Effects of Pravastatin on Murine Chronic Graft-Versus-Host Disease

Hyoung-Kyu Yoon,[1] Ji-Young Lim,[1] Tae-Jung Kim,[2] Chul-Soo Cho,[1] and Chang-Ki Min[1,3]

Background. Chronic graft-versus-host disease (CGVHD) is a serious and increasingly common complication after allogeneic (allo) hematopoietic stem-cell transplantation, but currently available therapies have demonstrated limited efficacy. Furthermore, the statins have been reported to be effective in various immune-mediated disease models, but their therapeutic potentials versus CGVHD have not been determined.

Methods. We used a B10.D2→BALB/c model of CGVHD, which differs at minor histocompatibility loci, to address the therapeutic effect of statins on the development of CGVHD. Pravastatin (PST, 30 mg/kg/day) was intraperitoneally injected for 5 days per week from the day of transplantation until 4 weeks after allo hematopoietic stem-cell transplantation.

Results. The onset of clinical cutaneous GVHD was significantly slower in PST-treated recipients than in allo-controls (36 days vs. 25 days, respectively, $P<0.05$), and pathologic changes in skin disease confirmed this clinical result. Animals injected with PST showed less submucosal fibrosis in lungs than allo-controls. In addition, collagen deposition in skin and lungs was markedly attenuated by PST treatment. PST also significantly reduced protein concentrations and numbers of inflammatory and epithelial cells in bronchoalveolar lavage fluid. Significantly lower numbers of donor $CD11b^+$ and $CD4^+$, but not $CD8^+$ cells, were observed in skin and bronchoalveolar lavage fluid after PST treatment. The protein concentrations of monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal T cell expressed and secreted (RANTES) in skin and lungs were substantially reduced in PST-treated animals when compared with allo-controls.

Conclusions. This study suggests that the CGVHD-protecting effect of PST involves the down-regulation of chemokines and the reduction of collagen synthesis.

Keywords: Chronic GVHD, Scleroderma, Pravastatin, Chemokines.

Allogeneic hematopoietic stem-cell transplantation (allo-HSCT) is an effective treatment for leukemia and genetic disorders. However, graft-versus-host disease (GVHD), opportunistic infections, and the relapse of underlying disease are major obstacles. Chronic GVHD (CGVHD) is an increasingly common complication of allo-HSCT, and its incidence has been reported to be as high as 80% in some series. Furthermore, its incidence is believed to be increasing, at least in part, because of the use of peripheral blood as a stem-cell source, nonmyeloablative conditioning, withdrawal of immunosuppression to induce antitumor activity, longer survival because of better supportive care, and increases in the numbers of patients receiving donor leukocyte infusions (*1, 2*). The treatment of CGVHD remains a formidable challenge. Even with a wide range of therapeutic options, CGVHD and infectious complications that arise during its management are major causes of late mortality among allo-HSCT recipients (*3*). Furthermore, the model systems developed to examine CGVHD are limited. Of these, the B10.D2 (H-2^d)→BALB/c (H-2^d), major histocompatibility complex-matched and minor histocompatibility antigen–mismatched model of GVHD shows characteristics resembling human CGVHD, such as a relatively late onset, skin fibrosis, alopecia, and lung fibrosis with increased collagen deposition (*4–6*).

The statins (3-hydroxy-3-methyl coenzyme reductase inhibitors) are a class of cholesterol-lowering drugs that reduce mortality resulting from cardiovascular disease and stroke, but they also have strong immunomodulatory effects on antigen-presenting cells (APCs) and T cells and interfere with the synthesis of L-mevalonate and its downstream isoprenoid metabolites (*7*). It has been demonstrated that some statins inhibit the productions of proinflammatory cytokines

and that they are beneficial in patients with chronic inflammatory disorders (*8, 9*). Recently, several studies on GVHD and statins have been performed using murine models of acute GVHD (AGVHD) with donor and recipient strains fully mismatched at major histocompatibility complex and minor histocompatibility loci (*10*). However, much remains unknown about the pathophysiology of CGVHD, and although AGVHD and CGVHD are similar in some respects, it seems likely that the two diseases have different initiation requirements and pathogenic mechanisms. More importantly from the experimental view, it is not obvious whether findings based on the AGVHD models apply to CGVHD syndrome (*11*). Moreover, few experimental studies have been conducted using CGVHD and statins, and thus, their molecular modes of action against the alloimmune reactions underlying CGVHD have not been clarified fully. In this study, we used a murine CGVHD model, which shares critical pathologic features with human CGVHD, to investigate the effects of pravastatin (PST) on the occurrence of CGVHD.

MATERIALS AND METHODS

Mice and Experimental Allo-HSCT

Female B10.D2 (H-2^d) and BALB/c (H-2^d) mice (8- to 12-week old) were purchased from Japan Shizuoka Institute for Laboratory Animals (Japan SLC, Shizuoka, Japan). B10.D2 and BALB/c mice were used as donors and recipients, respectively, for allo-HSCT to produce CGVHD (*5*). Briefly, recipient mice were lethally irradiated with 700 cGy using a Gammacel ^{137}Cs source. Approximately 6 hr later, they were injected intravenously through a tail vein with donor spleen (1.5×10^6/mouse) and bone marrow (BM) cells (3×10^6/mouse) suspended in Roswell Park Memorial Institute 1640 medium (WelGENE Inc., Daegu, South Korea) containing 10 U/mL heparin (Fisher Scientific, Pittsburgh, PA; referred to as the allo-control). PST was administered as described later after allo-HSCT (referred to as the allo-PST). In addition, radiated BALB/c recipient mice also received BALB/c spleen and BM cells (syngeneic [syn] HSCT, referred to as the syn-control). The dose of donor cells used in these experiments was determined using literature values (*5*) and by performing pilot experiments (data not shown). Degrees of clinical GVHD were assessed every 3 days based on the occurrences of skin lesions, such as alopecia and dermatitis.

PST Preparation and Treatment

PST (Merck, Darmstadt, Germany) was prepared as a 4 mg/mL stock solution. Briefly, 4 mg of PST was dissolved in 100 μL of saline, and the total volume of this solution was adjusted to 1 mL. PST (30 mg/kg/100 μL) was administered intraperitoneally (IP) for 5 consecutive days per week (from day 1 to 28). These injection conditions were chosen based on the findings of a pilot study (data not shown).

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed in situ four times with Hanks' balanced salt solution (35 mL/kg; pH 7.2–7.4, WelGENE Inc.), and total protein was determined in supernatant using the Bradford method (*12*), as recommended by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Pooled cell pellets were combined and resuspended in 1 mL of Hanks' balanced salt solution, and numbers of BAL cells was counted using a hemocytometer. Aliquots (100 μL) of cell suspensions were cytocentrifuged, and cytospin slides were stained with Diff-Quick for differential cell counting.

Preparation of Skin Cell Suspension

Small pieces of depilated skin were digested in Roswell Park Memorial Institute medium-10% fetal bovine serum (WelGENE Inc) containing 0.27% collagenase (Sigma-Aldrich, St. Louis, MO), 0.01% DNase (Sigma), and 1000 U/mL hyaluronidase (Worthington Biochemical Corp, Lakewood,

NJ) at 37ºC for 2 hr. The digested skin was filtered through 70-μm cell strainer Falcon (Bectin Dickenson Labware, Franklin, Lakes, NJ) to generate single-cell suspensions.

Histologic Analysis and Immunostaining

Formalin-fixed, paraffin-embedded tissue sections were hematoxylineosin (H&E) stained. Frozen lung and skin sections were immediately embedded in OCT embedding medium (Sakura, Ted Pella, Redding, PA) and cryosectioned for immunostaining. For collagen I staining, cryosections were first incubated with rabbit anti-mouse collagen I at a dilution of 1:200 (Chemicon International, Temecula, CA) and then with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA). After extensive washing, samples were counterstained with 4,6-diamidino-2-phenylindole (Sigma) and embedded in Vectashield (Vector Laboratories, Burlingame, CA). Degrees of CGVHD in skin were evaluated by measuring dermal thicknesses at the dermo-epidermal and dermis-subcutaneous fat junctions under a microscope. The presence of CGVHD in lungs was evaluated by assessing chronic airway rejection and obliterative bronchiolitis, as determined by Stewart et al. (*13*).

Flow Cytometry

To determine cell surface phenotypes, cells were stained with phycoerythrin-conjugated monoclonal antibodies (MoAbs) to CD4, CD8, and CD11b. All MoAbs were purchased from BD Pharmingen (San Diego, CA). Samples collected in heparin (50 μL) were incubated on ice for 30 min with mouse anti-mouse MoAbs and washed with phosphate-buffered saline (PBS; WelGENE Inc.) containing 0.05% sodium azide and fixed with PBS/1% paraformaldehyde. All samples were analyzed using a FACScan cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Protein Extraction and Measurements of Chemokines by ELISA and Western Blotting

Tissue samples were homogenized in 2 mL buffer solution ($1 \times$ PBS, 1% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate, and 1 tablet of Complete Protease Inhibitor Cocktail [Roche Diagnostics, Basel, Switzerland]), centrifuged at 3000 rpm for 20 min, and supernatants were harvested. Total protein concentrations in supernatant were determined using Bio-Rad Protein Assays (Bio-Rad Laboratories). Concentrations of regulated on activation normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1) (R&D Systems, Minneapolis, MN) were measured in supernatants of homogenized tissues. Assays were performed according to the manufacturer's protocol. Assay sensitivities were less than 2.0 pg/mL for RANTES and less than 9 pg/mL for MCP-1. ELISA plates were read using a microplate reader (Bio-Rad Laboratories). Protein sample from cell lysates, 40 μg, was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membranes and immunoblotted. MCP-1 antibody was purchased from Cell Signaling Technology (Beverly, MA), RANTES antibody from Abcam Inc. (Cambridge, MA), and donkey anti-rabbit IgG antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical Analysis

All values are expressed as means±SEMs. Statistical comparisons between groups were performed using the parametric independent sample *t* test if there were more than or equal to five animals per group or using the Mann-Whitney *U* test if there were less than five animals per group. Wilcoxon's rank test was used to analyze survival data. *P* values less than 0.05 were considered significant.

RESULTS

PST Attenuated Skin and Pulmonary Pathologic Lesions

Because there is growing evidence that statins have immunomodulatory activities, we asked whether PST could

prevent the development of CGVHD of lung and skin. PST (30 mg/kg) was administered IP for 5 consecutive days per week (from day 1 to 28) after transplantation. Lethally irradiated BALB/c mice were transplanted by tail vein injection with BM and spleen cells from B10.D2 mice and developed skin lesions with alopecia or scaling on ears, tails, and paws. Control BALB/c animals receiving syn transplants did not develop skin lesions. In vivo treatment with PST suppressed the incidence and severity of clinical skin CGVHD when compared with allo-controls injected with diluent. The onset of clinical cutaneous GVHD was significantly slower and less severe in the animals treated with PST than in the allo-controls (Fig. 1A, 36 days vs. 25 days, 80% vs. 50%, respectively, $P<0.05$). After day 60, all allo-recipients clinically bounced back from CGVHD regardless of PST treatment. Consistent with clinical results, histologic examinations revealed thick-

ening of the epithelial layer, loss of hair follicles and of sub-dermal fat, ulcers in epithelial and dermal layers, and heavy collagen deposition in the skin lesions of allo-controls. As shown in Figure 1(B), thickening of dermis in allo-controls was markedly increased compared with syn-controls. In stark contrast, allo-PST mice had much less severe lesions according to the pathologic categories mentioned earlier. All allo-controls exhibited the pathologic changes regardless of the presence of clinical cutaneous lesions. Findings of routine histopathologic sections showed that there was 30% increase in the dermal thickness in allo-controls compared with syn-controls. PST treatment significantly reduced dermal thickening compared with allo-controls at day 14; the increase of dermal thickness was just 18% compared with syn-controls (Fig. 1C). Dermal thickenings at 28 and 56 days in the allo-
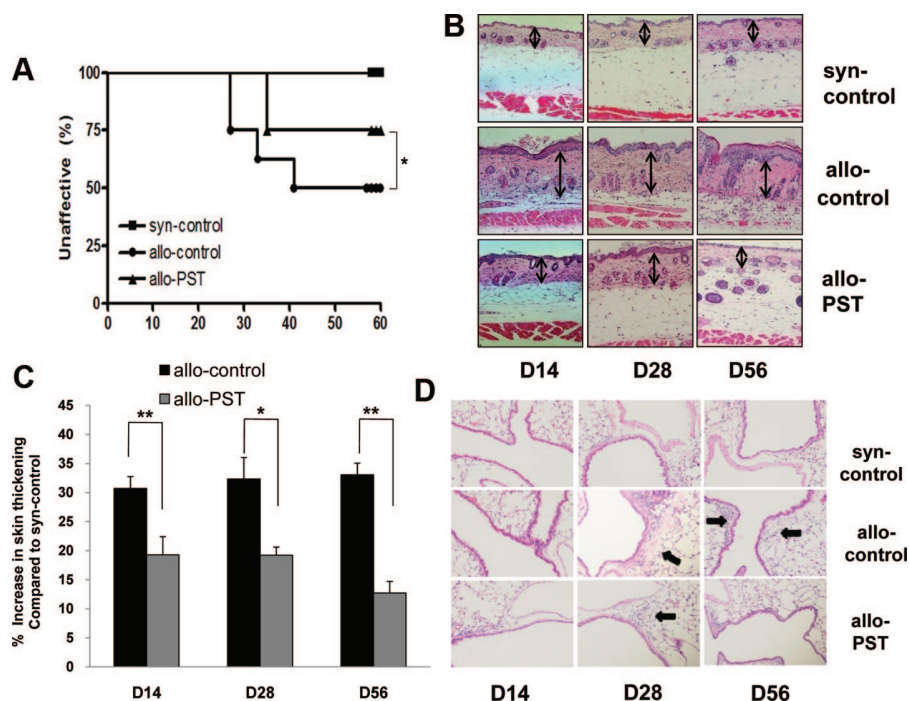

FIGURE 1. Pravastatin (PST) treatment ameliorated the severity of cutaneous and pulmonary chronic graft-versus-host disease (CGVHD). After being administered a lethal dose of radiation (700 cGy), BALB/c mice were transplanted with B10.D2 donor spleen (1.5×10^{6}/mouse) and bone marrow cells (3×10^{6}/mouse) as described in *Materials and Methods*. These mice were then administered (intraperitoneally) phosphate-buffered saline (the allogeneic [allo]-control, n=10) or PST (30 mg/kg, allo-PST, n=10) for 5 consecutive days per week (from day 1–28) post-hematopoietic stem-cell transplantation (HSCT). A control group of BALB/c recipient mice received BALB/c spleen and bone marrow cells (syngeneic [syn]-control, n=6). (A) The occurrence of skin CGVHD was monitored for 60 days after transplantation. Data obtained from two experiments were combined. *P less than 0.05 between the allo-control and allo-PST groups. (B) Representative histology of skin at days 14, 28, and 56 post-HSCT. Mice with CGVHD had thick skins with influx of infiltrates of mononuclear cells from day 14 post-HSCT. Syn-controls (BALB/c←BALB/c) showed no change in skin thickness or inflammation. PST markedly reduced skin thickenings and inflammatory cell infiltrations caused by CGVHD. (C) Pathology scores of skin for the representative mice. Bars represent means±SEMs of percentage increases in dermal thicknesses of the allo-PST (n=5) and allo-control groups (n=5), respectively, compared with those of the syn-control group (n=3). The paired *t* test revealed a significant difference between the allo-control and the allo-PST groups at days 14, 28, and 56. *P less than 0.05; **P less than 0.01. Dermal thicknesses were determined using hematoxylin-eosin (H&E)-stained tissue sections by image analysis, as described in *Materials and Methods*. (D) Histopathology of lungs in the syn-control, the allo-control, and the allo-PST groups was examined on days 14, 28, and 56 post-HSCT. H&E staining; magnification, ×400. Allo-controls had more severe sub-mucosal fibrosis than syn-controls. PST treatment markedly reduced fibrosis on day 56. However, fibrosis was observed in the allo-PST group on day 28, although it was less than that observed in the allo-controls. Syn-control mice did not develop submucosal fibrosis as determined by histology.
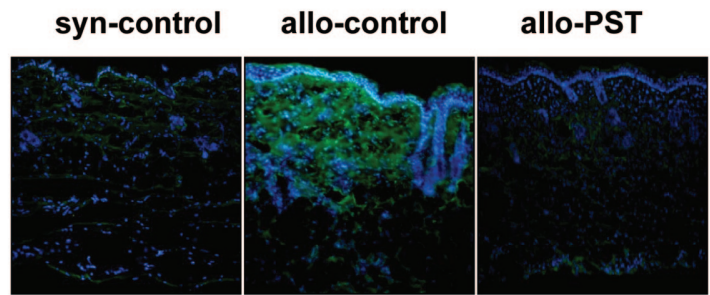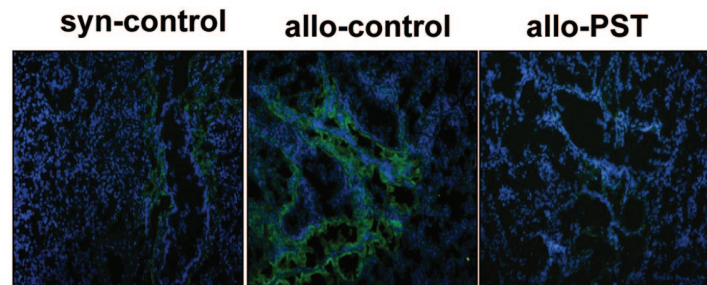
A skin

syn-control   **allo-control**   **allo-PST**

B lung

syn-control   **allo-control**   **allo-PST**

FIGURE 2. Pravastatin (PST) treatment prevented the deposition of type I collagen in skin and lungs. Tissue sections of skin (A) and lungs (B) from syngeneic (syn)-control, allogeneic (allo)-control, and allo-PST mice were stained with type 1 collagen. Images were acquired using an Olympus C-3000 zoom camera and processed using Image-Pro Plus, version 4 (Media Cybernetics, Inc., Bethesda, MD). Magnification, ×200.

PST mice were 20% and 13%, respectively, greater than those in syn-controls.

Because pulmonary fibrosis is a major cause of morbidity and mortality in CGVHD, we next asked whether our model would be useful for the study of lung and skin fibrosis. In the lungs, airway submucosal fibrosis was observed on days 28 and 56 in allo-controls. PST injection markedly reduced fibrosis on day 56 although some fibrosis was observed on day 28. Syn-controls did not develop GVHD clinically or have submucosal fibrosis by histologic examinations (Fig. 1D).

PST Treatment Inhibited Type 1 Collagen Deposition

Fibrosis, regardless of its cause, is characterized by extracellular matrix deposition of predominantly collagen type I (*14*). The progressive accumulation of connective tissue results in the destruction of the normal tissue architecture and internal organ failure. In CGVHD, the severity of skin and internal organ fibrosis is correlated with clinical disease course (*15*). Thus, we stained skin and lung tissues with anticollagen type I at day 28 after HSCT. As shown in Figure 2(A), collagen deposition was greater in the dermis of allo-controls compared with syn-controls, whereas dermal collagen deposition was markedly reduced by PST treatment. In the lungs, excessive lung collagen deposition was also noted in the interstitium of allo-controls when compared with syn-controls; pulmonary collagen deposition was also significantly diminished by PST treatment (Fig. 2B).

PST Reduced Skin and Lung Inflammation

We next evaluated the effects of PST on early cutaneous and pulmonary inflammation induced by CGVHD. PST-treated allo-recipients exhibited marked reduction in mean skin total cell counts versus allo-controls at days 14 and 28 after transplantation (Fig. 3A, $P<0.01$). In lungs, PST treat-
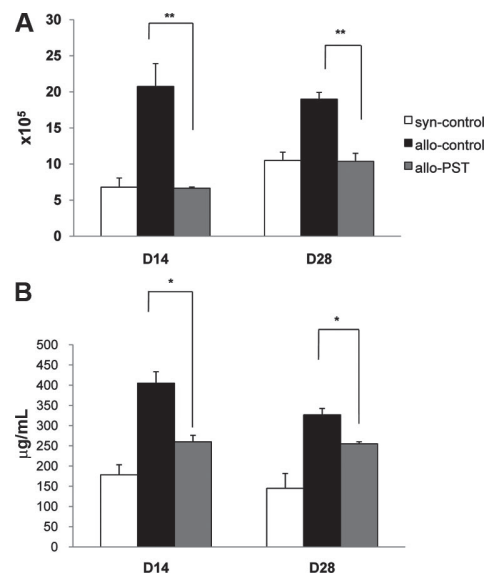

FIGURE 3. Effect of pravastatin (PST) on skin cell numbers (A) and protein concentrations of bronchoalveolar lavage (BAL) fluid (B). (A) Lethally irradiated BALB/c mice were transplanted with B10.D2 or BALB/c spleen and bone marrow cells, and skin cell suspensions were prepared, as described in *Materials and Methods*. The absolute numbers of mononuclear cells in skin cell suspensions were determined at each time point. *Open bars*, syngeneic (syn)-controls (n=3); *closed bars*, allogeneic (allo)-controls (n=5); and *gray bars*, allo-PST mice (n=5). Data are expressed as the means±SEM; **P less than 0.01. Data from one of two similar experiments are shown. (B) BAL fluid protein concentrations were analyzed using the Bradford method. *Open bars*, syn-controls; closed bars, allo-controls; gray bars, allo-PST mice. Data are expressed as means±SEM; n=5 animals per group. *P less than 0.05.

TABLE 1.   Reduction in the severity of lung inflammation by PST treatment

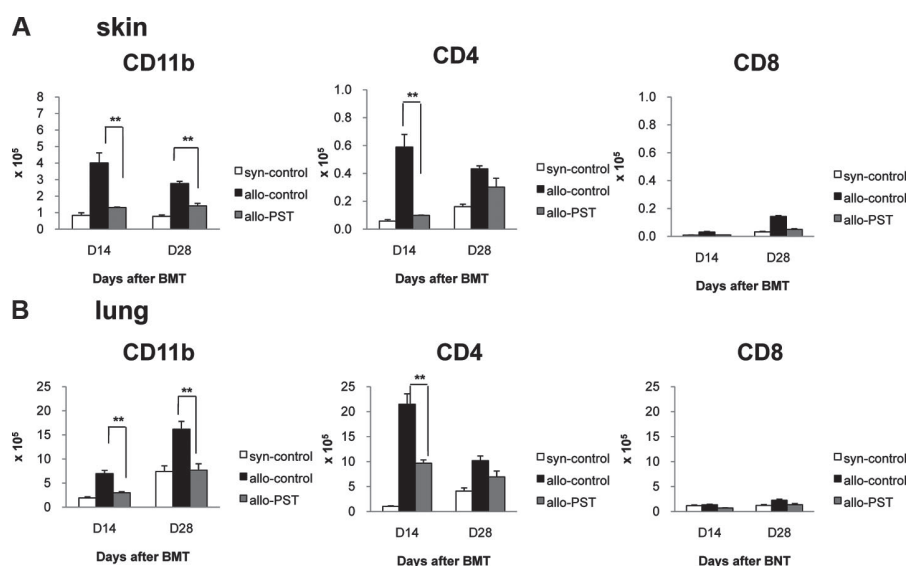| Group	Total cells (×10⁵)		Macrophages (×10⁵)		Lymphocytes (×10⁵)		Neutrophils (×10⁵)		Epithelial cells (×10⁵)	
	D14	D28	D14	D28	D14	D28	D14	D28	D14	D28
Syngeneic control	17.5 ± 1.6	29.4 ± 4.2	13.7 ± 1.4	21.3 ± 3.7	0.2 ± 0.0	0.8 ± 0.3	0.1 ± 0.0	0.3 ± 0.2	3.5 ± 0.4	$.0 \pm 1.1$
Allogeneic control	48.3 ± 5.0	82.0 ± 8.2	19.2 ± 5.1	54.9 ± 2.5	6.7 ± 0.9	9.8 ± 3.3	0.4 ± 0.2	0.4 ± 0.2	22.0 ± 2.4	17.5 ± 3.0
Allogeneic PST	26.2 ± 1.7^a	42.0 ± 6.0^a	15.6 ± 1.7	32.4 ± 4.9^a	2.3 ± 0.5^a	2.2 ± 0.5^b	0.3 ± 0.1	0.4 ± 0.2	9.5 ± 2.4^a	7.1 ± 1.0^a

Data are the mean±SEM (n=6).
[a] $P<0.01$ compared with the allogeneic control group.
[b] $P<0.05$.
PST, pravastatin.

FIGURE 4. Effect of pravastatin (PST) on numbers of CD11b⁺, CD4⁺, and CD8⁺ T cells in target organs. Cell numbers in skin (A) and bronchoalveolar lavage fluid (B) at days 14 and 28 posthematopoietic stem-cell transplantation were counted and phenotyped by flow cytometry, as described in *Materials and Methods*. *Open bars*, syngeneic (syn)-controls; *closed bars*, allogeneic (allo)-controls; and *gray bars*, allo-PST mice. The results are representative of two independent series of experiments. Results are means±SEMs; n=3 to 4 animals per group,**P less than 0.01. BMT, bone marrow transplantation.

ment significantly decreased total cell numbers in BAL fluid at all times when compared with allo-controls (Table 1). Moreover, PST treatment markedly reduced protein concentrations in BAL fluid (a marker of lung permeability) on days 14 and 28 (Fig. 3B, $P<0.05$). We next performed a microscopic examination of BAL fluid because BAL fluid cellularity can be used as a surrogate marker of lung inflammation caused by CGVHD (*16*) and performed differential counts of macrophages, lymphocytes, and neutrophils using morphologic criteria. As shown in Table 1, numbers of lymphocytes were significantly attenuated in the allo-PST group from day 14. Furthermore, alveolar macrophages and lymphocytes were significantly reduced in allo-PST animals at day 28. Neutrophil counts in BAL fluid were not different between the two groups, but the numbers of epithelial cells, which reflect degree of alveolar injury, were significantly lower at days 14 and 28 in the allo-PST group. These results indicate that PST treatment significantly attenuated skin and pulmonary inflammation caused by CGVHD.

It has been noted that initial target organ inflammation is caused primarily by CD11b⁺ monocytes and T cells (*17*, *18*). We performed flow cytometric analysis on cells in skin cell suspensions and BAL fluid using CD4, CD8, and CD11b surface markers. After allo-HSCT, the numbers of CD11b⁺

and CD4⁺ T cells, but not CD8⁺ cells, were markedly increased in skin and BAL fluid, and PST administration inhibited these increased infiltrations (Fig. 4A,B, respectively).

MCP-1 and RANTES Expression in Target Tissues Was Reduced by PST Administration

MCP-1 and RANTES have been shown to play the important roles by chemoattracting immunocompetent cells during pathogenesis of this CGVHD model (*5*, *6*). So, we hypothesized that the reduction in CGVHD caused by PST treatment would be because of the inhibition of chemokine expression, such as MCP-1 and RANTES. Accordingly, we measured the protein levels of MCP-1 and RANTES by ELISA in the skin and lung tissues to determine whether PST affects chemoattraction of these monocytes to the target tissues by influencing their expressions.

Although the concentrations of MCP-1 and RANTES were higher in allo-controls on days 14 and 28 compared with syn-controls, they were significantly reduced by PST treatment on day 14 but not on day 28. (Fig. 5A,B). In particular, the protein levels of RANTES in skin on day 28 were significantly reduced by PST treatment. Western blot analysis showed again that PST markedly reduced the chemokine ex-

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