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Altered Effector CD4$^+$ T Cell Function in IL-21R$^{-/-}$ CD4$^+$ T Cell-Mediated Graft-Versus-Host Disease

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We previously showed that transplantation with *IL-21R* gene-deficient splenocytes resulted in less severe graft-versus-host disease (GVHD) than was observed with wild type splenocytes. In this study, we 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 MLR and cytokine production by CD4$^+$ T cells were impaired. These results suggest that IL-21 might promote GVHD through enhanced production of effector CD4$^+$ T cells. Moreover, we found that CD25 depletion altered neither the impaired MLR 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. *The Journal of Immunology*, **2010, 185: 1920–1926.**

Interleukin-21 was discovered as a costimulatory 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 was also reported that IL-21 suppresses dendritic cell function (4) and increases hematopoietic progenitor cells (5). IL-21 is known to play critical roles in Ig production (6), whereas reports have differed regarding its contributions to Th1-, Th2-, and Th17-mediated effects and differentiation (6–15). IL-21 contributes to Th17 differentiation, but it 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 a systemic lupus erythematosus model mouse (the BXSB.6-Yaa$^+$/J) with high serum levels of IL-21 (16), the development of disease is abrogated when these mice are crossed to IL-21R knockout (KO) mice (17). In addition, autoimmune 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 *IL-21R* gene-disrupted splenocytes resulted in less severe GVHD than was seen with wild type (WT) splenocytes (22). We sought to elucidate the mechanism(s) for this observation; in this article, we 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 on a C57BL/6 background. 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 wk 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 university guidelines.

In vitro T cell stimulation and MLR

Cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine (Invitrogen), 50 μM 2-ME (Sigma-Aldrich), 0.1 mg/ml streptomycin, and 100 U/ml penicillin G (Invitrogen). Nonspecific pan T cell stimulation was performed using anti-CD3/CD28 beads for 3 d, according to the manufacturer's instructions (Dynal Biotech, Oslo, Norway). Alloantigen-specific T cell stimulation was induced by cocultivation of CD4 T cells with 30 Gy-irradiated splenocytes from C57BL/6-DBA2-F1 mice for 4 d.

GVHD models

We used IL-21R$^{-/-}$ bone marrow (BM) to eliminate the effects of WT T cells in BM. We compared transplantations with IL-21R$^{-/-}$ CD4$^+$ T cells versus WT CD4$^+$ T cells. C57BL/6-DBA2-F1 mice were irradiated with 11 Gy and injected i.v. with 5×10^6 IL-21R$^{-/-}$ BM and 5×10^6 purified CD4$^+$ T cells from WT or IL-21R$^{-/-}$ mice. The cells were purified using CD4 microbeads and AutoMACS (Miltenyi Biotec, Tokyo, Japan); the purity was >80–90%.

Pathological analysis

Two weeks after transplantation, mice were sacrificed; liver, skin, and intestine were subjected to formalin fixation, paraffin embedding, excision, and H&E staining. Photographs were taken with an Olympus BX51 microscope at ×400 magnification.

Flow cytometric analysis

Fc-block (BD Biosciences, San Jose, CA) was used to prevent nonspecific Ab binding to Fc receptors. Abs to CD4 (RM4-5), CD8 (Ly-2), CD25

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Abbreviations used in this paper: BM, bone marrow; GVHD, graft-versus-host disease; KO, knockout; Treg, regulatory T; WT, wild type.

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(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 a Cytofx/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions. Cells were stimulated with anti-mouse CD3/CD28 beads for 5 h in the presence of GolgiStop (BD Biosciences). The stimulation was omitted for Foxp3 intracellular staining. An LSR flow cytometer (BD Biosciences) was used for data collection, and data were analyzed using CellQuest software (BD Biosciences).

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 were 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 Ab, as described previously (24, 25). Briefly, a hybridoma producing anti-CD25 Ab (PC61; American Type Culture Collection, Manassas, VA) was cultured in serum-free medium (Protein-Free Hybridoma Medium-II from Invitrogen), and the Ab was purified from supernatant by ammonium sulfate precipitation and a PD10 column (GE Healthcare, Buckinghamshire, U.K.). The purified product was quantified using the Bradford assay (Bio-Rad, Hercules, CA) at OD595, and 1 mg was injected i.p. weekly from day 0 for 3 wk. Control rat nonspecific IgG was purchased from Invitrogen.

Quantitative RT-PCR

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

Statistical analysis

Kaplan–Meier plots were used to compare survival rates. The log-rank test was used to evaluate *p* values. Statistical analyses were performed using Stat Mate ver. 6 (ATMS, Tokyo, Japan). The Student *t* test was used; all error bars in this study represent SD, unless otherwise specified.

Results

Purified $CD4^+$ T cell transplantation and pathological analysis

Decreased GVHD was observed when we transplanted IL-21R–deficient splenocytes compared with WT bulk splenocytes (22). Although we sought to find molecular mechanism(s) for the ameliorated GVHD, no clue was immediately evident from the transplantation experiments (22). Thus, in this study, we used purified $CD4^+$ T cells instead of bulk splenocytes in an effort 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 WT and IL-$21R^{-/-}$ cells seemed to be greater than in the previous experiments using bulk splenocytes (22). All recipients of WT $CD4^+$ T cells died within 55 d, whereas those receiving IL-$21R^{-/-}$ $CD4^+$ T cells survived during this time period (Fig. 1*A*). Moreover, recipients of IL-$21R^{-/-}$ $CD4^+$ T cells recovered from body weight loss by day 14, but those receiving WT $CD4^+$ T cells did not recover and continued to lose weight (Fig. 1*B*). In recipients of IL-$21R^{-/-}$ $CD4^+$ T cells, pathological analysis showed markedly reduced infiltration into the regions surrounding bile ducts and portal veins and into the interstitial region of small intestine compared with the infiltration observed in recipients of WT $CD4^+$ T cells (Fig. 2, *upper* and *middle panels*). Apoptotic bodies near the surface area of crypts in the small intestine were barely visible in recipients

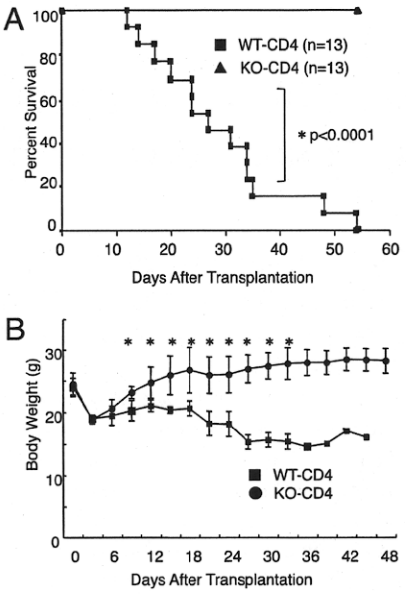

FIGURE 1. A role for IL-21 in $CD4^+$ T cell-mediated GVHD. *A*, Survival of recipients of WT and IL-$21R^{-/-}$ $CD4^+$ T cells. C57BL/6-DBA2-F1 mice were irradiated with 11 Gy and received 5×10^6 IL-$21R^{-/-}$ BM with 5×10^6 WT or IL-$21R^{-/-}$ $CD4^+$ T cells. Shown are combined data from two independent experiments. Thirteen recipients each for WT and IL-$21R^{-/-}$ $CD4^+$ T cells were analyzed. The log-rank test was used to calculate *p* values. *B*, Body weight after BM transplantation. Statistical significance was assessed with the Student *t* test.

of IL-$21R^{-/-}$ $CD4^+$ T cells, in contrast to recipients of WT $CD4^+$ T cells, in which apoptotic bodies were evident (Fig. 2, arrowheads in *middle panel*). No significant difference was observed in skin

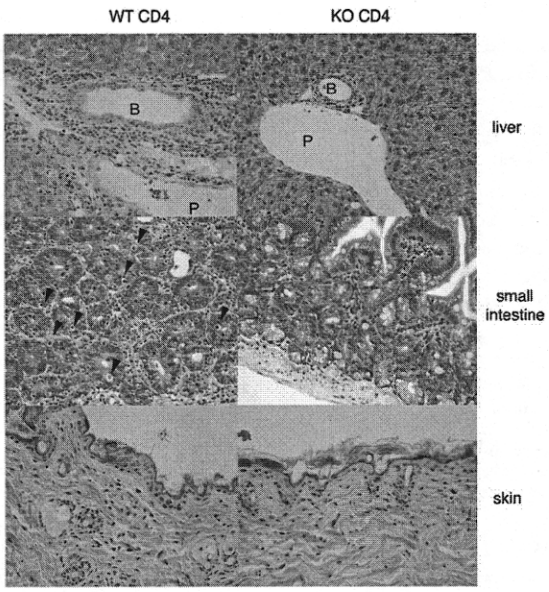

FIGURE 2. Pathological analysis of recipients. Liver, small intestine, and skin were stained with H&E (original magnification ×400). In recipients of WT $CD4^+$ T cells, cell infiltration is evident around the portal vein (P) and the bile duct (B) and into the interstitial region in small intestine. Arrowheads indicate apoptotic bodies near the surface of crypts. These changes were barely visible in recipients of IL-$21R^{-/-}$ $CD4^+$ T cells. Skin did not show any significant difference between recipients of WT and IL-$21R^{-/-}$ $CD4^+$ T cells. Shown is a representative result of six mice analyzed in each group. Only one recipient of IL-$21R^{-/-}$ $CD4^+$ T cells showed apoptotic bodies in the lumens of intestine and infiltration around the bile duct and portal vein, as was observed in the recipients of WT $CD4^+$ T cells.

pathology among recipients of WT 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 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. 1), we assessed T cell differentiation by cytokine production in the presence of cellular stimulation. Interestingly, at days 14 and 21 after transplantation, bulk splenocytes from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells 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 panels*). 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 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 WT versus IL-21R$^{-/-}$ CD4$^+$ T cells.

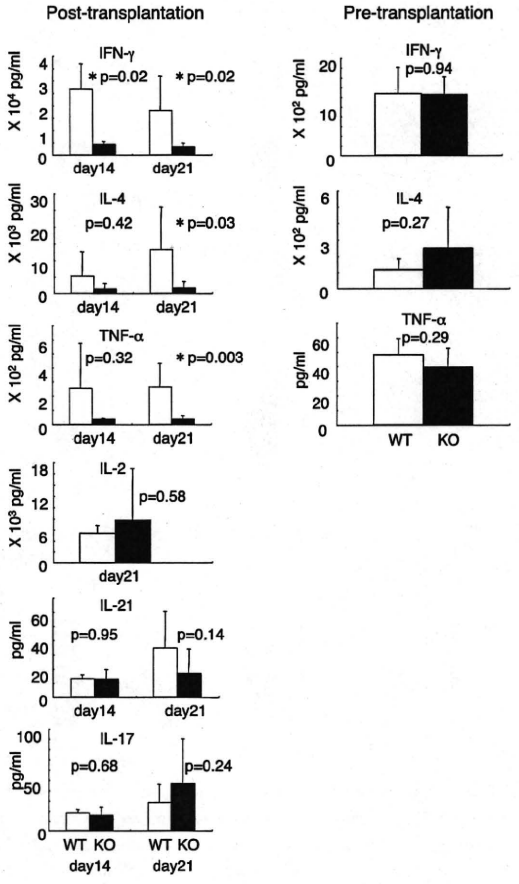

FIGURE 3. Cytokine production by bulk splenocytes before and after CD4$^+$ T cell transplantation. At days 14 and 21 after transplantation, splenocytes (5×10^5) were taken and stimulated with anti-CD3/CD28 Abs for 18 h. Concentrations of cytokines in the supernatants were determined by ELISA. Twelve or 13 recipients of WT CD4$^+$ T cells and 10 recipients of IL-21R$^{-/-}$ CD4$^+$ T cells were analyzed. Prior to transplantation, five WT and eight IL-21R$^{-/-}$ mice were analyzed. At days 14–21 after transplantation, the proportion of donor cells in the spleen was >95%. The Student *t* test was used to calculate *p* values. *Statistical significance ($p < 0.05$).

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 days 14–21 after transplantation. The number of donor H-2K^{d-}CD4$^+$ T cells was significantly lower in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells than in recipients of WT CD4$^+$ T cells (Fig. 4*A*; $p = 0.03$, Welch *t* test; $n = 15$ versus 12), although the ranges overlapped. Because 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 WT CD4$^+$ T cells (Fig. 4*B*). Moreover, posttransplantation, the levels

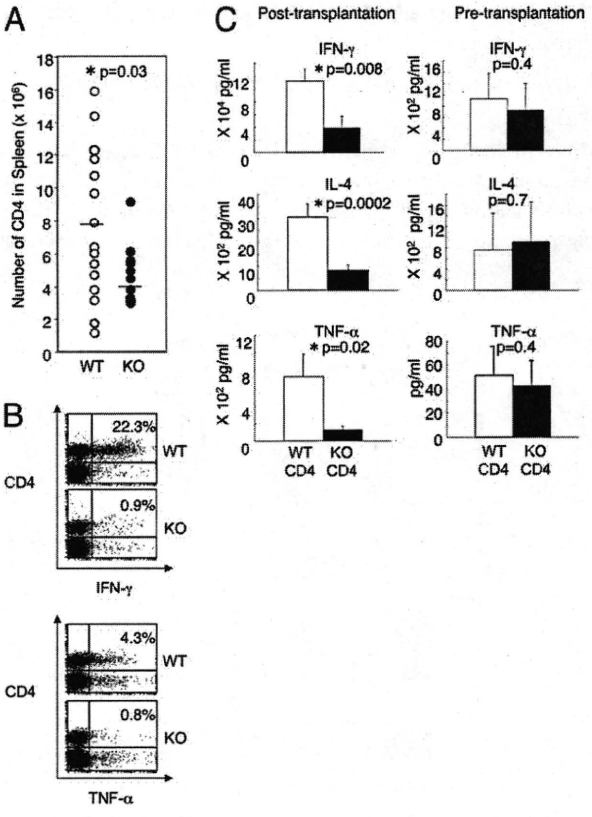

FIGURE 4. Cytokine production by splenic CD4$^+$ T cells before and after transplantation. *A*, Absolute number of donor H-2K^{d-}CD4$^+$ T cells in the spleen. The number of donor CD4$^+$ T cells was determined by multiplying the number of splenocytes by the percentage of H-2K^{d-}CD4$^+$ T cells. Each dot depicts the number of donor CD4$^+$ T cells in a mouse. Horizontal lines indicate the average. Fifteen recipients of WT CD4$^+$ T cells and 12 recipients of IL-21R$^{-/-}$ CD4$^+$ T cells were assessed. *B*, Intracellular staining of splenocytes after anti-CD3/CD28 stimulation. Splenocytes (1×10^6) were stimulated with anti-CD3/CD28 Abs for 5–6 h and stained with anti–IFN-γ or anti–TNF-α Ab in combination with anti-CD4 Ab. A total of three recipients in each group were analyzed, and a representative result is shown. *C*, Cytokine production by CD4$^+$ T cells in vitro. At days 14 or 21 after transplantation, splenic CD4$^+$ T cells (5×10^5) were purified and stimulated with anti-CD3/CD28 Abs for 18 h. Concentrations of cytokines in the supernatants were determined by ELISA. Twelve mice were analyzed in each group after transplantation. Five or six WT and eight or nine IL-21R$^{-/-}$ mice were analyzed before transplantation. *Statistical significance ($p < 0.05$).

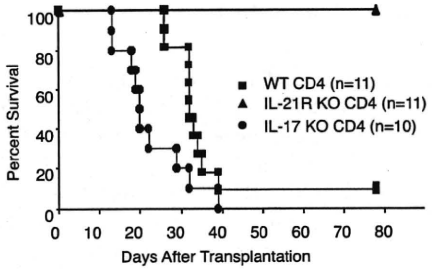

FIGURE 5. IL-17$^{-/-}$ CD4$^+$ T cells induced lethal GVHD. Survival of recipients of WT, IL-21R$^{-/-}$ (IL-21R KO), or IL-17$^{-/-}$ (IL-17 KO) CD4$^+$ T cells. Lethally irradiated (11 Gy) C57BL/6-DBA2-F1 mice were transplanted with 5×10^6 IL-21R KO BM and 5×10^6 WT, IL-21R KO, or IL-17 KO CD4$^+$ T cells. The data represent the combined results of two independent experiments.

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 compared with those receiving WT CD4$^+$ T cells (Fig. 4*C*, *left panels*). Before transplantation, IL-21R$^{-/-}$ CD4$^+$ T cells did not show any defect in IFN-γ, TNF-α, and IL-4 production (Fig. 4*C*, *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 it. 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 left panel*, bulk splenocytes from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells produced comparable amounts of IL-17 at days 14 and 21 after transplantation compared with mice receiving WT CD4$^+$ T cells. Moreover, we found that IL-17$^{-/-}$ CD4$^+$ T cells induced lethal GVHD analogous to WT 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 reflected by the survival of mice receiving IL-21R$^{-/-}$ CD4$^+$ T cells (Fig. 5).

Regulatory T cell number in spleen

We next determined the serum concentration of the major immunosuppressive cytokine, TGF-β1, at days 6–21 after transplantation. We found an increase in TGF-β1 only after transplantation (Fig. 6*A*; $p = 0.0003$ at day 14; $p = 0.01$ at day 21, Student 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 upregulated; in fact, it tended to be diminished (Supplemental Fig. 2), suggesting that the increase in serum TGF-β1 might be due to cells other than T cells. Because naive 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 cells (8), we also investigated whether more Treg cells were induced in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells. The proportion

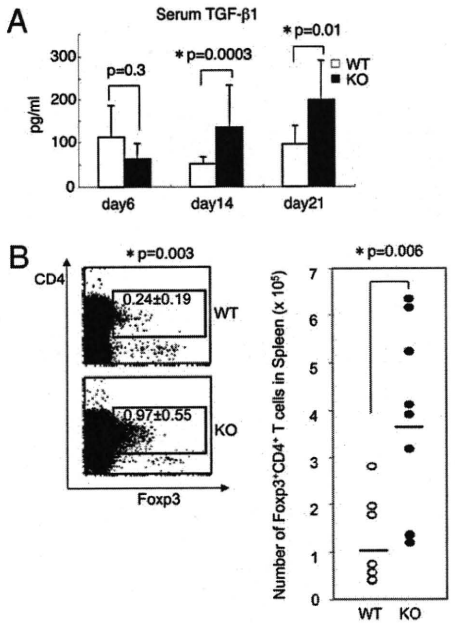

FIGURE 6. Increase in splenic Treg cells. *A*, Upregulation of serum TGF-β1. Serum TGF-β1 concentrations at the indicated day after transplantation were determined by ELISA. Three samples from recipients of WT CD4$^+$ T cells and 4 samples from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells at day 6, 23 samples from recipients of WT CD4$^+$ T cells and 25 samples from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells at day 14, and 8 samples from recipients of WT CD4$^+$ T cells and 7 samples from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells at day 21 were analyzed. *Statistical significance ($p < 0.05$). *B*, The percentage and absolute number of splenic Foxp3$^+$CD4$^+$ regulatory T cells at day 14 after transplantation. The left panel shows a representative flow cytometric result from eight or nine similar samples. The right panel indicates the number of all samples; the averages are indicated by the horizontal lines.

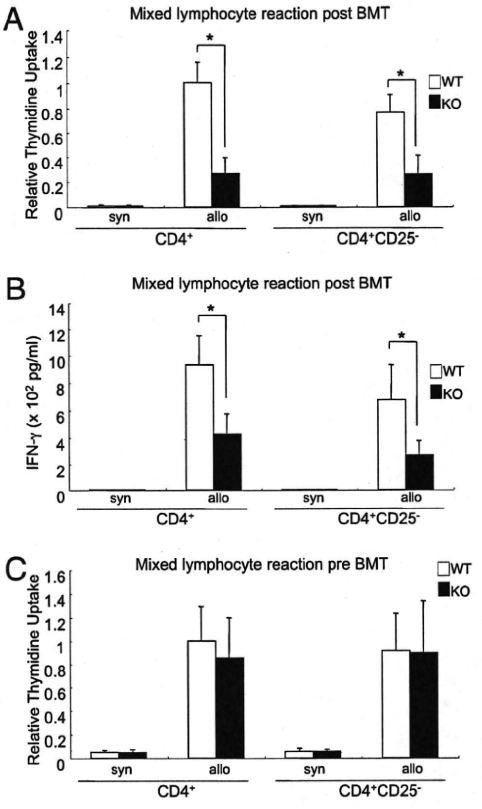

FIGURE 7. An impaired CD4 alloreaction is not dependent on CD25$^+$ CD4$^+$ T cells. CD4 alloreaction in vitro was impaired after transplantation, and this impairment was not restored by CD25$^+$ T cell depletion. *A*, At day 14 after transplantation, 1×10^5 sorter-purified splenic CD4$^+$ or CD25$^-$ CD4$^+$ T cells (>98% purity) were cultured with 4×10^5 irradiated allogeneic C57BL/6-DBA2-F1 splenocytes for 4 d. The cells were pulsed with 1 μCi of [^{3}H]thymidine for the last 24 h. Relative thymidine uptake to the value of WT CD4$^+$ T cells is depicted. *B*, Culture was the same as in *A*, but IFN-γ concentrations in the supernatants were determined by ELISA. *C*, Sorter-purified splenic CD4$^+$ or CD25$^-$CD4$^+$ cells from nontransplanted mice were cultured with irradiated allogeneic C57BL/6-DBA2-F1 splenocytes. Relative thymidine uptake to the number of WT CD4$^+$ T cells is depicted. *$p < 0.05$.

of splenic Foxp3$^+$CD4$^+$ Treg phenotype cells in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells was higher than in recipients of WT CD4$^+$ T cells, but the total percentage was still only ~1% (Fig. 6*B*, *left panel*). The absolute number was ~4-fold higher, but the actual number was only ~4×10^5 of the total number of splenocytes (~4×10^7) (Fig. 6*B*, *right panel*). In contrast to posttransplantation, pretransplantation splenocytes from IL-21R$^{-/-}$ mice did not show an increase in Foxp3$^+$CD4$^+$ T cells compared with cells from WT mice (Supplemental Fig. 3), suggesting that the increased Treg cell after transplantation was an induced Treg cell during GVHD reaction. For that reason, we did not deplete CD25$^+$ cells prior to transplantation.

CD25 depletion did not restore the suppressed alloreaction in vitro and did not exacerbate the ameliorated GVHD

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

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. 8*A*, 8*B*). In contrast, the severity of GVHD in recipients of WT CD4$^+$ T cells seemed to be slightly diminished by CD25$^+$ depletion (Fig. 8*A*, 8*B*). In this condition, as previously reported (30), the depletion efficacy of CD25$^+$CD4$^+$ T cells was >95% and that of Foxp3$^+$CD4$^+$ T cells was ≥50% (Fig. 8*C*, *upper* and *lower panels*). Interestingly, Foxp3 expression was higher in CD25$^-$CD4$^+$ T cells from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells than from recipients of WT CD4$^+$ T cells (Fig. 8*D*). Together with the results in vitro (Fig. 7), this suggests a relationship between the unresponsiveness of CD25$^-$CD4$^+$ T cells and greater expression of Foxp3.

Discussion

In this article, we reported evidence indicating that IL-21 is critical for the pathogenesis of CD4$^+$ T cell-mediated GVHD, at least in part because of its 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 showed no obvious difference. According to these results, T cell differentiation into Th1 and Th2 cells seemed to be altered in the absence of IL-21 during GVHD. Cytokines are believed to have positive and negative roles in GVHD. For example, although T cells from IFN-γ–deficient mice resulted in more severe GVHD (33–35), T cells from Stat4 (Th1)-deficient mice resulted in less severe GVHD than did T cells from WT mice with less severe colitis (36). In contrast to IFN-γ$^{-/-}$ 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 did those from WT 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

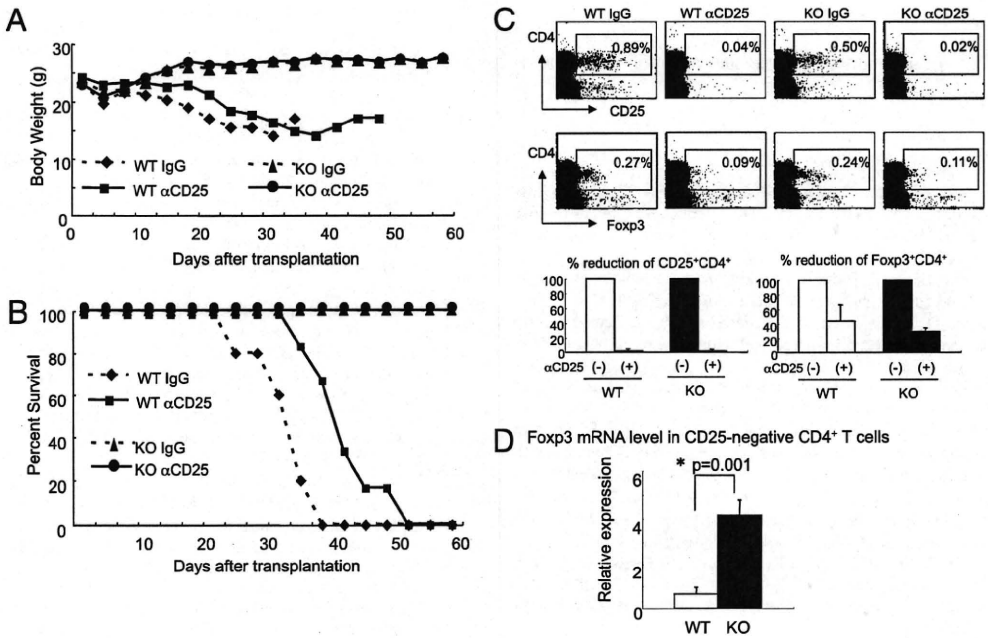

FIGURE 8. The ameliorated GVHD induced by IL-21R$^{-/-}$ CD4$^+$ T cells is not dependent on CD25$^+$CD4$^+$ T cells. The ameliorated GVHD induced by IL-21R$^{-/-}$ CD4$^+$ T cells was not exacerbated by depletion of CD25$^+$CD4$^+$ T cells. Body weight (*A*) and survival (*B*) of recipients are shown. Comparisons of recipients of WT CD4$^+$ T cells and IL-21R$^{-/-}$ CD4$^+$ T cells and additional comparisons with and without anti-CD25 Ab treatment were performed. Nonspecific rat IgG was used as the control Ab. *C*, Splenic CD25$^+$CD4$^+$ T cells and splenic Foxp3$^+$CD4$^+$ T cells at day 14 after transplantation with or without anti-CD25 Ab treatment were analyzed by flow cytometry (*upper two rows*). The lower panels indicate the mean reduction in the percentage of CD25$^+$CD4$^+$ and Foxp3$^+$CD4$^+$ cells from three similar results. *D*, Foxp3 mRNA level in CD25$^-$CD4$^+$ T cells at day 21 after transplantation. Cell sorter-purified CD25$^-$CD4$^+$ T cells were subjected to mRNA purification, reverse-transcriptase treatment, and TaqMan quantitative PCR. Relative value to β-actin is denoted.

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 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 early stages, 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 Treg cell differentiation (41–43) and between IL-21 and Treg 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 cell function and the increased number of Treg cells, it is possible that increased Treg cells suppress functional effector T cells. Alternatively, it is possible that effector differentiation itself is defective, and the resulting effector T cells cannot respond to alloantigen, 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 might be more consistent with the latter possibility, given that Treg cell 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 upregulation of Foxp3 in CD25$^-$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 that more than one mechanism can contribute to the attenuated GVHD.

Disclosures

K. Ozaki and W.J.L. 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, et al. 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: 11439–11444.
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., A. Hishiya, K. Hatanaka, H. Nakajima, G. Wang, P. Hwu, T. Kitamura, K. Ozawa, W. J. Leonard, and T. Nosaka. 2006. Overexpression of interleukin 21 induces expansion of hematopoietic progenitor cells. *Int. J. Hematol.* 84: 224–230.
6. Ozaki, K., R. Spolski, C. G. Feng, C. F. Qi, J. Cheng, A. Sher, H. C. Morse, 3rd, 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. Urban, Jr., 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, and 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. Cutting edge: 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, et al. 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. Morse 3rd, 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. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *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. Interleukin-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, and H. Matsu, RTatara, K. Sato, W. J. Leonard, and K. Ozawa. 2010. IL-21 is critical for GVHD 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, and T. Teshima. 2007. Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease. *Blood* 109: 1756–1764.
25. Aoyama, K., M. Koyama, K. Matsuoka, D. Hashimoto, T. Ichinohe, M. Harada, K. Akashi, M. Tanimoto, and T. Teshima. 2009. Improved outcome of allogeneic bone marrow transplantation due to breastfeeding-induced tolerance to maternal antigens. *Blood* 113: 1829–1833.
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, and 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: 1151–1164.
30. Liesz, A., E. Suri-Payer, C. Veltkamp, H. Doerr, C. Sommer, S. Rivest, T. Giese, and R. Veltkamp. 2009. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat. Med.* 15: 192–199.
31. Fujita, S., Y. Sato, K. Sato, K. Eizumi, T. Fukaya, M. Kubo, N. Yamashita, and K. Sato. 2007. Regulatory dendritic cells protect against cutaneous chronic graft-versus-host disease mediated through CD4+CD25+Foxp3+ regulatory T cells. *Blood* 110: 3793–3803.
32. Radojcic, V., M. A. Pletneva, H. R. Yen, S. Ivcevic, A. Panoskaltsis-Mortari, A. C. Gilliam, C. G. Drake, B. R. Blazar, and 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: 764–774.
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-versus-host disease reflect promotion of lymphohematopoietic graft-versus-host 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.

37. Schmaltz, C., O. Alpdogan, S. J. Muriglan, B. J. Kappel, J. A. Rotolo, E. T. Ricchetti, A. S. Greenberg, L. M. Willis, G. F. Murphy, J. M. Crawford, and M. R. van den Brink. 2003. Donor T cell-derived TNF is required for graft-versus-host disease and graft-versus-tumor activity after bone marrow transplantation. *Blood* 101: 2440–2445.
38. Yi, T., D. Zhao, C. L. Lin, C. Zhang, Y. Chen, I. Todorov, T. LeBon, F. Kandeel, S. Forman, and D. Zeng. 2008. Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. *Blood* 112: 2101–2110.
39. Kappel, L. W., G. L. Goldberg, C. G. King, D. Y. Suh, O. M. Smith, C. Ligh, A. M. Holland, J. Grubin, N. M. Mark, C. Liu, et al. 2009. IL-17 contributes to CD4-mediated graft-versus-host disease. *Blood* 113: 945–952.
40. Carlson, M. J., M. L. West, J. M. Coghill, A. Panoskaltsis-Mortari, B. R. Blazar, and J. S. Serody. 2009. In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. *Blood* 113: 1365–1374.
41. Wei, J., O. Duramad, O. A. Perng, S. L. Reiner, Y. J. Liu, and F. X. Qin. 2007. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. *Proc. Natl. Acad. Sci. USA* 104: 18169–18174.
42. Mantel, P. Y., H. Kuipers, O. Boyman, C. Rhyner, N. Ouaked, B. Rückert, C. Karagiannidis, B. N. Lambrecht, R. W. Hendriks, R. Crameri, et al. 2007. GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. *PLoS Biol.* 5: e329.
43. Hill, J. A., J. A. Hall, C. M. Sun, Q. Cai, N. Ghyselinck, P. Chambon, Y. Belkaid, D. Mathis, and C. Benoist. 2008. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. *Immunity* 29: 758–770.

High incidence of false-positive *Aspergillus* galactomannan test in multiple myeloma

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False-positive GM test in myeloma patients

Invasive aspergillosis (IA) remains one of the most significant causes of morbidity and mortality in patients with hematological malignancies undergoing chemotherapy and hematopoietic stem cell transplantation (HSCT), mainly due to the difficulty in its early diagnosis. Monitoring of galactomannan (GM) antigen, an exoantigen of Aspergillus, in the blood by sandwich ELISA is a useful and non-invasive method for early diagnosis of IA. The GM test has a sensitivity of 67-100% with a specificity of 81-99% in neutropenic patients and allogeneic transplant recipients (1-3). Although it has been widely used as a diagnostic criterion for IA (4, 5), one of the major limitations of this assay is false-positivity, particularly in pediatric patients (1), patients with graft-versus-host disease (GVHD) (6, 7), and those taking dietary GM (8, 9) or fungus-derived antibiotics, such as piperacillin-tazobactam (PIPC/TAZ) (10-12).

Multiple myeloma results from malignant proliferation of a single clone of plasma cells, which produces a monoclonal immunoglobulin. Opportunistic infection is a major cause of death in patients with myeloma (13, 14). The risk for infection primarily resides during periods of chemotherapy-induced neutropenia or in the terminal stages of the disease. Therefore, monitoring of Aspergillus is recommended during chemotherapy-induced neutropenia.

124 patients with hematological disorders hospitalized in our institution from April 2007 to September 2009 were analyzed retrospectively. The clinical characteristics of these patients are summarized in supplementary materials (Table 1) Twenty-seven patients had plasma cell associated disorders (IgG type

myeloma: 14, IgA type myeloma: 1, IgD type myeloma: 1, Bence-Jones type myeloma: 3, lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia: 3, plasma cell leukemia: 1, primary AL amyloidosis: 1, and POEMS syndrome: 2). The remaining 97 patients were diagnosed as acute leukemia (MDS/AML: 32, ALL: 15) or malignant lymphoma (n = 50). Out of the 124 patients, those receiving cytotoxic chemotherapy, autologous peripheral blood stem cell transplantation (PBSCT), and allogeneic HSCT were 81, 10, and 28, respectively. 111 patients received antifungal prophylaxis, mostly with FLCZ or ITCZ. Eight patients were administered PIPC/TAZ at sampling time points. Seventy patients were low-risk for the development of IA, while the remaining 54 patients were high-risk.

In 21 of the 124 (16.9%) patients, GM antigenemia was positive at least 2 consecutive times and their characteristics are shown in Table 1. However, only 7 of the 21 patients showed clinical features of IA and were diagnosed with probable IA in the lung (cases 1-7). Clinical features were relieved with treatment with VRCZ in these patients, confirming a diagnosis of IA. All of the 7 patients had received antifungal prophylaxis, but not antibiotics known to cause false-positive results, such as PIPC/TAZ (10-12), amoxicillin/clavulanic acid, or amoxicillin (17). Four of the 7 patients were high-risk for the developing IA and 3 were low-risk. Possible IA was diagnosed in 4 patients in the absence of positive GM results. On the other hand, no proven or probable IA was detected in 103 patients with negative GM antigenemia.

Fourteen of the 21 (66.7%) patients with positive GM antigenemia did not satisfy the diagnostic criteria of proven or probable IA (cases 8-21 in Table 2); thus their episodes were considered to be false-positive. None of the 14 patients were treated with antibiotics potentially causing false-positivity of GM test. Antifungal prophylaxis had been given in all 14 episodes (FLCZ in 5, ITCZ in 5, and MCFG in 4). These patients did not show any clinical features suggestive of IA. Chest CT scans did not show any abnormal findings in 10 patients, while 4 patients showed abnormalities in the lung which were not suspicious of IA. Diagnosis of these lung lesions were history of pneumoconiosis in one patient, proven bacterial pneumonia in one, and idiopathic interstitial pneumonia in the remaining 2 patients. These lesions were not deteriorated without antifungal treatment. (1→3)-β-D-glucan was negative in all patients showing false-positive GM test. With a median follow-up of 10 months (range: 1-19 months), these patients did not develop fungal infection without treatment. On the other hand, false negative GM results were obtained in 4 patients (3.2 %). In the current study, the sensitivity, specificity, PPV, and NPV of the GM ELISA test were 63.6% (7/11), 87.6% (99/113), 33.3% (7/21), and 96.1% (99/103), respectively.

Surprisingly, 11 out of the 14 patients showing the false-positive results had diagnosis of multiple myeloma. The false-positivity of GM antigenemia was significantly higher in myeloma patients (11/22, 50%) than those with other hematological malignancies (3/102, 2.9%) ($p < 0.001$). Moreover, in myeloma patients false-positive results were exclusively detected in those with IgG subtype. Thus, rate of false-positivity was extremely high in patients with IgG

myeloma (11/14, 78.6%). We could not find any difference in the characteristics of IgG myeloma patients with or without GM-false positivity, including serum levels of IgG (5083 ± 2077 mg/dl versus 4713 ± 3729 mg/dl). In addition, the GM test remained to be false-positive even after normalization of IgG levels by chemotherapy in 8 of the 11 patients. We also evaluated GM antigenemia in frozen serum samples collected prior to chemotherapy in 3 myeloma patients who showed false-positivity after chemotherapy to rule out the possibility that administration of myeloma-specific chemotherapy is associated with the false-positivity, and confirmed GM positivity prior to chemotherapy in these samples.

In a univariate logistic regression analysis, IgG myeloma and low risk category were strongly associated with false-positive GM antigenemia. Sex, type of treatment, antibiotics, corticosteroid usage, and serum levels of immunoglobulins were also significant or marginally associated with false-positivity. Multivariate analysis confirmed diagnosis of IgG myeloma as the only independent risk factor for false-positivity (odds ratio, 59.41; 95% confidence interval, 8.19 – 431.0; $p < 0.001$) (see supplementary materials, Table 2). In patients with other diseases, the GM assay showed a high sensitivity (7/11, 63.6%), specificity (96/99, 97.0%), PPV (7/10, 70%), and NPV (96/100, 96%). In contrast, for patients with IgG myeloma, specificity and PPV of the assay were very low (3/14, 21.4%, and 0/11, 0%), while NPV was 100% even in this cohort.

A recent meta-analysis addressing the accuracy of a GM assay for diagnosing IA confirmed the clinical usefulness of this test with a sensitivity of 71% and a specificity of 89% (18). Although our study demonstrated similar sensitivity (7/11:64%) and specificity (103/117:88%) of the GM test, PPV (33%) was lower, compared to previous studies that demonstrate 40-60% PPV (7, 19-21). This difference is due to an unexpectedly high incidence of GM antigen false-positivity (11.3%) in our study. It should be noted, however, that screening of GM antigen was performed less frequently in this study compared to previous studies, where GM antigenemia was evaluated 2 to 3 times per week (1, 2, 22), and such a frequent monitoring is ideal to assure the optimal PPV and NPV.

Diagnosis of multiple myeloma is a major risk factor for GM false-positivity. In particular, the false-positivity was exclusively observed in patients with IgG myeloma and was not observed in patients with other types of plasma cell disorders. These observations should be confirmed in a larger study because some studies previously reported the usefulness of GM antigen assay as a diagnostic tool for IA among patients with hematological malignancies including IgG myeloma (23, 24), and only small numbers of patients with plasma cell disorders other than IgG myeloma were included in this study. Low risk category of developing IA was a risk for false positive results in a univariate, but not multivariate analysis. IgG myeloma remained a strong risk for false positivity even after the compensation by the risk categorization.

Mechanisms of high frequency of GM false-positivity in myeloma

patients remain to be investigated. (1→3)-β-D-glucan, which is released from the fungal cell wall, is also widely used to support diagnosis of fungal infections and adopted as one of the microbiological criterion for probable IA in the revised EORTC/MSG definition (5). A previous study reported that high levels of immunoglobulins interfere with the measurement of (1→3)-β-D-glucan by causing precipitation of insolubilized proteins and increase the non-specific optical density levels of reaction fluid (25), although (1→3)-β-D-glucan was negative in patients showing false-positive GM test in this study. This phenomenon has not been reported in the GM assay. However, serum levels of IgG were not directly associated with the false-positivity; IgG levels did not differ between IgG myeloma patients with and without false-positivity. In addition, the GM test remained to be false-positive even after normalization of IgG levels by chemotherapy in 8 of the 11 patients.

Causative role of PIPC/TAZ, amoxicillin/clavulanic acid, and amoxicillin in GM false-positivity has been well documented (10-12, 17), therefore collection of samples prior to infusion of these antibiotics and the use of a relatively higher cut-off level (> 0.7) are recommended in patients receiving these agents (12). In this study, no patients with GM false-positivity received these antibiotics at the time of sampling. It has been hypothesized that dietary contamination by GM causes GM false-positivity by the translocation of dietary GM into the systemic circulation through the disrupted intestinal mucosal barrier, especially in patients with gastrointestinal GVHD after allogeneic HSCT (6, 7, 9). In our cohort, 1 (3.6%) of the 28 patients who underwent allogeneic HSCT showed

false-positivity. This patient with lymphoma had acute GVHD involving in the skin and intestine. A previous study demonstrated that false-positive results were preferentially observed in patients with febrile neutropenic sepsis (26), although subsequent study was unable to replicate this result (22). A recent study revealed that serum GM antigen levels was significantly higher in severely neutropenic patients (< 0.1 x 10^9/L) than in the other patients (27). However, we did not find such an association in this study.

In conclusion, the incidence of false-positive GM antigenemia was high in patients with IgG myeloma. Although the results should be confirmed in a prospective study including larger numbers of patients, positive results of GM antigenemia may be interpreted with caution, and intimate survey including CT scan or other microbiological markers will be recommended in myeloma patients.

References

1. Herbrecht R, Letscher-Bru V, Oprea C, Lioure B, Waller J, Campos F, Villard O, Liu KL, Natarajan-Ame S, Lutz P, Dufour P, Bergerat JP, Candolfi E. Aspergillus galactomannan detection in the diagnosis of invasive aspergillosis in cancer patients. J Clin Oncol 2002;20:1898-1906.

2. Maertens J, Verhaegen J, Lagrou K, Van Eldere J, Boogaerts M. Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. Blood 2001;97:1604-1610.

3. Wheat LJ. Rapid diagnosis of invasive aspergillosis by antigen detection. Transpl Infect Dis 2003;5:158-166.

4. Ascioglu S, Rex JH, de Pauw B, Bennett JE, Bille J, Crokaert F, Denning DW, Donnelly

JP, Edwards JE, Erjavec Z, Fiere D, Lortholary O, Maertens J, Meis JF, Patterson TF, Ritter J, Selleslag D, Shah PM, Stevens DA, Walsh TJ. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 2002;34:7-14.

5. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Munoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 2008;46:1813-1821.

6. Hamaki T, Kami M, Kanda Y, Miyakoshi S, Ueyama J, Morinaga S, Mutou Y. False-positive results of Aspergillus enzyme-linked immunosorbent assay in a patient with chronic graft-versus-host disease after allogeneic bone marrow transplantation. Bone Marrow Transplant 2001;28:633-634.

7. Asano-Mori Y, Kanda Y, Oshima K, Kako S, Shinohara A, Nakasone H, Kaneko M, Sato H, Watanabe T, Hosoya N, Izutsu K, Asai T, Hangaishi A, Motokura T, Chiba S, Kurokawa M. False-positive Aspergillus galactomannan antigenaemia after haematopoietic stem cell transplantation. J Antimicrob Chemother 2008;61:411-416.

8. Ansorg R, van den Boom R, Rath PM. Detection of Aspergillus galactomannan antigen in foods and antibiotics. Mycoses 1997;40:353-357.

9. Murashige N, Kami M, Kishi Y, Fujisaki G, Tanosaki R. False-positive results of Aspergillus enzyme-linked immunosorbent assays for a patient with gastrointestinal graft-versus-host disease taking a nutrient containing soybean protein. Clin Infect Dis 2005;40:333-334.

10. Sulahian A, Touratier S, Ribaud P. False positive test for aspergillus antigenemia related to concomitant administration of piperacillin and tazobactam. N Engl J Med 2003;349:2366-2367.

11. Viscoli C, Machetti M, Cappellano P, Bucci B, Bruzzi P, Van Lint MT, Bacigalupo A. False-positive galactomannan platelia Aspergillus test results for patients receiving piperacillin-tazobactam. Clin Infect Dis 2004;38:913-916.

12. Orlopp K, von Lilienfeld-Toal M, Marklein G, Reiffert SM, Welter A, Hahn-Ast C, Purr I, Gorschluter M, Molitor E, Glasmacher A. False positivity of the Aspergillus galactomannan Platelia ELISA because of piperacillin/tazobactam treatment: does it represent a clinical problem? J Antimicrob Chemother 2008;62:1109-1112.

13. Blade J, San Miguel JF, Fontanillas M, Esteve J, Maldonado J, Alcala A, Brunet S, Garcia-Conde J, Besalduch J, Moro MJ, Fernandez-Calvo J, Conde E, Font L, Gardella S, Carnero M, Carbonell F, Marti JM, Hernandez-Martin J, Ortega F, Besses C, Ribera JM, Trujillo J, Escudero ML, Rozman C, Estape J, Montserrat E. Increased conventional chemotherapy does not improve survival in multiple myeloma: long-term results of two PETHEMA trials including 914 patients. Hematol J 2001;2:272-278.

14. Augustson BM, Begum G, Dunn JA, Barth NJ, Davies F, Morgan G, Behrens J, Smith A, Child JA, Drayson MT. Early mortality after diagnosis of multiple myeloma: analysis of patients entered onto the United kingdom Medical Research Council trials between 1980 and 2002--Medical Research Council Adult Leukaemia Working Party. J Clin Oncol 2005;23:9219-9226.

15. Stynen D, Goris A, Sarfati J, Latge JP. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. Journal of clinical microbiology 1995;33:497-500.

16. Maertens JA, Klont R, Masson C, Theunissen K, Meersseman W, Lagrou K, Heinen C, Crepin B, Van Eldere J, Tabouret M, Donnelly JP, Verweij PE. Optimization of the cutoff value for the Aspergillus double-sandwich enzyme immunoassay. Clin Infect Dis 2007;44:1329-1336.

17. Mattei D, Rapezzi D, Mordini N, Cuda F, Lo Nigro C, Musso M, Arnelli A, Cagnassi S, Gallamini A. False-positive Aspergillus galactomannan enzyme-linked immunosorbent assay results in vivo during amoxicillin-clavulanic acid treatment. Journal of clinical microbiology 2004;42:5362-5363.

18. Pfeiffer CD, Fine JP, Safdar N. Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. Clin Infect Dis 2006;42:1417-1427.

19. Kawazu M, Kanda Y, Nannya Y, Aoki K, Kurokawa M, Chiba S, Motokura T, Hirai H, Ogawa S. Prospective comparison of the diagnostic potential of real-time PCR, double-sandwich enzyme-linked immunosorbent assay for galactomannan, and a (1-->3)-beta-D-glucan test in weekly screening for invasive aspergillosis in patients with hematological disorders. Journal of clinical microbiology 2004;42:2733-2741.

20. Alhambra A, Cuetara MS, Ortiz MC, Moreno JM, del Palacio A, Ponton J, del Palacio A. False positive galactomannan results in adult hematological patients treated with piperacillin-tazobactam. Rev Iberoam Micol 2007;24:106-112.

21. Lai CC, Hsu HL, Lee LN, Hsueh PR. Assessment of Platelia Aspergillus enzyme immunoassay for the diagnosis of invasive aspergillosis. Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi 2007;40:148-153.

22. Maertens J, Verhaegen J, Demuynck H, Brock P, Verhoef G, Vandenberghe P, Van Eldere J, Verbist L, Boogaerts M. Autopsy-controlled prospective evaluation of serial screening

for circulating galactomannan by a sandwich enzyme-linked immunosorbent assay for hematological patients at risk for invasive Aspergillosis. Journal of clinical microbiology 1999;37:3223-3228.

23. Miceli MH, Grazziutti ML, Woods G, Zhao W, Kocoglu MH, Barlogie B, Anaissie E. Strong correlation between serum aspergillus galactomannan index and outcome of aspergillosis in patients with hematological cancer: clinical and research implications. Clin Infect Dis 2008;46:1412-1422.

24. Woods G, Miceli MH, Grazziutti ML, Zhao W, Barlogie B, Anaissie E. Serum Aspergillus galactomannan antigen values strongly correlate with outcome of invasive aspergillosis: a study of 56 patients with hematologic cancer. Cancer 2007;110:830-834.

25. Yoshida K. [Recent advances of serodiagnosis for systemic fungal infections]. Nihon Ishinkin Gakkai zasshi = Japanese journal of medical mycology 2006;47:135-142.

26. Swanink CM, Meis JF, Rijs AJ, Donnelly JP, Verweij PE. Specificity of a sandwich enzyme-linked immunosorbent assay for detecting Aspergillus galactomannan. Journal of clinical microbiology 1997;35:257-260.

27. Cordonnier C, Botterel F, Ben Amor R, Pautas C, Maury S, Kuentz M, Hicheri Y, Bastuji-Garin S, Bretagne S. Correlation between galactomannan antigen levels in serum and neutrophil counts in haematological patients with invasive aspergillosis. Clin Microbiol Infect 2009;15:81-86.

Table 1. Characteristics of 21 patients with positive GM test

Case	Age /Sex	Disease	Treatment	GM (C.O.I)	Radiological / clinical findings	Diagnosis	Times / duration of false positivity	Antibiotics*	Antifungal agents*	Steroids	Neutrophils (x 10^9/L)	IgG (mg/dl)	IgA (mg/dl)	IgM (mg/dl)	Risk of IA
1	49/F	MDS/AML	CTx	1.1	nodules with halo	probable IA	-	CFPM	FLCZ	none	0.01	1419	227	50	High
2	63/M	MDS/AML	CTx	1.2	nodules	probable IA	-	MEPM, AMK	FLCZ	none	0	1253	238	62	High
3	50/F	AML	CTx	1.2	nodules	probable IA	-	MEPM, AMK	ITCZ	none	0	1301	293	235	High
4	57/F	MDS	allo-SCT	2.3	nodules	probable IA	-	CAZ, VCM	VRCZ	none	0.009	1198	256	120	High
5	63/M	ML	CTx	1.3	consolidation with pleural pain	probable IA	-	none	MCFG	DEXA	2.034	689	126	92	Low
6	57/F	ML	CTx	1.5	nodules	probable IA	-	CFPM	FLCZ	PSL	0.081	675	117	67	Low
7	59/F	ML	CTx	2.2	consolidation with pleural pain	probable IA	-	CZOP	MCFG	PSL	0.036	719	95	20	Low
8	70/M	ML	CTx	0.8	pneumoconiosis	F-P	7 / 7 months	none	FLCZ	PSL	5.096	1870	315	60	Low
9	52/M	ML	allo-SCT	2.5	bacterial pneumonia	F-P	2 / 1 month	CZOP, CPFX	ITCZ	mPSL	0.806	562	49	19	High
10	54/M	ML	CTx	0.9	negative	F-P	5 / 3 months	CFPM	FLCZ	none	0.395	691	46	10	Low
11	63/F	IgGκ MM	CTx	0.8	negative	F-P	2 / 1 month	none	ITCZ	DEXA	1.497	4770	25	27	Low
12	56/F	IgGκ MM	none	1.2	negative	F-P	3 / 2 months	CAZ	FLCZ	none	3.449	7196	5	38	Low
13	69/M	IgGκ MM	CTx	0.5	negative	F-P	4 / 4 months	none	MCFG	DEXA	0.72	3944	13	<10	Low
14	69/M	IgGκ MM	CTx	1.2	negative	F-P	6 / 3 months	none	FLCZ	DEXA	1.777	2385	6	28	Low
15	64/M	IgGκ MM	CTx	1.6	old inflammatory change	F-P	5 / 2 months	none	MCFG	PSL	1.919	3269	30	<10	Low
16	51/F	IgGκ MM	CTx	2.1	IP	F-P	23 / 15 months	none	ITCZ	PSL	3.791	8006	99	30	Low
17	64/M	IgGκ MM	CTx	1.6	negative	F-P	13 / 8 months	none	ITCZ	DEXA	0.899	3950	6	<10	Low
18	75/M	IgGκ MM	CTx	0.6	negative	F-P	2 / 1 month	MEPM	FLCZ	DEXA	0.823	8986	<5	<10	High
19	49/F	IgGκ MM	CTx	1.1	negative	F-P	8 / 3 months	none	MCFG	DEXA	0.724	4563	37	15	Low
20	74/F	IgGλ MM	CTx	0.7	negative	F-P	6 / 2 months	none	MCFG	none	1.451	3924	25	18	Low
21	56/M	IgGλ MM	CTx	1.1	negative	F-P	10 / 3 months	none	ITCZ	DEXA	1.605	4917	9	10	Low

*administrated at initial positive sampling, MDS : myelodysplastic syndrome, AML : acute myelogenous leukemia, ML : malignant lymphoma, MM: multiple myeloma, CTx : chemotherapy, allo-SCT : allogeneic stem cell transplantation, C.O.I : cut-off index, IP : interstitial pneumonia, F-P : false-positive, CFPM : cefepime, MEPM : meropenem, AMK : amikacin, CAZ : ceftazidime, VCM : vancomycin, CZOP : cefozopran, PIPC/TAZ : piperacillin/tazobactam, CPFX : ciprofloxacin, PSL : prednisolone, mPSL : methylprednisolone, DEXA : dexamethasone.