Induction of Mixed Chimerism With MHC-Mismatched but Not Matched Bone Marrow Transplants Results in Thymic Deletion of Host-Type Autoreactive T-Cells in NOD Mice

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OBJECTIVE—Induction of mixed or complete chimerism via hematopoietic cell transplantation (HCT) from nonautoimmune donors could prevent or reverse type 1 diabetes (T1D). In clinical settings, HLA-matched HCT is preferred to facilitate engraftment and reduce the risk for graft versus host disease (GVHD). Yet autoimmune T1D susceptibility is associated with certain HLA types. Therefore, we tested whether induction of mixed chimerism with major histocompatibility complex (MHC)-matched donors could reverse autoimmunity in the NOD mouse model of T1D.

RESEARCH DESIGN AND METHODS—Prediabetic wild-type or transgenic BDC2.5 NOD mice were conditioned with a radiation-free GVHD preventative anti-CD3/CD8 conditioning regimen and transplanted with bone marrow (BM) from MHC-matched or mismatched donors to induce mixed or complete chimerism. T1D development and thymic deletion of host-type autoreactive T-cells in the chimeric recipients were evaluated.

RESULTS—Induction of mixed chimerism with MHC-matched nonautoimmune donor BM transplants did not prevent T1D in wild-type NOD mice, although induction of complete chimerism did prevent the disease. However, induction of either mixed or complete chimerism with MHC-mismatched BM transplants prevented T1D in such mice. Furthermore, induction of mixed chimerism in transgenic BDC2.5-NOD mice with MHC-matched or -mismatched MHC $II^{-/-}$ BM transplants failed to induce thymic deletion of de novo developed host-type autoreactive T-cells, whereas induction of mixed chimerism with mismatched BM transplants did.

CONCLUSIONS—Induction of mixed chimerism with MHC-mismatched, but not matched, donor BM transplants re-establishes thymic deletion of host-type autoreactive T-cells and prevents T1D, with donor antigen-presenting cell expression of mismatched MHC II molecules being required. ***Diabetes* 60:555–564, 2011**

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Type 1 diabetes (T1D) is an autoimmune disease in which autoreactive T-cells attack the insulin-secreting islet β-cells and result in insulin deficiency and hyperglycemia (1–3). NOD mouse is still the best animal model for T1D, although the autoimmune abnormality in NOD mice does not totally reflect the abnormality in T1D patients (4–6). The autoimmunity in NOD mice and T1D patients is associated with particular major histocompatibility complex (MHC) or HLA loci such as $IA\beta^{g7}$ or HLA-DR (7–9). This particular genetic background is associated with central tolerance defects, in which autoreactive thymocytes are resistant to negative selection (10–12), as well as peripheral tolerance defects (13–17).

Transgenic expression of protective MHC II molecules in the thymus has been shown to prevent T1D development in mice (18–20). However, this approach cannot readily be translated to humans. Immunomodulation therapies such as administration of anti-CD3 have been shown to reverse new-onset T1D in mouse or ameliorate new-onset T1D in patients via induction of regulatory T-cells (21–25). However, the therapeutic benefit in patients appears to be limited in terms of duration (25). This indicates modulation of peripheral tolerance may not be sufficient for stable re-establishment of immune tolerance in T1D patients, because the defective thymus may constantly export autoreactive T-cells, which can overwhelm peripheral tolerance mechanisms. Therefore, a therapy that can re-establish both central and peripheral tolerance in T1D patients would appear optimal as a means to reverse the autoimmunity associated with T1D.

Indeed, autoimmune diseases such as T1D arise from abnormality in the immuno-hematological compartment, and a replacement of the system from a nonautoimmune individual can cure autoimmune T1D or vice versa (26). Therefore, previous studies have proposed that induction of mixed chimerism via hematopoietic cell transplantation (HCT) may provide a curative therapy for autoimmune diseases such as T1D (27). Although it was reported induction of mixed chimerism with bone marrow (BM) transplants from MHC-mismatched or MHC-matched nonautoimmune donors was able to prevent T1D development in NOD recipients conditioned with myelo- or nonmyeloablative total body irradiation (TBI) (28–32), as well as in recipients conditioned with a radiation-free anti-CD3-based regimen (33,34), the mechanisms whereby mixed chimerism reverses such autoimmunity remain largely

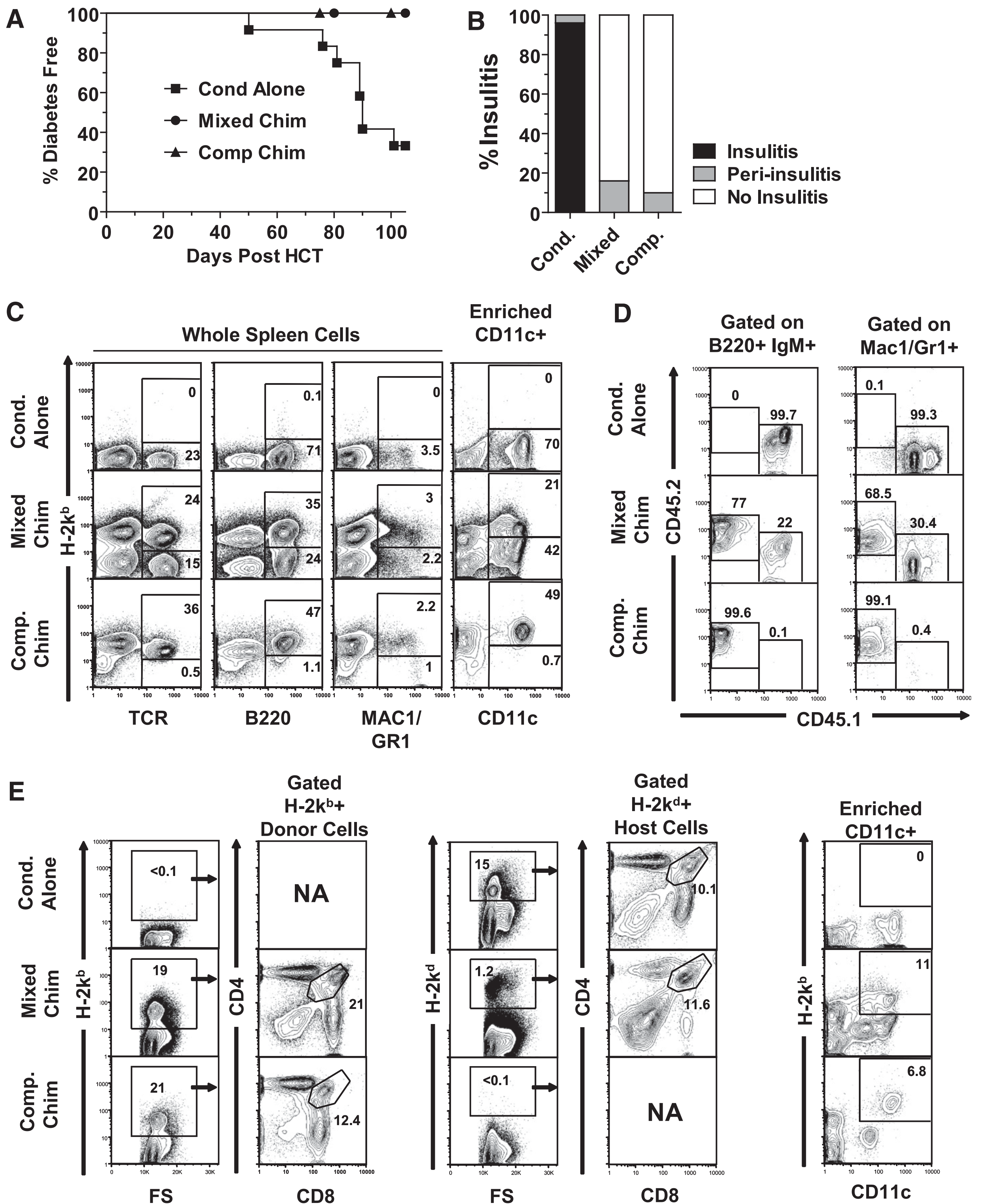

FIG. 1. Mixed and complete chimerism with MHC-mismatched BM transplants both prevented T1D in NOD mice. Wild-type NOD mice were conditioned with anti-CD3/CD8 (5 μg each) on days −10 and −5. On day 0, the conditioned mice were transplanted with graded doses of CD4$^+$ TCD splenocytes and whole BM from wild-type C57BL/6 donors to induce chimerism (<20 × 10^6 each for the mixed, ~50 × 10^6 each for the complete chimeras). Diabetes development was monitored weekly by both urine and blood glucose for up to 100 days. Thereafter, the recipient pancreas,

unknown. So-called mixed chimerism has been defined by the coexistence of donor- and host-type lymphocytes in the periphery such as in the blood or spleen, but it remains unclear whether the host-type cells in the mixed chimeric recipients are de novo developed after HCT or residual mature lymphocytes developed before HCT. In other words, it is not clear whether mixed chimerism can mediate deletion of de novo developed autoreactive T-cells. In addition, although MHC-matched HCT is preferred in clinical settings, it is not yet clear whether induction of mixed chimerism with MHC-matched donor transplants can mediate thymic deletion of de novo developed host-type autoreactive T-cells, because the defect in negative selection is associated with particular MHC II loci (7–9).

In the current study, we identified true mixed chimeras by measuring the donor and host-type T-cell precursors in the thymus as well as immature B and myeloid cells in the BM and we evaluated the impact of mixed and complete chimerism with MHC-matched or mismatched donor BM transplants.

RESEARCH DESIGN AND METHODS

Female NOD/LtJ and BDC2.5-NOD, wild-type C57BL/6, H-2^{g7} C57BL/6, and MHC $II^{-/-}$ C57BL/6 were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a pathogen-free room at City of Hope Research Animal Facilities (Duarte, CA). Animal use procedures were approved by our institutional committee.

Conditioning regimen and HCT. These procedures were described in our previous publications (33,34).

Flow cytometry and cell depletion/enrichment. Flow cytometry staining and analysis, CD11c enrichment, and $CD4^+$ T-cell depletion were performed as described (33–35). Phycoerythrin (PE)–labeled BDC2.5-mimotope tetramer (I-A^{g7} AHHPIWARMDA) and control-tetramer (I-A^{g7} PVSKMRMATPLLMQA) were obtained from NIH Tetramer Facility (Atlanta, GA).

Pancreatic analysis. Analysis of insulitis and β-cell surface area was performed as previously described (33).

Statistical analysis. Comparison of T1D development was evaluated using the log-rank test, and comparison of means was evaluated using unpaired two-tailed Student t tests with Prism version 5 (GraphPad, San Diego, CA).

RESULTS

Induction of mixed and complete chimerism with BM transplants from MHC-mismatched donors both prevented insulitis and T1D development. It was reported that induction of chimerism with BM cells from MHC-mismatched nonautoimmune donors prevented T1D development (28,30–34). However, it was not clear whether those chimeric recipients had mixed or complete chimerism, because in those reports chimerism was measured with peripheral blood mononuclear cells, and the mixed chimerism could consist of residual host-type T-cells without de novo developed host-type T-cells. To identify the true mixed chimerism, de novo developed donor- and host-type $CD4^+CD8^+$ thymocytes, as well as $B220^+IgM^+$ or $MAC1^+/Gr1^+$ BM resident cells, needed to be measured.

Therefore, we used a recently reported radiation-free and graft versus host disease (GVHD) preventative anti-CD3/CD8-based condition regimen (33–36) to induce mixed or complete chimerism by transplanting graded numbers of donor cells. Accordingly, 6-week-old NOD mice (H-$2k^d$, I-A^{g7}, CD45.1) were injected with anti-CD3 and anti-CD8 (5 μg/g each) on days −10 and −5. On day 0, the conditioned mice were intravenously injected with graded numbers (5–50 × 10^6) of BM and $CD4^+$ T-depleted spleen cells from MHC-mismatched C57BL/6 (H-$2k^b$, I-A^b, CD45.2) donors. The control mice were given conditioning therapy only. The recipients were checked for chimerism with blood mononuclear cells and monitored weekly for T1D development for up to 100 days after HCT. Thereafter, the pancreas, spleen, BM, and thymus of the recipients and control mice were harvested for evaluation of insulitis and confirmation of chimerism status.

TABLE 1
Percent donor- and host-type cells in spleen of mixed and complete chimeras given MHC-mismatched BM transplants

	$TCR\beta^+$	$B220^+$	$MAC1/Gr1^+$	$CD11c^+$
Mixed				
Donor-type	63.1 ± 13.8	51.6 ± 17.0	55.9 ± 14.6	29.1 ± 21.2
Host-type	36.9 ± 13.8	48.4 ± 17.0	44.1 ± 14.6	70.9 ± 21.2
Complete				
Donor-type	99.9 ± 0	99.6 ± 0.1	79.0 ± 2.4	98.0 ± 0.5
Host-type	0.1 ± 0	0.4 ± 0.1	21.1 ± 2.4	2.0 ± 0.5

Data are means ± SE; n = 4. Donor-type cells were defined as H-$2k^{b+}$, and host-type cells were defined as H-$2k^{b-}$.

Because we have reported that blood chimerism levels become stable in NOD recipients conditioned with the radiation-free anti-CD3-based regimen ~60 days after HCT (34), and this notion was confirmed with representative recipients in the current study (Supplementary Fig. 1 and Supplementary Table 1), we divided the recipients into mixed and complete chimeras after that time point. We found that the development of mixed and complete chimerism was associated with donor cell dose. In general, recipients given >25 × 10^6 MHC-mismatched donor cells all developed complete chimerism, and the majority of recipients given <20 × 10^6 donor cells developed mixed chimerism as judged by analysis of donor- and host-type T-cells, B-cells, and macrophages/granulocytes among blood mononuclear cells. We also found that, 100 days after HCT, 66% (8 of 12) of the control mice, but none of the mixed or complete chimeric recipients, developed T1D ($P < 0.01$, Fig. 1*A*). Although almost all of the residual islets in control mice had severe insulitis, none of the islets in the mixed and complete chimeric recipients had insulitis ($P <$ 0.01, Fig. 1*B*), although a small portion of them showed very minor peri-insulitis (Fig. 1*B*) and there was no significant difference between mixed and complete chimeric recipients ($P > 0.5$, Fig. 1*B*). A representative of the different types of insulitis is shown in Supplementary Fig. 2.

Furthermore, we confirmed the status of chimerism by examining the spleen, BM, and thymus of the recipients. We observed that, in the mixed chimeras, more than one-third of the T-cells, B-cells, macrophages, and granulocytes

spleen, BM, and thymus were harvested for evaluation of insulitis and chimerism status. There were 12 mice in each group combined from three replicate experiments. *A*: T1D development curve after HCT. *B*: Percent insulitis (n = 4–6). *C*: One representative spleen cell FACS profile of eight recipients examined with anti-H-$2K^b$ in each group. For DC patterns, spleens were digested with collagenase D and enriched using CD11c microbeads. *D*: One representative FACS profile of four recipients in each group examined with anti-CD45.2 and anti-CD45.1 for donor- or host-type $B220^+IgM^+$ or $Mac1^+/Gr1^+$ cells in the BM. *E*: Representative FACS profile of four recipients in each group examined with anti-H-$2K^b$ and anti-H-$2K^d$ for donor- and host-type thymocytes.

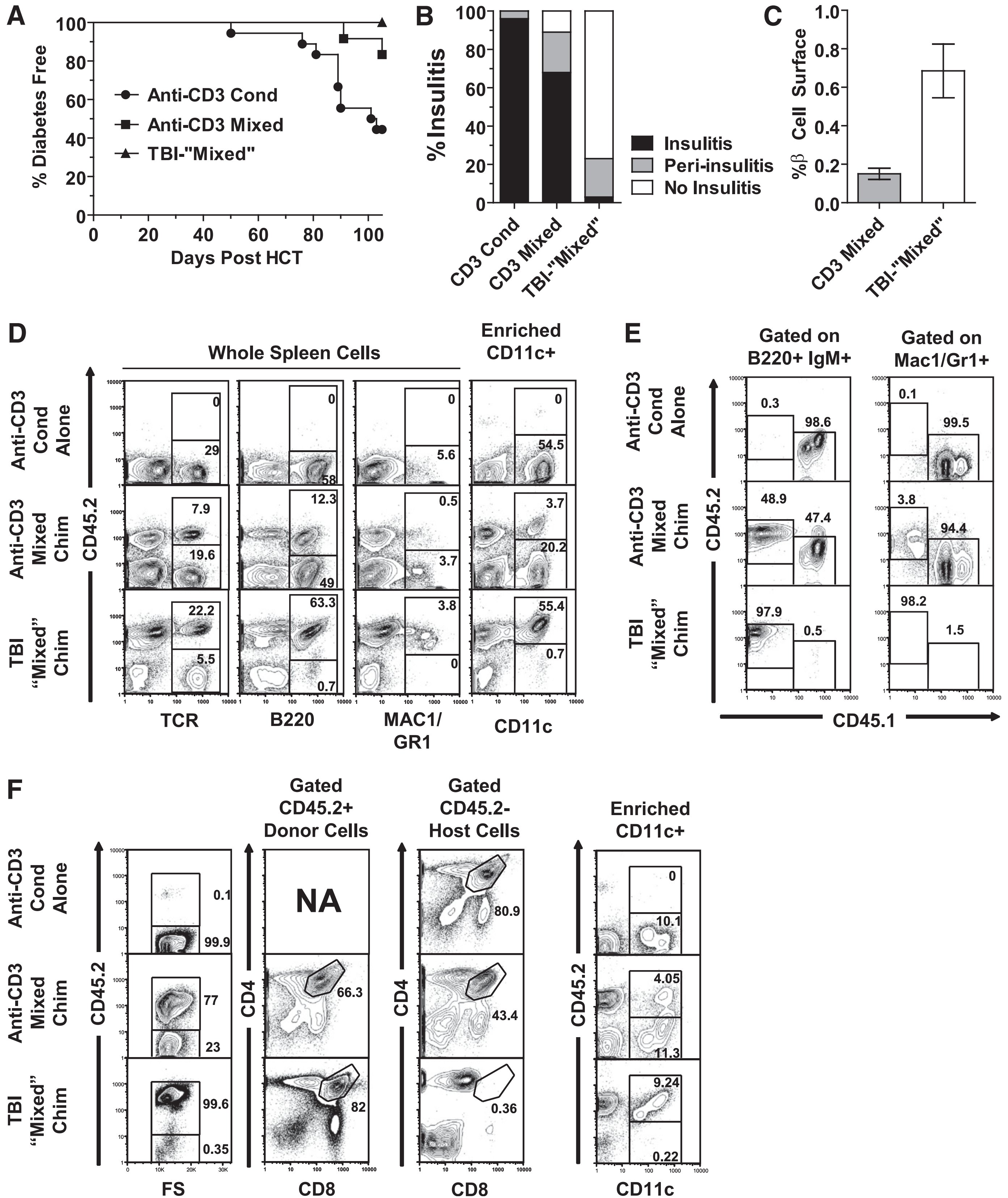

FIG. 2. Complete but not mixed chimerism with MHC-matched donor BM transplants prevented T1D. Wild-type NOD mice were conditioned with anti-CD3/CD8 on days −10 and −5. On day 0, the conditioned mice were transplanted with $CD4^+$-TCD splenocytes and BM (50×10^6 each) from MHC-matched H2-^{g7} C57BL/6 mice to induce mixed chimerism. To induce mixed chimerism with TBI conditioning, wild-type NOD were conditioned with 850cGy and transplanted with TCD BM (10×10^6). The control mice were given anti-CD3/CD8 conditioning only. Diabetes development was monitored weekly by both urine and blood glucose for up to 100 days after HCT. Thereafter, the recipient pancreas, spleen, BM, and thymus were harvested for evaluation of insulitis and chimerism status. *A*: Diabetes development curve after HCT (*n* = 12 for anti-CD3-conditioned chimeric recipients; *n* = 18 for conditioning alone: *n* = 6 for TBI-conditioned chimeric recipients). *B*: Percent insulitis (*n* = 4 for anti-CD3-conditioned

in the spleen were host-type. In contrast, in the complete chimeras, less than 1% of the T- and B-cells were host-type, although ~20% of the macrophage and granulocytes were host-type (Fig. 1*C* and Table 1). We should point out that those H-2^{b-} cells may not be true host-type cells, because we found that ~10% of the MAC-1/Gr-1^+ cells in the spleen of C57BL/6 mice are H-2^{b-} (Supplementary Fig. 3 and Supplementary Table 2). The mixed chimeric recipients had both donor- and host-type $B220^+IgM^+$ immature B-cells and $MAC1^{+/}Gr1^+$ myeloid cells in the BM, whereas the complete chimeras had only donor-type cells (Fig. 1*D*). Consistently, the mixed chimeric recipients had both donor- and host-type $CD4^+CD8^+$ thymocytes, and the complete chimeric recipients had only donor-type but no host-type thymocytes (Fig. 1*E*). Furthermore, after enrichment of $CD11c^+$ cells from thymus, we observed donor $CD11c^+$ dendritic cells (DCs) in the thymus of both mixed and complete chimeric recipients (Fig. 1*E*). This result is consistent with a previous report showing that donor-type DCs engraft in the thymus in the chimeric recipients as judged by immunohistochemistry (IHC) staining (37).

Taken together, these results indicate that induction of mixed chimerism with MHC-mismatched BM transplants is as efficient as induction of complete chimerism in prevention of insulitis and T1D.

Induction of mixed chimerism with BM transplants from MHC-matched donors did not prevent insulitis, although induction of complete chimerism did. It was reported by Beilhack et al. (29) that induction of mixed chimerism in lethally irradiated NOD recipients with BM cells from MHC-matched H-2^{g7} donors prevents T1D, but it was not clear whether those chimeric recipients were true mixed chimeras with de novo developed host-type T-cells. To clarify this issue, we performed a similar experiment by transplanting H-2^{g7} C57BL/6 (H-$2k^d$, I-A^{g7}, CD45.2) donor T-cell depleted (TCD) BM cells (10×10^6) into lethally irradiated NOD (H-$2k^d$, I-A^{g7}, CD45.1) recipients. In addition, we induced chimerism in anti-CD3/CD8-conditioned NOD recipients by transplanting BM and $CD4^+$ T-depleted spleen cells (50×10^6 each) from H-2^{g7} C57BL/6 donors. The control NOD mice were provided anti-CD3/CD8 conditioning only. The recipients and control mice were monitored for T1D development and checked for chimerism as described above.

We found that, consistent with the report of Beilhack et al. (29), the total body irradiation (TBI)-conditioned NOD recipients given TCD BM cells from MHC-matched H-2g7 C57BL/6 donors appeared to have so called mixed chimerism, as judged by the presence of both donor- and host-type T- and B-cells among blood mononuclear cells (data not shown), and no recipients developed T1D by 100 days after HCT, although ~61% (11/18) of control NOD given conditioning only developed T1D ($P < 0.01$, Fig. 2*A*). The anti-CD3/CD8-conditioned NOD recipients from BM and $CD4^+$ T-depleted spleen cells also showed mixed chimerism among blood mononuclear cells (data not shown), but ~17% (2/12) of them developed T1D by 100 days after HCT, although there was still a significant difference compared with NOD mice given conditioning only ($P < 0.05$, Fig. 2*A*). To further analyze the difference between the TBI-conditioned and the anti-CD3/CD8-conditioned chimeras, the pancreata of the chimeric recipients and control mice were evaluated for insulitis and their spleen, BM, and thymus were measured for chimerism. We found that, although the TBI-conditioned chimeric animals had little insulitis, the anti-CD3/CD8-conditioned chimeras had severe insulitis ($P < 0.01$, Fig. 2*B*) and marked reduction of β-cell surface as compared with the TBI-conditioned recipients ($P < 0.01$, Fig. 2*C*). These results indicate that insulitis is eliminated in TBI-conditioned but not in anti-CD3-conditioned chimeras.

Furthermore, we found that, although there were both donor- and host-type T-cells, B-cells, and macrophage/granulocytes among the spleen cells of TBI-conditioned or anti-CD3/CD8-conditioned chimeras, the donor-type cells were dominant in the former, which was consistent with the report of Beilhack et al. (29), but the host-type cells were dominant in the latter (Fig. 2*D* and Table 2). In addition the immature $B220^+IgM^+$ B-cells and Mac-1^+/Gr-1^+ myeloid cells in the BM were almost all donor-type in the TBI-conditioned recipients, although there were both donor- and host-type cells in the anti-CD3/CD8-conditioned chimeras (Fig. 2*E*). Consistently, although there were both donor- and host-type thymocytes in the TBI-conditioned chimeras, there were only donor-type but no host-type $CD4^+CD8^+$ thymocytes in the TBI-conditioned chimeras. In contrast, both donor- and host-type $CD4^+CD8^+$ thymocytes were present among the thymocytes of anti-CD3/CD8-conditioned chimeras (Fig. 2*F*). Finally, there was only donor-type $CD11c^+$ DCs in the thymus of TBI-conditioned chimeras, although there were both donor- and host-type $CD11c^+$ DCs in the anti-CD3/CD8 conditioned chimeras (Fig. 2*F*).

Taken together, the so-called mixed chimerism in lethally irradiated TBI-conditioned chimeras consists of radiation-resistant residual host-type but no de novo developed host-type hematological cells, and they are actually complete chimeras. In contrast, the mixed chimerism in anti-CD3/CD8-conditioned recipients consists of both residual and de novo developed host-type hematological cells, and they are truly mixed chimeras. These results indicate that induction of mixed chimerism with MHC-matched donor BM transplants cannot prevent insulitis, although induction of complete chimerism can.

Induction of mixed chimerism with BM transplants from MHC-mismatched but not matched donors resulted in thymic deletion of de novo developed host-type autoreactive T-cells. We next tested whether MHC-mismatched but not MHC-matched BM transplants were able to restore thymic negative selection of autoreactive T-cells in the mixed chimeric NOD mice, using T-cell receptor (TCR) transgenic BDC2.5-NOD mice (38). In addition, we tested whether donor cell expression of mismatched MHC II molecules was also required. Accordingly, anti-CD3-conditioned BDC2.5-NOD (H-$2k^d$, I-A^{g7}, CD45.1) mice were induced to develop mixed chimerism by injecting whole BM cells (50×10^6) from MHC-mismatched wild-type or MHC $II^{-/-}$ C57BL/6 (H-$2k^b$, I-A^b, CD45.2) or MHC-matched H-2^{g7} C57BL/6 (H-$2k^d$, I-A^{g7}, CD45.2) donors. The chimerism was evaluated by fluorescence-activated cell sorting (FACS) analysis of blood

chimeric recipients; *n* = 6 for TBI-conditioned chimeric recipients and conditioning alone). *C*: Percent β-cell surface area as compared with total pancreatic tissue surface area (*n* = 4–6). *D*: Representative FACS profiles showing spleen chimerism pattern 100 days after HCT (*n* = 12). For DC patterns, spleens were digested with collagenase D and enriched using CD11c microbeads. *E*: Representative FACS profiles showing donor- or host-type $B220^+IgM^+$ or $Mac1^+/Gr1^+$ cells in the BM (*n* = 4). *F*: Representative thymus chimerism pattern (*n* = 6–12).

TABLE 2
Percent donor- and host-type cells in spleen of chimeras given MHC-matched BM transplants

	TCRβ+	B220+	MAC1/Gr1+	CD11c+
Anti-CD3 mixed				
Donor-type	24.5 ± 2.2	16.3 ± 3.6	12.3 ± 3.0	10.8 ± 7.2
Host-type	75.5 ± 2.2	83.8 ± 3.6	87.7 ± 3.0	89.2 ± 7.2
TBI-mixed				
Donor-type	79 ± 0.9	98.5 ± 0.1	99.0 ± 0.2	97.1 ± 2.8
Host-type	21.0 ± 0.9	1.5 ± 0.1	1.0 ± 0.2	2.9 ± 2.8

Data are means ± SE; n = 4. Donor-type cells were defined as CD45.2, and host-type cells were defined as CD45.2$^-$.

mononuclear cells as described above (data not shown), and the recipients were monitored for T1D development.

We found that although 75% (9/12) of control BDC2.5-NOD mice given anti-CD3 conditioning only developed T1D, none (0/12) of the mixed chimeric recipients with MHC-mismatched BM showed T1D 160 days after HCT ($P < 0.01$, Fig. 3*A*). In addition, although the control mice had severe insulitis, the mixed chimeras with MHC-mismatched wild-type BM showed no insulitis ($P < 0.01$), although they showed mild peri-insulitis (Fig. 3*B*). It is of interest that induction of mixed chimerism with MHC-mismatched MHC II$^{-/-}$ or MHC-matched H-2^{g7} donor BM cells not only failed to prevent but markedly augmented T1D development, as compared with the control BDC2.5-NOD mice ($P < 0.01$, Fig. 3*A*). The mixed chimerism status in the different group was confirmed by flow cytometry analysis of the recipient spleen cells (Fig. 3*C*). These results indicate that induction of mixed chimerism with MHC-mismatched, but not MHC-matched, donor BM cells can prevent T1D development in transgenic autoimmune BDC2.5-NOD mice, and the expression of MHC II molecules by the MHC-mismatched donor cells is required for disease prevention.

We next tested whether there was a deletion of de novo developed host-type autoreactive T-cells in the thymus

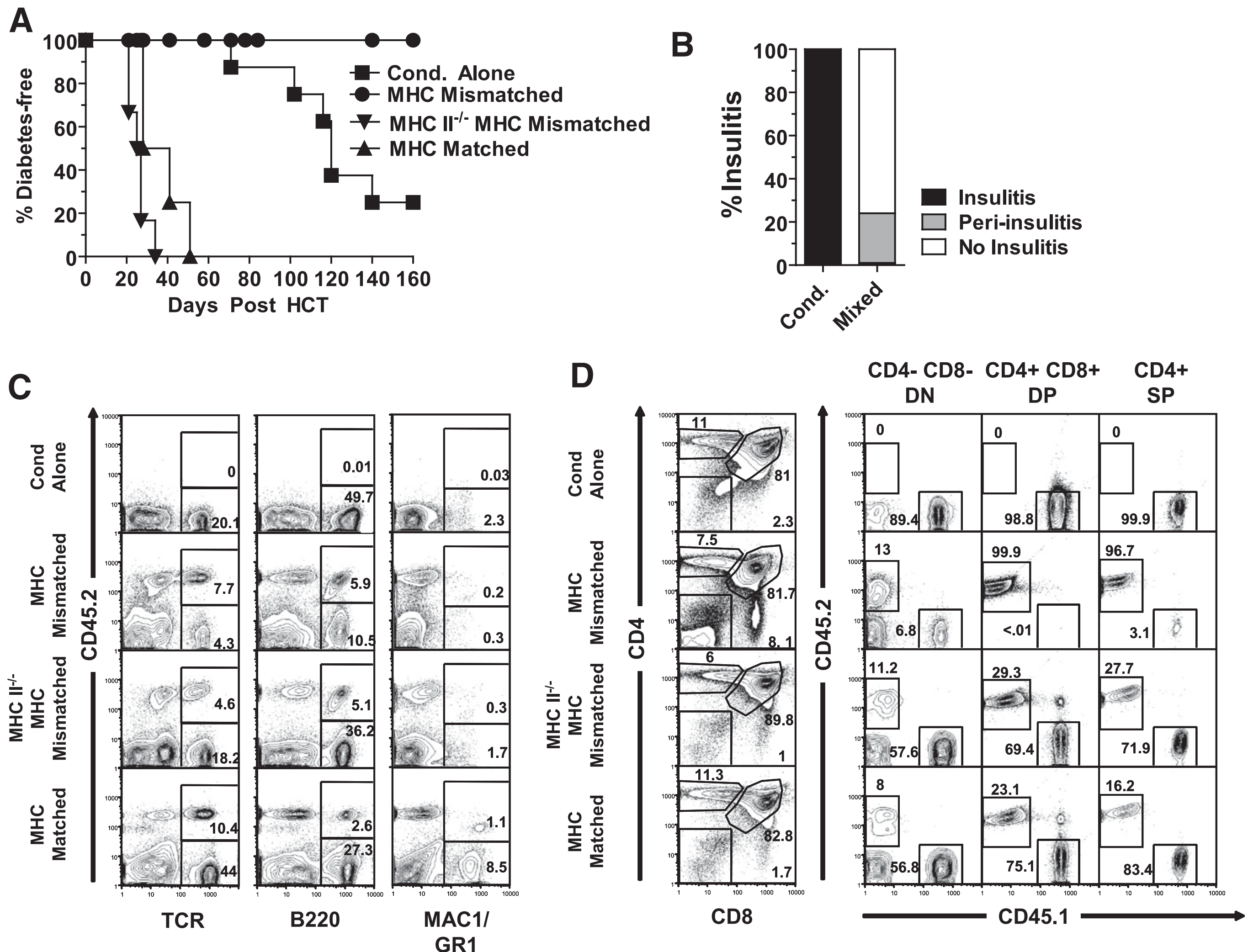

FIG. 3. Mixed chimerism with MHC-mismatched wild-type BM prevented T1D in transgenic BDC2.5-NOD mice. Transgenic BDC2.5-NOD mice were conditioned anti-CD3 (5 μg) on day −7. On day 0, the conditioned mice were transplanted with BM cells (50 × 10^6) from MHC-mismatched wild-type, MHC-mismatched MHC II$^{-/-}$ or MHC-matched H-2^{g7} C57BL/6 donors to induce mixed chimerism. Diabetes development was monitored weekly by both urine and blood glucose. *A*: Diabetes development curve after HCT (*n* = 12 for mice given conditioning alone or recipients given BM cells from MHC-mismatched wild-type donors; *n* = 7 for recipients given MHC-mismatched MHC II$^{-/-}$ donor BM cells; *n* = 8 for recipients given MHC-matched BM cells). *B*: Percent insulitis for recipients given MHC mismatched wild-type BM versus conditioning alone 120 days after transplantation (*n* = 4). *C*: Representative spleen chimerism pattern (*n* = 4). *D*: Representative thymus chimerism pattern (*n* = 4).

of the mixed chimeras given MHC-mismatched wild-type BM cells. We first compared the percentage of donor- and host-type thymocytes at $CD4^-CD8^-$, $CD4^+CD8^+$, and $CD4^+CD8^-$ stages (Fig. 3*D*). BDC2.5-NOD mice are a $CD4^+$ T transgenic mouse line (38), thus, we did not evaluate $CD4^-CD8^+$ thymocytes. We found that host-type thymocytes were present among $CD4^-CD8^-$, $CD4^+CD8^+$, and $CD4^+CD8^-$ in anti-CD3-conditioned control BDC2.5-NOD mice without HCT as well as in the mixed chimeras given MHC-mismatched MHC $II^{-/-}$ or MHC matched H-2^{g7} donor BM cells. In contrast, the host-type thymocytes in the mixed chimeras given MHC-mismatched donor BM cells were mainly present among $CD4^-CD8^-$ and $CD4^+CD8^-$ thymocytes, and they were nearly undetectable among total $CD4^+CD8^+$ thymocytes (Fig. 3*D*). However, after $CD45.1^+$ host-type cells were gated on, a small percentage of $CD4^+CD8^+$ cells were detected in those mixed chimeric recipients, although it was ~15-fold lower, as compared with control BDC2.5 mice or the mixed chimeras given MHC-mismatched MHC $II^{-/-}$ or MHC-matched donor BM cells ($P < 0.01$, Fig. 4*A*). Interestingly, the reduction of the percentage of $CD4^+CD8^+$ subset among host-type thymocytes in the mixed chimeras given MHC-mismatched wild-type donor BM cells was associated a more than 20-fold increase of $CD4^-CD8^-$ subsets ($P < 0.01$, Fig. 4*A*). These results indicate that host-type $CD4^-CD8^-$ thymocytes in the mixed chimeras given MHC-mismatched donor BM cells are prevented to develop into $CD4^+CD8^+$ stage.

Deletion of transgenic autoreactive thymocytes has been reported to take place at $CD4^-CD8^-$ stage (39,40). Thus we tested whether this is the case in the mixed chimeric BDC2.5-NOD recipients, using I-A^{g7}-mimotope-tetramer (BDC2.5-tetramer) to identify the autoreactive BDC2.5 T-cells, as previously described (41). We found that ~50% of the host-type $CD4^-CD8^-$ thymocytes in the control BDC2.5 mice given anti-CD3-conditioning only and the mixed chimeras given mismatched MHC $II^{-/-}$ or MHC-matched donor BM cells were BDC2.5-tetramer$^+$ and that there was no significant difference among the three groups (Fig. 4*A*). In contrast, the BDC2.5-tetramer$^+$ cells among the host-type $CD4^-CD8^-$ thymocytes in the mixed chimeras given MHC-mismatched wild-type donor BM cells were reduced by approximately fivefold as compared with control BDC2.5-NOD mice ($P < 0.01$, Fig. 4*A*). The yield of $CD4^-CD8^-$ BDC2.5-tetramer$^+$ thymocytes in the former group was also reduced approximately fivefold as compared with the latter ($P < 0.01$, Fig. 4*C*). In addition, we found that, although the majority of the residual host-type $CD4^+CD8^+$ or $CD4^+$ thymocytes and $CD4^+$ splenic cells in the mixed chimeras given MHC-mismatched wild-type donor BM cells were BDC2.5-tetramer$^+$, which was similar to the control BDC2.5 mice as judged by the percentage of BDC2.5-tetramer$^+$ cells (Fig. 4*B* and data not shown), the yield of the $CD4^+CD8^+$ and $CD4^+CD8^-$ BDC2.5-tetramer$^+$ thymocytes as well as the splenic $CD4^+$BDC2.5-tetramer$^+$ cells in those mixed chimeras was markedly reduced as compared with the control BDC2.5-NOD mice ($P < 0.01$, Fig. 4*D–F*). In contrast, induction of mixed chimerism with MHC-mismatched MHC $II^{-/-}$ or MHC-matched donor BM cells did not result in any reduction of the BDC2.5-tetramer$^+$ thymocytes or splenic $CD4^+$ T-cells as compared with control BDC2.5-NOD mice (Fig. 4*C–F*). These results indicate that induction of mixed chimerism with MHC-mismatched wild-type donor BM cells results in deletion of the de novo developed host-type autoreactive BDC2.5 transgenic T at $CD4^-CD8^-$ stage, and the deletion requires donor cell expression of mismatched MHC II molecules.

DISCUSSION

We believe this report represents novel findings by at least three considerations. First was our noting that induction of mixed chimerism with MHC-mismatched BM transplants from nonautoimmune donors is as effective as induction of complete chimerism in reversal of autoimmunity, elimination of insulitis, and prevention of T1D. Second, induction of mixed chimerism with MHC-matched BM transplants failed to reverse autoimmunity, eliminate insulitis, or prevent T1D development, although induction of complete chimerism with the matched BM transplants did. Finally, induction of mixed chimerism with MHC-mismatched but not MHC-matched BM transplant results in thymic deletion of de novo developed host-type autoreactive T-cells, and the deletion requires donor cell expression of MHC II molecules.

By measuring the chimerism of the de novo developed thymocytes, we clearly demonstrated that induction of mixed and complete chimerism with MHC-mismatched BM transplants was equally effective in reversal of autoimmunity in NOD mice. Previous reports stated that induction of so-called mixed chimerism in lethal or sublethally irradiated NOD recipients with BM cells from nonautoimmune donors prevented T1D (28,30–32). In those reports, so called mixed chimerism was defined by coexistence of donor- and host-type lymphocytes in the periphery of the chimeras, but it was not clear whether the host-type lymphocytes were de novo developed after HCT or residual host-type mature lymphocytes survived after TBI-conditioning. In the current study, we confirmed the mixed or complete chimerism by the presence or absence of de novo developed host-type $CD4^+CD8^+$ thymocytes as well as BM cells. We observed that, although a low level (i.e., ~1%) of host-type $CD4^+$ or $CD8^+$ lymphocytes were present in the periphery and thymus of the complete chimeras, there were no detectable $CD4^+CD8^+$ thymocytes in the complete chimeras. In contrast, both donor- and host-type $CD4^+CD8^+$ thymocytes were present in the thymus, and immature B- and myeloid cells were present in the BM of the mixed chimeras. We found that, even in the true mixed chimeric recipients with MHC-mismatched BM, insulitis was eliminated and T1D was prevented. Therefore, when MHC-mismatched donor BM transplantation is used, induction of mixed chimerism is sufficient for the cure of T1D autoimmunity. We believe this is of clinical significance since it has been reported that mixed chimeras with MHC-mismatched BM transplants provide superior immune function against infection (42,43).

Our studies also demonstrated that induction of mixed chimerism with MHC-matched BM transplants from nonautoimmune donors did not reverse autoimmunity or eliminate insulitis, although induction of complete chimerism did. Consistent with the report of Beilhack et al. (29), we observed that induction of chimerism in lethally irradiated NOD mice with TCD BM from MHC-matched H-2^{g7} C57BL/6 donors resulted in so-called mixed chimerism. However, we found that those chimeras did not have host-type $CD4^+CD8^+$ thymocytes or host-type BM resident $B220^+IgM^+$ or $MAC1^+/GR1^+$ cells, indicating that the host-type T- or B-lymphocytes were not de novo developed. Therefore, those so-called mixed chimeras were in fact complete chimeras. In contrast, we found that the true

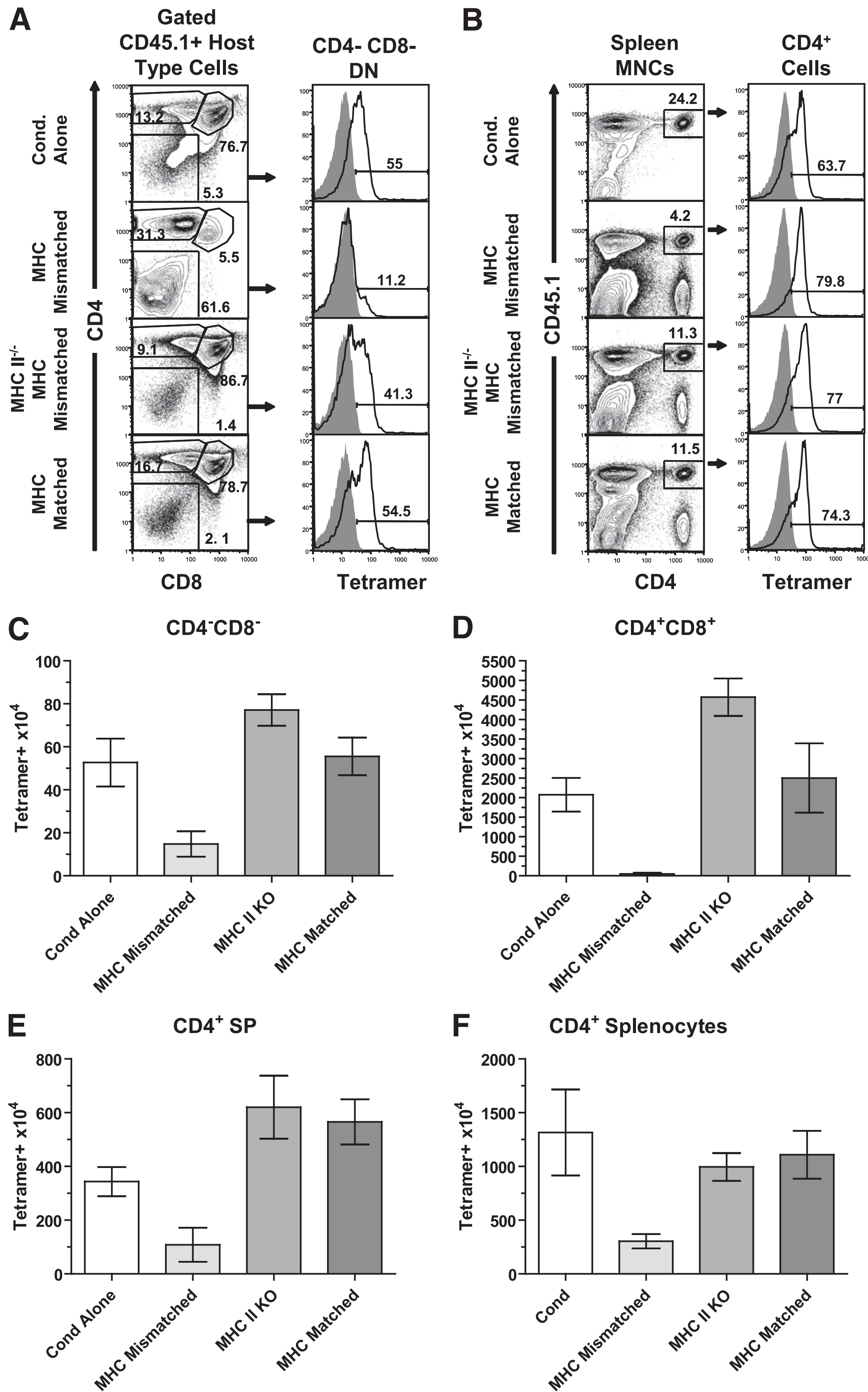

FIG. 4. Mixed chimerism with MHC-mismatched wild-type BM transplants mediated thymic deletion of de novo developed autoreactive T-cells. Transgenic BDC2.5-NOD mice were conditioned anti-CD3 (5 μg) on day −7. On day 0, the conditioned mice were transplanted with BM cells (50 × 10^6) to induce mixed chimerism. Diabetes development was monitored weekly by both urine and blood glucose. Mice were analyzed for clonal deletion of autoreactive T-cells at time of diabetes development, ~30–60 days after HCT. *A*: Representative FACS profiles showing clonal deletion of autoreactive T-cells in the thymus. $CD45.1^+$ host-type thymocytes were gated on and shown in CD4 versus CD8, and $CD4^-CD8^-$ cells were then gated and shown in histogram of BDC2.5-tetramer (solid line) versus control-tetramer (shaded area). *B*: Representative FACS profiles showing residual $tetramer^+$ T-cells in the spleen. Host-type $CD45.1^+CD4^+$ splenocytes were gated on and shown in histogram of BDC2.5-$tetramer^+$ (solid line) versus control-tetramer (shaded area) (*n* = 4). *C–F*: Yield of host-type $tetramer^+$ cells among $CD4^-CD8^-$, $CD4^+CD8^+$, and $CD4^+CD8^-$ thymocytes as well as $CD4^+$ splenocytes (*n* = 4).

mixed chimeras with MHC-matched BM transplants had both donor- and host-type $CD4^+CD8^+$ thymocytes as well as $B220^+IgM^+$ and $MAC1^+/GR1^+$ BM cells, and all of them had severe insulitis and reduced β-cell quantity, indicating that induction of mixed chimerism with MHC-matched donor BM cells is not able to reverse T1D autoimmunity.

Using approaches similar to those that have been used to evaluate thymic deletion of autoreactive T-cells mediated by hematopoietic cell-derived antigen-presenting cells (APCs) in previous publications (18,44,45), we demonstrated that induction of mixed chimerism with MHC-mismatched but not MHC-matched donor BM cells resulted in thymic deletion of de novo developed host-type autoreactive T-cells and that the deletion requires donor cell expression of MHC II molecules. We observed that de novo developed host-type autoreactive thymocytes in the mixed chimeric BDC2.5-NOD transgenic mice given MHC mismatch but not matched C57BL/6 BM cells were deleted before the $CD4^+CD8^+$ thymocyte stage when they started to express TCR, and the deletion required donor cell expression of mismatched MHC II molecules. This indicates that autoreactive T-cell interaction with donor-type APC via TCR-mismatched-MHC II complex play an important role in thymic negative selection of autoreactive T-cells in the mixed chimeras.

It was reported that the protection against T1D in (BDC2.5-NOD x C57BL/6) F1 mice ($H\text{-}2^{g7/b}$) was not associated with thymic deletion of the transgenic autoreactive T-cells but rather associated with positive selection of non-autoreactive T-cells expressing transgenic TCR coupling with endogenous Vαs (19). However, in the mixed chimeric BDC2.5 NOD mice, we found a marked reduction of NOD host-type thymocytes that express autoreactive TCR (Fig. 4), but no expansion of transgenic Vβ4 coupling with endogenous Vαs (Supplementary Fig. 4). Therefore, positive selection of nonautoreactive host-type T-cells by mismatched MHC II^b is unlikely to play a major role in reversal of autoimmunity in the mixed chimeric recipients. It was reported that MHC II-positive hematopoietic cells cannot mediate positive selection of $CD4^+$ T-lymphocytes (46). It is not surprising that tolerization mechanisms in mixed chimeras are different from F1 mice. In the chimeras, the MHC II ($I\text{-}A^b$) molecules are expressed by donor-type APC but not host-type thymic epithelial cells. The strong signals from TCR interaction with allo-MHC II may induce host-type thymocytes to go through apoptosis and result in deletion. In contrast, in F1 mice, the MHC II ($I\text{-}A^{g7/b}$) molecules are expressed by both thymic epithelial cells and APCs, in which they may be able to positively select non-autoreactive thymocytes with endogenous Vα and unable to mediate negative selection of autoreactive transgenic thymocytes.

We should also emphasize the association between failure in thymic deletion of the de novo developed host-type autoreactive T-cells and the failure in elimination of insulitis or prevention of T1D in the mixed chimeric recipients given MHC-matched $H\text{-}2^{g7}$ donor BM transplants. This indicates that re-establishing central tolerance in autoimmune T1D recipients is critical for prevention or reversal of autoimmunity. However, the significant delay of T1D onset in the mixed chimeric recipients given MHC-matched $H\text{-}2^{g7}$ donor BM transplants also indicates that peripheral tolerance mechanisms provided by nonautoimmune $H\text{-}2^{g7}$ donor cells play a significant role in regulating T1D development, although it is not sufficient to fully prevent autoimmunity. It was reported that non–MHC-associated loci from diabetes resistant mice mediated peripheral tolerance in autoimmune NOD mice (16,47).

In summary, we have identified the mechanisms wherein induction of mixed chimerism re-establishes the critical central tolerance in NOD mice. However, caution needs to be considered with regards to whether similar mechanisms operate in humans because of the far greater heterogeneity of this disease in humans (48). We recently reported that induction of mixed chimerism with MHC mismatched donor BM transplants under the radiation-free and GVHD preventative anti-CD3-based conditioning regimen can reverse new-onset T1D by augmenting residual β-cell expansion (33) as well as provide immune tolerance to donor islets (49). In addition, allogeneic donor T-cells in transplants have been shown not to cause GVHD in humans with mixed chimerism (50). Therefore, induction of mixed chimerism under the radiation-free anti-CD3-based conditioning regimen may represent a curative therapy for severe T1D.

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