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# Jagged2-signaling promotes IL-6-dependent transplant rejection

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**The Notch pathway is an important intercellular signaling pathway that plays a major role in controlling cell fate. Accumulating evidence indicates that Notch and its ligands present on antigen-presenting cells might be important mediators of T helper cell differentiation. In this study, we investigated the role of Jagged2 in murine cardiac transplantation by using a signaling Jagged2 mAb (Jag2) that activates recombinant signal-binding protein-Jκ. While administration of Jag2 mAb had little effect on graft survival in the fully allogeneic mismatched model BALB/c→B6, it hastened rejection in CD28-deficient recipients. Similarly, Jag2 precipitated rejection in the bm12→B6 model. In this MHC class II-mismatched model, allografts spontaneously survive for >56 days due to the emergence of Treg cells that inhibit the expansion of alloreactive T cells. The accelerated rejection was associated with upregulation of Th2 cytokines and proinflammatory cytokine IL-6, despite expansion of Treg cells. Incubation of Treg cells with recombinant IL-6 abrogated their inhibitory effects in vitro. Furthermore, neutralization of IL-6 in vivo protected Jag2-treated recipients from rejection and Jagged2 signaling was unable to further accelerate rejection in the absence of Treg cells. Our findings therefore suggest that Jagged2 signaling can affect graft acceptance by upregulation of IL-6 and consequent resistance to Treg-cell suppression.**

**Keywords:** Mice · Notch signaling · Regulatory T (Treg) cells · Rejection · Transplantation

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## Introduction

CD4$^+$ T helper cells (Th) play a central role in transplantation by orchestrating and regulating the adaptive alloimmune response. After encountering specific antigen, naïve CD4$^+$ T cells become activated and differentiate into various T-cell subtypes, such as T helper type 1 (Th1), Th2, Th17, or Treg cells [1]. The type of differentiation is determined by signals delivered by antigen-presenting cells (APCs) and the cytokine microenvironment [2]. Accumulating evidence indicates that Notch and its ligands on APCs might be important mediators of Th-cell differentiation [3–7].

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The Notch pathway is an important intercellular signaling pathway that plays a major role in controlling cell fate [3,5]. The Notch family consists of four highly conserved receptors (Notch1, 2, 3, and 4), which can interact with at least five Notch ligands (Jagged1 and 2; Delta1, 3, and 4). Within the immune system, the ligands are mainly expressed on APCs, whereas the Notch receptors are expressed on T cells [4,5,8]. At the molecular level, Notch is a heterodimeric surface receptor consisting of an extracellular ligand-binding region noncovalently associated with a transmembrane polypeptide with a long intracellular tail, which is cleaved during activation. This intracellular domain then translocates into the nucleus where it forms a complex with the ubiquitously expressed DNA-binding protein, recombinant signal-binding protein-J (RBP-J) that activates the expression of target genes [9]. Among the genes important for peripheral T-cell activation and differentiation, Notch has been shown to regulate the expression of nuclear factor-κB, T-bet, GATA-3, PI3K, ROR-γt, IL-6, and the interleukin-4 enhancer CNS2 [3,10–12].

Notch signaling during lymphoid development has been extensively studied [13, 14], however a role for Notch signaling during peripheral T-cell activation and differentiation has only been recently identified [3,15]. As an example, Delta1 expression on APCs was shown to promote differentiation of naïve $CD4^+$ T cells into the Th1-cell lineage, while Jagged1 and Jagged2 expression lead to Th2-cell differentiation [4]. Research from our lab revealed that Delta1 blockade was capable of prolonging graft survival in a fully allogeneic model when combined with B7/CD28 blockade by inhibiting alloreactive Th1 cells and cytotoxic T-cell generation [16]. However, the influence of Jagged-Notch signaling on Th-cell differentiation in the transplant setting, and the consequent effect on allograft outcome, remains to be determined. In this study, we examined the role of Jagged2 in murine cardiac transplantation. A Jagged2 signaling Ab (HMJ2–1) precipitated rejection of bm12 allografts and led to upregulation of IL-6 and Th2 cytokines. Using a neutralizing anti-IL-6 Ab or IL6KO recipients, we further showed that IL-6 was a major mediator of this accelerated rejection and Treg-cell depletion abrogated the precipitating effect of Jagged2 signaling. Our findings suggest an important role of Jagged2 signaling in breaking tolerance and precipitating rejection in alloimmunity.

## Results

### Jagged2 is upregulated on antigen-presenting cells (APCs) after transplantation

First, we examined the expression of Jagged2 on immune cells in naïve and cardiac transplant recipients both in acute rejection (BALB/c into B6) and in a chronic rejection model (bm12 into B6). Flow cytometric analysis using a biotinylated anti-Jagged2 mAb (HMJ2–1) revealed that Jagged2 was predominantly expressed on APCs, especially on dendritic cells ($CD11c^+$) and monocyte/macrophages ($CD11b^+$ $CD11c^-$) of naïve B6 mice (Fig. 1). Moreover, Jagged2 expression was significantly upregulated 7 days after transplantation in the recipients of bm12 hearts ($p < 0.01$), while it was only slightly increased in the rejecting recipients (Fig. 1). There was minimal expression of Jagged2 on $CD4^+$ T cells (0.86% ± 0.59), $CD8^+$ T cells (0.79% ± 0.28), and $Foxp3^+$ regulatory $CD4^+$ cells (0.36% ± 0.11). These flow cytometric findings were also confirmed by real-time PCR of MACS-sorted $CD11c^+$ and $CD11b^+$ cells (data not shown).

### Jagged2 signaling significantly accelerates cardiac allograft rejection

In order to explore the importance of Jagged2 in transplantation, we used a signaling Jagged2 mAb (HMJ2–1), which has been shown to increase Jagged2-Notch signaling through activation of RBP-Jκ [17]. Jagged2 mAb did not affect allograft survival in the BALB/c→B6 full MHC-mismatch model; all grafts were acutely rejected in both treated and control mice (Fig. 2A). In contrast, use of Jagged2 mAb significantly accelerated rejection in CD28-deficient B6 recipients, in which the lack of B7:CD28 costimulation results in a delay of graft rejection (Fig. 2B). Last, we tested this mAb in an MHC class II mismatched model where bm12 hearts are transplanted into B6 WT recipients [18–21]. In this transplant model, allografts spontaneously survive for >56 days due to the emergence of Treg cells that inhibit the expansion of alloreactive T cells [18,20,21]. While bm12 grafts in the control IgG group survived longer than 56 days ($n = 8$), cardiac grafts in the Jagged2-treated group showed an accelerated rejection ($n = 8$, MST = 35, $p = 0.0042$) (Fig. 2C). When analyzed 5 weeks after transplantation, allografts in the Jagged2-treated group demonstrated marked vasculopathy and fibrosis, and had higher rejection scores compared with those of the control group (Fig. 3), suggesting an aggressive alloimmune response. Jagged2 antibody had no effect on syngeneic B6-into-B6 transplant model, in which all grafts survived longer than 60 days (Supporting Information Fig. 1).

### Jagged2 antibody expands Treg cells

We chose the bm12 into B6 model to further investigate the mechanism of the accelerated rejection, since allograft survival in this model is dependent on immunoregulation by Treg cells [21]. First, we measured the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells, $CD4^+$, and $CD8^+$ effector/memory T cells in the spleens of recipients 7–14 days after transplantation. Surprisingly, despite an accelerated rate of rejection in the Jagged2-treated group, the number of Treg cells was increased compared with the control group (Fig. 4A). Even though the percentage of $CD4^+$ effector/memory (eff/mem) cells was also slightly expanded, there was no significant difference in the absolute CD4 eff/mem cell count between the two groups (Fig. 4B). In addition, the percentage of CD8 effector/memory cells was no different between groups (9.00 ± 0.68 and 10.82 ± 1.27; $p = 0.27$). As Jagged ligands have been involved in the activation of other cell types [22], we measured the percentage of dendritic cells, B cells, neutrophils,

 

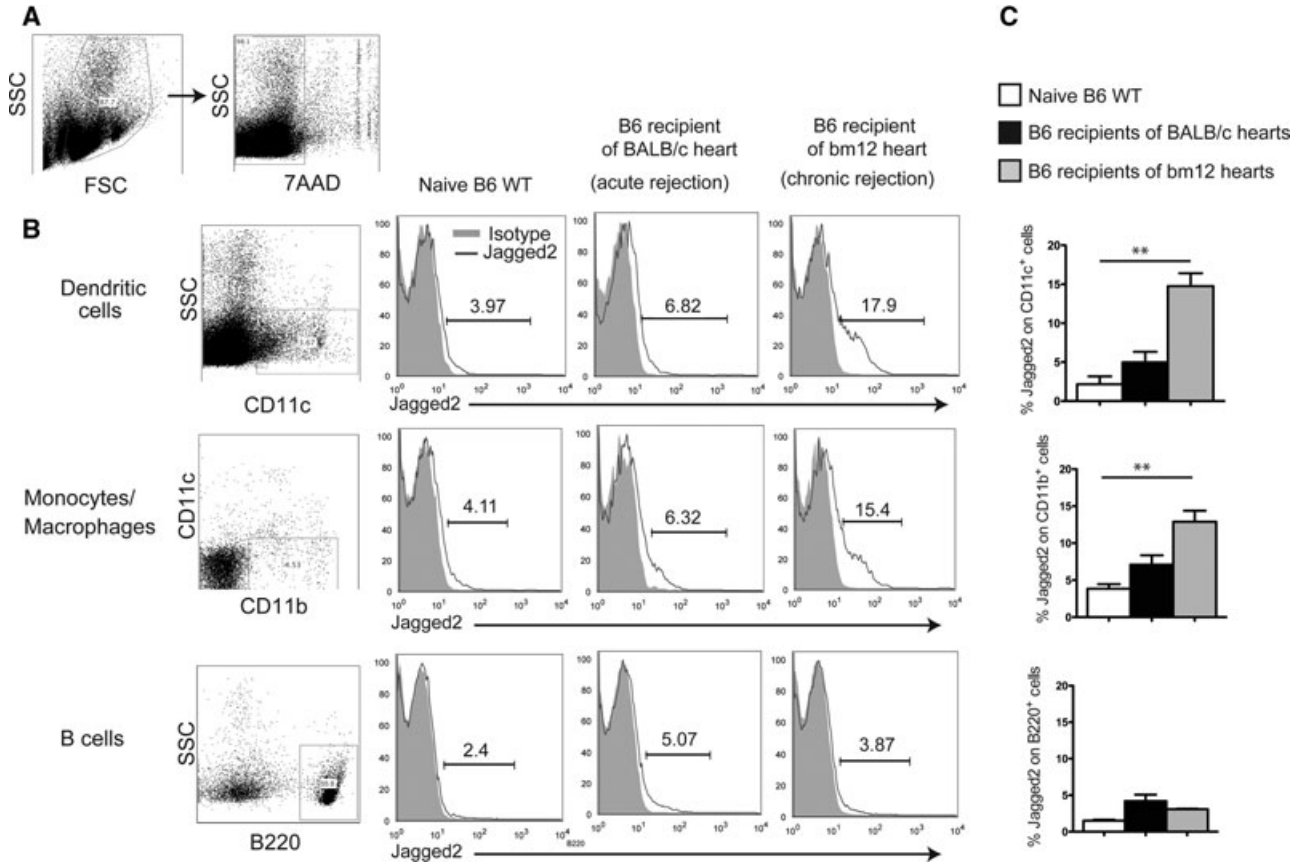


**Figure 1.** Expression of Jagged2 on immune cells of naïve and transplanted animals (Tx). (A) Representative dot plots of gating strategy to exclude dead cells by 7AAD staining. (B) Representative dot plots and histograms of Jagged2 expression on different cell subtypes from splenocytes of naïve B6 mice, recipients of BALB/c or bm1 2 allografts 7 days after transplantation. Biotinylated anti-Jagged2 Ab (HMJ2–1) or IgG control was used in combination with allophycocyanin-Streptavidin. Data shown are representative of two experiments performed. (C) The percentage of Jagged2 expression on different APCs is shown. Data are shown as mean + SEM of 6 per group and are from one experiment representative of two experiments performed. $^{**}p < 0.001$, Mann–Whitney nonparametric test.

monocyte/macrophages, and NK cells in both groups but found no significant differences among them (Fig. 4C). Finally, we evaluated the expression of activation markers on dendritic cells in order to evaluate if Jagged2 mAb had any effect in the maturation of these cells with consequent effects on T-cell activation [23]. We specifically measured the expression of CD80, CD86, and PDL1 on CD11c$^+$ cells, which showed similar percentages in the control and Jagged2-treated groups at both 7 and 14 days after transplantation (data not shown).

## Jagged2 signaling causes a marked increase in Th2 cytokines and IL-6

Since ectopic expression of Jagged ligands on DCs have been shown to induce Th2-cell differentiation in vitro [4], we assessed the effect of our signaling Jagged2 mAb on the cytokine signature of splenocytes from B6 recipients of bm12 allografts 2 weeks after transplantation by ELISPOT and Luminex. Indeed, the frequency of alloreactive T cells producing Th2 cytokines (IL-4, IL-5, and IL-13) were markedly increased in the Jagged2-treated group compared to controls ($p < 0.0001$) (Fig. 5). Moreover, IL-6 production was also significantly upregulated (Fig. 5A, $p = 0.0002$). Conversely, the frequency of IFN-γ-producing cells was low and only mildly elevated in the Jagged2 group (Fig. 5A). Other upregulated cytokines included MCP-1 (405 ± 108 versus 902 ± 114 pg/mL, $p = 0.0071$) and IL-10 (24.64 ± 4.41 versus 164.3 ± 16.35 pg/mL, $p < 0.0001$), while the levels of IL-17 (Fig. 5B), IL-2, TNF-α, and IL-9 were not different among groups (data not shown).

Since CD4$^+$ cells play a major role in the alloimmune response in this cardiac transplantation model, we decided to confirm the source of Th2 and IL-6 cytokines by isolating CD4$^+$ and CD8$^+$ cells from recipients of control- and Jagged2-treated groups and stimulating them with irradiated donor-type splenocytes. We observed a similar significant upregulation of IL-4 and IL-6 production by CD4$^+$ cells in the Jagged2-treated group (Fig. 6A), while as expected, IFN-γ was not significantly increased (103.8 ± 9 and 177 ± 27; $p = 0.12$) when compared to controls. Cytokine production by CD8$^+$ cells was not different between groups (Fig. 6A). Furthermore, CD4$^+$ cells isolated from the Jagged2-treated group demonstrated a marked increase in GATA-3 and IL-6 expression by real-time PCR (Fig. 6B), transcription factor, and cytokine, respectively, known to be regulated by the Notch pathway [4, 12]. Taken together, these results suggest that

 

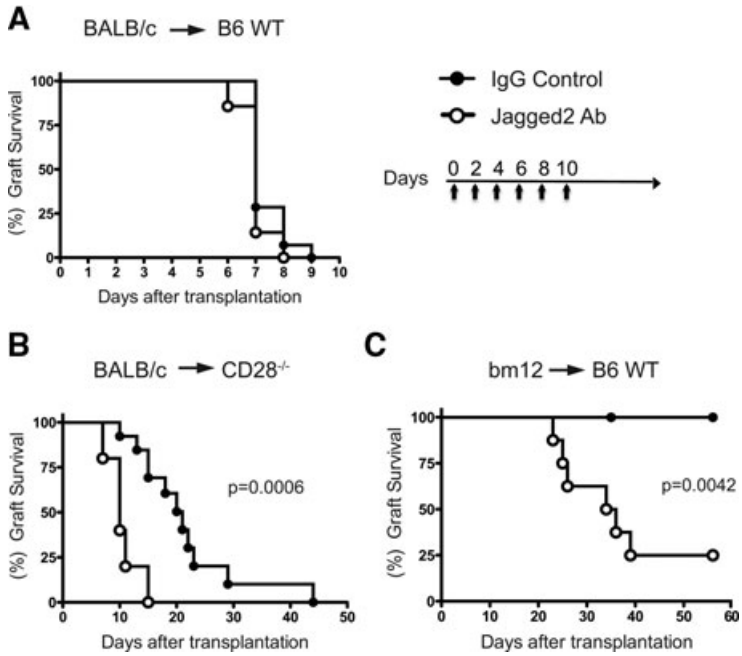


**Figure 2.** Effect of Jagged2 Ab on the survival of fully MHC-mismatch and class II-mismatch cardiac allografts. Hearts from BALB/c mice were transplanted into (A) B6 WT or (B) CD28$^{-/-}$ recipients, which were treated with Jagged2 Ab or control hamster IgG ($n$ = 6–10 on each group). (C) Hearts from bm12 mice were transplanted into B6 WT recipients, which were either treated with Jagged2 Ab or control IgG as above ($n$ = 8 on each group). The frequency of administration of mAb is depicted by arrow, illustrating days after transplantation.

Jagged2 mAb was able to preferentially upregulate Th2 cytokines and IL-6 in CD4$^+$ T cells.

## IL-6 neutralization prevents accelerated rejection resulting from Jagged2 mAb administration

Since Treg cells play a major role in graft acceptance on the bm12 into B6 transplant model [18, 20], we decided to investigate if the pro-inflammatory IL-6 could be the potential culprit of the accelerated rejection in this model [24, 25]. While the function of the Treg cells from both groups was similar when incubated with activated CD4$^+$ T cells in vitro (Fig. 7A), the addition of recombinant IL-6 significantly affected the inhibitory function of Treg cells in vitro (Fig. 7B). We then decided to neutralize IL-6 in our in vivo model in order to assess if accelerated rejection by Jagged2 Ab could be reversed. The administration of anti-IL-6 to B6 recipients treated with Jagged2 Ab was able to prevent the accelerated allograft rejection seen with Jagged2 Ab alone; all grafts survived for more than 8 weeks ($n = 6, p < 0.001$) (Fig. 7C). Furthermore, IL6KO recipients treated with Jagged2 Ab demonstrated prolonged graft survival (MST>56 days) (Fig. 7C), reinforcing our findings with the neutralization antibody approach. Last, Jagged2 Ab was unable to further accelerate rejection in the absence of CD25$^+$ Treg cells (Fig. 7D), suggesting a key role of IL-6 induction by Jagged2 mAb in promoting resistance to Treg-cell suppression and precipitating rejection in this Treg-cell-dependent cardiac transplant model.

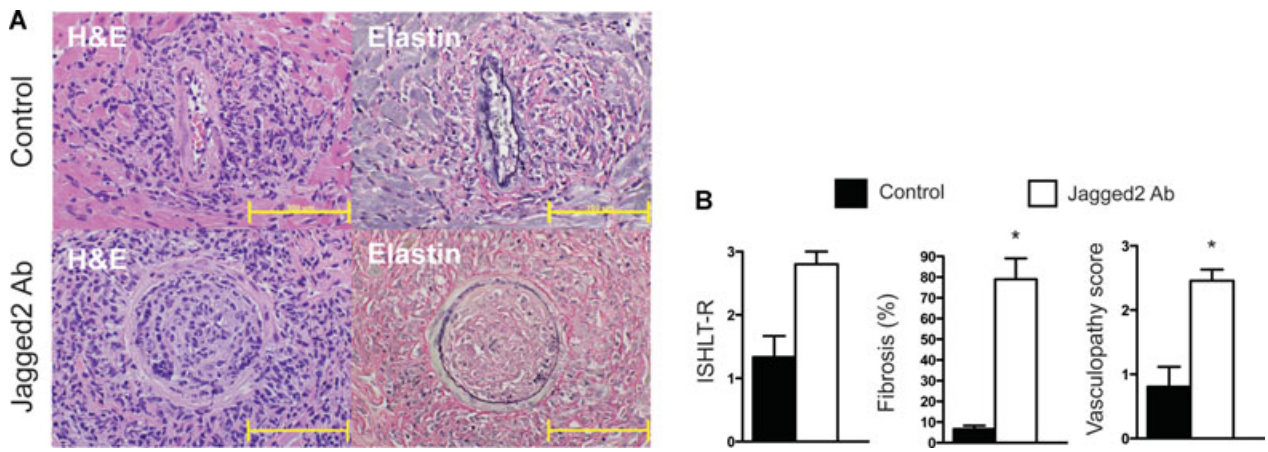


**Figure 3.** Histopathological findings of bm12 allografts in WT B6 recipients after Jagged2 Ab administration. (A) Representative photomicrographs of H&E and Elastin staining demonstrating the degree of vasculopathy on Jagged2-treated group compared with that of controls 5 weeks after transplantation. Scale bars, 100 μm. Data shown are representative of five samples evaluated. (B) At a similar time point, grafts were scored according to rejection, fibrosis, and vasculopathy. Data are shown as mean + SEM of 5 per group, *$p < 0.001$, Mann–Whitney nonparametric test.

 

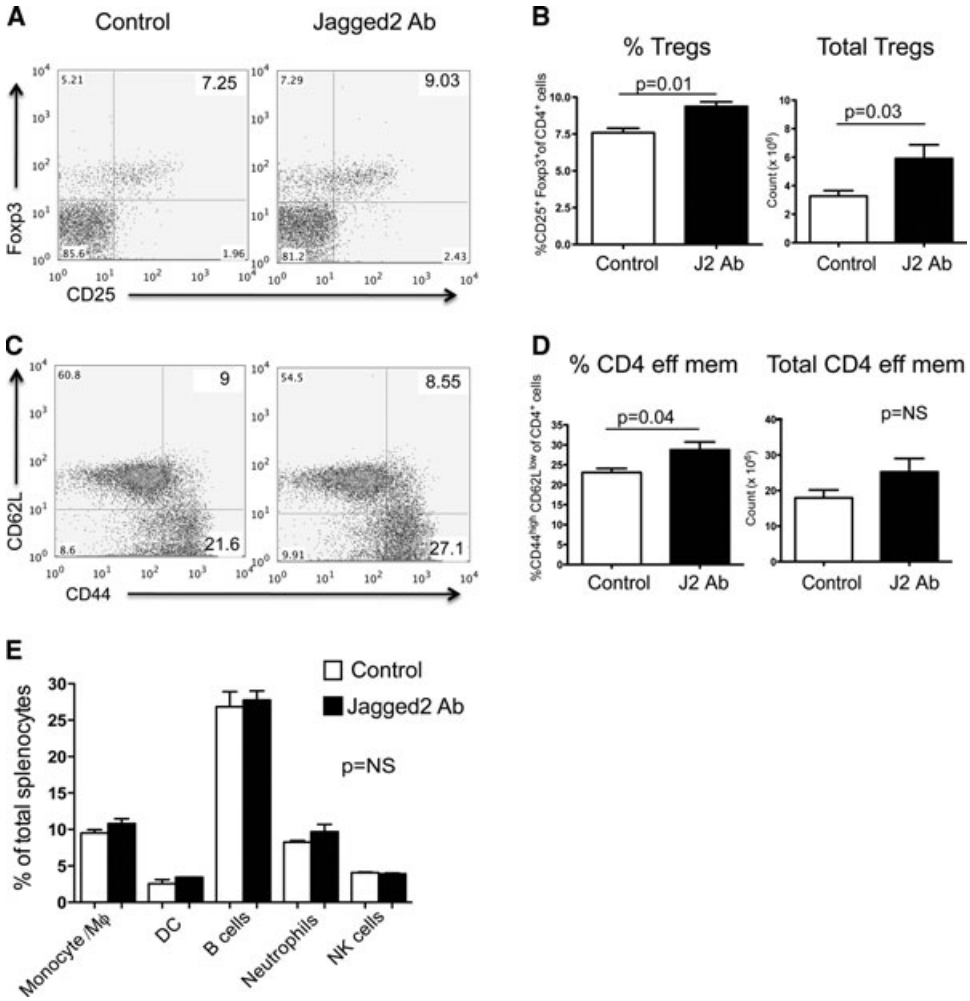


**Figure 4.** Effect of Jagged2 Ab on Treg cells and CD4$^+$ effector/memory cells. (A) Representative flow cytometry dot-plots of Treg cells (CD25$^+$Foxp3$^+$ of CD4$^+$ gated cells) from splenocytes of B6 WT recipients of bm12 hearts 14 days after transplantation. (B) The percentage and absolute count of Treg cells in IgG control and Jagged2-treated group is shown as mean +SEM of 4–5/group and are from one experiment representative of three experiments performed. (C) Representative flow cytometry dot-plots of CD4$^+$ effector memory cells (CD44$^{high}$CD62L$^{low}$ of CD4$^+$-gated cells) and (D) percentage and absolute count of CD4$^+$ effector/memory cells at same time point are shown as mean +SEM of 4–5/group. The absolute count was calculated by multiplying the total cell yield by the percentage of either Treg cells or CD4$^+$ effector/memory cells. (E) Percentage of other immune cells was determined on both groups by staining splenocytes with CD11b (monocyte/macrophage), CD11c (dendritic cells), B220 (B cells), Gr1 (neutrophils) and NK1.1 (NK cells) markers ($n = 4$–5/group). Data shown are from one experiment representative of three independent experiments performed.

## Discussion

Despite the accumulating evidence for the role of Notch signaling in T-cell differentiation [3–6], its importance in the transplant setting remains unclear. The various Notch ligands are induced on APCs by diverse environmental and microbial signals. While Delta1 and Delta4 ligands are induced on dendritic cells after exposure to bacteria, TLR ligands, or LPS [4, 26], Jagged1 and Jagged2 ligands are usually upregulated under Th2-cell-promoting stimuli, like in exposure to allergens or *Schistosoma mansoni* egg antigen [4, 12, 27–29]. This upregulation was shown to be dependent on c-Kit [12]. In this study, we demonstrated that the Notch ligand Jagged2 is predominantly expressed on dendritic cells and macrophages (Fig. 1), in agreement with prior published work on Notch ligands [8, 28, 30]. Moreover, Jagged2 expression was significantly upregulated after transplantation in B6 recipients of surviving bm12 allografts (Fig. 1) when compared with the BALB/c-into-B6 acute rejection model. This finding complements our prior work, in which Delta1 was the predominant Notch ligand upregulated during acute rejection [16]. The specific mechanism controlling Notch ligand upregulation and what elicits the difference in the two transplant models remains to be determined.

There is convincing data indicating that ectopic expression of Jagged ligands by artificial APCs can result in Th2-cell differentiation in vitro [4], while Jagged2$^{-/-}$ DCs are impaired in their ability to induce Th2 polarization in vitro [31]. Moreover, a stimulatory Jagged-Fc fusion protein can promote Th2-cell responses in the mouse model of experimental autoimmune encephalomyelitis (EAE), decreasing disease activity [32]. In contrast, the suppression of Jagged2 expression by small interfering RNA failed

 

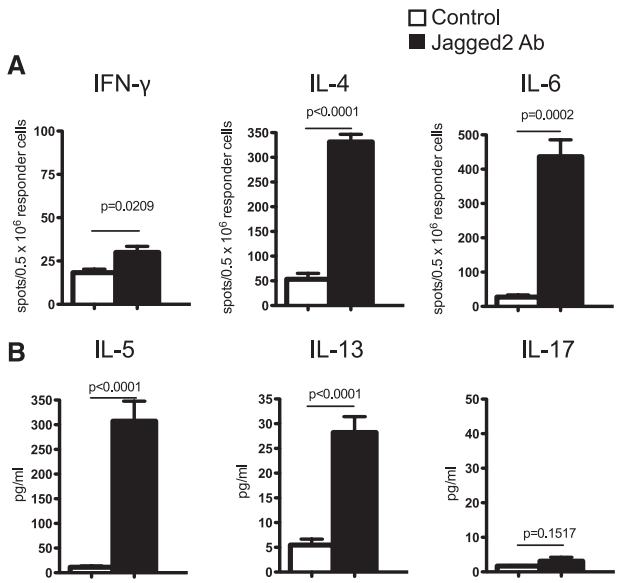


**Figure 5.** Cytokine production from splenocytes after allostimulation in vitro. Th1, Th2, and proinflammatory cytokines production by splenocytes of bm12 allograft recipients were assessed by (A) ELISPOT and (B) Luminex assay after in vitro stimulation with donor-irradiated splenocytes. Data are shown as mean + SEM of 4–5/group and are from one experiment representative of three experiments performed; Mann–Whitney nonparametric test.

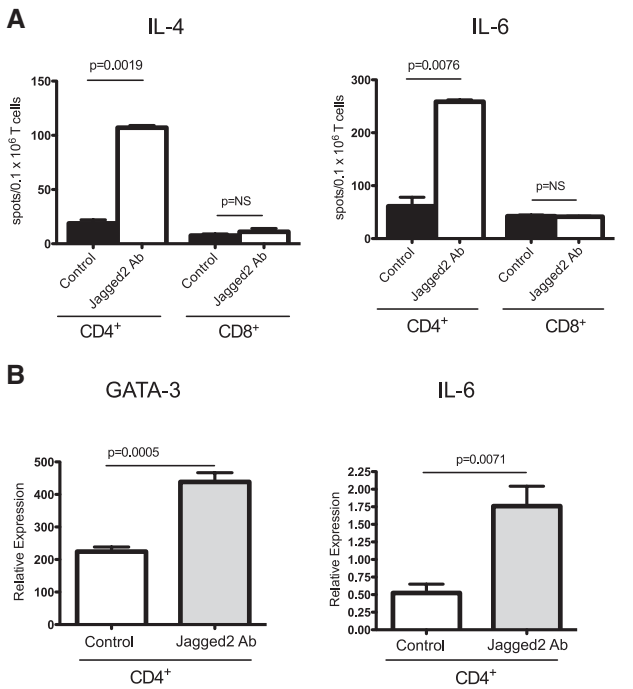


**Figure 6.** Cytokine production from CD4$^+$ and CD8$^+$ T cells after allostimulation in vitro. (A) CD4$^+$ and CD8$^+$ cells were isolated from splenocytes of B6 WT recipients of bm12 allografts 2 weeks after transplant and then stimulated with irradiated donor-type splenocytes for 48 h. The frequency of IL-4- and IL-6-producing alloreactive T cells was then determined by ELISPOT. (B) Prior to stimulation, some CD4$^+$ T cells were also processed for real-time PCR and evaluated for the expression of GATA-3 and IL-6. Data are shown as mean + SEM of triplicates and are from one experiment representative of two performed experiments.

to affect the ability of DCs to induce a Th2 phenotype, questioning the requirement of Jagged2 for a Th2 response [28]. Similarly, the presence of high amounts of IL-4 was able to override the requirements of Notch ligation to generate Th2 cells in vitro [15, 29, 33, 34]. However, since IL-4 is genetically downstream of Notch, the addition of exogenous IL-4, as occurs in traditional cytokine-mediated differentiation experiments, can overcome the requirement for Notch in Th2-cell differentiation and might explain some of the disparate conclusions [6, 35]. Nonetheless, the ability of Jagged signaling to promote Th2 differentiation has been linked to its direct regulation of *Gata3* expression, a key transcriptional regulator of Th2 polarization [11, 34], as well as to IL-4 transcription [36]. This has been shown to be independent of IL-4 receptor signaling [4].

We hypothesized that promoting Th2-cell differentiation in vivo would lead to prolongation of allograft survival in a full MHC-mismatched model. Our expectation of the "Th2" beneficial effect was based on prior observations suggesting that a Th1 response is the dominant phenotype in allograft rejection, while a Th2 response favors long-term survival in some transplant models [16,37–40]. Paradoxically, treatment with Jagged2 Ab accelerated allograft loss (Fig. 2B and C), despite a significant upregulation of Th2 cytokines (Fig. 5). It has been suggested that activation of the alloimmune response through the indirect pathway of antigen presentation preferentially leads to a Th2 response with an associated increase in alloantibody production, which may in turn, manifest as chronic rejection [41–43]. However, there is no evidence of antibody production in the bm12 into B6 model due to the close homology of the I-A$^b$ and I-A$^{bm12}$ molecules [18].

Treg cells have been shown to suppress the consequences of pathogenic effector T cells in the transplant setting [44, 45]. More specifically, the emergence of Treg cells in the bm12 into B6 MHC class II mismatched cardiac transplant model promotes long-term allograft survival through suppression of effector T cells [18, 20]. We have previously shown that by depleting Treg cells or reducing their generation, bm12 allografts are rapidly rejected in this model [20]. With regards to the Notch pathway, endogenous expression of Jagged2 by hematopoietic progenitors cells promoted Treg expansion in vivo, preventing autoimmune diabetes in NOD mice [46]. In our model, despite accelerated allograft rejection, the number of splenic Treg cells was increased after treatment with the Jagged2 Ab (Fig. 4A). Corroborating findings of Treg expansion were also seen in the EAE model using similar Jagged2 signaling Ab [17]. In transplantation, Treg expansion is reported to occur both in the setting of tolerance and acute rejection, and, in the latter, is most likely as a result of a "damage control response" [47, 48]. It is also worth noting that the expansion of Treg cells in Jagged2-treated recipients was small and by itself probably not enough to ensure protection from the aggressive alloimmune response.

The acceleration of cardiac graft rejection was also associated with higher IL-6 production. Interleukin-6 has been increasingly recognized to have potent effects in transplantation, with the capability of precipitating rejection [25,49–51]. Moreover, IL-6 in combination with TGF-β has been shown to promote differentiation of

 

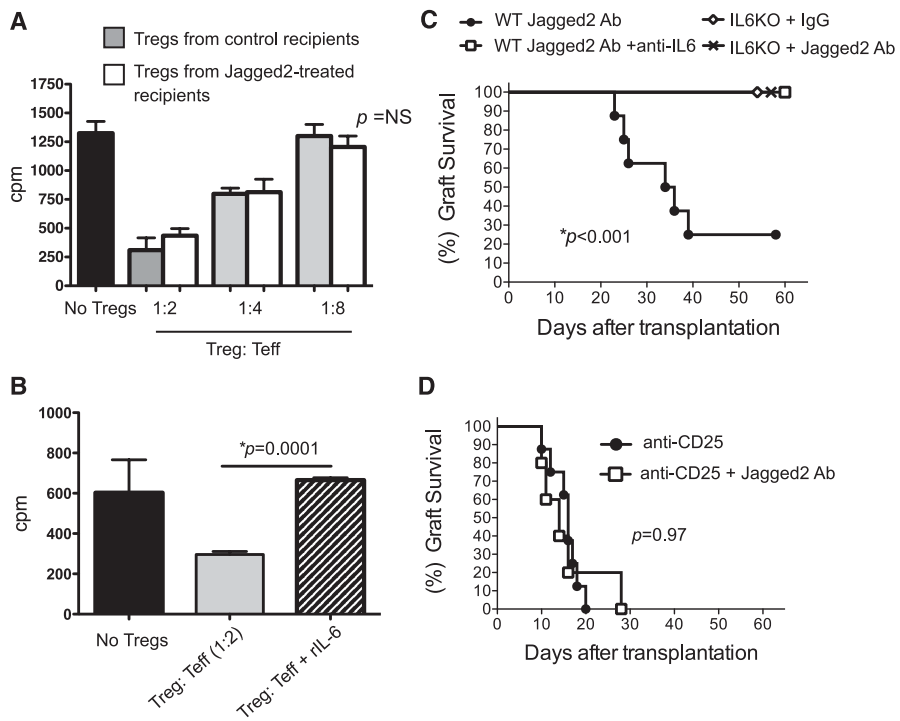


**Figure 7.** IL-6 and Treg-cell function upon Jagged2 Ab administration. (A) Isolated $CD4^+CD25^+$ Treg cells from recipients of IgG control or Jagged2 Ab 14 days after transplantation were incubated ex vivo with $CD4^+$ cells (Teff) and stimulated with irradiated allo-splenocytes for 72 h. The suppressive function was measured by the proliferation of $CD4^+$ cells in the presence of Treg cells at different ratios (1:0, 1:2, 1:4, 1:8). (B) Recombinant IL-6 was added to in vitro co-culture of Treg cells and Teff cells, in order to assess Treg-cell suppressive function. Data are shown as mean + SEM of triplicates and are from one experiment representative of three performed experiments. (C) Survival of bm12 allografts in WT recipients treated with anti-IL-6 Ab and Jagged2 Ab or in IL-6-deficient recipients (IL6KO) treated with Jagged2 Ab or IgG control ($n$ = 6 on each group, $p < 0.001$, *log-rank* test). (D) Survival of bm12 grafts in Treg-cell-depleted B6 WT recipients with or without Jagged2 mAb is shown ($n$ = 5–8, $p$ = 0.97, *log-rank* test). Anti-CD25 mAb (PC61) was administrated on days 6, and 1 before transplantation. (C and D).

naïve Th cells into Th17 cells and precipitate rejection in T-bet-deficient recipients that lack a type 1 T-cell response [52, 53]. Prior work has shown that interleukin-6 transcription could be activated by the Jagged2-Notch-signaling pathway through the binding of the Notch intracellular domain to CBF1/RBP-Jκ, which then becomes a transcription activator of numerous genes, including NFκB and IL-6 [10, 54]. Treatment with the Jagged2 signaling antibody did indeed significantly upregulate the production (Fig. 5) and transcription of IL-6 (Fig. 6B). In contrary to observed increase in Th17 cells upon Jagged2 signaling in the EAE model [17], IL-17 production was not affected by Jagged2 treatment in our transplant model (Fig. 5B). A possible explanation for our findings is that IL-6 was rendering T effector cells resistant to Treg suppression [24].

While Treg cells from control and anti-Jagged2 groups were equally functional in vitro (Fig. 7A), incubation of Treg cells with recombinant IL-6 abrogated their inhibitory effects in vitro (Fig. 7B), indicating a possible role of IL-6 in precipitating rejection in vivo. Corroborating with this hypothesis, treatment with anti-IL-6 Ab was able to reverse the accelerated rejection caused by administration of Jagged2 Ab. Additionally, IL6KO recipients had prolonged allograft survival despite administration of Jagged2 Ab (Fig. 7C), while depletion of Treg cells abolished the accelerated rejection effect of Jagged2 Ab (Fig. 7D), further suggesting the important role of IL-6 in Treg-cell function in this model. One proposed hypothesis is that IL-6 affects Treg-cell function through a decrease in Foxp3 transcription by inducing methylation of an upstream Foxp3 CpG island enhancer [55] or acetylation of Foxp3 protein [56]. Nonetheless, IL-6 could also have an important effect directly on effector T cells, promoting activation and proliferation that overcomes Treg inhibitory function [25]. One limitation of our study is the inability to prove that graft acceptance after IL-6 neutralization is exclusively related to the reestablishment of Treg-cell inhibitory function over T effector cells, and not through other potential effects of IL-6 blockade [57].

Taken together, our results indicate that Jagged2-signaling at the time of transplantation influences the pattern of cytokines produced by alloreactive T cells, with the capability of increasing Th2-cytokines and pro-inflammatory IL-6. In the MHC class II mismatched model, the abrogation of graft acceptance by signaling Jagged2 Ab likely results from Treg malfunction by IL-6 upregulation. These findings highlight for the first time the danger of inflammatory signals induced by Jagged2 signaling in breaking tolerance and precipitating rejection in alloimmunity.

## Materials and methods

### Mice

C57BL/6 ($H2^b$, B6), BALB/c ($H2^d$), CD28-deficient mice on B6 background (B6.129S2-*Cd28*$^{tm1Mak}$/J), B6.C-$H2^{bm12}$/KhEgJ (bm12), and B6.129S6-*Il6*$^{tm1Kopf}$ (IL-6 knockout on B6 background) mice were purchased from The Jackson Laboratory. All mice were 8–12 weeks of age and housed in accordance with institutional and NIH guidelines.

### Heterotopic heart transplantation

Vascularized heart grafts were placed in an intra-abdominal location using microsurgical techniques as described by Corry et al. [58]. 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 the median survival time (MST) in days.

## Antibodies and in vivo treatment protocol

Jagged2 Ab (HMJ2–1) mAb was generated as previously described [30]. These mAbs were manufactured and purified from the original hybridomas by a commercial source, BioXCell (West Lebanon, Lebanon, NH, USA). Control hamster IgG was also obtained from the same source. Cardiac allograft recipients were treated with mAbs ip at 500 μg at day 0, and 250 μg at days 2, 4, 6, 8, and 10 after transplantation. For the IL-6 depletion studies, B6 WT recipients were treated with 100 μg of anti-IL-6Ab ip (MP5–20F3; eBioscience) on the day of transplantation followed by days 1, 2, 3, 7, 10, and 14. Treg-cell depletion was performed with 250 μg rat anti-mouse CD25 mAb (PC61) at days -6, and -1 before transplantation [39].

## Measurement of cytokines by ELISPOT and Luminex assay

Splenocytes harvested at 14 days after transplantation from B6 WT recipients of bm12 heart allografts treated with hamster control IgG or Jagged2 Ab were restimulated by irradiated donor-type splenocytes in vitro. The ELISPOT assay (R&D Systems, Minneapolis, MN, USA) was adapted to measure the frequency of alloreactive T cells producing IFN-γ, IL-4, and IL-6, as described previously [20]. The frequencies of cytokine-secreting alloreactive cells were expressed as the number of cytokine-producing cells per $0.5 \times 10^6$ responder cells. For Luminex assay, cell-free supernatants of individual wells were removed after 48 h of incubation and analyzed by a multiplexed cytokine bead-based immunoassay using a preconfigured 21-plex mouse cytokine detection kit (Millipore) as described previously [52]. All samples were tested in triplicate wells. In addition to whole splenocytes, MACS-sorted $CD4^+$ and $CD8^+$ cells were also restimulated with irradiated donor-type splenocytes in order to elucidate the source of cytokine production (130–090–860 and 130–049–401; Miltenyi Biotec).

## Flow cytometry

Splenocytes from B6 WT recipients of bm12 hearts at 14 days after transplantation were stained with fluorochrome-labeled mAbs against CD4, CD8, NK1.1, CD11b, CD11c, B220, Gr1, CD62 ligand (CD62L), CD44, CD25, CD80, CD86, PDL1, 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 an FACSCalibur system (BD Biosciences) and analyzed using FlowJo software. In order to characterize Jagged2 expression, we first incubated splenocytes from naïve and transplanted animals 7 days after transplantation with anti-mouse CD16/CD32 purified (BD Biosciences) to block nonspecific binding to FcγR. Then cells were incubated with Armenian Hamster biotinylated anti-mouse Jagged2 Ab (HMJ2–1), generated as previously described [30], and control Hamster biotinylated IgG (eBioscience) for 15 min on ice. After washing the cells, APC Streptavidin was added. Washed cells were also stained for 7-AAD (BD Pharmingen) to exclude nonviable cells from flow cytometric analysis.

## Morphology

Cardiac graft samples from transplanted mice were harvested from rejecting (cessation of heartbeat by palpation) and long-term survivors (>56 days), then fixed in 10% formalin, embedded in paraffin, coronally sectioned, and stained with hematoxylin/eosin and elastin for evaluation of the degree of rejection according to International Society of Heart and Lung Transplantation guidelines [59] and for cellular infiltration and vasculopathy by light microscopy [60, 61]. An examiner blinded to the groups read all the samples (VV).

## In vitro suppression assays.

To assess the regulatory function of Treg cells in vitro, an MLR assay was set up in which $CD4^+$ T cells ($0.1 \times 10^6$) from B6 WT recipients of bm12 allografts were used as responder cells to irradiated bm12 splenocytes. Subsequently, $CD4^+CD25^+$ T cells from both IgG-treated and Jagged2-treated groups were added to each well at various ratios for 96 h (1:2, 1:4, 1:8, and 1:16). 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 Treg cells was assessed by the suppressive effect on the proliferation of responder cells. $CD4^+$ T cells and $CD4^+CD25^+$ T cells were isolated from splenocytes by magnetic activated sorting by a $CD4^+$ T cell and $CD4^+CD25^+$ Regulatory T Cell Isolation Kits (130–090–860 and 130–091–041, respectively; Miltenyi Biotec). The purity of T cells was estimated to be greater than 95% by FACS.

## Expression analysis by real-time PCR

MACS-sorted $CD4^+$, $CD11c^+$, and $CD11b^+$ cells from splenocytes of recipients treated with IgG control or Jagged2 Ab had their RNA purified using the Stratagene RNA kit and then transferred directly into the RT reagent using the Applied Biosystems TaqMan RT reagents. Samples were subjected to real-time PCR analysis on an Applied Biosystems PRISM 7000 Sequencer Detection System (Applied Biosystems) under standard conditions. Jagged2, GATA-3, and IL-6 were detected using commercially available assays (Applied Biosystems; Mm01325629_m1, Mm00484683_m1, and Mm00446190_m1, respectively). Relative mRNA abundance was normalized against GAPDH (Applied Biosystems). The $CD11b^+$ and $CD11c^+$ cells were isolated from splenocytes by magnetic activated sorting by positive selection with following kits: 130–049–601 and 130–052–001 (by Miltenyi Biotec).

 

### 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.

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*Abbreviation:* **RBP-J**: recombinant signal-binding protein-J

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