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IL-21 is critical for graft-versus-host disease in a mouse model

A Meguro[1], **K Ozaki**[1], **I Oh**[1], **K Hatanaka**[1], **H Matsu**[1], **R Tatara**[1], **K Sato**[1], **WJ Leonard**[2], and **K Ozawa**[1]

[1]Division of Hematology, Department of Medicine, Jichi Medical University Tochigi 329-0498, Japan

[2]Laboratory of Molecular Immunology, National Heart, Lung and Blood Institute National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Immunological effects of IL-21 on T-, B-, and NK-cells have been reported, but the role for IL-21 in graft-versus-host disease (GVHD) remains obscure. Here we demonstrate that morbidity and mortality of GVHD was significantly reduced after bone marrow transplantation with splenocytes from IL-21R$^{-/-}$ mice compared to those from wild type mice. To further confirm our observation, we generated a decoy receptor for IL-21. GVHD was again less severe in mice receiving bone marrow cells transduced with the IL-21 decoy receptor than control mice These results suggest that IL-21 critically regulates GVHD and that blockade of the IL-21 signal may represent a novel strategy for the prophylaxis for GVHD.

Keywords

GVHD; IL-21; BMT; hematopoietic stem cell transplantation

Introduction

IL-21 is a member of the common cytokine receptor gamma chain (γ_c) family that also includes IL-2, IL-4, IL-7, IL-9, and IL-15. The receptors for each of these cytokines are composed of more specific chain(s) and a γ_c chain.[1,2] The lack of functional γ_c causes X-linked severe combined immunodeficiency disease (X-SCID) in humans[3], which is characterized by a reduced number of T-cells and NK-cells, and a normal number but non-functional B cells. The phenotypes of IL-7 and IL-15 knockout mice suggest that the reduced number of T- and NK-cells are due to the lack of IL-7 and IL-15 signaling, respectively.[4,5] We previously proposed that the non-functionality of B-cells might be attributed to the lack of both IL-4 and IL-21, based on the observation that IL-4 and IL-21R double knockout mice exhibit a B-cell phenotype similar to that found in XSCID.[6] The switch from immunoglobulin IgM to IgG was impaired but still present in IL-21R$^{-/-}$ mice.[6]

Interleukin-21 (IL-21) was cloned as a co-stimulatory cytokine for T-cell proliferation and NK-cell expansion *in vitro*.[7] IL-21 is primarily produced by CD4 T-cells[7], and the receptor is primarily expressed on T, B, and NK cells.[7,8] It has been reported that IL-21 suppresses the function of dendritic cells[9] and increases hematopoietic progenitor cells.[10] IL-21 has been shown to play critical roles in immunoglobulin production.[6] IL-21 is not required but promotes Th17 differentiation in the presence of TGF-β[11–13] whereas reports have differed

Correspondence: Dr. K. Ozaki, Division of Hematology, Department of Medicine, Jichi Medical University, Yakushiji 3311-1, Shimotsuke-shi, Tochigi 329-0498, Japan, Phone: 81-285-58-7353, Fax: 81-285-44-5258, ozakikat@jichi.ac.jp.

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regarding its contributions to Th1- and Th2-differentiation[6,14–17]. Evidence for a relationship between IL-21 and autoimmune disease has been accumulating. For example, overexpression of IL-21 induces inflammation, and in the BXSB.6-Yaa^{+}/J mouse model of SLE, affected mice have high levels of IL-21[18] whereas IL-21R$^{-/-}$ BXSB.6-YAA^{+}/J mice no longer develop SLE.[19] In addition, IL-21R$^{-/-}$ NOD mice are resistant to the development of diabetes mellitus.[20,21]

Graft-versus-host disease (GVHD) is a serious complication following hematopoietic stem cell transplantation.[22] Severe GVHD is difficult to treat because of refractory characteristics and infectious complications resulting from immunosuppressive treatment.

Here, we have investigated the function of IL-21 in GVHD and demonstrate that IL-21 is critical for GVHD and that blockade of the IL-21 signal could lead to a treatment or prophylaxis for GVHD.

Methods

Mice

IL-21R$^{-/-}$ (KO) mice were generated previously.[6] Balb/c mice were purchased from Clea Japan (Tokyo, Japan). Perforin deficient mice and FasL deficient (g*ld/gld*) mice were purchased from Taconic (Hudson, NY) and Japan SLC (Shizuoka, Japan), respectively. All mice were housed in our mouse facility, which is regulated by an intramural small animal committee, and were treated in accordance with the guidelines of Jichi Medical University.

Mouse models of GVHD and GVL

Clinical symptoms of GVHD were scored as previously reported.[23]

GVHD group 1 (WT-BM and KO-SP vs. WT-BM and WT-SP)—Balb/c mice were irradiated with 8 Gy and injected intravenously with 5×10^6 WT-BM and 5×10^6 splenocytes (SP) from either WT or KO mice.

GVHD group 2 (KO-BM and KO-SP vs. KO-BM and WT-SP)—To delete the contaminated wild type T cells in BM, we used KO-BM.

Flow cytometric analysis

Fc-block® (BD Biosciences-Pharmingen, San Diego, CA) was used to prevent non-specific antibody binding to Fc receptors. Anti-CD4, CD8, H-2^{b}, and H-2^{d} antibodies were purchased from BD Biosciences-Pharmingen. A LSR flow cytometer (BD Biosciences-Immunocytometry Systems, San Jose, CA) was used for data collection, and the data were analyzed using CellQuest software (BD Biosciences-Immunocytometry Systems).

Decoy receptor of IL-21

The primers, 5'-TTCTAGCTACCAGCTGCAGGT-3' and 5'-TCCTGAAGTTCCTCATATTCA-3', were used to produce a truncated IL-21R lacking the region from box 1 to the C-terminus.[8] All nucleotide sequences were confirmed by sequencing. Extracellular expression of this receptor was confirmed by flow cytometric analysis using anti-IL-21-receptor polyclonal antibody (R&D Systems, Minneapolis, MN) and a secondary antibody conjugated with FITC (R&D Systems).

Retrovirus mediated transduction into BM

A retrovirus construct containing the decoy receptor of *IL-21*, was transfected into the packaging cell line, PLAT-E, and the viruses produced were transduced into pre-stimulated BM cells using Retronectin® according to the manufacturer's instructions (Takara, Osaka, Japan). We used 5–15 × 10^5 BM cells from 5-FU injected mice at the beginning of cultivation.

CFSE (Carboxyfluorescein diacetate succinimidyl ester) staining

Splenocytes were stained with CFSE for 15 minutes in PBS, washed, and injected into irradiated mice. Staining was performed according to the manufacturer's instructions (Invitrogen-Molecular prove, Carlsbad, CA).

Enzyme linked immunosorbent assay (ELISA)

ELISA kits for cytokines, IFN-γ, and IL-4 were purchased form BD Bioscience or R&D Systems. Concentrations were determined according to the manufacturer's instructions.

Statistical analysis

Kaplan-Meyer plots were used to compare survival rates. The Logrank test was used to evaluate p values. The Mann-Whitney U test was used to calculate p values for the GVHD clinical score. Statistical analyses were performed using "Stat Mate ver. 6" (ATMS, Tokyo, Japan). Unless otherwise specified, all error bars in this study are S.E.M.

Results

IL-21R$^{-/-}$ splenocytes (KO-SP) cause attenuated GVHD with wild type bone marrow

A well-established MHC-mismatch CD4 T cell-mediated GVHD model, C57BL/6 (H-2^b) → Balb/c (H-2^d) was used to evaluate the role for IL-21 in GVHD. Bone marrow cells (BM) and splenocytes were taken from donor C57BL/6 wild type (WT) or IL-21R KO mice and transplanted into irradiated Balb/c mice. Mice that were recipients of BM and splenocytes from WT mice became ill after 3–4 weeks and suffered from diarrhea, ruffled hair, a hunched posture, and diminished body weight. In contrast, mice transplanted from IL-21R KO mice showed attenuated GVHD symptoms and survived longer (Fig. 1A, p=0.0003, Logrank test). Strikingly, no mouse receiving the wild type splenocytes group survived more than 4 months, while 40 % of the mice recieving the IL-21R KO splenocytes survived for 4 months or longer. Consistent with this, clinical GVHD scores in mice transplanted from IL-21R KO-spenlocytes were significantly less than those in controls (Fig. 1B). In both groups, ~85% engraftment in peripheral blood was achieved, indicating that the difference was not attributable to a difference in engraftment.

KO-SP cause attenuated GVHD when combined with KO bone marrow

In the preceding experiments (Fig. 1A and 1B), we used un-fractionated wild type BM, which presumably contained IL-21R-expressing T cells that could respond to IL-21. To eliminate contaminating IL-21R$^{+/+}$ T cells from BM, we next used BM from IL-21R KO mice, in combination with splenocytes from either WT or KO mice. This resulted in enhanced differences between the groups receiving the wild type versus IL-21R KO-SP after two months: 0 % versus 60 % (Fig. 1C, p=0.0001 by Logrank test), respectively, confirming the former results (Fig. 1A and 1B) and a critical role for IL-21 in GVHD. Consistent with this, clinical GVHD scores in the wild type splenocyte group were higher than those in the IL-21R KO-SP group (Fig. 1D).

CD4/CD8 proliferation and serum cytokine levels

To investigate the mechanisms underlying the attenuated GVHD in IL-21R KO-SP transplanted mice, we first counted the number of CD4 and CD8 cells in the spleens 14 days after transplantation. In both IL-21R KO-SP and WT-SP transplanted mice, both cell types were similar in number (Table 1). Next, splenocytes were stained with CFSE to determine their proliferation in mice after transplantation. Both CD4 and CD8 cells from either WT-SP or IL-21R KO-SP transplanted mice showed similar proliferation profiles at day 3 (Fig. 2A). Serum concentrations of IFN-γ and IL-4 at day 14 (Fig. 2B), those at day 6 (Fig. 2C), and IFN-γ and IL-4 produced by recipients' splenocytes stimulated with allogeneic splenocytes *in vitro* were comparable between two groups (Fig. 2D).

Effects of additional CD4 or CD8 cells on survival

To investigate whether $CD4^+$ T cells are more important for GVHD than $CD8^+$ T cells in our system, we added either WT-$CD4^+$ or IL-21R KO-$CD4^+$ T cells onto the results-predictable transplantation with IL-21R KO-BM and IL-21R KO-SP (Fig 1C). The addition of WT-$CD4^+$ cells resulted in a significantly worse survival than seen with the addition of IL-21R KO-$CD4^+$ cells (Fig. 3A, p=0.0024, Logrank method). In contrast to this, the difference between WT-$CD8^+$ and IL-21R KO-$CD8^+$ T cells did not reach statistical significance (Fig 3B, p=0.09, Logrank method), although they have similar tendency, suggesting that WT-$CD4^+$ rather than WT-$CD8^+$ cells is more important for GVHD at least in our conditions.

A decoy receptor of IL-21 ameliorates GVHD

To develop a treatment or prophylaxis model in mice and exclude the possibility that the ameliorated GVHD is due to an intrinsic defect in the IL-21R KO-splenocytes, we designed a decoy receptor of IL-21, which contains its signal sequence, extracellular domain, and transmembrane domain, but a truncated intracellular domain. The cDNA encoding this truncated IL-21R was produced by polymerase chain reaction, sequenced, and its extracellular expression in 293 cells was confirmed by flow cytometric analysis using an anti-IL-21R antibody (Fig. 4A). In 293 cells, we confirmed that the IL-21R decoy reduced IL-21 concentration in medium *in vitro* (Fig. 4B). Using retroviral transduction, we expressed the decoy receptor in IL-21R KO-BM cells and transplanted them concomitantly with wild type splenocytes. We performed three independent experiments and evaluated the survival. Two weeks after transplantation, transduction efficiencies were determined in peripheral blood by measuring GFP-positive cells, and were found to be approximately 30–70% (Fig. 4C). In an additional experiment, in which transduction efficiency was 40%, the serum concentration of IL-21 was not detectable in decoy receptor transduced BM recipients, whereas those transduced with the empty vector showed 22 pg/ml in average (Fig. 4D), indicating that decoy receptor significantly diminished serum IL-21 *in vivo*. Although most of the expression was transient, decoy IL-21R-transduced BM resulted in prolonged survival compared to empty-vector transduced BM (Fig. 4E, p=0.003, Logrank test), suggesting that blockade of the IL-21 signal might be an effective treatment or prophylaxis for GVHD. These results are consistent with the results shown above (Fig. 1), and support the hypothesis that IL-21 promotes GVHD.

The attenuated GVHD appeared to be independent of cytotoxic molecules

The significance of cytotoxic molecules, such as perforin and granzyme B in GVHD has been reported.[24] To investigate the relationship between these molecules and the attenuated GVHD, we sought to see the levels of expression of these molecules in $CD4^+$ and $CD8^+$ T cells. No clear difference between WT-SP and IL-21R KO-SP transplanted mice was detected (Fig. 5A). Moreover, we used a combination of IL-21 decoy receptor and *perforin*

deficient mice. The IL-21 decoy receptor-transduced BM improved survival of recipients with splenocytes from *perforin* deficient mice, suggesting that the mechanism of the attenuated GVHD is primarily independent of perforin *in vivo* (Fig 5B). The Fas/FasL system is another cytotoxic molecule and was reported to be important for GVHD rather than GVL.[25,26] To investigate a possible role for Fas/FasL in the attenuated GVHD, we analyzed the effect of IL-21 decoy receptor transduced BM on survival of recipients with splenocytes from FasL deficient (*gld/gld*) mice and found enhanced survival, suggesting that the mechanism of the attenuated GVHD is also primarily independent of Fas/FasL (Fig 5C).

Discussion

Here, we report that IL-21 is critical for GVHD. Splenocytes from IL-21R$^{-/-}$ (KO) mice induced less severe GVHD than those from wild type mice. Moreover, a decoy receptor for IL-21 attenuates GVHD and prolongs survival, confirming the former experiments with KO mice and suggesting that a possible clinical application of blocking IL-21 for the treatment or prophylaxis of GVHD.

Serum IFN-γ and IL-4 concentrations, CD4/CD8 T cell proliferation at day 3 after transplantation, and the number of CD4$^+$/CD8$^+$ T cells in spleens of recipients at day 14 after transplantation, all appeared to be comparable between two groups at least under the conditions we have tested. In addition, the decoy IL-21R also ameliorated GVHD induced by *FasL*-deficient and *perforin*-deficient splenocytes, suggesting that Fas/FasL and perforin are not crucial for the mechanism of the attenuated GVHD by IL-21R KO splenocytes. However, we could not rule out the possibility that the other transplantation conditions might make the difference more profound and lead to interpretation of the mechanisms. Results of additional CD4$^+$/CD8$^+$ experiments suggested the importance of CD4$^+$ cells. We are now investigating the process by using other transplantation models, such as purified CD4$^+$ T cell transplantation.

Although we have shown that IL-21 is critical for GVHD in two different systems using both IL-21R KO mice and decoy receptor for IL-21, the molecular mechanisms by which blocking IL-21 attenuates GVHD are not clear at this point.

Because the transplantation model we used is an MHC-mismatch CD4$^+$ cell-mediated GVHD, a role for IL-21 in both CD8$^+$ cell-mediated GVHD and MHC-matched GVHD is possible and an area for future investigation.

Here we opened a new insight for mechanisms of GVHD. These results would help understanding the mechanisms of GVHD and developing a treatment for GVHD. In fact, we could demonstrate that IL-21 blockade is a potentially novel approach for the treatment or prophylaxis of GVHD in mouse model. Further *in vivo* and *in vitro* studies, including humans, are required to potentially translate these results from mouse models to human diseases.

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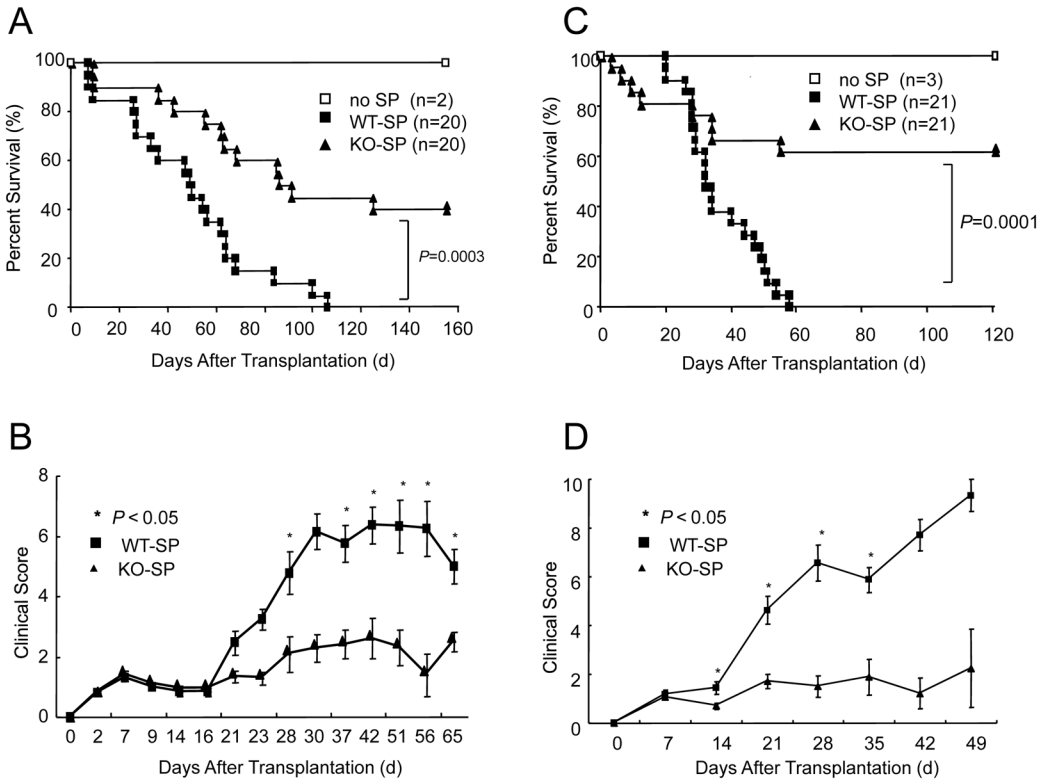

Figure 1.

IL-21R$^{-/-}$ (KO) splenocytes ameliorated GVHD. (**A and B**) Survival and clinical score of GVHD with bone marrow cells from wild type C57BL/6 mice (WT-BM). Balb/c recipients were transplanted with 5×10^6 WT-BM and either 5×10^6 splenocytes from IL-21R KO (Katsu—I would just use KO because that's how the text reads and what is on the figures) C57BL/6 mice (KO-SP) or WT-SP. Open squares (n=2), filled squares (n=20), and filled triangles (n=20) indicate transplants without splenocytes as controls, with WT-SP, and with IL-21R KO-SP, respectively. The combined results of three independent experiments are shown. P values were calculated by the Logrank method. **(C and D)** Survival and clinical scores of GVHD with IL-21R KO-BM. Balb/c recipients were transplanted with 5×10^6 IL-21R KO-BM and either 5×10^6 IL-21R KO-SP or WT-SP. Open squares (n=3), filled squares (n=21), and filled triangles (n=21) indicate transplants without splenocytes as controls, with WT-SP, and with IL-21R KO-SP, respectively. The combined results of three independent experiments are shown. P values were calculated by the Logrank method.

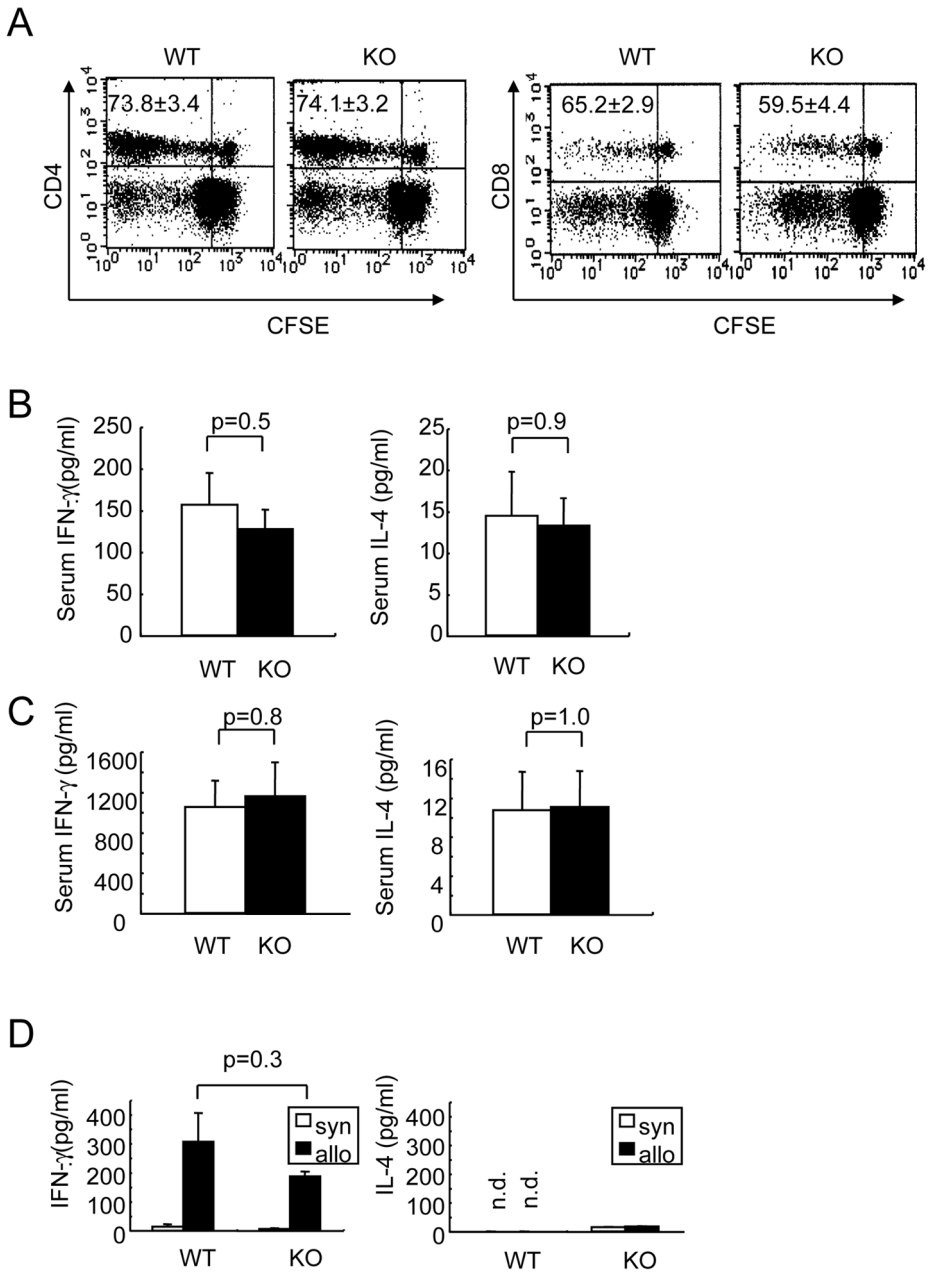

Figure 2.

Proliferation and cytokine production after transplantation. (**A**) Flow cytometric analysis of CD4/CD8 proliferation *in vivo* by CFSE staining. Splenocytes (H-2^b, 1×10^8) from either wild type or IL-21R KO mice were stained with CFSE and transplanted into irradiated Balb/c mice; three days later, splenocytes were collected and analyzed. Donor cells were selected by gating on H-2^{d-}. The numbers at left quadrants were the percentage of dividing cells in CD4 or CD8 cells (mean ± S.E.M.). Total number of recipients analyzed was 6 mice in each group. (**B**) Serum cytokine concentrations at day 14 after transplantation. Balb/c recipients were transplanted with 5×10^6 IL-21R KO-BM and either 5×10^6 IL-21R KO-SP or WT-SP. Serum was sampled at day 14 after transplantation, and cytokine concentrations of IFN-γ (**left panel**) and IL-4 (**right panel**) were determined by ELISA. Total number of

recipients analyzed was more than 6 mice in each group. (**C**) Serum cytokine concentrations at day 6 after transplantation. C57BL/6-DBA2 F1 recipients were transplanted with 1×10^8 splenocytes from wild type or IL-21R KO mice. Serum was sampled at day 6 after transplantation, and cytokine concentrations of IFN-γ (**left panel**) and IL-4 (**right panel**) were determined by ELISA. Total number of recipients analyzed was 5 mice in each group. (**D**) Transplantations were performed as in (**C**). At day 6, splenocytes (1×10^5) from recipients of WT-SP or IL-21R KO-SP were incubated with 30 Gy irradiated 4×10^5 splenocytes from C57BL/6 (syngeneic) or C57BL/6-DBA2 F1 (allogeneic) mice. After 72 hours, cytokine concentrations in the supernatants were determined by ELISA. Total number of recipients analyzed was 5 mice in each group.

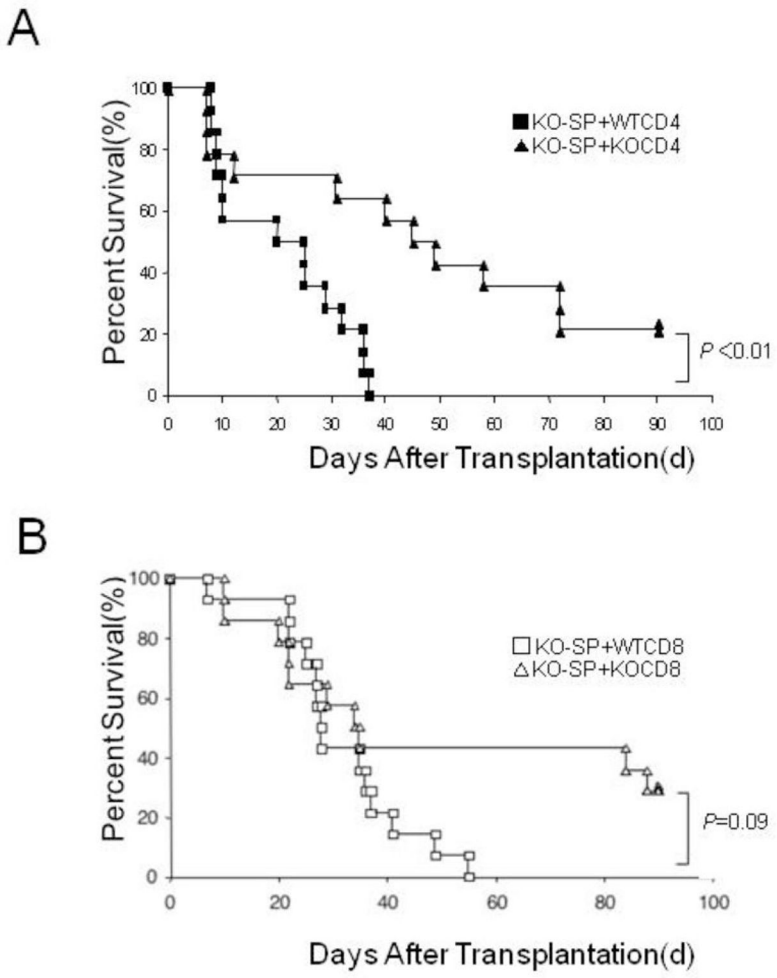

Figure 3.

Effects of additional CD4 or CD8 T cells on survival. (**A**) Survival curve with addition of either WT- or IL-21R KO-CD4 cells. Transplantations were performed as in Fig. 1C. CD4 T cells (5×10^5) from either WT or KO mice were transplanted concomitantly with KO-BM (5×10^6) and KO-SP (5×10^6). (**B**) Survival curve with addition of either WT- or IL-21R KO-CD8 cells. CD8 T cells (5×10^5) from either WT or IL-21R KO mice were transplanted with IL-21R KO-BM (5×10^6) and IL-21R KO-SP (5×10^6). The combined results of three independent experiments are shown. P value was calculated by Logrank method.

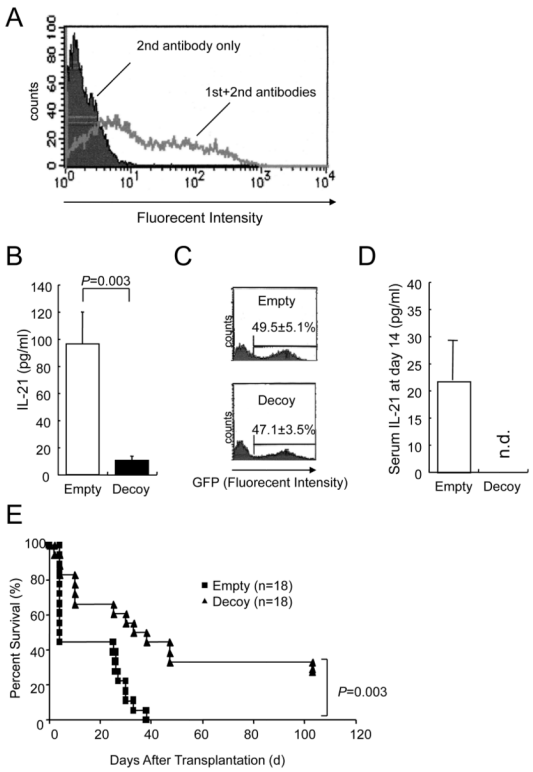

Figure 4.

Forced expression of IL-21 decoy receptor ameliorated GVHD. (**A**) Expression of the IL-21 decoy receptor in the 293 cells. (**B**) In vitro activity of IL-21 decoy receptor. 293 cells were transduced with either the IL-21 decoy receptor or empty vector, and then recombinant mouse IL-21 (1000 pg/ml) was added to the medium. IL-21 levels were determined by ELISA after 18 hours. Shown is mean ± S.D. from three independent experiments (p=0.003). (**C**) Transduction efficiency in peripheral blood two weeks after transplantation. Transduction efficiency was determined by flow cytometric analysis. Transduced cells are GFP-positive since the vector contains IRES (internal ribosomal entry site)-GFP. (**D**) Serum concentration of IL-21 at day 14 after transplantation. In this particular experiment, transduction efficiency was ~40% in both groups. Total mice analyzed were four empty vector transduced BM recipients and five decoy receptor transduced BM recipients. (**E**) Survival curve after forced expression of the IL-21 decoy receptor. Balb/c recipients were irradiated with 8 Gy and transplanted with wild type splenocytes (5×10^6) and either empty-vector or decoy-receptor transduced BM (starting dose at 5×10^5). Filled squares and filled triangles indicate transplantions with empty-vector transduced BM (n=18) and decoy-receptor transduced BM (n=18), respectively. The combined results of three independent experiments are shown (p=0.003, Logrank test).

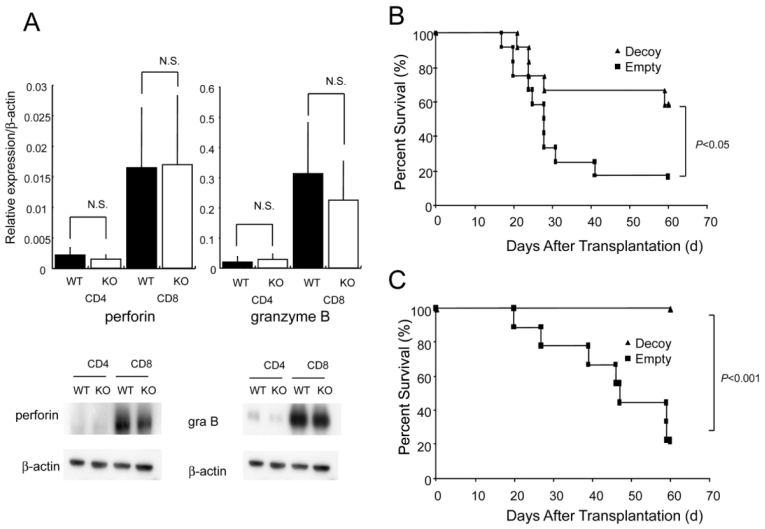

Figure 5.

The ameliorated GVHD is dependent on neither FasL nor perforin. (**A**) Perforin and granzyme B expression in CD4/CD8 cells. C57BL/6-DBA2 F1 recipients were transplanted with 1×10^8 splenocytes from wild type or IL-21R$^{-/-}$ mice. tRNA and protein were harvested from CD4 or CD8 cells purified from transplanted mice at day 6 after transplantation. Results of RT-qPCR and Western blot analysis are shown in upper and lower panel, respectively. (**B**) Survival curve with splenocytes from perforin deficient mice. C57BL/6-DBA2 F1 recipients were irradiated with 11 Gy and transplanted with either empty-vector or decoy-receptor transduced BM (final cell number: $3–3.2 \times 10^6$) with splenocytes from perforin deficient mice ($3.75–5 \times 10^7$). Filled squares and filled triangles indicate transplantations with empty-vector transduced BM (n=9) or decoy-receptor transduced BM (n=9), respectively. The combined results of two independent experiments are shown. *P* value was calculated by the Logrank method. Transduction efficiencies two weeks after transplantation in peripheral blood were around 30–70% like Fig. 4C. (**C**) Survival curve with splenocytes from FasL deficient mice (*gld/gld* mice). C57BL/6-DBA2 F1 recipients were irradiated with 11 Gy and transplanted with either empty-vector or decoy-receptor transduced BM (final cell number: $3.2–3.5 \times 10^6$) with splenocytes from *gld/gld* mice ($8–10 \times 10^7$). Filled squares and filled triangles indicate transplantations with empty-vector transduced BM (n=12) or decoy-receptor transduced BM (n=12), respectively. The combined results of two independent experiments are shown. The P value was calculated by the Logrank method.

Table 1

Donor $CD4^+/CD8^+$ T-cell numbers in spleen at day 14 after transplantation.

	$CD4^+$	**$CD8^+$**
WT spleen (n=6)	$2.9 \pm 1.2^*$	$3.2 \pm 1.3^*$
KO spleen (n=10)	$3.7 \pm 1.1^*$	$3.3 \pm 1.2^*$
P value	N.S.	N.S.

N.S.=not significant ($p \leq 0.05$) by Student's t-test;

*mean ± S.E.M. ($\times 10^{-6}$) from three independent experiments