autoantibody differ between WT and CD28TM-Tg hosts, and that this caused the change of target tissues. To investigate this, we performed indirect immunofluorescence staining using serum from WT and CD28TM-Tg hosts on the tissues from Rag2−/− mice. As shown in Fig. 8, the staining patterns were obviously different. Sera from WT hosts contained high levels of anti-dsDNA IgG and mainly reacted with the nuclei (Fig. 8A–C). Sera from CD28TM-Tg hosts showed no reactivity against nuclei, but recognized skin Ags, especially hair follicles (Fig. 8A). In kidney, the serum recognized the ductal-like structure between the cortex and medulla (Fig. 8B). The sera from CD28TM-Tg hosts did not react with liver (Fig. 8C). These results suggested that altered target Ag of autoantibodies changed a target tissue. Furthermore, donor CD4+ T cells possibly had different Ag specificities between WT and CD28TM-Tg hosts, and this might alter their tissue tropisms. In summary, cGVHD phenotypic change due to CD28 deficiency in host T cells is due to the defect in host Tregs via the cytoplasmic C-terminal proline-rich motif of CD28. Phenotype may be mediated by altered cytokine production of donor CD4+ T cells and altered target Ags.

Discussion

CD28 costimulation in host T cells played an important role in the pathogenesis of cGVHD in a mouse model. CD28-deficient hosts showed SSc-type cGVHD, but WT hosts showed SLE-type cGVHD. Development of SSc-type cGVHD was caused by CD28 deficiency in host T cells and was due to an absence of signal transduction through the C-terminal proline-rich motif in the cytoplasmic tail of CD28. This signaling pathway is reportedly required by both Tconv and Tregs for IL-2 production/proliferation and for thymic development, respectively. Our adoptive transfer experiments showed that the SSc-like disease was due to defective host Tregs only. Host CD28 signaling controlled the pathogenesis of cGVHD, depending on the status of host Tregs. In these cells, the defect altered cytokine production in donor CD4+ T cells and the target Ag specificity of autoantibodies. These changes might contribute to the development of distinct patterns of pathogenesis in cGVHD. Therefore, the status of host Tregs can determine the allogeneic immune responses.

The pivotal roles of Tregs in the development and maintenance of immune self-tolerance have been well documented (42). In mice, donor or host Tregs prevent the onset and progression of SSc- and SLE-type cGVHD (33, 35, 43). It was thought that Tregs functioned as immunosuppressors in allogeneic immune responses and in autoimmune responses. However, this study revealed a novel role for Tregs in cGVHD pathogenesis, wherein they governed the phenotype of cGVHD.

A deficiency of CD28 signal affects Treg development in the thymus and Treg homeostasis in peripheral lymphoid tissues, and reduces Treg numbers (10, 11). The number of host Tregs could determine cGVHD phenotype. But impairment of Treg function must also be considered. In this study, Tregs from CD28TM-Tg mice inhibited the proliferation of Tconv in vitro to a lesser extent than did Tregs from WT mice. Also, although Treg numbers remain normal, Treg-specific CD28 deletion causes autoimmune disease (12), suggesting that CD28 costimulation plays a crucial role in the suppressive activity of Tregs, as well as in their development. CD28-deficient Tregs express less CCR6 on their surfaces (12), with CCR6 important for recruiting Tregs into inflammatory tissues (44). Consistent with this, Tregs in CD28TM-Tg mice showed less surface CCR6 expression. Moreover, skin-homing receptor CCR4 expression on their Tregs was reduced. This defect in homing of Tregs might be responsible for the manifestation of skin disease in CD28-deficient hosts. Thus, in Tregs, both reduced suppressive activity and impaired migration may disrupt immune homeostasis. CD28 costimu-


FIGURE 5. In CD28TM-Tg hosts, a defect in host CD4+CD25+ T cells causes SSc-type cGVHD. Seven days before GVHD induction, TM-Tg hosts were added back with WT T cells, either unfractionated (Unfrac. WT) \((6 \times 10^7)\), or depleted for CD4+ \((3 \times 10^7)\), CD8+ \((3 \times 10^7)\), NK1.1+ \((6 \times 10^7)\), or unfractionated TM-Tg T cells (Unfrac. TM) \((6 \times 10^7)\). (A) Average clinical skin GVHD scores. Statistical comparisons are as follows: *p < 0.05 for TM-Tg hosts that added back with CD4+-depleted cells versus unfractionated WT T cells; p > 0.05 (not significance) for TM-Tg hosts that were added back with CD8+- and NK1.1+-depleted T cells versus unfractionated WT T cells. (B) Representative skin cGVHD histology (H&E). Scale bars, 50 μm. Data are representative of two independent experiments performed with four to five mice per group. TM-Tg hosts were added back with either unfractionated WT CD4+ T cells (Unfrac. CD4) \((3 \times 10^7)\) or WT CD25+-depleted CD4+ T cells \((3 \times 10^7)\). (C) Average clinical skin GVHD scores of added-back and unadded back TM-Tg hosts. (D) Representative skin cGVHD histology (H&E stain). Scale bars, 50 μm. (E) Data (means ± SEM) represent pathological GVHD scores for skin tissues obtained from host mice in (A) and (C). (F) Serum levels of anti-dsDNA IgG in hosts 8 wk after donor cells transfer. Data are representative of two independent experiments with five or more mice per group. *p < 0.05 for TM-Tg hosts that were added back with unfractionated CD4+ T cells versus CD25+-depleted CD4+ T cells and unadded back TM-Tg hosts.

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We did not observe acceleration of the onset of skin disease (Supplemental Fig. 1), indicating that donor Tregs did not influence the pathogenesis of SSc-like cGVHD. Although the mechanism remains unclear, the timing of distribution to skin, or the amount of donor Tregs, may account for these observations.
In contrast with WT hosts, CD28TM-Tg hosts developed SSc-type cGVHD, not SLE-type, suggesting that the status of host Tregs controls the phenotype of cGVHD. This may occur as follows. We observed deposition of IgG1 subclass in glomeruli and high serum IgE in CD28TM-Tg hosts. In CD28TM-Tg hosts, the level of Th2 cytokines was increased in local lesions, in serum, and in the donor T cells response. We therefore hypothesized that augmented donor Th2 responses due to defective host Tregs led to fibrosis in the skin and internal organs. Many studies have suggested that Th2 cytokines promote fibrosis (45). Inhibition of IL-4 reduced the development of dermal fibrosis in a putative mouse model of SSc (46), whereas the expression of IL-4 mRNA and the infiltration of IL-4–producing T cells were observed in the skin of SSc patients (47, 48). These reports support our hypothesis. Furthermore, Tian et al. (49) reported a relation between Th cell subsets and frequency of Tregs when they studied Treg depletion. Th2 cells were sharply increased by high depletion of Tregs but were not increased by low depletion. This suggests that the number of Tregs may regulate the Th2 response in allogeneic immune responses and in autoimmune responses. Thus, in CD28TM-Tg hosts, the defect in Tregs may obviate the suppression of the Th2 response in allogeneic immune responses, leading to fibrosis in the skin and internal organs. In summary, the status of host Tregs controls Th cell differentiation of donor CD4+ T cells and contributes to the pathogenesis of cGVHD.

In CD28TM-Tg hosts, but not in WT hosts, tissues such as skin, tongue, and pancreas become targeted. Abs recognized different target Ags in the two hosts. The Ag specificities of donor CD4+ T cells were perhaps altered by the status of host Tregs and may change the target tissue. Treg-deficient NOD.Cd28−/− mice develop both pancreatic exocrine disease and pancreatic endocrine disease. Pancreatic endocrine disease has been observed in WT NOD mice. Pancreatic amylase becomes an additional target Ag because of Treg deficiency, not because of Ag spreading (50). This indicates that Tregs regulate the Ag specificity of effector T cells. This supports our speculation that in CD28TM-Tg hosts, the target Ags of donor CD4+ T cells may be altered. Genetic variation such as MHC and minor histocompatibility Ag are thought to affect alloreactivity, morbidity, and mortality of GVHD (51). Kaplan et al. (52) demonstrated that a difference of target Ags, decided by MHC haplotype, determined GVHD phenotype in a murine model. In this study, we found that differences in host Treg status also determine cGVHD phenotype. These observations suggested that different repertoires of donor CD4+ T cells, specific for dif-
FIGURE 7. Donor CD4+ T cell properties in WT versus CD28TM-Tg hosts 5 wk after donor cells transfer. To distinguish donor and host cells, we used a congenic marker CD45.1. Donor cells from bm12 mice (CD45.1+) were transferred to host mice (CD45.1+CD45.2+) and were analyzed after 5 wk. (A) Cytokine production of CD45.2+ donor CD4+ T cells was measured upon restimulation of splenocytes with PMA plus ionomycin. Representative dot plots show the percentage of cytokine+ cells in donor CD4+ T cells. (B) Mean ± SEM of cytokine+ cells within donor CD4+ T cells from spleen. (C) Serum cytokine level of [bm12 to WT] (closed circle) and [bm12 to TM-Tg] (open circle) mice measured with Multiplex. Each dot represents one mouse. Data are combined from two independent experiments with four mice per group. Means are indicated by a horizontal bar. (D) Ifng and Il4 gene expression, normalized to Gapdh, of skin and kidney. (E) Representative images show CD4 (green), CD45.2/host (red), and Hoechst/nucleus (blue) staining of skin and kidney. Thus, donor or host CD4+ cells were shown as green or yellow, respectively. Scale bar, 50 μm. (F) Donor (CD45.2+) CD4+ T cells, infiltrating into skin and kidney of hosts, were analyzed by flow cytometry. Flow-cytometry plots are gated on live total lymphocytes (CD45+). Data represent four mice per group from two independent experiments. *p < 0.05, **p < 0.01 for WT hosts versus TM-Tg hosts.
ferent Ags, were selectively expanded, depending on the state of host Tregs. In this study, host Tregs seem not to suppress SLE-type cGVHD. Add-back of total CD4+ T cells including CD25+CD4+ Tregs, as opposed to CD25+-depleted CD4+ T cells, into CD28TM-Tg hosts increased anti-dsDNA autoantibody. In addition, in the [DBA/2→BALB/c] cGVHD murine model, which develops both SSc- and SLE-type symptoms, add-back of Tregs caused a limited reduction of anti-dsDNA autoantibody compared with sclerodermatous skin damage (35). A recent report shows that Treg depletion severely reduces follicular Th cells and germinal center B cell responses to influenza virus (53). Consistent with this report, in our study, the donor follicular Th cell population in CD28TM-Tg hosts was lower than in WT hosts (data not shown). Taken together, host Tregs might favor increasing SLE-type disease rather than inhibiting it. In humans, HCT is increasingly performed using nonmyeloablative conditioning regimens in an attempt to reduce its toxicity (4), but this increases the incidence of lupus-like glomerulonephritis with immune complex deposition (54–57). Our findings suggest that the increased frequency of host Tregs may explain this.

In SLE patients and in some murine models (39), in addition to immune complex deposition in the glomeruli of kidney, immune complex deposition at the epidermal–dermal junction of skin is also observed. Zhang et al. (35) reported that in the [DBA/2→BALB/c] cGVHD murine model, when recipient mice received total body irradiation as preconditioning, skin lesions with immune complex deposition were observed. In the [DBA/2→(B6×DBA/2)F1] model, van Elven et al. (58) described that when DBA/2 donor cells were repeatedly (four times) transferred into F1 recipient mice, immune complexes were deposited in the skin. In contrast, in our [bm12→B6] murine model, neither skin lesions nor immune complex deposition in the skin were observed. Furthermore, in the [DBA/2→(B6×DBA/2)F1] model, when DBA/2 donor cells were injected once, immune complex was deposited in kidney but not in skin (data not shown). The differences between our model without immune complexes and the models with immune complexes in skin can be explained by induction of a massive allogeneic immune response by repeated donor cell transfers, or by injury to blood vessels in the skin by irradiation. Compared with kidney, a larger allogeneic immune response or promoted extravasation of immune complex from injured blood vessels might be required for deposition of immune complex in the skin.

Previously, we and others reported that CD28 signaling in donor T cells was necessary for the development of SLE-type cGVHD, because blockade of CD28 by CTLA4-Ig prevented it (29, 59). This study demonstrated that when host CD28 signaling is deficient, the abnormality in host Tregs (but not Tconvs), converted cGVHD symptoms from SLE-type to SSc-type. This suggests that CTLA4-Ig treatment might have not only a beneficial effect, but also a negative effect because of impaired Treg homeostasis. Indeed, in some murine cardiac and skin transplant models, treatment with CTLA-4Ig shortened allograft survival and reduces Tregs (60).

Our results suggested that Lck signaling through the C-terminal proline-rich motif inhibited SSc-type cGVHD, but PI3K and Itk signaling were not necessary. Other signaling pathways via as yet unidentified motifs might protect SSc-type cGVHD because disease status in cPA hosts was milder than in CD28TM-Tg hosts. Regarding donor T cell CD28 costimulation: signaling pathways
via three motifs, the YMMN and two proline-rich motifs, were respectively necessary for expansion and survival of donor T cells and induction of aGVHD (Ref. 28 and S. Ogawa and R. Abe, unpublished observations). Such observations suggest that pharmacological inhibitors of the CD28 signaling molecules driven by the YMMN motif and the N-terminal proline-rich motifs (not the C-terminal proline-rich motif) might prevent cGVHD. A recent report indicated that CD28 and Itk signaling regulated the trafficking of autoreactive T cells into tissues in autoimmune disease models (61). Pharmacological inhibition of Itk prevented infiltration into tissues. Rapamycin, which can inhibit the PI3K/Akt/mTOR pathway through the YMMN motif, has efficacy on SSc-type cGVHD patients (62), preserving Treg expansion and function (63, 64).

In conclusion, our study found that CD28 signaling in host T cells is important in the pathogenesis and phenotype of cGVHD. The status of host Tregs qualitatively impacts on the differentiation of donor CD4+ T cells and on the Ag specificity of the allogeneic response, ultimately leading to the development of various phenotypes of cGVHD.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1

Depletion of Treg cells from donor cells did not accelerate the onset of SSc-like cGVHD in CD28-deficient hosts

CD28TM-Tg hosts were injected with donor bm12 unfractionated spleen cells or CD25+ cell-depleted spleen cells.

Graph shows the incidence of skin disease with four mice per group.