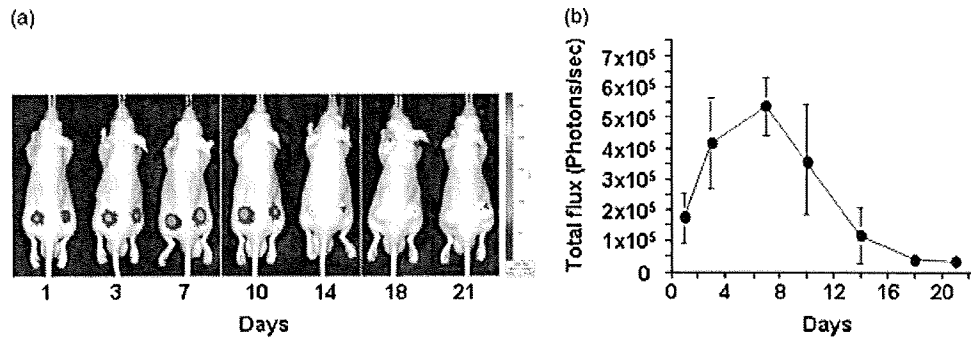

Figure 3. Survival of MSCs in subcutaneous 9L tumor. (a) Typical luminescence signals of luciferase-expressing MSCs measured using an *in vivo* imaging system. Stable luciferase-expressing MSCs mixed with an equal number of nontransduced 9L cells were subcutaneously inoculated into the bilateral dorsal region of nu/nu mice. (b) Time course of the quantified luminescence levels at tumor sites of the mice ($n = 4$)

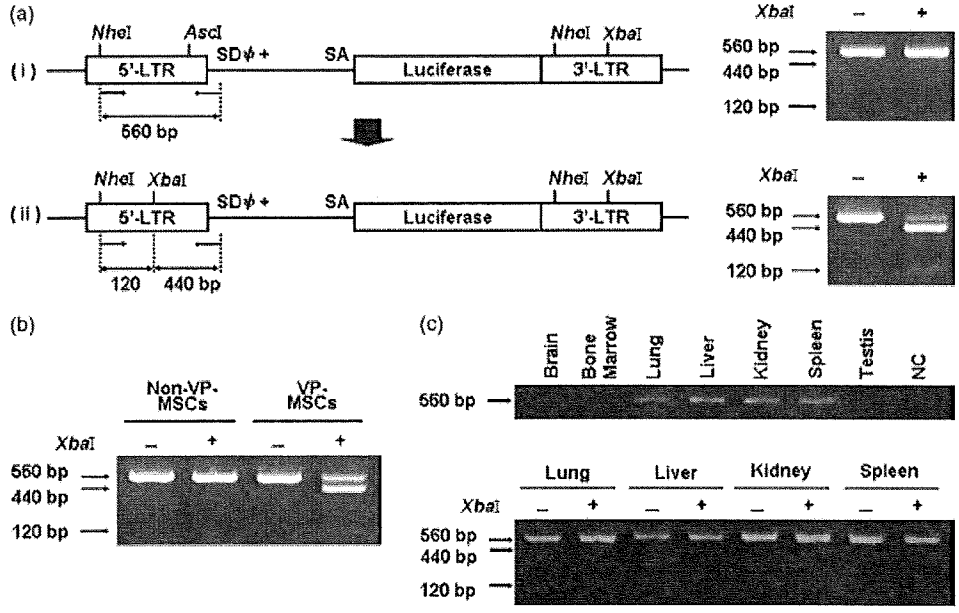

Figure 4. Tumor-specific transduction with progeny retroviral vectors in the VP-MSC system. (a) The diffrence in 5′-LTR sequences between MSCs (VP- and non-VP-) and target cells transduced with progeny retroviruses produced from VP-MSCs. The 5′-LTR sequence of transgene in VP-MSCs and non-VP-MSCs does not contain the *Xba*I site. On the other hand, the 5′-LTR sequence of transgene in target cells transduced with progeny retrovirus produced from VP-MSCs contains the *Xba*I site because this 5′-LTR is the copy of 3′-LTR (containing the *Xba*I site) in retroviruses. Therefore, the presence of two fragments (440 bp + 120 bp) after *Xba*I digestion of PCR products from the 5′-LTR region (560 bp) indicates that retroviral vector-mediated gene transfer occurred. (b) *Xba*I digestion pattern of the PCR products from the 5′-LTR region of the transgene in the tumor periphery at 21 days after MSC administration. The typical *Xba*I digested pattern indicates that transduction of the tumors with progeny retroviruses occurred. (c) The 5′-LTR region in the host tissues was examined by PCR/*Xba*I digestion at day 21 (upper panel). No typical *Xba*I digested pattern was observed in the normal tissues (lower panel), indicating the absence of retroviral vector-mediated gene transfer

retroviruses. In addition, normal tissues in the VP-MSC group were also examined PCR/*Xba*I digestion. Although the 5′-LTR region was amplified in several normal tissues (Figure 4c, upper panel), no typical *Xba*I digested pattern was observed, suggesting that gene transfer did not occur in such normal tissues/organs (Figure 4c, lower panel).

Anticancer effects of VP-MSCs *in vivo*

During the continuous infusion of GCV, tumor growth was significantly suppressed in the VP-MSC group compared to that in the non-VP-MSC, untransfected MSC or non-MSC control groups (Figure 5a). No difference in the tumor growth was observed without GCV administration (Figure 5b).

Discussion

In the present study, we developed MSCs that produce progeny retroviral vectors locally, which enable enhanced and tumor-specific transduction. Systemic delivery of modified MSCs enhanced transgene expression in the

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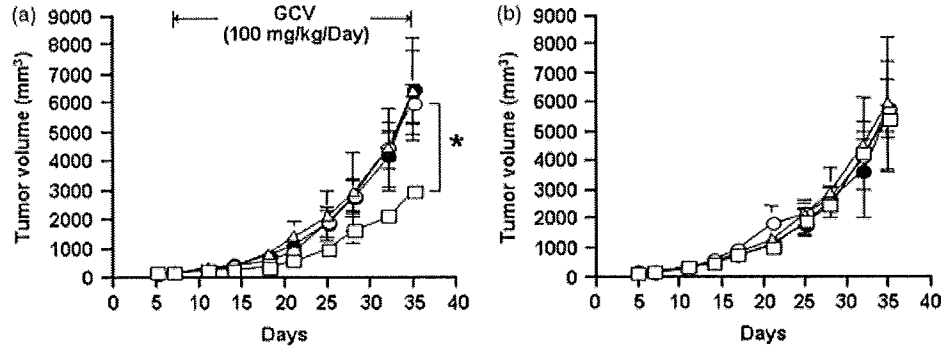

Figure 5. Anticancer effects of VP-MSCs *in vivo*. (a) Suppression of tumor growth after VP-MSC administration under GCV treatment. MSCs were injected into the left ventricular cavity immediately after subcutaneous inoculation of 9L cells into the bilateral dorsal region of Balb/c nu/nu mice (day 0). The mice were continuously administered GCV (100 mg/kg/d) from days 7–35 through intraperitoneal osmotic pumps. The tumor volume was measured periodically. (b) No change was observed in tumor growth without GCV treatment. The groups were: no MSCs (○); MSCs without genetic modification (●); MSCs nucleofected with an HSV-*tk*-expressing plasmid (non-VP-MSCs, Δ); and MSCs nucleofected with the retroviral vector components pLTR-*tk*, pGag-pol, and pVSV-G (VP-MSCs, □) ($n = 4$, for each group). *$p < 0.05$ versus non-VP-MSC group

9L tumors in mice. VP-MSC administration further augmented local transgene expression, leading to significant suppression of tumor growth compared to non-VP-MSC administration using a HSV-*tk*/GCV system.

Recently, nucleofection, an electroporation-based nonviral transfection technique, has been shown to be effective in transfection of MSCs [14]. In the present study, we found that nucleofection is also effective in preparing MSCs with a vector-producing property compared to other nonviral methods.

We demonstrated that systemic MSC administration enhanced transgene expression at the 9L tumor sites in mice, suggesting selective accumulation of MSCs at the tumor. Furthermore, the vector-producing property augmented amplification and expression of the luciferase gene *in vivo*. Immunostaining studies have demonstrated marked production of the luciferase protein at the tumors in the VP-MSC group. These results suggest that VP-MSCs can locally produce substantial amount of recombinant proteins not only through their homing ability, but also through the enhancement of transgene amplification and expression.

Our results may raise an important question about the origin of bioluminescence and immunostaining signals in the late phase: are the signals derived from VP-MSCs or transduced 9L cells? We demonstrated that most MSCs in the vicinity of 9L glioma cells were eliminated within 14 days *in vivo*. On the other hand, administration of VP-MSCs, but not non-VP-MSCs, further augmented the transgene expression at the tumor site after 14 days. These results suggest that luciferase signals in glioma at day 21 were mainly derived from 9L tumor cells transduced with progeny retroviral vectors.

The GCV-dependent anticancer effect and *tk* gene amplification in the VP-MSC group was significantly greater than that in the non-VP-MSC group *in vitro*. The IC_{50} value of GCV when it was used in concert with VP-MSCs was far lower (approximately 1 : 250) than when it was used with non-VP-MSCs. This anticancer effect of HSV-*tk*-expressing VP-MSCs *in vivo* was considered to be due to TK expression, and not due to the oncolytic properties of the progeny retrovirus because no anticancer effect was observed in the VP-MSC group without GCV treatment.

Although the effect of VP-MSCs was significantly greater than that of non-VP-MSCs, it was still partial. MSCs are suitable for repeated administration because they have little immunogenicity due to the lack of costimulatory molecule expression [7,15–17]. In addition, in the present study, VP-MSCs were administered on the same day of tumor cell inoculation. In our preliminary data, VP-MSCs have limited therapeutic effects on established palpable 9L tumor (data not shown). Preferential accumulation of VP-MSCs at the tumor periphery may explain their partial anticancer effects. Repeated administration of VP-MSCs would also be required for the treatment of established tumors.

Finally, we assessed the biodistribution and replication of progeny retrovirus to ensure the safety and specificity of our system. In the present study, after 21 days of systemic VP-MSC administration, PCR analysis revealed weak amplification of a 5′-LTR sequence in the bone marrow, lungs, kidneys and spleen. When the non-VP- and VP-MSCs were inoculated into the tumor-bearing mice, an IVIS imaging study showed that definite gene expression from MSCs was limited to the tumor site (Figures 2b and 2c). VP-MSCs expressing HSV-*tk* are considered to localize to tumor tissues in the same way. Although a small amount of transgene was detected in several normal tissues by PCR analysis, the *Xba*I-digestion pattern revealed that the transgene was derived from inoculated MSCs (Figure 4c). These results indicate that, even if a small number of injected VP-MSCs were remained in normal tissues, transduction by progeny retrovirus did not occur in these normal tissues. On the other hand, the PCR/*Xba*I digestion experiments showed that retrovirus-mediated gene transfer occurred in the vicinity of tumors. These results ensure the safety and tumor-specific transduction of the VP-MSC system.

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In conclusion, this is the first study to demonstrate the effectiveness and safety of systemic administration of VP-MSCs in tumor-bearing mice. VP-MSCs exert their function through *in situ* retroviral vector production and expression of transgenes after accumulation at tumors. Although our system needs to be improved further, the present findings will contribute to the development of more efficient cancer gene therapy using MSCs as a platform.

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References

1. Ram Z, Culver KW, Oshiro EM, *et al.* Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 1997; **3**: 1354–1361.
2. Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000; **11**: 2389–2401.
3. Hall B, Dembinski J, Sasser AK, *et al.* Mesenchymal stem cells in cancer: tumor-associated fibroblasts and cell-based delivery vehicles. *Int J Hematol* 2007; **86**: 8–16.
4. Nakamizo A, Marini F, Amano T, *et al.* Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 2005; **65**: 3307–3318.
5. Ozawa K, Sato K, Oh I, *et al.* Cell and gene therapy using mesenchymal stem cells (MSCs). *J Autoimmun* 2008; **30**: 121–127.
6. Karnoub AE, Dash AB, Vo AP, *et al.* Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007; **449**: 557–563.
7. Maitra B, Szekely E, Gjini K, *et al.* Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation. *Bone Marrow Transplant* 2004; **33**: 597–604.
8. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
9. Hall B, Andreeff M, Marini F. The participation of mesenchymal stem cells in tumor stroma formation and their application as targeted-gene delivery vehicles. *Handb Exp Pharmacol* 2007; 263–283.
10. Le Blanc K, Ringden O. Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**: 321–334.
11. Studeny M, Marini FC, Champlin RE, *et al.* Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res* 2002; **62**: 3603–3608.
12. Okada T, Caplen NJ, Ramsey WJ, *et al.* In situ generation of pseudotyped retroviral progeny by adenovirus-mediated transduction of tumor cells enhances the killing effect of HSV-tk suicide gene therapy in vitro and in vivo. *J Gene Med* 2004; **6**: 288–299.
13. Nakamura K, Ito Y, Kawano Y, *et al.* Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. *Gene Ther* 2004; **11**: 1155–1164.
14. Aluigi M, Fogli M, Curti A, *et al.* Nucleofection is an efficient nonviral transfection technique for human bone marrow-derived mesenchymal stem cells. *Stem Cells* 2006; **24**: 454–461.
15. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005; **105**: 1815–1822.
16. Le Blanc K, Rasmusson I, Sundberg B, *et al.* Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004; **363**: 1439–1441.
17. Barry FP, Murphy JM, English K, *et al.* Immunogenicity of adult mesenchymal stem cells: lessons from the fetal allograft. *Stem Cells Dev* 2005; **14**: 252–265.

Altered effector CD4^{+} T cell function in IL-21R$^{-/-}$ CD4^{+} T cell-mediated graft-versus-host disease[1]

Running title: Effector function and GVHD induced by IL-21R$^{-/-}$ CD4^{+} T cells

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Key words: Cytokine, GVHD, Interleukin-21, IL-21

Abstract

We previously showed that transplantation with interleukin-21 receptor (IL-21R) gene-deficient splenocytes resulted in less severe GVHD than was observed with wild type splenocytes. We now here sought to find mechanism(s) explaining this observation. Recipients of donor CD4^{+} T cells lacking IL-21R exhibited diminished GVHD symptoms, with reduced inflammatory cell infiltration into the liver and intestine, leading to prolonged survival. After transplantation, CD4^{+} T cell-numbers in the spleen were reduced and mixed lymphocyte reaction and cytokine production by CD4^{+} T cells were impaired. These results suggest that IL-21 might promote GVHD through enhancing production of effector CD4^{+} T cells. Moreover, we found that CD25 depletion altered neither the impaired mixed lymphocyte reaction in vitro nor the ameliorated GVHD symptoms in vivo. Thus, the attenuated GVHD might be caused by an impairment of effector T-cell differentiation itself rather than by an increase in regulatory T-cells and suppression of effector T-cells.

Introduction

Interleukin-21 (IL-21) was discovered as a co-stimulatory cytokine for T-cell proliferation and NK-cell expansion *in vitro* (1,2). IL-21 is produced by activated CD4 T-cells (1), and its receptor is expressed on T, B, and NK cells (1,3). It has also been reported that IL-21 suppresses dendritic cell function (4) and increases hematopoietic progenitor cells (5). IL-21 is known to play critical roles in immunoglobulin production (6), whereas reports have differed regarding its contributions to Th1-, Th2-, and Th17-mediated effects and differentiation (6-15). Regarding Th17, IL-21 contributes to Th17 differentiation but may not be required for this process (7,9,14,15). A relationship between IL-21 and autoimmune disease has been established. Overexpression of IL-21 induces inflammation, and in an SLE model mouse (the BXSB.6-Yaa^{+}/J mouse) with high serum levels of IL-21 (16), development of disease is abrogated when these mice are crossed to IL-21R KO mice (17). In addition, autoimmune nonobese diabetic (NOD) mice do not develop diabetes in the absence of IL-21 signaling (18-20).

Graft-versus-host disease (GVHD) is a major complication following hematopoietic stem cell transplantation (21), sometimes with a fatal outcome. Previously, we showed that transplantation with interleukin-21 receptor (IL-21R) gene-disrupted splenocytes resulted in less severe GVHD than was seen with wild type splenocytes (22). We sought to elucidate the mechanism(s) for this observation and now demonstrate dysregulated effector function of activated CD4^{+} T cells in IL-21R$^{-/-}$ mice.

Materials and Methods

Mice

IL-21R$^{-/-}$ and IL-17$^{-/-}$ mice were generated previously (6,23). Both were C57BL/6 background. Both male and female mice were used as donors. C57BL/6-DBA2-F1 male mice were purchased from Clea Japan (Tokyo, Japan). All mice used in experiments were 6-12 weeks old. All mice were housed in a Jichi Medical University mouse facility, which is regulated by an intramural small animal committee, and were treated in accordance with the university guidelines.

In vitro T-cell stimulation and mixed lymphocyte reaction

Cells were cultured in RPMI1640 (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal calf serum (Sigma, St. Louis, MO), 2 mM L-glutamine (Invitrogen), 50 μM 2-mercaptoethanol (Sigma), 0.1 mg/mL streptomycin, and 100 U/mL penicillin G (Invitrogen). Non-specific pan T-cell stimulation was performed using anti-CD3/CD28 beads for three days according to the manufacturer's instructions (Dynal Biotech ASA, Oslo, Norway). Allo-antigen-specific T-cell stimulation was induced by co-cultivation of CD4 T-cells with 30 Gy irradiated splenocytes from C57BL/6-DBA2-F1 mice for four days.

GVHD models

To eliminate effects of wild type T cells in BM, we used IL-21R$^{-/-}$ BM. We compared transplantations with IL-21R$^{-/-}$ vs. wild type CD4^{+} T cells. C57BL/6-DBA2-F1 mice were irradiated with 11 Gy and injected intravenously with 5 x 10^6 IL-21R$^{-/-}$ BM and 5 x 10^6 purified CD4^{+} T cells from either wild type or IL-21R$^{-/-}$ mice. The cells were purified using CD4-microbeads and AutoMACS® (Milltenyi Biotec K.K., Tokyo, Japan; purity was >80-90 %).

Pathological analysis

Two weeks after transplantation, mice were sacrificed and liver, skin, and intestine were subjected to formalin fixation, paraffin embedding, excision and hematoxylin-eosin staining. Photographs were taken with an Olympus BX51 microscope using X400 magnification.

Flow cytometric analysis

Fc-block® (BD Biosciences, San Jose, CA) was used to prevent non-specific antibody binding to Fc receptors. Antibodies to CD4 (RM4-5), CD8 (Ly-2), CD25 (7D4), H-2^{b} (AF6-88.5), H-2^{d} (SF1-1.1), IFN-γ (XMG1.2), and TNF-α (MP6-XT22) were purchased from BD Biosciences and anti-Foxp3 (FJK-16a) was from eBioscience (San Diego, CA). Intracellular staining was performed with Cytofx/Cytoperm® kit (BD Biosciences) according to the manufacturer's instruction. Cells were stimulated with anti-mouse CD3/CD28 beads for 5 hours in the presence of GolgiStop® (BD Biosciences). For Foxp3 intracellular staining, the stimulation was omitted. A LSR flow cytometer (BD Biosciences) was used for data collection, and data were analyzed using CellQuest software (BD Biosciences).

Enzyme linked immunosorbent assay (ELISA)

ELISA kits for IL-2, IL-4, and IFN-γ were from BD Biosciences and ELISA kits for IL-21, IL-17, TNF-α, and TGF-β1 were from R&D Systems (Minneapolis, MN). Concentrations were determined according to the manufacturer's instructions.

CD25 depletion in vitro and in vivo

In vitro purification of CD4^{+} T cells and depletion of the CD25^{+}CD4^{+} subpopulation was performed by cell-sorting using a FACSAria® (BD Biosciences), which yielded highly pure populations (>98%). In vivo CD25 depletion was performed by injecting anti-CD25 antibody as described previously (24, 25). Briefly, a hybridoma producing anti-CD25 antibody (PC61, American Type Culture Collection) was cultured in serum-free medium (PFHM-II from Invitrogen) and the antibody was purified from supernatant by ammonium sulfate precipitation and a PD10 column (GE Healthcare, UK). The purified product was quantified by the Bradford assay (Bio-Rad, Hercules, CA) at OD595 and 1 mg was injected intraperitoneally weekly from day 0 for 3 weeks. Control rat non-specific IgG was purchased from Invitrogen.

RT-quantitative PCR

At day 21 after bone marrow transplantation, CD25-negative CD4^{+} T cells were purified by cell-sorting from either recipients of wild type or IL-21R$^{-/-}$ CD4^{+} T cells, and RNA was isolated (RNeasy®, Qiagen, Valencia, CA), reverse transcribed using SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen), and PCR-amplifed using TaqMan® Gene Expression Assay's primer for mouse Foxp3 (Mm00475156) and β-actin (Mm00607939) and an ABI Prism 7700 sequence detection System (PE Applied Biosystems, Foster, CA, USA).

Statistical analysis

Kaplan-Meier plots were used to compare survival rates. The Logrank test was used to evaluate p values. Statistical analyses were performed using "Stat Mate ver. 6" (ATMS, Tokyo, Japan). The Student's t-test was used and all error bars in this study are S.D., unless otherwise specified.

Results

Purified CD4^{+} T cell-transplantation and pathological analysis

As noted above, decreased GVHD was observed when we transplanted IL-21R-deficient as compared to wild type bulk splenocytes (22). Although we sought to find molecular mechanism(s) for the ameliorated GVHD, no clue was immediately evident from the transplantation experiments performed (22). Thus, here we used purified CD4^{+} T cells instead of bulk splenocytes in this study, hoping to augment the differences observed. We used a well-known model of CD4^{+} T cell-mediated GVHD (26), in which C57BL/6 mice were donors and C57BL/6-DBA2-F1 mice were recipients. In this model, the difference between wild type and IL-21R$^{-/-}$ cells indeed appeared to be greater than in the previous experiments with bulk splenocytes (22). All recipients of wild type CD4^{+} T cells died within 55 days whereas those receiving IL-21R$^{-/-}$ CD4^{+} T cells survived during this time period (**Fig. 1A**). Moreover, recipients of IL-21R$^{-/-}$ CD4^{+} T cells recovered from body weight loss by day 14 but those receiving wild type CD4^{+} T cells did not recover and moreover continued to lose body weight (**Fig. 1B**). In recipients of IL-21R$^{-/-}$ CD4^{+} T cells, pathological analysis showed markedly reduced infiltration into region surrounding bile ducts and portal veins, and into the interstitial region of small intestine, as compared to the greater infiltration

observed in recipients of wild type CD4^{+} T cells (**Fig. 2, upper and middle panels**). Apoptotic bodies near the surface area of crypts in the small intestine could barely be seen in recipients of IL-21R$^{-/-}$ CD4^{+} T cells, in contrast to recipients of wild type CD4^{+} T cells, in which apoptotic bodies were evident (**Fig. 2, middle panel, see arrowheads**). No significant difference was observed in skin pathology among recipients of wild type CD4^{+} and IL-21R$^{-/-}$ CD4^{+} T cells. These results suggested that IL-21 might be essential for CD4-mediated GVHD, at least in this setting.

Normal cytokine production by the splenocytes after transplantation is dependent on IL-21

The above observations suggested that IL-21-mediated donor CD4^{+} T cell activation was involved in the exacerbation of GVHD. Because we could not find any significant difference in serum cytokine concentrations after transplantation (**Supplemental Fig. S1**), we assessed T cell differentiation by cytokine production in the presence of cellular stimulation. Interestingly, at day 14 and 21 after transplantation, bulk splenocytes from recipients of IL-21R$^{-/-}$ CD4^{+} T cell exhibited defective cytokine production, with decreased levels of IFN-γ, TNF-α, and IL-4; in contrast, levels of IL-2, IL-17, and IL-21 were not significantly diminished (**Fig. 3, left six panels, p values are indicated**). Before transplantation, IL-21R$^{-/-}$ CD4^{+} T cells did not show any significant defect in IFN-γ, IL-4, or TNF-α production (**Fig. 3, right three panels**), suggesting that the defect was acquired after transplantation. This defect in effector T cell function might represent a mechanism for the difference in the development of GVHD by mice receiving wild type versus IL-21R$^{-/-}$ CD4^{+} T cells.

CD4^{+} T cells were responsible for the low production of cytokines.

To elucidate the basis for diminished cytokine production, we examined the number of donor CD4^{+} T cells in the spleen at day 14-21 after transplantation. The number of donor H-2K^{d}-negative CD4^{+} T cells was significantly lower in recipients of IL-21R$^{-/-}$ CD4^{+} T cells than in recipients of wild type CD4^{+} T cells (**Fig. 4A, p=0.03, Welch t-test, n=15 vs 12**), although the ranges overlapped. As it is thought that donor T cells proliferate in secondary lymphoid organs such

as the spleen and then infiltrate into target organs (27), the reduced number of CD4$^+$ T cells in the spleen is consistent with the reduced infiltration into the liver and small intestine, as shown above (**Fig. 2**). To identify the cells responsible for defective cytokine production, we performed intracellular staining and ELISA with purified CD4$^+$ T cells. After anti-CD3/CD28 stimulation, the proportion of IFN-γ^+ and TNF-α^+ cells in splenic CD4$^+$ T cells was lower in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells than in those receiving wild type CD4$^+$ T cells (**Fig. 4B**). Moreover, post-transplantation, the levels of IFN-γ, TNF-α, and IL-4 production were significantly diminished with splenic purified CD4$^+$ T cells from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells as compared to those receiving wild type CD4$^+$ T cells (**Fig. 4C, left three panels**). Before transplantation, IL-21R$^{-/-}$ CD4$^+$ T cells did not show any defect in IFN-γ, TNF-α, and IL-4 production (**Fig. 4C, right panels**).

IL-17 production and GVHD induced by IL-17$^{-/-}$ CD4$^+$ T cells

Although IL-21 is not essential for Th17 differentiation, IL-21 can promote Th17 differentiation. To evaluate the effect of IL-21$^{-/-}$ CD4$^+$ T cell-transplantation on IL-17 production, we measured IL-17 after transplantation. As shown in **Fig 3, bottom panel**, bulk splenocytes from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells produced comparable amounts of IL-17 at day 14 and 21 after transplantation, as compared to mice receiving wild type CD4$^+$ T cells. Moreover, we found that IL-17$^{-/-}$ CD4$^+$ T cells induced lethal GVHD analogous to wild type CD4$^+$ T cells (if anything, death occurred earlier), suggesting that IL-17 is dispensable for this process, in contrast to the essential role of IL-21 as indicated by the survival of mice receiving IL-21R$^{-/-}$ CD4$^+$ T cells (**Fig. 5**).

Regulatory T cell (Treg) number in spleen

We next determined the serum concentration of the major immunosuppressive cytokine, TGF-β1 at day 6-21 after transplantation. We found an increase of TGF-β1 only after transplantation (**Fig. 6A, p=0.0003 at day 14, p=0.01 at day 21, Student's t-test**). In splenocytes from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells, the production of TGF-β1 and IL-10 by in vitro T cell stimulation was not up-regulated and in fact tended to be diminished (**Supplemental Fig. S2**), suggesting that the increase of serum TGF-β1 might

be due to cells other than T cells. As naïve T cells can differentiate into regulatory T (Treg) cells in the presence of TGF-β1 (28) and it was reported that IL-21$^{-/-}$ T cells were predisposed to differentiate into Treg (8), we also investigated whether more Treg cells were induced in recipients of IL-21R$^{-/-}$ CD4^{+} T cells. The proportion of splenic Foxp3^{+}CD4^{+} Treg phenotype cells in recipients of IL-21R$^{-/-}$ CD4^{+} T cells was higher than that in recipients of wild type CD4^{+} T cells, but the total percentage was still only ~1% (**Fig. 6B, left panel**). The absolute number was ~4 folds higher but the actual number was only ~4 x 10^{5} out of the total number of splenocytes, ~4 x 10^{7} (**Fig. 6B, right panel**). In contrast to post-transplantation, pre-transplantation-splenocytes from IL-21R$^{-/-}$ mice did not show an increase of Foxp3^{+}CD4^{+} T cells as compared to cells from wild type mice (**Supplemental Fig. S3**), suggesting that the increased Treg after transplantation was an induced Treg during GVHD reaction. For that reason, we did not deplete CD25^{+} cells prior to transplantation.

CD25 depletion did not restore the suppressed allo-reaction in vitro and did not exacerbate the ameliorated GVHD

To investigate the importance of Treg cells in diminishing GVHD, we performed a mixed lymphocyte reaction, which corresponds to allo-reaction in vitro, with or without CD25^{+}CD4^{+} T cells. As Foxp3 is an intracellular protein and Foxp3 staining cannot be used to purify or deplete Treg cells, anti-CD25 antibody is widely used for this purpose (9, 29-32). The impaired mixed lymphocyte reaction of IL-21R$^{-/-}$ CD4^{+} T cells after transplantation was not restored by CD25 depletion (**Fig. 7A**), nor was the impaired IFN-γ production by IL-21R$^{-/-}$CD4^{+} T-cells in a mixed lymphocyte reaction (**Fig. 7B**). Moreover, analogous to cytokine production by anti-CD3/CD28 stimulation (**Fig. 3**), IL-21R$^{-/-}$ CD4^{+} T cells before transplantation were not defective for allo-reaction (**Fig. 7C**).

Consistent with the in vitro experiments above, CD25^{+} depletion in vivo did not alter the severity of GVHD in recipients of IL-21R$^{-/-}$ CD4^{+} T cells, without altering the body weight loss and survival (**Fig. 8A and 8B**). In contrast, the severity of GVHD in recipients of wild type CD4^{+} T cells appeared to be slightly diminished by CD25^{+} depletion (**Fig. 8A and 8B**). In this condition, as previously

reported (30), the depletion efficacy of CD25^{+}CD4^{+} T cells was more than 95 % and that of Foxp3^{+}CD4^{+} T cells was at least 50 % (**Fig. 8C upper and lower panels**). Interestingly, Foxp3 expression was higher in CD25-negative CD4^{+} T cells from recipients of IL-21R$^{-/-}$ CD4^{+} T cells than from recipients of wild type CD4^{+} T cells (**Fig. 8D**). Together with results in vitro (**Fig. 7**), this suggests a relationship between unresponsiveness of CD25-negative CD4^{+} T cells and higher expression of Foxp3.

Discussion

Here, we have reported evidence indicating that IL-21 is critical for the pathogenesis of CD4^{+} T cell-mediated GVHD, at least in part due to effects on CD4 differentiation. In this study, we focused on CD4^{+} T cell-mediated GVHD; a role for IL-21 in CD8^{+} T cell-mediated GVHD remains to be investigated.

We found a profound defect in T cell effector function only after transplantation, although serum cytokine concentrations had no obvious difference. According to these results, T cell differentiation into Th1 and Th2 cells appeared to be altered in the absence of IL-21 during GVHD. Cytokines are believed to have both positive and negative roles in GVHD. For example, whereas T cells from IFN-γ deficient mice developed more severe GVHD (33-35), T cells from Stat4 (Th1) deficient mice exhibited less severe GVHD than T cells from wild type mice with less severe colitis (36). In contrast to IFN-$\gamma^{-/-}$ T cells, T cells from IL-4 deficient mice induced less severe GVHD (34); analogously, T cells from Stat6 (Th2) deficient mice induced less severe GVHD than those from wild type mice (36). T cells from TNF-α deficient mice developed less severe GVHD with less severe colitis (37). Our data suggest a strong correlation between the defect in effector function in recipients of IL-21R$^{-/-}$ CD4^{+} T cells and the attenuated phenotype of GVHD, indicating a role for IL-21 in this process.

IL-21 as well as IL-6 induces Th17 differentiation in the presence of TGF-β, suggesting a possible involvement of IL-17 in the phenotype we observed. However, our results with IL-17$^{-/-}$ CD4^{+} T cells demonstrated that IL-17 was dispensable for CD4^{+} T cell-mediated GVHD, indicating that the attenuated GVHD in recipients of IL-21R$^{-/-}$ CD4^{+} T cells was not due to an IL-17-related defect. During the preparation of this manuscript, a role for IL-17 in GVHD was reported (38-40). These reports varied but one report suggested that the lack of IL-17 promotes GVHD (38). Another report suggested that IL-17$^{-/-}$ CD4^{+} T cells can ameliorate GVHD only at the beginning of GVHD, which suggested a promoting effect for IL-17 at an early stage of GVHD (39). The third report suggested that ex vivo differentiated Th17 cells induced skin and lung GVHD (40). Thus, the role of IL-17 may be complex and dependent on the specific experimental conditions.

Because there are reciprocal relationships between Th1/Th2 and regulatory

T cell differentiation (41-43) and between IL-21 and regulatory T cell differentiation (8), we investigated the level of Treg cells in the spleens of recipients. Foxp3^{+}CD4^{+} T cells were increased in percentage and absolute number but still represented only ~1% of splenocytes. Regarding the relationship between the defective effector T cells function and the increased number of Treg cells, it is possible that increased Treg cells suppress functional effector T-cells, but alternatively, it is also possible that effector differentiation itself is defective and the resulting effector T cells cannot respond to allo-antigen, analogous to the situation in T cell anergy, and that the increased Treg cell number is also a result of a dysregulated differentiation. Our results here might be more consistent with the latter possibility, given that Treg depletion by anti-CD25 treatment did not alter the results in vitro and in vivo, although the efficiency of depletion of Foxp3^{+}CD4^{+} T cells in vivo was incomplete. It is also conceivable that the up-regulation of Foxp3 in CD25-negative CD4^{+} T cells (which would not be removed by CD25^{+} depletion) in the absence of IL-21 signaling might result in unresponsiveness or poor-responsiveness of effector T cells, and also that more than one mechanism can contribute to the attenuated GVHD.

Disclosures

Drs. Katsutoshi Ozaki and Warren J. Leonard are inventors on patents and patent applications related to IL-21.

References

1. Parrish-Novak J., S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Madden, W. Xu, J. West, S. Schrader, S. Burkhead, M. Heipel, C. Brandt, J. L. Kuijper, J. Kramer, D. Conklin, S. R. Presnell, J. Berry, Shiota F, Bort S, Hambly K, Mudri S, Clegg C, Moore M, Grant FJ, Lofton-Day C, T. Gilbert, F. Rayond, A. Ching, L. Yao, D. Smith, P. Webster, T. Whitmore, M. Maurer, K. Kaushansky, R. D. Holly, and D. Foster. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature. 408: 57-63.

2. Spolski R., and W. J. Leonard. 2008. Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu Rev Immunol. 26: 57-79.

3. Ozaki K., K. Kikly, D. Michalovich, P. R. Young, and W. J. Leonard. 2000. Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. Proc. Natl. Acad. Sci. USA 97: 11439-114344.

4. Brandt K., S. Bulfone-Paus, D. C. Foster, and R. Rückert. 2003. Interleukin-21 inhibits dendritic cell activation and maturation. Blood. 102: 4090-4098.

5. Ozaki K., Hishiya A, Hatanaka K, Nakajima H, Wang G, Hwu P, Kitamura T, Ozawa K, Leonard WJ, Nosaka T. 2006. Overexpression of IL-21 induces expansion of hematopoietic progenitor cells. Int. J. Hemat. 84; 224-230.

6. Ozaki K., R. Spolski, C. G. Feng, C. F. Qi, J. Cheng, A. Sher, H. C. 3rd Morse, C. Liu, P. L. Schwartzberg, and W. J. Leonard. 2002. A critical role for IL-21 in regulating immunoglobulin production. Science. 298: 1630-1634.

7. Zhou L., I. I. Ivanov, R. Spolski, R. Min, K. Shenderov, T. Egawa, D. E. Levy, W. J. Leonard, and D. R. Littman. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat. Immunol. 8: 967-974.

8. Nurieva R., X. O. Yang, G. Martinez, Y. Zhang, A. D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S. S. Watowich, A. M. Jetten, and C. Dong. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature. 448: 480-483.

9. Korn T., E. Bettelli, W. Gao, A. Awasthi, A. Jäger, T. B. Strom, M. Oukka, and V. K. Kuchroo. 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 448: 484-487.

10. Wurster A. L., V. L. Rodgers, A. R. Satoskar, M. J. Whitters, D. A. Young, M. Collins, and M. J. Grusby. 2002. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells. J. Exp. Med. 196: 969-977

11. Pesce J., M. Kaviratne, T. R. Ramalingam, R. W. Thompson, J. F. Jr Urban, A. W. Cheever, D. A. Young, M. Collins, M. J. Grusby, and T. A. Wynn. 2006. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. J. Clin. Invest. 116: 2044-2055.

12. Fröhlich A., B. J. Marsland, I. Sonderegger, M. Kurrer, M. R. Hodge, N. L. Harris, M. Kopf. 2007. IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. Blood. 109: 2023-2031.

13. Strengell M., T. Sareneva, D. Foster, I. Julkunen, and S. Matikainen. 2002. IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. J.Immunol. 169: 3600-3605.

14. Coquet, J. M., S. Chakravarti, M. J. Smyth, and D. I. Godfrey. 2008. IL-21 is not essential for Th17 differentiation or experimental autoimmune encephalomyelitis. J. Immunol. 180: 7097-7101.

15. Sonderegger, I., J. Kisielow, R. Meier, C. King, and M. Kopf. 2008. IL-21 and IL-21R are not required for development of Th17 cells and autoimmunity in vivo.

Eur. J. Immunol. 38:1833-1838.

16. Ozaki, K., R. Spolski, R. Ettinger, H. P. Kim, G. Wang, C. F. Qi, P. Hwu, D. J. Shaffer, S. Akilesh, D. C. Roopenian, H. C. 3rd Morse, P. E. Lipsky, and W. J. Leonard. 2004. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. J. Immunol. 173: 5361-5371.

17. Bubier, J. A., T. J. Sproule, O. Foreman, R. Spolski, D. J. Shaffer, H. C. 3rd Morse, W. J. Leonard, and D. C. Roopenian. 2009. A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB- Yaa mice. Proc. Natl. Acad. Sci. U S A. 106: 1518-1523.

18. King, C, A. Ilic, K. Koelsch, and N. Sarvetnick. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. 2004. Cell. 117: 265-277.

19. Spolski R., M. Kashyap, C. Robinson, Z. Yu, and W. J. Leonard. 2008. IL-21 signaling is critical for the development of type I diabetes in the NOD mouse. Proc. Natl. Acad. Sci. USA 105: 14028-14033.

20. Sutherland A. P., T. Van Belle, A. L. Wurster, A. Suto, M. Michaud, D. Zhang, M. J. Grusby, and M. von Herrath. 2009. IL-21 is required for the development of type 1 diabetes in NOD mice. Diabetes. 58: 1144-1155

21. Shlomchik W. D. 2007. Graft-versus-host disease. Nat. Rev. Immunol. 7: 340-352.

22. Meguro A., K. Ozaki, I. Oh, K. Hatanaka, H. Matsu, Tatara, R. K. Sato, W. J. Leonard, and K. Ozawa. 2010. IL-21 is critical for graft-versus-host disease in a mouse model. Bone Marrow Transplant. 45: 723-729.

23. Nakae S., Y. Komiyama, A. Nambu, K. Sudo, M. Iwase, I. Homma, K.

Sekikawa, M. Asano, and Y. Iwakura. 2002. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. Immunity. 17: 375-387.

24. Sakoda Y, D. Hashimoto, S. Asakura, K. Takeuchi, M. Harada, M. Tanimoto, T. Teshima. 2007. Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease. Blood. 109(4):1756-64

25. Aoyama K, M. Koyama, K. Matsuoka, D. Hashimoto, T. Ichinohe, M. Harada, K. Akashi, M. Tanimoto, T. Teshima. 2009. Improved outcome of allogeneic bone marrow transplantation due to breastfeeding-induced tolerance to maternal antigens. Blood. 113(8):1829-33

26. Teshima T., G. R. Hill, L. Pan, Y. S. Brinson, M. R. van den Brink, K. R. Cooke, and J. L. Ferrara. 1999. IL-11 separates graft-versus-leukemia effects from graft-versus-host disease after bone marrow transplantation. J. Clin. Invest. 104: 317-325.

27. Beilhack A., S. Schulz, J. Baker, G. F. Beilhack, C. B. Wieland, E. I. Herman, E. M. Baker, Y. A. Cao, C. H. Contag, and R. S. Negrin. 2005. In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. Blood. 106: 1113-1122.

28. Sakaguchi S, T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. Cell. 133: 775-787.

29. Sakaguchi S, N. Sakaguchi, M . Asano, M. Itoh, M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 155:1151-64.

30. Liesz A, E. Suri-Payer, C. Veltkamp, H. Doerr, C. Sommer, S. Rivest, T. Giese, R. Veltkamp. 2009. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. Nat Med. 15(2):192-9

31. Fujita S, Y. Sato, K. Sato, K. Eizumi, T. Fukaya, M. Kubo, N. Yamashita, K. Sato. 2007. Regulatory dendritic cells protect against cutaneous chronic graft-versus-host disease mediated through CD4+CD25+Foxp3+ regulatory T cells. Blood. 110(10):3793-803

32. Radojcic V, MA. Pletneva, HR. Yen, S. Ivcevic, A. Panoskaltsis-Mortari, AC. Gilliam, CG. Drake, BR. Blazar, L. Luznik. 2010. STAT3 signaling in CD4+ T cells is critical for the pathogenesis of chronic sclerodermatous graft-versus-host disease in a murine model. J Immunol. 184:764-74.

33. Yang Y. G., B. R. Dey, J. J. Sergio, D. A. Pearson, and M. Sykes. 1998. Donor-derived interferon gamma is required for inhibition of acute graft-versus-host disease by interleukin 12. J Clin Invest. 102: 2126-2135

34. Murphy W. J., L. A. Welniak, D. D. Taub, R. H. Wiltrout, P. A. Taylor, D. A. Vallera, M. Kopf, H. Young, D. L. Longo, and B. R. Blazar. 1998. Differential effects of the absence of interferon-gamma and IL-4 in acute graft-versus-host disease after allogeneic bone marrow transplantation in mice. J Clin Invest. 102: 1742-1748.

35. Wang H., W. Asavaroengchai, B. Y. Yeap, M. G. Wang, S. Wang, M. Sykes, and Y. G. Yang. 2009. Paradoxical effects of IFN-{gamma} in graft-vs.-host (GVH) disease reflect promotion of lymphohematopoietic GVH reactions and inhibition of epithelial tissue injury. Blood. 113: 3612-3619.

36. Nikolic B., S. Lee, R. T. Bronson, M. J. Grusby, and M. Sykes. 2000. Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. J Clin Invest. 105: 1289-1298.