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ORIGINAL ARTICLE

Interleukin-33 prolongs allograft survival during chronic cardiac rejection

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allograft survival, antibody-mediated rejection, cardiac transplantation, interleukin-33, myeloid-derived suppressor cells, Treg.

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Summary

Interleukin-33 (IL-33) stimulates the generation of cells and cytokines characteristic of a Th2 immune response. We examined the effects of IL-33 on allografted heart tissue in a chronic cardiac rejection model, including analysis of the peripheral myeloid and lymphoid compartments. B6.C-H2bm12/KhEg hearts were transplanted into MHC class II-mismatched C57Bl/6J mice; IL-33 was administered daily. Cells from allografts and spleens were isolated for flow cytometry and cultured for cytokine production; some tissues were used for immunohistochemistry. Animals treated with IL-33 showed significantly longer allograft survival, which was associated with a distinct cytokine profile produced by graft-infiltrating cells. Proinflammatory IL-17A production was decreased with IL-33 treatment, while increased levels of IL-5, IL-10, and IL-13 were observed. After IL-33 therapy, flow cytometry showed a direct induction of $CD4^+$ $Foxp3^+$ Treg, whereas the number of $B220^+$ $CD19^+$ B cells, and circulating, as well as allograft deposited, alloantibodies was reduced. Following IL-33 treatment, a significant decrease in graft-infiltrating $CD11b^{high}Gr1^{high}$ granulocytes coincided with a significant increase in $CD11b^{high}Gr1^{intermediate}$ myeloid-derived suppressor cells (MDSC). In conclusion, IL-33 treatment in the setting of chronic rejection promotes the development of a Th2-type immune response that favors MDSC and Treg expansion, reduces antibody-mediated rejection (AMR), and ultimately, prolongs allograft survival.

Introduction

IL-33 is a recently identified member of the IL-1 family and signals through its receptor ST2, which activates NF-κB and MAP-kinases [1,2]. ST2 is principally expressed on Th2 and mast cells, but also on basophils, natural killer cells, eosinophils, and myeloid progenitor cells [3–6]. It has been shown that IL-33 plays an important role in the pathophysiology of asthma, allergies, sepsis, arthritis, and inflammatory bowel disease by induction of Th2 cytokines and activation of mast cell-mediated inflammation [1,3,7–10]. Furthermore, it has been shown that IL-33 inhibits the development of atherosclerosis by inducing a switch from Th1 to Th2 cell differentiation and reducing macrophage foam cell formation [11,12].

In the context of transplantation, a Th1 response with upregulation of proinflammatory cytokines like IL-2, TNF-α, and IFN-γ is known to be principally responsible for allograft rejection [13,14]. In the absence of a Th1 immune response it has been shown that a T cell producing the proinflammatory cytokine IL-17A mediates allograft rejection [15]. However, these pathways leading to allograft rejection can be influenced. After blocking an acute graft-deteriorating rejection through anti-CD4 mAb, a change in T-cell homeostasis is thought to be involved in the regulatory process [16]. This is supported by the finding that exogenous administration of the Th2

cytokines IL-4 and IL-10 results in prolongation of allograft survival [17]. Thus, knowing that IL-33 is a strong inducer of a potentially beneficial Th2 response in the organ transplantation setting, we decided to test IL-33 therapy in a chronic murine heart rejection model. Besides monitoring for heart transplant survival, immune cells from transplanted heart tissue were profiled for differential phenotype and cytokine production patterns consistent with Th2 responses and immune regulation.

Material and methods

Heart transplantation and IL-33 treatment

An MHC class II mismatched model with female B6.C-H2bm12/KhEg (bm12) mice as donors and female C57Bl/6J mice as recipients was used (The Jackson Laboratory, Bar Habor, ME USA) [18]. Vascularized cardiac allografts were transplanted into the abdomen using a microsurgical technique as previously described by Corry *et al.* [19]. To increase the rejection intensity, we performed a reduced donor heart perfusion via the abdominal vena cava with cold 0.9% saline (3 ml) containing 500 IE heparin. Allograft failure was defined as complete cessation of a palpable heart beat, which was confirmed by laparotomy.

Foxp3-GFP-reporter mice (C57Bl/6J background) that express GFP under control of the Foxp3 gene promoter were used for Treg induction experiments (The Jackson Laboratory).

IL-33 protein

Purified biotinylated IL-33 protein was produced as described by Ali *et al.* by transformed *Escherichia coli* BL21 (DE3) [20]. Starting at day 5 after transplantation 1 μg of IL-33 in 100 μl PBS or only PBS was administered i.p. daily. Experiments were approved by the local animal commission of the University of Regensburg, and regional government authorities.

Histology

Cardiac allografts were harvested at day 20, and, in the IL-33 group, additionally at day 50 after transplantation. Formalin fixed, paraffin embedded heart and spleen tissue was sectioned for hematoxylin and eosin (H&E) staining. For immunohistochemical staining 2 μm sections were incubated with 5% albumin bovine (Biomol, Hamburg, Germany) for 1 h at RT. After washing with PBS (with 0.3% Triton-X-100; Sigma, Munich, Germany), sections were stained with monoclonal rat anti-mouse Ki-67 antibody (Dako M7249, Glostrup, Denmark) or purified Foxp3 rat anti-mouse antibody (14-5773 eBioscience, San Diego, CA USA) for 1 h at RT. After rinsing with PBS/Triton, sections were incubated for 1 h at RT with ready-to-use biotinylated goat anti-rat IgG (sc-3826 Santa Cruz Biotechnology, Heidelberg, Germany). For determination of IgG deposition in allografts, sections were stained with ready-to-use biotinylated goat anti-mouse IgG antibody (ab 64255, Abcam, Cambridge, UK). Streptavidin-HRP reagent (ImmunoBioScience, Everett, WA, USA) was applied for 15 min, followed by 3,3'-diamino-benzidine tetrahydrochlorhydrate (Merck, Darmstadt, Germany) for 5 min and finally washed with distilled water. The slides were counterstained with hematoxylin. Sections were analyzed by light microscopy. For quantitative analysis, positive cells from three random high-power fields (HPF; 20x magnification) were counted. Histologic evaluation of cardiac allograft rejection was performed according to the revised 2004 ISHLT grading system [21].

Cell isolation from cardiac grafts and spleens

Cardiac tissue was minced in 10 ml of RPMI 1640 medium with 10% fetal calf serum, 600 U/ml collagenase II (Roche Diagnostics, Mannheim, Germany) and desoxyribonuclease I (DNase, Sigma). This mixture was shaken at RT for 2 h and supernatant was flushed through a 100 μm nylon cell strainer (Schubert & Weiss, Munich, Germany). Remaining tissue was again digested in 5 ml of RPMI-collagenase-DNase solution at 37°C and strained through a 100 μm nylon strainer. Splenic tissue was minced and strained through a 100 μm nylon strainer. Digested cell suspensions were centrifuged for 5 min at 1500 rpm (4°C). To remove red blood cells, the pellet was treated with ACK lysis buffer (Lonza Walkersville, Walkersville, MD, USA) and incubated for 2 min at RT. After centrifugation, cells were suspended in HBSS medium (Gibco, NY, USA) and counted.

ELISA

Cardiac allografts were harvested at day 20 and graft-infiltrating cells were isolated; 5×10^5 cells/500 μl medium were cultured and stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies. Supernatants were removed after 48 h and levels of IL-5, IL-10, IL-13, IL-17A, and IFN-γ were determined in duplicate by ELISA (R&D Systems, Minneapolis, MN, USA; eBioscience, San Diego, CA, USA; BD Biosciences, San Jose, CA, USA).

Flow cytometry

Analyses were performed using a FACSCalibur or FACSCanto II flow cytometer (BD Biosciences). Data were obtained using BD CellQuest Pro acquisition software (BD Biosciences) and analyzed via FlowJo software (Tree

Star Inc., Ashland, OR, USA). Cell isolates were blocked with 1% mouse serum (Dako, Glostrup, Denmark) and stained with appropriate nonoverlapping conjugated monoclonal antibodies (anti-Gr1 antibody from Miltenyi Biotec, Bergisch Gladbach, Germany; all other antibodies from eBioscience). Foxp3 staining was carried out by first fixing and permeabilizing cells with Cytofix/Cytoperm solution (BD Pharmingen, San Diego, CA, USA). To determine if IL-33 directly or indirectly causes Treg induction, we reconstituted C.129S7Rag1^{tm1Mom}/J (Rag1$^{-/-}$) mice (Charles River, Sulzfeld, Germany) with 5×10^6 i.p. injected, MACS-sorted, CD4$^+$ cells from spleens of either wild-type or MyD88$^{-/-}$ C57Bl/6J mice (Oriental Bioservice Inc., Nishikyogoku, Japan). Reconstituted mice were treated with IL-33 for 20 days. Then splenic cells were harvested for flow cytometric evaluation. For cell cycle analysis of Treg, Vybrant Dye Cycle Violet Stain (V35003, Invitrogen, Carlsbad, California, USA) was used.

Serum alloantibody measurement

For analysis of alloantibodies, serum from C57Bl/6J recipients grafted with bm12 donor hearts was collected at day 20 after transplantation. 5×10^5 splenocytes isolated from bm12 mice per tube were incubated with recipient serum for 1 h. Cells were stained using appropriate nonoverlapping conjugated rat anti-mouse CD3, IgG and IgM antibodies and analyzed by flow cytometry. The mean fluorescence intensity of CD3$^+$ cells with respect to anti-IgG or anti-IgM fluorescence was determined and compared between the groups.

Statistical analysis

Kaplan-Meier graphs were constructed for survival analyses. Group comparisons were made using the log-rank (Mantel–Cox) test. ELISA data are presented as means ± SD, and comparisons between the values were performed using the two-tailed Student's *t*-test. The level of significance was set at a probability of $P < 0.05$.

Results

IL-33 prolongs allograft survival following cardiac allograft transplantation

We performed heterotrophic cardiac transplantation in an MHC class II mismatched model. Starting at postoperative day 5 we administered IL-33 or control solution. Allograft function was evaluated daily by palpation. In controls a median allograft survival of 21.5 days was observed. The IL-33-treated group showed a statistically significant prolonged allograft survival to >50 days (Fig. 1a).

IL-33 prevents coronary allograft vasculopathy

To detect changes in the allograft architecture H&E staining was performed on heart transplants after 20 days in control, and after 20 and 50 days in IL-33-treated mice. In controls we found signs of chronic allograft rejection with perivascular leucocytic infiltration, destruction of cardiac muscle structure, and coronary allograft vasculopathy (CAV) with complete or nearly complete obstruction of the vessels (Fig. 1b). In contrast, under IL-33 treatment we detected reduced leucocytic infiltration and normal cardiac muscle architecture. In addition, no CAV was observed. Control mice predominantly showed severe Grade 3R rejection according to the revised 2004 ISHLT grading system [21]. Mild Grade 1R allograft rejection was observed in the majority of IL-33-treated mice. These histologic differences were statistically significant (Fig. 1c). Similar results were found in sections from IL-33-treated recipients on postoperative day 50.

IL-33 induces splenomegaly

We also examined spleens from these mice to investigate IL-33 effects on the peripheral myeloid and lymphoid compartment. IL-33-injected mice showed splenomegaly, in contrast to the normal spleen size found in controls (Fig. 1d). This significant difference was confirmed when counting cells from dissociated spleens, where more than twofold the number of cells was present in IL-33-treated mice. In addition to the splenomegaly, we noted at laparotomy that mice treated with IL-33 had accumulated significant amounts of intraperitoneal fluid.

IL-33 induces cell proliferation in the spleen, but not in cardiac allografts

As increased numbers of spleen cells gave rise to the assumption that IL-33 affects cell proliferation in the periphery, we evaluated the proliferation rate of cells in cardiac allografts and spleens by staining tissue sections for the proliferation marker Ki-67. Based on the number of Ki-67 positive cells/HPF in cardiac allografts, an increased proliferation rate was observed in the IL-33-treatment group, though the difference compared with the control group was not significant (Fig. 2a and b). In contrast, in spleens of IL-33-treated mice, a significantly higher proliferation rate was detected compared with controls (Fig. 2c and d). These results suggest that IL-33 is a strong inducer of cell proliferation in the peripheral cell compartment.

IL-33 changes the cytokine expression of cardiac allograft-infiltrating cells

For surveying the effects of IL-33 treatment on the cytokine environment within allografts, graft-infiltrating cells

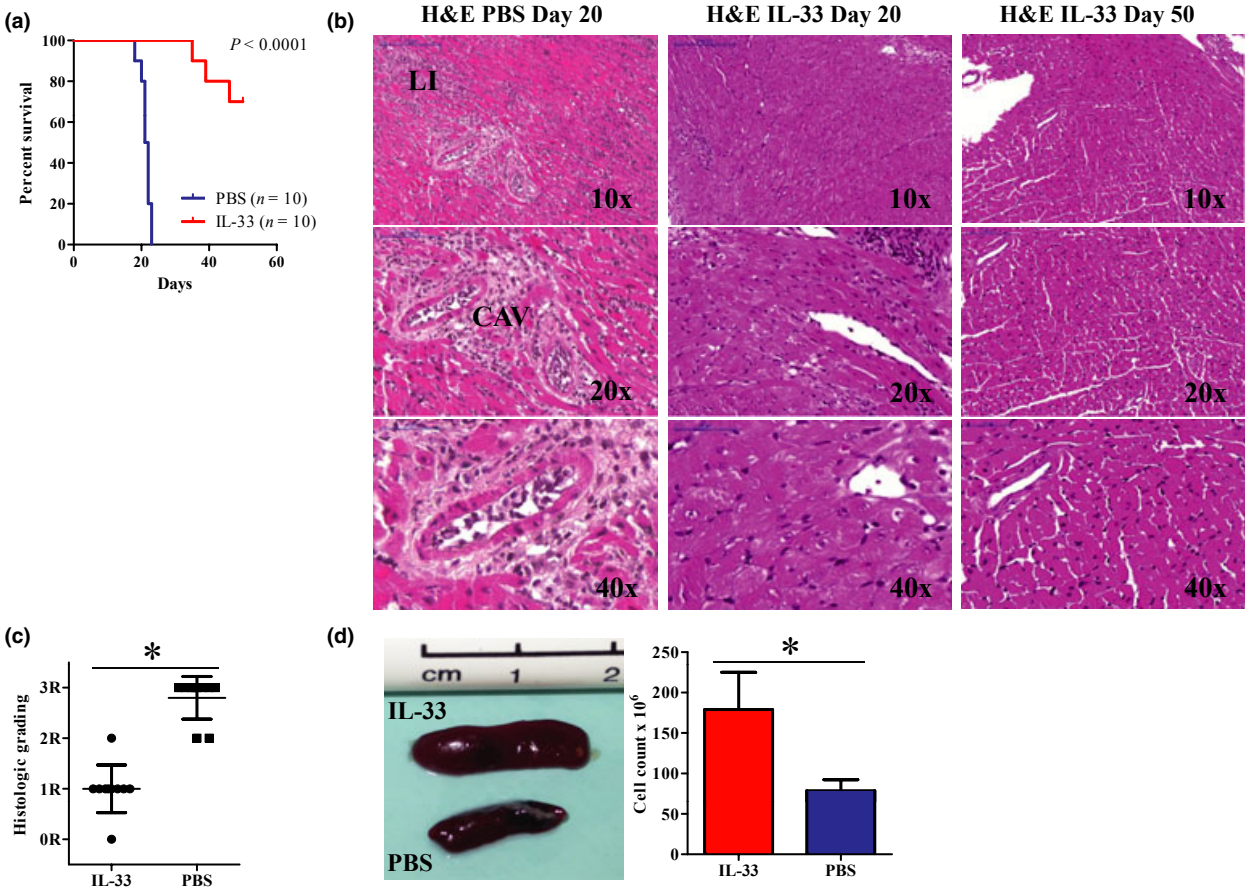

Figure 1 IL-33 treatment prolongs allograft survival and preserves cardiac muscle architecture in an MHC class II mismatched model. (a) Heart transplant survival (bm12 donor hearts to C57Bl/6J recipient mice) from mice treated with IL-33 or control PBS (Kaplan-Meier; $P < 0.0001$ between groups). (b) H&E staining of representative cardiac sections at postoperative days 20 and 50 from mice treated with IL-33 or control PBS (CAV coronary allograft vasculopathy; LI perivascular leucocytic infiltration). (c) Histologic grading of allograft rejection according to the revised 2004 ISHLT grading system in H&E stained sections of cardiac allografts treated with IL-33 or control PBS ($N = 10$ per group; $^*P < 0.05$). (d) IL-33 induces splenomegaly and significantly increases absolute cell numbers in the spleen. Absolute cell numbers were counted from seven mice per group ($^*P < 0.05$).

were harvested from transplanted hearts on postoperative day 20. These cells were cultured and analyzed for cytokine production by ELISA. Regarding IFN-γ levels, we found no significant differences between the IL-33-treatment group and the controls (Fig. 3). However, IL-33 induced significant increases in IL-5, IL-10, and IL-13 production from graft-infiltrating cells. In contrast, IL-17A production was strongly down-regulated in graft-infiltrating cells from IL-33-treated mice. These experiments suggest that while IL-33 treatment has little effect on the local Th1 cell response, it promotes polarization of the local immune response towards a Th2 phenotype, and at the same time inhibits Th17 reactivity. The molecular mechanism underlying changes in cytokine expression is unclear. However, it should be noted that the IL-33 receptor ST2 is generally expressed on T cells (Fig. S1).

IL-33 decreases antibody-mediated rejection

To examine the effect of IL-33 treatment on B cells, which are also important in the setting of chronic allograft rejection, we analyzed cells from study animal spleens by flow cytometry for changes in B cell numbers. After IL-33 therapy, we detected a significant decrease in the number of $B220^+$ $CD19^+$ B cells in the spleen (Fig. 4a and b). To test if the reduction in B cell numbers that was detected in 4 independent experiments led to decreased antibody-mediated rejection (AMR), allograft sections were stained for IgG deposition in control and in IL-33-treated mice. In controls we found high levels of IgG deposited in the allografts compared with low IgG levels in the IL-33-treated mice (Fig. 4c). These findings suggest that IL-33 treatment not only influences B cell

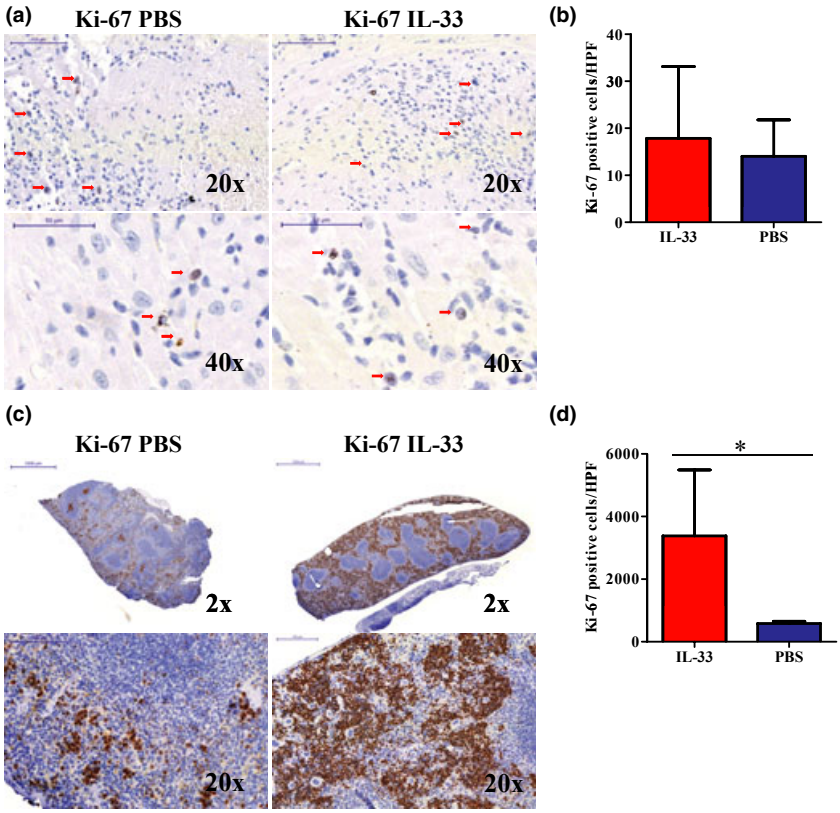

Figure 2 IL-33 treatment induces cell proliferation in the spleen. (a and b) Ki-67 staining of representative cardiac sections at postoperative day 20 from mice treated with IL-33 or control PBS. Ki-67 positive cells per high power field were counted from at least 8 mice per group. Red arrows indicate examples of Ki-67 positive cells. (c and d) Ki-67 staining of representative spleen sections at postoperative day 20 from mice treated with IL-33 or control PBS. Ki-67-positive cells per high power field were counted from at least 6 mice per group (*$P < 0.05$).

numbers in the periphery, but also reduces antibody deposition in the graft.

We further tested whether decreased B cell numbers are associated with reduced alloantibody production. We incubated donor bm12 splenocytes with C57Bl/6J recipient serum obtained on day 20 after transplantation. FACS analysis of $CD3^+$ cells in 5 independent experiments revealed significantly lower IgG and IgM alloantibody binding on cells incubated with serum from IL-33-treated mice (Fig. 4d and e). Altogether, these results suggest that IL-33 reduces AMR in cardiac allografts in this model.

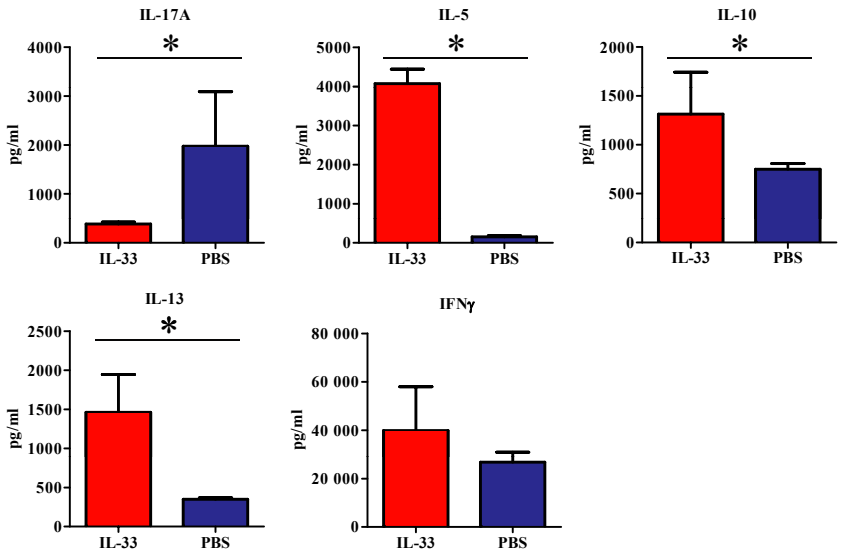

Figure 3 IL-33 treatment significantly changes the cytokine expression in graft-infiltrating cells. Graft-infiltrating cells were isolated on day 20 after transplantation and cells from 5 mice per group were pooled. 5×10^5 cells/500 µl were placed in cultures and stimulated for 48 h. Cytokines were determined in supernatants by ELISA (*$P < 0.05$). Experiments were conducted three times independently.

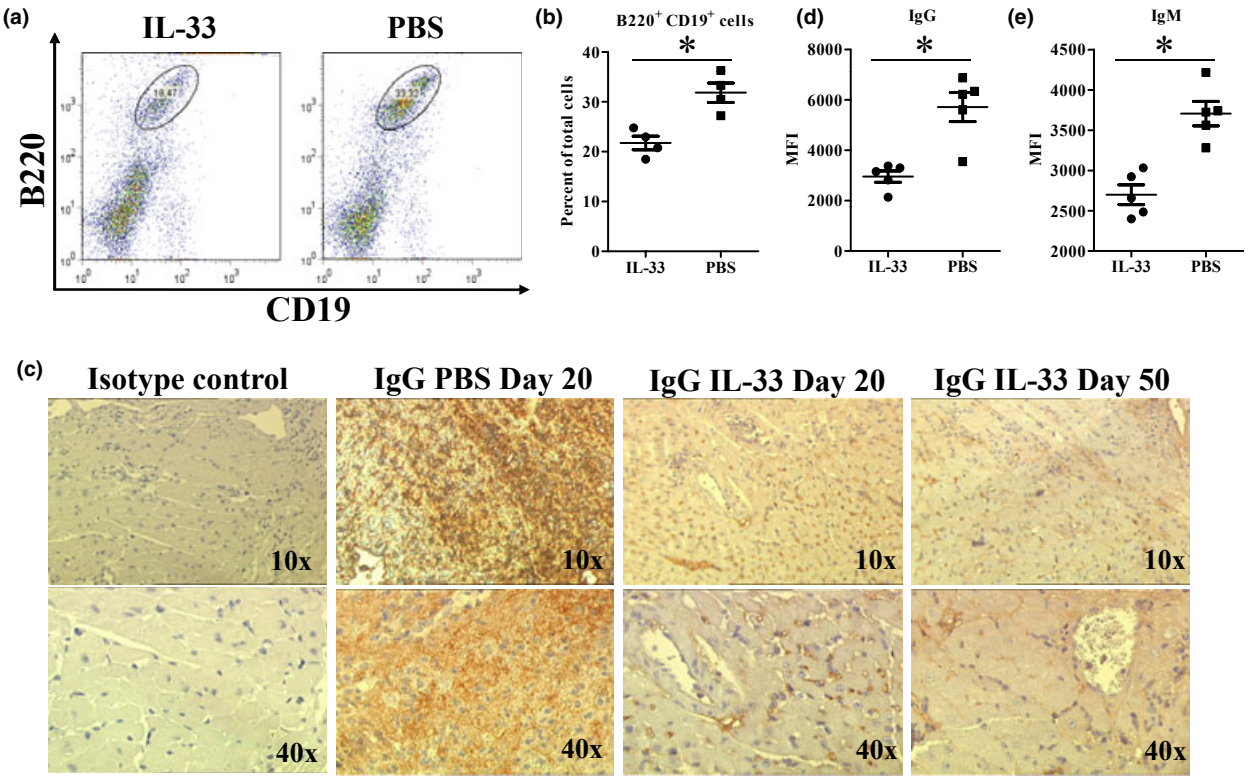

Figure 4 IL-33 treatment reduces B cells and AMR. (a) Representative flow cytometric diagram of $B220^+$ $CD19^+$ cells isolated from the spleen of mice treated with IL-33 or control PBS. (b) $B220^+$ $CD19^+$ cells shown as percent of total cells. Data was obtained from 4 independent experiments. One dot represents pooled splenic cells from 5 mice per group (*$P < 0.05$). (c) IgG staining of representative cardiac sections at postoperative day 20 and 50 from mice treated with IL-33 or control PBS. (d and e) Flow cytometric analysis of IgG and IgM alloantibodies in the serum on day 20 of allogeneic transplanted mice treated with IL-33 or control PBS (MFI mean fluorescence intensity; $N = 5$ per group; *$P < 0.05$).

IL-33 directly induces the proliferation of $Foxp3^+$ Treg in spleens and cardiac allografts

We suspected that Treg may play an important role in prolonged allograft survival after IL-33 therapy. FACS analysis of splenic cells from Foxp3-GFP mice showed that while IL-33 treatment had little influence on the relative number of $CD4^+$ T cells (data not shown), the same $CD4^+$ T-cell population showed an approximate doubling of $Foxp3^+$ Treg on day 20 post-transplantation (Fig. 5a). Further characterization of this phenomenon was conducted by cell-cycle analysis. Flow cytometric evaluation demonstrated that upon IL-33 treatment, $Foxp3^+$ Treg start to proliferate, as approximately 10% of those cells leave the resting G_0 and G_1 phases and enter the active S and G_2 phases (Fig. 5b).

To discriminate if IL-33 directly or indirectly increases the Treg pool we generated an *in vivo* situation in which all cells, except T cells, were able to respond to IL-33 stimulation. This was achieved by reconstitution of $Rag1^{-/-}$ mice with $CD4^+$ T cells from $MyD88^{-/-}$ mice, as MyD88 is a necessary component for IL-33-induced intracellular signaling. In contrast to $Rag1^{-/-}$ mice reconstituted with MyD88-deficient T cells, in $Rag1^{-/-}$ mice reconstituted with wild-type T cells, both antigen-presenting cells and T cells are able to respond to IL-33 therapy [22]. Following 20 days of IL-33 treatment, FACS analysis of isolated splenic cells demonstrated a significant increase in $CD4^+$ $CD25^+$ $Foxp3^+$ Treg in $Rag1^{-/-}$ mice reconstituted with wild-type, but not with MyD88-deficient, T cells (Fig. 5c–e and Fig. S2). This finding suggests that IL-33 directly induces Treg proliferation and that the proliferative effect is not mediated by antigen-presenting cells or soluble factors derived from them.

The increase in Treg was confirmed through immunohistochemical analysis of spleens, where IL-33-treated animals showed a significant, and similar, twofold increase in $Foxp3^+$ cell numbers/HPF, compared with controls (Fig. 6a and b). We also tested for Treg in allografts 20 days post-transplantation. As a result of the limited availability of leucocytes from transplanted hearts, we were only able to perform analyses by immunohistochemistry. As in the spleen, IL-33 treatment significantly increased the number of $Foxp3^+$ cells/HPF at postoperative day 20,

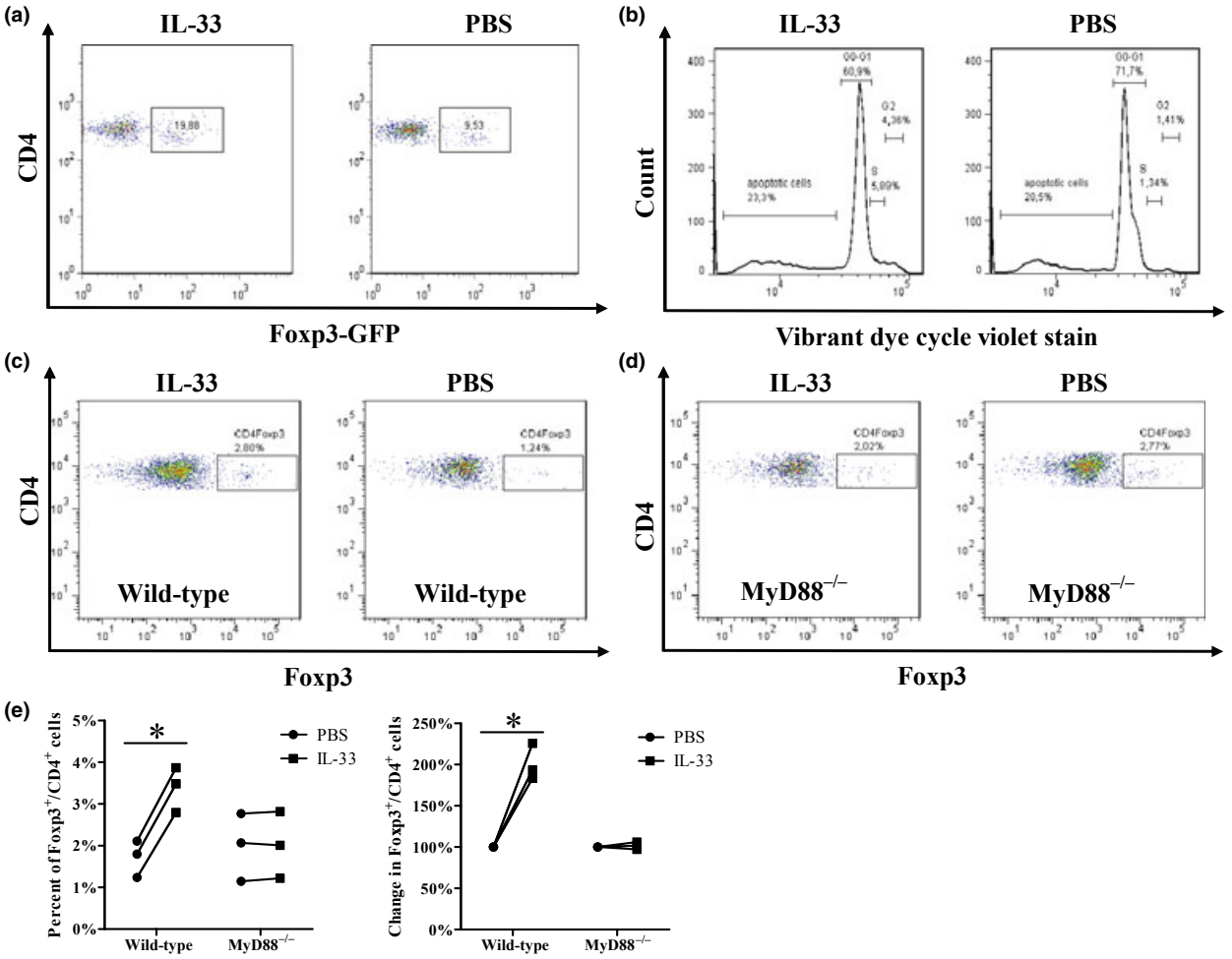

Figure 5 IL-33 directly induces Treg. (a) Flow cytometric analysis of $CD4^+$ Foxp3-GFP^+ cells isolated from the spleen of Foxp3-GFP-reporter mice treated with IL-33 or control PBS. (b) Flow cytometric cell cycle analysis of $CD4^+$ $Foxp3^+$ cells from C57Bl/6J mice treated with IL-33 or control PBS. (c and d) Flow cytometric analysis of $CD4^+$ $Foxp3^+$ cells obtained from $Rag1^{-/-}$ mice reconstituted with wild-type or $MyD88^{-/-}$ $CD4^+$ T cells treated with IL-33 or control PBS. (e) Flow cytometric analysis of Foxp3 expression of all $CD4^+$ cells obtained from $Rag1^{-/-}$ mice reconstituted with wild-type or $MyD88^{-/-}$ $CD4^+$ T cells. Mice were treated with IL-33 or control PBS. Left diagram shows $CD4^+$ $Foxp3^+$ cells as percentage of all $CD4^+$ cells. Right diagram shows change of $CD4^+$ $Foxp3^+$ cell numbers after IL-33 therapy in relation to mice treated with control PBS. Panel includes three independent experiments as presented in c and d. Each dot represents results from pooled splenic cells of five mice per group ($*P < 0.05$).

versus control transplants at postoperative days 12 (data not shown) and 20; the increase in Treg remained steady out to day 50 after transplantation (Fig. 6c and d). Together, our results suggest that Treg, directly induced by IL-33, both locally in the allograft and in the periphery, play a role in the immunological protection afforded by IL-33 treatment.

IL-33 strongly induces $CD11b^{high}Gr1^{intermediate}$ myeloid-derived suppressor cells

We hypothesised that prolonged allograft survival may also be because of an induction of myeloid-derived suppressor cells (MDSC) by IL-33 therapy. MDSC potentially have tolerogenic capacity and can therefore affect allograft rejection. To test this idea graft-infiltrating cells were examined for $CD11b^{high}Gr1^{intermediate}$ MDSC 20 days after transplantation. In the IL-33 group we observed a conspicuous expansion of cells with a high forward and side scatter (Fig. 7a). These cells were $CD45^+$ and therefore represent leucocytes (Fig. S3). Further, a significant proportion of these cells was $CD11b^+Gr1^+$ and, additionally, expresses the ST2 receptor (Fig. 7b). This suggests that these cells can directly be influenced by the ST2 receptor ligand IL-33.

Comparing graft-infiltrating cells, a shift from $CD11b^{high}Gr1^{high}$ granulocytes under PBS treatment to $CD11b^{high}Gr1^{intermediate}$ MDSC with IL-33 therapy was

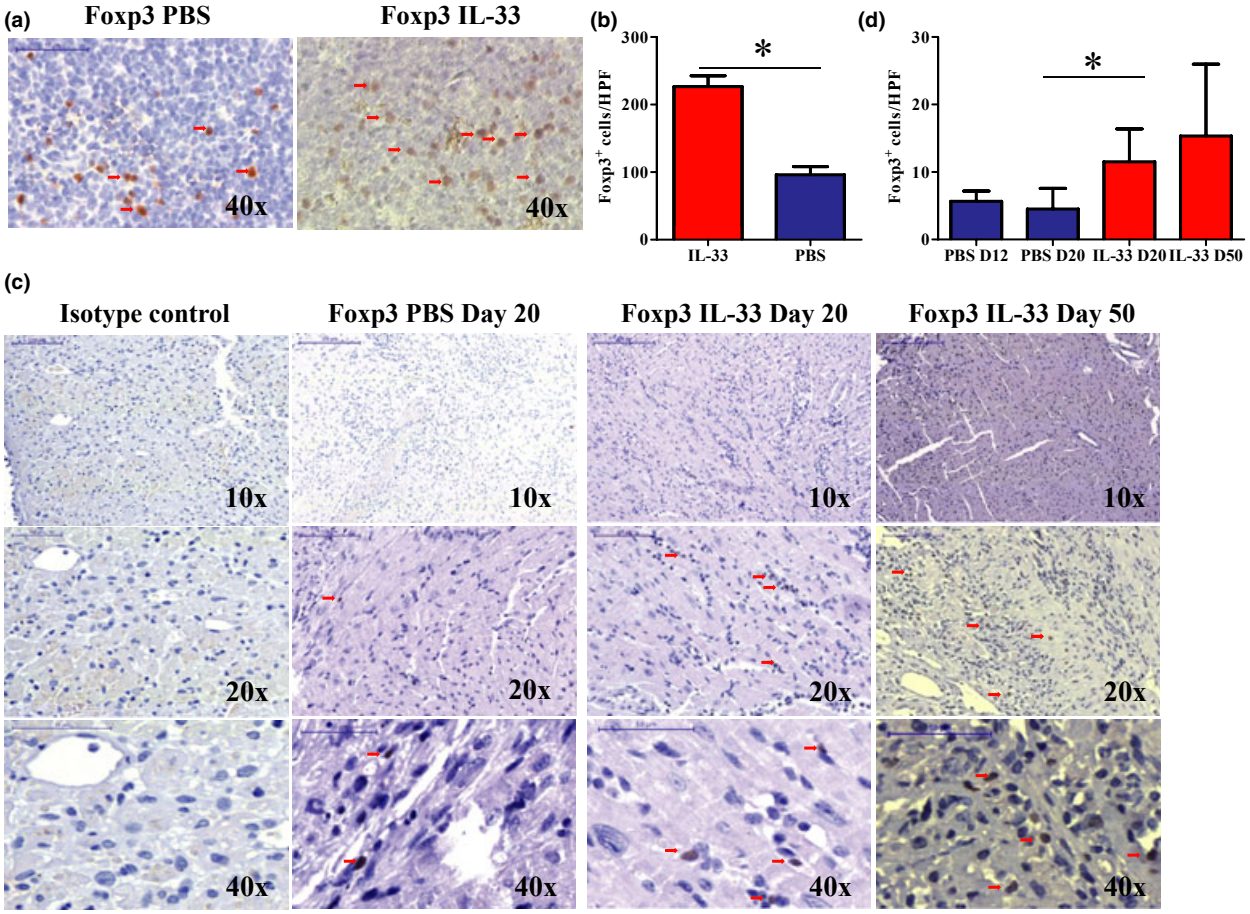

Figure 6 IL-33 induces accumulation of Foxp3$^+$ Tregs in spleens and cardiac allografts. (a and b) Foxp3 staining of representative spleen sections on postoperative day 20 of mice treated with IL-33 or control PBS. Foxp3$^+$ cells per high power field were counted from eight mice per group (*$P < 0.05$). (c and d) Foxp3 staining of representative cardiac allograft sections on postoperative day 20 and day 50 from mice treated with IL-33 or control PBS. Foxp3$^+$ cells per high power field were counted from eight mice per group (PBS day 20 versus IL-33 day 20;*$P < 0.05$). Red arrows indicate examples of Foxp3-positive cells.

evident. More specifically, amongst all CD11b^{high} cells isolated from the cardiac allografts we found that in controls approximately one-third of these cells were CD11b^{high}Gr1^{high} and approximately two-thirds were CD11b^{high}Gr1^{intermediate}, versus 2.6% of these cells being CD11b^{high}Gr1^{high} and 87.1% CD11b^{high}Gr1^{intermediate} in IL-33-injected mice (Fig. 7c). Further evaluations regarding spleen, bone marrow and peritoneal fluid revealed similar results. In particular, in spleens we found a shift from two-thirds CD11b^{high}Gr1^{high} and one-third CD11b^{high}Gr1^{intermediate} cells in the controls to 16% CD11b^{high}Gr1^{high} and 67% CD11b^{high}Gr1^{intermediate} cells out of all CD11b^{high} cells in the IL-33 group. Analysis of four independent experiments, each experiment being conducted using pooled splenic cells from five mice per group, showed significant differences (Fig. 7d–f). Bone marrow of control mice also contained two-thirds CD11b^{high}Gr1^{high} and one-third CD11b^{high}Gr1^{intermediate} cells compared with an opposite relation with one-third CD11b^{high}Gr1^{high} and two-thirds CD11b^{high}Gr1^{intermediate} cells under IL-33 treatment. (Fig. 7g) Interestingly, we found a striking enrichment of CD11b^{high}Gr1^{intermediate} cells in peritoneal fluid that normally accumulated in IL-33-treated mice; (Fig. 7h) not enough ascites was available in control mice to perform a comparative analysis. These results suggest that the increase in MDSC under IL-33 treatment might contribute to improved allograft survival.

Discussion

Allograft rejection is the result of a complex interplay between tolerogenic mechanisms and factors promoting rejection. In this setting, it is generally accepted that naïve T-helper cells can differentiate into Th1, Th2, Th17, and Treg depending on the local cytokine milieu. These cells

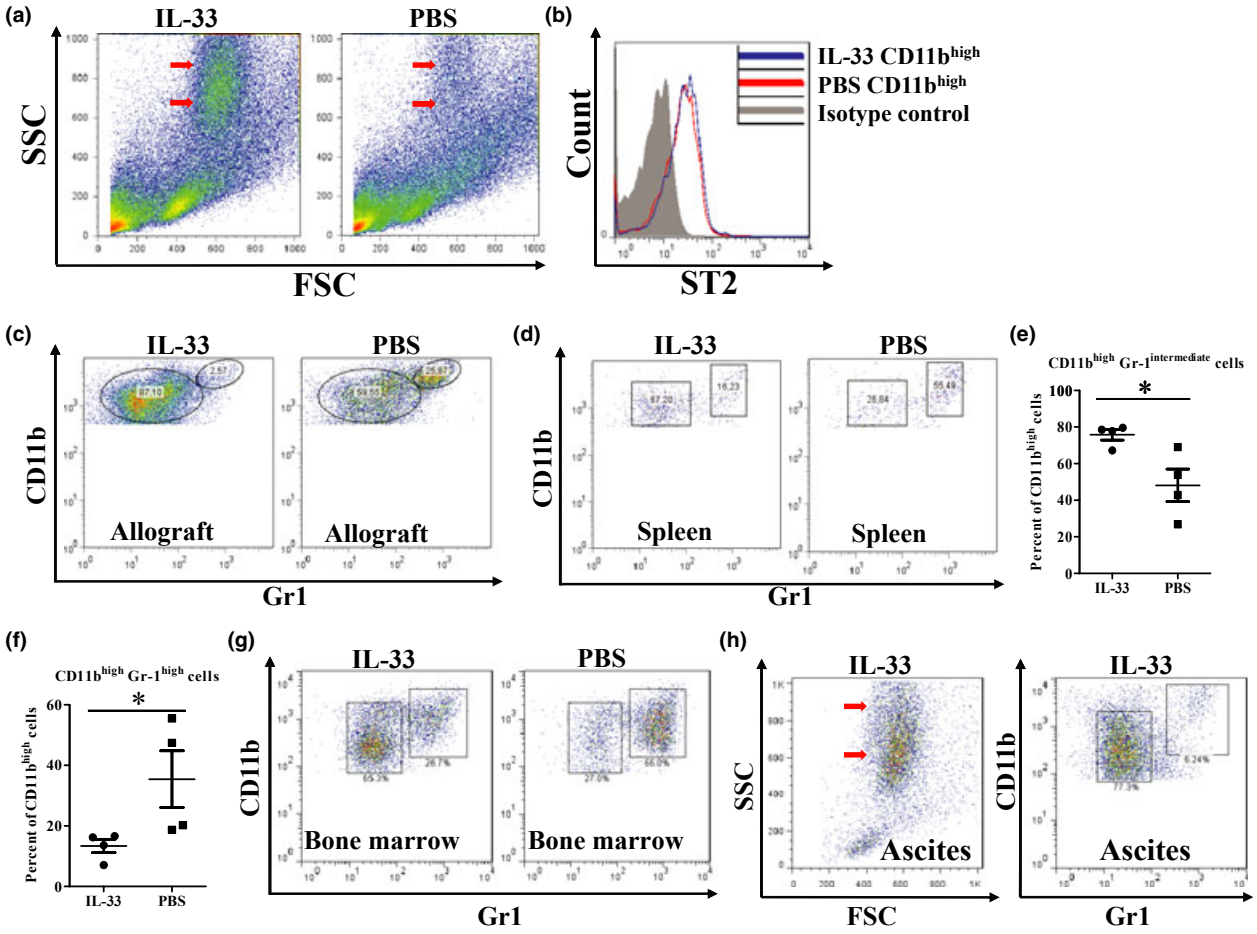

Figure 7 IL-33 treatment induces CD11b^{high}Gr1^{intermediate} MDSC and downregulates CD11b^{high}Gr1^{high} granulocytes. (a) Flow cytometric analysis of FSC and SSC of cells isolated from the cardiac allografts of mice treated with IL-33 or control PBS. (b) Flow cytometric analysis of ST2 expression on CD11b^{high} cells isolated from the spleen of mice treated with IL-33 or control PBS. (c) Flow cytometric analysis of CD11b^{high}Gr1^{intermediate} or CD11b^{high}Gr1^{high} cells isolated from cardiac allografts of mice treated with IL-33 or control PBS. (d) Flow cytometric analysis of CD11b^{high}Gr1^{intermediate} or CD11b^{high}Gr1^{high} cells isolated from the spleen of mice treated with IL-33 or control PBS. (e and f) CD11b^{high}Gr-1^{intermediate} and CD11b^{high} Gr-1^{high} cells expressed as percent of CD11b^{high} cells. Data shown is obtained from 4 independent experiments. One dot represents pooled splenic cells of 5 mice per group (*$P < 0.05$). (g) Flow cytometric analysis of CD11b^{high}Gr1^{intermediate} or CD11b^{high}Gr1^{high} cells isolated from bone marrow of mice treated with IL-33 or control PBS. (h) Flow cytometric analysis of CD11b^{high}Gr1^{intermediate} or CD11b^{high}Gr1^{high} cells isolated from ascites of mice treated with IL-33.

can either be identified by their specific transcription factors, T-bet for Th1, GATA-3 for Th2, RORγt for Th17, and Foxp3 for Treg, or by the cytokines they produce [23]. Th1 cells are involved in acute allograft rejection, [24,25] where Th1 responses are characterized by the production of IL-2, IL-12, IFNγ, and TNF [26]. In the clinical reality of early post-transplant rejection driven by Th1 cells, these responses can be effectively inhibited with standard immunosuppressors like cyclosporine, rapamycin or tacrolimus [27]. However, side effects of long-term pharmacological immunosuppression and late graft loss caused by chronic rejection remain unsolved problems [28].

In the absence of a Th1-mediated alloimmune response Th17 cells drive a proinflammatory response that accelerates chronic allograft rejection and CAV. This was demonstrated in a chronic rejection model using T-bet$^{-/-}$ mice by Yuan *et al.* In this context IL-17A has been shown to be the key cytokine for reduced allograft survival time [15]. Booth *et al.* also found IL-17A to be involved in chronic rejection [29]. Further studies underlined the importance of Th17 cells in rejection, as antibody blockade of IL-6 resulted in reduced IL-17A transcript levels and attenuated cardiac allograft rejection [30]. In our study we were able to demonstrate a strong downregulation of the proinflammatory cytokine IL-17A

in allografts under IL-33 treatment. The effect of IL-33 on IL-17A can be one of the reasons for the observed prolonged allograft survival.

Th2 immune responses are mainly characterized by an induction of the cytokines IL-4, IL-5, IL-10 and IL-13. Th2 cells normally control immune reactions to extracellular parasites and are involved in the pathophysiology of asthma and other allergic inflammatory diseases [31]. It is also reported that under certain circumstances Th2 cytokines, especially IL-4 and IL-13, are able to prolong allograft survival [32–34]. Previous results have shown that IL-33 induces Th2 cytokines in the periphery [9]. In this setting IL-33 administration potentially aggravates the course of allergic-induced airway inflammation by an IL-4-independent Th2 differentiation [7]. In contrast to the results from Yin *et al.* who found an IL-33-induced Th1 to Th2 polarization in the periphery responsible for prolonged allograft survival in an acute rejection model [35], our studies show an IL-33-mediated Th17 to Th2 switch; moreover, we show that this switch occurs within the allograft itself, and in the situation of a chronic rejection. IL-33 treatment induced a reduction in pathogenic IL-17A production by graft-infiltrating cells, which was combined with an upregulation of IL-5, IL-10, and IL-13 production by graft-infiltrating cells. These results support the contention that graft-infiltrating cells shift phenotypically under these circumstances from $CD4^+$ Th17 to $CD4^+$ Th2 cells.

Alloantibody responses to an allograft are another major reason for allograft injury and rejection. In our study, the novel finding of IL-33-mediated reduction of $B220^+$ $CD19^+$ B cells in the periphery and decreased alloantibody production suggest that IL-33 treatment decreases the humoral response directed against the cardiac allograft. This is important especially in the setting of chronic rejection with the development of CAV; indeed, this type of rejection is typical for the MHC class II-mismatched model we used in our study. Russel *et al.* were able to demonstrate that CAV in the cardiac allografts is dependent on antibodies to donor cells [36]. Together our study suggests IL-33 treatment serves to diminish antibody-mediated vasculopathy associated with chronic rejection.

A major finding of our study was the strong induction of $CD4^+$ $Foxp3^+$ Treg by IL-33. Our results give rise to the assumption that IL-33 induces an expansion of the Treg pool by directly mediating Treg proliferation. This direct effect of IL-33 on $Foxp3^+$ Treg mediated by the ST2 receptor is a novel finding regarding the influence of the cytokine milieu on Treg homeostasis and might even impact future therapeutic options in terms of allograft rejection. Importantly, a doubling of $CD4^+$ $Foxp3^+$ Treg was observed in allografts, accentuating the balance within the graft to an immunoregulatory environment. Immunohistochemistry for Ki-67 showing an increased proliferation rate in the spleen and some increase of proliferation in the allograft suggests that IL-33-mediated changes in lymphocyte homeostasis mostly take place in the periphery, and to a lesser degree within the allograft. It has been shown previously that adequately prestimulated $CD4^+$ $Foxp3^+$ regulatory T cells can prevent acute and chronic cardiac allograft rejection [37–39]. Allograft tolerance can be achieved either by immunotherapy with *ex vivo*-expanded $CD4^+$ $CD25^+$ $Foxp3^+$ natural Treg, *ex vivo*-induced $CD4^+$ $CD25^+$ $Foxp3^+$ inducible Treg or by *in vivo* induction of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg [40]. In the setting of *in vivo* expansion of Treg it has been shown that 'alternatively activated' DCs were 'tolerogenic' and were able to induce Treg *in vitro* and *in vivo* [40].

Complementing our finding of directly-mediated enhanced Treg development with IL-33 treatment, we also detected the expansion of regulatory MDSCs both in the graft and peripherally in the spleen. Supporting our observation, it has been previously shown that IL-33 promotes *in vitro* DC generation in a GM-CSF-dependent manner [41]. These $CD11b^{high}Gr1^+$ cells have been described as MDSCs [42]. Amongst this cell population are three distinct types of cells with different immunosuppressive capacities [43]. $CD11b^{high}Gr1^{low}$ cells showed some immunosuppressive potential in selective tumor models and $CD11b^{high}Gr1^{high}$ cells demonstrated some minor tolerogenic capacity *in vitro*, but not *in vivo*. However, the third population, $CD11b^{high}Gr1^{intermediate}$ cells, were immunosuppressive both *in vitro* and *in vivo* following adoptive transfer [44]. Interestingly, $CD11b^{high}$ $Gr1^{intermediate}$ MDSCs may promote tolerance through the induction of $CD4^+$ $Foxp3^+$ Treg, which has been described by Serafini *et al.* using an A20 B-cell lymphoma model [45]. In this context MDSCs likely contribute to tumor immune evasion by restraining T-cell activation [44,46,47]. Consistent with this hypothesis, in cancer patients Lechner *et al.* have demonstrated a positive correlation with increased MDSC levels and aggressive disease with poor prognosis [48]. The importance of MDSC has also been shown in transplant tolerance studies [49,50]. Mechanistically, MDSCs have been shown to inhibit T-cell activation by depleting cystin and cystein, and by downregulating L-selectin expression on $CD4^+$ and $CD8^+$ T cells [51,52]. After kidney or liver transplantation, it has been suggested that MDSCs promote stable, antigen-specific, tolerance through NO-dependent pathways [49,50,53].

In conclusion our study shows that IL-33 treatment prolongs allograft survival in a chronic transplant rejection model. We propose that IL-33 mediates this positive effect by inducing regulatory MDSCs and Treg; IL-33 additionally enhances allograft survival by reducing B

cell-dependent AMR. Another potential pro-tolerance mechanism of IL-33 demonstrated in our study is related to the reduction of Th17 responses and the induction of Th2 cytokines. Together, our results indicate that IL-33 may be exploitable as a future therapeutic intervention to reduce chronic rejection in organ transplantation.

Authorship

SMB: analysis and interpretation of data, study concept and design, drafting of the manuscript. GS: acquisition of data, technical material support. WF: contributed important reagents. HJS: critical revision of the manuscript for important intellectual content. EKG: analysis and interpretation of data, critical revision of the manuscript for important intellectual content. SF-F: analysis and interpretation of data, study concept and design, drafting of the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The ST2 receptor is expressed on Th1, Th2, Th17 and Treg. Flow cytometric analysis of ST2 expression of Th1, Th2, Th17 and Treg compared with an isotype control. Methods: Cells out of the spleens of naïve C57Bl/6J mice were isolated. Then naïve $CD4^+$ $CD25^-CD62L^+$ T cells were stimulated with CD28, IL-12, IL-21 and anti-IL-4 for Th1, with CD28, IL-4, anti-IL-12 and anti-IFNγ for Th2, with CD28, TGFβ and IL-6 for Th17 and with CD28, TGFβ and IL-2 for Treg and cultured in CD3-coated cell culture plates for 96 h. After restimulation with PMA/ionomycin we analyzed these cells by flow cytometry and tested for ST2 expression.

Figure S2 (a and b) Flow cytometric analysis of $CD4^+$ $CD25^+$ cells obtained from $Rag1^{-/-}$ mice reconstituted with wild-type or $MyD88^{-/-}$ $CD4^+$ T cells treated with IL-33 or control PBS. All FACS blots shown are representative of three independently conducted experiments; each experiment was conducted with pooled splenic cells from five mice per group.

Figure S3 Flow cytometric analysis of splenic cells of mice treated with IL-33 or control PBS. (a) Appropriate gating in the FSC/SSC diagram. (b) Indentification of $CD45^+$ and $CD45^-$ cells. (c) $CD45^+$ cells represent leucocytes and include $CD11b^+$ cells, whereas $CD45^-$ cells represent parenchymal cells and do not contain $CD11b^+$ cells. (d) 'Back-gating' demonstrates that those leucocyte cells are in the appropriate gate in the FSC/SSC diagram.

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