



Anti-Interleukin-6 Promotes Allogeneic Bone Marrow Engraftment and Prolonged Graft Survival in an Irradiation-Free Murine Transplant Model

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Transfer of recipient regulatory T cells (Tregs) induces mixed chimerism and tolerance in an irradiation-free bone marrow (BM) transplantation (BMT) model involving short-course co-stimulation blockade and mTOR inhibition. Boosting endogenous Tregs pharmacologically *in vivo* would be an attractive alternative avoiding the current limitations of performing adoptive cell therapy in the routine clinical setting. Interleukin-6 (IL-6) potently inhibits Treg differentiation and its blockade was shown to increase Treg numbers *in vivo*. Therefore, we investigated whether IL-6 blockade can replace adoptive Treg transfer in irradiation-free allogeneic BMT. Treatment with anti-IL-6 instead of Treg transfer led to multi-lineage chimerism (persisting for ~12 weeks) in recipients of fully mismatched BM and significantly prolonged donor skin (MST 58 days) and heart (MST > 100 days) graft survival. Endogenous Foxp3⁺ Tregs expanded in anti-IL-6-treated BMT recipients, while dendritic cell (DC) activation and memory CD8⁺ T cell development were inhibited. Adding anti-IL-17 to anti-IL-6 treatment increased Treg frequencies, but did not further prolong donor skin graft survival significantly. These results demonstrate that IL-6 blockade promotes BM engraftment and donor graft survival in non-irradiated recipients and might provide an alternative to Treg cell therapy in the clinical setting.

Keywords: transplantation, tolerance, chimerism, interleukin-6, regulatory T cells, bone marrow transplantation, co-stimulation blockade

INTRODUCTION

Long-term allograft survival without the need of permanent immunosuppression remains an important goal in transplantation medicine (1). In this context, the co-transplantation of allogeneic bone marrow (BM) for the induction of mixed chimerism (i.e., co-existence of donor and recipient hematopoietic cells) evolved to be an attractive strategy (2). Several murine mixed chimerism protocols have been successfully translated to non-human primate (NHP) studies and several pilot trials performed in renal transplant patients demonstrate the clinical relevance of chimerism-based tolerance. However,

the need of myelosuppressive pre-conditioning with its associated risks is a major challenge to more widespread clinical application (3–5).

Our group has recently developed a regimen free of myelosuppression that induces chimerism and long-term tolerance through a combination of donor BM transplantation (BMT) and recipient regulatory T cell (Treg) therapy under short-course CB (anti-CD40L and CTLA4-Ig) and of mTOR inhibition (6–9). Even though individualized cell therapy is a promising emerging therapeutic option (10, 11), its translation into clinical routine is impeded by numerous hurdles (12, 13). Hence, it is a desirable goal to find pharmacological alternatives that can be substituted for Treg cell therapy (14). Various promising protocols build on the principle of boosting thymically derived (t) Tregs *in vivo* (15) or the conversion of non-regulatory CD4⁺ T cells into peripherally derived (p) Tregs (16). With regard to non-cytotoxic BMT, our group could recently show that IL-2 complexes cannot replace adoptive Treg therapy (17) and thus alternative strategies need to be developed for this setting.

The pro-inflammatory cytokine interleukin-6 (IL-6) is a critical player in T cell and B cell (allo)immunity (18, 19). IL-6 is known for its versatile immunological functions, including its role as a key regulator of the Treg/Th17 balance where it prevents Treg development while promoting the differentiation of naïve T cells into Th17 cells (20, 21). IL-6 also promotes the activation and survival of effector T cells (22) and regulates *in vivo* dendritic cell (DC) differentiation through STAT3 (23). Moreover, it promotes T_{FH} lineage commitment, plasma cell progression, and high affinity antibody production (24, 25). Previously, it was shown that IL-6 deficiency can delay CD4-mediated cardiac allograft rejection (26–28). The role of IL-6 after allogeneic BMT is not fully understood yet, but it was shown that elevated serum levels correlate with acute graft-vs-host disease (GvHD) (29–31). Inhibition of the IL-6 signaling pathway reduces the severity of GvHD due to the augmentation of thymic-dependent and independent Treg reconstitution (32). Recently, we found that the co-transfer of high numbers of donor T cells triggers CB-resistant rejection of donor BM through IL-6-dependent bystander activation (33). Furthermore, disrupting the IL-6 signaling pathway with a humanized anti-IL-6 receptor (IL-6R) monoclonal antibody (tocilizumab) is approved for clinical use in Castleman's disease, rheumatoid and juvenile arthritis (34–36). Notably, anti-IL-6R has been successfully tested as part of a delayed-tolerance-induction protocol in NHPs where long-term lung allograft survival was achieved *via* mixed chimerism with a non-myeloablative T cell depleting regimen (37). Moreover, tocilizumab revealed promising potential as GvHD prophylaxis after allogeneic stem-cell transplantation in a phase I/II trial (38). Several additional antibodies targeting the IL-6R or IL-6 itself are currently under clinical development (39–41).

To date, suitable protocols that achieve allogeneic BM engraftment without the need of cytoreductive therapy are still limited. The combination of CB, mTOR inhibition, and Treg therapy seems to be a promising approach. To increase clinical applicability, we investigated whether anti-IL-6 administration could obviate the need for Treg cell therapy in this non-cytotoxic murine mixed chimerism model.

MATERIALS AND METHODS

Mice

Female C57BL/6 (B6, recipient, H-2^b), BALB/c (donor, H-2^d), and C3H/N (third party, H-2^k) mice were purchased from Charles River Laboratories (Sulzfeld, Germany), housed under specific pathogen-free conditions, and used for experiments between 6 and 12 weeks of age. Congenic B6.SJL-Ptprca Pepcb/BoyJ (CD45.1⁺) and B10.D2 mice [donor, MHC mismatched, minor antigen (mHAg) matched to B6] were purchased from Jackson Laboratory (ME, USA) and bred in our own facility. Skin and heart transplantations were performed under controlled anesthesia with an intraperitoneally (i.p.) injected mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg). All experiments were approved by the local review board of the Medical University of Vienna and the Austrian Federal Ministry of Science, Research and Economy and were performed in accordance with national and international guidelines of laboratory animal care (permission number GZ: BMWFW-66.009/0377-WF/V/3b/2016 and GZ: BMWFW-66.009/0303-WF/V/3b/2016).

BM Transplantation and *In Vivo* Antibody Treatment Regimens

Groups of age-matched C57BL/6 recipients received 20×10^6 unseparated BM cells or splenocytes [as donor-specific transfusion (DST) control] from BALB/c donors by injection into the tail vein together with CB consisting of anti-CD154 mAb (1 mg, d0, anti-CD40L, MR1, BioXcell), hCTLA4-Ig (0.5 mg, d2, abatacept, Bristol Myers Squibb), and a short-course of rapamycin (0.1 mg, d-1, d0, and d2, LC Laboratories) (6). Indicated recipients were additionally treated with an anti-IL-6 mAb (1 mg on days -1, 1, and 3 and then 0.1 mg every other day until day 13, MP5-20F3, BioXcell) (27, 28). Where indicated, groups of mice received in addition (0.5 mg on days -1, 1, and 3 and 0.1 mg every other day until day 13) anti-IL-6R (15A7) or anti-IL-17A (BZN035, Novartis Pharma AG) (42, 43).

Skin and Heart Transplantation

Full thickness tail skin from BALB/c, B10.D2, or C3H/N mice was grafted to recipient flanks (lateral thorax wall) 5 weeks after BMT and visually inspected thereafter at short intervals. Grafts were considered to be rejected when less than 10% of the graft remained viable (6). Cervical heterotopic heart transplantation was performed 5 weeks after BMT, as described previously (44). Briefly, the recipient's right external jugular vein and common carotid artery were everted over a cuff. The donor heart was harvested and flushed in a retrograde fashion with 4 mL HTK solution (Custodiol, Koehler Chemie, Alsbach-Haenlien, Germany) through the aortic arch. The pulmonary artery was connected with the external jugular vein and the aortic trunk with the common carotid artery. Heart allograft survival was determined by visual inspection and palpation at least twice weekly during long-term follow-up. End of graft survival was defined as complete cessation of heartbeat and was confirmed by histological analysis.

Histology

Skin and heart grafts were harvested at the end of the experiment and fixed in 7.5% formalin. Paraffin blocks were sectioned and stained with hematoxylin and eosin (H&E) according to standard protocols. Histological slides were then scanned by Aperio ScanScope scanner (Aperio Technologies, Inc., Vista, CA, USA) and analyzed with ImageScope software. Additionally, grading of skin grafts was performed according to Banff 2007 working classification of skin-containing composite tissue allograft pathology and hearts were scored according to the International Society for Heart and Lung Transplantation (ISHLT) 2005 guidelines for cellular rejection by a pathologist blinded to the experimental background of samples.

Mixed Lymphocyte Reaction (MLR) and Suppression Assay

Regarding MLR 5×10^5 congenic C57BL/6 CD45.1⁺ responder cells (unseparated splenocytes) were cultured for 5 days in RPMI1640 supplemented with 10% FCS (Linaris), PenStrep (100 U Penicillin, 100 μ g Streptomycin/ml; Sigma), 10 mM Hepes (MP Biomedicals), 1 mM Sodium Pyruvate (Sigma), and $1 \times$ non-essential amino acids (Sigma). Responder cells were stimulated with equal numbers of stimulator cells (unseparated splenocytes) from BALB/c (allogeneic) and C57BL/6 (self) or with Medium. Proliferation was measured by staining Ki-67 in responding (CD45.1⁺) CD4⁺ and CD8⁺ T cells after 5 days of culture. For the *in vitro* suppression assay, a 96-well plate was pre-coated overnight with 1 μ g/ml anti-CD3 (145-2C11, BioXcell) diluted in PBS. On the next day, 4×10^5 naïve responder CD45.1⁺ splenocytes (VPD 450 pre-labeled) were cocultured with indicated numbers of Tregs (for a responder:Treg ratio of 1:0, 1:1, 2:1, 4:1, 8:1, and 16:1) isolated on day 14 from BMT recipients (receiving CB and rapamycin) either with or without anti-IL-6. Tregs were purified by magnetic bead separation using the MACS CD4⁺CD25⁺ regulatory T-cell Isolation Kit (Miltenyi Biotec). Purity of separated population was >90%. After 4 days of incubation responder cell proliferation was assessed by measuring the dilution of VPD450. The percentage of suppression was calculated using the formula: [(% proliferation Tresp. cells alone – % of Tresp. cells treated with Treg)/% of Tresp. cells alone] \times 100.

Flowcytometric Analysis

Three-color flow cytometric analysis of multi-lineage chimerism was performed as described previously (45, 46). Briefly, chimerism was calculated as the net percentage of donor MHC class I⁺ [H2-D^d-BIO (34-2-12)] in different leukocyte lineages {myeloid cells [Mac-1-FITC (M1/70)], B cells [CD19-PE (6D5)], CD4⁺ cells [CD4-APC-Cy7 (RM4-5)], and CD8⁺ cells [CD8-PE-Cy7 (53-6.7)]}. CD44-BIO (IM7), CD62L-PE (MEL-14), CD11c-BIO (N418), CD80-PERCP-Cy5 (16-10A1), CD86-FITC (GL-1), MHCII-PE-Cy7 (M5/114.15.2), CD40-PE (3/23), CD3-PerCP-Cy5 (17A2), SAV-APC, CD45.1-BIO (A20), CD45.2-BIO (104), and IgG-PE (Poly4053) were purchased from BioLegend (San Diego, CA, USA). For intracellular staining, cells were permeabilized with the Foxp3/Transcription

Factor Staining Buffer Set from eBioscience according to the manufacturer's specification. Anti-mouse/rat Foxp3 APC (FJK-16s) and anti-mouse/rat Ki-67 PE-Cy7 (SolA15) were obtained from eBioscience. Flow cytometric analysis was performed with a BD FACS Canto II, and data were analyzed using FlowJo (10.0.8) software.

Donor-Specific Antibodies (DSA)

5×10^5 naïve recipient or donor thymocytes were incubated with 15 μ l serum of indicated mice for 30 min at 37°C after heat inactivation of complement as described earlier (6). Briefly, binding of donor-reactive IgG to thymocytes was assessed by staining with anti-IgG (PE) and was measured by flow cytometry.

Statistical Analysis

The statistical analyses were performed with SPSS 23.0 and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between chimerism levels were compared using ANOVA. Error Bars represent SD and statistics were performed with a two-sided Student's *t*-test with equal variances. Skin and heart allograft survival was calculated according to the Kaplan–Meier product limit method and compared between groups using the log-rank test. Cardiac rejection scores were compared by using Fisher's exact test. A *p*-value < 0.05 was considered statistically significant.

RESULTS

Anti-IL-6 Promotes Allogeneic BM Engraftment and Prolongs Donor Skin Graft Survival

To investigate whether IL-6 blockade can replace Treg cell therapy in an irradiation-free BMT setting, C57BL/6 mice received fully mismatched BALB/c BM, CB (anti-CD40L mAb and CTLA4-Ig), rapamycin and in addition anti-IL-6 (instead of adoptive Treg transfer). All mice receiving anti-IL-6 developed early multi-lineage chimerism (within the myeloid and B cell lineages) that persisted for up to 12 weeks (18/18 chimeric at week 2, 6/18 mice remained chimeric at week 12, pooled results from three independent experiments). Noticeably, T cell chimerism appeared in most anti-IL-6 treated mice between weeks 6 and 9 and lasted until week 12 (Figures 1A,B), whereas no T cell chimerism was detectable in mice without anti-IL-6 (week 9: 14/18 with anti-IL-6 vs 0/10 without, *p* = 0.017). Among all measured leukocyte lineages, the myeloid population showed the highest chimerism levels over the whole period, peaking at week 2. In contrast, almost all control mice without anti-IL-6 (and without Treg transfer) lost myeloid (and B cell, data not shown) chimerism by week 3, or shortly thereafter (week 3: 1/10 chimeric vs 18/18 chimeric with anti-IL-6, *p* = 0.021) (Figure 1C), consistent with our previous experience (6).

To determine whether anti-IL-6 leads to donor-specific tolerance, donor skin was transplanted ~5 weeks post-BMT (i.e., 22 days after the last dose of anti-IL-6). Donor skin graft survival was significantly prolonged in anti-IL-6-treated mice

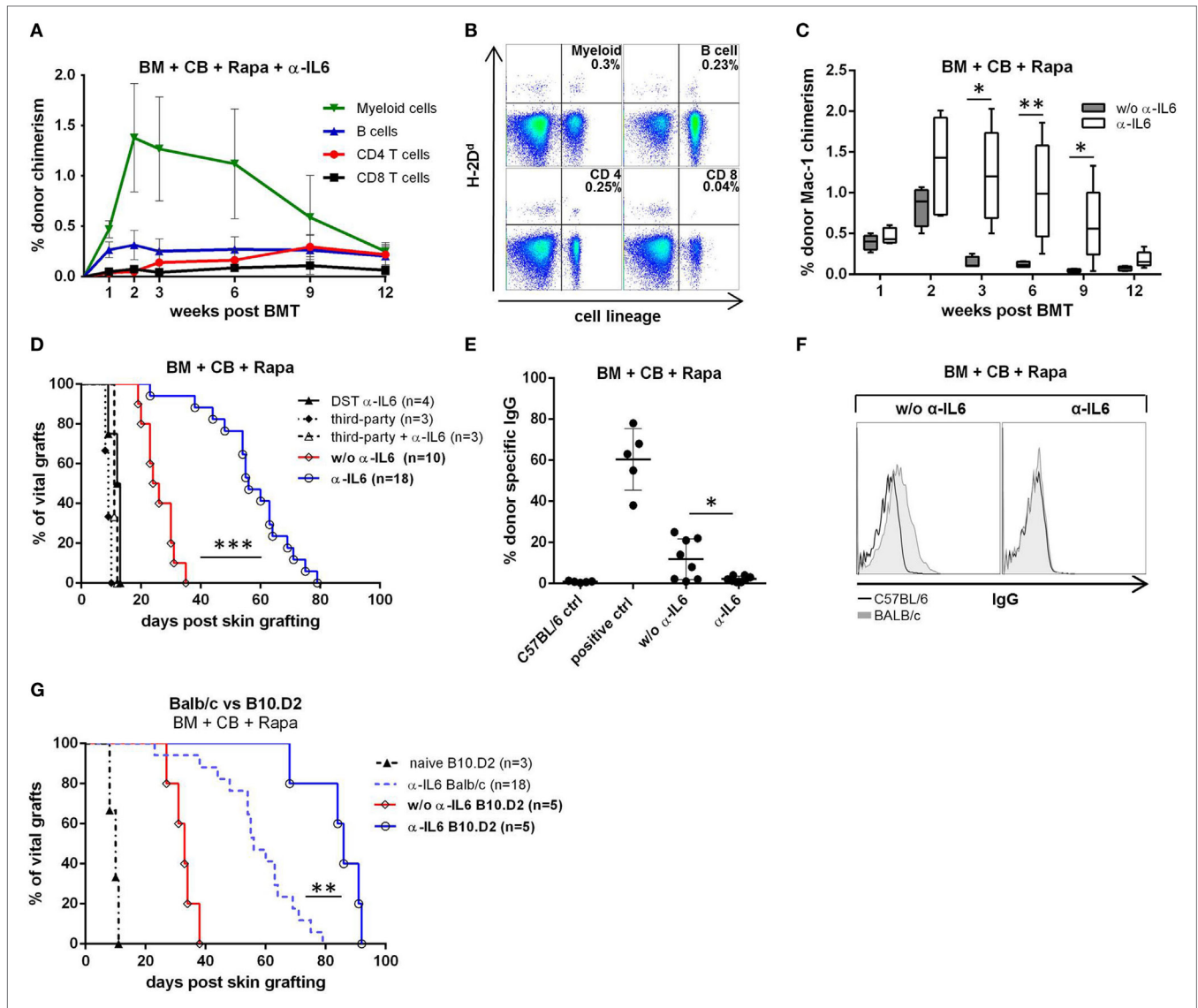


FIGURE 1 | Enhanced bone marrow (BM) engraftment and prolonged skin graft survival with anti-IL-6. C57BL/6 recipients received fully mismatched BALB/c BM, co-stimulation blockade (CB), and rapamycin (Rapa). Additionally, indicated groups received anti-IL-6 mAb (α -IL6). Data shown in (A,C,D) are pooled from three independent experiments. (A) Mean percentage donor (H-2D^d) chimerism among peripheral blood leukocyte lineages was followed over time in anti-IL-6 treated recipients ($n = 18$). (B) Two-color flow cytometry plots of lineage specific chimerism are shown for one representative anti-IL-6-treated recipient at week 12. (C) Percentage myeloid chimerism with and without anti-IL-6 is shown over time as box and whisker plots ($n = 18$ vs 10). (D) Survival of donor and third-party skin grafted 5 weeks post-BMT or splenocyte transfer [donor-specific transfusion (DST) α -IL-6] is depicted for the indicated groups. (E) IgG binding to BL/6 (C57BL/6 control, $n = 5$) or BALB/c (i.e., donor, with or w/o anti-IL-6, $n = 8$ /grp) thymocytes was measured by flow cytometry 100 days post skin grafting. Serum from sensitized untreated BL/6 mice was used as positive control ($n = 5$). (F) Representative histograms of bound IgG are shown for anti-IL-6-treated or untreated mice. (G) Survival of B10.D2 skin grafted onto BALB/c BM recipients with or without anti-IL-6 is shown (note: the α -IL-6 Balb/c group is the same shown in panel D). Naive B10.D2 control represents untreated BL/6 mice that received B10.D2 skin ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

[median survival time (MST) 58 days, $n = 18$] compared to control mice without anti-IL-6 (MST 24 days, $n = 10$, $p = 0.0001$). Third-party skin grafts were rapidly rejected in all groups (MST 11 and 10.5 days, respectively). To test whether the observed prolongation of BALB/c donor skin graft survival after BMT with anti-IL-6 depends on (transient) BM engraftment, a group of mice was treated with the same anti-IL-6 regimen but received donor splenocytes instead of BM (DST α -IL-6, $n = 4$). DST recipients promptly rejected donor skin (MST = 12.5 days), suggesting that

BM engraftment is essential for graft prolongation and that DST does not suffice (Figure 1D). No DSA were detectable in sera of anti-IL-6 treated recipients 100 days post skin grafting, while more than half of mice without anti-IL-6 presented detectable levels of DSA (0/8 vs 5/8, $p = 0.019$), indicating that donor-specific humoral immunity is prevented in anti-IL-6 treated mice (Figures 1E,F).

Minor histocompatibility antigens (mHAg) are a barrier to tolerance induction through chimerism (47, 48). To investigate

whether mHAg disparities are responsible for the late rejection of donor skin grafts in anti-IL-6-treated recipients, B10.D2 skin was grafted instead of BALB/c skin. Survival of B10.D2 grafts was significantly prolonged compared to BALB/c grafts (MST 86 vs 58 days, $p = 0.0014$) but all grafts were eventually rejected (Figure 1G), suggesting that mHAg disparities are not solely responsible for late rejection.

These results show that anti-IL-6 prolongs donor skin graft survival due to enhanced allogeneic BM engraftment.

Anti-IL-6 Leads to Long-term Donor Heart Graft Acceptance

Skin grafts are generally considered to be the most stringent test for tolerance, while vascularized heart grafts are better suited to assess chronic rejection. Therefore, additional groups were transplanted with donor hearts 5 weeks post-BMT. Remarkably, in anti-IL-6-treated BMT recipients all donor hearts remained viable with a palpable heart beat for the length of the follow-up ($n = 4$, 100 days), whereas 3/4 recipients from the group without anti-IL-6 rejected their grafts (MST 88.5, $p = 0.04$) (Figure 2A). Histopathologic examination revealed lower rejection scores in mice treated with anti-IL-6 [median ISHLT score 2 (anti-IL-6) vs 4 (w/o anti-IL-6)] (Figure 2C). Heart grafts from mice without anti-IL-6 showed severe signs of cellular rejection, including extensive lymphocytic infiltrations, myocardial fibrosis, and necrosis, while three of four grafts from anti-IL-6 treatment demonstrated only mild to moderate signs of rejection, including lower rates of pericardial inflammation and intima arteritis (Figure 2B).

Hence, these data demonstrate that anti-IL-6 treatment leads to long-term acceptance of donor heart grafts in an irradiation-free BMT setting.

IL-6 Blockade Induces Endogenous Treg Expansion and Proliferation after Allogeneic BMT

Regulatory T cells modulate immune responsiveness to alloantigens, thereby modulating graft survival (49). To test whether the chimerism- and tolerance-promoting effects of anti-IL-6

administration are associated with *in vivo* boosting of endogenous Tregs, we measured the frequency, number, and proliferation of Foxp3⁺CD4⁺ T cells. Anti-IL-6 was associated with significantly increased frequencies of Foxp3 Tregs in blood (on days 14 and 21 post-BMT) (Figures 3A,B) and spleen (not shown), with an approximately 30% increase in absolute Treg numbers in spleen (Figure 3C). Moreover, Treg proliferation was elevated, as measured by the proliferation marker Ki-67 (Figures 3D,E). As it has been reported that CD4⁺CD25⁺ Tregs isolated from IL-6-deficient mice are more potent than those from WT mice (27), an *in vitro* suppression assay with polyclonally activated CD45.1⁺ splenocytes as responders and descending numbers of CD45.2⁺CD4⁺CD25⁺ Tregs isolated from BMT recipients treated either with or without anti-IL-6 was performed. Tregs from both groups suppressed the proliferation of responder T cells to a comparable degree in a dose-dependent manner (Figure 3F).

These data provide evidence that anti-IL-6 therapy leads to a quantitative expansion of endogenous Tregs, but does not qualitatively alter their suppressive potency.

DC Activation and CD8⁺ Memory T Cell Generation Are Reduced after Anti-IL-6 Therapy

Dendritic cells play a central role in alloimmunity through the presentation of alloantigens to naïve and memory T cells. IL-6 influences the maturation and activation of DCs (23). Therefore, recipient spleens of anti-IL-6-treated and untreated recipients were isolated on day 7 and 14 post-BMT and the DC population (CD45⁺CD11c⁺CD3⁻) was analyzed by flow cytometry. The number of DCs was significantly reduced in anti-IL-6-treated mice compared to the untreated group (~20% less at day 14, $p = 0.018$) (Figure 4A). Moreover, anti-IL-6-treated mice showed a significantly lower expression of CD80 and CD86 (anti-IL-6 CD86: 12%, CD80: 15% vs without anti-IL-6 CD86: 17% and CD80: 21%, $p \leq 0.05$), suggesting reduced DC activation (MHCII and CD40 were similarly expressed in both groups) (Figures 4B,C).

Interleukin-6 is also important for the expansion and survival of effector T cells and the persistence of immunological memory

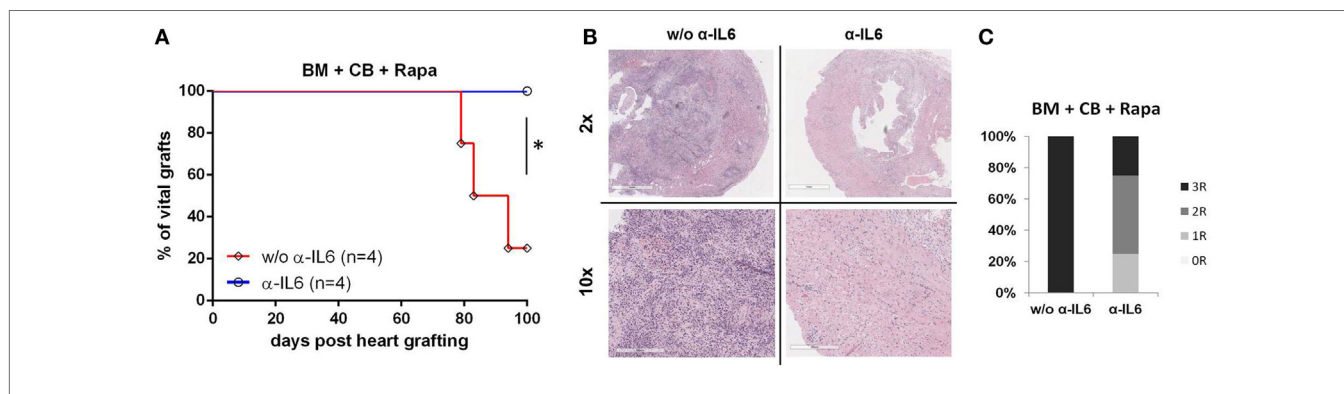
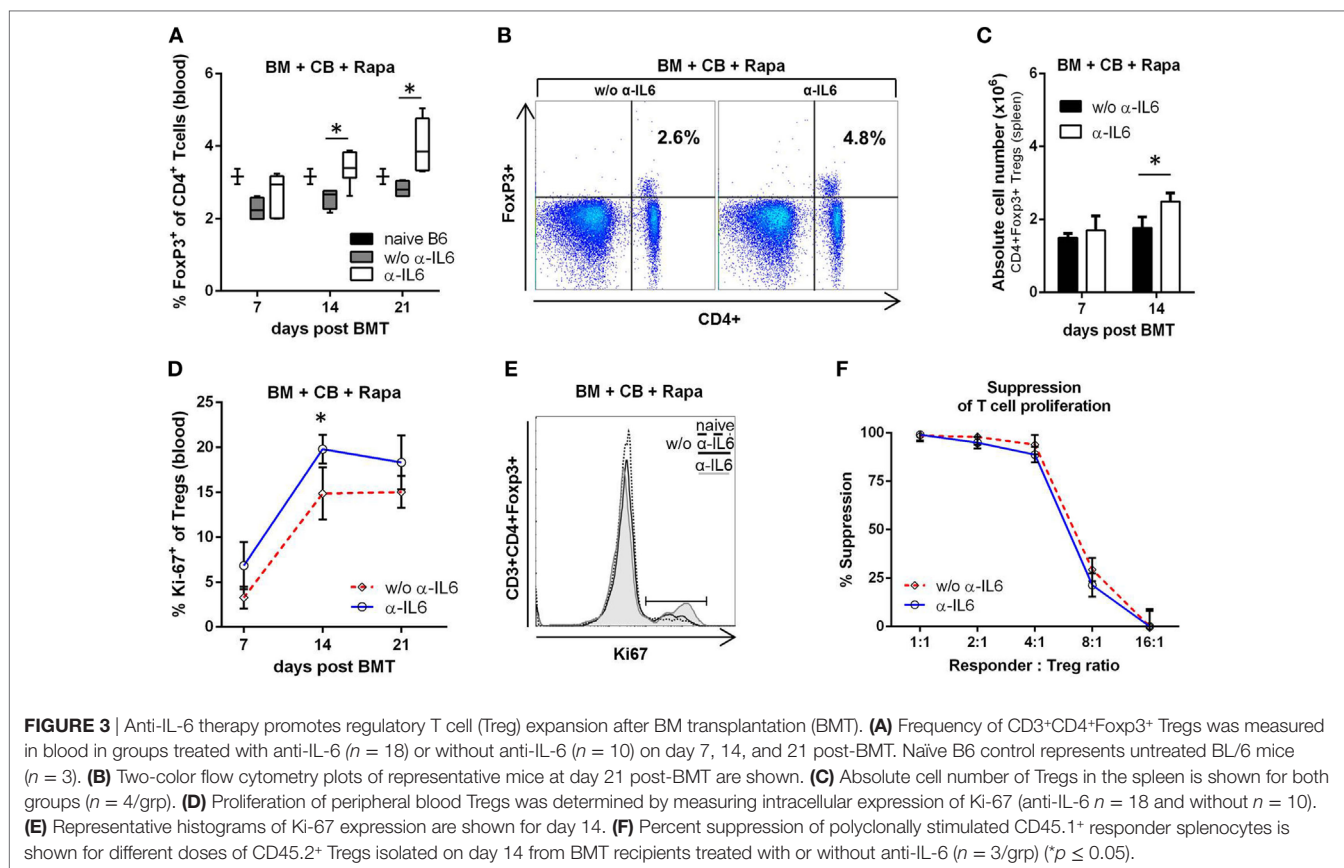


FIGURE 2 | Long-term heart graft survival with anti-IL-6 BALB/c bone marrow (BM) recipients under CB, Rapa, and with or without anti-IL-6-received donor heart grafts 5 weeks post-BMT. Grafts were harvested 100 days post-transplantation for histopathologic analysis. (A) Survival of donor hearts in BM transplantation recipients treated with or without anti-IL-6. (B) Representative H&E images of heart grafts [original magnifications 2x (top) and 10x (bottom)] are shown. (C) Clinical International Society for Heart and Lung Transplantation rejection score of donor hearts from both groups ($n = 4/\text{grp}$) ($*p \leq 0.05$).



(22). Thus, we followed the development of memory T cell subsets over time. In mice treated with anti-IL-6 the frequency of naïve CD8⁺ T cells (CD62L^{high}/CD44^{low}) remained constant at around 60% (similar to pre BMT levels), whereas it declined in mice not receiving anti-IL-6 (Figures 4D,F). In parallel, the frequency of CD8⁺ effector memory T cells (T_{EM}, CD62L^{low}/CD44^{high}) remained relatively stable in the anti-IL-6 group and was significantly lower compared to the group without anti-IL-6 with the most pronounced difference at week 3 (~30% reduction, $p \leq 0.001$) (Figures 4E,F). In contrast, generation of memory CD4⁺ T cells was not affected (data not shown). To investigate whether the tempered CD8⁺ T_{EM} development affects the “second-set” allograft response, a second donor skin graft was transplanted 3 weeks after the rejection of the first donor graft. Both groups rejected second donor skin grafts with similar kinetics as first grafts were rejected in untreated mice (Figure 4G). 2 weeks after second graft rejection low but detectable DSA levels appeared in anti-IL-6-treated mice (3/5), which were absent after rejection of the first graft (0/8, $p = 0.039$) (Figure 4H). In addition, an *in vitro* MLR with splenocytes, harvested from BM recipients after rejection of the first skin graft, revealed no significant difference regarding proliferation of both CD4⁺ and CD8⁺ T cells whether recipients were treated with anti-IL-6 or not, which is consistent with the *in vivo* results of skin graft rejection (Figure 4I).

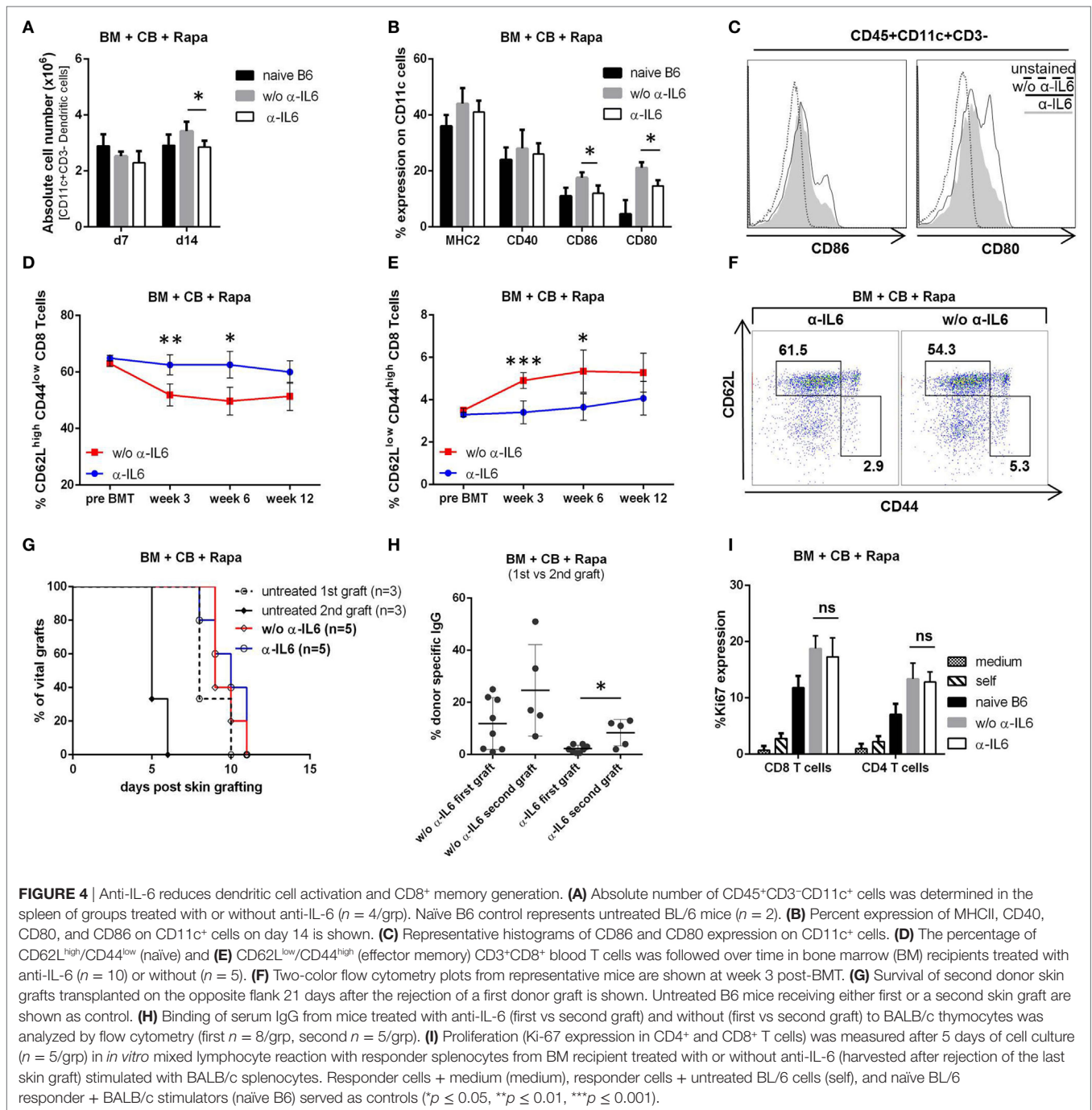
Therefore, we conclude that anti-IL-6 reduces the expansion and activation of DCs and impairs CD8⁺ T_{EM} generation during

BMT. However, this is not sufficient to delay rejection of a second skin graft.

Adding Anti-IL-17 to Anti-IL-6 Therapy Further Elevates Treg Frequency but Does Not Extend Skin Graft Survival

Anti-IL-6 prolongs, but does not extend skin graft survival indefinitely in our model. Previously it has been demonstrated that IL-6 KO and IL-6R KO mice display phenotypic differences which might be explained by the fact that soluble IL-6R shares 60% homology with the IL-12p40 subunit which results in low-affinity interaction with IL-12 and IL-23 (50, 51). Furthermore, distinct outcomes were seen in a murine contact burn model where combinational treatment of anti-IL-6 and anti-IL-6R showed a significant reduction of the inflammatory response compared to anti-IL-6 or anti-IL-6R alone (52). Besides IL-6, increased serum IL-17 levels could be found in several murine acute and chronic allograft rejection models and it was shown that elevated IL-17 levels promote rejection in part by suppressing Treg expansion (53, 54). Thus, we asked whether the combination with anti-IL-17 or anti-IL-6R provides an additional benefit to anti-IL-6 treatment with regard to inducing long-term skin graft acceptance.

Anti-IL-17 and anti-IL-6R further increased the percentage of blood Tregs on day 21 post-BMT when added to anti-IL-6 treatment (anti-IL-17: $p^* = 0.039$ and anti-IL6R: $p = 0.059$;



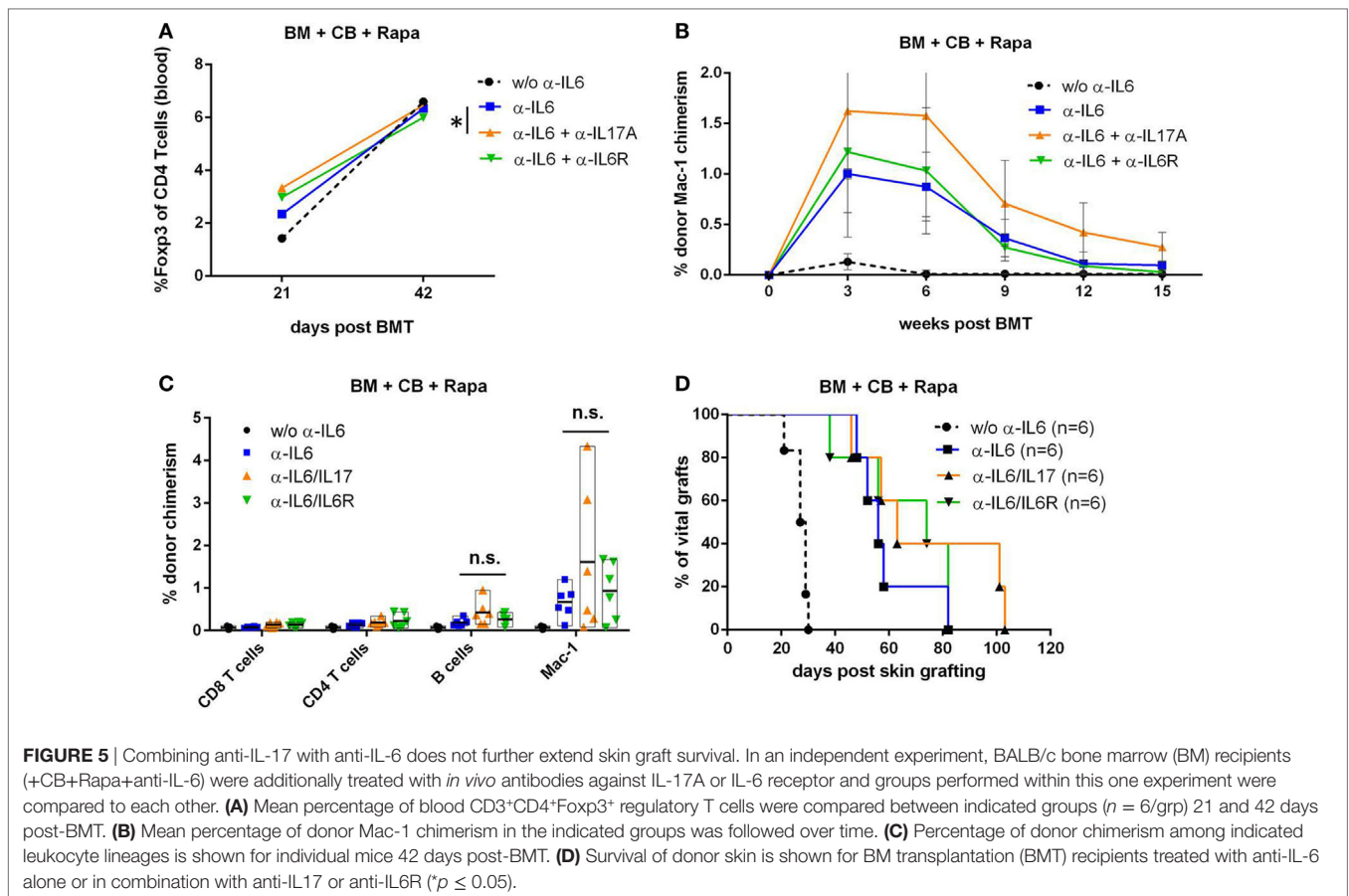
n = 6/grp) (Figure 5A). In contrast, anti-IL-17 alone showed no difference compared to anti-IL-6 (data not shown). Myeloid chimerism levels were numerically higher at all measured time points in mice treated with anti-IL-6 and anti-IL-17 compared to mice treated with anti-IL-6 alone (Figure 5B), with the clearest difference at week 6 (Figure 5C). There was, however, only a slight, non-significant prolongation of donor skin graft survival with addition of anti-IL-17 or anti-IL-6R (Figure 5D).

Taken together, these data suggest that the combination of anti-IL-6 and anti-IL-17 further expands endogenous blood

Tregs and slightly improves BM engraftment, but is not sufficient to achieve permanent donor skin graft acceptance.

DISCUSSION

Although human pilot trials have shown promising results of tolerance induction *via* mixed chimerism (3, 5, 55), its widespread clinical translation is still a challenging process due to the need of myelosuppressive and cytotoxic recipient pre-conditioning. So far, the engraftment of clinically realistic BM doses in fully



mismatched strain combinations remains a considerable challenge without the use of irradiation or cytotoxic antibody/drug treatment (56–58). Targeting pro-apoptotic factors such as Bcl-2 (with ABT-737) and the combination of Treg therapy with BM transplantation are the most effective ways to induce mixed chimerism without cyto-reductive conditioning in mice developed so far (6, 59). In the present study, we demonstrate that IL-6 blockade exhibits a synergistic effect with CB and rapamycin which can largely replace adoptive Treg therapy in a murine non-myelosuppressive chimerism-based tolerance model.

Our data show that blocking IL-6 in conjunction with CB and mTOR inhibition promotes BM engraftment leading to transient multi-lineage chimerism. Anti-IL-6 treatment substantially prolonged donor skin graft survival and led to permanent donor heart graft survival with well-preserved histology. Moreover, the development of DSA was prevented with anti-IL-6. Treg transfer as performed in previous studies led to donor multi-lineage chimerism peaking around month 2 with a gradual decline thereafter, whereas anti-IL-6 therapy also led to early multi-lineage chimerism but with an earlier peak around week 2. However, Treg therapy associated chimerism remained relatively stable for the length of follow-up (up to 7 months post-BMT) with higher levels at all measured time points (e.g., myeloid chimerism ~1.5% with anti-IL-6 vs ~5% with Treg therapy at week 2 post-BMT). Moreover, Treg transfer led to permanent donor skin (vs MST 58 with anti-IL-6) and heart graft survival (similar to anti-IL-6) (6, 8).

The pro-tolerogenic effect of anti-IL-6 occurred only in the context of (transient) BM engraftment, as no such effect was seen when DST with donor splenocytes instead of BM was given. Since heart grafts survived until the end of the observation period while skin grafts were gradually rejected (although both express the same donor MHC molecules), tissue-specific antigens could be one reason for this difference in outcomes. However, it cannot be ruled out that the heart allografts would have been rejected at later times due to different effector mechanisms accounting for rejection of distinct tissues. mHAg disparities have a profound role in organ transplantation (60) and our group recently demonstrated that they significantly reduce the success rate of a CB-based chimerism protocol in inducing donor-specific tolerance (48). mHAg-matched donor skin showed prolonged survival but was not accepted indefinitely. Thus, mHAg disparities are not the only factor limiting donor skin graft survival under anti-IL-6 treatment and it is possible that the rejection of skin allografts was due to incomplete tolerance induction under the protocol used.

We found that chimerism and extended donor graft survival were associated with a quantitative increase in endogenous Treg numbers. It has been reported that isolated CD4⁺CD25⁺ cells from IL-6 deficient mice showed a greater potency to suppress IFN- γ production than wild-type Tregs (27). However, we observed no difference in the suppressive capacity of Tregs isolated from BMT recipients after anti-IL-6 treatment. IL-6 was previously

shown to prolong T cell survival *via* maintenance of Bcl-2 expression and Fas ligand downregulation (61, 62). In addition, it could be demonstrated that IL-6 is an essential mediator for antigen-specific T cell priming (22). Moreover IL-6 KO recipients receiving an allogeneic heart graft presented a significant reduction in CD8⁺ T_{EM} generation (27). In the line with this, our results show that IL-6 blockade reduces the maturation of naïve CD8⁺ T cells to T_{EMs}. Additionally, proliferation and priming of antigen-specific CD8⁺ T cells can be suppressed by Tregs (63), thus the decreased population of CD8⁺ T_{EMs} might also be related to the increased Treg levels seen upon anti-IL-6 treatment in our model. However, this effect was not sufficient to measurably affect the survival of a second donor skin graft. IL-6 also regulates DC differentiation and maturation (23, 64). Circulating DCs express MHC class II and low levels of CD40, CD80 and CD86 (65). In contrast, following stimulus, migrating DCs lose their ability to capture Ags, upregulate co-stimulatory molecules and secrete pro-inflammatory cytokines which further increase the inflammatory response (66). Our data show that anti-IL-6 reduces the expansion and activation of DCs during allogeneic BMT.

In a murine contact burn model the treatment of anti-IL-6 together with anti-IL-6R indicated further benefits in the clearance of the inflammatory response (52). Thus, we hypothesized that a combination of both antibodies (anti-IL6/anti-IL-6R) might improve the efficacy described above. Although the addition of anti-IL-6R further elevated Treg frequencies, chimerism levels were not significantly different to anti-IL-6 treatment alone, neither was skin graft survival. Previous studies have also suggested a synergistic effect of IL-6 blockade with blockade of other pro-inflammatory cytokines (67, 68). Combining anti-IL-6 and anti-IL-17 led to significantly increased Treg levels and a trend regarding higher myeloid chimerism levels. However, permanent

chimerism or donor skin graft acceptance was not achieved, suggesting that other yet to be defined mechanisms need to be targeted in anti-IL-6 treated recipients.

Collectively, we conclude that anti-IL-6 therapy leads to BM engraftment and transient chimerism, with associated prolonged donor skin graft acceptance and indefinite donor heart survival. Thus, targeting IL-6 in combination with CB and mTOR inhibition represents a promising strategy for use in chimerism-based tolerance protocols.

ETHICS STATEMENT

All experiments were approved by the local review board of the Medical University of Vienna and the Austrian Federal Ministry of Science, Research and Economy and were performed in accordance with national and international guidelines of laboratory animal care (permission number GZ: BMWFW-66.009/0377-WF/V/3b/2016 and GZ: BMWFW-66.009/0303-WF/V/3b/2016).

AUTHOR CONTRIBUTIONS

All authors have actively contributed to the study. NG and TW designed all experiments, analyzed and interpreted the data, and wrote the manuscript. NG, MM, BM, NP, AF, LU, SM, RH, and MW performed experiments. HR performed histopathological staining and analysis. All authors approved the final version of the manuscript.

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Conflict of Interest Statement: The author declares that the complete study was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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