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Blockade of Notch Ligand Delta1 Promotes Allograft Survival by Inhibiting Alloreactive Th1 Cells and Cytotoxic T Cell Generation

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The Notch signaling pathway has been recently shown to contribute to T cell differentiation in vitro. However, the in vivo function of Notch signaling in transplantation remains unknown. In this study, we investigated the importance of Delta1 in regulating the alloimmune response in vivo. Delta1 expression was upregulated on dendritic cells and monocytes/macrophages upon transplantation in a BALB/c into B6 vascularized cardiac transplant model. Whereas administration of anti-Delta1 mAb only slightly delayed survival of cardiac allografts in this fully MHC-mismatched model, it significantly prolonged graft survival in combination with single-dose CTLA4-Ig or in CD28 knockout recipients. The prolongation of allograft survival was associated with Th2 polarization and a decrease in Th1 and granzyme B-producing cytotoxic T cells. The survival benefit of Delta1 blockade was abrogated after IL-4 neutralization and in STAT6KO recipients, but was maintained in STAT4KO recipients, reinforcing the key role of Th2 cell development in its graft-prolonging effects. To our knowledge, these data demonstrate for the first time an important role of Delta1 in alloimmunity, identifying Delta1 ligand as a potential novel target for immunomodulation in transplantation. ***The Journal of Immunology*****, 2011, 187: 000–000.**

The adaptive immune system has permitted the development of specific defense mechanisms against a large variety of pathogens and abnormal cells. In transplantation, both $CD4^+$ and $CD8^+$ T cells play a dominant role in cell-mediated immunity against alloantigens and have been shown to be of key importance in allograft rejection (1–3). After Ag recognition, naive $CD4^+$ T cells can differentiate into Th1, Th2, Th17, or regulatory T cells, whereas naive $CD8^+$ T cells can become CTLs. The type of differentiation is determined by signals delivered by APCs and the cytokine microenvironment (4). Accumulating evidence indicates that Notch and its ligands on APCs might be important mediators of T cell differentiation (5–11).

The Notch pathway is an important intercellular signaling pathway that plays a major role in controlling cell fate (5, 7). 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 (6, 7, 12). 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 CSL/RBP-Jκ/MAML/p300, which activates the expression of target genes (7, 13).

Depending on the predominant Notch ligand expressed on APCs, different target genes are activated by Notch signaling. As an example, Delta1-expressing dendritic cells (DCs) have increased ability to activate naive $CD4^+$ T cells and promote Th1 cell development in vitro via upregulation of T-bet and IFN-γ, and inhibition of IL-4R signaling (6, 11). In contrast, Jagged-expressing APCs lead to Th2 cell differentiation via enhancement of GATA3 transcription (6). Finally, transfected Delta1 on DCs are also able to direct differentiation of naive $CD8^+$ T cells into CTLs by promoting granzyme B and eomesodermin (Eomes) transcription (10, 14).

In transplantation, a Th1 response is the dominant phenotype in allograft rejection, whereas a Th2 response favors long-term survival in some transplant models (15–17). Therefore, we decided to study whether blockade of Delta1 could potentially tip the balance toward a favorable T cell subtype and improve allograft survival. In this study, we demonstrate that an anti-Delta1 blocking mAb is able to prolong allograft survival in a fully MHC-mismatched model of cardiac transplantation, in particular with blockade of the B7:CD28 costimulation. This effect was characterized by an

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; Eomes, eomesodermin; KO, knockout; MST, median survival time; MZB, marginal zone B; sCTLA4-Ig, single-dose CTLA4-Ig; WT, wild-type.

increase in Th2 polarization and a decrease in Th1 and cytotoxic T cells. Finally, the prolongation in allograft survival was shown to require the presence of Th2 cytokines, in particular IL-4. These data lead us to conclude that Delta1 signaling is important in alloimmunity in vivo, and blockade of this pathway could be a potential additional target to improve graft outcome.

Materials and Methods

Mice

C57BL/6 (H-2^b, B6), BALB/c (H-2^d), CD28-deficient mice on B6 background (B6.129S2-*Cd28*^{tm1Mak}/J), and STAT4-deficient and STAT6-deficient mice on BALB/c background (C.129S2-*Stat4*^{tm1Gru}/J and C.129S2-*Stat6*^{tm1Gru}/J, respectively) were purchased from The Jackson Laboratory. All mice were 8–12 wk of age and housed in accordance with institutional and National Institutes of Health guidelines.

Heterotopic heart transplantation

Vascularized heart grafts were placed in an intra-abdominal location using microsurgical techniques, as described by Corry et al. (18). 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.

Abs and in vivo treatment protocol

The anti-Delta1 (HMD1-5) mAb was generated, as previously described (19). This mAb was manufactured and purified from the original hybridoma by a commercial source, BioXCell (West Lebanon, NH). Control hamster IgG was also obtained from the same source. Cardiac allograft recipients were treated with mAbs i.p. at 500 μg at day 0, and 250 μg at days 2, 4, 6, 8, and 10 after transplantation. CTLA4-Ig is a fusion protein composed of a human IgG1 Fc fused to the extracellular domain of CTLA4, which was purchased from Bristol-Myers-Squibb. CTLA4-Ig was given i.p. at day 2 after transplantation (250 μg). For IL-4 neutralization, 1 mg anti–IL-4 mAb (11B11) was administered on days 0, 1, 3, 5, and 8 after transplantation (BioXCell).

Measurement of cytokines by ELISPOT and Luminex assay

Splenocytes harvested at 10–14 d after transplantation from B6 recipients of BALB/c heart allografts were restimulated by irradiated donor-type splenocytes. The ELISPOT assay (R&D Systems, Minneapolis, MN) was adapted to measure the frequency of alloreactive T cells producing granzyme B, as described previously (20). The frequencies of cytokine-secreting alloreactive cells were expressed as the number of cytokine-

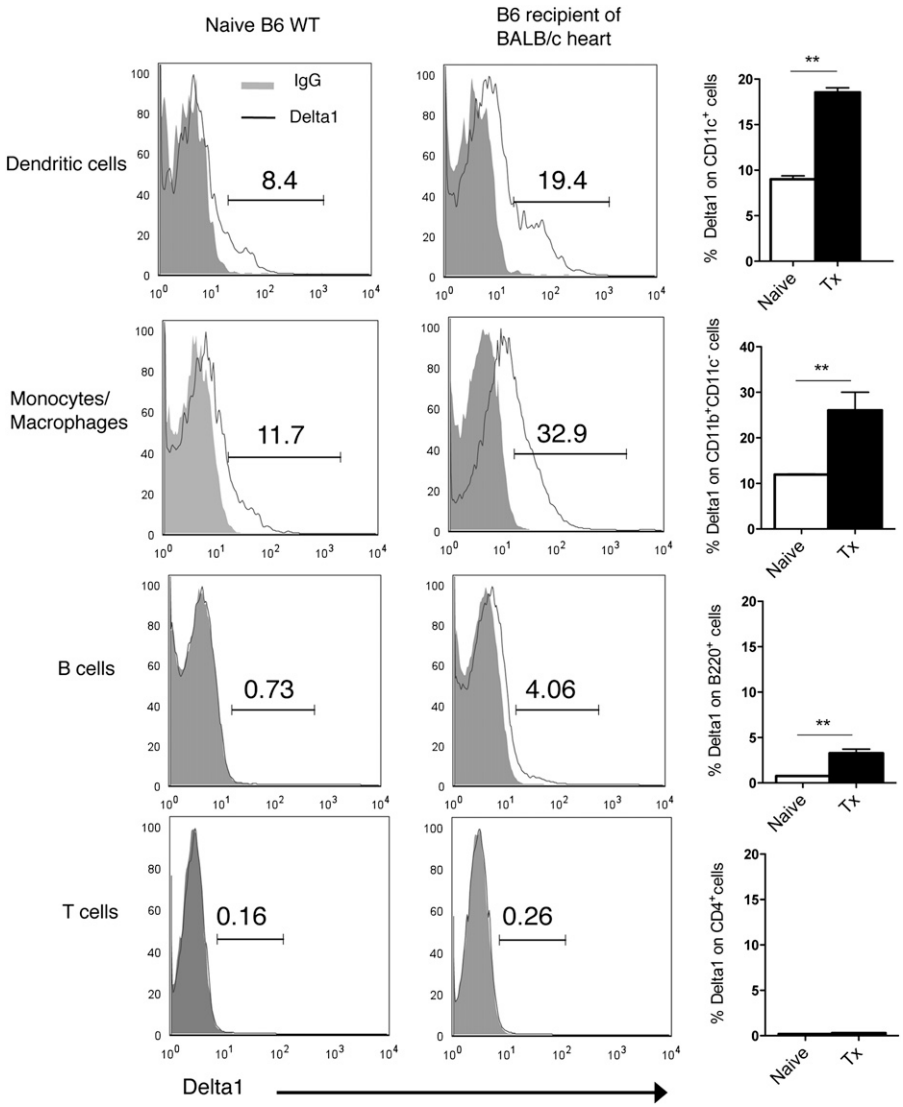

FIGURE 1. Expression of Delta1 on APCs is significantly upregulated upon transplantation. By using a biotinylated anti-Delta1 Ab, we were able to demonstrate a predominant expression of Delta1 on APCs, mainly DCs ($CD11c^+$) and monocytes/macrophages ($CD11b^+CD11c^-$). *Left and middle columns*, Representative histograms of Delta1 expression on different cell subtypes from splenocytes of naive B6 mice or B6 recipients of BALB/c allografts 7 d after transplantation (Tx). Isotype control IgG is shown with solid gray line. *Right column*, Demonstrates the significant upregulation of Delta1 in the setting of transplantation on APCs ($n = 6$ each group) ($^{**}p < 0.001$).

producing cells per 0.5×10^6 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 (21). All samples were tested in triplicate wells.

Flow cytometry

Splenocytes from recipients at 10–14 d after transplantation were stained with fluorochrome-labeled mAbs against CD4, CD8, CD11b, CD11c, B220, CD62 ligand (CD62L), CD44, CD25, CD80, CD86, PDL-1, CD1d, CD5, CD21, and FoxP3 (BD Biosciences, San Jose, CA). 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. To characterize Delta1 expression, we first incubated splenocytes from naive and transplanted animals 7 d after transplantation with anti-mouse CD16/CD32 purified (BD Biosciences) to block nonspecific binding to FcγR. Then cells were incubated with biotinylated anti-mouse Delta1 (HMD1-5) and Delta4 (HMD4-2) Abs, generated as previously described (19), and biotinylated control hamster IgG (eBioscience) for 15 min on ice. After washing the cells, allophycocyanin-streptavidin was added. Washed cells were also stained for 7-aminoactinomycin D (BD Pharmingen) to exclude nonviable cells from flow cytometric analysis. For Notch receptors' characterization on T cells, the following fluorochrome-conjugated Abs were used: Notch1 PE (HMN1-12), Notch2 allophycocyanin (HMN2-35), Notch3 PE (HMN3-133), and Notch4 allophycocyanin (HMN4-14), with respective isotype hamster IgG controls (Biolegend) (19).

Intracellular cytokine staining

To isolate cells from the allografts, hearts were excised, minced, and digested with ~500 U/ml collagenase (Worthington Biochemical) for 30 min at 37°C. Cells were then mashed through 70-μm filters, and RBCs were lysed. Cells (0.5×10^6) were resuspended in HL-1 medium (BioWhittaker); supplemented with 1% L-glutamine (BioWhittaker), 1% penicillin (BioWhittaker), and 10% FCS (BioWhittaker); and restimulated with PMA (5 ng/ml; Sigma-Aldrich) plus ionomycin (500 ng/ml; Sigma-Aldrich); and brefeldin A (10 μg/ml; Sigma-Aldrich) was added. Cells were incubated for 4 h at 37°C. After staining for the surface markers (CD4 and CD8), cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences), according to the manufacturer's instructions, and incubated with PE-conjugated anti–IFN-γ (XMG1.2) and allophycocyanin-conjugated anti–IL-17 (eBio17B7) for 30 min at 4°C. A gate was set on $CD4^+$ and $CD8^+$ cells, and the percentage of IFN-γ and IL-17 cells was determined by flow cytometric analysis.

Ex vivo cytotoxicity assay

Splenocytes from control and anti-Delta1–treated recipients were harvested after transplantation, and $CD8^+$ T cells were isolated by MACS (CD8a; Ly-2 microbeads from Miltenyi Biotec). B cells from allogeneic BALB/c mice were used as target cells after cell sorting with CD19 microbeads (Miltenyi Biotec) with >95% purity. For the cytotoxicity assay, $CD8^+$ T cells and allogeneic target cells were incubated for 6 h at 1:1 and 5:1 E:T cell ratio at 37°C, followed by staining of target cells by fluorochrome-B220 Ab (allophycocyanin; eBioscience) and ethidium/calcein AM, according to protocol from LIVE/DEAD viability/cytotoxicity kit (Invitrogen) (22). The percentage of calcein AM negative (apoptotic cells) and ethidium positive (dead cells) was determined by flow cytometry to differentiate apoptotic/dead target cells from viable ones. The specific cytotoxicity of $CD8^+$ cells was calculated as follows: percentage of specific cytotoxicity = (percentage of dead/apoptotic target cells in the presence of effector cells) − (percentage of dead/apoptotic target cells alone) (23).

Morphology

Cardiac graft samples from transplanted mice were harvested from rejecting (cessation of heartbeat by palpation) mice and at 7–14 d after transplantation when some of the grafts started to reject. Grafts were then fixed in 10% formalin, embedded in paraffin, transversely sectioned, and stained with H&E stain and Elastin Van Gieson stain. Immunoperoxidase staining using CD4 and CD8 Abs was also performed on smaller pieces of frozen sections following air drying and fixation with acetone. For each immunoperoxidase Ab, the number of stained leukocytes per high power field was recorded by averaging the number of positively stained leukocytes in the most affected five high power fields. Acute cellular rejection was semiquantitatively graded (0R-3R) using the revised International Society of Heart and Lung Transplantation classification (24). Because the revised International Society of Heart and Lung Transplantation histologic schema was proposed to grade small endomyocardial biopsies rather than resection specimens, additional parameters were used to further assess the severity and extent of the histologic changes. The extent of the cellular infiltration (percentage of inflammation) and myocyte loss was assessed and expressed as the percentage of surface areas involved by the aforementioned processes (25, 26). A transplant pathologist (I. Batal) blinded to the groups read all the samples.

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. Student *t* test was used for comparison of means. A p value <0.05 was considered statistically significant.

Results

Delta1 expression is upregulated on APCs in an acute rejection model

First, we decided to characterize Delta1 and Delta4 ligand expression in the naive and transplant setting. We analyzed their

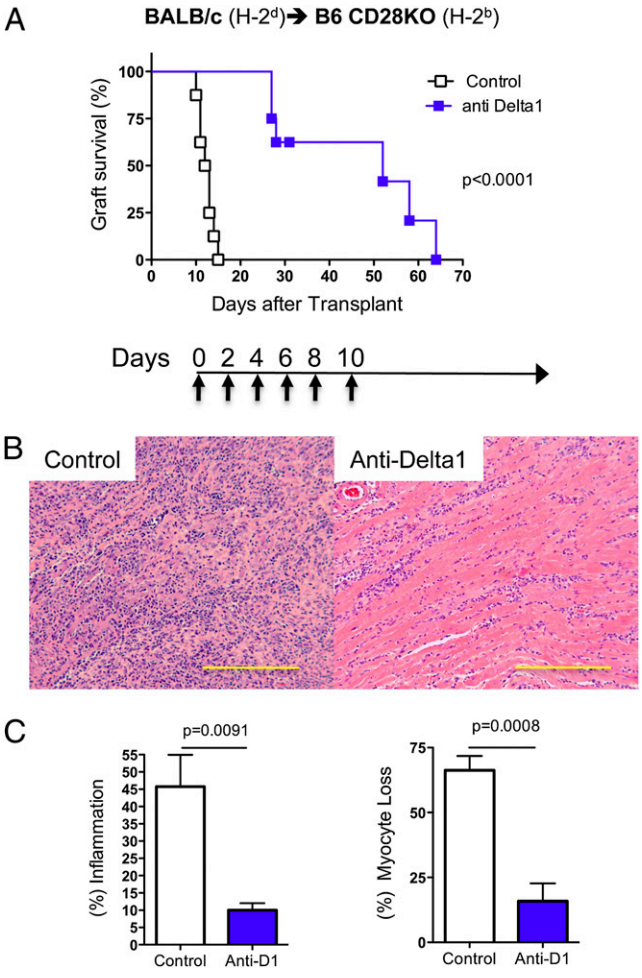

FIGURE 2. Anti-Delta1 mAb delays rejection of BALB/c cardiac allografts in CD28KO recipients. The hearts from BALB/c mice were transplanted into CD28KO recipients on B6 background, which were treated with anti-Delta1 mAb or control hamster IgG. *A*, Whereas control-group cardiac grafts had a MST of 12 d ($n = 8$), the anti-Delta1–treated group had a delayed rejection with MST of 52 d ($n = 8$, $p < 0.0001$). Days of administration of mAb are depicted on arrow below survival curves. *B*, Representative photomicrographs of H&E staining demonstrate more prominent cellular infiltrate in the control group compared with anti-Delta1 at 2 wk after transplantation. *C*, At a similar time point, scoring of pathology samples from both groups showed a significantly higher extent of interstitial inflammation and myocyte loss in the control group ($n = 5$ each group). Scale bars, 200 μm.

expression on hematopoietic cells from lymph nodes and spleens of naive B6 wild-type (WT) animals by flow cytometry. Interestingly, Delta1 was predominantly expressed on DCs ($CD11c^+$) and monocytes/macrophages ($CD11b^+CD11c^-$), whereas it was minimally present on B cells and T cells (Fig. 1). In contrast, Delta4 expression was low in all hematopoietic cells (e.g., $CD11c^+$, 0.41 ± 0.08; Supplemental Fig. 1). Next, we performed cardiac transplants in a fully allogeneic model of cardiac transplantation and noted that Delta1 expression was significantly upregulated upon transplantation, especially close to the time of allograft rejection (7 d) (Fig. 1), whereas Delta4 did not significantly vary (Supplemental Fig. 1). This observation suggests a potential role of Delta1 in allograft rejection; however, it does not prove a direct contribution of this Notch ligand to the alloimmune response. Because Delta1 ligand signals through Notch receptors, we also evaluated the expression of these receptors on naive and transplanted T cells. Notch2 was the predominant receptor on $CD4^+$ cells (18.96% ± 0.33), whereas $CD8^+$ T cells expressed similar amounts of both Notch2 and Notch3 receptors (~19%) (Supplemental Fig. 2). Both Notch2 and Notch3 receptors were upregulated upon transplantation on T cells, whereas Notch1 and Notch4 remained low (<1%) in both naive and transplant settings (Supplemental Fig. 2).

Delta1 blockade prolongs cardiac graft survival

To evaluate whether Delta1 plays a role in allograft rejection, we decided to use a Delta1 blocking mAb (HMD1-5) in vivo. We transplanted BALB/c hearts into B6 recipients and administered either hamster IgG control or anti-Delta1 mAb for 10 d (500 μg on day 0; 250 μg on days 2, 4, 6, 8, and 10). Although control animals had a MST of 7 d, anti-Delta1–treated group had a slight delayed graft survival (MST = 9 d, $n = 6$, $p = 0.0005$). However,

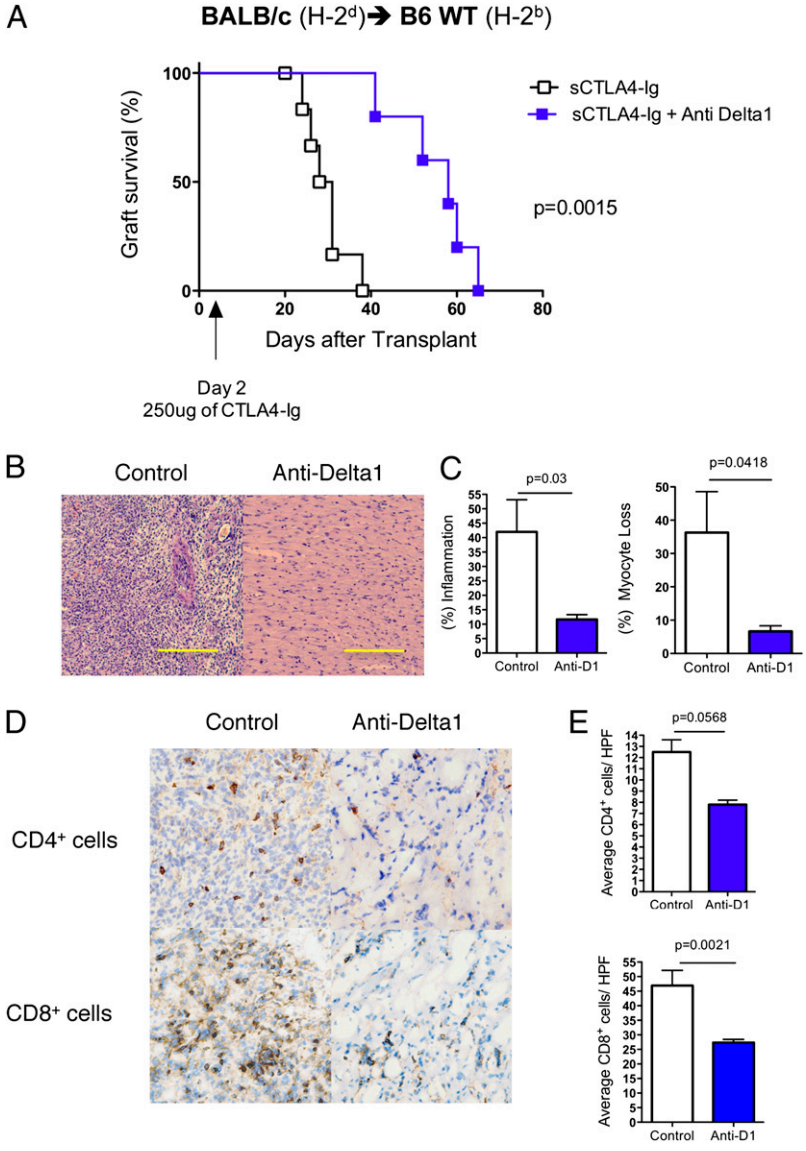

FIGURE 3. Anti-Delta1 mAb prolongs BALB/c cardiac graft survivals in synergy with sCTLA4-Ig in B6 WT recipients. The hearts from BALB/c mice were transplanted into B6 WT recipients, which were treated with a single dose of CTLA4-Ig (sCTLA4-Ig) in combination with either anti-Delta1 mAb or control hamster IgG. *A*, Whereas control cardiac grafts had a MST of 29 d ($n = 7$), the anti-Delta1–cotreated group had a delayed rejection with MST of 58 d ($n = 7$, $p = 0.0015$). *B*, Representative photomicrographs of H&E staining demonstrate more prominent cellular infiltrate in the control group compared with the anti-Delta1–treated group at 2 wk after transplantation. *C*, At a similar time point, scoring of pathology samples from both groups showed a significantly higher extent of interstitial inflammation and myocyte loss in the control group ($n = 5$ each group). *D*, Representative photomicrographs of $CD4^+$- and $CD8^+$-stained sections showed less $CD8^+$ and to a lesser extent $CD4^+$ infiltration in the combined treated group with significantly less T cells/high power field (HPF) as depicted on *E* (original magnification ×20). Scale bars, 200 μm.

when CD28 knockout (KO) mice were used as recipients, Delta1 blockade significantly delayed allograft rejection (MST = 52 d versus 12 d in controls, $n = 8$, $p < 0.0001$) (Fig. 2*A*). A similar finding was also observed when B6 WT recipients were treated with anti-Delta1 in combination with a single dose of CTLA4-Ig (250 μg on day 2) (MST = 58 versus 29 d in sCTLA4-Ig alone, $n = 7$, $p < 0.0001$) (Fig. 3*A*). These data suggest that brief Delta1 blockade is able to significantly delay allograft rejection, especially in the absence of strong B7:CD28 costimulation.

Anti-Delta1 decreases cellular infiltration and myocyte loss of cardiac allografts

To elucidate the delayed rejection in anti-Delta1–treated group, we started by analyzing cardiac grafts at 2 wk after transplantation, when control grafts started to be rejected. Although control grafts had intense inflammatory infiltrates and myocyte losses, Delta1 blockade on CD28KO recipients led to significantly less myocyte loss and inflammatory infiltrates (Fig. 2*B*, 2*C*), with lower rejection scores by International Society of Heart and Lung Transplantation classification (1.8 ± 0.1 versus 2.4 ± 0.24, $p < 0.05$). Similar picture was also observed in B6 WT recipients treated with sCTLA4-Ig and anti-Delta1 (Fig. 3*B*, 3*C*). Furthermore, the latter grafts had less $CD4^+$ and $CD8^+$ infiltrating cells when analyzed by immunohistochemistry (Fig. 3*D*, 3*E*). These findings suggest that Delta1 blockade leads to a decrease in the intensity of the alloimmune response.

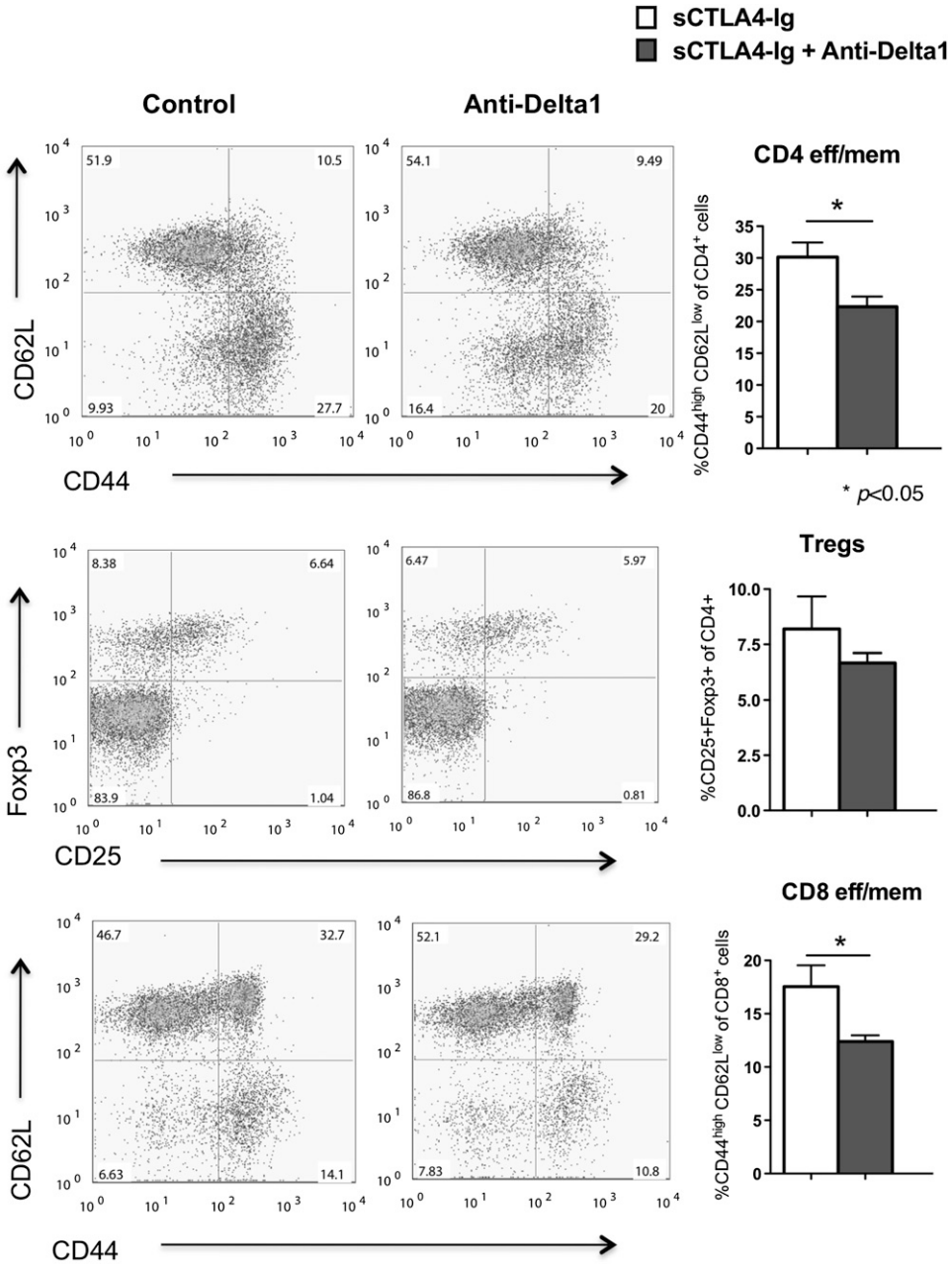

FIGURE 4. Lower frequency of CD4 eff/mem and CD8 eff/mem in the combined sCTLA4-Ig/anti-Delta1–treated group. Flow cytometry analysis of splenocytes from B6 WT recipients of BALB/c hearts 14 d after transplantation showed that anti-Delta1 Ab significantly decreased the percentage of $CD4^+CD44^{high}CD62L^{low}$ and $CD8^+CD44^{high}CD62L^{low}$ cells (CD4 and CD8 eff/mem, respectively) when compared with sCTLA4-Ig alone. $CD4^+CD25^+Foxp3^+$ regulatory T cells were no different between groups ($p = 0.3820$). *Left panels*, Representative examples of dot plots. Data are representative of three independent experiments. *$p < 0.05$.

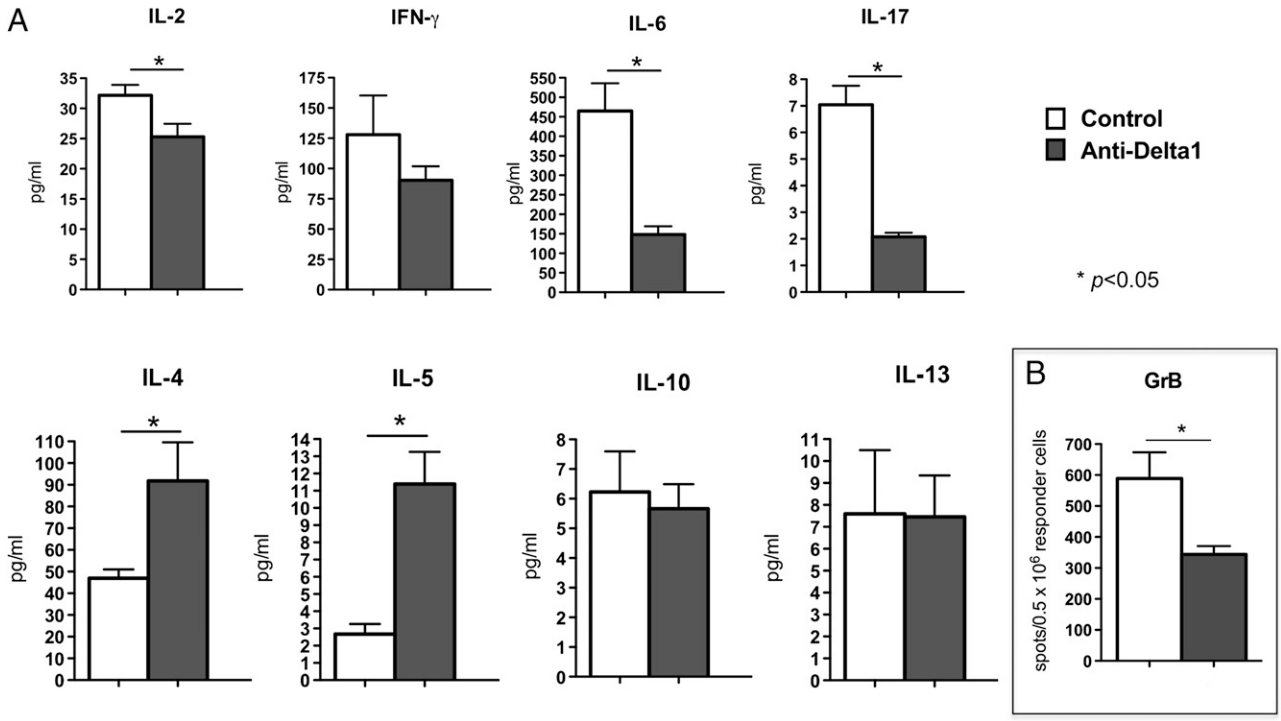

FIGURE 5. Anti-Delta1 mAb upregulates Th2 cytokines. *A*, Donor-specific Th1, Th2, and proinflammatory cytokine production of splenocytes from CD28KO recipients of BALB/c allografts was assessed 14 d after transplantation by Luminex assay. Anti-Delta1 treatment led to a significant increase in the production of Th2 cytokines (IL-4, IL-5), whereas it decreased the production of IL-2, IL-6, and IL-17. *B*, The frequency of GrB-producing cells was also decreased on the Delta1 blockade group by ELISPOT. There was a trend toward lower IFN-γ, but it did not reach statistical significance. Data are representative of three independent experiments and indicate the mean of triplicate results in each experiment (*$p < 0.05$).

Anti-Delta1 reduced the frequency of CD4 and CD8 effector/memory cells

To investigate the mechanisms of reduced alloimmunity in response to Delta1 blockade, we first measured the frequency of lymphocyte subtypes in transplanted groups 14 d after transplantation. Anti-Delta1 administration resulted in a significant decrease in both CD4 and CD8 effector/memory T cells ($CD44^{high}$ $CD62L^{low}$) in CD28KO recipients ($p = 0.001$) (Supplemental Fig.

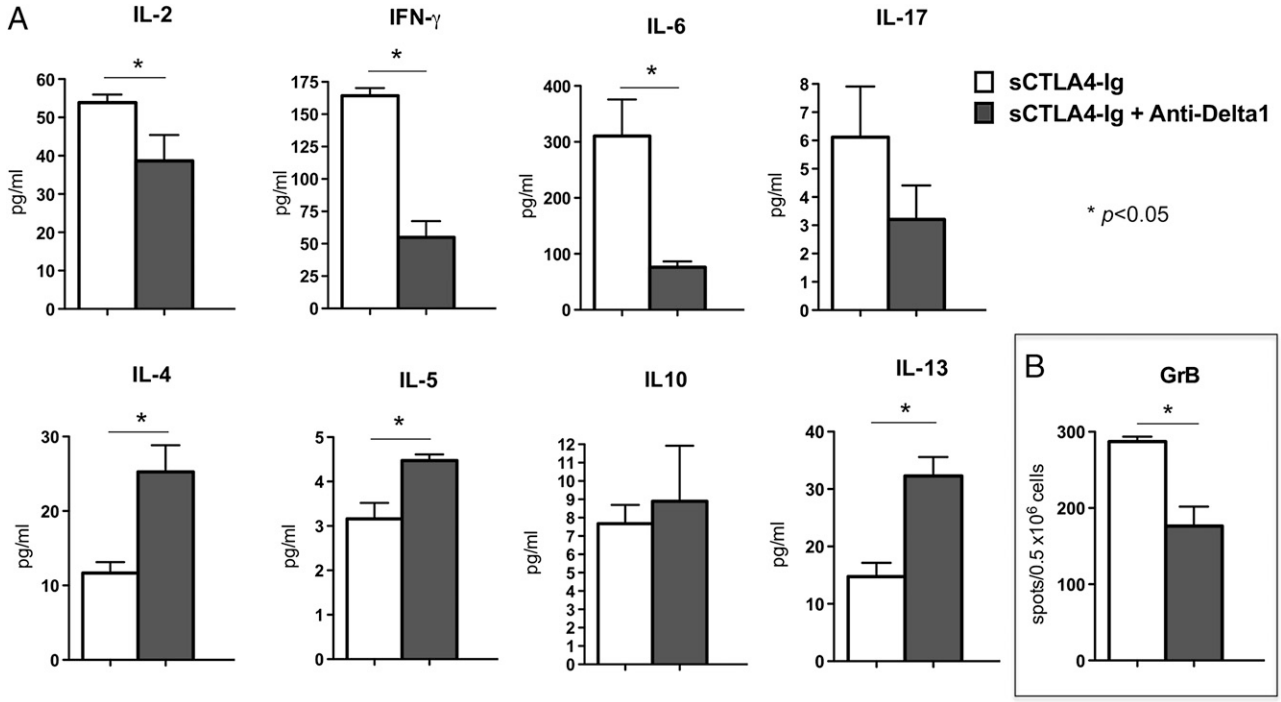

FIGURE 6. Combination of sCTLA4-Ig and anti-Delta1 lowered GrB and IFN-γ production, although it increased Th2 cytokines. *A*, Donor-specific cytokine production of splenocytes from B6 WT recipients of BALB/c allografts was assessed 14 d after transplantation by ELISPOT and Luminex assay. GrB (*B*), IL-2, IFN-γ, and IL-6 were significantly decreased in the combined sCTLA4-Ig/anti-Delta1 group, whereas Th2 cytokines (IL-4, IL-5, and IL-13) were increased when compared with the sCTLA4-Ig alone group. Data are representative of three independent experiments and indicate the mean of triplicate results in each experiment (*$p < 0.05$).

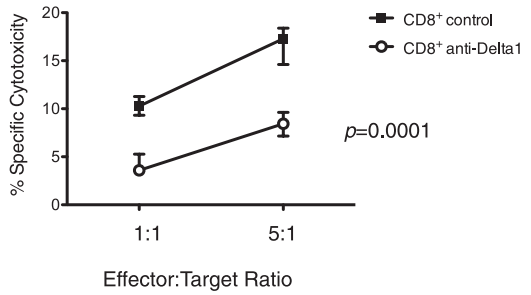

FIGURE 7. Cytotoxicity assay. $CD8^+$ T cells from control and anti-Delta1–treated recipients were incubated with allogeneic target cells for 6 h at E:T ratios of 1:1 and 5:1, and cytotoxicity activity of these T cells was measured using a LIVE/DEAD viability/cytotoxicity kit (Invitrogen). Percentage of specific cytotoxicity with either control or anti-Delta1 $CD8^+$ T cells was calculated, as described in *Materials and Methods* (n = 3–4, $p < 0.0001$). Data are representative of three independent experiments.

3) as well as in B6 WT recipients cotreated with sCTLA4-Ig ($p < 0.05$) when compared with controls (Fig. 4). Regulatory T cells were not significantly different between groups (Fig. 4). Furthermore, the percentage of APC subtypes was similar between groups as well as the activation status of DCs, as assessed by CD80, CD86, and PDL-1 expression (Supplemental Fig. 4*A*). Because Delta1 has been shown to be important in the development of marginal zone B (MZB) cells, we also checked the frequency of this subpopulation in the spleens of treated and control animals. Despite a lower trend in percentage of total B cells as well as subtypes (regulatory B cells and MZB cells), there was no statistically significant difference between anti-Delta1 group and controls (Supplemental Fig. 4*B*). In summary, anti-Delta1 lowered the frequency of effector/memory CD4 and CD8 cells, whereas it did not affect the activation status of DCs or other APC subtypes.

Anti-Delta1 mAb tipped the balance toward Th2 cells

Next, we sought to characterize the influence of Delta1 blockade on Th cell phenotype in lymph nodes and spleens of CD28KO recipients 2 wk after transplantation. The anti-Delta1–treated group showed increased production of IL-4 and IL-5, whereas IFN-γ and IL-17 levels were decreased, as measured by Luminex bead-based assay (Fig. 5*A*). Similar observation was also noted in B6 WT recipients cotreated with sCTLA4-Ig and anti-Delta1 (Fig. 6*A*); however, the decrease in IFN-γ was more dramatic in this model. Moreover, Delta1 blockade also led to lower frequency of granzyme B-producing cells, as demonstrated by ELISPOT (Figs. 5*B*, 6*B*). This was confirmed by an in vitro cytotoxicity assay, in which $CD8^+$ T cells from anti-Delta1–treated group demonstrated lower cytotoxicity activity against allogeneic target cells when compared with controls (8% apoptotic/dead cells versus 16% on controls, $p = 0.0001$) (Fig. 7).

Finally, when lymphocytes infiltrating the allografts were isolated and cultured with irradiated donor cells, the frequency of both $CD4^+$ and $CD8^+$ IFN-γ^+ T cells was significantly lower in the anti-Delta1 group (Fig. 8). These results indicate that Delta1 blockade leads to polarization into Th2 cells and decreases Th1 and cytotoxic T cells, potentially explaining the delay in allograft rejection.

Anti-Delta1 prolongation of allograft survival is dependent on Th2 cytokines

Th1 and Th2 development is dependent on STAT4 and STAT6 signaling, respectively (27). To evaluate whether Delta1 signaling may induce Th1 differentiation indirectly via suppression of Th2 cells through IL-4 signaling blockade, we decided to dissect what was the dominant beneficial consequence of Delta1 blockade. Because STAT4KO mice were only commercially available on BALB/c background, we inverted the strains for this experiment. First, we confirmed the effect of anti-Delta1 mAb in this strain combination by demonstrating a slight prolongation of graft survival on BALB/c recipients of B6 hearts treated with anti-Delta1 mAb (MST = 11) when compared with controls (MST = 9, n = 5/group, $p = 0.0025$), similarly to what was observed on the BALB/c into B6 model. By transplanting B6 hearts into STAT4KO or STAT6KO recipients on BALB/c background, we were able to observe that prolongation of allograft survival was only maintained in STAT4KO treated with anti-Delta1 (Fig. 9*A*), whereas STAT6KO recipients had similar accelerated rejection as controls (Fig. 9*B*). Furthermore, neutralization of IL-4 abrogated the

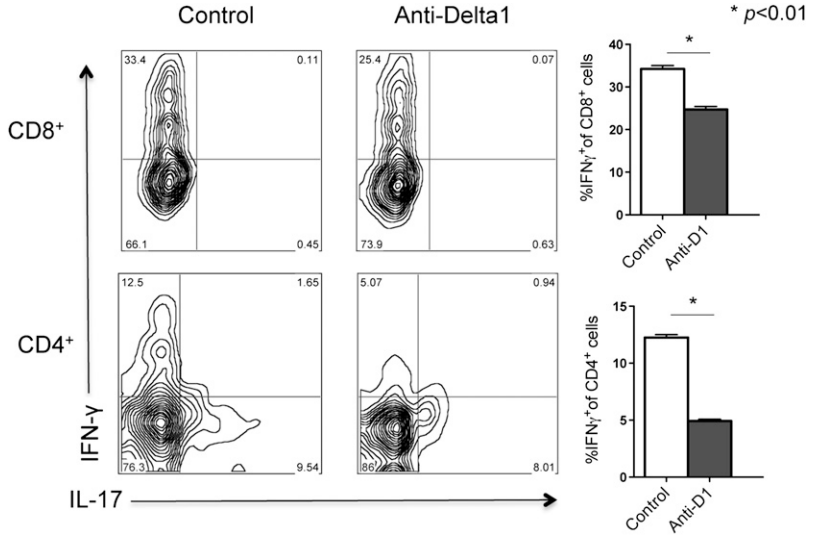

FIGURE 8. Lower frequency of IFN-γ–producing $CD4^+$ and $CD8^+$ T cells isolated from heart allografts cotreated with anti-Delta1. Lymphocytes were isolated from heart allografts 2 wk after transplantation and were restimulated in vitro for 4 h. Subsequent intracellular cytokine staining by flow cytometry revealed a lower frequency of IFN-γ–producing T cells in the group treated with both sCTLA4-Ig and anti-Delta1 compared with control (only sCTLA4-Ig). Contour plots are representative examples. Graphs on the *right* are representative of three independent experiments (*$p < 0.01$).

prolonging effects of Delta1 blockade in CD28KO recipients of BALB/c hearts (MST = 52 versus 13 in the cotreated group with anti-Delta1 and anti–IL-4 mAb, $n = 5$, $p = 0.0002$) (Fig. 9*C*). These findings suggest that the improved survival by Delta1 blockade is mainly dependent on Th2 polarization.

Discussion

The role of the Notch pathway in T cell differentiation in vitro has been increasingly explored lately. In particular, Delta1 ligands expressed on DCs have been shown to promote Th1 cell development and CTL differentiation (6, 10, 11, 14), whereas Delta4 promotes IL-17 production (28). Nevertheless, little is known about the contribution of Notch ligands in the transplant setting in vivo, especially of Delta1, because both Th1 and CTLs are known to play a major role in allograft rejection (15–17, 29). In this study, we demonstrated that Delta1 is strongly upregulated upon transplantation in APCs in an acute cardiac rejection model (Fig. 1), whereas Delta4 remains mainly unchanged. The expression of Delta1 on DCs is induced by several stimuli, including infection with virus and bacteria, exposure to TLR ligands, and CpG-containing DNA (6, 11, 30). In alloimmunity, multiple stimuli might act in synergy in the upregulation of Delta1, especially TLR ligands released after surgical trauma and ischemic injury (31, 32). Based on observations of Delta1 expression after syngeneic cardiac transplants in which Delta1 was not as significantly upregulated as in allotransplants (data not shown), other unknown stimuli must be present to contribute to the dominant Delta1 expression upon transplantation.

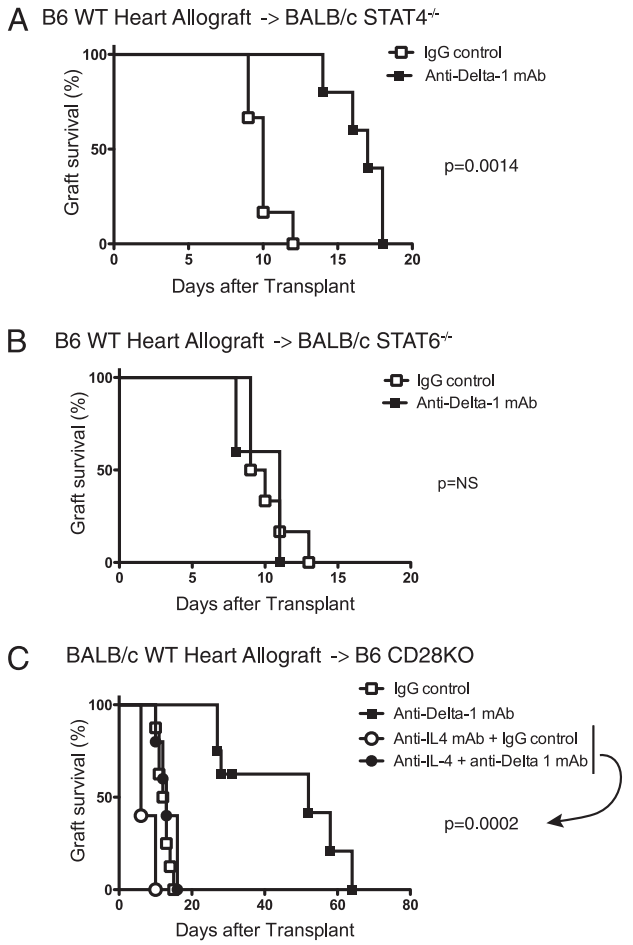

FIGURE 9. Prolongation of allograft survival on anti-Delta1–treated group is dependent on Th2 cytokines. The hearts from B6 mice were transplanted into STAT4KO (*A*) or STAT6KO (*B*) recipients on BALB/c background, which were treated with anti-Delta1 mAb or control hamster IgG. Whereas anti-Delta1 delayed allograft rejection in STAT4KO recipients ($n = 6$, $p = 0.0014$), it did not affect graft survival in STAT6KO recipients ($n = 6$, $p = 0.87$). *C*, The hearts from BALB/c mice were transplanted into CD28KO recipients on B6 background, which were treated with anti–IL-4 mAb in combination with either anti-Delta1 mAb or control hamster IgG. IL-4 neutralization abrogated the prolonging effect of Delta1 blockade (MST = 13 versus 52 in controls, $n = 5$/group, $p = 0.0002$).

Delta1 deficiency in mice is an embryonically lethal condition because Delta1 plays a major role in the compartmentalization of somites during embriogenesis (33). Cre-transgenic mice with selective deletion of Delta1 after birth demonstrated that the proportion of T and B cells was normal in both spleens and lymph nodes when compared with age-matched controls (34), suggesting that Delta1 is dispensable for postnatal T cell development in the thymus. However, a subpopulation of B cells, splenic MZB cells, was significantly decreased in these Delta1-null mice (34). Later, it was shown that despite the essential role of Delta1 for the maintenance of MZB cells in normal mice, anti-Delta1 blocking Ab did not significantly affect MZB cells in lupus-prone (New Zealand Black × New Zealand White)F_1 mice (19), indicating that other signals might contribute to MZB cell maintenance. Similarly to these findings, we noticed that MZB cell subpopulation was not significantly decreased in the transplant setting at both 7 and 14 d after transplantation (Supplemental Fig. 4*B*), whereas it was decreased in the naive B6 mice that received anti-Delta1 without a transplant (data not shown). These findings suggest that Delta1 blockade does not reduce the alloimmune response through MZB cell depletion or by affecting T cell development; however, it could be affecting T cell activation and/or differentiation.

T cell activation requires the TCR engagement and costimulatory signals. Adding to this complexity, Notch proteins have emerged as potentiators of TCR signaling (5). Notch signaling has been shown to promote the nuclear retention of the NF-κB proteins p50 and p65 (35, 36) and to be involved in a positive feedback loop that regulates IL-2 production and CD25 expression (37). Furthermore, chemical γ-secretase inhibitors that block Notch signaling decrease T cell proliferation significantly (36). Corroborating with these findings, blockade of Delta1 in our in vivo model led to a decrease in the frequency of both CD4 and CD8 effector/memory cells, suggesting that Delta1 signaling might contribute to T cell proliferation. On the contrary, the transfection of small interfering RNAs into DCs against different Notch ligands led to enhanced IFN-γ production in MLR experiments with $CD4^+$ T cells (38). These discrepancies seem to depend on the experimental system used and will only be resolved by performing experiments with conditionally gene-targeted mice or perhaps further development of in vivo small interfering RNA technologies.

$CD8^+$ T cells have been reported to be major contributors of chronic rejection (29, 39), and its different requirements for costimulation (3) might pose a challenge to the development of tolerogenic strategies. The differentiation of naive $CD8^+$ T cells into a functional CTL requires the induction of the two following key genes: T-bet and Eomes (40). CTLs can then mediate direct cell killing via granzyme B, perforin, or Fas ligand expression. Delta1-expressing DCs have increased capacity to promote CTL differentiation in vitro via interaction with Notch2 on naive $CD8^+$ T cells (10). After this interaction, Notch2 intracellular domain migrates to the nucleus in combination with CREB1 to regulate granzyme B expression (10). Furthermore, Cho et al. (14) have

shown that Notch1 is also able to directly regulate the expression of Eomes, perforin, and granzyme B via binding of the promoters of these crucial effector molecules. Reinforcing these findings, Delta1 blockade decreased the frequency of granzyme B-producing cells in our transplant models (Figs. 5*B*, 6*B*) and led to lower cytotoxicity activity of $CD8^+$ T cells ex vivo against alloantigen target cells (Fig. 7), suggesting an important role of Delta1–Notch interaction in the regulation of cytolytic effector function of $CD8^+$ T cells. Nonetheless, we realize that our Ab approach is unable to define whether Notch signaling is truly required to mount an efficient CTL response in vivo.

In sharp contrast to our findings, Delta1-transfected L cells (carrying both MHC class I and II molecules) injected into recipients 14 d before transplantation were able to prolong cardiac allograft survival in another study (41). This inhibition of rejection was CD8 dependent and presumably due to the emergence of regulatory IL-10–producing $CD8^+$ cells. However, this conclusion was based on in vitro findings using Delta1-Fc, and not Delta1-transfected L cells like the in vivo model, raising concern about their conclusion. Moreover, the experimental approach undertaken with preinjection of cells in combination with MHC molecules and the properties of Delta1 on L cells could account for the differences observed. Nonetheless, we did not observe any effect of Delta1 blockade on IL-10 production in our experiment in vivo.

Different approaches have been undertaken to prove the role of Delta1 in Th1 cell differentiation, including transfection of Delta1 on APCs (6, 11) and use of Delta1-Fc proteins (30, 42). Furthermore, a neutralization Ab specific for Delta1 ligand (HMD1-5), the same clone used in this study, was able to decrease the frequency of Th1 cells and reduce the severity of disease in a model of experimental autoimmune encephalomyelitis (42). In agreement with these reports, our anti-Delta mAb was able to decrease Th1 and increase Th2 cells in murine cardiac transplantation, in particular when B7:CD28 pathway was blocked (Figs. 5, 6), suggesting an important role of Delta1 signaling in the determination of Th cell polarization in vivo.

It is believed that once Delta1 ligand interacts with the Notch receptor on $CD4^+$ T cells, it activates the transcription of Tbx21 via a RBP-J–dependent mechanism, which encodes the Th1 cell-specifying transcription factor T-bet (43). Moreover, Sun et al. (11) have shown that Delta1 signaling could also induce a preferential Th1 response by inhibiting IL-4R signaling, suggesting that it is actually the suppression of Th2 development that favors Th1 polarization. Interestingly, our results with IL-4 neutralization and STAT4- and STAT6-deficient recipients go in support of this hypothesis, in which the dominant effect of Delta1 signaling is possibly through its suppressive effect on Th2 cell development. In neutral conditions, Th cells seem to favor Th2 polarization through GATA3 expression (44), and Delta1 affects IL-4 responsiveness to allow Th1 cell development (11). In the transplant setting, Th2 response favors long-term survival in some transplant models (15–17, 45), and tipping the balance from a Th1 to a Th2 response via Delta1 blockade demonstrated to be effective in reducing the alloimmune response. Among the Th2 cytokines, IL-4 was demonstrated in our model to be specifically important for the protective effect of Delta1 blockade, because neutralization of IL-4 abrogated the prolonging graft effect of anti-Delta1. However, the mechanism by which Delta1-initiated signaling blocks Th2 cell development remains to be established.

Taken together, our findings support a key role of Delta1 in the regulation of Th cell polarization and CTL differentiation in transplantation (Fig. 10). We were able to show that Delta1 is significantly upregulated on APCs upon transplantation, and, using an Ab approach, a brief blockade of Delta1 was able to decrease CTLs and tip the balance toward a Th2 response, in synergy with B7:CD28 blockade. This effect was shown to be dependent on Th2 cytokines, in particular IL-4, suggesting a protective role of Th2 polarization in delaying rejection. In face of the poor long-term outcomes of current immunosuppressive drugs in organ transplantation, the identification of beneficial effects of Delta1 blockade might help in the development of potential novel targets for tolerogenic strategies in alloimmunity.

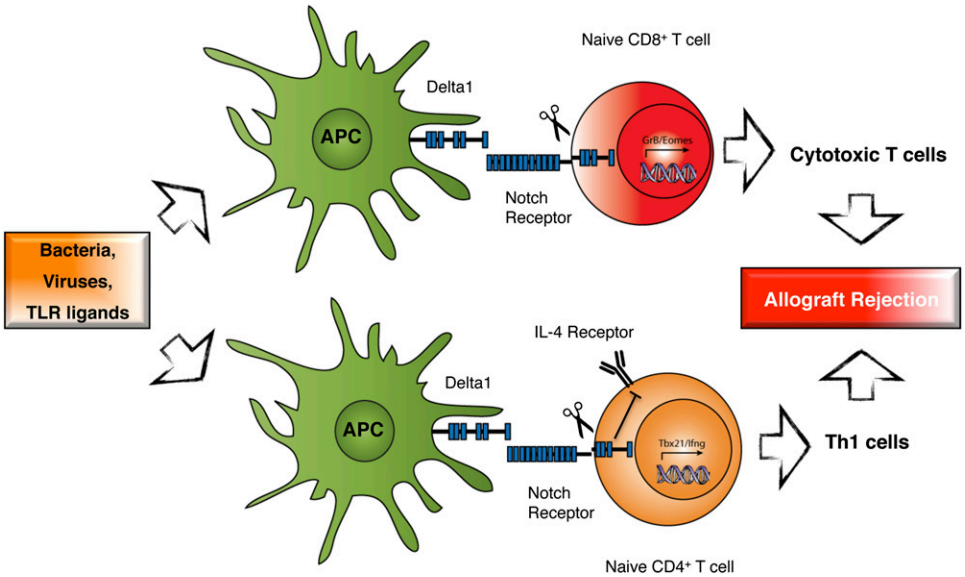

FIGURE 10. The role of Delta1 in T cell differentiation in alloimmunity. Bacteria, virus, and TLR ligands are some of the known stimuli that can induce the expression of Delta1 by APCs, which in turn promote the capacity of APCs to induce cytotoxic $CD8^+$ T (CTL) cells and Th1 cell differentiation. When Delta1 interacts with the Notch receptor on T cells, it leads to sequential cleavage of the transmembrane region of Notch, resulting in the release of the Notch intracellular domain that translocates to the nucleus. In combination with cofactors (data not shown), it activates the transcription of Notch target genes, including GrB and Eomes on $CD8^+$ T cells and Tbx21 and Ifng on $CD4^+$ T cells. Delta1–Notch activation also leads to inhibition of IL-4 signaling on naive $CD4^+$ T cells, suppressing Th2 cell differentiation. As a result, the induction of Th1 cells and CTLs promotes rejection in the transplant setting.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Trambley, J., A. W. Bingaman, A. Lin, E. T. Elwood, S. Y. Waitze, J. Ha, M. M. Durham, M. Corbascio, S. R. Cowan, T. C. Pearson, and C. P. Larsen. 1999. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. *J. Clin. Invest.* 104: 1715–1722.
2. Vu, M. D., F. Amanullah, Y. Li, G. Demirci, M. H. Sayegh, and X. C. Li. 2004. Different costimulatory and growth factor requirements for CD4+ and CD8+ T cell-mediated rejection. *J. Immunol.* 173: 214–221.
3. Halamay, K. E., R. L. Kirkman, L. Sun, A. Yamada, R. C. Fragoso, K. Shimizu, R. N. Mitchell, and D. B. McKay. 2002. CD8 T cells are sufficient to mediate allorecognition and allograft rejection. *Cell. Immunol.* 216: 6–14.
4. Kapsenberg, M. L. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat. Rev. Immunol.* 3: 984–993.
5. Osborne, B. A., and L. M. Minter. 2007. Notch signalling during peripheral T-cell activation and differentiation. *Nat. Rev. Immunol.* 7: 64–75.
6. Amsen, D., J. M. Blander, G. R. Lee, K. Tanigaki, T. Honjo, and R. A. Flavell. 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117: 515–526.
7. Maillard, I., T. Fang, and W. S. Pear. 2005. Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu. Rev. Immunol.* 23: 945–974.
8. Yuan, J. S., P. C. Kousis, S. Suliman, I. Visan, and C. J. Guidos. 2010. Functions of notch signaling in the immune system: consensus and controversies. *Annu. Rev. Immunol.* 28: 343–365.
9. Radtke, F., N. Fasnacht, and H. R. Macdonald. 2010. Notch signaling in the immune system. *Immunity* 32: 14–27.
10. Maekawa, Y., Y. Minato, C. Ishifune, T. Kurihara, A. Kitamura, H. Kojima, H. Yagita, M. Sakata-Yanagimoto, T. Saito, I. Taniuchi, et al. 2008. Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nat. Immunol.* 9: 1140–1147.
11. Sun, J., C. J. Krawczyk, and E. J. Pearce. 2008. Suppression of Th2 cell development by Notch ligands Delta1 and Delta4. *J. Immunol.* 180: 1655–1661.
12. Yamaguchi, E., S. Chiba, K. Kumano, A. Kunisato, T. Takahashi, T. Takahashi, and H. Hirai. 2002. Expression of Notch ligands, Jagged1, 2 and Delta1 in antigen presenting cells in mice. *Immunol. Lett.* 81: 59–64.
13. Kubo, M. 2007. Notch: filling a hole in T helper 2 cell differentiation. *Immunity* 27: 3–5.
14. Cho, O. H., H. M. Shin, L. Miele, T. E. Golde, A. Fauq, L. M. Minter, and B. A. Osborne. 2009. Notch regulates cytolytic effector function in CD8+ T cells. *J. Immunol.* 182: 3380–3389.
15. Sho, M., A. Yamada, N. Najafian, A. D. Salama, H. Harada, S. E. Sandner, A. Sanchez-Fueyo, X. X. Zheng, T. B. Strom, and M. H. Sayegh. 2002. Physiological mechanisms of regulating alloimmunity: cytokines, CTLA-4, CD25+ cells, and the alloreactive T cell clone size. *J. Immunol.* 169: 3744–3751.
16. Waaga, A. M., M. Gasser, J. E. Kist-van Holthe, N. Najafian, A. Müller, J. P. Vella, K. L. Womer, A. Chandraker, S. J. Khoury, and M. H. Sayegh. 2001. Regulatory functions of self-restricted MHC class II allopeptide-specific Th2 clones in vivo. *J. Clin. Invest.* 107: 909–916.
17. Waaga-Gasser, A. M., M. R. Grimm, J. Lutz, V. Lange, S. M. Lenhard, B. Aviles, J. E. Kist-van Holthe, T. Lebedeva, D. Samsonov, D. Meyer, et al. 2009. Regulatory allospecific T cell clones abrogate chronic allograft rejection. *J. Am. Soc. Nephrol.* 20: 820–830.
18. Corry, R. J., H. J. Winn, and P. S. Russell. 1973. Primarily vascularized allografts of hearts in mice: the role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation* 16: 343–350.
19. Moriyama, Y., C. Sekine, A. Koyanagi, N. Koyama, H. Ogata, S. Chiba, S. Hirose, K. Okumura, and H. Yagita. 2008. Delta-like 1 is essential for the maintenance of marginal zone B cells in normal mice but not in autoimmune mice. *Int. Immunol.* 20: 763–773.
20. Yang, J., L. V. Riella, O. Boenisch, J. Popoola, S. Robles, T. Watanabe, V. Vanguri, X. Yuan, I. Guleria, L. A. Turka, et al. 2009. *Paradoxical functions of* B7: CD28 costimulation in a MHC class II-mismatched cardiac transplant model. *Am. J. Transplant.* 9: 2837–2844.
21. Yuan, X., J. Paez-Cortez, I. Schmitt-Knosalla, F. D'Addio, B. Mfarrej, M. Donnarumma, A. Habicht, M. R. Clarkson, J. Iacomini, L. H. Glimcher, et al. 2008. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *J. Exp. Med.* 205: 3133–3144.
22. Liu, J., N. Gong, X. Huang, A. D. Reynolds, R. L. Mosley, and H. E. Gendelman. 2009. Neuromodulatory activities of CD4+CD25+ regulatory T cells in a murine model of HIV-1-associated neurodegeneration. *J. Immunol.* 182: 3855–3865.
23. Correia, M. P., A. V. Costa, M. Uhrberg, E. M. Cardoso, and F. A. Arosa. 2011. IL-15 induces CD8+ T cells to acquire functional NK receptors capable of modulating cytotoxicity and cytokine secretion. *Immunobiology* 216: 604–612.
24. Stewart, S., G. L. Winters, M. C. Fishbein, H. D. Tazelaar, J. Kobashigawa, J. Abrams, C. B. Andersen, A. Angelini, G. J. Berry, M. M. Burke, et al. 2005. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. *J. Heart Lung Transplant.* 24: 1710–1720.
25. Harada, H., A. D. Salama, M. Sho, A. Izawa, S. E. Sandner, T. Ito, H. Akiba, H. Yagita, A. H. Sharpe, G. J. Freeman, and M. H. Sayegh. 2003. The role of the ICOS-B7h T cell costimulatory pathway in transplantation immunity. *J. Clin. Invest.* 112: 234–243.
26. Shimizu, K., U. Schönbeck, F. Mach, P. Libby, and R. N. Mitchell. 2000. Host CD40 ligand deficiency induces long-term allograft survival and donor-specific tolerance in mouse cardiac transplantation but does not prevent graft arteriosclerosis. *J. Immunol.* 165: 3506–3518.
27. Shuai, K., and B. Liu. 2003. Regulation of JAK-STAT signalling in the immune system. *Nat. Rev. Immunol.* 3: 900–911.
28. Mukherjee, S., M. A. Schaller, R. Neupane, S. L. Kunkel, and N. W. Lukacs. 2009. Regulation of T cell activation by Notch ligand, DLL4, promotes IL-17 production and Rorc activation. *J. Immunol.* 182: 7381–7388.
29. Fischbein, M. P., J. Yun, H. Laks, Y. Irie, M. C. Fishbein, B. Bonavida, and A. Ardehali. 2002. Role of CD8+ lymphocytes in chronic rejection of transplanted hearts. *J. Thorac. Cardiovasc. Surg.* 123: 803–809.
30. Maekawa, Y., S. Tsukumo, S. Chiba, H. Hirai, Y. Hayashi, H. Okada, K. Kishihara, and K. Yasutomo. 2003. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* 19: 549–559.
31. Kaczorowski, D. J., A. Nakao, K. P. Mollen, R. Vallabhaneni, R. Sugimoto, J. Kohmoto, K. Tobita, B. S. Zuckerbraun, K. R. McCurry, N. Murase, and T. R. Billiar. 2007. Toll-like receptor 4 mediates the early inflammatory response after cold ischemia/reperfusion. *Transplantation* 84: 1279–1287.
32. Tesar, B. M., D. Jiang, J. Liang, S. M. Palmer, P. W. Noble, and D. R. Goldstein. 2006. The role of hyaluronan degradation products as innate alloimmune agonists. *Am. J. Transplant.* 6: 2622–2635.
33. Hrabě de Angelis, M., J. McIntyre, II, and A. Gossler. 1997. Maintenance of somite borders in mice requires the Delta homologue DII1. *Nature* 386: 717–721.
34. Hozumi, K., N. Negishi, D. Suzuki, N. Abe, Y. Sotomaru, N. Tamaoki, C. Mailhos, D. Ish-Horowicz, S. Habu, and M. J. Owen. 2004. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. *Nat. Immunol.* 5: 638–644.
35. Shin, H. M., L. M. Minter, O. H. Cho, S. Gottipati, A. H. Fauq, T. E. Golde, G. E. Sonenshein, and B. A. Osborne. 2006. Notch1 augments NF-kappaB activity by facilitating its nuclear retention. *EMBO J.* 25: 129–138.
36. Palaga, T., L. Miele, T. E. Golde, and B. A. Osborne. 2003. TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. *J. Immunol.* 171: 3019–3024.
37. Adler, S. H., E. Chiffoleau, L. Xu, N. M. Dalton, J. M. Burg, A. D. Wells, M. S. Wolfe, L. A. Turka, and W. S. Pear. 2003. Notch signaling augments T cell responsiveness by enhancing CD25 expression. *J. Immunol.* 171: 2896–2903.
38. Stallwood, Y., E. Briend, K. M. Ray, G. A. Ward, B. J. Smith, E. Nye, B. R. Champion, and G. J. McKenzie. 2006. Small interfering RNA-mediated knockdown of notch ligands in primary CD4+ T cells and dendritic cells enhances cytokine production. *J. Immunol.* 177: 885–895.
39. Lunsford, K. E., P. H. Horne, M. A. Koester, A. M. Eiring, J. P. Walker, H. L. Dziema, and G. L. Bumgardner. 2006. Activation and maturation of alloreactive CD4-independent, CD8 cytolytic T cells. *Am. J. Transplant.* 6: 2268–2281.
40. Williams, M. A., and M. J. Bevan. 2007. Effector and memory CTL differentiation. *Annu. Rev. Immunol.* 25: 171–192.
41. Wong, K. K., M. J. Carpenter, L. L. Young, S. J. Walker, G. McKenzie, A. J. Rust, G. Ward, L. Packwood, K. Wahl, L. Delriviere, et al. 2003. Notch ligation by Delta1 inhibits peripheral immune responses to transplantation antigens by a CD8+ cell-dependent mechanism. *J. Clin. Invest.* 112: 1741–1750.
42. Elyaman, W., E. M. Bradshaw, Y. Wang, M. Oukka, P. Kivisäkk, S. Chiba, H. Yagita, and S. J. Khoury. 2007. JAGGED1 and delta1 differentially regulate the outcome of experimental autoimmune encephalomyelitis. *J. Immunol.* 179: 5990–5998.
43. Minter, L. M., D. M. Turley, P. Das, H. M. Shin, I. Joshi, R. G. Lawlor, O. H. Cho, T. Palaga, S. Gottipati, J. C. Telfer, et al. 2005. Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21. *Nat. Immunol.* 6: 680–688.
44. Zhu, J., B. Min, J. Hu-Li, C. J. Watson, A. Grinberg, Q. Wang, N. Killeen, J. F. Urban, Jr., L. Guo, and W. E. Paul. 2004. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat. Immunol.* 5: 1157–1165.
45. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170: 2081–2095.