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MHC-mismatched mixed chimerism restores peripheral tolerance of noncross-reactive autoreactive T cells in NOD mice

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Autoimmune type 1 diabetes (T1D) and other autoimmune diseases are associated with particular MHC haplotypes and expansion of autoreactive T cells. Induction of MHC-mismatched but not -matched mixed chimerism by hematopoietic cell transplantation effectively reverses autoimmunity in diabetic nonobese diabetic (NOD) mice, even those with established diabetes. As expected, MHC-mismatched mixed chimerism mediates deletion in the thymus of host-type autoreactive T cells that have T-cell receptor (TCR) recognizing (cross-reacting with) donor-type antigen presenting cells (APCs), which have come to reside in the thymus. However, how MHC-mismatched mixed chimerism tolerizes host autoreactive T cells that recognize only self-MHC–peptide complexes remains unknown. Here, using NOD.Rag1$^{-/-}$.BDC2.5 or NOD.Rag1$^{-/-}$.BDC12-4.1 mice that have only noncross-reactive transgenic autoreactive T cells, we show that induction of MHC-mismatched but not -matched mixed chimerism restores immune tolerance of peripheral noncross-reactive autoreactive T cells. MHC-mismatched mixed chimerism results in increased percentages of both donor- and host-type Foxp3^{+} Treg cells and up-regulated expression of programmed death-ligand 1 (PD-L1) by host-type plasmacytoid dendritic cells (pDCs). Furthermore, adoptive transfer experiments showed that engraftment of donor-type dendritic cells (DCs) and expansion of donor-type Treg cells are required for tolerizing the noncross-reactive autoreactive T cells in the periphery, which are in association with up-regulation of host-type DC expression of PD-L1 and increased percentage of host-type Treg cells. Thus, induction of MHC-mismatched mixed chimerism may establish a peripheral tolerogenic DC and Treg network that actively tolerizes autoreactive T cells, even those with no TCR recognition of the donor APCs.

type 1 diabetes | autoreactive T cells | mixed chimerism | peripheral tolerance | hematopoietic cell transplantation

Type 1 diabetes (T1D) is a tissue-specific autoimmune disease resulting from autoimmune dysfunction and destruction of pancreatic islet β cells that produce insulin, which leads to insufficient insulin production and hyperglycemia (1–3). The nonobese diabetic (NOD) mouse is the preclinical model closest to T1D in humans and has provided an invaluable understanding of basic immune pathogenesis, genetic, and environmental risk factors and immune-targeting strategies (4). T1D pathogenesis in both NOD mice and humans is associated with a particular MHC haplotype: H2-IA^{g7} with H2-K^{d}D^{b} in NOD mice (5–7) and HLA-DQ8 or -DR4 with HLA-A2 in humans (8, 9). In both NOD mice and humans, T1D is mediated by autoreactive CD4^{+} T cells in collaboration with CD8^{+} T cells, B cells, dendritic cells (DCs), and other innate immune cells (4, 10, 11), although the severity of insulitis differs significantly in NOD mice and T1D patients (12, 13). Autoreactive CD4^{+} T cells that recognize autoantigens derived from proinsulin, GAD65 peptides, or abnormal insulin peptides are known to play a central role in the progress of T1D pathogenesis (14–17), although β cell stress and defective innate immune cells also play an initial role in T1D pathogenesis (18, 19).

The autoreactive pathogenic CD4^{+} T cells in NOD mice that recognize low-affinity autoantigens presented by H2-A^{g7} MHC II are those that escape from thymic negative selection and are exported into the periphery (20–22). Due to defective peripheral immune tolerance mechanisms, such as quantitative and qualitative decline of Treg function (23, 24), autoreactive CD4^{+} T cells are activated and play a central role in propagating and perpetuating autoimmune responses by promoting epitope spreading and CD8^{+} T-mediated autoimmunity (25). Autoreactive CD4^{+} T cells in NOD mice manifest cross-reactivity either by expression of more than one T-cell receptor (TCR) or by a single TCR recognizing more than one antigen presented by H2-A^{g7} self-MHC (26, 27). The autoreactive CD4^{+} T cells with more than one TCRα may derive from inefficient allelic exclusion of TCRα, and these dual TCR cells may interact with mismatched MHC II on the donor antigen presenting cells (APCs) (28–31).

Interactions between CD4^{+}Foxp3^{+} Treg cells and tolerogenic DCs play a critical role in maintaining peripheral tolerance and preventing activation of autoreactive T cells (32). Treg cells include thymus-derived natural Treg (tTreg) cells and peripheral antigen-specific Treg (pTreg) cells converted from conventional T (Tcon) cells during activation and differentiation. tTreg cells

Significance

Mixed chimerism has shown good potential to cure some autoimmune diseases and prevent tissue rejection. It is known that MHC-mismatched but not -matched mixed chimerism effectively tolerizes autoreactive T cells, even those noncross-reactive T cells that do not directly recognize donor-type antigen presenting cells [i.e., dendritic cells (DCs)]. How this is accomplished remains unknown. These studies have shown that tolerizing peripheral residual host-type noncross-reactive autoreactive T cells requires engraftment of donor-type DCs and involves a host-type DC-mediated increase in donor-type Treg cells, which associates with restoration of tolerogenic features of host-type plasmacytoid DCs and expansion of host-type Treg cells. This study suggests a previously unrecognized tolerance network among donor- and host-type DCs and Treg cells in MHC-mismatched mixed chimeras.

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facilitate systemic immune tolerance, and pTreg cells augment tissue-specific immune tolerance (33, 34). DCs include CD11b$^+$ myeloid DC, CD8$^+$ lymphoid DC, and B220$^+$PDCA-1$^+$ and B220$^+$PDCA-1$^-$ plasmacytoid dendritic cell (pDC) (32, 35–37). Resting DCs are tolerogenic and can tolerize activating T cells (37). tTreg cells help maintain DCs' tolerogenic status via their expression of cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) and secretion of IL-10 and TGF-β (38); in turn, tolerogenic DCs augment tTreg expansion and generation of pTreg cells via their expression of programmed death-ligand 1 (PD-L1) and production of indoleamine 2,3-dioxygenase (IDO) (39–41). Although Treg cells in T1D were found to be defective in suppression of autoimmunity (42) and Tcon cells in T1D were found to be resistant against Treg suppression (43, 44), in vivo expansion of Treg cells by administration of low-dose IL-2 or transfer of in vitro expanded Treg cells from T1D patients was able to ameliorate T1D in mice and humans (45).

CD11c^{int}B220$^+$PDCA-1$^+$ pDCs play a complicated role in T1D initiation and regulation (46, 47). Immature pDCs are often CCR9$^+$ and exhibit poor immunostimulatory ability, and their interaction with peripheral T cells often favors the generation of pTreg cells due to their production of IDO (37, 40). pDCs express TLR7 and TLR9 and produce large amounts of IFN-α in response to viral infection (48, 49). pDC production of IFN-α was associated with their role in initiating T1D pathogenesis (46), and their production of IDO was associated with their ability to control insulitis in NOD mice (47). However, the factors that dictate pDCs' pathogenic or protective role in T1D remain unclear.

Many therapies have been reported to prevent development of T1D, and dozens of therapies have been reported to reverse new-onset T1D in mice; however, none of the therapies were able to reverse new-onset T1D in patients (50). The reasons for the difficulties in reversing autoimmunity in T1D patients remain unclear. It was proposed that combination therapy that can eliminate the activated/memory autoreactive T cells and reestablish central and peripheral tolerance may be required for cure of autoimmunity in T1D patients (51). Indeed, autologous hematopoietic stem cell transplantation (HCT) with nonmyeloablative conditioning that can eliminate most activated/memory T cells has been reported to reverse new-onset T1D in some patients, although others showed relapse (52).

We and others have reported that induction of MHC-mismatched but not -matched mixed chimerism under nonmyeloablative conditioning is able to cure autoimmunity and reestablish central and peripheral tolerance of T and B cells in NOD mice with prediabetic, new-onset, or late-stage T1D (53–56). No other regimen has been reported to restore immune tolerance in NOD mice with late-stage diabetes. We have also reported that MHC-mismatched but not -matched mixed chimerism was able to mediate deletion of cross-reactive autoreactive T cells with dual TCRs in the thymus (26). However, it remains unknown why MHC-mismatched mixed chimerism is required for tolerizing noncross-reactive autoreactive T cells that recognize only self-MHC–antigen complexes; it is also unknown how MHC-mismatched mixed chimerism can tolerize the noncross-reactive autoreactive T cells that do not directly interact with the mismatched MHC.

Transgenic BDC2.5 NOD mice have transgenic CD4$^+$ T cells that recognize antigens derived from chromogranin (57, 58); BDC12-4.1 NOD mice have transgenic CD4$^+$ T cells that recognize antigens derived from proinsulin (59, 60). Importantly, when used as hosts for HCT and induction of mixed chimerism, it is found that there is a population of T cells that express dual TCRs (transgenic TCRβ coupled with transgenic TCRα or endogenous TCRα), and the transgenic T cells with dual TCRs do recognize the mismatched MHC II on donor APCs (26, 60). Induction of MHC-mismatched but not -matched mixed chimerism is able to tolerize both cross-reactive T cells that recognize mismatched MHC and noncross-reactive transgenic autoreactive CD4$^+$ T cells in BDC2.5 NOD mice (26). MHC-mismatched mixed chimerism is expected to mediate deletion of cross-reactive autoreactive CD4$^+$ T cells that possess dual TCRs in the thymus (26), but how MHC-mismatched mixed chimerism also tolerizes the noncross-reactive CD4$^+$ T cells that do not directly interact with the mismatched MHC remains unknown.

In these studies, we have used transgenic BDC2.5-Rag1$^{-/-}$ or BDC12-4.1-Rag1$^{-/-}$ mice that develop both autoimmune insulitis and T1D. Because productive TCR rearrangement requires Rag1, they only express the autoreactive transgenic TCRα and TCRβ, and they do not have the cross-reactive autoreactive T cells that have dual TCRs (26, 59, 60). Using these transgenic lines as recipients, we found that induction of MHC-mismatched but not -matched mixed chimerism effectively prevented development of insulitis or T1D; induction of MHC-mismatched but not -matched mixed chimerism results in an increase of both donor- and host-type Treg percentages as well as restoration of tolerogenic features of host-type pDC with their expression of high-level PD-L1. Therefore, induction of MHC-mismatched mixed chimerism may reestablish the peripheral tolerance network consisting of donor- and host-type DCs and Treg cells, which can effectively control autoimmunity.

Results

Induction of MHC-Mismatched but Not -Matched Mixed Chimerism Effectively Tolerizes Peripheral Host-Type Noncross-Reactive Autoreactive CD4$^+$ T Cells That Do Not Possess Dual TCRs. Our recent publication showed that NOD.Rag1$^{+/+}$.BDC2.5 mice have CD4$^+$ T cells with dual TCRs of transgenic Vα1Vβ4 and nontransgenic Vα2Vβ4. The autoreactive T cells with dual TCRs can interact with mismatched MHC, and induction of MHC-mismatched but not matched mixed chimerism led to deletion of the cross-reactive autoreactive T cells in the thymus (26). All CD4$^+$ T cells in BDC2.5-Rag1$^{-/-}$ and BDC12-4.1-Rag1$^{-/-}$ mice are transgenic T cells. They do not contain cross-reactive autoreactive T cells that can directly interact with mismatched MHC II. Consistently, we observed that induction of MHC-mismatched mixed chimerism in BDC2.5-Rag1$^{-/-}$ (Fig. S1*A*) or BDC12-4.1-Rag1$^{-/-}$ (Fig. S1*D*) host mice did not lead to reduction of percentage of host-type CD4$^+$CD8$^+$ double positive (DP) thymocytes, and their percentage of DP thymocytes was similar to that of nonchimeric mice given conditioning alone or MHC-matched mixed chimeras (Fig. S1 *B* and *E*), indicating that MHC-mismatched mixed chimerism does not mediate thymic deletion of autoreactive T cells that do not express cross-reactive TCRs. However, induction of MHC-mismatched mixed chimerism markedly reduced insulitis and prevented T1D development in both BDC2.5-Rag1$^{-/-}$ and BDC12-4.1-Rag1$^{-/-}$ mice, especially in BDC2.5-Rag1$^{-/-}$ mice (Fig. S1 *C* and *F*), which aggressively develop insulitis. Induction of MHC-matched mixed chimerism only partially reduced insulitis in both transgenic mice (Fig. S1 *C* and *F*). These results indicate that MHC-mismatched but not -matched mixed chimerism effectively tolerizes peripheral noncross-reactive autoreactive T cells, although the matched mixed chimerism can have some effect.

Next, we investigated how MHC-mismatched mixed chimerism tolerizes noncross-reactive autoreactive T cells in BDC2.5-Rag1$^{-/-}$ and BDC12-4.1-Rag1$^{-/-}$ mice. Since all of the T cells in those transgenic mice are the transgenic autoreactive CD4$^+$ T cells, we evaluated the changes in total CD4$^+$ T cells. We first measured the activation status and cytokine production profiles of the CD4$^+$ T cells in the mixed chimeras to determine whether the residual transgenic autoreactive T cells were tolerized with anergy/exhaustion, since they were not able to mediate insulitis. Anergic/exhausted T cells are known to up-regulate expression of programmed cell death protein 1 (PD-1), down-regulate expression of IL-7Rα, and down-regulate production of cytokines (61–63). Compared with CD4$^+$ T cells of control BDC2.5-Rag1$^{-/-}$ mice given conditioning alone, splenic and pancreatic lymph node (PanLN) CD4$^+$ T cells from MHC-mismatched mixed chimeras had about threefold reduction of CD62L^{lo}CD44^{hi} effector memory T (Teff) cells (Fig. 1*A*), and the Teff cells up-regulated expression of PD-1, down-regulated expression of IL-7Rα (Fig. 1*B*), and down-regulated production of IFN-γ (Fig. 1*C* and Fig. S2*A*). In addition, similar changes in PD-1 and IL-7Rα expression as well as IFN-γ production were observed with autoreactive CD4$^+$ T cells in MHC-mismatched mixed chimeric BDC12-4.1-Rag1$^{-/-}$ mice (Figs. S2*B* and S3). IL-17 production of T cells from both chimeras was too low to be evaluated (Fig. S2).

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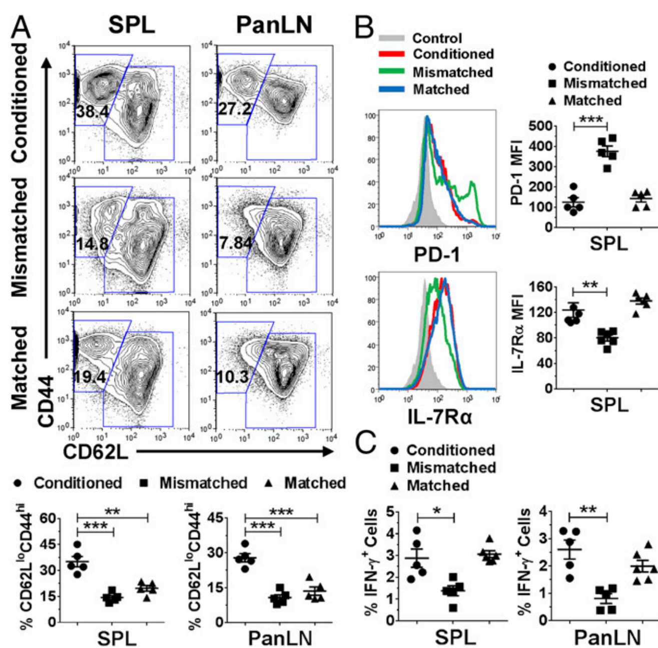

Fig. 1. Induction of MHC-mismatched mixed chimerism tolerizes the peripheral noncross-reactive autoreactive $CD4^+$ T cells in BDC2.5-$Rag1^{-/-}$ mice. After conditioning with anti-CD3 (5 mg/kg), mixed chimerism was induced in 2-wk-old female BDC2.5-$Rag1^{-/-}$ (H2-K^d, H2-D^b, H2-A^{g7}, CD45.1) mice by transplanting with BM (50×10^6) and $CD4^+$ T-depleted SPL cells (10×10^6) from MHC-mismatched C57BL/6 (H2-K^b, H2-D^b, H2-A^b, CD45.2) or MHC-matched congenic C57BL/6 (H2-K^d, H2-D^b, H2-A^{g7}, CD45.2) donors, respectively. At day 60 after HCT, the percentage of residual host-type Teff cells and the expression of surface markers were measured by flow cytometry in SPL and PanLN from control mice given anti-CD3 conditioning alone (conditioned), MHC-mismatched mixed chimeras (mismatched), and MHC-matched mixed chimeras (matched). (*A*) Representative flow cytometry pattern (*Upper*) and percentage (*Lower*) of host-type $CD62L^{lo}CD44^{hi}$ Teff cells in SPL and PanLN after gating on $CD45.1^+TCR\beta^+CD4^+$ cells ($n = 5$). Percentages of host- vs. donor-type $CD62L^{lo}CD44^{hi}$ Teff cells in SPL are 14.5 vs. 82.5% (mismatched chimeras) and 19.8 vs. 56.9% (matched chimeras; $n = 5$). Percentages of host- vs. donor-type $CD62L^{lo}CD44^{hi}$ Teff cells in PanLN are 10.8 vs. 76.9% (mismatched chimeras) and 13.6 vs. 38.8% (matched chimeras; $n = 5$). (*B*) The surface expression of PD-1 and IL-7Rα on gated host-type $CD62L^{lo}CD44^{hi}$ Teff cells in SPL. Representative histograms (*Left*) and mean fluorescence intensities (MFIs; *Right*) of PD-1 and IL-7Rα expression are shown ($n = 5$–6). (*C*) Percentages of host-type IFN-γ^+ cells in SPL and PanLN after gating on $CD45.1^+TCR\beta^+CD4^+$ cells ($n = 5$–6). Means ± SEM are shown. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

In contrast, although induction of MHC-matched mixed chimerism also reduced the percentage of Teff cells by about twofold in BDC2.5-$Rag1^{-/-}$ mice (Fig. 1*A*), interestingly, the residual Teff cells did not significantly up-regulate expression of PD-1, down-regulate expression of IL-7Rα, or down-regulate production of IFN-γ compared with control mice given conditioning alone (Fig. 1 *B* and *C*). Similarly, no reduction of PD-1 or IL-7Rα expression was observed in MHC-matched mixed chimeric BDC12-4.1-$Rag1^{-/-}$ mice (Fig. S3*B*), although IFN-γ production of $CD4^+$ T cells in the matched chimeras was reduced compared with nonchimeric mice (Figs. S2*B* and S3*C*). These results indicate that MHC-matched mixed chimerism can induce partial anergy/exhaustion of noncross-reactive T cells in the periphery of BDC2.5-$Rag1^{-/-}$ or BDC12-4.1-$Rag1^{-/-}$ mice, but only MHC-mismatched mixed chimerism effectively induces anergy/exhaustion of the noncross-reactive T cells in the periphery and prevents induction of T1D.

Induction of MHC-Mismatched but Not -Matched Mixed Chimerism Effectively Augments Expansion of Host-Type pTreg and Donor-Type tTreg Cells and Enhances Their Expression of CTLA-4. $CD4^+Foxp3^+$ Treg cells include the $Helios^+$ thymic-derived tTreg and the $Helios^-$ pTreg cells converted from $CD4^+$ Tcon cells in the periphery (64, 65). Both tTreg and pTreg cells play important roles in maintaining peripheral tolerance (64), and their expression of CTLA-4 plays an important role in their suppressor function (38). In addition, interaction of CD80 on Treg cells with PD-L1 on APCs augments tTreg survival and expansion (66), and interaction of CD80 but not PD-1 on activating $CD4^+$ Tcon cells with PD-L1 on APCs may also augment Tcon cells' conversion to pTreg cells (67). Thus, we measured the percentages and expression levels of CTLA-4, CD80, PD-1, and Helios on host- and donor-type Treg cells in the mixed chimeras.

Compared with control BDC2.5-$Rag1^{-/-}$ mice given conditioning only, the percentage of host-type Treg cells in the spleen (SPL) and PanLN of MHC-mismatched chimeras increased by more than threefold, but there was little increase in the matched mixed chimeras (Fig. 2*A*). The host-type Treg cells in the mismatched mixed chimeras up-regulated expression of CTLA-4, CD80, and PD-1, although the host-type Treg cells in the matched chimeras did not up-regulate CTLA-4 or CD80; only expression of PD-1 was up-regulated (Fig. 2*B*). Interestingly, host-type Treg cells in the mismatched and matched chimeras both had reduced expression levels of Helios and reduced percentage of $Helios^+$ tTreg cells (Fig. 2*B* and Fig. S4*A*). Additionally, similar changes of host-type Treg cells were observed in the mixed chimeric BDC12-4.1-$Rag1^{-/-}$ mice (Fig. S5). These results indicate that MHC-mismatched but not -matched mixed chimerism increases the percentage of host-type Treg cells and their expression of CTLA-4 and CD80. The lack of percentage increase of host-type Treg cells in the matched mixed chimeras is associated with their lack of up-regulation of CD80.

However, the percentage of donor-type Treg cells in the SPL and PanLN of both mismatched and matched mixed chimeras of BDC2.5-Rag-$1^{-/-}$ mice was increased, but the increase in the mismatched recipients was significantly higher than that in the matched recipients compared with the percentage of Treg cells in H2-A^b C57BL/6 or congenic H2-A^{g7} C57BL/6 donor mice before HCT (Fig. 3*A*). The donor-type Treg cells in both mismatched and matched mixed chimeras up-regulated CTLA-4, CD80, and PD-1, and no significant difference was observed (Fig. 3*B*). Interestingly, the expression levels of Helios and percentages of $Helios^+$ donor-type tTreg cells were increased in both mismatched and matched mixed chimeras (Fig. 3*B* and Fig. S4*B*). Furthermore, there was an increase in the percentage of tTreg cells among donor- but not host-type $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes in both mixed chimeras, although the increase of tTreg percentage was approximately two- to fourfold higher in the MHC-mismatched compared with -matched mixed chimeras (Fig. S6).

Additionally, similar increases in the percentage of donor-type tTreg cells and their up-regulation of CTLA-4 and PD-1 were also observed in mixed chimeric BDC12-4.1-Rag-$1^{-/-}$ mice (Fig. S7). These results indicate that both MHC-mismatched and -matched mixed chimerism augment thymic production of donor-type tTreg cells and their expression of CTLA-4 and PD-1 in the periphery. Taken collectively, MHC-mismatched but not -matched mixed chimerism effectively increases the percentage of host-type pTreg cells and their expression of CTLA-4 and CD80; MHC-mismatched mixed chimerism also markedly augments thymic production of donor-type tTreg cells in the thymus compared with matched mixed chimerism, although matched mixed chimerism can also augment donor-type tTreg production. In addition, both mismatched and matched mixed chimerism augment donor-type tTreg cells expression of CTLA-4 and PD-1.

Induction of MHC-Mismatched but Not -Matched Mixed Chimerism Up-Regulates Host-Type Plasmacytoid DC Expression of PD-L1. pDCs are identified as $CD11c^{int}B220^+PDCA\text{-}1^+$ and $CD11c^{int}B220^+PDCA\text{-}1^-$ (35, 37). PD-L1 is up-regulated by tolerogenic DCs (68), and PD-L1 on DCs was reported to augment pTreg differentiation (69, 70). Our previous work showed that host-type APC expression of PD-L1 augmented tTreg expansion early after HCT via interaction with CD80 on donor tTreg cells (66).

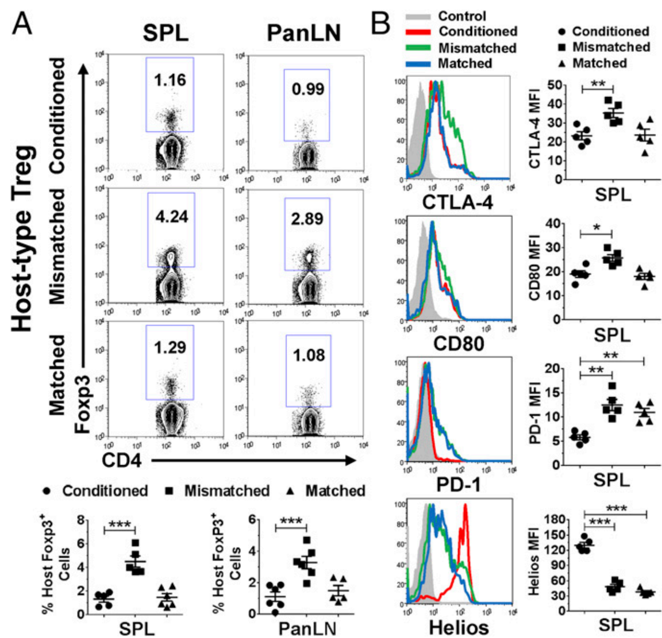

Fig. 2. Induction of MHC-mismatched mixed chimerism augments expression of host-type pTreg cells and enhances their expression of CTLA-4 in BDC2.5-Rag1$^{-/-}$ mice. As described in Fig. 1, mixed chimerism was induced in BDC2.5-Rag1$^{-/-}$ mice using MHC-mismatched or -matched C57BL/6 donors. At day 60 after HCT, the percentage of host-type Foxp3^{+} T cells and the expression of surface markers were measured by flow cytometry in SPL or PanLN. (*A*) Representative flow cytometry pattern (*Upper*) and percentage (*Lower*) of host-type CD4^{+}Foxp3^{+} T cells after gating on CD45.1^{+}TCRβ^{+} cells in SPL and PanLN (n = 5–6). (*B*) The surface expression of CTLA-4, CD80, PD-1, and Helios on host-type CD45.1^{+}TCRβ^{+}CD4^{+}Foxp3^{+} T cells in SPL. Representative histograms (*Left*) and mean fluorescence intensities (MFIs; *Right*) of CTLA-4, CD80, PD-1, and Helios expression are shown (n = 5). Means ± SEM are shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Thus, we evaluated the impact of induction of mixed chimerism on host- and donor-type pDC subset changes and their expression of PD-L1. We observed that pDCs in control BDC2.5-Rag1$^{-/-}$ mice given conditioning alone were predominantly CD11c^{int}B220^{+}PDCA-1^{+}; similarly, pDCs in MHC-matched and MHC-mismatched mixed chimeras were also predominantly CD11c^{int}B220^{+}PDCA-1^{+} (Fig. 4*A*). However, MHC-mismatched mixed chimeras had reduced percentages of pDCs, and the residual pDCs expressed higher levels of PD-L1 and MHC II, although there was no significant difference in their expression of CD80 and CD86 compared with the control mice. However, MHC-matched mixed chimeras did not have significant changes in the percentage of host-type pDCs or their expression of PD-L1, MHC II, CD80, or CD86 (Fig. 4*A*). Similar changes of host-type DCs were also observed in MHC-mismatched and -matched mixed chimeras of BDC12-4.1-Rag1$^{-/-}$ mice (Fig. S8). These results indicate that induction of MHC-mismatched but not -matched mixed chimerism can modulate host-type pDCs and restore their tolerogenic feature of high expression levels of PD-L1.

It is of interest that, although there were both CD11c^{int}B220^{+} PDCA-1^{+} and CD11c^{int}B220^{+}PDCA-1^{-} DCs in both H2-A^{b} and H2-A^{g7} C57BL/6 donor mice before HCT, donor-type DCs seemed to be predominantly CD11c^{int}B220^{+}PDCA-1^{-} in both mismatched and matched mixed chimeras compared with before HCT (Fig. 4*B*). CD11c^{int}B220^{+}PDCA-1^{-} pDCs also expressed PD-L1, although the PD-L1 expression levels did not seem to be higher than before transplantation (Fig. 4*B*). These results indicate that induction of MHC-matched and -mismatched mixed chimerism has a similar impact on donor-type DCs but that only induction of MHC-mismatched mixed chimerism leads to host-type pDC expression of high levels of PD-L1.

Next, using Foxp3-GFP donor-type Treg cell and histo-immunofluorescent staining, we tested whether donor-type Treg cells could interact with MHC-mismatched host-type DCs, as interactions between Treg cells and pDCs play a critical role in maintaining the tolerance feature of pDCs (71). Indeed, we found that donor-type Treg cells did touch both host- and donor-type DCs in the SPL of MHC-mismatched mixed chimeras (Fig. 4*C*). This observation suggests that donor-type Treg cells in MHC-mismatched chimeras not only interact with donor-type DCs but also, interact with host-type DCs. Taken collectively, donor-type Treg cells in MHC-mismatched chimeras may be able to interact with both donor- and host-type DCs, and this interaction may contribute to restoration of the tolerogenic features of host-type pDC in NOD mice.

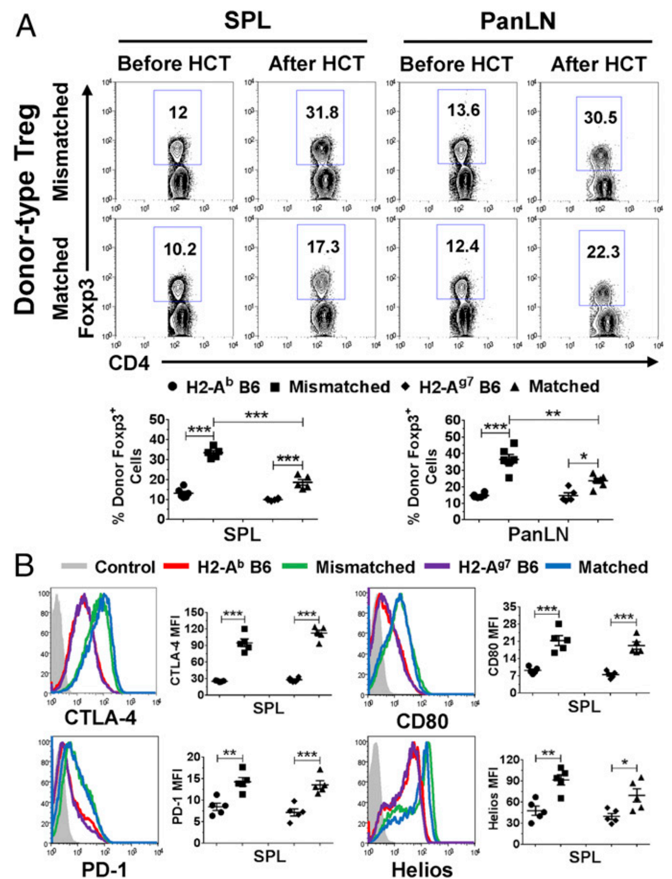

Fig. 3. Induction of MHC-mismatched mixed chimerism augments expression of donor-type tTreg cells and enhances their expression of CTLA-4 in BDC2.5-Rag1$^{-/-}$ mice. As described in Fig. 1, mixed chimerism was induced in BDC2.5-Rag1$^{-/-}$ mice using MHC-mismatched or -matched C57BL/6 donors. At day 60 after HCT, the percentage of donor-type Foxp3^{+} T cells and the expression of surface markers were measured by flow cytometry in SPL or PanLN. (*A*) Representative flow cytometry pattern (*Upper*) and percentage (*Lower*) of donor-type CD4^{+}Foxp3^{+} T cells in SPL and PanLN after gating on CD45.2^{+}TCRβ^{+} cells (n = 5–6). The flow cytometry pattern and percentage of TCRβ^{+}CD4^{+}Foxp3^{+} T cells in SPL and PanLN of wild-type H2-A^{b} C57BL/6 (H2-A^{b} B6) and congenic H2-A^{g7} C57BL/6 (H2-A^{g7} B6) mice were taken as "before HCT" control. (*B*) The surface expression of CTLA-4, CD80, PD-1, and Helios on donor-type CD45.2^{+}TCRβ^{+}CD4^{+}Foxp3^{+} T cells in SPL. Representative histograms (columns 1 and 3) and mean fluorescence intensities (MFIs; columns 2 and 4) of CTLA-4, CD80, PD-1, and Helios expression are shown (n = 5). The histograms and MFIs of CTLA-4, CD80, PD-1, and Helios in H2-A^{b} C57BL/6 and H2-A^{g7} C57BL/6 mice were taken as before HCT control. Means ± SEM are shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

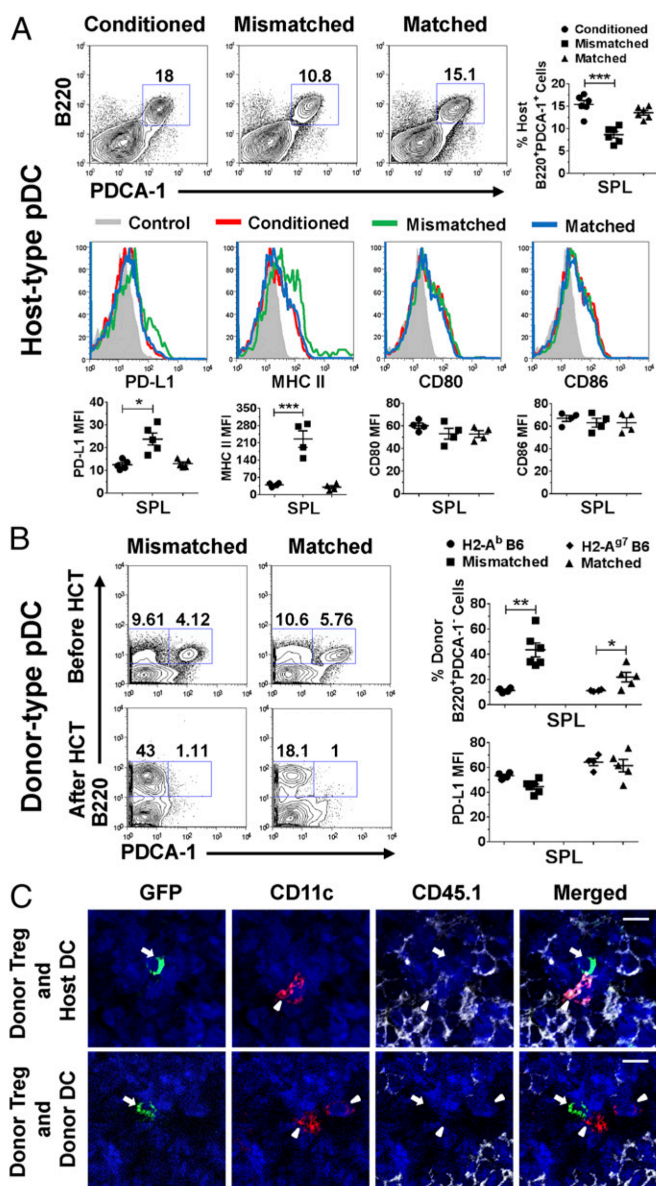

Fig. 4. Induction of MHC-mismatched mixed chimerism modulates host-type pDCs and up-regulates their expression of PD-L1 in BDC2.5-Rag1$^{-/-}$ mice. As described in Fig. 1, mixed chimerism was induced in BDC2.5-Rag1$^{-/-}$ mice using MHC-mismatched or -matched C57BL/6 donors. At day 60 after HCT, the percentage of host- and donor-type pDCs and the expression of surface markers were measured by flow cytometry in SPL. (*A*) Representative flow cytometry pattern (*Top Left*) and percentage (*Top Right*) of host-type B220$^+$PDCA-1$^+$ pDCs in SPL after gating on CD45.1$^+$CD11c^{int} cells (n = 6). Representative histograms (*Middle*) and mean fluorescence intensities (MFIs; *Bottom*) of PD-L1, MHC II, CD80, and CD86 expression on host-type B220$^+$PDCA-1$^+$ pDCs are shown (n = 4–5). (*B*) Representative flow cytometry pattern (*Left*) of donor-type B220$^+$PDCA-1$^-$ and B220$^+$PDCA-1$^+$ pDCs in SPL after gating on IgM$^-$IgD$^-$CD45.2$^+$CD11c^{int} cells. Percentage (*Upper Right*) of donor-type B220$^+$PDCA-1$^-$ pDCs and MFIs (*Lower Right*) of their PD-L1 expression are shown (n = 4–6). The flow cytometry patterns and MFIs of pDCs in H2-A^b C57BL/6 and H2-A^{g7} C57BL/6 mice were taken as before HCT control. (*C*) As described in Fig. 1, mixed chimerism was induced in BDC2.5-Rag1$^{-/-}$ mice using MHC-mismatched Foxp3 (ki)-DTR-EGFP C57BL/6 donors on day 0. Frozen SPL sections from recipients at day 60 after HCT were stained for GFP (green), CD11c (red), CD45.1 (white), and DAPI (blue), and merged staining is also shown. Arrows point to Treg cells, and arrowheads point to DCs. One representative staining pattern in SPL is shown from three replicated experiments. Means ± SEM are shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (Scale bars, 10 μm.)

Tolerizing Peripheral Noncross-Reactive Autoreactive T Cells Requires Engraftment of Donor-Type DCs and Relative Expansion of Donor-Type Treg Cells, Which Are in Association with Up-Regulating Expression of PD-L1 by Host-Type DCs and Relative Expansion of Host-Type Treg Cells. As described above, transgenic noncross-reactive autoreactive CD4$^+$ T cells in the SPL and PanLN of MHC-mismatched mixed chimeric BDC2.5-Rag1$^{-/-}$ or BDC12-4.1-Rag1$^{-/-}$ mice were anergic/exhausted (Fig. 1 and Fig. S3), which was associated with an increased percentage of donor- and host-type Treg cells as well as up-regulation of PD-L1 by host-type DCs. To further validate the role of donor- and host-type Treg cells and DCs in the maintenance of peripheral tolerance in MHC-mismatched chimeras, we first tested using in vitro cultures. Sorted host-type CD4$^+$ T cells that contain both Tcon cells and Treg cells from MHC-mismatched mixed BDC12-4.1-Rag1$^{-/-}$ chimeras were cocultured with (*i*) host-type DCs pulsed with proinsulin peptide with or without the presence of donor-type CD4$^+$ T cells that contains both Tcon cells and Treg cells from the same recipients, (*ii*) donor-type DCs pulsed with proinsulin peptide with the presence of donor-type T cells, or (*iii*) host-type DCs pulsed with proinsulin peptide with the presence of donor-type T cells and donor-type DCs.

We found that host-type T cells proliferated in response to host-type DC/peptide stimulation, although there was no response to donor-type DC/peptide stimulation. Presence of donor-type T cells and/or donor-type DCs had no impact (Fig. S9). These results indicate that anergic/exhausted CD4$^+$ T cells from the tolerant MHC-mismatched chimeras can still proliferate in vitro in response to autoantigen/DC stimulation, suggesting that the anergy/exhaustion status of autoreactive T cells in the mixed chimeras is actively maintained. These results also suggest that the dynamic interactions among donor- and host-type Treg cells and DCs cannot be easily revealed with in vitro assays.

Next, we used an in vivo system to dissect the requirement for maintaining tolerance status of noncross-reactive host-type autoreactive CD4$^+$ T cells. Host- or donor-type T cells were transferred into adoptive NOD-SCID recipients with or without preengraftment of donor-type DCs. A mixture of sorted host- and donor-type CD4$^+$ T cells (0.25×10^6 each), which contains host- and donor-type Treg cells, from the SPL of the primary mixed chimeric BDC2.5-Rag1$^{-/-}$ mice was injected into either adoptive NOD-SCID mice or NOD-SCID mice preengrafted with donor-type wild-type or MHC II$^{-/-}$ DCs. Surprisingly, the injected T cells in mice without donor APCs rapidly induced T1D within 20 d after cell transfer, and the mice showed severe insulitis (Fig. 5*A* and Fig. S10*A*). The diabetic NOD-SCID recipients had only host-type DCs and Mac1/Gr1 cells in bone marrow (BM) (Fig. S11); they had both donor- and host-type CD4$^+$ T cells, but very few donor- and host-type Treg cells (Fig. 5 *B*, *Top* and *C*).

In contrast, the injected T cells did not induce any T1D or insulitis in the adoptive recipients with preengrafted wild-type donor-type DCs that express H2-A^b (Fig. 5*A* and Fig. S11). However, the majority (four of seven) of adoptive recipients with MHC II$^{-/-}$ donor-type DCs that did not express H2-A^b developed T1D, and the residual recipients showed severe insulitis (Fig. 5*A* and Figs. S10*A* and S11). In addition, we found that there was a marked increase in the percentage of Treg cells among donor- or host-type CD4$^+$ T cells in the adoptive recipients with preengrafted wild-type donor-type DCs compared with the adoptive recipients with MHC II$^{-/-}$ donor-type DCs or without donor-type DCs (Fig. 5 *B*, *Middle* and *Bottom* and *C*). These results indicate that inhibition of autoreactive CD4$^+$ T cells and prevention of induction of T1D require relative expansion of donor-type Treg cells and engraftment of donor-type DCs that express MHC II.

Furthermore, we tested the role of host-type DCs in expansion of donor- and host-type Treg cells by transferring host-type T cells alone or host-type T plus donor-type T cells into the NOD-SCID mice with donor-type APCs only (Fig. 5*D* and Fig. S11). None of the recipients showed T1D development (Fig. 5*D*). Consistently, almost no host-type T cells were detectable in those adoptive recipients (Fig. 5*E*). Although

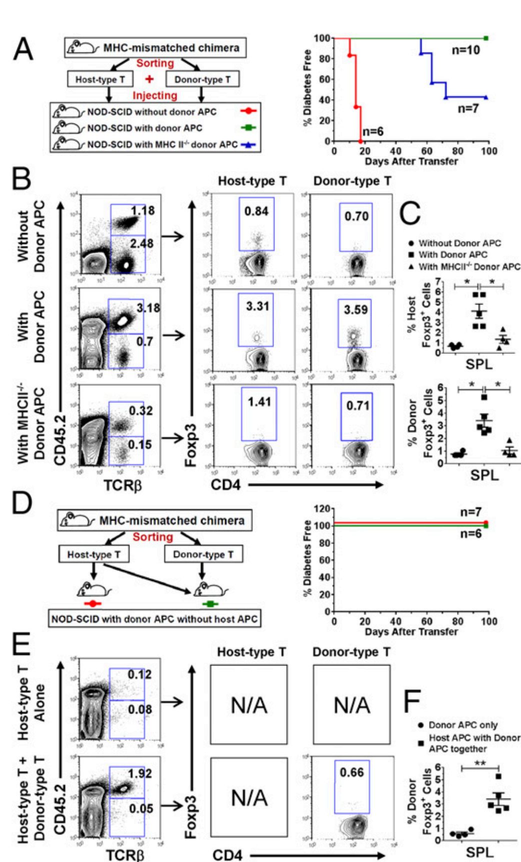

Fig. 5. Coengraftment of donor- and host-type DCs is required for tolerizing peripheral noncross-reactive autoreactive T cells. As described in Fig. 1, mixed chimerism was induced in BDC2.5-Rag1$^{-/-}$ mice using MHC-mismatched C57BL/6 donors. At day 60 after HCT, the recipient SPLs were harvested to sort the host-type T cells (CD45.1^{+}TCRβ^{+}CD4^{+}) and donor-type T cells (CD45.2^{+}TCRβ^{+}CD4^{+}) for transferring into different NOD-SCID adoptive recipients. (*A*–*C*) We first engrafted NOD-SCID (H2-K^{d}, H2-D^{b}, H2-A^{g7}, CD45.1) mice with donor-type DCs by transplanting BM cells from Rag2$^{-/-}$ C57BL/6 (H2-K^{b}, H2-D^{b}, H2-A^{b}, CD45.2) mice. NOD-SCID mice were irradiated with 100 cGy and reconstituted with 8 × 10^{6} BM from Rag2$^{-/-}$ C57BL/6 or MHC II$^{-/-}$ Rag2$^{-/-}$ C57BL/6 mice. At day 30 after HCT, the coexistence of host- and donor-type Mac1/Gr1^{+} cells in the peripheral blood was determined by flow cytometry in those NOD-SCID mice. The coexistence of host- and donor-type CD11c^{+} DCs was confirmed by checking BM cells in three to four NOD-SCID recipients. Thereafter, host- and donor-type T cells (0.25 × 10^{6} each) sorted from MHC-mismatched mixed chimeric BDC2.5-Rag1$^{-/-}$ mice were injected into the following mice: NOD-SCID mice without donor APCs and NOD-SCID mice preengrafted with donor APCs from Rag2$^{-/-}$ or MHC II$^{-/-}$ Rag2$^{-/-}$ C57BL/6 mice. Diabetes development was monitored weekly by both urine and blood glucose for up to 100 d. At the end of the experiment, the percentages of host- and donor-type T cells and Treg cells in SPL were measured by flow cytometry. (*A*) Experimental scheme (*Left*) and diabetes development curve (*Right*) of NOD-SCID recipients. (*B*) Representative flow cytometry pattern of host- and donor-type T cells (*Left*) and Treg cells (*Right*) in SPL of NOD-SCID recipients without donor APCs, with donor APCs, and with MHC II$^{-/-}$ donor APCs. The arrows indicate the gating strategy. (*C*) Percentages of host-type (*Upper*) and donor-type (*Lower*) TCRβ^{+}CD4^{+}Foxp3^{+} T cells in SPL of NOD-SCID recipients (*n* = 4–5). (*D*–*F*) NOD-SCID mice were irradiated with 200 cGy and reconstituted with 12 × 10^{6} BM from Rag2$^{-/-}$ C57BL/6 mice to establish NOD-SCID mice with total replacement of host-type DCs with donor-type DCs. At day 30 after HCT, complete replacement of host-type Mac1/Gr1^{+} cells with donor type in the peripheral blood was detected by flow cytometry in those NOD-SCID mice. Replacement of host-type CD11c^{+} DCs with donor type was confirmed by checking BM cells in three to four NOD-SCID recipients. Thereafter, host-type T cells (0.25 × 10^{6} each) alone or host-type T cells (0.25 × 10^{6} each) plus donor-type T cells (0.25 × 10^{6} each) sorted from MHC-mismatched mixed chimeric BDC2.5-Rag1$^{-/-}$ mice were injected into NOD-SCID mice with the complete donor APCs. Diabetes development was monitored weekly by both urine and blood glucose for up to 100 d. At the end of experiment, the percentages of host- and donor-type T cells and Treg cells in SPL were measured by flow cytometry. (*D*) Experimental scheme (*Left*) and diabetes development curve (*Right*) of NOD-SCID recipients. (*E*) Representative flow cytometry pattern of host- and donor-type T cells (*Left*) and Treg cells (*Right*) in SPL of NOD-SCID recipients. The arrows indicate the gating strategy. (*F*) Percentage of Treg cells in SPL of NOD-SCID recipients with donor-type APCs only and with host- and donor-type APCs together (*n* = 4–5). Means ± SEM are shown. N/A, not available. *$P < 0.05$; **$P < 0.01$.

donor-type CD4^{+} T cells were detectable in those adoptive recipients, the percentage of donor-type Treg cells was markedly lower than that in the recipients with both donor- and host-type DCs (Fig. 5 *E* and *F*). These results indicate that (*i*) survival of host-type CD4^{+} T and Treg cells requires the presence of host-type DCs and (*ii*) expansion of donor-type Treg cells requires the presence of host-type DCs.

To further test the impact of donor-type Treg cells on host-type DC PD-L1 expression and host-type Treg expansion, host-type CD4^{+} T cells (0.25 × 10^{6}) containing both Tcon cells and Treg cells were injected alone or together with sorted donor-type Foxp3-GFP^{+}CD4^{+} Treg cells (0.1 × 10^{6}) from the same mixed chimeric BDC2.5-Rag1$^{-/-}$ mice into adoptive NOD-SCID mice preengrafted with donor-type DCs. Host-type CD4^{+} T cells alone induced T1D in all recipients (six of six), although none of recipients (zero of six) given both host-type CD4^{+} T cells and donor-type Treg cells showed T1D or insulitis (Fig. 6*A* and Fig. S10*B*). In addition, although injection of host-type CD4^{+} T cells alone resulted in little host-type Treg expansion, coinjection with donor-Treg cells led to expansion of host-type Treg cells (Fig. 6 *B* and *C*) as well as decrease of percentage of host-type CD11c^{int}B220^{+}PDCA-1^{+} pDCs (Fig. 6*D*), with residual pDCs up-regulating expression of PD-L1 (Fig. 6*E*). These results indicate that donor-type Treg cells are required for modulation of host-type pDCs and augmentation of their expression of PD-L1; donor-type Treg cells are also required for expansion of host-type Treg cells in the MHC-mismatched chimeras.

Discussion

Using transgenic BDC2.5-Rag1$^{-/-}$ (and also BDC12-4.1-Rag1$^{-/-}$) mice that possess only noncross-reactive autoreactive CD4^{+} T cells that do not directly interact with mismatched MHC II, we have shown that tolerizing noncross-reactive autoreactive CD4^{+} T cells (which recognize only self-MHC–peptide complexes) by induction of MHC-mismatched mixed chimerism requires coengraftment of donor- and host-type DCs and relative expansion of donor-type tTreg cells that can interact with MHC-mismatched host-type DCs, which are in association with host-type DC expression of high-level PD-L1 and relative expansion of host-type pTreg cells. Our observations are consistent with those of a very recent publication: that hematopoietic stem cells in NOD mice have a defect in expression of PD-L1 and that up-regulation of the stem cell expression of PD-L1 via gene therapy or pharmacological agents can prevent autoimmunity in NOD mice (72).

We observed that residual noncross-reactive autoreactive CD4^{+} T cells from MHC-mismatched but not -matched mixed chimeras had an anergy/exhaustion phenotype, with up-regulated expression of PD-1 and down-regulated expression of IL-7Rα as well as reduced production of proinflammatory IFN-γ. However, the anergic/exhausted autoreactive CD4^{+} T cells from the tolerant NOD mice with MHC-mismatched mixed chimerism still proliferated vigorously in vitro in response to syngeneic DC/autoantigen stimulation. Addition of donor- and host-type Treg cells did not suppress proliferation. These anergic/exhausted T cells also induced T1D in NOD-SCID mice in the absence of preengrafted donor-type DCs or in the absence of donor-type Treg cells. These observations indicate that the tolerant status of autoreactive T cells in MHC-mismatched chimeras is actively maintained by a network consisting of donor- and host-type DCs and Treg cells and that each of the four elements may be indispensable. These observations also suggest that in vitro proliferation assays are not suitable for evaluating the in vivo tolerance status of the noncross-reactive autoreactive T cells.

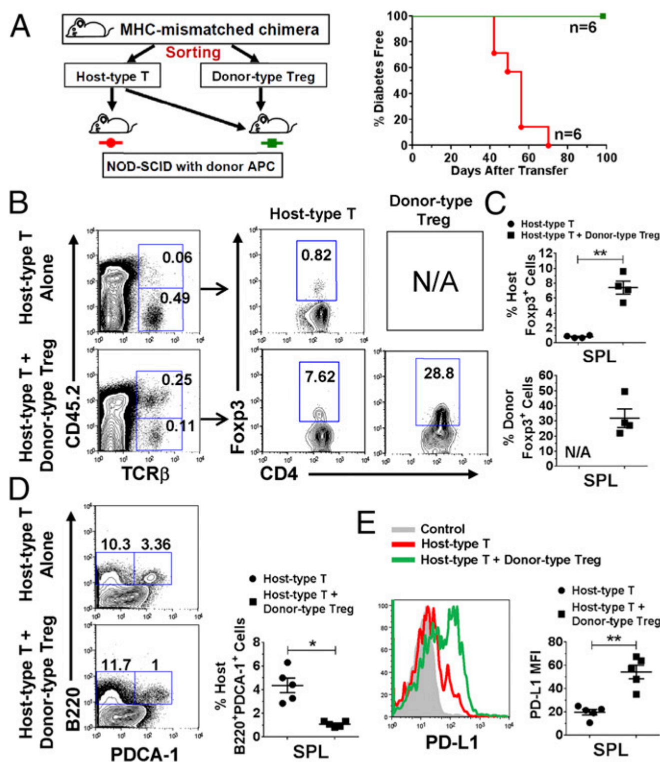

Fig. 6. Donor-type Treg cells are required for up-regulation of host-type DC expression of PD-L1 and tolerization of peripheral noncross-reactive autoreactive T cells. As described in Fig. 1, mixed chimerism was induced in BDC2.5-Rag1$^{-/-}$ mice using MHC-mismatched Foxp3 (ki)-DTR-EGFP C57BL/6 donors. At day 60 after HCT, the recipient SPLs were harvested to sort host-type T cells (CD45.1$^+$TCRβ$^+$CD4$^+$) and donor-type Treg cells (CD45.2$^+$TCRβ$^+$CD4$^+$Foxp3-GFP$^+$) for transferring into adoptive NOD-SCID recipients that were preengrafted with donor-type APCs as described in Fig. 5 *A*–*C*. Thereafter, host-type T cells (0.25 × 10^6 each) alone or host-type T cells (0.25 × 10^6 each) plus donor-type Treg cells (0.1 × 10^6 each) sorted from MHC-mismatched mixed chimeric BDC2.5-Rag1$^{-/-}$ mice were injected into the NOD-SCID mice preengrafted with donor APCs. Diabetes development was monitored weekly by both urine and blood glucose for up to 100 d. At the end of experiment, the percentages of host- and/or donor-type T cells, Treg cells, and pDCs were measured by flow cytometry in SPL. (*A*) Experimental scheme (*Left*) and diabetes development curve (*Right*) of NOD-SCID recipients. (*B*) Representative flow cytometry pattern of host- and donor-type T cells (*Left*) and Treg cells (*Right*) in SPL. The arrows indicate the gating strategy. (*C*) Percentage of host-type (*Upper*) and donor-type (*Lower*) Treg cells in SPL of NOD-SCID recipients (n = 4). (*D*) Representative flow cytometry pattern (*Left*) and percentage (*Right*) of host-type CD45.1$^+$CD11c^{int}B220$^+$PDCA-1$^+$ pDCs in SPL. (*E*) The histograms (*Left*) and mean fluorescence intensities (MFIs; *Right*) of PD-L1 expression on host-type CD45.1$^+$CD11c^{int}B220$^+$PDCA-1$^+$ pDCs are also shown (n = 5). Means ± SEM are shown. N/A, not available. *P < 0.05; **P < 0.01.

We observed that, in MHC-mismatched chimeras, there was an relative expansion of donor-type tTreg cells and relative expansion of host-type pTreg cells in the peripheral SPL and PanLN as judged by increased percentages of total Treg cells in both, increased percentage of Helios$^+$ tTreg cells among donor-type Treg cells, and increased percentage of Helios$^-$ pTreg cells among host-type Treg cells. The increase of donor-type tTreg cells was most likely due to increased production of tTreg cells in the thymus, because the percentage of tTreg cells was significantly increased among donor-type CD4$^+$CD8$^+$ (DP) and CD4$^+$CD8$^-$ single positive (SP) thymocytes in MHC-mismatched mixed chimeras compared with that in the thymus of donor mice before HCT.

The increased thymic production of C57BL/6 (H2-A^b) donor-type tTreg cells may result from positive selection of donor-type Treg cells by the H2-A^{g7}–donor antigen complex on host-type DCs in the thymic medullary. We observed a markedly increased percentage of tTreg cells among CD4$^+$CD8$^+$ (DP) and CD4$^+$CD8$^-$ (SP) thymocytes in the thymus of MHC-mismatched mixed chimeric NOD (H2-A^{g7}) mice but not in the MHC-mismatched mixed chimeric SJL/J (H2-A^S) mice (73), suggesting that host expression of H2-A^{g7} plays an important role. In addition, the percentage of donor-type tTreg cells was also significantly increased in MHC-matched mixed chimeric NOD mice given transplants from congenic H2-A^{g7} C57BL/6 mice, although it was less than in the MHC-mismatched mixed chimeras, suggesting that H2-A^{g7} presenting minor antigens from C57BL/6 donors also play an important role. Based on these observations, we theorize that the donor-type (H2-A^b) T cells are first positively selected by host-type cortical epithelial cells that express H2-A^{g7} MHC, and then, Foxp3 expression is augmented at the thymic medullary by interaction with host-type APCs that present donor-type "alloantigens." MHC–alloantigen complexes often have high affinity for TCRs (53, 74), and the high-affinity TCR interaction with thymic medullar APCs can augment tTreg development in the thymus, although it augments negative selection of Tcon cells (64, 74). In addition, donor-type tTreg cells could interact with host-type DCs in the periphery, and this interaction might lead to donor-type tTreg cells up-regulating expression of CTLA-4 and their

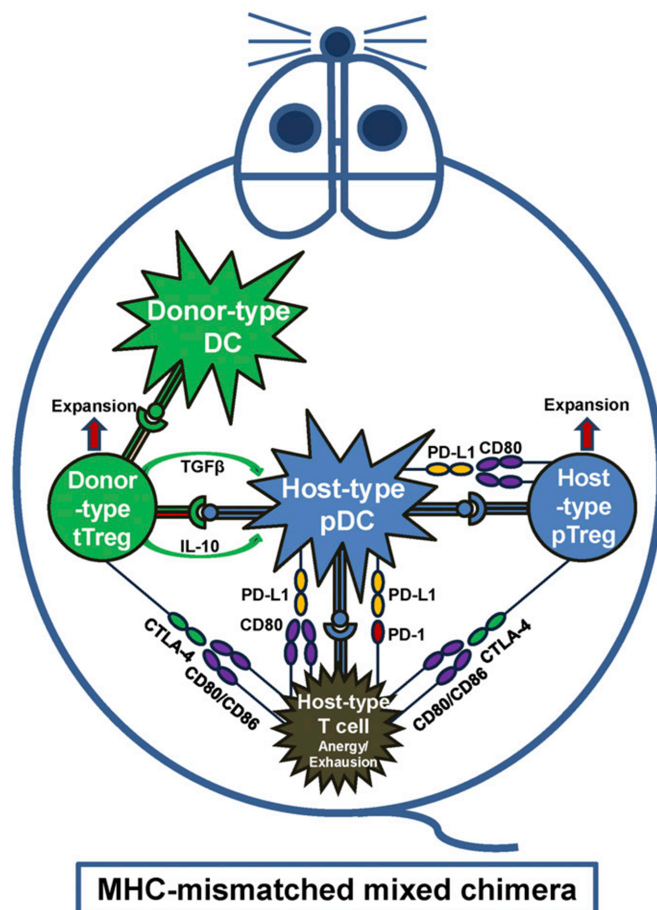

Fig. 7. Hypothetical diagram depicts the interactions among donor-type tTreg cells and DCs and among host-type pTreg cells and DCs. In MHC-mismatched chimeras, donor-type tTreg cells interact with donor-type DCs in the periphery, leading to donor-type tTreg cell activation and expression of CTLA-4. The donor-type tTreg cells interact with host-type pDCs and trigger pDCs to up-regulate expression of PD-L1 via tTreg cell secretion of TGF-β and IL-10, restoring the tolerogenic feature of pDCs. The host-type tolerogenic pDCs interact with host-type pTreg cells and augment their expansion via PD-L1: CD80. Finally, donor-type tTreg cells, host-type pTreg cells, and tolerogenic host-type DCs work synergistically via CTLA-4 and PD-L1 to tolerize noncross-reactive autoreactive T cells.

expansion. This hypothesis can also explain why donor-type tTreg cells failed to expand in the adoptive NOD-SCID recipients that did not have a donor- or host-type hematopoietic system.

We observed an expansion of host-type Helios^{-} pTreg cells in the periphery of MHC-mismatched but not -matched mixed chimeras. This pTreg expansion may result from host-type pTreg interaction with host-type DCs, especially with pDCs via PD-L1/CD80 interactions. We found that, in the MHC-mismatched chimeras, host-type pDCs expressed high levels of PD-L1 and that the host-type Treg cells expressed high levels of CD80, PD-1, and CTLA-4. In contrast, in the MHC-matched chimeras, host-type pDCs did not express high levels of PD-L1, and host-type Treg cells expressed high levels of PD-1 but not CD80 or CTLA-4. Although PD-L1–mediated signaling was reported to augment expansion of pTreg cells (70, 75), PD-L1/PD-1 interaction was found to inhibit pTreg expansion (67). We previously reported that interaction of CD80 on activated Treg cells with PD-L1 on APCs augmented Treg cell survival and expansion (66), although the same interaction augmented activated Tcon cell apoptosis (76). Therefore, expression of high levels of PD-L1 on host-type pDCs may contribute to the expansion of host-type antigen-specific pTreg cells in MHC-mismatched chimeras.

We observed that, in MHC-mismatched mixed chimeras, host-type Treg expansion required the presence of both donor-type Treg cells and host-type DCs. Also, we found that host-type pDC expression of high levels of PD-L1 required the presence of donor-type Treg cells. These observations indicate that donor-type Treg cells may play an important role in modulating MHC-mismatched host-type DCs. As discussed above, donor-type Treg cells may recognize host-type H2-A^{g7} DCs that present minor antigens derived from donor-type hematopoietic cells. The interaction between donor-type Treg cells and donor-type DCs may lead to donor-type Treg cells up-regulating expression of CTLA-4. The interaction between donor-type Treg cells and host-type pDCs may lead to reduction of host-type pDCs and up-regulation of expression of PD-L1 by the residual host-type pDCs. The activated donor-type Treg cells may eliminate the activated pathogenic pDCs, resulting in reduction in the percentage of pDCs. We previously reported that activated tTreg cells killed APCs to down-regulate autoimmune-like chronic graft-versus-host disease (GvHD) (77). The donor-type tTreg cells may up-regulate host-type pDC expression of PD-L1 via their production of TGF-β, because TGF-β from tTreg cells is known to regulate expression of PD-L1 (78). Expression of high levels of PD-L1 is a feature of tolerogenic DCs that can augment conversion of Tcon cells to pTreg cells as well as augment expansion of pTreg cells as mentioned above. It is of interest to note that, in MHC-matched mixed chimeras, there was no expansion of host-type pTreg cells or up-regulation of PD-L1 by host-type pDCs. This observation further indicates that donor-type tTreg cells that can interact with mismatched host-type MHC II play a critical role in restoring the host-type peripheral immune tolerance network of pDCs and Treg cells.

We reported that MHC-mismatched but not -matched mixed chimerism was able to effectively prevent and reverse T1D in NOD mice (53, 79). Other than our previous report showing that MHC-mismatched but not -matched mixed chimerism was able to mediate deletion of cross-reactive autoreactive T cells with dual TCRs (53, 79), we also found differences in transgenic NOD mice of noncross-reactive autoreactive T cells: (*i*) MHC-mismatched mixed chimerism causes stronger augmentation of thymic production of donor-type tTreg cells compared with the matched mixed chimerism, (*ii*) MHC-mismatched but not -matched mixed chimerism augments expansion of the peripheral pTreg cells and their expression of CTLA-4 as well as augments host-type pDC expression of PD-L1, and (*iii*) MHC-mismatched but not -matched mixed chimerism leads to residual autoreactive T-cell anergy/exhaustion, with manifestation of up-regulating expression of PD-1 and down-regulating expression of IL-7Rα.

In summary, we have shown that both donor-type DCs and Treg cells are required to tolerize the noncross-reactive autoreactive T cells that cannot directly interact with donor-type MHC in the MHC-mismatched mixed chimeras. In addition, we have also found that the tolerance status is in association with host-type pDC expression of high levels of PD-L1 and expansion of host-type pTreg cells. Here, we propose an overarching hypothesis to link our findings together. As depicted in Fig. 7, after induction of MHC-mismatched mixed chimerism, the thymus of the chimera increases production of donor-type tTreg cells that can interact with MHC-matched donor-type and/or MHC-mismatched host-type DCs. Donor-type tTreg interaction with donor-type DCs in the periphery leads to their activation and up-regulation of CTLA-4 expression; the donor-type tTreg cells interact with host-type pDCs via their secretion of TGF-β and IL-10 to up-regulate pDC expression of PD-L1 and restore their tolerogenic features. The host-type tolerogenic pDC interaction with host-type Tcon cells and pTreg cells via their PD-L1 with CD80 on the T cells leads to expansion of host-type pTreg. Finally, donor-type tTreg cells, tolerogenic host-type DCs, and host-type pTreg cells work synergistically via their surface PD-L1 or CTLA-4 to tolerize the noncross-reactive autoreactive T cells. Other interactions among costimulatory and coinhibitory receptors may also be involved in the interactions, although they are not shown.

In conclusion, MHC-mismatched mixed chimerism not only is able to mediate deletion of cross-reactive autoreactive T cells in the thymus, as shown in our previous publications (26, 53), but also, is able to tolerize the noncross-reactive autoreactive T cells in the periphery, as shown in these studies. In both situations, mismatched donor MHC II plays a critical role. Therefore, induction of MHC-mismatched mixed chimerism is a powerful approach for reestablishing central and peripheral immune tolerance in autoimmune mice. Whether a particular donor-type MHC is required and whether haploidentical mixed chimerism is able to restore immune tolerance remain important questions.

Materials and Methods

NOD.Rag1$^{-/-}$, NOD.Rag1$^{+/+}$.BDC2.5, NOD.Rag1$^{-/-}$.BDC12-4.1, NOD-SCID, congenic H2-A^{g7} C57BL/6, MHC II$^{-/-}$ C57BL/6, Rag2$^{-/-}$ C57BL/6, and Foxp3 (ki)-DTR-EGFP C57BL/6 were purchased from the Jackson Laboratory. Wild-type H2-A^{b} C57BL/6 mice were purchased from the National Cancer Institute. All mice were maintained in a pathogen-free room at the City of Hope (COH) Animal Research Center. The experimental procedures were approved by the COH Institutional Animal Care and Use Committee. Induction of mixed chimerism, flow cytometry staining and analysis, histopathology and immunofluorescence staining, T-cell proliferation assay, T-cell transfer to NOD-SCID recipients, and statistical analysis were described in our previous publications (26, 53, 79, 80) and are discussed in *SI Materials and Methods*.

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