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Deleterious Effect of CTLA4-Ig on a Treg-Dependent Transplant Model

L. V. Riella, T. Liu, J. Yang, S. Chock, T. Shimizu, B. Mfarrej, I. Batal, X. Xiao, M. H. Sayegh and A. Chandraker*

Renal Division, Transplantation Research Center, Brigham and Women's Hospital, Children's Hospital Boston, Harvard Medical School, Boston, MA
**Corresponding author: Anil Chandraker, achandraker@rics.bwh.harvard.edu*

Blockade of the B7:CD28 costimulatory pathway has emerged as a promising therapy to prevent allograft rejection. However, results from the belatacept phase III clinical trial demonstrated a higher rejection rate when compared to cyclosporine, raising concern about potential deleterious effects of this agent. In this study, we investigated the consequences of B7:CD28 blockade by hCTLA4Ig on regulator T cell (Treg) generation in different major histocompatibility complex (MHC) mismatch transplant models. Administration of hCTLA4Ig significantly decreased the amount of Tregs in B6 WT animals and this effect was predominant in thymus-induced Tregs (Helios$^+$). Although hCTLA4Ig prevented rejection in a fully allogeneic mismatch model, it accelerated rejection in a MHC class-II mismatch model (MST = 26, $p < 0.0001$), in which long-term allograft survival is dependent on Tregs. This accelerated rejection was associated with a marked reduction in thymus-induced Tregs and led to a higher effector/regulatory T-cell ratio in secondary lymphoid organs and in the allograft. This study confirms the importance of the B7:CD28 pathway in Treg homeostasis in an *in vivo* transplant model and suggests that hCTLA4Ig therapy may be deleterious in circumstances where engraftment is dependent on Tregs.

Key words: Costimulation, MHC class II, mice, regulatory T cells, rejection

Abbreviations: hCTLA4-Ig, human cytotoxic T-lymphocyte antigen 4; MHC, major histocompatibility complex; Tregs, regulatory T cells.

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Introduction

T cells play a central role in transplantation, orchestrating the alloimmune response that ultimately leads to graft destruction. To be fully activated after antigen presentation, T cells require an antigen-independent signal provided by costimulatory molecules (1). The B7:CD28 costimulation has emerged as one of the most important costimulatory pathways in T-cell activation, as evidenced by *in vitro* studies in which T-cell activation through TCR stimulation in the absence of CD28 costimulation resulted in T-cell anergy and/or apoptosis of the responding T cell (2). Therefore, targeting this pathway presents a potential opportunity to induce transplant tolerance.

Nevertheless, advancement in the understanding of the B7:CD28 pathway has led to some unexpected discoveries. CD28 signaling has been demonstrated to have an essential role in the maintenance and generation of regulatory T cells (Tregs) (3), raising concerns about the consequences of blockade of this pathway in Tregs in alloimmunity. Moreover, B7 interaction with both CD28 and CTLA4 has been shown to be important in Th17 suppression (4,5). Although Tregs are considered protective to the allograft, Th17 cells have emerged as potential contributors to graft rejection and may exhibit resistance to current immunosuppressive drugs (6,7).

Interestingly, results from the phase III clinical trial with belatacept demonstrated a higher rejection rate in the belatacept-treated groups (~20%) when compared to the cyclosporine group (7%; 8), raising concern about the potential deleterious effects of this agent. Initial studies by our group in a rodent model showed that B7- or CD28-deficient recipients of bm12 cardiac allografts developed an accelerated rejection because of a decrease in Treg generation and maintenance (9).

In this study, we investigate the consequences of B7:CD28 blockade by hCTLA4Ig on Treg generation and suppression of Th17 cells in alloimmunity using different murine mismatch cardiac transplant models. hCTLA4-Ig was found to be deleterious in a major histocompatibility complex (MHC) class II mismatch model dependent on Tregs for allograft survival, precipitating rejection through a decrease in Tregs. Thymus-induced Tregs (Helios$^+$) were the predominant subpopulation of Tregs affected by B7:CD28 blockade, although peripherally induced Tregs (Helios$^-$) were not altered. These hypothesis-generating findings have clinical relevance with the recent approval of belatacept as an immunosuppressive drug by the FDA, because it is important to identify conditions in which this agent may have deleterious effects.

Material and Methods

Mice

C57BL/6 ($H2^b$, B6), B6.C-$H2^{bm12}$/KhEgJ ($H2^{bm12}$) and BALB/c ($H\text{-}2^d$) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). GFP-Foxp3 Knock In (Foxp3-GFPKI) mice were a kind gift of Dr. Rudensky (University of Washington). All mice were 8–12 weeks of age and housed in accordance with institutional and National Institutes of Health guidelines.

Heterotopic heart transplantation

Vascularized heart grafts were placed in an intraabdominal location as described by Corry et al. (10). Bm12 ($H2^{bm12}$) and BALB/c ($H\text{-}2^d$) mice were used as heart donors, although B6 ($H2^b$) mice were used as recipients. Graft function was assessed by palpation of the heartbeat. Rejection was determined by complete cessation of palpable heartbeat and was confirmed by direct visualization after laparotomy. Graft survival is shown as median survival time (MST) in days.

Antibodies and in vivo *treatment protocol*

hCTLA4-Ig, a fusion protein composed of a human IgG1 fused to the extracellular domain of CTLA4, was purchased from Bristol–Myers–Squibb (New York, NY, USA). Cardiac allograft recipients were treated with hCTLA4-Ig intraperitoneally (i.p.) according to the following protocol: 0.1 mg of mAb on the day of transplantation and 0.05 mg on days 2, 4 and 6 after transplantation. For the delayed dosing group, CTLA4-Ig was administered on days 14, 16, 18 and 20 with similar dosing. Neutralizing anti-IL-17 mAb (MAB421; R&D Systems, Minneapolis, MN, USA) was administered at a dose of 100 μg i.p. daily on days 0, 1, 2 and 3, followed by every other day injection until day 13 after transplantation (11).

For the experiments on naïve mice, rat antimouse CD25 mAb (PC61) was given for 2 doses 5 days apart (250 μg), although hCTLA4-Ig, anti-CD154 and anti-CTLA4 mAb were given for four doses 2 days apart (100 μg then 50 μg), with recovering of lymphoid organs one day after the last dose of Ab. Long-term follow-up to evaluate the frequency of Tregs and $Foxp3^+Helios^{+/-}$ cells was performed on GFPFoxp3-Ki mice on a B6 background with administration of hCTLA4Ig as above, with subsequent *ex vivo* analysis on days 4, 7 and 14 after the last injection.

ELISPOT assay

Splenocytes recovered at 7–10 days after transplantation from B6 recipients of bm12 and BALB/c heart allografts were restimulated by irradiated donor-type splenocytes and cultured for 24–48 h. Additional soluble anti-CD3 stimulation was used under some conditions (0.4 μg/mL). The ELISPOT assay (R&D Systems) was adapted to measure the frequency of alloreactive T cells producing IFN-γ , IL-4 and IL-17, as reported previously (9). The frequencies of cytokine-secreting alloreactive cells were expressed as the number of cytokine-producing cells per 0.5×10^6 responder cells. All samples were tested in triplicate wells.

Flow cytometry

Splenocytes from B6 recipients of bm12 or BALB/c grafts at 7–10 days after transplantation were stained with fluorochrome-labeled mAbs against CD4, CD8, L-selectin (CD62L), CD44, CD25 and FoxP3 (BD Biosciences, San Jose, CA, USA). Intracellular FoxP3 staining was performed using the Cytofix/Cytoperm intracellular staining kit. Flow cytometry was performed with a FACSCalibur system (BD Biosciences) and analyzed using FlowJo software, assessing regulatory T cells ($CD4^+CD25^+FoxP3^+$) as well as $CD4^+$ and $CD8^+$ effector memory cells ($CD44^{high}$ $CD62L^{low}$ phenotype). The ratio of CD4 eff/mem/Tregs was calculated by dividing the total count of CD4 eff/mem cells by those of Tregs. Leukocytes were isolated from heart allografts and restimulated *in vitro* with PMA and ionomycin as previously described (12). After staining for CD4 surface marker, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and incubated with PE-conjugated anti-IFN-γ (XMG1.2) and APC-conjugated anti-IL-17 (eBio17B7) for 30 min at 4°C. A gate was set on $CD4^+$, and the percentage of IFN-γ and IL-17 cells was determined by flow cytometric analysis.

In vitro *suppression assay*

To assess the regulatory function of Tregs *in vitro* in the setting of hCTLA4-Ig, an MLR assay was set up in which $CD4^+$ $Foxp3^-$ T cells (0.05×10^6) from GFP-Foxp3KI mice were cultured with syngeneic irradiated antigen-presenting cells (T cell depleted splenocytes) (0.05×10^6), 1 μg/mL anti-CD3 and $CD4^+Foxp3^+$ cells at various ratios for 72 h (1:2, 1:4, 1:8) with either IgG control or hCTLA4-Ig at an optimized concentration of 5 μg/mL. Cells were pulsed with [^{3}H] thymidine (1 μCi/well) for the final 16 h of incubation and incorporation of [^{3}H] thymidine was measured with a microbeta liquid scintillation counter. The regulatory function of Tregs was assessed by the suppressive effect on the proliferation of effector T cells. $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells were isolated from splenocytes by flow cell sorting with greater than 99% purity.

Adoptive cell transfer experiments

10×10^6 Thy1.2 $CD4^+$ cells from B6 WT naïve mice were labeled with CFSE (Invitrogen) and transferred into congenic B6 WT mice (Thy1.1) followed by treatment with IgG or CTLA4-Ig on days 1, 2 and 4 (0.1 mg). Spleens and lymph nodes were then recovered 5 days after adoptive transfer and the proliferation of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells was determined by the dilution of CFSE in these gated subpopulations (13). $CD4^+$ T cells were isolated from splenocytes by magnetic activated cell sorting (130-090-860; Miltenyi Biotec, Auburn, CA, USA). The purity of T cells was estimated to be greater than 95% by FACS.

Morphology

Cardiac graft samples from transplanted mice were recovered at 1 and 3 weeks after transplantation as well as at the time of rejection and/or 56 days after transplantation. Samples were fixed in 10% formalin, embedded in paraffin, coronally sectioned and stained with hematoxylin and eosin. Immunohistochemistry was performed on 5-mm-thick, formalin-fixed, paraffin-embedded tissue sections with anti-CD3 (CMC363; Cell Marque, Rocklin, CA, USA; 1:1500, EDTA) and anti-Foxp3 (14–5773; eBioscience, San Diego, CA, USA; 1:25, citrate) primary Abs. All of the stained slides were scanned using an Aperio ScanScope XT (Aperio Technologies, Vista, CA, USA). Images were analyzed using ImageScope software (version 10.0.35.1800; Aperio Technologies) and a standard analysis algorithm (nuclear version 9.0; Aperio Technologies) with appropriate modifications for cell and nuclear sizes. An examiner blinded to the groups read all of the samples (I.B.).

Statistics

Graft survival was expressed graphically using the Kaplan–Meier method, and statistical differences in survival between the groups were assessed by the log-rank test. The Mann–Whitney nonparametric test was used for comparison of means. A $p < 0.05$ was considered statistically significant.

Results

hCTLA4-Ig significantly decreases natural Tregs on naïve mice

The B7:CD28 costimulatory pathway has been shown to be important for Treg maintenance (3). To evaluate the effect of hCTLA4-Ig on Tregs, we administered hCTLA4Ig to naïve B6 wild-type mice and measured Tregs 1 day after a course of 4 doses. Administration of hCTLA4-Ig decreased by half the percentage and total amount of $CD4^+Foxp3^+$ T

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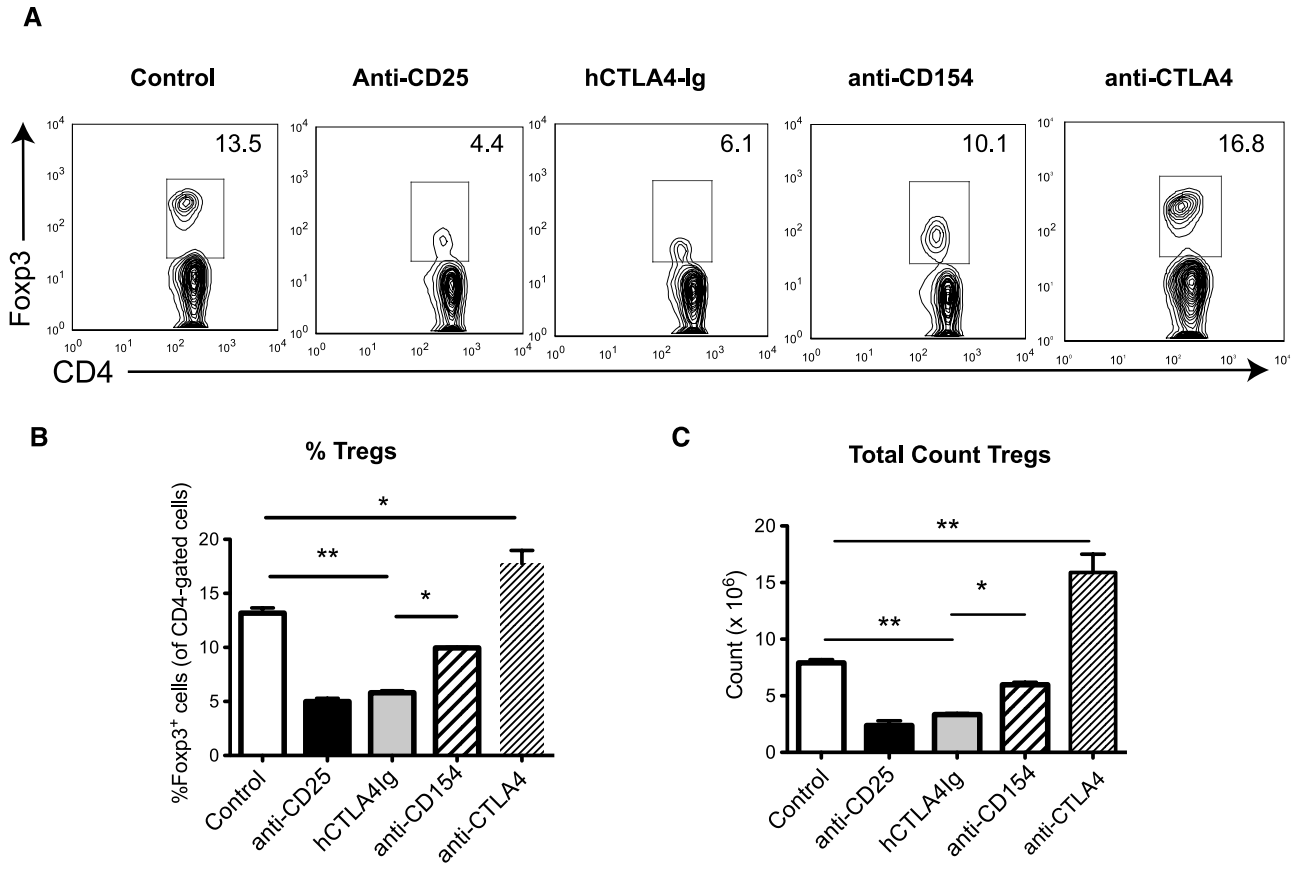

Figure 1: Decreased numbers of regulatory T cells by flow cytometry in naïve B6 wild-type mice treated with hCTLA4Ig. (A) Representative flow cytometry plots of regulatory T cells ($CD4^+Foxp3^+$) in naïve controls and in mice after a short course of mAb administration protocol as detailed on Materials and Methods. (B) Column graphs with average percentage of $CD4^+Foxp3^+$ in different groups (n = 5 per group). (C) Total cell count of $CD4^+Foxp3^+$, calculated by multiplying the total cell yield from spleens by the percentage of $CD4^+Foxp3^+$ in the five groups depicted (n = 5). *$p < 0.001$, **$p < 0.0001$.

cells (Tregs) in naïve mice ($3.34 \times 10^6 \pm 0.09$) compared with untreated controls (vs. $7.9 \times 10^6 \pm 0.29$, $p = 0.0001$; Figure 1). A similar reduction was also seen with anti-CD25 mAb administration ($2.37 \times 10^6 \pm 0.41$), although blockade of the CD40:CD154 costimulatory pathway through administration of anti-CD154 mAb (MR1) had less of an effect on Tregs (Figure 1). Because hCTLA4-Ig could also block B7:CTLA4 interaction, we also administered anti-CTLA4 mAb to naïve mice. Interestingly, blockade of CTLA4 led to a significant increase in the number of Tregs (Figure 1), opposite to the effect of hCTLA4-Ig. This confirms that B7:CD28 blockade affects Treg homeostasis in naïve wild-type mice.

Regulatory T cells have been shown to undergo homeostatic proliferation in the steady state (13,14). To evaluate the effect of hCTLA4-Ig on the proliferation of Tregs, we transferred 10×10^6 Thy1.2 $CD4^+$ cells labeled with CFSE into congenic B6 WT mice (Thy1.1) followed by treatment with IgG or CTLA4-Ig. As shown in Figure 2A, B7:CD28 blockade inhibits the homeostatic proliferation of Tregs at 5 days after cell transfer. Finally, to dissect if hCTLA4-Ig could also be affecting Treg function via blockade of B7:CTLA4 pathway, we conducted an *in vitro* suppression assay and observed that hCTLA4-Ig did not significantly affect Treg inhibitory function on T-cell proliferation (Figure 2C).

$Helios^+$ regulatory T cells are significantly reduced after B7:CD28 costimulation blockade

Expression of Helios has been proposed to help in the differentiation of thymic derived from peripherally induced $Foxp3^+$ regulatory T cells (15). To investigate the effect of hCTLA4-Ig treatment on $Helios^+$ Tregs, we evaluated the expression of Helios on $Foxp3^+$ Tregs of naïve GFP-Foxp3KI mice in the absence of exogenous antigen over time. hCTLA4Ig predominantly reduced the number of $Helios^+$ Tregs compared to $Helios^-$ Tregs (Figure 3). This effect was persistent even 2 weeks after last hCTLA4-Ig injection. This corroborates with the belief that B7:CD28 is particularly important for the homeostasis of thymus-induced Tregs ($Helios^+Foxp3^+$).

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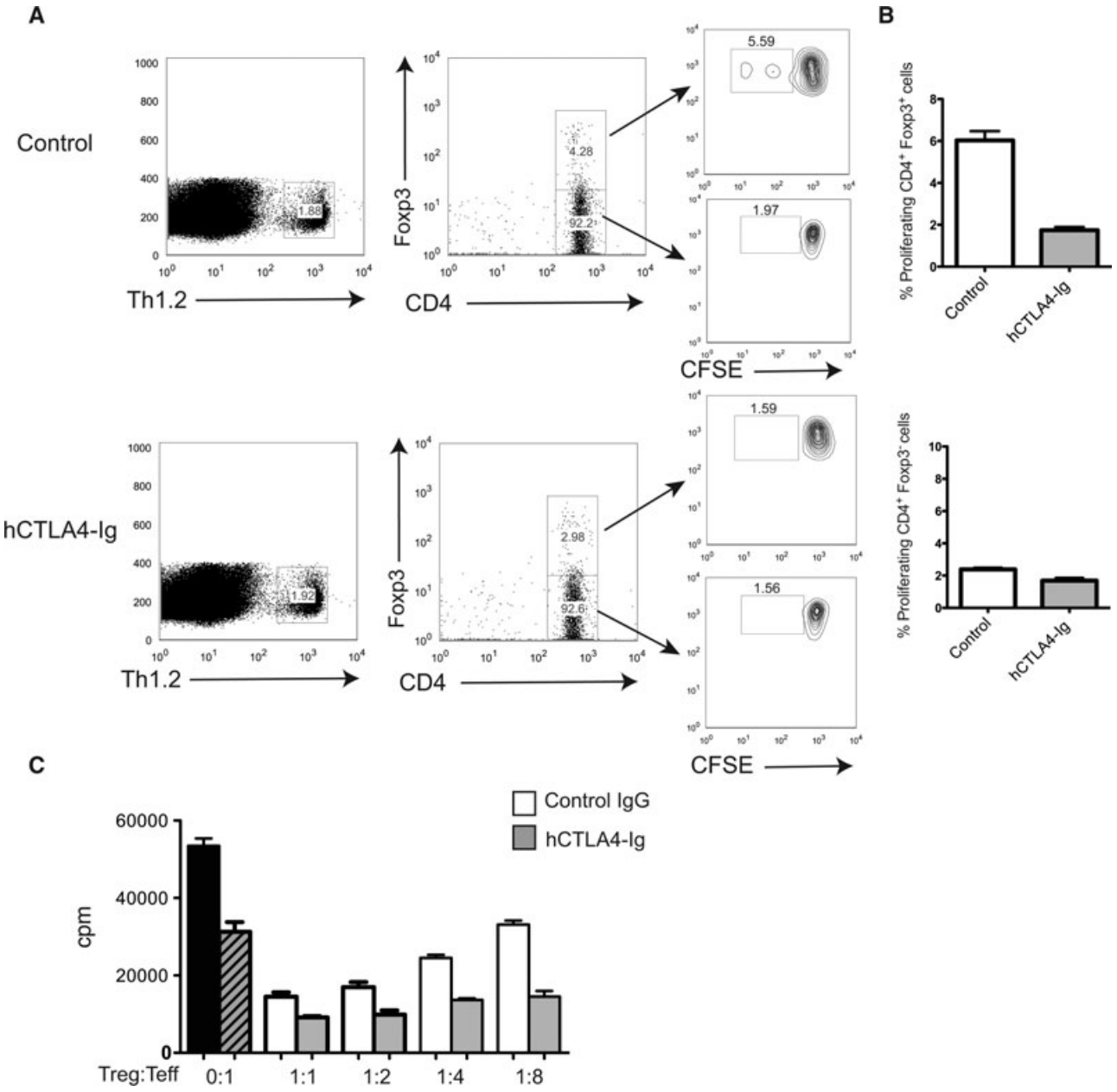

Figure 2: Effect of hCTLA4Ig on Tregs' homeostatic proliferation *in vivo* and in Tregs' function *in vitro.* (A) 10×10^6 Thy1.2 $CD4^+$ cells labeled with CFSE were transferred into congenic B6 WT mice (Thy1.1) followed by treatment with IgG or hCTLA4Ig and recovering of cells 5 days later. Representative flow cytometry plots of CFSE staining on Th1.2 selected cells, after gating on regulatory T cells ($CD4^+Foxp3^+$). (B) Percentage of proliferating $CD4^+Foxp3^+$ in controls and hCTLA4 group, compared with $CD4^+Foxp3^-$ cells (n = 4 on each group). (C) *In vitro* suppression assay of Tregs ($CD4^+Foxp3^+$ flow sorted cells) in combination with T effector cells ($CD4^+Foxp3^-$) at different ratios in the presence of hCTLA4-Ig or control IgG, as described in Material and Methods.

hCTLA4-Ig administration has opposite effects on the alloimmune response in a fully allogeneic versus a single MHC class-II mismatch model

hCTLA4-Ig has been shown to be an effective agent in suppressing acute graft rejection in some animal transplant models, however it was incapable of inducing long-term tolerance by itself in primates (16). To evaluate possible mechanisms, we decided to test hCTLA4-Ig in different MHC mismatch transplant models with corresponding different alloreactive T-cell pool sizes. Interestingly, although hCTLA4-Ig prolonged graft survival in the fully mismatched "BALB/c into B6" model (MST > 60 days vs. 7.5 days in controls; Figure 4C), it precipitated rejection in the MHC class II mismatched "bm12 into

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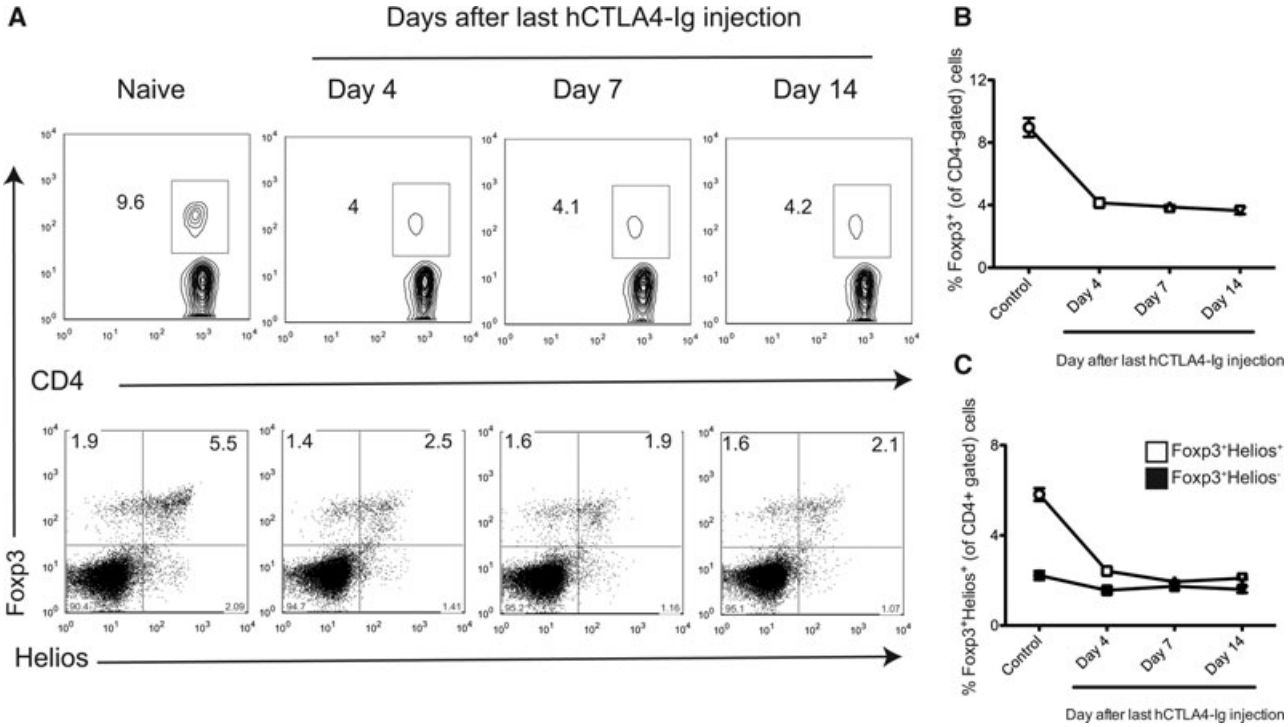

Figure 3: Persistent reduction of Foxp3$^+$Helios$^+$CD4$^+$ cells after hCTLA4-Ig administration. Naive GFP-Foxp3KI mice were treated with 4 consecutive doses of hCTLA4-Ig and followed for up to 2 weeks. (A) Representative flow cytometry plots of Helios/Foxp3 expression on CD4-gated cells on days 4, 7 and 14 after last hCTLA4-Ig injection compared to naïve mouse. (B) Frequency of CD4$^+$Foxp3$^+$ cells overtime after hCTLA4-Ig injections. (C) Frequency of Foxp3$^+$Helios$^+$ of CD4-gated cells overtime (n = 3 on each group).

B6″ model (MST = 26 days vs. >60 days in controls; Figure 4A). In the latter model, the alloreactive T-cell pool size is small and long-term survival is dependent on Treg generation (9,17). hCTLA4-Ig predominantly inhibited Tregs and led to a higher effector/regulatory T-cell ratio (**p = 0.004) in the MHC class II mismatch model (Figure 4B). Among regulatory T cells, thymus-induced Helios$^+$Foxp3$^+$ T cells were the predominant cell type affected by B7:CD28 blockade (7.3 ± 0.48% vs. 12.3 ± 0.27% on controls, n = 4, p = 0.02), although peripherally induced Tregs (Helios$^-$Foxp3$^+$CD4$^+$ cells) were not significantly altered (Figure 5).

In the presence of a larger alloreactive T-cell pool size (BALB/c into B6), hCTLA4-Ig had a dominant effect on the effector T cells rather than on Tregs (Figure 4D). Moreover, delayed administration of hCTLA4Ig starting at day 14 after transplantation also precipitated rejection in the MHC class II mismatch model (Figure 4A). In contrary, hCTLA4-Ig did not accelerate rejection in a minor mismatch model in which hearts from 129 mice were transplanted into B6 WT recipients (MST > 60 days, n = 6). These data demonstrate a deleterious impact of B7:CD28 blockade on alloimmunity, irrespective of timing, in the setting of a single MHC class II mismatch with associated reduction of thymus-induced Tregs.

hCTLA4-Ig leads to significant cellular rejection and higher CD3$^+$/Foxp3$^+$ ratio in bm12 allografts

To further investigate the mechanism of rejection of bm12 allografts in B6 recipients treated with hCTLA4-Ig, we recovered cardiac allografts 2–3 weeks after transplantation, at which time point the hearts started to demonstrate worsening function. Recipients of bm12 allografts treated with hCTLA4Ig had a more intense cellular infiltrate when compared to controls (Figure 6A). Moreover, there was a higher ratio of CD3/Fox3 cells by immunohistochemistry in the hCTLA4-Ig treated group (Figure 6B). However, the absolute count of Foxp3$^+$ cells/high power field was higher in the rejecting graft from the hCTLA4Ig-treated group when compared to control allografts at a similar time point (Figure 6B), corroborating recent data suggesting that Foxp3$^+$ cells in the graft correlate better with rejection rather than favorable outcomes (18).

B7:CD28 blockade increased alloreactivity in B6 recipients of bm12 allografts

In addition to its role in Treg maintenance, B7:CD28 interaction has also been shown to be important in Th17 suppression (4,5). To evaluate the consequence of the decrease in Tregs and possible effect on Th17 cells, we analyzed the frequency of alloreactive cytokine-producing splenocytes by ELISPOT. B6 WT recipients of bm12 grafts

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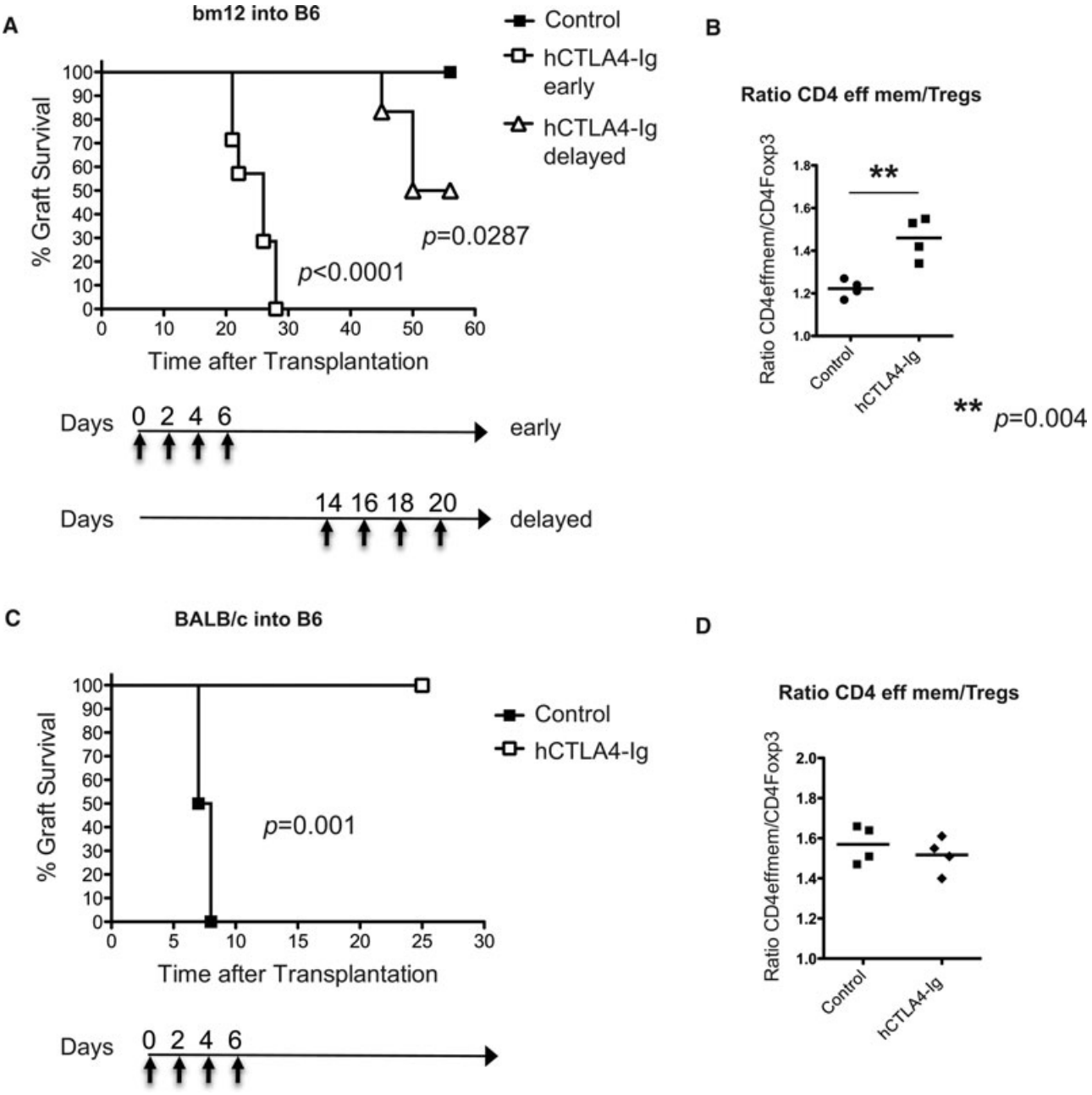

Figure 4: Opposite survival effects of hCTLA4-Ig administration in a fully allogeneic mismatch model versus a MHC class II mismatch. (A) Survival of bm12 allografts transplanted into B6 WT recipients treated with hCTLA4-Ig with two protocols and controls: early after transplantation or delayed, as depicted by horizontal arrows (n = 7–8 per group). (B) Ratio of CD4 eff/mem cells to Tregs from splenocytes of recipients of bm12 allografts in controls and early treated hCTLA4-Ig groups (1 week after transplantation). (C) Survival of BALB/c allografts transplanted into B6 recipients treated with early course of hCTLA4-Ig and controls (n = 6 per group). (D) Ratio of CD4 eff/mem cells to Tregs from splenocytes of recipients of BALB/c allografts in controls and early treated hCTLA4-Ig groups (1 week after transplantation). **p = 0.004.

treated with hCTLA4-Ig demonstrated a similar frequency of IFN-γ-producing T cells when compared to controls (42 ± 8 vs. 57 ± 14, p = 0.39; Figure 7A), however an increase in both IL-17- and IL-4-producing cells was noticed (*p < 0.03; Figure 7A). Corroborating the findings on secondary lymphoid organs, when lymphocytes infiltrating the allografts were isolated and restimulated *ex vivo* for intracellular cytokine determination, the frequency of

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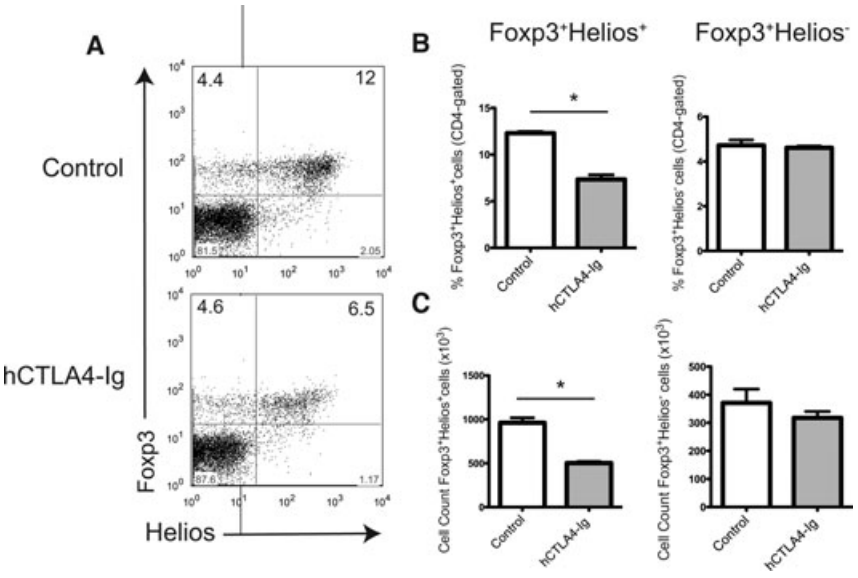

Figure 5: Foxp3$^+$Helios$^+$CD4$^+$ subpopulation was significantly reduced in the hCTLA4-Ig-treated group. (A) Representative flow cytometry plots of Helios and Foxp3 expression on CD4-gated cells of recipients of bm12 allografts 10 days after transplantation. (B) Average frequency of Foxp3$^+$Helios$^{+/-}$ CD4-gated cells on controls and hCTLA4-Ig group. (C) Total cell counts of Foxp3$^+$Helios$^{+/-}$ CD4-gated cells (n = 4, *p = 0.02).

IL-17-producing CD4 cells were increased in the hCTLA4-Ig-treated group compared to controls (Figure 6C), although IFN-γ-producing CD4 cells were similar between groups (Figure 6D). Despite the increase in Th17 cells both in the spleen and allograft, neutralization of IL-17 in B6 recipients of bm12 allografts treated with hCTLA4-Ig was not able to delay rejection (MST = 23 vs. 26 days; n = 4; p = 0.85; 11), questioning the pathogenic role of Th17 in the accelerated rejection. In contrast, the dominant cytokine effect of hCTLA4-Ig in B6 recipients of BALB/c hearts was a five-fold decrease in the frequency of IFN-γ alloreactive T cells (957 ± 80 vs. 175 ± 40, p = 0.02), although other cytokines were not significantly different (Figure 7B). In conclusion, hCTLA4-Ig may be deleterious to allograft survival in a MHC class II mismatch setting by decreasing natural Tregs and consequently disrupting immune regulation.

Discussion

The B7:CD28 pathway has emerged as a promising target to control the alloimmune response. The development of CTLA4-Ig, a soluble recombinant immunoglobulin fusion protein composed of an extracellular domain of human CTLA4 with an IgG heavy chain tail, has generated great interest as a potential immunosuppressive biological agent (19). However, recent discoveries regarding the B7:CD28 pathway have led to some concerns about the tolerogenic effect of CTLA4-Ig (1). Indeed, the phase III clinical trial with belatacept revealed a higher rejection rate in the belatacept-treated groups (22% in the more intensive arm and 17% on the less intensive arm) when compared to the cyclosporine group (7%), despite similar graft survival at 1 year (8). Moreover, the rejection in the

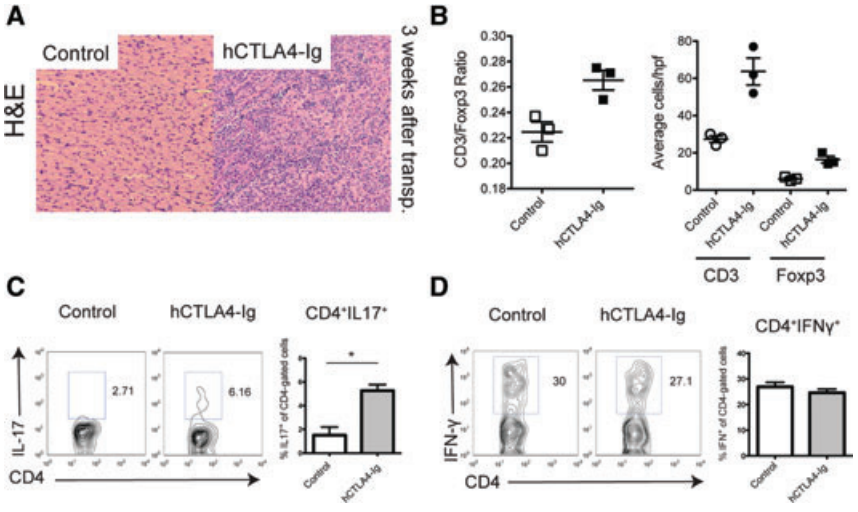

Figure 6: Histopathology and infiltrating leukocytes analysis of cardiac allografts. (A) Hematoxylin and eosin of bm12 allografts 3 weeks after transplantation demonstrated significant cellular infiltration in the hCTLA4-Ig treated group, although less infiltrate was seen in controls. (B) Quantification of the ratio and average of CD3 and Foxp3 cells by immunohistochemistry stains per high power field (hpf). (C, D) Representative flow cytometry plots and frequency of IFN-γ- and IL-17-producing CD4$^+$ T cells isolated from heart allografts treated with hCTLA4Ig or controls 3 weeks after transplantation (n = 3 on each group; *p = 0.02).

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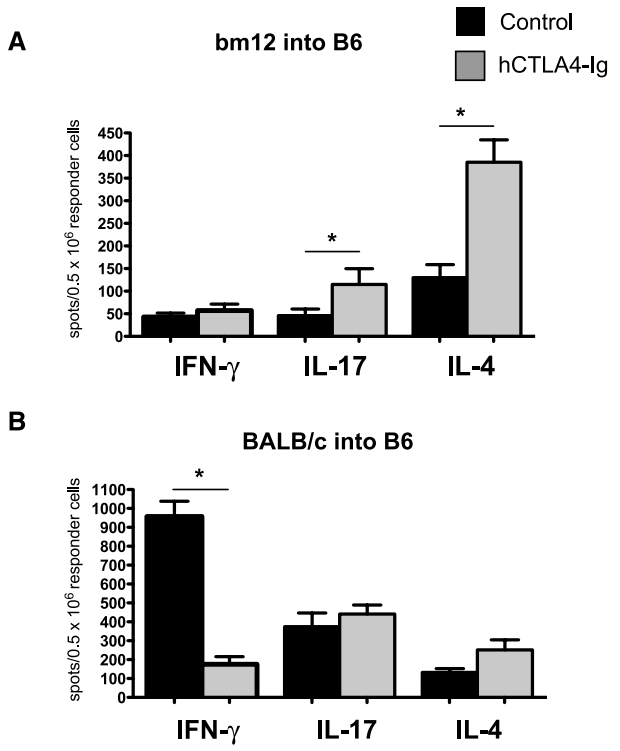

Figure 7. Alloreactive T-cell cytokine profile by ELISPOT. Splenocytes derived from recipients of either bm12 or BALB/c grafts 7–10 days after transplantation were cultured with irradiated donor cells. (A) Recipients of bm12 allografts treated with CTLA4-Ig demonstrated a significant increase in the frequency of both IL-17- and IL-4-producing alloreactive T cells, although IFN-γ was no different from controls. (B) Recipients of BALB/c allografts treated with CTLA4-Ig showed a decrease in IFN-γ-producing T cells but no difference between IL-17 and IL-4. Data are representative of at least three independent experiments using a minimum of $n = 4$ mice per group. All measurements were done in triplicates ($^*p < 0.03$).

belatacept groups was much more severe, with Banff scores ≥IIA (8).

The initial simplistic view of the positive B7:CD28 costimulatory pathway has become more complex when B7:CD28 interaction was shown to be not only important for the activation of effector T cells, but also essential for the maintenance and generation of regulatory T cells (3,20). In transplantation, Tregs play an indispensable role in both the induction and maintenance of allograft tolerance and the achievement of tolerance seems to dependent not only in the prevention of an effector T-cell response but also in the promotion and maintenance of Tregs (21). We have previously shown that both CD28-deficient as well as B7.1/2-double-deficient mice have a significant lower number of Tregs when compared with controls and deficiency of either B7.1/2 or CD28 leads to a paradoxical accelerated rejection in a MHC class II mismatch model of cardiac transplantation (9). The rate of rejection was inversely related to the amount of Tregs in the recipient (9).

In our current study, we further explored the potential deleterious effects of interfering with B7:CD28 pathway, but now with the use of hCTLA4-Ig in different MHC mismatch models of cardiac transplantation. In humans, treatment with hCTLA4-Ig has been shown to decrease Tregs' frequency in patients with rheumatoid arthritis and in kidney transplant recipients (22,23). The first striking observation was that hCTLA4-Ig depleted by half the number of Tregs from naïve mice after only a few doses and this reduction in Tregs was persistent even 2 weeks after the last injection (Figures 1 and 3). This depletion was as significant as the one obtained with anti-CD25 mAb, although blocking another positive costimulatory pathway CD40:CD154 did not lead to such a profound effect (Figure 1). Tregs undergo homeostatic proliferation in the steady state (13,14) and blockade of B7:CD28 interaction inhibited this proliferation (Figure 2A), contributing to the reduction in Tregs (Figure 1).

Although hCTLA4-Ig was capable of prolonging allograft survival in a fully allogeneic cardiac transplant model (Figure 4C), it precipitated rejection in an MHC class II mismatch model (Figure 4A). In this model, allografts spontaneously survive for >56 days because of the emergence of Tregs that inhibit the expansion of alloreactive T cells (9,17). Blockade of B7:CD28 had a predominant effect on Tregs, leading to a higher Teff/Treg ratio (Figure 4B). This observation corroborated with our previous findings with B7- or CD28-deficient recipients (9). Furthermore, delayed administration of hCTLA4-Ig also abrogated survival (Figure 4A), suggesting that even late blockade might be harmful. In contrast, hCTLA4-Ig had a predominant effect on T effector cells in the fully allogeneic model (Figure 4D), raising the question that if in a setting of a MHC class II mismatch or in settings with a smaller alloreactive T cells' pool size, B7:CD28 blockade might have a deleterious effect.

Natural and induced regulatory T cells have been suggested to independently contribute to tolerance (24,25). Helios, an Ikaros transcription factor family, has been proposed as a maker of thymus-induced Tregs (natural Tregs) as compared to peripherally induced ones (Helios$^-$; 15). Blockade of B7:CD28 interaction predominantly decreased natural Tregs, although peripherally induced Tregs (iTregs) were minimally affected. A recent report even suggests that the B7:CD28 pathway might have opposing roles in natural versus induced Tregs, with evidence that strong CD28 costimulation might suppress the generation of iTregs (26). Further studies are still needed to investigate these findings in the transplant setting.

Among T helper cells that are involved in the alloimmune response, IFN-γ-producing T cells (Th1) are known to play a major role in allograft rejection (1). More recently, effector

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T cells that produce IL-17 (Th17) have been characterized and linked to allograft rejection as well (6). Importantly, B7:CD28 interaction was shown to suppress the development of Th17 cells and blockade of this pathway with hCTLA4-Ig facilitated both murine and human Th17 differentiation *in vitro* (4). Furthermore, B7:CTLA4 interaction has been shown to be capable of inhibiting Th17 cell differentiation in another study and suppressing the development of Th17-mediated autoimmunity (5). We did also observe a slightly higher frequency of IL17-producing cells in the hCTLA4-Ig-treated group, however the difference was only significant in the MHC class II mismatch model. The reason for this difference requires further investigation, but it might be related to the intensity of the Th1-response (higher T-bet transcription) elicited by the fully allogeneic mismatch graft, which has a negative regulatory effect on Th17-cell development (11). In aggregate, these findings suggest that hCTLA4-Ig might promote Th17 differentiation; however, neutralization of IL-17 was not capable of delaying rejection, questioning the pathogenic role of this T-subtype in transplantation. Additional investigation, including analysis of Th17 cells in rejecting allografts in the belatacept-treated groups, is warranted.

A Th2 response has been proposed to be protective in some transplant models, although Th1 cells have been linked to acute graft rejection (27,28). Despite the increase in Th2 cytokines in recipients of bm12 grafts treated with hCTLA4-Ig, an accelerated rejection was observed. This is in agreement with other reports where an increased Th2 response was also capable of producing graft rejection in certain settings (29–31).

In addition to blocking B7:CD28 interaction, CTLA4-Ig also interferes with B7 interaction with CTLA4. CTLA4 is an indispensable negative regulator of peripheral T-cell function and it binds with much higher affinity to B7.1/B7.2 ligands than CD28 (10–20×) and preferentially to B7.1 (32). In transplantation, CTLA4 engagement has been shown to be required for the induction of peripheral T-cell tolerance (33). In alloimmunity, we have also previously shown that anti-CTLA4 Ab can break tolerance by precipitating severe rejection (34). By blocking B7.1 and B7.2 ligands, CTLA4-Ig not only interferes with activation of naïve T cells through CD28, but it also affects CTLA4 signaling, which is required for the suppressive function of Tregs (35). Indeed, Treg-specific CTLA4 deficiency results in the development of autoimmune diseases and systemic lymphoproliferation (35). Nonetheless, blockade of B7:CD28 with hCTLA4-Ig did not significantly affect Treg function *in vitro* (Figure 2C), suggesting that complementary mechanisms might preserve Treg suppressive function in physiologic conditions (wild-type setting).

A limitation of using the bm12 into B6 model is related to the degree of MHC class II disparity between the B6 wild-type and the bm12 mouse, because they differ solely by 3 amino acids in the the hypervariable region of Aβ chain (36). Therefore, observations on this model cannot necessarily be translated to other MHC class II disparities, in which the differences in the MHC molecules might lead to higher immunogenicity. Nevertheless, the key role of Tregs in transplant tolerance in this model creates an interesting platform to study immune regulation. Other models, like the minor mismatch 129 into B6, do not carry such a dependence on Tregs for graft survival and consequently, are not negatively affected by hCTLA4-Ig.

Taken together, this study highlights the potential consequences of blockade of the B7:CD28 pathway with CTLA4-Ig on natural Treg homeostasis and Th17 suppression in alloimmunity, in particular in the setting of a MHC class II mismatch transplant model. Our results are clinically relevant, as prolonged and intensive B7:CD28 blockade appears to have a deleterious effect on Tregs' development.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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