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RESEARCH ARTICLE

IL-21 accelerates xenogeneic graft-versus-host disease correlated with increased B-cell proliferation

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ABSTRACT

Graft-versus-host disease (GVHD) is a prevalent and potential complication of hematopoietic stem cell transplantation. An animal model, xenogeneic GVHD (X-GVHD), that mimics accurately the clinical presentation of GVHD would provide a tool for investigating the mechanism involved in disease pathogenesis. Murine models indicated that inhibiting IL-21 signaling was a good therapy to reduce GVHD by impairing T cell functions. We sought to investigate the effect of exogenous human IL-21 on the process of X-GVHD. In this study, human IL-21 was expressed by hydrodynamic gene delivery in BALB/c-Rag2$^{-/-}$ IL-2Rγc$^{-/-}$ (BRG) immunodeficient mice which were intravenously transplanted human peripheral blood mononuclear cells (hPBMCs). We found that human IL-21 exacerbated X-GVHD and resulted in rapid fatality. As early as 6 days after hPBMCs transplanted to BRG mice, a marked expansion of human CD19^{+} B cells, but not T cells, was observed in spleen of IL-21-treated mice. Compared with control group, IL-21 induced robust immunoglobulin secretion, which was accompanied by increased accumulation of CD19^{+} CD38^{high} plasma cells in spleen. In addition, we demonstrated that B-cell depletion was able to ameliorate X-GVHD. These results are the first to find *in vivo* expansion and differentiation of human B cells in response to IL-21, and reveal a correlation between the expansion of B cells and the exacerbation of xenogeneic GVHD. Our findings show evidence of the involvement of B cells in X-GVHD and may have implications in the treatment of the disease.

KEYWORDS IL-21, B cell, xenogeneic GVHD, immunodeficient mice, immunoglobulin

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative option for a variety of malignant hematological diseases (Welniak et al., 2007). Although HSCT has been successfully used for treatment of these diseases, but it is not without risk. The major complication following HSCT is the development of graft-versus-host disease (GVHD), sometimes with a fatal outcome (Shlomchik, 2007). An animal model, xenogeneic GVHD (X-GVHD), that mimics accurately the clinical GVHD, would help to develop therapies and provide a tool for investigating the mechanism involved in disease pathogenesis. Neither CB17-*scid* nor NOD-*scid* immunodeficent mice engrafted with human PBMCs provided a reproducible model of xenogeneic GVHD (Shultz et al., 2007). BALB/c- Rag2$^{-/-}$ IL-2Rγc$^{-/-}$ (BRG) mice, which have no B, T, or NK cells and no leakiness, showed improved human PBMCs engraftment compared with NOD-*scid* mice (van Rijn et al., 2003; Shultz et al., 2007; Ito et al., 2009; Shultz et al., 2011). Recently, BRG mice which transferred with hPBMCs (hu-PBMC BRG mice) were used as a suitable platform to explore the effect of novel GVHD therapy agents, such as immunoglobulin or regulatory T cells (Tregs) (Mutis et al., 2006; Gregoire-Gauthier et al., 2012).

T cells have been identified as a key player in the graft-versus-host reaction and both donor CD4 and CD8 T cells have crucial roles in the pathogenesis of GVHD (Shlomchik, 2007; Blazar et al., 2012). Because of their ability to differentiate into various effector T cell subsets—Th1 cells, Th2 cells, and Th17 cells, CD4 T cells were considered as potential targets for treatment of GVHD in the clinic (Blazar et al., 2012). Recently many approaches, such as T cell depletion (anti-

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thymocyte globulin, anti-CD7 antibody, etc.), T cell activation inhibition (PKC inhibitors and antibodies specific for CD2, CD3 or CD147) and induction of Treg cells (Bortezomib, low-dose IL-2 or HDAC inhibitors) have been used in clinical trials for the prevention or treatment of GVHD (van Oosterhout et al., 2000; Lopez et al., 2006; Evenou et al., 2009; Blazar et al., 2012). However, these approaches usually leave patients at risk of complications such as infection or cancer relapse. Recent studies showed an association between high numbers of donor B cells and the development of both acute and chronic GVHD (Shimabukuro-Vornhagen et al., 2009). And treatment with rituximab—a CD20-specific monoclonal antibody that depletes B cells before HSCT, reduced GVHD incidence (Christopeit et al., 2009). In addition, IVIG (intravenous immunoglobulin) was reported to prevent xenogeneic GVHD by inhibiting the reconstitution of B cells, but not T cells (Gregoire-Gauthier et al., 2012). Therefore, specific approaches which targeted to B cells might be beneficial for the treatment of GVHD.

IL-21 is produced by $CD4^+$ T cells and NKT cells, which could promote the activation, differentiation, maturation of NK cells, B cells, $CD8^+$ and $CD4^+$ T cells, dendritic cells, and macrophages (Spolski and Leonard, 2008). Previous reports showed that blocking IL-21 signaling, either by genetically depletion or via neutralizing mAbs, reduced disease severity in murine models of GVHD (Bucher et al., 2009; Meguro et al., 2010; Hanash et al., 2011; Meguro et al., 2011). Similar results were obtained in xenogeneic GVHD model by injecting a neutralizing anti-human IL-21 mAb (Hippen et al., 2012). Disease amelioration was related with a decrease in the numbers of IFN-γ-secreting $CD4^+$ T cells and an increase in the proportion of $CD4^+$ Tregs. Using Treg-depleted donor cells, Bucher et al demonstrated that the increased frequency of Tregs in anti-IL-21 mAb treating group was due to the conversion of $CD4^+CD25^-$ T cells into inducible regulatory T cells (Bucher et al., 2009). However, in xenogeneic GVHD model the effect of IL-21 on B cells has not been reported. Therefore, we wondered the effect of IL-21 on human B cells in xenogeneic GVHD.

In this work, we treated $Rag2^{-/-}IL\text{-}2R\gamma c^{-/-}$ immunodeficieny mice with hPBMCs intravenously to establish the X-GVHD model, and human IL-21 was systemically expressed using a hydrodynamics-based gene delivery technique. We found that administration of IL-21 plasmid DNA resulted in high levels of circulating IL-21 *in vivo*, which exacerbated the process of X-GVHD. Surprisingly, B cells in IL-21 group augmented on day 7, while there was no increase in the quantity of T cells in spleen. Furthermore, compared with control group, IL-21 induced higher hIgM and hIgG, and increased the accumulation of $CD19^+$ $CD38^{high}$ plasma cells in spleen. Finally, we demonstrated that B-cell depletion was able to abolish X-GVHD. Collectively, we found that IL-21 treatment could promote B cell expansion, plasma cell differentiation, and immunoglobulin secretion, which were correlated with the accelerated X-GVHD progression. Our finding may provide an outlook on the therapeutic prospect for GVHD.

RESULTS

Administration of hIL-21 plasmid resulted in high levels of expression *in vivo*

A full-length hIL-21 gene was amplified by RT-PCR from human PBMCs and then ligated into PTT3-Flag plasmid. We determined the kinetic expression level of hIL-21 in BRG mouse serum after intravenous hydrodynamic injection of PTT3-hIL-21-Flag plasmid DNA into the tail vein. As shown in Fig. 1A, one day after a single dose of 20 μg of PTT3-hIL-21-Flag plasmid, a high level of IL-21 was detected in BRG mouse serum (about 40,000 pg/mL) by a sandwich double antibody ELISA. Serum levels of hIL-21 decreased quickly but were still higher than 500 pg/mL on day 5 and returned to baseline on day 7. No detectable hIL-21 was found in serum from naïve mice and mice that received injections of the same amount of control plasmid DNA. Importantly, we did not observe obvious toxicity such as weight loss and capillary leaking, caused by the *in vivo* expression of hIL-21 plasmid DNA (data not shown).

Human IL-21 exacerbated the process of xenogeneic GVHD

To determine whether IL-21 could enhance GVHD, we chose an X-GVHD model using BRG mice. We expressed hIL-21 protein in BRG mice by hydrodynamic injection. 1 day after injection of IL-21 or control plasmid, the mice were sub-lethally irradiated and then injected with hPBMCs on the same day. The kinetic expression of IL-21 in hu-PBMC BRG mice was similar to that in naïve BRG mice (data not shown). Consistent with earlier reports, morbid mice in two groups developed hunched posture, inactivity, and labored breathing. As shown in Fig. 1B and 1C, hu-PBMC BRG mice in IL-21 group exhibited accelerated weight loss and significantly faster disease development compared with control group (median survival time, 14 days versus 31.5 days, $P < 0.01$). And BRG mice that were injected hIL-21 plasmid without human PBMCs displayed 100% survival (Fig. 1B). These results indicated that hIL-21 exerted an effect on hPBMCs to exacerbate the process of X-GVHD.

Human IL-21 induced B cell engraftment and robust secretion of immunoglobulin

Next, we wonder the effect of IL-21 on human immune cell sub-populations *in vivo*. Flow cytometric analysis of hu-PBMC-BRG mouse splenocytes was performed after plasmid administration. Because serum hIL-21 can sustain as long as 7 days, hu-PBMC-BRG mice were sacrificed on day 7. In agreement with previous studies (van Rijn et al., 2003), the majority of engrafted cells in BRG mice were $CD3^+$ T cells and $CD19^+$ B cells (>90%, Fig. 2A). Surprisingly, the percentage of $CD19^+$ B cells in spleen significantly increased in IL-21-treated group compared with control group (23.5 ± 2.5 versus 5.1 ± 0.5, $P < 0.001$), but the percentage of $CD3^+$ T cells decreased in IL-21-treated group (67.8 ± 3.2 versus 87.3 ± 1.8, $P < 0.001$, Fig. 2A). Because the total number of human lymphocytes

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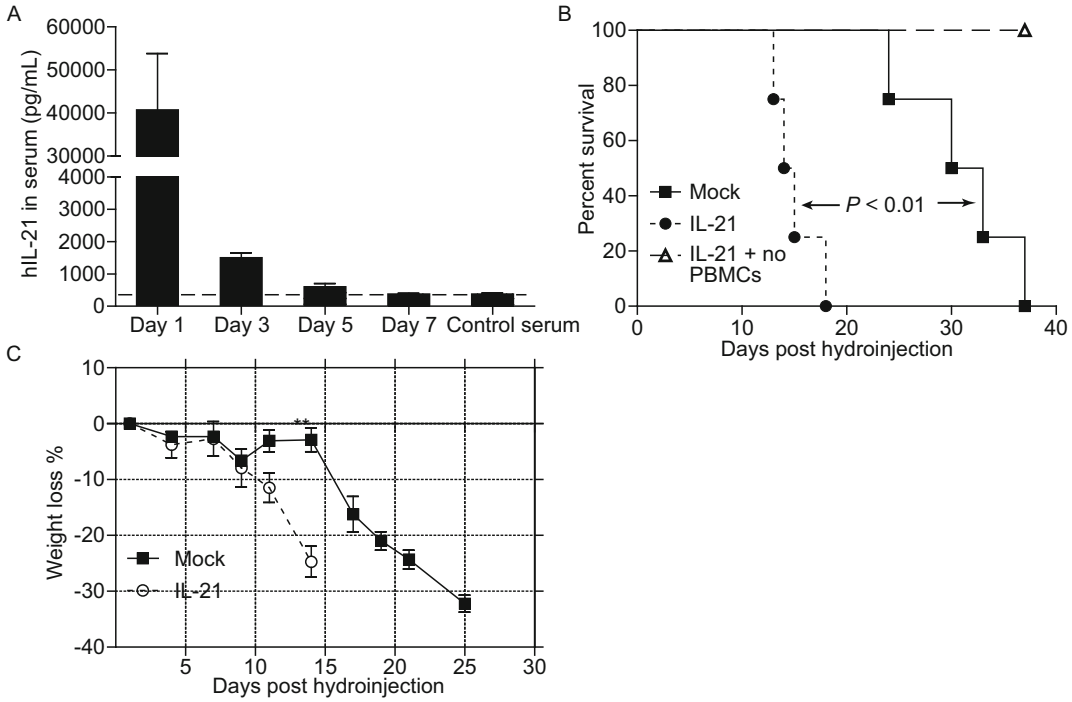

Figure 1. Human IL-21 exacerbates the process of xenogeneic GVHD. (A) Naïve Rag2$^{-/-}$IL-2γc$^{-/-}$ mice were hydrodynamic injected with PTT3-hIL21-Flag plasmid (n = 5) or mock control plasmid (n = 4) on day 0. The concentrations of hIL-21 in serums from IL-21 treated mice were measured at indicated time points. Serums from mice treated with mock control plasmid after 1 day were used as control serums. (B and C) Rag2$^{-/-}$IL-2γc$^{-/-}$ mice were hydrodynamic injected with PTT3-hIL21-Flag plasmid (dashed line) or mock control plasmid (solid line) on day 0. On day 1, mice were irradiated and intravenously transfused with 1.0 × 10^7 freshly isolated human PBMC. Mice receiving PTT3-hIL-21-Flag plasmid but without PBMCs transfer were set as a control (long dashed line). The weight loss was measured twice a week. (B) Survival of hIL-21 treated mice (median survival time = 14, n = 4) was significantly shorter than that of control mice (median survival time = 31.5, n = 4, Long-rank test, P < 0.01). (C) The average weight loss is shown as a percentage of starting weight (unpaired Student t test, **P < 0.01). Each data point represents the mean SEM. Data shown are representative of three experiments.

increased after IL-21 plasmid administration (Fig. 2B), the absolute number of B cells and T cells was even more profound than the percentage. As shown in Fig. 2B, the absolute number of human B cells was still remarkably higher in IL-21 group than that in control group. However, there was no difference in the absolute number of human T cells between mice receiving IL-21 plasmid and control plasmid. Besides cell number, we further analyzed the cytokine producing ability of human T cells involved in X-GVHD. Human Th1 and Th2 cytokines were measured in serum of mice on day 7 (Fig. 2C). Differences in human IFN-γ, TNF-α, IL-10, IL-5, and IL-4 levels were not observed between IL-21-treated group and control group. Collectively, these observations suggested that the expression of hIL-21 *in vivo* had no obvious biological effects on T cells during the early stage of X-GVHD.

Because autoantibodies play a major role in many kinds of autoimmune disorders, such as SLE (Sherer et al., 2004), rheumatoid arthritis (Nielen et al., 2004), and so on, we hypothesized that the expanded B cells in IL-21-treated mice might produce more antibodies which were specific to recipient tissues. The concentrations of human IgG and IgM in serum were determined by ELISA, and we found that the levels of both kinds of immunoglobulin in IL-21-treated mice were about 20–30 times higher than those in control group (IgG, 44.31 ± 9.3 versus 1.9 ± 0.6 μg/mL; IgM, 59.6 ± 13.6 versus 1.6 ± 0.6 μg/mL, respectively, Fig. 2D). Taken together, our results indicated that human IL-21 could enhance B cell engraftment and immunoglobulin secretion in X-GVHD.

Human IL-21 promoted B cell proliferation and immunoglobulin secretion during the early stage of X-GVHD

In order to determine whether IL-21 promote B cell proliferation or survival after transfer, we further detected the kinetics of human B cells in hu-PBMC-BRG mice treated with hIL-21 plasmid. Human B cells in spleen were evaluated every two days after injection of IL-21 or control plasmid. As shown in Fig. 3A and 3B, there was no difference in CD19$^+$ B cells between IL-21 group and control group on day 2 and day 4, but the percentage and absolute number of B cells were significantly higher in IL-21 group than those in control group on day 6. Since the absolute number of B cells decreased quickly in

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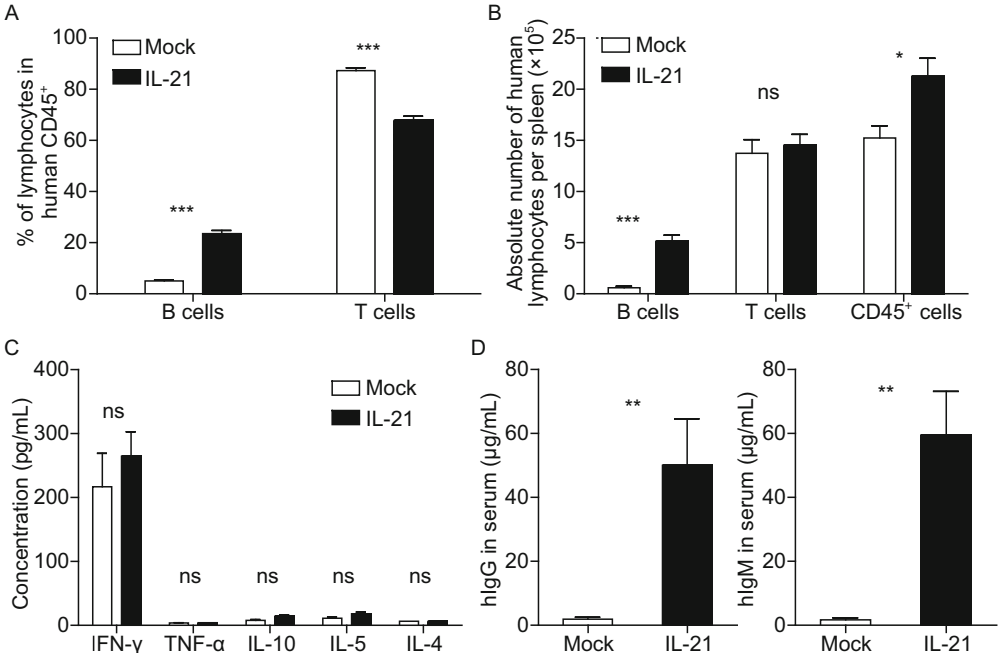

Figure 2. Human IL-21 induces B cells engraftment and robust secretion of immunoglobulins. Hu-PBMC BRG mice were treated with hIL-21 plasmid (n = 4) or control plasmid (n = 4) as described in Fig. 1. (A and B) Lymphocytes were prepared from spleens on day 7, and then stained with antibodies specific for human CD45, CD3, and CD19. Samples were analyzed by flow cytometry to calculate the percentages and absolute number of human T and B lymphocytes. (C and D) On day 7, serums were harvested and analyzed the presence of human cytokines (C) and the concentration of human IgG and IgM (D) as described in materials and methods. Data shown are representative of three experiments. Unpaired Student t test was performed, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

both groups from day 2 to day 4, IL-21 may have no effect on B cell survival. And after day 4, B cells in both groups began to proliferate, and IL-21 showed obvious promotive effect on B cells proliferation. We also detected the human immunoglobulin levels in hu-PBMC-BRG mice, which reflected the state of activated B cells. Before day 4, the IgG and IgM in both control group and IL-21 group were at a very low level (Fig. 3C and 3D). It is notable that large amounts of IgG and IgM were detected in IL-21 group as early as day 6, and the levels of immunoglobulin only slightly raised in control mice. These results suggested that IL-21 could promote B cell proliferation but not survival after PBMC transfer, and IL-21 might contributed to the differentiation of B cells.

IL-21 enhanced the differentiation of human B cells into plasma cells *in vivo*

It is well accepted that the frequency of plasma cells generally is correlated with the amount of Ig production *in vivo* (Ettinger et al., 2005), so the capacity of IL-21 to induce plasma cells differentiation was explored. 7 days after plasmid administration, flow cytometric analysis of hu-PBMC-BRG mouse splenocytes was performed. $CD19^+$ $CD45^+$ cells were gated and assessed for the expression of CD38. As shown in Fig. 4A, only a small fraction (5.4%) of freshly isolated peripheral B cells were $CD19^+$ $CD38^{high}$ plasma cells, which agreed with previous reports (Ettinger et al., 2008). After 6 days *in vivo*, the percentage of $CD19^+$ $CD38^{high}$ plasma cells was significantly higher in IL-21 treated group than that in control group (79.8 ± 1.6 versus 61.3 ± 4.7, Fig. 4B). And the absolute number of plasma cells was also higher in IL-21 group, compared with control group (Fig. 4C). Taken together, B cells could differentiate into plasma cells in the model of X-GVHD, and IL-21 could accelerate the process of plasma cell differentiation.

B cells were important to the process of X-GVHD

Given the significant expansion and differentiation of human B cells after IL-21 treatment, we next wondered the role of B cells in X-GVHD. We intravenously injected pre-irradiated BRG mice with PBMCs or non-B cell PBMCs which were isolated from PBMCs (Fig. 5A). Following injection, we assessed the development of X-GVHD by recording body weight every 3 days. As shown in Fig. 5B and 5C, BRG mice transferred with non-B cell PBMCs did not exhibit weight loss until day 25 and survived long-term without evidence of GVHD, whereas mice transferred with PBMCs developed lethal X-GVHD. These results demonstrated that B-cell depletion reduced the incidence of X-GVHD.

DISCUSSION

Since the physiological effect of IL-21 on B cells *in vivo* has

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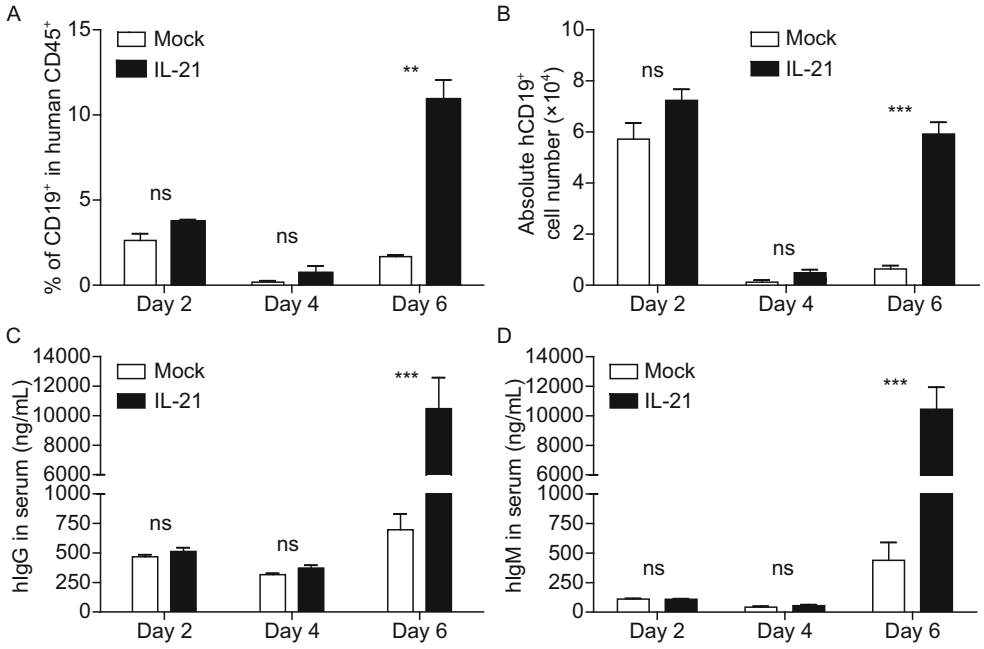

Figure 3. Human IL-21 promotes B cells proliferation and immunoglobins secretion during the early stage of X-GVHD. Hu-PBMC BRG mice were treated with hIL-21 plasmid or control plasmid as described in Fig. 1. Lymphocytes were prepared from spleens at indicated time periods (n = 3, IL-21 group and control group, respectively), and then stained with antibodies specific for human CD45 and CD19. Samples were analyzed by flow cytometry to calculate the percentages (A) and absolute accumulation (B) of human B lymphocytes. The presence of human IgG (C) and IgM (D) in mice (n = 7, IL-21 group and control group, respectively) serum was determined as described in materials and methods. Data shown are representative of three experiments. Unpaired Student t test was performed, $^{**}P < 0.01$, $^{***}P < 0.001$.

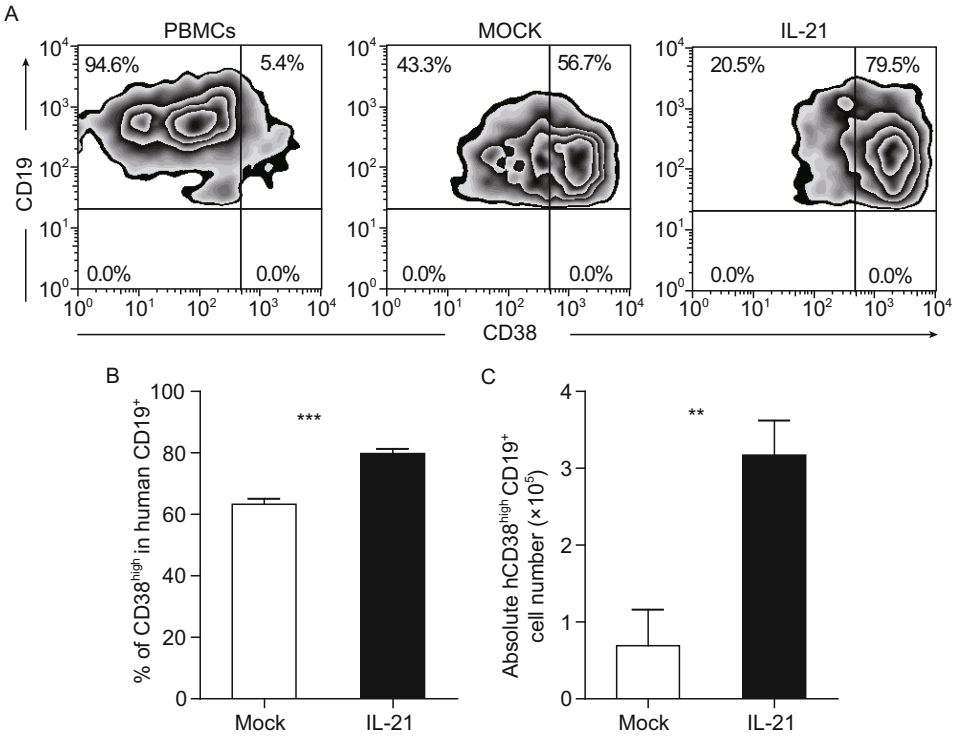

Figure 4. IL-21 enhances the differentiation of human B cells into plasma cells *in vivo*. Hu-PBMC BRG mice were treated with hIL-21 plasmid (n = 7) or control plasmid (n = 7) as described in Fig. 1. Lymphocytes were prepared from spleens on day 7, and then stained with antibodies specific for human CD45, CD38, and CD19. (A) Human $CD45^+CD19^+$ B cells were gated and the percentage of $CD38^{high}$ plasma cell was shown. The percentage of plasma cells in healthy donor PBMCs before transfer was shown as a control. One representative percentage (B) and absolute number (C) of human plasma cells from two experiments were shown. Unpaired Student t test was performed, $^{**}P < 0.01$, $^{***}P < 0.001$.

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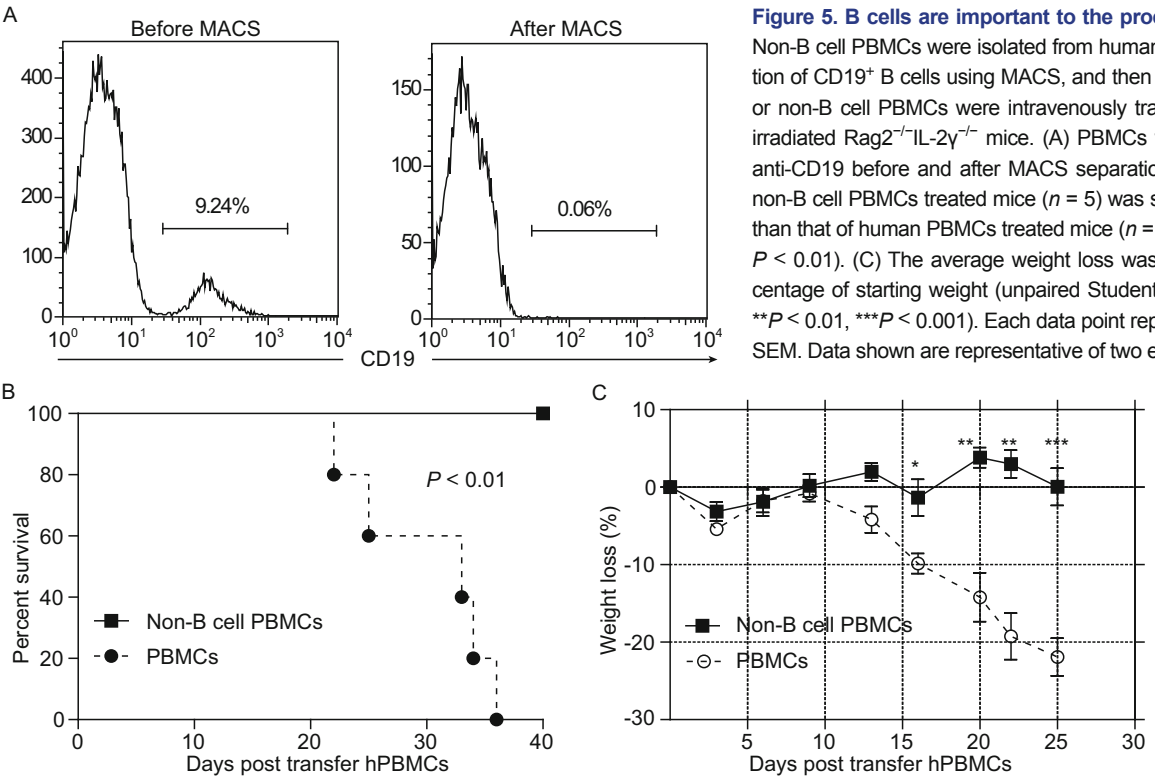

Figure 5. B cells are important to the process of X-GVHD. Non-B cell PBMCs were isolated from human PBMC by depletion of $CD19^+$ B cells using MACS, and then 1.0×10^7 PBMCs or non-B cell PBMCs were intravenously transferred into pre-irradiated $Rag2^{-/-}IL\text{-}2\gamma^{-/-}$ mice. (A) PBMCs were stained with anti-CD19 before and after MACS separation. (B) Survival of non-B cell PBMCs treated mice ($n = 5$) was significantly longer than that of human PBMCs treated mice ($n = 5$, Long-rank test, $P < 0.01$). (C) The average weight loss was shown as a percentage of starting weight (unpaired Student t test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Each data point represents the mean SEM. Data shown are representative of two experiments.

not been well described, in this study, we expressed human IL-21 by hydrodynamic injection in an X-GVHD model using hu-PBMC BRG mice. We found that human IL-21 expression exacerbated X-GVHD and resulted in rapid fatality. In the early stage of X-GVHD, a marked expansion of human $CD19^+$ B cells, but not T cells, was observed in IL-21 treated mice. The number of $CD19^+$ $CD38^{high}$ plasma cells also increased, accompanied by robust IgM and IgG production after IL-21 treatment. Our results firstly revealed the *in vivo* effects of IL-21 on B cells in X-GVHD model.

Previous studies showed that T cells played a major role in the progression of GVHD (Shlomchik, 2007), and IL-21 was demonstrated to promote the proliferation and cytokine secretion of both CD4 and CD8 cells (Leonard and Spolski, 2005). In xenogeneic GVHD model, neutralizing anti-IL-21 mAb could attenuate GVHD by inhibiting CD4 T cell function (Hippen et al., 2012). In our model, when hIL-21 was highly expressed in the early stage of X-GVHD, the number of human T cells decreased during the first 2–3 days. Then the number of T cells began to rise, which was similar to the kinetics of B cells (Fig. 3B). But the T-cell dynamic changes were nearly the same between IL-21 and control groups during the early stage of GVHD (data not shown). Besides cell number, we also analyzed the levels of cytokines which could reflect T cell functions in mice serum. And we found no significant difference in human IFN-γ, TNF-α, IL-10, IL-5, and IL-4 levels on day 7. Taken together, in the early phase of our X-GVHD model, the effect of IL-21 on T cells was not obvious. IL-21 has pleiotropic effects on NK cells, such as enhanced proliferation and increased cytotoxicity (Spolski and Leonard, 2008). Previous report showed that NK cells were very low in all compartments during the process of xenogeneic GVHD (van Rijn et al., 2003). And in our xenogeneic GVHD model, $CD56^+$ $CD3^-$ NK cells were almost absent in spleen of IL-21-treated group and control group on day 7. Therefore, these results indicated that IL-21 showed little help to NK cells in the early stage of xenogeneic GVHD. In addition, IL-21 is also a critical regulator of Th17 development (Spolski and Leonard, 2008). Because of the low number of human T cells in spleen on day 7, it is difficult for us to identify Th17 cells by their ability to express IL-17 after *ex vivo* stimulation. If possible, we will detect Th17 cells at the late stage of X-GVHD in the future.

The impact of IL-21 on human B-cell responsiveness was addressed *in vitro*, and all of the experiments were carried out, in which human B cells were isolated and stimulated with IL-21 in combination with anti-CD40 and/or anti-IgM, which mimic the BCR-antigen and B cell-T cell interaction (Ettinger et al., 2008; Konforte et al., 2009). Notably, IL-21 was found to induce proliferation and differentiation of human B cells isolated from PBMCs and spleen (Ettinger et al., 2005; Good et al., 2006). In our xenogeneic GVHD model, B cells were activated and proliferated after engaging mouse tissues with the help of human T cells. The number of human B cells and plasma cells also increased in hIL-21 group compared with control group. These results suggested that IL-21 treatment could promote B cell expansion and plasma cell differentiation *in vivo*, which was consistent with the studies *in vitro*. In addition, as early as 5 days after human PBMCs injection, large amounts of hIgG

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and hIgM could be assayed in IL-21 group, consistent with the *in vitro* studies that IL-21 efficiently induced Ig production from purified B cells stimulated with anti-CD40 and/or anti-IgM. Therefore, the physiological function of IL-21 on B cells *in vivo* is similar to that on isolated B cells stimulated *in vitro*.

Yoshizaki A et al. found that the severity of EAE increased when transferring Il21r$^{-/-}$ CD1d^{hi} CD5^{+} B cells. They demonstrated that *ex vivo* IL-21 drove the development and expansion of CD1d^{hi} CD5^{+} B-cell, which produced IL-10 to inhibit disease symptoms when transferred into mice with established autoimmune disease (Yoshizaki et al., 2012). In our xenogeneic GVHD model, we found robust B cell proliferation and differentiation in IL-21-treated group, but we did not detect such B10 cells because of the very low number of human B cells. If B10 cells had been induced by exogenous IL-21, the T cell immune response should be inhibited. However, our results showed that the process of xenogeneic GVHD was accelerated after IL-21 treatment (Fig. 1B and 1C). In addition, based on our cytokine secretion results, the levels of IL-10 and IFN-γ in serum were similar between IL-21-treated group and control group, which indicated that the IL-10 producing B10 cells and the functions of T cells were not affected by IL-21. Taken together, the effect of IL-21 on B cells might vary between autoimmune EAE model and our xenogeneic GVHD model.

B cells in PBMCs mainly consist of naïve B cells and memory B cells. And naïve and memory B cells in PBMCs react differently to the antigen. In our experiments, there were about 60% naive B cells and 30% memory B cells in PBMCs, which varied among different donors. In X-GVHD model, both naïve B cells and memory B cells were injected into BRG mice, but we did not examine the difference of proliferation and differentiation between naïve and memory B cells in response to IL-21. Previous study showed that IL-21R was expressed on naïve B cells, but not on memory B cells (Good et al., 2006). Although memory B cells lacked IL-21R, it was rapidly induced following activation *in vitro* (Good et al., 2006). This may allow memory B cells to proliferate to the same extent as naïve B cells in *in vitro* culture. So we hypothesized that both naïve B cells and memory B cells in response to IL-21 proliferated in X-GVHD model.

We were also eager to know whether IL-21 had direct effect on B cells to accelerate X-GVHD and tried hard to solve this problem. However, it is important to note that the mechanism of GVHD is very complex, and CD4^{+} T cells, CD8^{+} T cells, and Th17 cells have crucial roles in the pathogenesis of GVHD (Blazar et al., 2012), which may compensate for the loss of B cells. So we did not prove this hypothesis and we will pay attention to it in the future experiments at the late stage of GVHD. Clinical trials showed that treatment with a CD20-specific antibody that depletes B cells, reduced GVHD incidence (Shimabukuro-Vornhagen et al., 2009; Sarantopoulos et al., 2011). In an allogeneic GVHD model, researchers found that the recipients given B-cell-depleted donor spleen (B220^{-} splenocytes) cells did not develop chronic GVHD over more than 40 days (Zhang et al., 2006). These studies indicated that donor B cells played an important role in the process of GVHD. And in our xenogeneic GVHD model, we also observed that the BRG mice given non-B cell PBMCs did not exhibit GVHD symptoms for 25 days. This result is consistent with previous report in allogeneic GVHD, and suggests that B cells were important to xenogeneic GVHD. We suppose that B cells act as APCs to augment T cell immune responses, which might be proved in the future.

Because autoantibodies play a major role in many kinds of autoimmune disorders, such as SLE, RA, and so on (Nielen et al., 2004; Sherer et al., 2004), we hypothesize that the expanded B cells in IL-21 treating mice may produce more antibodies which are specific to recipient tissues. Antibody production is one of the most important ways to protect us against infection and malignancy. When the immune system is weak and ineffective, autoantibodies, which directed at the body's own (self) cellular components, are produced. Most autoimmune diseases are caused when specific autoantibodies develop and begin to injure the body's tissues and cells. In our X-GVHD model, after engaging mouse tissues, human B cells were activated and proliferated, and then differentiated into plasma cells. High levels of IgM and IgG were produced by plasma cells which should be specific to mice tissues. For mice, these antibodies were actually autoantibodies. Because the production of autoantibodies was correlated with the progress of GVHD, we hypothesize that autoantibody is an important factor in the occurrence of GVHD. We are trying to confirm this by detecting or staining the complexes formed by autoantibodies and autoantigens.

The ability of IL-21 on proliferation of B cells indicates that it may have clinical application in conditions of immunodeficiency. For example, severe deficiency of memory B cells was observed in immunocompromised individuals, such as patients with immunodeficient states (Warnatz et al., 2002). Therefore, careful injection of IL-21 has the potential to restore the B cell compartment of these patients.

Taken together, our results firstly defined the effect of IL-21 on B cells in *in vivo* model. In X-GVHD model which well mimic the clinical presentation of GVHD, we found that IL-21 treatment could promote B cell expansion, plasma cell differentiation, and immunoglobulins secretion, which were related with the exacerbation of xenogeneic GVHD. Specifically targeting B cells or immunoglobulin may provide new approaches for the treatment of GVHD.

MATERIALS AND METHODS

Immunodeficient mice

BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice were kindly provided by Pro. Lieping Chen, Department of Immunobiology, Yale University School of Medicine, New Haven, USA. Male and female BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice used were between 8–12 weeks of age. The mice were maintained under specific pathogen-free conditions in the animal facility at the Institute of Biophysics, Chinese Academy of Sciences.

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Plasmid *in vivo* transfection

The IL-21 cDNA was cloned from stimulated human PBMCs and inserted into PTT3-Flag to construct the IL-21 expression plasmid, while the mock PTT3-Flag was used as control plasmid in the study. To exclude endotoxin contamination, large scale preparation of PTT3-hIL-21-Flag and the control PTT3-flag plasmid DNA was purified by the EndoFree Plasmid Mega kit (Qiagen, Germany). Plasmid DNA was transfected *in vivo* by using a hydrodynamic-based gene transfer technique. Briefly, 20 μg/mouse plasmid DNA was diluted in 2 mL of PBS (about 0.1 mL/g body weight) and injected into the tail vein using a 27-gauge needle and syringe within a time period of 5–8 s. The dynamic expression of IL-21 *in vivo* was confirmed by detecting IL-21 concentration in the serum with an ELISA kit from eBioscience (88-7216, San Diego, CA, USA) at indicated time points.

Cell isolation and xenogeneic graft-versus-host disease (X-GVHD) model

Human PBMCs were isolated from heparinized blood of healthy donors by Ficoll-Hypaque density centrifugation, and then resuspended in PBS. For B cell depletion, PBMC were stained with FITC conjugated mAb specific for human CD19, followed by anti-FITC microbeads (Miltenyi, Bergisch Gladbach, Germany). The magnetically labeled B cells are depleted by retaining them on a MACS Column in the magnetic field of a MACS Separator, while the unlabeled non-B cell PBMC pass through the column. BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice were hydrodynamic injected with PTT3-hIL21-Flag plasmid or mock control plasmid on day 0. On day 1, mice firstly received total body irradiation with a single dose of 3.5 Gy and then 1.0 × 10^7 freshly isolated human PBMCs or non-B cell PBMC were intravenously injected via the tail vein on the same day. Body weight loss and other GVHD symptoms including hunched posture, ruffled fur, reduced mobility, and tachypnoea were measured.

Measurement of human immunoglobulins and cytokines

The concentrations of human IgG and IgM in serum of mice were determined using double antibody sandwich ELISA. All of these detection antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-hIgG (I1886) and peroxidase conjugated anti-human IgG (A0170) were used for hIgG detection. Anti-human IgM (I2386) and peroxidase conjugated anti-human IgM (A0420) were used for hIgM detection.

The concentrations of human interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-4, IL-5, IL-10 were measured using the human Th1/Th2 Cytokine Bead Array (BD pharmingen, San Diego, CA, USA).

FACS analysis

Mice were sacrificed at indicated time point. Cells were harvested from the spleens and stained with antibodies specific for human CD45, CD3, CD19, and/or CD38. Samples were analyzed by flow cytometry to calculate the percentages and absolute accumulation of human lymphocytes.

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ABBREVIATIONS

BRG, BALB/c- Rag2$^{-/-}$ IL-2Rγc $^{-/-}$; GVHD, graft-versus-host disease; PBMCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; X-GVHD, xenogeneic graft-versus-host disease

COMPLIANCE WITH ETHICS GUIDELINES

Xiaoran Wu, Yi Tan, Qiao Xing and Shengdian Wang declare that they have no conflict of interest.

Human PBMCs were obtained from healthy donors, which was approved by the Ethics Committees of the Institute of Biophysics, Chinese Academy of Sciences. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5).

All institutional and national guidelines for the care and use of laboratory animals were followed.

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