IL-6 Blockade Attenuates the Development of Murine Sclerodermatous Chronic Graft-Versus-Host Disease

Doanh Le Huu[1], Takashi Matsushita[1], Guihua Jin[1], Yasuhito Hamaguchi[1], Minoru Hasegawa[1], Kazuhiko Takehara[1] and Manabu Fujimoto[1]

Systemic sclerosis (scleroderma) is a connective tissue disease characterized by excessive extracellular matrix deposition in the skin and visceral organs. Serum IL-6 levels are reported to be elevated in human scleroderma and chronic graft-versus-host disease (cGVHD) patients. IL-6 blockade using anti-IL-6 receptor mAb (anti-IL-6R mAb) results in amelioration of the pathologic symptoms of some autoimmune diseases such as rheumatoid arthritis and juvenile idiopathic arthritis. In this study, we examined the effects of anti-IL-6R mAb on either prevention or treatment of murine sclerodermatous cGVHD (Scl-cGVHD). We found that serum IL-6 levels in Scl-cGVHD mice gradually increased after bone marrow transplantation. Administration of anti-IL-6R mAb attenuated the development of severe Scl-cGVHD and fibrosis and resulted in an increase in $CD4^+CD25^+FoxP3^+$ regulatory T cells. However, treatment of established Scl-cGVHD with anti-IL-6R mAb showed no effects on disease severity. The effects of anti-IL-6R mAb were mostly inhibited by anti-CD25 mAb. Together, our results indicate that IL-6 has an important role in the pathogenesis of Scl-cGVHD. IL-6 blockade may be an effective approach for preventing Scl-cGVHD and treating cGVHD and scleroderma in humans.

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INTRODUCTION

Systemic sclerosis (SSc) is a connective tissue disease characterized by excessive extracellular matrix deposition in the skin and visceral organs (Varga and Abraham, 2007). The etiology and pathogenesis of SSc are so far unknown (Katsumoto *et al.*, 2011). Chronic graft-versus-host disease (cGVHD) emerges from alloreactive reactions between donor-derived immune cells and host cell populations, and donor T cells have a major role in the pathogenesis of this disease. On the basis of clinical similarities between sclerodermatous cGVHD (Scl-cGVHD) and SSc-transplantation of B10.D2 ($H\text{-}2^d$) bone marrow and splenocytes across minor histocompatibility loci into sublethally irradiated BALB/c ($H\text{-}2^d$) recipients has become a well-established animal model for human Scl-cGVHD and SSc (Jaffee and Claman, 1983; Toshiyuki, 2005; Ferrara and Reddy, 2009).

[1]*Department of Dermatology, Faculty of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan*

Correspondence: Takashi Matsushita, Department of Dermatology, Faculty of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan. E-mail: t-matsushita@derma.m.kanazawa-u.ac.jp

Abbreviations: anti-IL-6R mAb, anti-IL-6 receptor mAb; BMT, bone marrow transplantation; cGVHD, chronic graft-versus-host disease; CBA, Cytometric Bead Array; MCP, monocyte chemotactic protein-1; mRNA, messenger RNA; Tregs, regulatory T cells; SSc, systemic sclerosis; RT–PCR, reverse transcriptase PCR; TNF-α, tumor necrosis factor-α

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IL-6 is a widely expressed effector cytokine that is produced by activated T cells, B cells, and macrophages (Scheller and Rose-John, 2006). IL-6 inhibits the induction of TGF-β-induced regulatory T cells (Tregs), whereas IL-6 induces Th17 differentiation in the presence of TGF-β (Bettelli *et al.*, 2006). Of note, it has been shown that the absence of Tregs results in IL-6 production, which leads to the development of GVHD-associated autoimmunity (Chen *et al.*, 2007).

The serum IL-6 levels were elevated in patients with diffuse SSc (Stuart *et al.*, 1995; Hasegawa *et al.*, 1998; Sato *et al.*, 2001; Matsushita *et al.*, 2006). In this regard, serum IL-6 levels may be a useful indicator of disease activity in SSc (Stuart *et al.*, 1995; Hasegawa *et al.*, 1998, 2011; Scala *et al.*, 2004).

Blockade of IL-6 signaling by anti-IL-6 receptor mAb (anti-IL-6R mAb) has positive effects on several experimental inflammatory diseases, such as experimental autoimmune encephalomyelitis, polymicrobial sepsis, and autoimmune kidney disease (Mihara *et al.*, 1998; Serada *et al.*, 2008; Mukaino *et al.*, 2010). Furthermore, administration of anti-IL-6R mAb was effective in patients with autoimmune diseases such as rheumatoid arthritis and juvenile idiopathic arthritis (Nishimoto *et al.*, 2008). For these reasons, we examined the relationship between IL-6 and Scl-cGVHD development, and the effects of anti-IL-6R mAb on murine Scl-cGVHD initiation and progression.

RESULTS

Serum IL-6 gradually increases during the course of cGVHD development

Alteration of skin cytokine messenger RNA (mRNA) expression in murine Scl-cGVHD has been reported, with a

mixed Th1/Th2-like cytokine profile present in early fibrosis, characterized by an initial Th1-like predominance that preceded a Th2-like profile (Zhou *et al.*, 2007).

To investigate how serum inflammatory cytokines are altered during the course of Scl-cGVHD, IL-6, IL-10, monocyte chemotactic protein-1 (MCP-1), IFN-γ, tumor necrosis factor-α (TNF-α), and IL-12p70 levels were measured by Cytometric Bead Array (CBA) on days 7, 14, 21, 28, and 35 after bone marrow transplantation (BMT; Figure 1a). Although TNF-α and IFN-γ reached a peak at day 28 after BMT, the level of IL-6 gradually increased throughout the 35-day examination period after BMT (Figure 1a). Whereas IL-12 and IL-10 did not change during cGVHD development, MCP-1 increased 7 days post transplantation (Figure 1a), with $P<0.01$ compared with BALB/c mice without BMT (Data not shown).

Blockade of IL-6/IL-6R interactions after administration of anti-IL-6R mAb

To determine whether blockade of IL-6R would result in an alteration in the levels of circulating inflammatory cytokines, we blocked IL-6 signaling using a mAb that binds to both membrane and soluble components of the IL-6R. Either anti-IL-6R mAb (MR16-1) or control rat IgG was injected into recipients on the day of BMT. Serum samples were harvested on day 7 following BMT for examination of proinflammatory cytokine levels. These data revealed a significant increase in IL-6 levels in the MR16-1-treated group (Figure 1b) but no significant differences in the rest of cytokine levels compared with the control group (Figure 1b). A similar increase in serum IL-6 levels after MR16-1 injection had been previously reported and was postulated to result from decreased cytokine clearance as a consequence of circulating IL-6 being unable to bind to IL-6Rs (Uchiyama *et al.*, 2008; Chen *et al.*, 2009).

Blockade of IL-6 attenuated cGVHD severity in a preventive model of Scl-cGVHD, but not in a therapeutic model

The current study showed that serum IL-6 levels gradually increased during Scl-cGVHD development. Thus, we conducted studies to determine whether treatment with MR16-1 would result in prevention or in treatment of

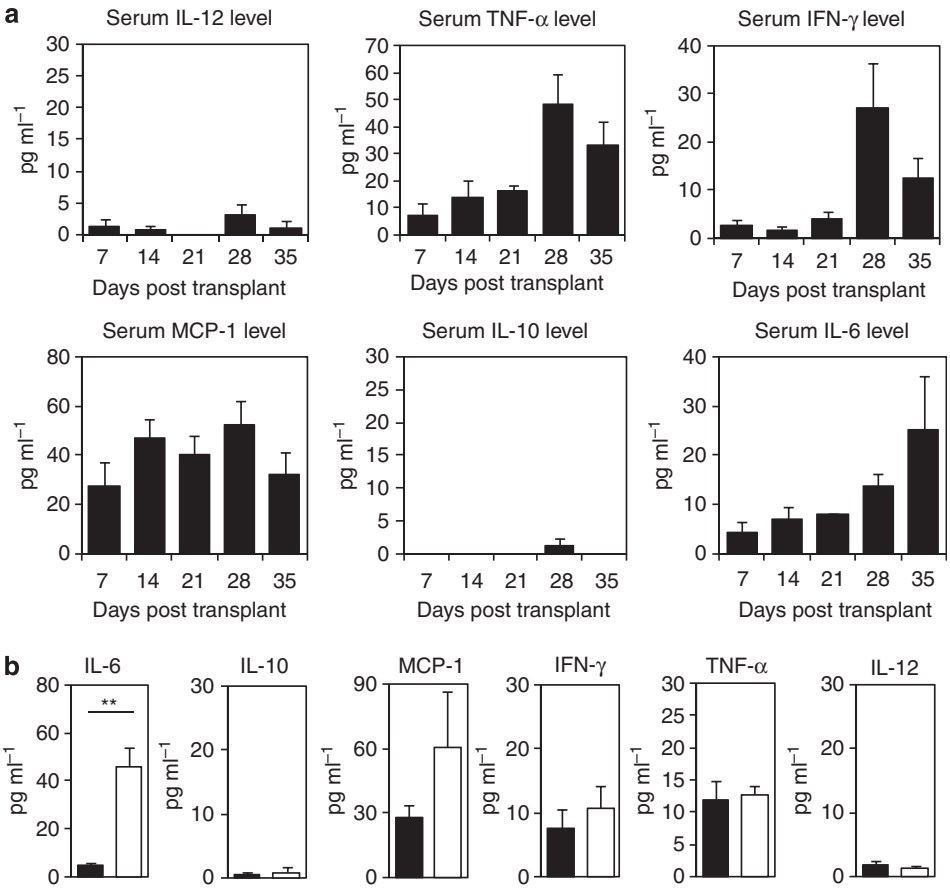

Figure 1. Changes in serum inflammatory cytokines levels during development of sclerodermatous chronic graft-versus-host disease (Scl-cGVHD). BALB/c (H-2^d) recipients were irradiated (800 cGy) and transplanted with B10.D2 (H-2^d) donor 10×10^6 T-cell-depleted bone marrow (TCD-BM) and 10×10^6 unfractionated splenocytes. (**a**) Serum samples were collected every 7 days after bone marrow transplantation (BMT). IL-12p70, tumor necrosis factor-α (TNF-α), IFN-γ, monocyte chemotactic protein-1 (MCP-1), IL-10, and IL-6 levels were measured by Cytometric Bead Array. (**b**) Allogeneic BMT recipients were treated with MR16-1 (anti-IL-6R mAb, □) or with control rat IgG (■) on day 0 at a dose of 2 mg per mouse. Seven days after BMT, serum IL-6, IL-10, MCP-1, IFN-γ, TNF- α, and IL-12p70 concentrations were measured by Cytometric Bead Array. Each value indicates mean (± SEM) results from three to four mice. $^{**}P<0.01$.

cGVHD. During preventive application of MR16-1, both treated and control recipients showed similar early declines and recovery in body weight (Figure 2a). However, once skin lesions developed, MR16-1-treated mice experienced less body weight loss than controls, with weight loss of 10 ± 0.02% vs. 21 ± 0.02% at day 30 and 14 ± 0.01% vs. 26 ± 0.01% at day 42 (Figure 2a, $P = 0.01$). Similarly, MR16-1-treated mice developed less skin disease, as evidenced by their improved skin score curve (Figure 2b). For example, the average control versus MR16-1 skin score was 4.76 ± 0.40 vs. 2.95 ± 0.42 at day 30, and 5.74 ± 0.52 vs. 3.77 ± 0.56 at day 42 after BMT, respectively (Figure 2b, $P < 0.05$).

TGF-β is a key mediator of tissue fibrosis in SSc and includes three mammalian isoforms (Ihn, 2008). TGF-β1 is the primary isoform responsible for collagen upregulation leading to skin fibrosis (McCormick *et al.*, 1999). In our MR16-1 preventive model, plasma TGF-β1 levels were found to be elevated in the control group relative to the MR16-1-treated group (Figure 2d). In contrast, there were no significant differences in body weight or skin score when MR16-1 was administered therapeutically on day 25 after BMT (Figure 2e and f).

The observed trends in skin disease were also verified by histopathology. Skin histopathological scores, dermal thickness, skin total soluble collagen, and collagen deposition of skin, lung, and liver were measured as described in Materials and Methods. In the preventive model, histopathological scores, dermal thicknesses, skin soluble collagen, and fibrosis area in skin, lung, and liver were lower in the MR16-1-treated group than in the control group ($P < 0.05$; Figure 3b, d, f, and g). However, in the therapeutic model, the MR16-1-treated group had scores indistinguishable from the control group (Figure 3c and e).

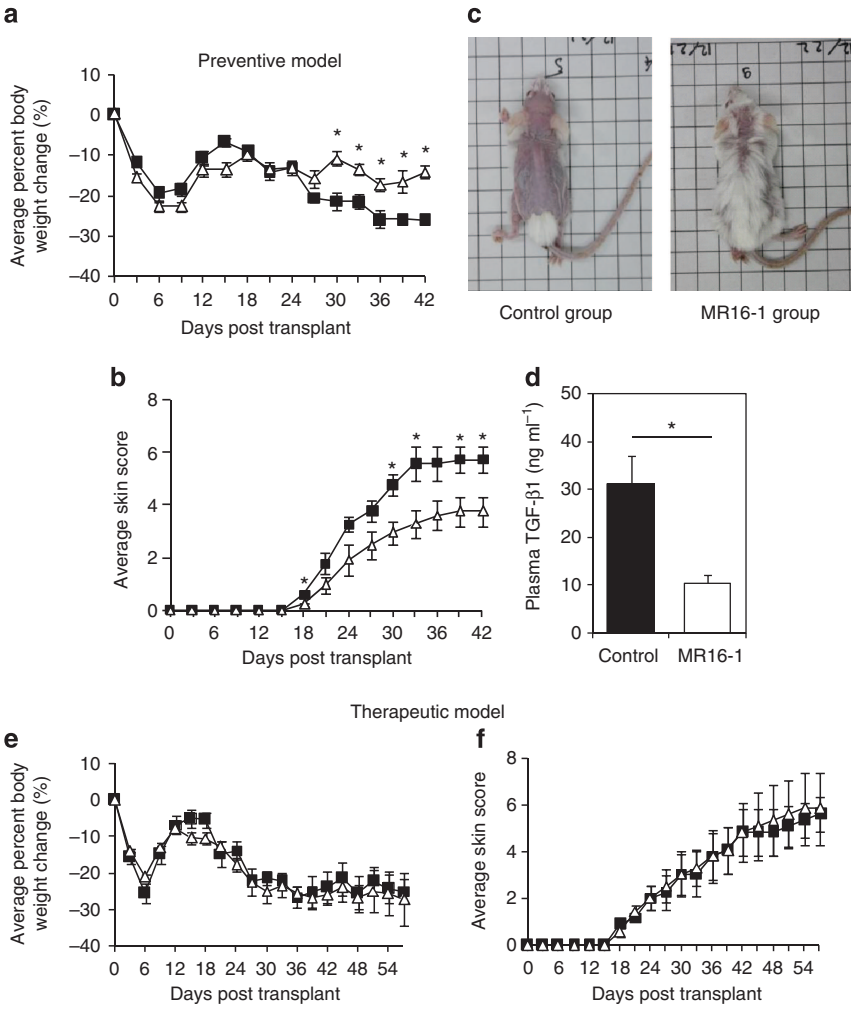

Figure 2. MR16-1 treatment attenuated chronic graft-versus-host disease (cGVHD) severity in a preventive model but did not alter in a therapeutic model. Preventive model: recipients were treated with MR16-1 (△) or control (■) on day 0 at a dose of 2 mg per mouse and then weekly at a dose of 0.5 mg per mouse. (**a**) Body mass changes and (**b**) skin scores were assessed every 3 days. (**c**) Representative photographs were taken on day 42 after bone marrow transplantation (BMT). (**d**) Plasma TGF-β1 level was measured by ELISA 42 days after BMT. Therapeutic model: recipients were treated with MR16-1 (△) or control (■) on day 25 at a dose of 2 mg per mouse and then weekly at a dose of 0.5 mg per mouse. (**e**) Body mass changes and (**f**) skin scores were assessed every 3 days. Each value indicates mean (± SEM) results from five mice. *$P < 0.05$.

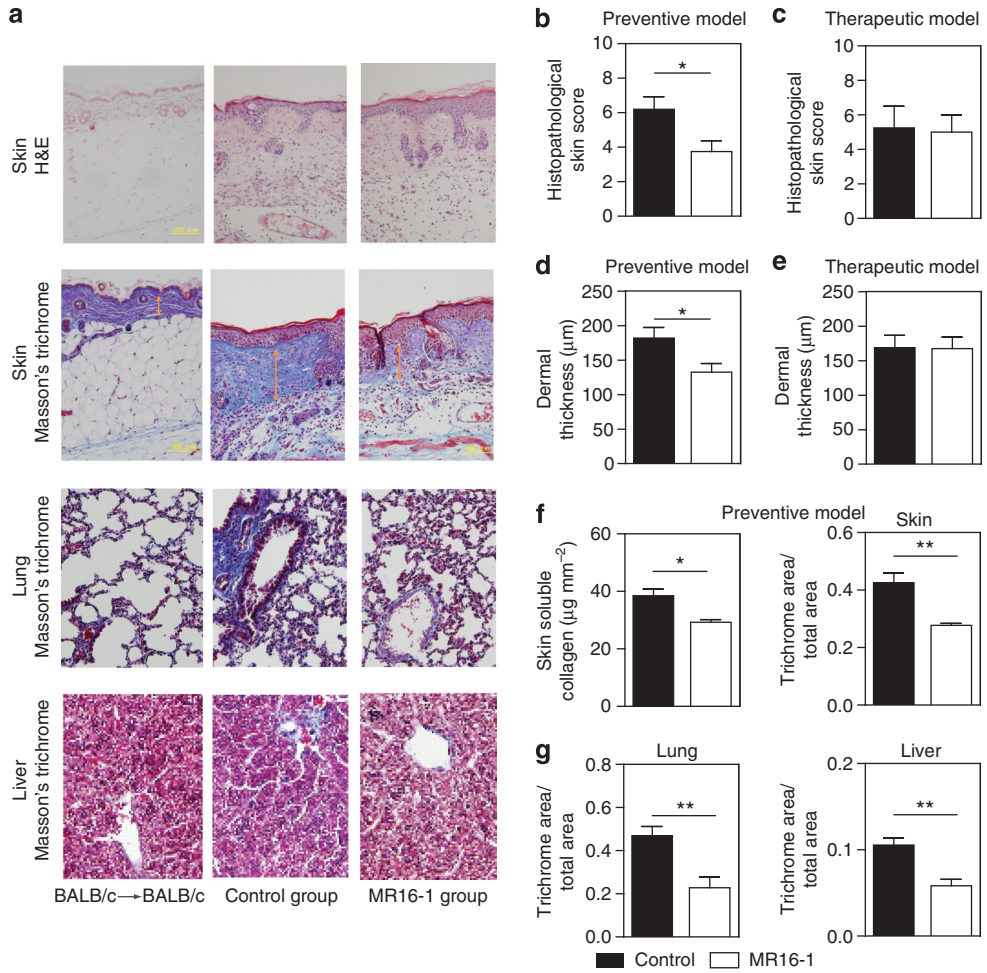

Figure 3. Improved fibrosis scores in MR16-1-treated animals in the preventive model. Slides were scored and skin soluble collagen was measured as described in Materials and Methods. (**a**) Representative photomicrographs of histopathological changes from syngeneic (BALB/c→BALB/c), control, and MR16-1-treated groups. Sections were stained with hematoxylin and eosin (H&E) or Masson's trichome. Arrows indicate the dermal thickness. Bar = 100 μm. Histopathological scores were analyzed at the end time point from (**b**) preventive and (**c**) therapeutic models. (**d**, **e**) Skin fibrosis was compared by determining dermal thickness, (**f**) soluble collagen, and trichrome area/total area. (**g**) Lung and liver fibrosis was estimated by the ratio of trichrome area/total area. Each value indicates mean (±SEM) results from four to five mice. *$P<0.05$, **$P<0.01$.

Attenuation of cGVHD by blockade of IL-6 is correlated with increased Treg numbers

As IL-6 inhibits the differentiation of Tregs that negatively regulate some autoimmune diseases including GVHD (Scheller and Rose-John, 2006; Serada *et al.*, 2008), we analyzed the number of Tregs ($CD4^+CD25^+Foxp3^+$ T cells) in preventive model. In both early-stage (14 days after BMT) and late-stage (42 days after BMT) disease, an increase in Treg numbers was found in the MR16-1-treated group compared with the control group ($P=0.03$, Figure 4a and b). In our therapeutic model, Treg numbers were no different between control and MR16-1 groups at day 57 (Figure 4c).

Regulatory B cells (IL-10-producing B cells) are able to influence the progression of some inflammatory diseases such as contact hypersensitivity, rheumatoid arthritis, and encephalomyelitis (Watanabe *et al.*, 2007; Lampropoulou *et al.*, 2008; Matsushita *et al.*, 2008; Fujimoto, 2010). Splenic regulatory B cells have been recently identified within a unique $CD1d^{hi}CD5^+$ B-cell subset (Yanaba *et al.*, 2008). To determine whether B cells and $CD1d^{hi}CD5^+$ B cells change after IL-6 blockade, we analyzed B-cell and $CD1d^{hi}CD5^+$ B-cell numbers in MR16-1-treated and control mAb-treated mice. MR16-1 treatment had no effects on total B-cell and $CD1d^{hi}CD5^+CD19^+$ B-cell numbers in either our preventive model or in our therapeutic model (Figure 4e–g). Splenic IL-10-producing B cells were also analyzed, but there was no significant difference in the frequency of IL-10-producing B cells between MR16-1-treated and control groups (Figure 5a and b).

TNF-α has a role in all phases of GVHD pathophysiology to mediate both direct tissue damage and indirect tissue damage caused by cytotoxic lymphocytes (Levine, 2011). To investigate whether TNF-α, IFN-γ, IL-6, or IL-17 cytokine production by $CD4^+$ T cells changed after treatment with MR16-1 in Scl-cGVHD, splenic single-cell suspensions from mice were stimulated for 4 hours. Splenic $CD4^+$ T cells in

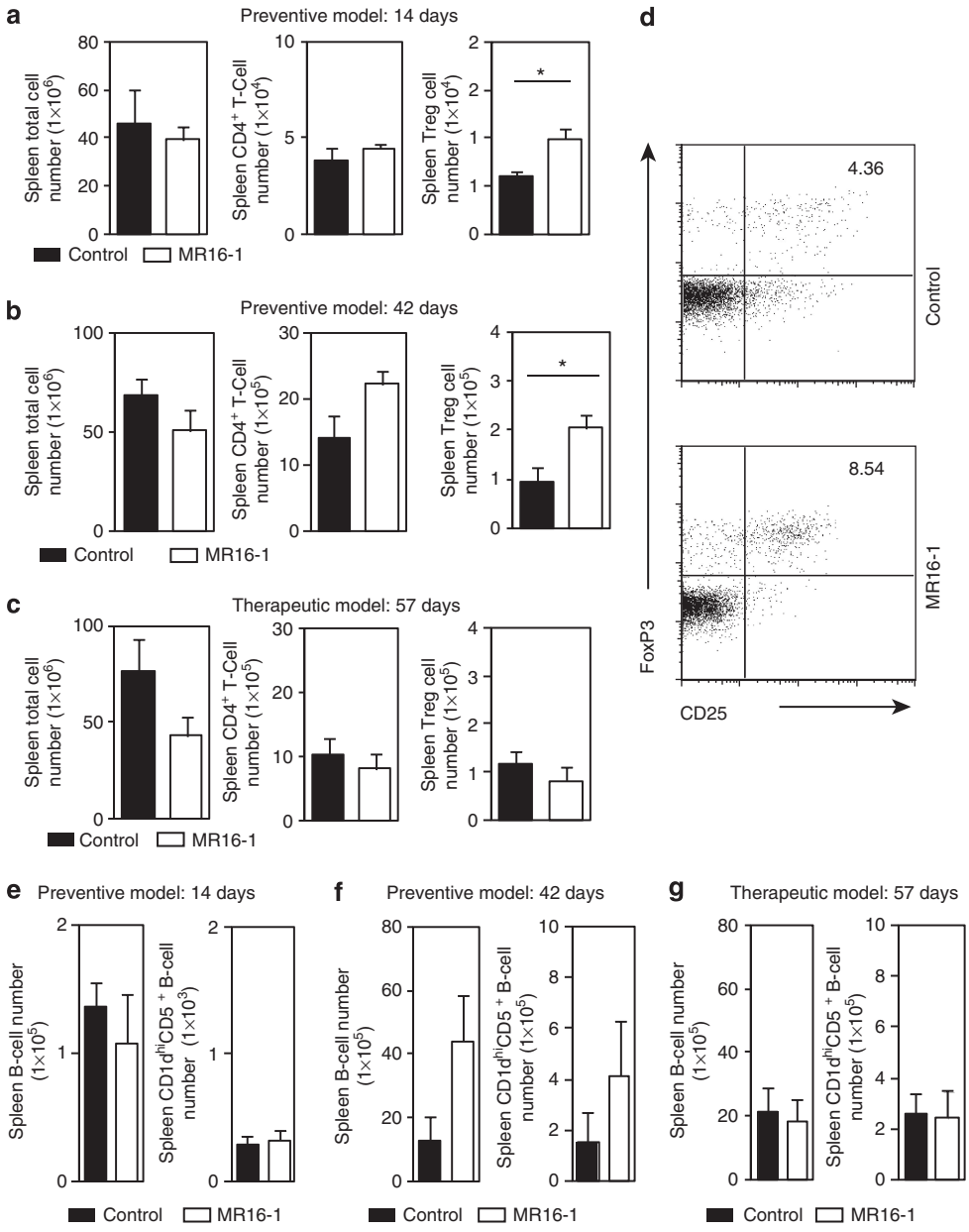

Figure 4. Elevated regulatory T cell (Treg) numbers in MR16-1-treated animals in the preventive model. Tregs in each experimental group. Total splenic cellularity, absolute number of splenic $CD4^+$ T cells, and Tregs are shown (**a**) 14 days and (**b**) 42 days following bone marrow transplantation (BMT) in the preventive model, and (**c**) 57 days after BMT in the therapeutic model. $CD1d^{hi}CD5^+CD19^+$ B cells in each experimental group. Absolute number of splenic $CD19^+$ B cells and $CD1d^{hi}CD5^+CD19^+$ B cells (**e**) 14 days and (**f**) 42 days after BMT in the preventive model, and (**g**) 57 days after BMT in the therapeutic model. Each value indicates mean (±SEM) results from four mice. $^*P<0.05$.

MR16-1-treated animals had significantly reduced TNF-α production compared with animals in the control group ($P=0.04$), whereas we observed no statistical differences in IFN-γ, IL-6, or IL-17 levels in the two groups (Figure 5d).

To determine whether skin cytokine/chemokine changes after MR16-1 treatment in a preventive model, skin mRNA expressions were assessed by real-time reverse transcriptase PCR (RT–PCR). At 42 days after BMT, the mRNA expressions of IL-2, IL-10, and IL-17A were similar in both groups. By contrast, the expressions of IFN-γ, TNF-α, IL-6, IL-18, TGF-β1, CCL2, CCL3, and CCL5 were significantly lower in the MR16-1-treated group than in the control group (Figure 5e).

Anti-CD25 mAb treatment inhibited MR16-1's attenuation in Scl-cGVHD

To determine how depleting MR16-1-induced increase of Tregs influences the Scl-cGVHD severity, Tregs were depleted by intraperitoneal injection of anti-mouse CD25 mAb at days 0, 7, 21, and 35 in the MR16-1-treated group. The number of splenic $CD4^+$ T cells and $CD4^+FoxP3^+$ T cells in the MR16-1+anti-CD25-treated group were

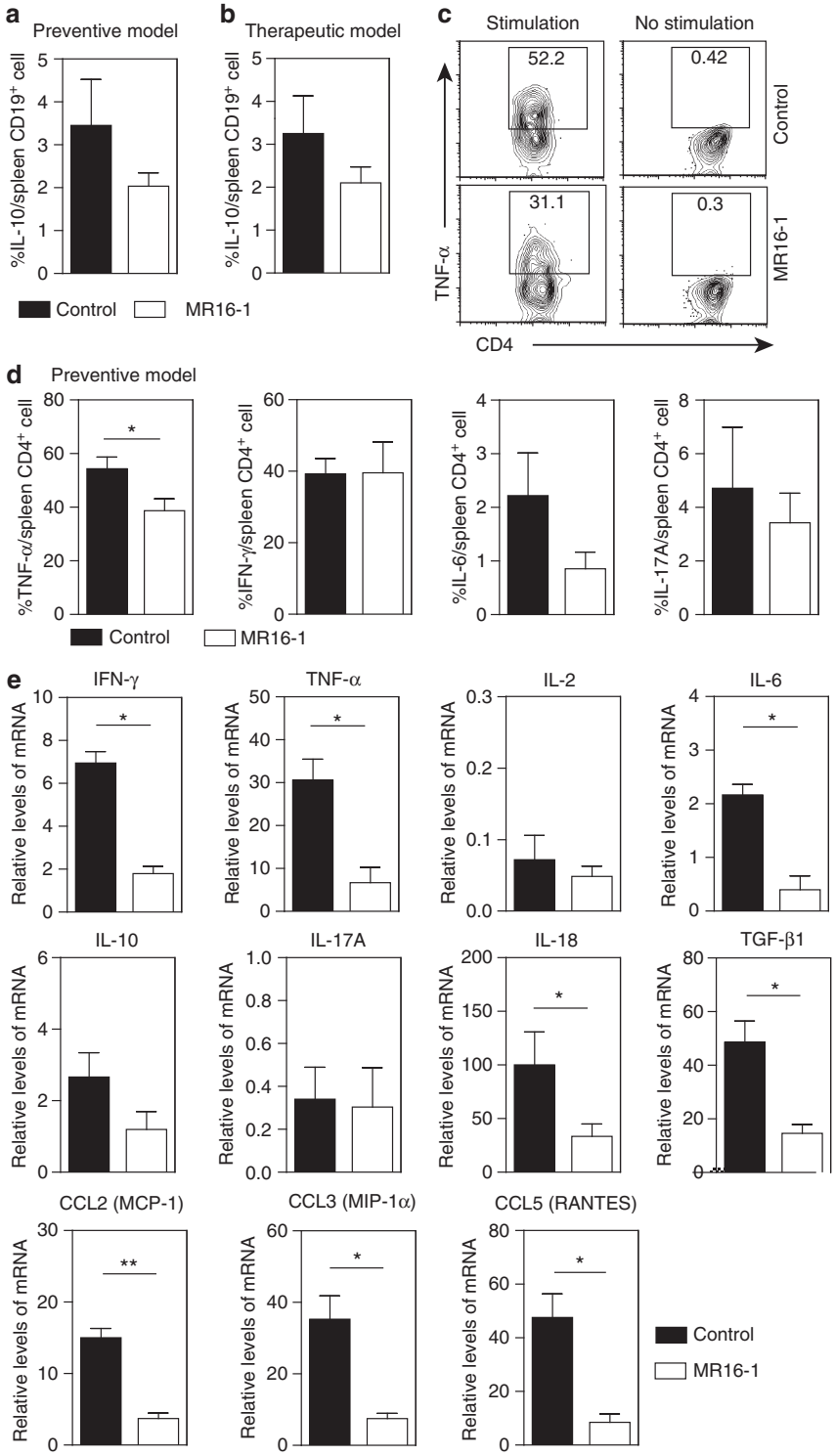

Figure 5. A decrease in tumor necrosis factor-α (TNF-α)-producing CD4$^+$ T cells and low levels of TNF-α, IFN-γ, IL-6, IL-18, TGF-β1, CCL2, CCL3, and CCL5 mRNA in the preventive model. Splenic single-cell suspensions were stimulated as described in Materials and Methods. Percentages of IL-10-producing CD19$^+$ B cells from (**a**) preventive and (**b**) therapeutic models. (**c**) From the CD4$^+$ T-cell gate, results represent one mouse from the MR16-1-treated group or control group, indicating the percentages of TNF-α-producing CD4$^+$ T cells with/without stimulation. (**d**) Cytokine production by CD4$^+$ T cells in the preventive model. (**e**) Expression of cytokine/chemokines was measured by real-time quantitative reverse transcriptase (RT–PCR) analysis. Each value indicates mean (±SEM) results from four to five mice. *$P<0.05$, **$P<0.01$.

significantly lower than that in the MR16-1-treated group ($0.46 \pm 0.15 \times 10^6$ and $0.49 \pm 0.19 \times 10^5$ vs. $1.4 \pm 0.25 \times 10^6$ and $2.27 \pm 0.38 \times 10^5$, $P = 0.016$ and $P = 0.006$), and similar to the control group (Figure 6d and e). Tregs ($CD4^+CD25^+FoxP3^+$) were mostly depleted by anti-CD25 mAb in the MR16-1 + anti-CD25 group. Thus, MR16-1 + anti-CD25-treated group had an equivalent body weight change and skin score to the control group and more body weight loss and less skin score than the MR16-1-treated group (Figure 6a and b).

Both MR16-1 + anti-CD25- and MR16-1-treated groups have similar histopathological scores and dermal thickness (6.86 ± 0.63 vs. 7 ± 0.58, $P = 0.7816$), which were higher than those of the MR16-1 group (4.8 ± 0.58). Skin trichrome area ratios and soluble collagen levels also were significantly higher in the MR16-1 + anti-CD25 and control group than in the MR16-1 group (Figure 6c).

Therefore, anti-CD25 mAb treatment elicits that elevation of Tregs is sufficient to demonstrate the effect of anti-IL6R mAb on Scl-cGVHD.

DISCUSSION

In Scl-cGVHD, tissue damage results in the upregulation of inflammatory mediators and chemoattractants in target tissues, and in adhesion molecules in monocytes and donor-derived T cells (Chu and Gress, 2008). Here, we show that MCP-1 increased 7 days post transplantation (Figure 1a), which could promote infiltration of donor-derived mononuclear cells, such as monocytes, activated macrophages, and T cells into target tissues. Coincident with mononuclear cell infiltration, local host tissue damage leads to production of proinflammatory cytokines, such as TNF-α (Figure 1a), that augment the interaction between APCs, such as dendritic cells and donor T cells (Levine, 2011).

Fibrotic changes of the skin and other organs including the gastrointestinal tract, lung, and liver are the phenotypic hallmarks in Scl-cGVHD, which are detectable ~21 days post transplantation (Claman *et al.*, 1985; McCormick *et al.*, 1999). In contrast to autoantibody production observed in the systemic lupus erythematosus cGVHD model, induction of TGF-β1 and Th1 and Th2 cytokines is a specific characteristic of Scl-cGVHD (Zhang *et al.*, 2002; Zhou *et al.*, 2007; Chu and Gress, 2008). IFN-γ is a prominent cytokine in the early phase of murine Scl-cGVHD, confirmed by semiquantitative RT-PCR analysis of total skin RNA on day 21 (Zhang *et al.*, 2002). In Scl-cGVHD cutaneous tissue, there is a mixed Th1/Th2-like cytokine profile in early fibrosis with a Th1-like predominance early and Th2-like profile later (Zhou *et al.*, 2007). In this study, we showed that serum Th1 cytokines such as TNF-α and INF-γ were present at high levels on day 28 post transplantation (Figure 1a).

Previous studies demonstrated an increase of serum and target tissue IL-6 concentrations 7 days after allogeneic BMT (Zhou *et al.*, 2007; Chen *et al.*, 2009; Tawara *et al.*, 2011). Similarly, we found a significant increase of serum IL-6 levels (10-fold higher) 7 days post transplantation using anti-IL-6R mAb (Figure 1b). Notably, serum IL-6 levels correlate with cGVHD severity. Blockade of IL-6 signaling with anti-IL-6R mAb demonstrated salutary effects in mouse models of several inflammatory diseases (Serada *et al.*, 2008). Although serum IL-6 levels at later stages of Scl-cGVHD were increased compared with those detected at early stages, our current study indicates that blockade of IL-6 signaling reduces the severity of Scl-cGVHD only if anti-IL-6R mAb is administered during early stages of the disease (Figures 2 and 3). Using blockade of IL-6 after Scl-cGVHD was already established did not result in any changes of body weight or skin and histopathological scores compared with administration of control rat IgG. Moreover, we also demonstrated that IL-6 has a role in disease pathogenesis only in early stages of cGVHD, whereas IL-6 is present as a result of the inflammatory process and fibrosis in later stages of the disease.

In the current study, the reduction of GVHD severity when blocking IL-6 signaling early in the course of disease was associated with increased numbers of Tregs (Figure 4a and b). Prior studies support the idea that reconstitution of Tregs, which have an important role in regulating T cell–mediated transplantation tolerance, is severely impaired in both acute and chronic GVHD (Zorn *et al.*, 2005; Rieger, 2006; Chen *et al.*, 2007; Zhu *et al.*, 2010). Blocking IL-6 binding to IL-6R promotes an increase in the number of donor Tregs as a result of peripheral Treg conversion (Chen *et al.*, 2009). In our study, an increase of Tregs appeared early after blockade of IL-6 in Scl-cGVHD (Figure 4a). Recent studies have shown that whereas IL-6 suppresses TGF-β1-induced Treg generation IL-6 and TGF-β1 promote the generation of Th17 cells from naive T cells (Bettelli *et al.*, 2006; Veldhoen *et al.*, 2006; Kimura *et al.*, 2007). Interestingly, we found that IL-17-producing $CD4^+$ T cells and skin mRNA levels of IL-17A were not different between MR16-1-treated and control groups (Figure 5d and e). Furthermore, in our preventive model, these results suggest that the increase in Treg numbers results from early elevated TGF-β1 and blockade of IL-6 signaling. The effect of MR16-1 on Scl-cGVHD appeared mostly inhibited by anti CD25 mAb (Figure 6). Thus, Tregs that were reported to increase after using MR16-1 may have an important role in attentuating the severity and fibrosis in Scl-cGVHD.

Our current study demonstrates that IL-6 has an important role in the pathogenesis of Scl-cGVHD. Therefore, blockade of IL-6 may be an effective method for preventing Scl-cGVHD, and may prove efficacious in the treatment of cGVHD and scleroderma at early stages of disease in humans.

MATERIALS AND METHODS

Bone marrow transplantation

Eight- to twelve-week-old male B10.D2 ($H\text{-}2^d$) and female BALB/c ($H\text{-}2^d$) mice were used as donors and recipients, respectively. Bone marrow was T-cell depleted with anti-Thy1.2 microbeads (Miltenyi Biotech, Auburn, CA). BALB/c recipients were irradiated with 800 cGy by an MBR-1520R machine (Hitachi, Tokyo, Japan). The recipients were injected via the tail vein with 10×10^6 T-cell-depleted bone marrow and 10×10^6 splenocytes in 0.5 ml of phosphate-buffered saline. The Committee on Animal Experimentation of Kanazawa University Graduate School of Medical Science approved all studies and procedures.

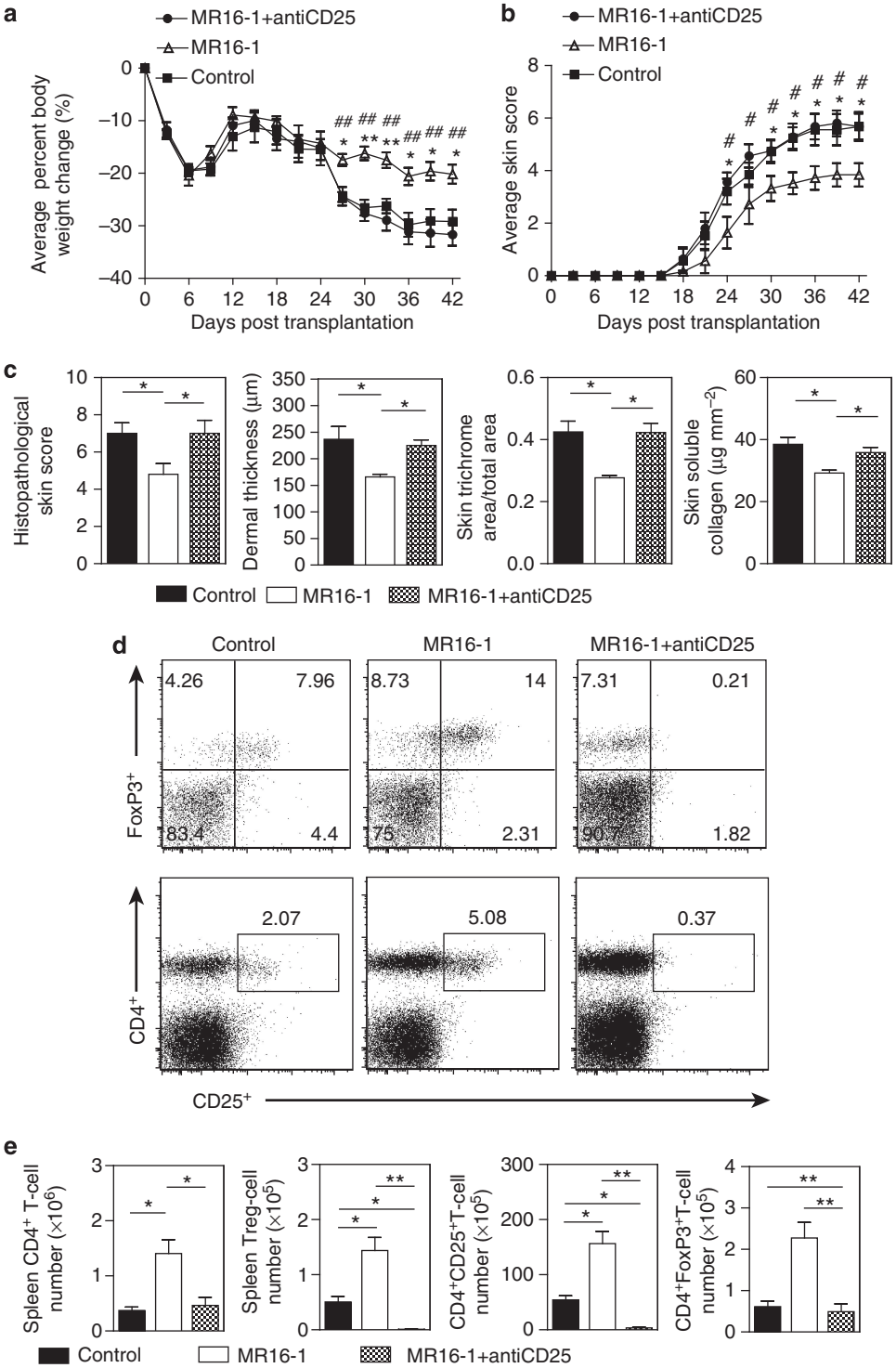

Figure 6. Anti-CD25 mAb treatment inhibited MR16-1's attenuation in sclerodermatous chronic graft-versus-host disease (Scl-cGVHD). According to the preventive model described in Materials and Methods, MR16-1-treated recipients were injected intraperitoneally with 250 μg per mouse of anti-CD25 mAb at days 0, 7, 21, and 35. (**a**) Body mass changes and (**b**) skin scores were assessed every 3 days. (**c**) Histopathological skin score, dermal thickness, skin trichrome area/total area, and skin soluble collagen were analyzed 42 days post bone marrow transplantation (BMT). (**d**) Representative of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) and $CD4^+CD25^+$ T cells in each experimental group. (**e**) Absolute number of splenic $CD4^+$ T cells, ($CD4^+CD25^+FoxP3^+$) Tregs, $CD4^+CD25^+$ T cells, and $CD4^+FoxP3^+$ T cells are shown. Each value indicates mean (± SEM) results from four to six mice. $^{*,\#}P<0.05$, $^{**,\#\#}P<0.01$.

CBA and ELISA

Serum samples were obtained at 7, 14, 21, 28, and 35 days after BMT. Serum IL-6, IL-10, MCP-1, IFN-γ, TNF-α, IL-12p70, and plasma TGF-β1 levels were measured by either the CBA Mouse Inflammation Kit (BD Biosciences, San Jose, CA) or by the ELISA mouse TGF-β1 kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

Flow cytometry

The following mAbs were used: FITC-, PE-, PE-Cy5-, PE-Cy7-, or PerCP-Cy5.5-conjugated mAbs to mouse CD4 (RM4-5), CD19 (1D3), CD25 (MF-14), CD5 (53-7.3), CD1d (1B1) (BD Biosciences).

Splenic single-cell suspensions were stained for 20 minutes for two-, three-, or four-color immunofluorescence analysis at 4 °C using mAbs at predetermined optimal concentrations. Intracellular staining for FoxP3 (FJK-16 s, eBioscience, San Jose, CA) was performed using the Cytofix/Cytoperm kit (BD Biosciences). Stained samples were analyzed on a FACSVantage SE (BD Biosciences). Data were analyzed using the FlowJo (Tree Star, Ashland, OR) software.

Reagents

MR16-1, a rat IgG1 mAb against the murine IL-6R, was provided by Chugai Pharmaceutical (Tokyo, Japan). LEAF anti-mouse CD25 mAb (clone PC61) was obtained from BioLegend (San Diego, CA). Control rat IgG, supplied by MP Biomedicals, LLC (Solon, OH), was used as a control and was administered at the same dose and times as MR16-1 and/or LEAF anti-mouse CD25 mAb. MR16-1 and control rat IgG1 were resuspended in phosphate-buffered saline before injection.

Chronic GVHD

In the preventive model, recipients were injected intraperitoneally with a loading dose of 2 mg per mouse of MR16-1 or control rat IgG on the day of BMT, and then injected weekly thereafter at a dose of 0.5 mg per mouse for 6 weeks. In the therapeutic model, recipients were injected intraperitoneally with a loading dose of 2 mg per mouse of MR16-1 or with control rat IgG on day 25 after BMT. Mice were then injected weekly with 0.5 mg antibody per mouse for 5 weeks. For depletion of Tregs, MR16-1-treated recipient mice were injected intraperitoneally with 250 μg per mouse of anti-mouse CD25 mAb at days 0, 7, 21, and 35.

Intracellular cytokine staining

Briefly, purified cell resuspensions (2×10^6 cells per ml) were stimulated for 5 hours at 37 °C with lipopolysaccharide (10 mg ml^{-1}, Sigma-Aldrich, St Louis, MO), phorbol 12-myristate 13-acetate (50 ng ml^{-1}; Sigma-Aldrich), ionomycin (500 ng ml^{-1}; Sigma-Aldrich), and monensin (2 mM; eBioscience) for the detection of IL-10 production by B cells, and stimulated for 4 hours at 37 °C with phorbol 12-myristate 13-acetate (50 ng ml^{-1}), ionomycin (1 μg ml^{-1}), and brefeldin A (3 μM; BioLegend) for the detection of cytokine production by T cells. After cell-surface staining with anti-CD19 mAb or anti-CD4 mAb, the cells were washed, fixed, and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences); cells were then stained with propidium iodide–conjugated anti-IL-10 (JES5-16E3) mAb from BioLegend or propidium iodide–conjugated anti-TNF-α (MP6-XT22), IFN-γ (XMG1.2), IL-6 (MP5-20F3), and IL-17A (TC11-18H10) mAbs from eBiosciences.

Reverse transcriptase PCR

Total RNA was isolated from frozen skin specimens using RNeasy spin columns (Qiagen, Hilden, Germany) and digested with DNase I (Qiagen) to remove chromosomal DNA. Total RNA was reverse-transcribed to a complementary DNA using a reverse transcription system with random hexamers (Promega, Southampton, UK). Cytokine mRNAs were analyzed using real-time RT–PCR quantification (Applied Biosystems, Foster City, CA). Sequence-specific primers and probes were designed with predeveloped TaqMan assay reagents (Applied Biosystems). Real-time RT–PCR was performed on an ABI Prism 7000 sequence detector (Applied Biosystems). GAPDH was used to normalize the mRNA. The relative expression of real-time RT–PCR products was determined according to the $\Delta\Delta C_t$ method to compare target gene and GAPDH mRNA expression.

GVHD skin score

Clinical cGVHD score was modified as previously described (Anderson, 2003). The following scoring system was used: healthy appearance = 0; skin lesions with alopecia equal or less than 1 cm^2 in area = 1; skin lesions with alopecia 1 to 2 cm^2 in area = 2; skin lesions with alopecia 2–5 cm^2 in area = 3; skin lesions with alopecia 5–10 cm^2 in area = 4; skin lesions with alopecia 10–15 cm^2 in area = 5; skin lesions with alopecia 15–20 cm^2 in area = 6; and skin lesions with alopecia more than 20 cm^2 in area = 7. In addition, animals were assigned 0.4 points for skin disease (lesions or scaling) on tail, and 0.3 points each for lesions on ears and paws. The minimum score was 0, and the maximum score was 8. Final scores for dead animals were kept in the data set for the remaining time points of the experiment.

Histological analysis

Skin samples taken from the interscapular region, lung, and liver were fixed in 10% formalin and embedded in paraffin. Sections (6 μm in thickness) were stained with hematoxylin and eosin and Masson's trichrome for the detection of collagen deposition. Slides of skin were scored by a dermatopathologist (blinded to experimental groups) on the basis of epidermal interface changes, dermal collagen thickness, mononuclear cell inflammation, subdermal fat loss, and follicular dropout with scores from 0 to 2 for each category. The minimum score was 0, and the maximum score was 10 (Anderson, 2003). Dermal thickness was defined as the thickness of skin from the top of the granular layer to the junction between the dermis and intradermal fat (Tanaka *et al.*, 2010). Collagen deposition was quantified on trichrome-stained section as the ratio of blue-stained area to total stained area of total staining using the Adobe Photoshop CS4 analysis tool.

Sircol soluble collagen assay

Total soluble collagen was quantified using the Sircol soluble collagen assay (Biocolor, Newtownabbey, UK). Briefly, 6-mm skin samples were homogenized in acid-pepsin solution (0.5 M acetic acid containing 1 mg ml^{-1} pepsin) over 2 nights at 4 °C. After centrifugation, 1 ml of Sircol dye was added to 100 μl of supernatant and incubated for 30 minutes. After removing the suspension, droplets were dissolved in 1 ml Sircol alkali reagent and relative absorbance was measured at 555 nm.

Statistics

All data are shown as mean ± SEM. The significance of differences between sample means was determined with the Student's *t*-test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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