Stem Cell Transplantation within Tumors Is Induced by Hematopoietic Mediated Blockade of Regulatory T Cells

Takeshi Udagawa, Kenta Narumi, Koji Suzuki, Kouichirou Aida, Reina Miyakawa, Yoshinori Ikarashi, Atsushi Makimoto, Tatsuya Chikaraishi, Teruhiko Yoshida and Kazunori Aoki

J Immunol 2013; 191:3440-3452; Prepublished online 21 August 2013; doi: 10.4049/jimmunol.1201454

http://www.jimmunol.org/content/191/6/3440

Supplementary Material

http://www.jimmunol.org/content/suppl/2013/08/21/jimmunol.1201454.DC1

Why The JI?

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Speedy Publication! 4 weeks from acceptance to publication

*average

References

This article cites 44 articles, 19 of which you can access for free at: http://www.jimmunol.org/content/191/6/3440.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Vascular Endothelial Growth Factor-D–Mediated Blockade of Regulatory T Cells within Tumors Is Induced by Hematopoietic Stem Cell Transplantation

Takeshi Udagawa,*†,1 Kenta Narumi,*† Koji Suzuki,*‡ Kouichirou Aida,*† Reina Miyakawa,* Yoshinori Ikarashi,* Atsushi Makimoto,‡ Tatsuya Chikaraishi,‡ Teruhiko Yoshida,§ and Kazunori Aoki*  

Lymphopenia-induced homoeostatic proliferation of T cells after autologous hematopoietic stem cell transplantation (HSCT) skews the T cell repertoire by engaging tumor-associated Ags, leading to an induction of antitumor immunity. However, how HSCT alters the immuno-suppressive microenvironment in the tumors is unknown. In this study, we first analyzed the kinetics of regulatory T cells (Tregs) in the tumors after syngeneic HSCT. Unexpectedly, the frequency of CD4+ cells expressing Foxp3 was increased in the spleens, whereas the frequency was clearly decreased in the tumors after HSCT. The origin of reconstituted CD4+ and Foxp3+ cells in the tumors was mainly from the expansion of transferred splenic T cells. Then, to examine the mechanism of Treg suppression after HSCT, we isolated CD11c+ cells from tumors. A large amount of Treg-inhibitory cytokine IL-6 was secreted from the CD11c+ cells in the tumors, but not in the spleens in the recipient mice. Furthermore, to understand what factor affects the activity of CD11c+ cells in the tumors after HSCT, we analyzed the expression of various cytokines/chemokines with mouse cytokine Ab arrays, and noticed that VEGF-D concentration was increased in the tumors in the early period after HSCT. The CD11c+ cells produced IL-6 in response to VEGF-D stimulation, and an administration of VEGF receptor-3 neutralizing Ab significantly suppressed the production of IL-6 from CD11c+ cells accompanied with the increase of Tregs in the tumors of HSCT recipients. Autologous HSCT creates an environment that strongly supports the enhancement of antitumor immunity in reconstituted lymphoepithelial recipients through the suppression of Tregs.  

CD4⁺CD25⁻Foxp3⁺ Tregs can play a critical role in suppressing immune reaction (15, 16). Unexpectedly, we found that the frequency of CD4⁺ T cells expressing Foxp3 was significantly decreased in the tumors but not in the spleens after HSCT, and that the decrease of Tregs was dependent on IL-6 produced by dendritic cells (DCs) in the tumors.

To examine what factor affects the activity of DCs in the tumors after HSCT, we analyzed the expression of various cytokines, and we found that the vascular endothelial growth factor (VEGF)-D level in the tumors was elevated in HSCT recipients. VEGF-D is a secreted factor that binds to the vessel and lymphatic receptor, VEGF receptor (VEGFR)-3, which is thought to be a signal for lymphangiogenesis (17). In this study, we also showed that VEGF-D is responsible for IL-6 production by DCs in tumors in HSCT recipients.

**Materials and Methods**

**Animals and tumor cell lines**

Seven- to 9-wk-old female BALB/c (H-2b) and C57BL/6 (H-2b) mice were purchased from Charles River Japan (Kanagawa, Japan). Female C57BL/6-Tg (CAG-EGFP) mice (H-2b), which ubiquitously express GFP, and C57BL/6 × BALB/c F1 mice (H-2b/H-2d) were purchased from Japan SLC (Hamamatsu, Japan). Animal studies were carried out according to the Guideline for Animal Experiments of the National Cancer Center Research Institute and approved by the Institutional Committee for Ethics in Animal Experimentation. CT26 and Meth-A (American Type Culture Collection, Rockville, MD) are BALB/c-derived colon and fibrosarcoma cell lines, respectively, and Pan02 (National Cancer Institute, Frederick, MD) is a C57BL/6-derived pancreatic cancer cell line. Cells were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS (ICN Biomedicals, Irvine, CA), 2 mM l-glutamine, and 0.15% sodium bicarbonate.

**HSCT and tumor inoculation**

Nine- to 10-wk-old syngeneic mice received a lethal (9 Gy) irradiation on the day of transplantation. The irradiated mice were injected i.v. with 5 × 10⁶ BM cells and 2 × 10⁶ splenic T cells from donor syngeneic mice. BM cells were isolated from donors by flushing each femur and tibia with RPMI 1640 medium supplemented with 5% FBS, and splenic cells were prepared by macerating the spleens. The transfer of T cells (splenocytes) is crucial to induce tumor immunity (8). After lysis of the erythrocytes, splenic cells were incubated with anti-Thy-1.2 immunomagnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) at 4°C for 15 min, followed by selection of T cells by AutoMACS (Miltenyi Biotec GmbH). CT26 (1 × 10⁶), Meth-A (5 × 10⁶), or Pan02 cells (5 × 10⁶) were injected s.c. into the legs of the mice. The tumor volume was calculated using the formula: tumor volume = 1/2 × ([the shortest diameter]² × [the longest diameter]). Data are presented as mean ± SD.

To examine the origin of Tregs in the tumors, we performed three types of transplantation with EGFP-transgenic mice: 1) splenic T cells isolated from EGFP-transgenic mice and BM cells isolated from naive mice, 2) splenic T cells isolated from naive mice and BM cells isolated from EGFP-transgenic mice, and 3) splenic T cells and BM cells isolated from EGFP-transgenic mice. We consider the EGFP⁺ fraction of CD4⁺ and Foxp3⁺ cells as the expansion of transferred cells in the graft and in those with EGFP⁺ splenic T cells and EGFP⁺ BM cells as the de novo generation from marrow progenitor cells. In the transplanted mice with EGFP⁺ splenic T cells and EGFP⁺ BM cells, EGFP⁺ cells were considered as the residual host cells. The total of percentages of three origins was ~100%, and the percentage of each origin was calculated compared with their total.

**ELISpot assay**

An IFN-γ ELISpot kit (BD Biosciences, San Jose, CA) was used according to the manufacturer’s instructions. In brief, splenocytes (1 × 10⁵) and mitomycin C-treated tumor cells (1 × 10⁶) were cocultured per well in 96-well plates precoated with mouse IFN-γ (BD Biosciences) for 20 h at 37°C in RPMI 1640 medium in triplicate. After washing the wells, a biotinylated anti-mouse IFN-γ Ab (5 μg/ml) was added and followed by incubation for 2 h at room temperature. Then, a streptavidin-HRP solution was added and incubated for 1 h at room temperature. After the addition of an aminoethyl carbazole substrate solution, spots were counted under a stereomicroscope.

**Tetramer staining and FACS analysis**

The CT26-specific H-2LΔ MuVAgp70 (AH-1) peptide tetramer was purchased from BD Biosciences. For cell staining, the manufacture’s protocol was followed. Abs against the following molecules coupled to the indicated fluorochromes were purchased from BD Biosciences: FITC–anti-CD4, allophycocyanin–anti-Foxp3, and PE–anti-CD11c. An anti–VEGF-3 (FLT-4) Ab was purchased from R&D Systems (Minneapolis, MN). Tumors and spleens were harvested from the mice, and tumors were prepared with gentleMACS dissociator (Miltenyi Biotec GmbH). After washing, cells were incubated with mAbs in a total volume of 100 μl PBS with 5% FBS for 30 min at 4°C and then fixed. Cells were analyzed by FACS (FACSCalibur; BD Biosciences). Irrelevant IgG mAbs were used as a negative control. Ten thousand live events were acquired for analysis.

**Immunohistochemistry**

Immunostaining was performed using streptavidin-biotin-peroxidase complex techniques (Nichirei, Tokyo, Japan). Consecutive cryostat tissue sections (6 μm) were mounted on glass slides and fixed in 95.5% ethanol for 20 min. After blocking with normal rabbit serum, the sections were stained with rat anti-mouse CD4, CD8, and Foxp3 Abs (BD Biosciences). Parallel negative controls with Abs of the same isotype were examined in all cases. The sections were counterstained with methyl green.

**Isolation of CD11c⁺ cells and T cell proliferation assay**

DCs were isolated using mouse CD11c Microbeads and AutoMACS magnetic sorters (Miltenyi Biotec GmbH) from tumors of HSCT mice at 2 wk after transplantation or from tumors of control non-HSCT mice, and designated as HSCT-CD11c⁺ or control CD11c⁺, respectively. The flow cytometry showed that ~90% of isolated cells express CD11c and that ~80% of the isolated CD11c⁺ cells are negative for CD14 (macrophage marker), suggesting that a major population of isolated CD11c⁺ cells are DCs. CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells were isolated from the spleen of naive BALB/c mice using mouse CD4 pre-enrichment kit, mouse CD25 selection kit, and AutoMACS magnetic sorter (Miltenyi Biotec GmbH). These populations were stained with anti-Foxp3 Ab, and flow cytometry revealed that ~80% of CD4⁺CD25⁻ T cells expressed Foxp3, CD4⁺CD25⁺ T cells were isolated in a 96-well plate (1 × 10⁵/well) with the supernatant of CD11c⁺ cells, 0.5 μg/ml anti-CD3 Ab and CD4⁺CD25⁺ T cells (5 × 10⁵/well) for 72 h at 37°C with [H]thymidine (Perkin-Elmer Japan, Kanagawa, Japan) added for the last 12 h of culture. Plates were then harvested with a Packard harvester onto glass fiber filters, and the filters were dried. Thirty microliters of MicroScint 20 scintillation fluid was added to each well, and the plates were read with a Packard Top-Count plate reader (Packard Instrument Company, Meriden, CT).

**Cytokine Ab array**

CT26 s.c. tumors were isolated 2 wk after HSCT and were lysed with RIPA buffer (10 mM Tris-HCl [pH 7.4], 1% deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 0.2 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) and mouse cytokine Ab arrays (G series 3 and 4; RayBiotech, Norcross, GA) were used for cytokine detection (a total of 96 kinds). We processed the cytokine array slides according to the manufacturer’s recommendation. In brief, the slides were blocked by incubation with the blocking buffer at room temperature for 30 min and incubated with the sample at room temperature for 1 h. Slides were washed and incubated with biotin-conjugated Abs at room temperature for 2 h. Finally, the slides were washed and incubated with Alexa Fluor 555–conjugated streptavidin at room temperature for 1 h, and the signals were detected using a microarray scanner (Innoven, Brabrand, Denmark). The data of cytokine Ab arrays were deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/; accession no. E-MTAB-1761).

**Statistical analysis**

Comparative analyses of the data were performed by the Student t test, using SPSS statistical software (SPSS Japan, Tokyo, Japan). A p value <0.05 was considered significant.

**Results**

Syngeneic HSCT recovers a proliferative activity of lymphocytes suppressed by tumors

First, to examine whether syngeneic HSCT could induce an antitumor effect, we injected BALB/c mice s.c. with CT26 colon cancer cells shortly after lethal (9 Gy) irradiation; then we infused BM and T cells into the mice to avoid the direct effect of radiation on the
tumor cells. This model simulates the situation where patients have minimal residual cancer after standard therapy. Tumor growth was significantly suppressed in HSCT recipients (Fig. 1A, left panel) as previously reported (18, 19). No overt toxicity was observed for the treated mice, including their blood chemistry. HSCT with BM and splenic T cells isolated from nude mice did not show the tumor growth suppression as compared with non-HSCT mice (Fig. 1A, right panel), indicating that the antitumor effect is not mediated by a nonspecific effect of irradiation or lymphocyte infusion. In addition to the minimal tumor model, we inoculated the CT26 or Meth-A cells on the legs of BALB/c mice 1 wk before HSCT as a pre-established tumor model. The tumor growth was more strongly suppressed in the HSCT recipients (Fig. 1B). Furthermore, the experiment was repeated in the pre-established tumor model, including control groups in which nude mouse BM cells with or without nude mouse or BALB/c splenic T cells are transferred. The result showed that the tumor growths in nude mouse BM cells with no splenocytes, and in those with nude mouse splenocytes, presented similar tumor growth with the non-HSCT mice, whereas the transplantation of nude mouse BM cells and BALB/c splenic T cells significantly suppressed the CT26 tumor growth (Fig. 1C), suggesting that the transferred splenocytes are crucial to induce an antitumor effect, which was compatible with a previous report (8).

It is known that tumor progression results in systemic immune suppression possibly because of the induction of immunosuppressive cells and cytokines (20–22). To examine how syngeneic HSCT affects the systemic immune suppression in tumor-bearing mice, we inoculated CT26 cells at the time of HSCT and we analyzed the proliferation of CD3^+ T cells during 3 wk after HSCT. There is a significant difference of lymphocyte proliferation activity in HSCT mice compared with non-HSCT mice at 3 wk after HSCT (Fig. 1D), suggesting that HSCT can generally maintain the proliferative activity of lymphocytes. An ELlSpot assay showed that HSCT significantly increased the number of IFN-γ spots in response to tumor cells but not to syngeneic lymphocytes (Fig. 1E). The tetramer assay also showed that the number of CT26-specific AH-1–responsive CD8^+ T cells was significantly increased at 3 wk after the transplantation (Fig. 1F), indicating that syngeneic HSCT enhances the proliferation of tumor-responsive lymphocytes.

Inhibition of Tregs in tumors after syngeneic HSCT

The effect of HSCT on the immune-tolerant microenvironment developed in the tumors is not clearly known. Therefore, we examined the dynamics of Tregs, which are one of the main factors in creating immunosuppression in the periphery and tumor after HSCT. First, we examined whether CD4^+CD25^+ Tregs were involved in the in vivo growth of the tumor cells used in this study. We i.v. injected anti-mouse CD25 Ab (purified from PC61 hybridoma) (23) and then inoculated CT26, Meth-A, and Pan02 cells in the syngeneic mice. The growth of all three s.c. tumors in the spleens and tumors after HSCT. Therefore, there are three possibilities about the origin of Tregs in HSCT recipients: de novo generation from hematopoietic stem cells, expansion of Tregs present in the graft, and residual host Tregs. To examine the origin of Tregs, we performed three types of transplantation with EGFP-transgenic C57BL/6 mice: 1) EGFP^+ splenic T cells and EGFP^+ BM cells, 2) EGFP^+ splenic T cells and EGFP^+ BM cells, and 3) EGFP^+ splenic T cells and EGFP^+ BM cells. EGFP^+ cells per CD4^+ cells or Foxp3^+ cells were examined in the spleens and Pan02 tumors by flow cytometry at 2 and 3 wk after HSCT. In the spleens, 40–60% of CD4^+ T cells and Foxp3^+ cells were considered to be de novo generation from BM progenitor cells, and 30% were the expansion of transferred cells. In the tumors, ~70% of CD4^+ T cells and Foxp3^+ cells were the expansion of transferred Foxp3^+ cells, and <30% were de novo generation from marrow progenitor cells (Fig. 3A).

Furthermore, to confirm the result in CT26 tumors, we made an EGFP-transgenic F1 mouse (H-2^bd) by mating a C57BL/6-Tg (CAG-EGFP) mouse with a BALB/c mouse. CT26 cells were inoculated on the right legs of (C57BL/6 × BALB/c)F1 mice shortly after a lethal dose of irradiation, and three types of transplantation were performed. The flow cytometry showed that in the tumors, 60–70% of CD4^+ T cells and Foxp3^+ cells were the expansion of transferred cells, and <30% were de novo generation from marrow progenitor cells (Supplemental Fig. 3), which were similar to Pan02 tumors in C57BL/6 mice (Fig. 3A). The results suggest that the main origin of CD4^+ T and Foxp3^+ cells is different between the spleens and tumors after HSCT.

Our future aim is to use the Treg suppression in tumors by HSCT to enhance antitumor immunity. In the clinical setting, a larger decrease of Tregs would be required to achieve a strong clinical effect. Because Foxp3^+ cells in the tumors were mainly derived from the expansion of cells present in the graft, we examined whether the depletion of Tregs from the graft enhances the antitumor effect of HSCT. The depletion of CD25^+ cells from the graft significantly enhanced the antitumor effect against CT26 and Meth-A s.c. tumors (Fig. 3B), suggesting that Tregs in the graft have a role in suppressing the HSCT-mediated antitumor immunity.
FIGURE 1. Syngeneic HSCT suppressed the growth of s.c. tumors. (A) Growth suppression of s.c. tumors in syngeneic HSCT mice in a minimal tumor model. The BALB/c and BALB/c nude mice received a lethal dose of irradiation, followed by a transfusion of BM and splenic T cells derived from BALB/c and BALB/c nude mice, respectively. CT26 cells were inoculated into right legs (number of animals per each group: \( n = 7 \)). The transplanted BALB/c nude mice were i.v. injected with an asialo–GM-1 Ab every 5 d after HSCT to deplete NK cells. Shown is a representative of at least two separate experiments with similar results. (B) Growth suppression of s.c. tumors in syngeneic HSCT mice in a pre-established tumor model. CT26 or Meth-A cells were inoculated on the legs of BALB/c mice 1 wk before HSCT (\( n = 7 \)). (C) Antitumor effect by the transferred splenic T cells in a pre-established tumor model. CT26 tumors are engrafted 1 wk before HSCT, including control groups in which nude mouse BM cells with or without nude mouse splenic T cells or BALB/c splenic T cells are transferred (\( n = 5 \)). The transplanted nude mice were i.v. injected with an asialo–GM-1 Ab every 5 d. (D) The increase of proliferating activity of CD3+ cells in the HSCT mice in the minimal tumor model. The splenocytes were isolated 1–3 wk after HSCT, and \( 5 \times 10^6 \) CFSE-labeled splenocytes were cultured with CD11c+ cells in CD3-coated 24-well plates. After 48 h, the proliferating fraction of CD3+ cells was evaluated with anti-CD3+ Ab (BD Biosciences) by flow cytometry (\( n = 3 \)). The proliferating cells were defined as a single or more round of division, and the proliferating fractions during 1–3 wk were compared with those at 0 wk. Data are representative of two separate experiments with similar results. (E) The increase of IFN-\( \gamma \)+ cells in response to stimulation of CT26 cells by enzyme-linked immunosorbent spot assay. Two weeks after HSCT, splenocytes were isolated from HSCT mice and non-HSCT mice, and cocultured with CT26 cells or control lymphocytes (\( n = 3 \)). (F) The increase of tumor-specific CD8+ T cells in HSCT mice. The splenocytes were isolated 2 and 3 wk after HSCT, and CT26-specific AH-1-tetramer+ cells were analyzed by flow (Figure legend continues)
Upregulation of IL-6 in tumors after syngeneic HSCT

We next examined whether HSCT influences the activity of DCs in the tumors, because these cells have a vital role in the modulation of immune response and immune tolerance (24). To analyze the cytokine expression profile of the CD11c+ cells, we collected CD11c+ cells from the CT26 tumors 2 wk after HSCT in the minimal tumor model, cultured the cells in vitro for 24 h, and measured the expression of various cytokines in the medium. The HSCT-CD11c+ cells produced a large amount of immune-stimulatory cytokines such as IL-1β, IL-6, and IL-12 compared with the control CD11c+ cells isolated from CT26 tumors of non-HSCT mice (Fig. 4A), as shown in our previous report (18, 19), which may enhance the proliferation and activation of lymphocytes in the HSCT mice. The expression of immunosuppressive cytokine TGF-β was not changed in the HSCT-CD11c+ cells as compared with the control CD11c+ cells (Fig. 4A). The expression of another immune-suppressive cytokine IL-10 was elevated in HSCT-CD11c+ cells. The enhanced IL-12 production in the HSCT-CD11c+ cells may promote the production of IL-10 in some population of the CD11c+ cells as a negative feedback mechanism (25).

IL-6 is of particular interest, because, when released from DCs, it is critical for overcoming Treg-mediated immune suppression (26). In the pre-established model, the CD11c+ cells in the tumors of the HSCT mice produced a significant amount of IL-6 (Fig. 4B). To examine whether the CD11c+ cells inhibit the suppressive activity of Tregs, we isolated CD11c+ cells from regional lymph nodes and tumors in the minimal tumor model, and CD4+CD25+ T cells (target) and CD4+CD25– Tregs were cocultured with the supernatant of CD11c+ cells in mouse anti-CD3 T cell activation plates. When Tregs were cocultured with the supernatant of control CD11c+ cells, Tregs effectively suppressed the proliferation of target T cells. The coculture with the supernatant of HSCT-CD11c+ cells inhibited the suppressive activity of Tregs for effector T cells, and the addition of anti–IL-6 Ab significantly restored the suppressive activity of Tregs (Fig. 4C). The CD11c+ supernatant and the IL-6 blocking Ab did not influence the baseline proliferation of target cells (Fig. 4C). Furthermore, another assay using CFSE also showed that the supernatant of HSCT-CD11c+ cells significantly inhibits the Treg-suppressive activity compared with that of CD11c+ cells in a dose-dependent manner (Fig. 4D). These results indicated that the critical factor of CD11c+ cell–mediated Treg inhibition is IL-6 produced from the activated CD11c+ cells.

To understand the mechanism that a frequency of Tregs per CD4+ T cells was significantly decreased in the tumors, but not in the spleens, in HSCT mice, we isolated CD11c+ cells from the CT26 tumors and spleens 2 wk after HSCT. The ELISA showed that tumor CD11c+ cells produced a significant amount of IL-6, as shown in Fig. 4A, whereas the IL-6 production from splenic CD11c+ cells was minimal even in the HSCT mice (Fig. 4E), suggesting that the tumor microenvironment activates CD11c+ cells to produce IL-6 after HSCT.

VEGF-D–mediated IL-6 production in CD11c+ cells after HSCT

To examine what factor affects the activity of CD11c+ cells in the tumors after HSCT, we analyzed the expression of various cytokines with mouse cytokine Ab arrays containing a total of 96 cytokines/chemokines. The assay showed that 40 cytokines were upregulated in the tumors of HSCT mice >3-fold compared with the non-HSCT mice (Fig. 5A). The tumor-infiltrating lymphocytes, tumor cells, and stromal cells are considered to be the source of these cytokines. Among them, we excluded the cytokines produced from the lymphocytes, because they may be caused by the consequence of the immune reaction, but not the initial cause of the immune reaction in the tumors. We then selected several cytokines produced from stromal cells such as SDF-1α, M-CSF, and VEGF-D or arbitrarily expressed cytokines such as Lungkine and Resistin. Finally, we focused on VEGF-D, because it has been reported that some populations of DCs express the VEGF-3 (27, 28). In fact, the flow cytometry showed that a significant population of CD11c+ cells in tumors of non-HSCT mice and HSCT mice expressed VEGF-3 (Fig. 5B), suggesting that a population of CD11c+ cells in the tumors expresses VEGF-3.

To compare the VEGF-D expression, we harvested the tumors and spleens during 0–3 wk after tumor inoculation. In HSCT mice, the VEGF-D concentration was low in the spleens, whereas the VEGF-D level in the tumors was significantly elevated 1 wk after HSCT (Fig. 5C). In non-HSCT mice, the concentration of VEGF-D was not significantly changed in the tumors and spleens. Although the concentration of VEGF-A in the tumors increased during 0–3 wk, there was no difference between VEGF-A expression in tumors of HSCT mice and those of non-HSCT mice (Fig. 5C). The results suggest that the elevation of the VEGF-D level in tumors is associated with HSCT, and that VEGF-A expression is correlated with tumor growth.

To examine whether the CD11c+ cells produce IL-6 in response to VEGF-D stimulation, we isolated CD11c+ cells from the spleens of HSCT mice and non-HSCT mice. Although the splenic CD11c+ cells produced IL-6 in response to VEGF-D, the splenic CD11c+ cells isolated from HSCT mice showed a lower production of IL-6 compared with the cells isolated from non-HSCT mice (Fig. 5D). The HSCT-CD11c+ cells, which were isolated from tumors of HSCT mice, produced a significant amount of IL-6 as shown in Figs. 4A and 4D, and the addition of recombinant VEGF-D protein enhanced IL-6 expression in a dose–response manner (Fig. 5E). In addition to IL-6, the CD11c+ cells stimulated with VEGF-D produced significant amounts of keratinocyte chemoattractant and TNF-α (data not shown). VEGF-C, which is another ligand for VEGFR-3, slightly elevated the IL-6 production in HSCT-CD11c+ cells (data not shown).

VEGF-3 blocking suppressed VEGF-D–mediated Treg inhibition

The human VEGF-D shows similar affinity for both VEGFR-2 and VEGFR-3; in contrast, mouse VEGF-D binds only to VEGFR-3 (29). To verify whether VEGF-D–VEGFR-3 signaling in CD11c+ cells in tumors has an important role in inducing antitumor effect of HSCT, we administered HSCT mice with CT26 tumors with anti-mouse VEGFR-3 neutralizing Ab (mF4-31C1 (28); ImClone Systems/Eli Lilly, New York, NY). The IL-6 production in HSCT-CD11c+ cells isolated from CT26 tumors was significantly decreased with the treatment of VEGFR-3 neutralizing Ab (Fig. 6A). The flow cytometry showed that the frequency of Foxp3+ per CD4+ T cells was significantly elevated in the tumors of HSCT mice treated with VEGFR-3 Ab (Fig. 6B). Furthermore, its administration significantly enhanced the growth of CT26 s.c. tumors in HSCT mice (Fig. 6C).
FIGURE 2. Frequency of Foxp3+ cells per CD4+ T cells was decreased in tumors of HSCT recipients. (A) Decrease of Foxp3+ cell number in tumors after HSCT. The fresh-frozen sections of tumors at 2 and 4 wk after HSCT were processed for immunohistochemistry with anti-mouse CD4, CD8, and Foxp3 Abs. Positive cells were counted in 10 representative high-power view fields (×400) under microscope. (B) Kinetic analysis of total cell numbers of CD4+ cells and Foxp3+ cells in the spleen after HSCT. The splenocytes were isolated from mice with or without inoculated tumors at 2–5 wk after HSCT, and the cell numbers of CD4+ and Foxp3+ cells were analyzed and calculated by flow cytometry (n = 4). Data are representative of two separate experiments with similar results. (C) The frequency of Foxp3+ cells per CD4+ cells in the spleens and tumors after HSCT. Spleens and tumors were harvested at 2–5 wk after HSCT, processed into single-cell suspension, and the percentage of CD4+Foxp3+ cells was analyzed by flow cytometry (n = 4). In the tumors, 100,000 live cells were analyzed for flow cytometry. We gated the lymphocyte regions in FACs plots and developed spots in two dimensions. Data are representative of two separate experiments with similar results. The p values provided show differences in non-HSCT mice with tumors (open square) versus HSCT mice with tumors (filled circle). (D) Frequency of Foxp3+ cells per CD4+ cells after HSCT in the pre-established tumor model. Spleens and tumors were harvested during 0–5 wk after HSCT, and the percentage of CD4+Foxp3+ cells was analyzed by flow cytometry (n = 4).
Next, the VEGFR-3 Ab was applied to a Meth-A tumor model. In Meth-A tumors also, HSCT significantly suppressed the tumor growth, and the administration of the VEGFR-3 Ab enhanced the growth of Meth-A tumors in HSCT mice (Supplemental Fig. 4A) and increased the frequency of Foxp3+ cells per CD4+ cells in the tumors of HSCT mice (Supplemental Fig. 4B).

VEGFR-3 Ab was then combined with an anti–IL-6R Ab (clone MR16-1; Chugai Pharmaceutical, Gotemba, Japan) to block the IL-6–IL-6R signaling, and an anti-CD25 Ab to deplete the Tregs in HSCT mice. In the IL-6R Ab–treated HSCT mice, CD11c+ cells produced a significant amount of IL-6, but the Treg frequency and tumor growth were increased, whereas in the IL-6R Ab and VEGFR-3 Ab–treated HSCT mice, the expression of IL-6 from CD11c+ cells was decreased, and the Treg frequency and the tumor growth were increased (Fig. 6D–F), suggesting that irrespective of VEGFR-3 Ab, IL-6–IL-6R signaling has an important role in suppressing Tregs in tumors after HSCT. In the CD25 Ab–treated HSCT mice, CD11c+ cells produced a significant amount of IL-6 and the Treg frequency and tumor growth were increased, whereas in the IL-6R Ab and VEGFR-3 Ab–treated HSCT mice, the expression of IL-6 from CD11c+ cells was decreased, and the Treg frequency and the tumor growth were increased (Fig. 6D–F), suggesting that irrespective of VEGFR-3 Ab, IL-6–IL-6R signaling has an important role in suppressing Tregs in tumors after HSCT. In the CD25 Ab–treated HSCT mice, CD11c+ cells produced a significant amount of IL-6 and the Treg frequency and tumor growth were increased, whereas in the IL-6R Ab and VEGFR-3 Ab–treated HSCT mice, the expression of IL-6 from CD11c+ cells was decreased, and the Treg frequency and the tumor growth were increased (Fig. 6D–F), suggesting that irrespective of VEGFR-3 Ab, IL-6–IL-6R signaling has an important role in suppressing Tregs in tumors after HSCT. In the CD25 Ab–treated HSCT mice, CD11c+ cells produced a significant amount of IL-6 and the Treg frequency and tumor growth were increased, whereas in the IL-6R Ab and VEGFR-3 Ab–treated HSCT mice, the expression of IL-6 from CD11c+ cells was decreased, and the Treg frequency and the tumor growth were increased (Fig. 6D–F), suggesting that irrespective of VEGFR-3 Ab, IL-6–IL-6R signaling has an important role in suppressing Tregs in tumors after HSCT.

Finally, to further show that the effect of a VEGF-D–VEGFR-3 interaction on Treg numbers in tumors is dependent on IL-6 levels in tumors. The overall results suggested that VEGF-D–mediated IL-6 production from CD11c+ cells decreases the frequency of Tregs in the tumors of HSCT mice, leading to the induction of antitumor immunity (Fig. 7D).

**Discussion**

In this study, we demonstrated that the frequency of Tregs was significantly decreased in the tumors in the HSCT recipients as compared with the non-HSCT mice. In a clinical trial of autologous HSCT-mediated immunotherapy, Borrello et al. (30) reported a phase 2 clinical study of GM-CSF–secreting cellular vaccine in combination with HSCT as postremission therapy for acute myeloid leukemia, and autologous HSCT-mediated immunotherapy for solid tumors is not practiced at present in the clinical setting. However, autologous HSCT is able to create a suitable tumor microenvironment for the enhancement of antitumor immunity induced by cancer immune therapy as shown in this study. In fact, we reported that an intratumoral allogeneic MHC gene transfer...
and type I IFN gene transfer resulted in marked tumor suppression when administered in the early period of syngeneic HSCT (18, 19, 31), and we are planning to apply the combination therapy of immune therapy and HSCT for solid cancers. The solid tumor models used in this study may be important as a preclinical study of HSCT-mediated immune therapy.
Because HP leads to a break in tolerance against self-Ags, the induction of autoimmunity against tumors could theoretically promote T cell response not only against tumor cells, but also against host normal cells, which may cause an autoimmune reaction. However, no overt toxicity was observed for the recipient mice. The immunogenic DCs at the tumor sites were able to capture both tumor-associated Ags and normal self-Ags shared by tumor and normal cells, whereas resting host DCs away from the tumor site present only normal self-Ags and may induce tolerance or exhaustion of host-reactive T cells (32, 33). Alternatively, the percentage of Foxp3+ cells per CD4+ T cells in the spleen was clearly elevated at an early phase after syngeneic HSCT (Fig. 2C) as
FIGURE 6. Administration of anti–VEGFR-3 Ab suppressed the IL-6 production from CD11c+ cells in tumors in the minimal tumor model. An irrelevant rat IgG was used as an isotope control Ab. (A) Suppression of IL-6 production from CD11c+ cells in tumors treated with anti–VEGFR-3 Ab. Anti–VEGFR-3 neutralizing Ab (800 μg) was i.p. injected into HSCT mice every 4–5 d after HSCT. At 2 wk after HSCT, the CD11c+ cells were isolated and cultured in 96-well plates for 24 h. The IL-6 concentration in the medium was measured by a cytokine assay (Meso Scale Discovery; n = 4). Data are representative of two separate experiments with similar results. (B) The increase of Foxp3+ cells in HSCT tumors by the administration of anti–VEGFR-3 Ab. Tumors were harvested at 2 and 3 wk after HSCT, processed into single-cell suspension, and the percentage of Foxp3+ cells per CD4+ cells was analyzed by flow cytometry (n = 4). Data are representative of two separate experiments with similar results. (C) Tumor growth in HSCT mice treated with anti–VEGFR-3 Ab. Tumor volumes were measured at indicated days (n = 5). Arrows denote i.p. injection of anti–VEGFR-3 Ab. Data are representative of three separate experiments with similar results. (D) IL-6 production from CD11c+ cells in CT26 tumors treated with anti–VEGFR-3, anti–IL-6R, and anti–CD25 Abs in HSCT mice. Anti–IL-6R neutralizing Ab (1000 μg) was i.p. injected into HSCT mice every 7 d after HSCT. Anti-CD25 neutralizing Ab (500 μg) was i.v. injected into HSCT mice at 7 d after HSCT. At 2 wk after HSCT, the CD11c+ cells were isolated from the CT26 tumors treated with Abs and were cultured in 96-well plates for 24 h. The IL-6 concentration in the medium was measured by ELISA (n = 3; R&D Systems). (E) The percentage of Foxp3+ cells in HSCT tumors treated with Abs. CT26 tumors were harvested at 2 wk after HSCT, processed into single-cell suspension, and the percentage of Foxp3+ cells per CD4+ cells was analyzed by flow cytometry (n = 3). (F) Tumor growth in HSCT mice treated with Abs. Tumor volumes were measured at indicated days (n = 6).
previously reported (34). The finding suggests that among CD4 + T cells, Tregs rapidly proliferate during HP in the body, which might protect patients against autoimmunity after autologous HSCT.

Why is the frequency of Tregs decreased in the tumors after HSCT? An analysis of the cytokine profile showed that the HSCT-CD11c+ cells produce a large amount of IL-6, as well as other immune-stimulatory cytokines (Fig. 4A). It has been reported that IL-6 increases methylation of upstream Foxp3 enhancer and represses the Foxp3 transcription in natural Tregs (35). IL-6 is central to T cell plasticity; it helps to convert Foxp3 + Tregs into IL-17–secreting T (Th17) cells and potently abolishes conversion of conventional T cells into induced Tregs, and in its absence, no other cytokine can substitute for this inhibition (36). In this study, we also demonstrated that the supernatant of HSCT-CD11c+ cells isolated from tumors suppress the activity of Tregs (Fig. 4B, 4C), which may inhibit the reconstitution of an immune-tolerant microenvironment within the tumor.

Unexpectedly, the IL-6 production was elevated in HSCT-CD11c+ cells in the tumors, but not in the spleens, which may protect patients against autoimmunity after autologous HSCT.

Why is the frequency of Tregs decreased in the tumors after HSCT? An analysis of the cytokine profile showed that the HSCT-CD11c+ cells produce a large amount of IL-6, as well as other immune-stimulatory cytokines (Fig. 4A). It has been reported that IL-6 increases methylation of upstream Foxp3 enhancer and represses the Foxp3 transcription in natural Tregs (35). IL-6 is central to T cell plasticity; it helps to convert Foxp3+ Tregs into IL-17–secreting T (Th17) cells and potently abolishes conversion of conventional T cells into induced Tregs, and in its absence, no other cytokine can substitute for this inhibition (36). In this study, we also demonstrated that the supernatant of HSCT-CD11c+ cells isolated from tumors suppress the activity of Tregs (Fig. 4B, 4C), which may inhibit the reconstitution of an immune-tolerant microenvironment within the tumor.

Unexpectedly, the IL-6 production was elevated in HSCT-CD11c+ cells in the tumors, but not in the spleens, which may provide an explanation for the difference of Treg frequency between the tumors and spleens. We hypothesized that the tumor microenvironment causes CD11c+ cells to produce IL-6 in response to VEGF-D stimulation, and an administration of VEGF-3 neutralizing Ab significantly suppressed the production of IL-6 from CD11c+ cells and increased the frequency of Tregs per CD4+ T cells in the tumors of HSCT recipients. This study showed that VEGF-D may regulate the Tregs via IL-6 levels produced by DCs, which may inhibit the reconstitution of an immune-tolerant microenvironment for the enhancement of antitumor immunity.

VEGF-D belongs to the VEGF/PDGF family, which consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. It has been reported that VEGF-D is involved in adult inflammation-associated lymphangiogenesis (17), wound healing (37), and tumor metastasis (38), being produced by a variety of cells residing at inflamed sites, including macrophages, DCs, neutrophils, mast cells, fibroblasts, and tumor cells (39–41). VEGF-D activates VEGFR-3 expressed on lymphatic endothelium in adult tissues. Regarding DCs, it was reported that VEGF-3 was expressed in the CD11c+ cells in the cornea and transplanted heart (27, 28), and that the expression was considered to implicate trafficking of DCs in draining lymph nodes (42). The induction of lymphatic vessels should enhance the delivery of DCs to the
lymph nodes, which contributes to inflammation. In addition, as shown in this study, VEGF-D enhanced the production of proinflammatory cytokine IL-6 from CD11c+ cells and suppressed Tregs, which may also contribute to the induction of inflammation. In contrast, VEGF-A, which is a master regulator of vascular endothelial cell biology, is abundantly expressed by a large percentage of solid tumors, and VEGF-A causes a defect in the functional maturation of DCs from progenitors (43). Thus, the role of VEGF-D may be different from VEGF-A in the immune environment in tumors of HSCT recipients. Because VEGF-D is produced by a variety of cells residing at inflamed sites, the next step in research may include the determination of cell types expressing VEGF-D in tumors after HSCT and the mechanism to enhance VEGF-D expression in the cells.

It has been reported that VEGF-D is involved in tumor metastasis (38, 44). Stacker et al. (38) showed that VEGF-D induces the formation of lymphatics within tumors generated from s.c. inoculation of 293EBNA cells stably expressing VEGF-D, and that the expression of VEGF-D in tumors leads to their spread to lymph nodes. In contrast, in this study, distant metastases were not recognized in the HSCT mice. ELISA showed that the VEGF-D level was increased at 1 wk after HSCT, then gradually decreased and returned to the normal level at 3 wk after HSCT. The formation of lymphatics and distant metastasis may require the long-term expression of VEGF-D, or the antitumor immunity induced by HSCT may inhibit the distant metastasis. Laakkonen et al. (44) reported that Ab interference with VEGF-R-3 function can block lymphangiogenesis and inhibit the growth of several human tumor xenografts in immune-compromised mice, whereas we showed that the administration of anti–VEGF-R-3 Ab increased tumor growth in immune-competent mice. The effect of VEGF-R-3 signaling block on the tumor growth might be determined based on the balance between the inhibition of lymphangiogenesis and the construction of an immune-tolerant microenvironment within tumors.

This study showed that IL-6 is a key factor in suppressing Tregs in tumors after HSCT. Differentiation of Th17 cells is dependent on the specific combination of IL-6 and TGF-β cytokines. Although Th17 cells play critical roles in the pathogenesis of inflammatory and autoimmune diseases, recent accumulation of data suggests the importance of Th17 cells to tumor immunity (45). Therefore, we are planning to examine the role of Th17 in inducing tumor immunity after HSCT in clarifying the mechanism of tumor immunity.

In conclusion, autologous HSCT is a promising immunotherapy for solid cancers, because of the activation of tumor-specific immunity, suppression of the immune-tolerant environment, and safety profile. This study provides a rationale for future clinical trials combining immune therapies such as cancer vaccine, cytokine therapy, and immune cell and gene therapy with autologous HSCT.

Acknowledgments
We thank ImClone Systems/Eli Lilly for providing the mF4-31C1 Ab, Chugai Pharmaceutical Co. for providing the MR16-1 Ab, and Vical Inc. for providing the DMRIE-DOPE liposome.

Disclosures
The authors have no financial conflicts of interest.

References


