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MHC-Mismatched Mixed Chimerism Mediates Thymic Deletion of Cross-Reactive Autoreactive T Cells and Prevents Insulitis in Nonobese Diabetic Mice

Jeremy J. Racine,*†‡ Mingfeng Zhang,*†‡ Miao Wang,*‡ William Morales,§ Christine Shen,§ and Defu Zeng*†‡

Type 1 diabetic NOD mice have defects in both thymic negative selection and peripheral regulation of autoreactive T cells, and induction of mixed chimerism can effectively reverse these defects. Our recent studies suggest that MHC-mismatched mixed chimerism mediates negative selection of autoreactive thymocytes in wild-type NOD and TCR-transgenic NOD.Rag1+/-.BDC2.5 mice. However, it remains unknown how mismatched I-A\textsubscript{b} MHC class II can mediate deletion of autoreactive T cells positively selected by I-A\textsubscript{g7}. In the present study, we directly tested the hypothesis that mismatched MHC class II in mixed chimeras mediates deletion of cross-reactive autoreactive thymocytes. We first identify that transgenic BDC2.5 T cells from NOD.Rag1+/-.BDC2.5 but not NOD.Rag1−/−.BDC2.5 mice possess cross-reactive TCRs with endogenous TCR chains; MHC-mismatched H-2\textsuperscript{b} but not matched H-2\textsuperscript{g7} mixed chimerism mediates thymic deletion of the cross-reactive transgenic T cells in NOD.Rag1+/-.BDC2.5 mice. Second, by transplanting T cell–depleted (TCD) bone marrow (BM) cells from NOD.Rag1+/-.BDC2.5 or NOD.Rag1−/−.BDC2.5 mice into lethally irradiated MHC-mismatched H-2\textsuperscript{b} C57BL/6 or MHC-matched congenic B6.H-2\textsuperscript{g7} recipients, we demonstrate that NOD.Rag1+/-.BDC2.5 BM-derived cross-reactive transgenic T cells, but not NOD.Rag1−/−.BDC2.5 BM-derived non–cross-reactive transgenic T cells, can be positively selected in MHC-mismatched H-2\textsuperscript{b} thymus, Third, by cotransplanting NOD.Rag1+/-.BDC2.5 TCD BM cells with BM cells from MHC-mismatched T cell–deficient C57BL/6 mice into lethally irradiated MHC-mismatched B6.H-2\textsuperscript{g7} recipients, we demonstrate that thymic deletion of the cross-reactive transgenic T cells is dependent on MHC-mismatched donor BM-derived APCs but not on donor BM-derived T cells. Taken together, our studies indicate that MHC-mismatched mixed chimerism can mediate thymic deletion of cross-reactive autoreactive T cells that express more than one TCR. The Journal of Immunology, 2015, 194: 407–417.

In autoimmune type 1 diabetes (T1D), improper thymic selection leads to the presence of autoreactive T cells in the periphery that destroy the insulin producing β cells of the pancreas (1–3). Years of work in the NOD mouse model of T1D has indicated that appearance of the autoreactive clones in the periphery is due to the failure of negative selection in the thymus (4–7). This enriched autoreactive T cell repertoire has been linked to the I-A\textsubscript{g7} MHC class II (MHC II) molecule (8–10). The mutation in I-A\textsubscript{g7} (8) results in an MHC II that is both a promiscuous peptide binder and a weak peptide binder (11–14). Taken together, this has led to the hypothesis that there is insufficient MHC-TCR signaling in the thymus to properly deliver a negatively selecting signal. More recent work has proposed that negative selection is not defective in NOD mice, but that endogenous defects within NOD T cells leads to an altered positive selection event (15), which could lead to an enhanced positive selection of autoreactive clones compared with nonautoimmune backgrounds.

Regardless of the mechanism by which autoreactive clones are produced in the thymus of NOD mice, several groups have shown that negative selection of these autoreactive T cells can be induced by introduction of protective MHC. Using backcrossing experiments, negative selection of the NY4.1 TCR-transgenic clone was observed when dendritic cells (DCs) expressed I-A\textsubscript{b}, I-A\textsubscript{d}/I-E\textsubscript{d}, and I-A\textsubscript{b}1, whereas expression of other MHC such as I-A\textsubscript{d} or I-A\textsubscript{b} triggered only partial or no negative selection (4). Additionally, the CD8+ T cell clone A14 was negatively selected by expression of H-2\textsuperscript{b} when the protective MHC II molecules were expressed on >50% of DCs (16). Using a gene therapy approach, introduction of I-A\textsubscript{b}1 or I-A\textsubscript{b}1 (to pair with endogenous I-A\textsubscript{b}–chains) led to the negative selection of BDC15 tetramer-reactive T cells in the thymus (7). However, these approaches are not currently a clinically relevant means by which to introduce protective MHC molecules.

Induction of mixed chimerism is a clinically relevant means by which protective MHC can be introduced into T1D patients. The Illdstad and Shizuru groups reported that induction of chimerism with donor whole BM cells or purified BM stem cells reversed autoimmunity in myeloablatively conditioned NOD mice (17–19). Our group and those of Sykes and Illdstad reported that induction
of mixed chimerism under nonmyeloablative conditioning regimens reversed autoimmunity and eliminated insulitis in NOD mice (20–25). Furthermore, we found that induction of mixed chimerism with MHC-mismatched but not MHC-matched donor BM was capable of reversing autoimmunity and eliminating insulitis in NOD mice (20, 26). We linked this reversal of autoimmunity to the ability of MHC-mismatched transplantation to induce the apparent negative selection of autoreactive T cells using the NOD.BDC2.5 TCR transgenic mouse model (26). In this system, we observed the drop in CD4^+CD8^+ double-positive (DP) thymocytes that is often observed in negative selection of transgenic T cell clones, as well as the overall reduction of BDC2.5 thymocytes and splenocytes. However, previous work by others with the BDC2.5 clone indicated that this I-A^b-restricted clone (as originally isolated) did not have cross-reactivity to I-A^b DCs (27). Additionally, in I-A^b/I-A^b heterozygous NOD.BDC2.5 mice, the BDC2.5 T cell did not appear to undergo negative selection, and there was an expansion of protective BDC2.5 cells with endogenous Vα2 expression (28).

Our observations, coupled with work from other groups studying negative selection, led us to ask several questions about the mechanisms involved in the tolerance of BDC2.5 T cells: How can introduction of a protective MHC via hematopoietic cell transplantation (HCT) delete an autoreactive TCR-transgenic clone previously considered non–cross-reactive to I-A^b? Is the introduction of MHC-mismatched donor APCs sufficient to initiate negative selection of BDC2.5 T cells? In the present study, we found that donor APCs in the MHC-mismatched mixed chimeras can induce thymic negative selection of host-type cross-reactive autoreactive T cell clones with more than one TCR.

**Materials and Methods**

**Mice**

NOD.Cg-Tg(TcrBDC2.5,TcrbBDC2.5)1DoiDoi (H-2K^b, I-A^b^2, CD45.1) is cited herein as NOD.BDC2.5-Rag1+/+; C57BL/6-J (H-2K^b, I-A^b^2, CD45.2) is cited as B6.Rag2+/+; B6;129P2-Rag2tm1Cgn/J is cited as NOD.Rag1+/+. All of the above mice or breeders were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were purchased from the National Institutes of Health Tetramer Facility (Atlanta, GA). FITC-labeled CD45.2 (104), CD45.1 (A20), and Vβ4 (Kt4), PE-labeled Mac1 (H57-597), and B220 (RA3-6B2) mAbs is very limited, and we used anti-TCR Vα2 as a representative nontransgenic Vα-chain.

**Flow cytometry and cell depletion**

PE-labeled BDC2.5 mimotope tetramer (I-A^b–restricted clone previously considered non–cross-reactive to I-A^b) is cited as NOD.Rag1+/+.BDC2.5 mice, which is dependent on donor cell expression of mismatched MHC II molecules. It has been proposed that diabetogenic T cells possess promiscuous TCRs that have cross-reactivity (40–43), although some autoreactive T cell clones (i.e., Vβ4Vα1 BDC2.5) were shown to be non–cross-reactive (27). Interestingly, we found that transgenic T cells from NOD.Rag1+/+.BDC2.5 (hereafter BDC2.5-Rag1+/+) but not NOD.Rag1+/+.B6 (hereafter BDC2.5-Rag1+/+) mice were able to proliferate in response to MHC-mismatched H-2^b DC background (Fig. 1A). These results suggest that transgenic autoreactive BDC2.5 T cells from the Rag1+/+ but not Rag1−/− background can be cross-reactive to H-2^b.

**Results**

**Transgenic BDC2.5 T cells from NOD.Rag1+/+.BDC2.5 mice exhibit cross-reactivity, and induction of MHC-mismatched mixed chimerism mediates thymic deletion of the transgenic T cells**

We recently reported that MHC-mismatched mixed chimerism mediated thymic deletion of host-type autoreactive T cells in TCR-transgenic NOD.Rag1+/+.BDC2.5 mice, which is dependent on donor cell expression of mismatched MHC II molecules. It has been proposed that diabetogenic T cells possess promiscuous TCRs that have cross-reactivity (40–43), although some autoreactive T cell clones (i.e., Vβ4Vα1 BDC2.5) were shown to be non–cross-reactive (27). Interestingly, we found that transgenic T cells from NOD.Rag1+/+.BDC2.5 (hereafter BDC2.5-Rag1+/+) but not NOD.Rag1+/+.B6 (hereafter BDC2.5-Rag1+/+) mice were able to proliferate in response to MHC-mismatched H-2^b DC background (Fig. 1A). These results suggest that transgenic autoreactive BDC2.5 T cells from the Rag1+/+ but not Rag1−/− background can be cross-reactive to H-2^b.

**Because Rag1+/+ TCR-transgenic mice tend to have endogenous α-chain rearrangement due to incomplete allelic exclusion of TCRα-chains (44), we tested whether BDC2.5 T cells from a Rag1+/+ background express nontransgenic TCRα. Because the availability of anti-mouse TCR Vα mAbs is very limited, and we and others have observed a large percentage of Vα2 T cells among mouse T cells (45), and BDC2.5 in particular (15, 26), we used anti-TCR Vα2 as a representative nontransgenic Vα-chain. Indeed, we found that some CD4^+ T cells in BDC2.5-Rag1+/+ mice expressed nontransgenic Vα2 TCR, although none of the CD4^+ T cells in BDC2.5-Rag1−/− mice expressed the nontransgenic Vα2 TCR (Fig. 1B). Because BDC2.5 mimotope tetramer was reported to be able to identify BDC2.5 autoreactive T cells (46), the tetramer was used to identify transgenic autoreactive CD4^+ T cells in the spleen of BDC2.5-Rag1+/+ and BDC2.5-Rag1−/− mice. We found that the tetramer staining intensity of CD4^+ T cells from BDC2.5-Rag1+/+ mice is much broader than that of CD4^+ T cells from BDC2.5-Rag1−/− mice, but the latter was higher than the former. Thus, CD4^+ T cells from BDC2.5-Rag1−/− mice were used to set a threshold for tetramer^+CD4^+ T cells (Fig. 1C). We observed that the nontransgenic TCR Vα2^+ T cells existed among...
FIGURE 1. Rag1 sufficiency conveys cross-reactivity to BDC2.5 T cells, and cross-reactive transgenic T cells are negatively selected in MHC-mismatched H-2b mixed chimeric BDC2.5. (A) Enriched CD4+ T cells from wild-type NOD mice and TCR-transgenic BDC2.5-Rag1+/+ or BDC2.5-Rag1−/− NOD mice were stimulated in culture with C57BL/6 or NOD DCs for 3 d. The stimulation index (mean ± SE) measured with [3H]TdR incorporation of three replicate experiments is shown. **p = 0.0095. (B) Representative staining pattern of thymocytes (top) or splenocytes (bottom) gated on living CD4+CD8−Vβ4+ MNCs showing FSC versus Vα2 (n=4). (C) In the top panel, gated CD4+Vβ4+ splenocytes from BDC2.5-Rag1+/+ mice are shown with binding to BDC2.5 mimotope tetramer (black line). The filled-in gray line represents tetramer− control. The light gray line is tetramer staining of BDC2.5-Rag1−/− T cells, which are all tetramerhi, and are used to separate tetramerhi from tetramerlo. In the bottom panel, gated CD4+Vβ4+ tetramerhi or tetramerlo cells are shown as nontransgenic Vα2 versus FSC. (D) CD4+BDC2.5 tetramer+ T cells from the spleen of BDC2.5-Rag1+/+ mice were sorted into tetramerhi and tetramerlo T cells. The sorted T cells were stimulated with C57BL/6 DCs (0.1 × 106 each/well). The proliferation was measured by [3H]thymidine incorporation. Stimulation indexes (mean ± SE) from three replicate experiments are shown. (E) Six-week-old TCR-transgenic BDC2.5-Rag1+/+ mice were conditioned with anti-CD3 (5 µg/g) 5 d prior to transplantation with 50 × 106 whole BM cells from C57BL/6 donor mice. Ten-day-old transgenic BDC2.5-Rag1−/− mice were conditioned with anti-CD3 (5 µg/g) 5 d prior to transplantation with 10 × 106 CD4+ splenocytes and (Figure legend continues)
tetramer<sub>hi</sub> and tetramer<sub>lo</sub> populations in BDC2.5-Rag1<sup>+/-</sup> mice (Fig. 1C). Additionally, both tetramer<sub>hi</sub> and tetramer<sub>lo</sub> BDC2.5 T cells from BDC2.5-Rag1<sup>+/-</sup> mice proliferated to MHC-mismatched DC stimulation (Fig. 1D). These results indicate that the cross-reactivity imbued upon BDC2.5 T cells in a Rag1<sup>+/+</sup> background likely comes from endogenous α-chain rearrangement, and both autoreactive TCR<sub>hi</sub> and autoreactive TCR<sub>lo</sub> cells are capable of expressing endogenous TCRs and having alloreactivity.

Finally, we induced mixed chimerism in BDC2.5-Rag1<sup>+/-</sup> or BDC2.5-Rag1<sup>-/-</sup> mice. We found that induction of mixed chimerism with BM cells from MHC-mismatched C57BL/6 (H-2<sup>b</sup>) donors mediated thymic deletion of BDC2.5 transgenic T cells in BDC2.5-Rag1<sup>+/-</sup> but not in BDC2.5-Rag1<sup>-/-</sup> mice, as indicated by a marked reduction of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes in the former mice (Fig. 1E). These results indicate that MHC-mismatched mixed chimerism mediates deletion of cross-reactive autoreactive T cells that probably express more than one TCR.

H-2<sup>b</sup>-restricted transgenic BDC2.5 T cells from a Rag1<sup>+/-</sup> but not Rag1<sup>-/-</sup> background are positively selected in the thymus of H-2<sup>b</sup>-mismatched C57BL/6 recipients

It was previously shown that the diabetogenic H-2<sup>b</sup>-restricted 4.1 T cell clone was negatively selected by H-2<sup>b</sup>-expressing medul- lary APCs in an autoantigen-independent manner (4), but 4.1 TCR-transgenic T cells could not be positively selected by H-2<sup>b</sup> expressed on cortical epithelial cells (47). Additionally, it has been reported that autoreactive transgenic BDC2.5 T cells could not be positively selected in I-A<sup>β</sup>/I-Ab mice (28). Thus, we tested whether transgenic BDC2.5 T cells (CD45.1<sup>+</sup>, H-2<sup>b</sup>) from either a Rag1<sup>+/-</sup> or Rag1<sup>-/-</sup> background could be positively selected in the thymus of C57BL/6 recipients (CD45.2<sup>+</sup>, H-2<sup>b</sup>). Accordingly, TCD BM cells from BDC2.5-Rag1<sup>+/-</sup> or BDC2.5-Rag1<sup>-/-</sup> mice were transplanted into lethally irradiated (1100 cGy total body irradiation [TBI]) C57BL/6 recipients. Twenty through sixty days after HCT, we checked initial chimerism status by measuring the percentage of donor-type CD45.1<sup>+</sup> Mac1<sup>+</sup>/Gr-1<sup>+</sup> macrophages among total blood MNCs. (A) Representative staining patterns of percentage CD45.1<sup>+</sup> among Mac1/Gr-1<sup>+</sup> blood cells. (B) Representative staining patterns of percentage CD45.1<sup>+</sup> among TCR<sup>β+</sup> blood cells. (C) Percentage CD45.1<sup>+</sup> Mac1/Gr-1<sup>+</sup> blood cells (mean ± SE, n = 3–4/group/time point). (D) Percentage CD45.1<sup>+</sup> TCR<sup>β+</sup> blood cells (mean ± SE, n = 3–4/group/time point).
The lack of CD45.1<sup>−/−</sup> rism, and the pancreas was harvested for measurement of insulitis. BM cells can give rise to transgenic BDC2.5 T cells in the MHC-mismatched recipients. The recipients given TCD BM from both BDC2.5-Rag1+/+ and BDC2.5-Rag1−/− donors had a clear de novo–developed CD45.1+ TCR<sup>b+</sup> population (~10% of total MNCs) in the spleen, whereas few (~0.1% of total MNCs) CD45.1+ TCR<sup>b+</sup> cells were observed in the BDC2.5-Rag1−/− chimeras (p < 0.001, Fig. 3B). These results indicate that transgenic BDC2.5 T cells in the Rag1−/− background arrest at the DP stage in MHC-mismatched C57BL/6 recipients.

Sixty to 90 d after HCT, the thymus, spleen, and BM of the chimeras were harvested for confirmation of donor-type chimerism, and the pancreas was harvested for measurement of insulinitis. The lack of CD45.1<sup>−/−</sup> or CD45.2<sup>−/−</sup> host-type Mac-1<sup>+</sup>/Gr-1<sup>+</sup> cells in spleen and BM indicates the status of complete chimerism in both chimeric groups (Supplemental Fig. 3), as described in our previous publications (26, 29–31). The recipients given TCD BM from BDC2.5-Rag1+/+ donors had ~75% CD4<sup>+</sup>CD8<sup>+</sup> DP thymocytes and ~5% CD4<sup>+</sup>CD8<sup>+</sup> (single-positive [SP]) thymocytes; in contrast, the recipients given TCD BM from a BDC2.5-Rag1−/− donors had ~95% DP thymocytes but <2% SP thymocytes (p < 0.001, Fig. 3A). Furthermore, the BDC2.5-Rag1+/+ chimeras had a clear de novo–developed CD45.1+TCR<sup>b+</sup> population (~10% of total MNCs) in the spleen, whereas few (~0.1% of total MNCs) CD45.1+TCR<sup>b+</sup> cells were observed in the BDC2.5-Rag1−/− chimeras (p < 0.001, Fig. 3B). These results indicate that nontransgenic V<sub>a</sub>-chains can be positively selected by mismatched I-A<sup>b</sup> thymic cortical epithelial cells. It is of interest that cross-reactive BDC2.5 T cells positively selected by I-A<sup>b</sup> cortical epithelial cells did not appear to be negative selected by H-2<sup>d</sup>–expressing APCs in the medulla or by I-A<sup>d</sup>–expressing medullary thymic epithelial cell (mTECs), because there was a higher percentage of nontransgenic V<sub>a</sub>2+ TCRs among CD4<sup>+</sup>CD8<sup>+</sup> thymocytes as compared with CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (p < 0.05, Fig. 3E).

**FIGURE 3.** Cross-reactive T cells from BDC2.5-Rag1+/+ TCD BM develop in the thymus of C57BL/6 mice, are exported to the periphery, and retain autoreactivity. (A) In the left panel, representative staining patterns of gated CD45.1+ thymocytes show CD4 versus CD8 and gating on CD4+CD8<sup>+</sup> and CD4<sup>+</sup> SP thymocytes. In the right panel, the percentage of positively selected CD4<sup>+</sup> SP thymocytes is shown (mean ± SE, n = 6–7/group). (B) In the left panel, representative staining patterns of percentage CD45.1+TCR<sup>b+</sup> among total splenocytes are shown. In the right panel, the percentage of CD45.1+TCR<sup>b+</sup> among total splenocytes is shown (mean ± SE, n = 6–7/group). (C) Representative (one of four) staining of tetramer staining in the spleen of BDC2.5-Rag1+/+→C57BL/6 chimeras. Gated CD45.1+CD4<sup>+</sup>V<sub>B</sub>4<sup>+</sup> cells show BDC2.5 mimotope tetramer (solid line) versus control tetramer (filled gray area). (D) Insulitis levels in BDC2.5-Rag1+/+→C57BL/6 chimeras and BDC2.5-Rag1−/−→C57BL/6 chimeras. (E) Representative staining pattern of gated CD45.1+CD4<sup>+</sup> thymocytes and splenocytes showing endogenous V<sub>a</sub> usage in BDC2.5-Rag1+/+→C57BL/6 chimeras. **p = 0.015, ***p = 0.0006, ****p < 0.0001.

Next, we evaluated the development of cross-reactive BDC2.5-Rag1+/+ T cells and non–cross-reactive BDC2.5-Rag1−/− T cells in an MHC-matched but minor Ag-mismatched B6.H-2<sup>d</sup> (CD45.2<sup>+</sup>) recipients by transplanting TCD BM cells from BDC2.5-Rag1+/+ or BDC2.5-Rag1−/− donors. As with MHC-mismatched C57BL/6 recipients, we monitored the development...
of T cell chimerism in the peripheral blood 20–60 d after HCT. Unlike the case in C57BL/6 recipients, both chimeric groups had development of CD45.1+ BDC2.5 T cells. By 60 d after HCT, CD45.1+ BDC2.5 T cells among blood T cells reached 60% in recipients of BDC2.5-Rag1+/+ TCD BM and reached 30% in recipients of BDC2.5-Rag1−/− TCD BM (Fig. 4A, 4B).

By 60 d after HCT, both chimeric groups had similar percentage (~85%) of CD4+CD8+ thymocytes and similar percentage (~12%) of CD4+CD8− thymocytes, indicating positive selection of these clones in the B6.H2g7 background (Fig. 4C). Additionally, both groups had peripheral TCRβ+ T cells, indicative of thymic output of the T cell clones (Fig. 4D). Combining the findings shown in Figs. 2–4, the results indicate that cross-reactive BDC2.5-Rag1+/+ T cells are not able to negatively select either cross-reactive or non–cross-reactive BDC2.5 autoreactive T cells.

Cross-reactive BDC2.5-Rag1+/+ T cells that are positively selected by I-Ag7 cortical epithelial cells are negatively selected by MHC-mismatched I-Aβ APCs in the medulla

As mentioned above, induction of mixed chimerism in BDC2.5-Rag1+/+ mice induced thymic deletion of transgenic autoreactive BDC2.5 T cells as indicated by reduction of CD4+CD8− thymocytes (Fig. 1). However, there are two complicating factors in this primary recipient model. First, reduction in DP thymocytes, although seemingly negative selection of negative section might be confounded by clonal competition for limited positive selection niches (48). Second, analysis of peripheral T cells to determine negative selection capacity of cross-reactive clones is complicated by the existence of T cells that existed prior to HCT. To definitively evaluate whether donor APCs induced negative selection of cross-reactive BDC2.5-Rag1+/+ T cells, we used BM cells from B6.Rag2−/− or B6.TCRβ−/− donor mice to avoid the influence of competition with de novo–developed C57BL/6 T cells in the mixed chimeras. Additionally, we attempted to mimic the mixed chimerism situation in wild-type NOD mice in which thymocytes are positively selected by H-2β7 cortical epithelial cells. To avoid the complicating factor of pre-existing BDC2.5 T cells in the thymus and periphery, we chose to use B6.H-2β7 recipients as our recipient mouse of choice.

First, we established mixed chimeras with TCD BM cells from BDC2.5-Rag1+/+ (CD45.1+) mice plus graded numbers of TCD BM cells from B6.Rag2−/− or B6.TCRβ−/− (CD45.2+) donors into lethally irradiated B6.H2g7 (CD45.2+) recipients. Sixty to 90 d after HCT, chimerism and thymic deletion were measured. Chimeric status was confirmed by analyzing the percentage of BM and splenic macrophages as well as thymic DCs as described above (Supplemental Fig. 4) and in our previous publications (26, 29–31). In the control chimeras given BDC2.5-Rag1+/+ TCD BM alone (no B6-APC), the percentage of CD4+CD8+ thymocytes was >75%; in contrast, in the mixed chimeras given BDC2.5-Rag1+/+ TCD BM plus B6.Rag2−/− or B6.TCRβ−/− TCD BM (±B6-APC), the percentage of CD4+CD8+ thymocytes was significantly decreased (p < 0.01, Fig. 5A). Interestingly, the residual CD4+CD8+ thymocytes in different individual recipients varied from 1 to 60%, and the effect of Rag2−/− or TCRβ−/− BM was similar (Fig. 5A). The variation in deletion of BDC2.5 CD4+CD8+ thymocytes correlated with the percentage of B6-type thymocytes in the thymus. When CD45.2+ thymocytes represented <10% of the total thymocytes, there was a small but significant reduction of

FIGURE 4. Both cross-reactive and non–cross-reactive T cells derived from BDC2.5-Rag1+/+ or BDC2.5-Rag1−/− TCD BM cells are positively selected in B6.H2g7 recipients and are exported to the periphery. B6.H2g7 mice (CD45.2+, H-2β7) were irradiated with 1100 cGy and reconstituted with 5 × 106 TCD BM from BDC2.5-Rag1+/+ and BDC2.5-Rag1−/− (CD45.1, H-2β7) mice. Blood was harvested at various time points after HCT and analyzed for percentage of donor-type CD45.1+ T cells. (A) Representative staining patterns of percentage CD45.1+ among TCRβ+ blood cells. (B) Percentage CD45.1+ among TCRβ+ blood cells (mean ± SE, n = 5–7/group). (C) In the left panel, representative staining patterns of gated CD45.1+ thymocytes show CD4 versus CD8 and gating on CD4+CD8+ and CD4+ SP thymocytes. In the right panel, the percentage of positively selected CD4+ SP thymocytes is shown (mean ± SE, n = 5–7/group). (D) In the left panel, representative staining patterns of percentage CD45.1+ TCRβ+ among total splenocytes cells are shown. In the right panel, the percentage of CD45.1+ TCRβ+ among total splenocytes is shown (mean ± SE, n = 6–7/group).
BDC2.5 CD4⁺CD8⁺ thymocytes; the presence of >10% of B6 donor thymocytes was associated with dramatic reduction of BDC2.5 CD4⁺CD8⁺ thymocytes (Fig. 5B).

Second, we established mixed chimeras with TCD BM cells from BDC2.5-Rag1⁻/⁻ (CD45.1⁺) donors plus graded numbers of BM cells from B6.Rag2⁻/⁻ (CD45.2⁺) into lethally irradiated B6.H2g7 (CD45.2⁺) recipients. As was the case with recipients of BDC2.5-Rag1⁻/⁻ TCD BM, 60–90 d after HCT, chimera status and thymic deletion were measured. Chimeric status was confirmed by analyzing the percentage of BM and splenic macropaghes as well as thymic DCs as described above (Supplemental Fig. 4) and in our previous publications (26, 29–31). Unlike recipients of BDC2.5-Rag1⁻/⁻ TCD BM, there was only a small percentage drop when B6 APCs were present in the thymus (Fig. 5C). Despite obtaining a large number of chimeras (>12), only two recipients had C57BL6 donor-type (CD45.2⁺) chimerism >10%. Regardless of whether CD45.2⁺ chimerism status was <10% or >10% in the thymus, there was little difference in the percentage of DP thymocytes (Fig. 5D).

Finally, we evaluated the effect of thymic negative selection of cross-reactive BDC2.5 T cells on peripheral T cells. We found that the thymic deletion of BDC2.5-Rag1⁻/⁻ CD4⁺CD8⁺ T cells in the presence of B6 APCs was associated with depletion of BDC2.5 tetramer⁺ T cells that expressed nontransgenic endogenous Vα2⁺ TCR in the spleen (p < 0.05, Fig. 6A). As expected, the BDC2.5-Rag1⁻/⁻ T cells lacked Vα2 expression regardless of the presence or absence of B6 APCs (p > 0.05, Fig. 6B). Taken together, these results indicate that, in the absence of T cell clonal competition, expression of mismatched I-Aβ MHC by thymic medullary APCs is able to mediate negative selection of cross-reactive BDC2.5-Rag1⁻/⁻ autoreactive T cells that were positively selected by I-Aβ⁺ cortical epithelial cells.

FIGURE 5. Cross-reactive but not non–cross-reactive autoreactive cells selected on I-Aβ⁺ are deleted in the presence of MHC-mismatched H-2b APCs. B6.H2g7 recipients were irradiated and reconstituted with BDC2.5-Rag1⁻/⁻ (A and B) or BDC2.5-Rag1⁻/⁻ TCD BM (CD45.1, H-2k) (C and D) alone (No B6-APC) or with additional TCD BM cells (5–15 × 10⁶) from B6.Rag2⁻/⁻ or B6.TCRβ⁻/⁻ (CD45.2, H-2b) mice (+B6-APC). Sixty to 90 d after HCT, mice were euthanized and thymuses were analyzed for thymocyte populations. (A and C) Comparison of DP cells in recipients of BDC2.5-Rag1⁻/⁻ with and without B6 APCs. Filled square indicates B6 APC from B6.TCRβ⁻/⁻ mice; open square, B6 APC from B6.Rag2⁻/⁻ mice (n = 7–17/group). (B and D) Graphical representation of CD45.1⁺CD4⁺CD8⁺ percentage versus percentage of CD45.2⁺ thymus chimerism level. For (A), comparing all +B6-APC to No B6-APC, ****p < 0.0001.

Discussion

In the present study, we have identified that transgenic autoreactive BDC2.5 T cells from Rag1⁻/⁻ background possess endogenous TCRs (i.e., Vα2⁺ TCR) that mediate cross-reactivity. BDC2.5 T cells from the Rag1⁻/⁻ background do not posses endogenous TCRs and lack cross-reactivity. MHC-mismatched mixed chimerism can delete the autoreactive T cells with endogenous TCRs.

By a series of transfer experiments with TCD BM from BDC2.5-Rag1⁻/⁻ or BDC2.5-Rag1⁻/⁻ TCR-transgenic mice into MHC-mismatched C57BL6 or MHC-matched B6.H2g7 recipients, we have observed that BDC2.5 autoreactive T cells with endogenous TCRs can be positively selected by I-Aβ cortical epithelial cells in I-Aβ⁺ C57BL6 recipient thymus, but they are not negatively selected by I-Aβ medullary epithelial cells. In contrast, the BDC2.5 autoreactive T cells with endogenous TCRs that are positively selected by I-Aβ⁺ cortical epithelial cells in B6.H2g7 mice can be negatively selected by I-Aβ medullary APCs derived from B6.Rag2⁻/⁻ or B6.TCRβ⁻/⁻ donor cells. Finally, induction of MHC-mismatched mixed chimerism with H-2b donor BM mediates thymic deletion of cross-reactive BDC2.5-Rag1⁻/⁻ autoreactive T cells that express endogenous TCRs. However, induction of MHC-mismatched mixed chimerism with H-2k BM cannot mediate the negative selection of non–cross-reactive BDC2.5-Rag1⁻/⁻ autoreactive T cells.

The present observations provide potential novel insights into how MHC-mismatched mixed chimerism mediates thymic deletion of host-type autoreactive T cells. We recently observed that MHC-mismatched but not MHC-matched mixed chimerism was capable of reversing autoimmunity in wild-type and TCR-transgenic NOD.BDC2.5 mice (26). It remained unclear how MHC-mismatched I-Aβ⁺ donor APCs can delete autoreactive thymocytes that typically recognize I-Aβ⁺ and have previously been identified as being I-Aβ⁻ restricted (27). In the present study, we observed that the
The figure 6 shows the presence of endogenous α-chain rearrangement correlated with the BDC2.5 autoreactive T cells’ ability to be negatively selected in the thymus. This negative selection correlated with the ability of Rag1<sup>+/+</sup> T cells to proliferate on MHC-mismatched APCs in an alloreactive manner. The alloreactivity, that is, cross-reactivity, of the autoreactive BDC2.5 TCR-transgenic T cells was associated with their expression of endogenous TCRα (i.e., Vα<sup>2</sup> TCR). It is of interest that in graft-versus-host disease studies, it has been revealed that many alloreactive T cells actually contain dual or multiple TCRs (49, 50), a state that appears to resemble transgenic mice derived BDC2.5 T cells to rearrange endogenous TCR<sub>α</sub> chain and in BDC2.5b/b mice, no positive selection of BDC2.5 cells was observed, whereas only non-transgenic BDC2.5 tetramer<sup>+</sup> cells developed in I-A<sub>b</sub>/I-Ab mice (28). Thus, how could I-A<sub>b</sub> DCs mediate negative selection of the BDC2.5 T cells that recognize I-A<sub>g7</sub> in the mixed chimeras may have cross-reactivity to other MHC types other than H-2<sup>b</sup>. Whether different allo-MHC types mediate deletion of autoreactive T cells that recognize different self-MHC also needs to be addressed in future studies.

Second, the original BDC2.5 clone did not exhibit any cross-reactivity to I-A<sub>b</sub> (27). It has also been reported that in BDC2.5<sup>5N<sub>g7</sub></sup> mice, no negative selection of the BDC2.5 clone was observed, and in BDC2.5<sup>5b/b</sup> mice, no positive selection of BDC2.5 cells was observed (28). Thus, how could I-A<sub>b</sub> DCs mediate negative selection of the BDC2.5 T cells that recognize I-A<sub>e7</sub> in BDC2.5 mice with I-A<sub>b</sub> mixed chimerism? The ability of BM-derived BDC2.5 T cells to rearrange endogenous TCR<sub>α</sub> contributes to the deletion. Our present study showed that unlike the situation in which BDC2.5 is bred into a I-A<sub>b</sub>/I-Ab<sub>b</sub> background, transplantation of BDC2.5-Rag1<sup>+/+</sup> TCD BM cells into I-A<sub>b</sub> recipients was able to give rise to immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes as well as mature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes that were exported into the periphery. Additionally, whereas only non-transgenic BDC2.5 tetramer<sup>+</sup> cells developed in I-A<sub>b</sub>/I-Ab<sub>b</sub> mice (15), the T cells that developed in (BDC2.5-Rag1<sup>+/+</sup>→C57BL/6) complete chimeras retained a BDC2.5 tetramer binding TCR, and all T cells appeared to be BDC2.5 tetramer<sup>+</sup> and were capable of expressing dual or multiple TCRs. It is unlikely that all the transgenic T cells expressed endogenous TCR<sub>α</sub>. Thus, it is unlikely that MHC-mismatched mixed chimerism will deplete all the autoreactive transgenic T cells. Additionally, owing to limited availability of anti-V<sub>α</sub> mAb, we only used anti-Vα2 as an example in the present study; it is possible that other Vα-chains have differential ability to be negatively selected. We also cannot rule out the possibility that the residual autoreactive BDC2.5 T cells in the mixed chimeras may have cross-reactivity to other MHC types other than H-2<sup>b</sup>.
inducing insulitis. In contrast, BDC2.5-Rag1\(^{+/−}\) TCD BM was not able to give rise to mature CD4\(^+\)CD8\(^+\) thymocytes that could be exported to the periphery to induce insulitis. These results indicate that the ability to rearrange endogenous \(\alpha\)-chains and express dual or multiple TCRs allows BM-derived BDC2.5-Rag1\(^{+/−}\) T cells to be positively selected by mismatched I-A\(^b\) MHC II in the thymus of C57BL/6 recipients. In other words, the positive selection of the BDC2.5 T cells in the I-A\(^b\) recipients is mediated by the TCRs with endogenous \(\alpha\)-chain rearrangement.

The presence of autoreactive BDC2.5 T cells and induction of insulitis in the (BDC2.5-Rag1\(^{+/−}\)→C57BL/6) complete chimeras also suggest that any negative selection mediated by I-A\(^{b+}\) C57BL/6 medullary epithelial cells is not sufficient to delete the autoreactive BDC2.5-Rag1\(^{+/−}\) T cells that have been positively selected by I-A\(^{b+}\) cortical epithelial cells. Alternatively, I-A\(^{b+}\) DCs deleted BDC2.5-Rag1\(^{+/−}\) T cells that were first positively selected by I-A\(^{b+}\) cortical epithelial cells in mixed-chimeric B6.H2\(^{b+}\) recipients. This suggests that deletion of BDC2.5 autoreactive T cells requires thymic DC expression of an MHCII that is not also involved in positive selection of the T cells; in other words, the positive and negative selection of the BDC2.5 T cells can be mediated by different TCRs, and the negative selection of BDC2.5 T cells in the MHC-mismatched H-2\(^b\) recipients is mediated by TCRs with dual TCR\(\alpha\)-chains.

Third, because we were working with a TCR transgenic system, any apparent negative selection event could have been due to a failure of the transgenic T cells to outcompete WT T cells for positive selection niches. In the present study, we have demonstrated that deletion of autoreactive BDC2.5 T cells in the MHC-mismatched H-2\(^b\) mixed chimeras occurred in the absence of donor BM-derived TCR\(\alpha\)\(\beta\) cells. We observed that when cross-reactive BDC2.5-Rag1\(^{+/−}\) T cells were first selected on I-A\(^{b+}\), and then encounter I-A\(^b\) in the medulla, I-A\(^{b+}\) APCs alone were sufficient to delete the thymocytes that express endogenous TCR\(\alpha\)-chains, as indicated by deletion of nontransgenic V\(\alpha\)2\(^+\) TCR. This negative selection phenotype also manifested itself in the periphery as a loss of those T cells expressing nontransgenic V\(\alpha\)2 in the mice in which I-A\(^{b+}\) DCs were present.

Taken together, these data indicate that MHC-mismatched donor APCs can delete autoreactive T cells that express more than one TCR.

Note that our current experimental system cannot determine whether minor Ags (in MHC-matched transplants) affect the negative selection of cross-reactive BDC2.5 T cells. However, owing to the inability of B6.H2\(^{b+}\) mTECs to negatively select BDC2.5 T cells, it is unlikely that mismatched minor Ags play an important role in mediating negative selection. Additionally, how MHC-mismatched H-2\(^b\) mixed chimeras tolerizes the autoreactive T cells that do not express dual or multiple TCRs needs to be determined in future studies.

In summary, induction of mixed chimerism with MHC-mismatched donor BM is a novel regimen that can cure autoimmune diseases such as T1D on multiple fronts, including re-establishing thymic negative selection (26), elimination of peripheral autoreactive T cells and insulitis (20–26), and tolerizing autoreactive B cells (29). The present study is focused on dissecting the mechanism of restoring thymic negative selection. Based on our observations with transgenic NOD.BDC2.5 mice, we propose a novel mechanism whereby MHC-mismatched mixed chimerism mediates deletion of autoreactive T cells. As depicted in Fig. 7, in nonchimeric autoimmune thymus, T cells with dual TCRs are positively selected by one TCR in the cortex. The dual TCR situation decreases the frequency of autoreactive TCRs on a single cell surface and allows for autoreactive T cells to escape negative selection in the medulla, as previously proposed (53, 55). In the thymus of MHC-mismatched (i.e., H-2\(^b\)) mixed chimeras, both TCRs on the autoreactive thymocytes that express dual TCRs can interact with the mismatched MHC II on donor APCs in the medulla. The strong affinity of TCR–allo-MHC interaction leads to deletion of the immature autoreactive thymocytes. Future studies are needed to find out the requirement of donor-recipient MHC combinations that can mediate thymic deletion of host-type autoreactive T cells; future studies are also needed to test this hypothesis in wild-type (i.e., non-TCR-transgenic) animal models and human patients.

**FIGURE 7.** Hypothetical diagram of MHC-mismatched mixed chimerism deleting autoreactive T cells with dual TCRs. In the thymus of autoimmune individuals, autoreactive T cells with dual TCRs are positively selected with one TCR in the thymic cortex and escape from negative selection in the medulla owing to lower density of the autoreactive TCR. In the MHC-mismatched mixed chimeras, both TCRs on an autoreactive T cell can interact with the allogeneic MHC II of donor DCs in the medulla, and strong interaction leads to apoptosis of the autoreactive T cells.
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References


