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Development of Either Split Tolerance or Robust Tolerance along with Humoral Tolerance to Donor and Third-Party Alloantigens in Nonmyeloablative Mixed Chimeras

William F. N. Chan,* Haide Razavy,+ Bin Luo,2+ A. M. James Shapiro,+ and Colin C. Anderson3*†

Hematopoietic chimerism is considered to generate robust allogeneic tolerance; however, tissue rejection by chimeras can occur. This “split tolerance” can result from immunity toward tissue-specific Ags not expressed by hematopoietic cells. Known to occur in chimeric recipients of skin grafts, it has not often been reported for other donor tissues. Because chimerism is viewed as a potential approach to induce transplantation tolerance, we generated mixed bone marrow chimerism in the tolerance-resistant NOD mouse and tested for split tolerance. An unusual multilevel split tolerance developed in NOD chimeras, but not chimeric B6 controls. NOD chimeras demonstrated persistent T cell chimerism but rejected other donor hematopoietic cells, including B cells. NOD chimeras also showed partial donor alloreactivity. Furthermore, NOD chimeras were split tolerant to donor skin transplants and even donor islet transplants, unlike control B6 chimeras. Surprisingly, islet rejection was not a result of autoimmunity, since NOD chimeras did not reject syngeneic islets. Split tolerance was linked to non-MHC genes of the NOD genetic background and was manifested recessively in F1 studies. Also, NOD chimeras but not B6 chimeras could generate serum alloantibodies, although at greatly reduced levels compared with nonchimeric controls. Surprisingly, the alloantibody response was sufficiently cross-reactive that chimerism-induced humoral tolerance extended to third-party cells. These data identify split tolerance, generated by a tolerance-resistant genetic background, as an important new limitation to the chimerism approach. In contrast, the possibility of humoral tolerance to multiple donors is potentially beneficial. * The Journal of Immunology, 2008, 180: 5177–5186.

Establishing chimerism by bone marrow transplantation (BMT) has been thought to induce the most robust form of immunological tolerance to alloantigens (1–4) because it takes advantage of central tolerance, consistent with the way the immune system has likely evolved to eliminate most of the potentially destructive anti-self-responses (5). However, it is clear that even with chimerism, whether induced naturally or otherwise (6–11), it is possible for donor tissues to be rejected (known as “split tolerance”). We recently confirmed and extended this observation to murine chimeras generated by more than one clinically relevant protocol (12). Importantly, split tolerance appears to be possible in mixed chimeras (10–12) that are more clinically favored than full chimeras. Most past studies demonstrated split tolerance in chimeras that maintained donor hematopoietic cells but rejected donor skin transplants, the cause of which was likely immunity toward polymorphic tissue-specific Ags expressed by donor skin but absent from their bone marrow cells (10, 12–18). Although split tolerance involving tissues other than skin has not often been reported in murine chimeras, in a canine model split tolerance was observed in which chimeric recipients rejected donor hearts (19). Furthermore, pancreatic isoantigens were identified in rabbits (20). These findings suggest the possibility that split tolerance involving non-skin Ags may occur. In addition, potential host resistance to tolerance induction, or a general defect in self-tolerance, could lead to a state of split tolerance with or without involvement of donor tissue-specific Ags. We investigated whether this might be the case with NOD mice, whose background possesses the genetically dissociable traits of tolerance resistance (21–23) and autoimmune-mediated β cell destruction (24). Since chimerism is viewed to be a potential approach toward islet transplantation tolerance in type 1 diabetics, the potential for increased split tolerance in the NOD mouse model of human type 1 diabetes therefore requires full evaluation. Surprisingly, we found that NOD chimeras lacking β cell autoimmunity, unlike chimeric B6 controls, developed multiple levels of split tolerance such that donor T cells survived, but other donor hematopoietic cells including B cells as well as donor skin and islet transplants were rejected. Split tolerance was mediated by non-MHC genes.

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‡ Abbreviations used in this paper: BMT, bone marrow transplantation; KO, knock-out; Treg, regulatory T.
of the NOD genetic background. We also identified that, in mixed chimeras, humoral tolerance to donor alloantigens could be extended to third-party alloantigens.

Materials and Methods

Animals

Adult C3H (H-2b), C57BL/6 (B6; H-2d), and DBA/2 (H-2b) mice were purchased from the National Cancer Institute-Frederick. FVB (H-2q), B6.NOD (B6.g7; H-2g7), NOD.B10 (H-2\(^b\)), and NOD (H-2\(^b\)) mice were purchased from The Jackson Laboratory. BALB/c (H-2d) mice were purchased locally from the Health Sciences Laboratory Animal Services. C3H.129S6(B6)-Rag2tm1Fwa (C3H-RAG-KO) mice were purchased from Taconic Farms. B6.129S7-Rag1tm1Mom (NOD-RAG-KO) mice, as well as the F1 progeny of NOD and B6 or NOD and BALB/c crosses, were bred at the University of Alberta. B cell-deficient NOD mice (25) were provided by Dr. D. Serreze (The Jackson Laboratory, Bar Harbor, ME) and bred on site. Nondiabetic female NOD mice were used at 7–9 wk of age. All care and handling of animals were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Chimerism induction protocol and BMT

To induce chimerism in NOD or B6 mice, nondiabetic recipients were given a donor-specific transfusion of 20 million unmodified C3H spleen cells administered i.p. on day –7, anti-CD40L (MR1), and CTLA-4-Ig (each at 0.25 mg i.p.; Bio Express) on days –7, –5, –3, 0, 2, 4, and 6, busulfan (either provided by Outerbridge or purchased from Calgene) at 20 mg/kg i.p. on day –1, and sirolimus (Wyeth-Ayerst Pharmaceuticals) at 3 mg/kg i.p. and daily from days 0 to 28. Twenty or 40 million unmodified C3H bone marrow cells harvested from femur and tibia were injected i.v. on day 0.

Skin transplantation

Full-thickness tail skin grafts were transplanted onto the lateral thoracic wall of anesthetized recipients. Grafts were secured with sutures and protected with gauze and bandage for a minimum of 7 days. Health of donor skin was monitored by visual and tactile inspection. The day of skin rejection was defined as graft necrosis of < 100%.

Islet isolation and transplantation, nephrectomy, and glucose monitoring

Islet isolation was conducted as previously described (26). NOD and B6 mice were made diabetic by a single i.p. injection of streptozotocin (Sigma-Aldrich) at 200–225 mg/kg. Diabetes was confirmed by a blood glucose of > 20.0 mmol/L at least two readings from separate days) or up to 43 consecutive measurements from untreated and treated NOD mice until disease onset (n = 12). Mice were divided into groups of control non-chimeric, donor type, and third-party recipients, respectively. Four days later, recipient spleens were harvested and CFSE dilution in the transferred cells was examined by flow cytometry gating on TCR\(\beta^+\) cells. We did not observe any significant signs of graft-vs-host reaction other than splenomegaly in our recipients. Total splenocyte numbers were also determined, by counting on a hemacytometer to calculate the absolute number of T cells recovered.

Serum alloantibody detection

To detect alloantibodies in recipient serum, at > 14 wk after BMT, we immunized NOD and B6 chimeras as well as control nonchimeric NOD and B6 mice with 10 million \(\gamma\)-irradiated (1500 rad) or nonirradiated C3H whole spleen cells i.p. More than 3 wk later, we secondarily immunized them with 10 million irradiated FVB whole spleen cells i.p. Three to 4 weeks after each immunization, animals were tail-bled and sera were harvested. Sera from immunized NOD or B6 chimeras, immunized nonchimeric controls, or unimmunized naive controls were incubated with syngeneic, donor-type and third-party spleen cells. Binding of serum IgG Abs
to spleen cells was detected using FITC-conjugated Fcγ-specific rabbit anti-mouse IgG F(ab’)2, (Jackson ImmunoResearch Laboratories) and analyzed by flow cytometry.

**Statistical analysis**

The two-tailed Student t test was used for comparison of means between two groups. The log rank test was used to compare survival curves. One-way ANOVA and Tukey’s multiple comparison test were used to compare three or more means. All statistical analyses were done using Prism 4 (GraphPad Software) with statistical significance defined as p < 0.05.

**Results**

**Split tolerance toward donor hematopoietic lineages develops in tolerance-resistant NOD mice**

Using an irradiation-free, costimulation-blockade-based nonmyeloablative protocol, we induced mixed hematopoietic chimerism in NOD mice and, for comparison, in B6 mice that are not known for tolerance resistance or autoimmunity using fully mismatched bone marrow and tested for subsequent tolerance induction to donor tissues. From three independent experiments (Fig. 1, A–D), we induced mixed chimerism in 26 of 27 NOD mice (vs 100% success in B6 mice). In the majority of mice that we monitored chimerism for up to 32 wk after BMT, mixed chimerism was maintained both in NOD (Fig. 1, A–C) and B6 (Fig. 1D) mice and never showed a tendency to become full chimerism. Moreover, in those NOD and B6 chimeras that we sacrificed between 37 and 58 wk after BMT, all still contained mixed chimerism (Table I). Only 1 of 26 NOD chimeras completely lost chimerism eventually (Fig. 1, D). In general, the chimerism level was higher in B6 than in NOD mice and it was not increased by injecting twice as many donor cells into the NOD recipients (Fig. 1C vs Fig. 1, A and B).

We examined the lineage composition of donor cells present in NOD and B6 chimeras between 15 and 17 wk after BMT and found that the chimerism was multilineage. We detected, in PBMCs, various donor-type cells phenotypically consistent with T (TCRβ+ cells) of both sublineages (CD4+ or CD8+), B (CD19+) cells, NK (DX5+) cells, macrophages (CD11b+), and dendritic cells (CD11c+). In this period, chimerism in B6 mice was significantly higher than that in NOD mice. Additionally, we noted significant differences in the levels of most donor-type hematopoietic lineages found in B6 and NOD chimeras, with consistently lower levels in the latter mice (Fig. 1E). Between 23 and 40 wk after BMT, we failed to detect donor DX5+, CD11b+, or CD11c+ cells (except for TCRβ+ cells) in the spleen of most of the nine NOD chimeras that we analyzed, while multilineage chimerism was still detectable even in the blood of B6 chimeras at >32 wk after BMT (n = 8) and confirmed subsequently in the spleen (n = 2; W. F. Chan, unpublished observations). When we tracked the level of donor lymphocytes over time, we saw a steady persistence of donor T cells but a precipitous decline and an eventual complete loss of B cell chimerism in the peripheral blood of NOD but not B6 chimeras (Fig. 2A), suggesting that NOD chimeras were split tolerant to the different donor hematopoietic lineages. Subsequent analysis of chimerism in the spleen and bone marrow of NOD and B6 chimeras completely lost chimerism eventually (Fig. 1, D). In general, the chimerism level was higher in B6 than in NOD mice and it was not increased by injecting twice as many donor cells into the NOD recipients (Fig. 1C vs Fig. 1, A and B).

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eight NOD chimeras, between 23 and 40 wk after BMT, revealed a low to undetectable level of donor B cells in all mice (Table II). Furthermore, in two such chimeras, we also failed to detect donor B cells in the peritoneum and the peripheral lymph nodes (our unpublished observations). The absence of donor B cells was not simply due to down-regulation of the donor MHC class I molecule (K^b) or the B cell marker (CD19) that we stained for, because even when using a different set of markers for donor vs recipient cells (CD45.2 vs CD45.1, respectively) and additional B cell markers (B220, CD21, CD22, surface IgM, and the donor MHC class II molecule I-A^k), we failed to detect donor B cells in all tissues examined (our unpublished observations).

Table III. \( \beta \) expression on peripheral blood T cells from FVB to NOD mixed chimeras

<table>
<thead>
<tr>
<th>Chimera (n = 4)</th>
<th>Control (n = 2)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Donor cells (all)^a</td>
<td>4.43 ± 3.75^b</td>
<td>NA</td>
</tr>
<tr>
<td>% Donor B cells^1</td>
<td>1.61 ± 1.41</td>
<td>NA</td>
</tr>
<tr>
<td>% Donor T cells^1</td>
<td>5.31 ± 4.30</td>
<td>NA</td>
</tr>
<tr>
<td>% ( \beta ) 6^* CD4^+</td>
<td>7.89 ± 0.05^c,e</td>
<td>7.94 ± 0.49^d</td>
</tr>
<tr>
<td>% ( \beta ) 6^* CD4^-</td>
<td>9.21 ± 0.34^d,e</td>
<td>9.83 ± 0.40</td>
</tr>
<tr>
<td>% ( \beta ) 10^* CD4^-</td>
<td>2.61 ± 0.17</td>
<td>2.75 ± 0.32</td>
</tr>
<tr>
<td>% ( \beta ) 17^* CD4^-</td>
<td>4.45 ± 0.29</td>
<td>4.75 ± 0.37</td>
</tr>
<tr>
<td>% ( \beta ) 17^* CD4^-</td>
<td>0.23 ± 0.07</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td>% ( \beta ) 17^* CD4^-</td>
<td>0.00 ± 0.00</td>
<td>0.05 ± 0.00</td>
</tr>
</tbody>
</table>

^a Expressed as a percentage of all cells, all B cells, or all T cells, respectively.
^b All data are expressed as mean ± SEM.
^c NA, Not applicable.
^d \( \beta \) percentages reflect D^\*HL\* TCR\( \beta ^{-}\)-gated (recipient type) cells.
^e No statistical significance (p > 0.05) was found between chimeric and control NOD mice in CD4^+ or CD4^− recipient T cells expressing \( \beta \) 6 or \( \beta \) 10.
observations). However, despite the absence of multilineage mixed chimerism, we were able to detect donor T cells in the thymus and spleen of long-term chimeric NOD mice, confirming the systemic nature of donor chimerism (Fig. 2B). To investigate whether this chimerism could lead to deletion of donor-specific T cells, we generated another cohort of NOD chimeras using FVB mice as the source of donor bone marrow. In this combination, the frequency of recipient \( \text{V}^\beta_{10} ^+ \) T cells expressing the relevant \( \text{V}^\beta \) for binding to FVB-derived superantigen (27) can be monitored, and their decrease in our chimeras would suggest deletion of donor-reactive T cells. Table III shows that deletion of donor superantigen-reactive T cells was not evident at 11 or 19 wk after BMT in the NOD mixed chimeras, even in the presence of donor B cells that express donor-type MHC class II molecules. NOD chimeras were, however, able to delete in a normal fashion \( \text{V}^\beta_{17} ^+ \) T cells that recognize the corresponding endogenous superantigen, while preserving \( \text{V}^\beta_{6} ^+ \) T cells that are normally present in NOD mice.

**Split tolerance extends to donor skin and islets in NOD mixed chimeras lacking overt islet autoimmunity**

Although B6 mice appeared fully tolerant of donor hematopoietic cells, NOD recipients appeared tolerant of donor T cells but not donor B cells and other non-T cells. To further test the extent of split tolerance, we examined whether the NOD mixed chimeras could reject donor skin and islets. Based on our previous work (12) and that of other investigators (11, 28), showing that split tolerance occurs in MR1-treated nonautoimmune mixed chimeras given donor skin grafts late but not early after bone marrow infusion, we determined whether this would also be the case in our NOD chimeras. At 1–2 days or 14 wk after BMT, we challenged NOD and B6 chimeras with donor and third-party skin grafts. Although NOD chimeras acutely rejected third-party skin transplants as expected, they also rapidly rejected donor-type skin given late, indicating split tolerance (Fig. 3A). B6 mice made chimeric by the same protocol also rejected donor and third-party skin transplants given at 14 wk. Similar to our previous data with a different MR1-based protocol (12), B6 chimeras demonstrated long-term acceptance of donor-type skin grafts given early after BMT. Surprisingly, NOD chimeras transplanted with donor skin at this early time instead rejected the grafts (rejection was delayed compared with late skin grafting, \( p < 0.01 \)), demonstrating split tolerance to skin could be reduced in B6 but not NOD mice by giving the graft early (Fig. 3A).

Having shown that NOD mice appear to have a greater propensity for split tolerance to both donor skin, and certain lineages of donor hematopoietic cells, we next investigated whether split tolerance extended to donor islets. Even at moderate levels, mixed chimerism in nonautoimmune mice led to robust tolerance to strongly mismatched donor islet transplants (29), suggesting that split tolerance may be less of an issue for islets than skin. However, either islet autoimmunity or tolerance resistance of NOD mice could potentially alter the outcome. We found with our protocol that transplantation of fully mismatched bone marrow did not universally abrogate diabetes (Fig. 3B). Nevertheless, the general rate of diabetes was significantly reduced in chimeras, reducing the likelihood that any islet rejection we might observe would be due to autoimmunity. Furthermore, we gave high-dose streptozotocin to induce diabetes in those mixed chimeras that remained nondiabetic before islet transplantation, a treatment that is known to prevent islet autoimmunity in NOD mice (30). Despite the presence of mixed chimerism, NOD chimeras rejected donor islets as rapidly as control nonchimeric NOD mice (Fig. 3C), indicating that split tolerance in relation to tissue transplants was not exclusive to skin. Even donor islets transplanted at the time of bone marrow infusion were rejected by NOD chimeras, despite high levels of chimerism in some of these mice both during and after rejection (Fig. 3D). However, syngeneic islets were accepted long-term when transplanted into NOD chimeras that had either rejected donor islets or were not previously challenged, indicating an absence of islet-specific autoimmunity (Fig. 3C). Although NOD chimeras were split tolerant to donor islets, B6 chimeras showed long-term acceptance of donor islets but not third-party islets (Fig. 3C); donor islet acceptance even occurred in the one B6 recipient with long-term low-level chimerism (<4%; Fig. 1D). Control naive B6 mice acutely rejected donor and third-party islets (Fig. 3C). Thus, although we showed that mixed chimerism was achieved in NOD mice and that they demonstrated an even more pronounced split tolerance to donor skin than nonautoimmune-prone mice, there was additionally split tolerance to donor islets; surprisingly, this was not a result of islet autoimmunity.

**NOD mixed chimeras show partial donor alloreactivity and humoral tolerance extends to third-party cells**

Because islet-specific autoimmunity was not the cause of split tolerance in NOD chimeras, we tested whether they may demonstrate residual alloreactivity to the donor (tolerance resistance). Between 20 and 32 wk after BMT, we performed in vivo proliferation assays by transferring CFSE-labeled spleen cells from naive or chimeric NOD mice to NK cell-depleted syngeneic, donor-type, and third-party RAG-KO recipients. A comparable degree of proliferation by chimeric and control cells was observed in the third-party recipients, which was at least 10-fold greater than the background.
homeostatic proliferation of chimeric and control cells when transferred into syngeneic RAG-KO recipients. In contrast, we observed more undivided chimeric NOD cells than nonchimeric control B cells in the donor type recipient, based on lower T cell recovery and a higher percentage of T cells with undiluted CFSE (undivided cells; Fig. 4). However, proliferation and recovery of chimeric NOD cells were still significantly higher in the donor-type recipient than in the syngeneic recipient, indicating alloreactivity was not completely abrogated in the chimeras. Hence, chimerism induction in NOD mice led to a partial loss of donor-specific alloreactivity.

Because our NOD mixed chimeras were eventually devoid of donor B cells and other donor MHC class II-expressing hematopoietic cells (Fig. 2A, Table II, and our unpublished observations), we tested for their production of donor alloantibodies and compared their response with that of B6 chimeras that were able to maintain donor class II-expressing cells. When we assessed serum Ab binding to TCRβ⁺ (top) vs TCRβ⁻ (bottom) syngeneic (light gray filled) or donor (black line) spleen cells following incubation with serum from C3H-immunized nonchimeric NOD controls (n = 8) or NOD chimeras (n = 5; chimeras with detectable alloantibodies). C, Nonchimeric NOD and B6 mice (n = 3) were immunized with C3H spleen cells. Their sera were then tested for reactivity to C3H cells (dark gray filled) and different third-party cells (thin black line, FVB; dotted black line, BALB/c; thick black line, NOD cells for B6 sera or B6 cells for NOD sera) and compared with syngeneic cells (light gray filled). Sera were obtained from these mice before immunization and tested similarly (naive NOD and naive B6). As a negative control, B cell-deficient NOD mice (NOD-μMT; n = 3) were immunized and their sera tested. D, C3H-immunized NOD (n = 4) and B6 (n = 3) chimeras from A as well as nonchimeric NOD and B6 control mice (n = 2) were further immunized with FVB spleen cells. Sera were harvested and tested for Ab reactivity to C3H (solid black line) and FVB (dotted black line) cells as compared with syngeneic cells (light gray filled). A–D, Representative plots are shown.
FIGURE 6. Non-MHC genes of the NOD background control split tolerance to donor hematopoietic cells without altering Treg cell frequency. NOD.B10, B6.g7, (NOD × B6)F1, and (NOD × BALB/c)F1 mice (n = 3–4) were made chimeric with C3H bone marrow cells by our chimerism induction protocol. PBMCs were analyzed up to 15 wk after BMT for chimerism. A, Overall donor cells; B, donor T cells; and C, donor B cells. Mean and SEM are shown at each time point. Only NOD.B10 chimeras lost donor B cells. D, All groups of chimeras were analyzed for the frequency of potential Treg cells in their peripheral blood at the time when NOD.B10 chimeras lost donor B cells. The bar graph depicts mean and SEM for each group. There was no significant difference in Treg cell frequency between chimeras with and without donor B cells.

Interestingly, immunization of nonchimeric NOD and B6 mice with C3H spleen cells resulted in production of alloantibodies that strongly cross-reacted with cells from multiple third-party strains (Fig. 5C). We therefore examined whether the alloantibody tolerance generated in C3H to B6 or NOD chimeras resulted in B cell tolerance to a third-party strain. Fig. 5D shows that B cell complete tolerance or partial tolerance in B6 and NOD mixed chimeras, respectively, extended to third-party alloantigens.

Non-MHC genes control split tolerance development in NOD mixed chimeras

To begin investigating the mechanism responsible for the tolerance resistance of NOD chimeras that was associated with multiple levels of split tolerance, we took a genetic approach and generated three different types of mixed chimeras using our induction protocol: 1) NOD.B10 chimeras, carrying the NOD background genes but expressing H-2Kb; 2) B6.g7 chimeras, carrying the B6 background genes but expressing H-2Kq; and 3) chimeras in which the recipient background was the F1 generation of crosses between NOD and B6 or BALB/c mice (Fig. 6A). As shown in Fig. 6, B and C, split tolerance to hematopoietic cells previously demonstrated by NOD chimeras (Fig. 2) was reproduced only in NOD.B10 chimeras, indicating that the non-MHC genes of the NOD background but not the H-2Kq MHC haplotype contributed to this outcome. Moreover, crossing NOD mice with tolerance-susceptible B6 or BALB/c mice abrogated split tolerance when chimerism was induced, indicating that this phenotype was recessive to that associated with full tolerance. To begin to determine how genetics may influence split tolerance, at the time when NOD.B10 chimeras lost donor B cells completely, we analyzed the frequency of potential regulatory T (Treg) cells in their peripheral blood and compared that to the frequency observed in B6.g7 chimeras as well as in F1 chimeras. We found no significant differences, suggesting that split tolerance to hematopoietic cells could not be attributed to an altered frequency of cells with a Treg phenotype (Fig. 6D).

Discussion

Mixed chimerism is a promising approach to induce transplantation tolerance in the clinic because the tolerance induced is the most robust tolerance so far achieved experimentally and because less toxic conditioning regimens have now been developed. However, although the strength of chimerism-induced tolerance is well appreciated (1–4), a serious potential pitfall of chimerism-induced tolerance, split tolerance (8–15), has received little attention in recent years despite the potential problems it may pose for this approach. We considered that split tolerance may be even more likely to occur in current chimerism protocols; there is a push for milder (clinically feasible) conditioning regimens that leave more of the recipient T cell compartment intact, and hence success becomes more dependent on peripheral tolerance in both direct and indirect donor-reactive T cells. We have shown that stable mixed chimeras induced by nonmyeloablative approaches with few exceptions generate split tolerance, where donor hematopoietic cells persist and donor skin grafts are rejected (12). This split tolerance was attributed to a response against tissue-specific Ags since skin rejection could be prevented by giving the skin graft early, providing a source of donor skin Ags during the “tolerance-promoting” treatment with MR1 Ab. However, unlike B6 chimeras, the NOD chimeras rejected donor skin grafts even when the grafts were given early (Fig. 3A). It therefore seemed likely to us that autoimmunity, or genetic traits associated with autoimmunity or tolerance resistance, may heighten the potential for split tolerance via additional mechanisms.

Indeed, our NOD recipients could be more resistant to chimerism-induced tolerance of islets due to islet autoimmunity (31) and their known resistance to tolerance induction strategies (21–23). However, although islet-specific autoimmunity could lead to donor islet rejection by chimeras, as suggested in a previous study (32), the lack of syngeneic islet rejection in our study indicated that autoimmunity could not explain the split tolerance. The remaining low-level alloreactivity to the donor, detected in NOD chimeras, could instead explain their increased split tolerance as compared with B6 chimeras. As B6 chimeras maintained donor B cells and other donor MHC class II-expressing cells while lacking alloantibodies, this suggests that NOD chimeras failed to become tolerant to donor class II but not donor class I (donor T cells persisted); studies on the specificity of the alloantibodies produced should help clarify this issue. We therefore propose that the “tolerance-resistant” phenotype of the NOD mouse (21–23) prevented the MR1-based chimerism induction protocol from generating complete tolerance of Ags in donor hematopoietic cells; a sufficient number of anti-donor CD4 T cells likely remained responsive and induced split tolerance. This seems to be a likely possibility, since...
CD4 T cells appear to play a prominent role in the immune reactivity of NOD mice (33–35).

Since donor T cells were the only cell type that the NOD chimeras maintained at a stable low level while other hematopoietic cells and nonhematopoietic tissues were rejected, this also potentially suggests a difference in susceptibility among various cells and tissues to recipient effector mechanisms. In this regard, the indirect pathway of rejection by CD4 T cells is unusually potent in NOD mice (33, 34). Hence, we have recently investigated the issue of differential tissue sensitivity to CD4-mediated indirect rejection in a TCR-transgenic model. These studies showed that CD4 T cells are able to indirectly reject donor B cells, islets, and skin, while in the same recipients the donor T cells are resistant to this pathway of rejection (submitted for publication). These data parallel those found here in the NOD chimeras, suggesting a possible mechanism for the multiple levels of split tolerance we have observed.

Notwithstanding, we took a genetic approach and determined that the non-MHC genes of the NOD genetic background contributed to the split tolerance toward donor hematopoietic cells in NOD chimeras. NOD.B10 mixed chimeras but not B6g7t chimeras showed loss of donor B cells while maintaining donor T cells (Fig. 6, A–C), an observation that clearly attributes split tolerance to genetic elements of the NOD background outside of the MHC. Interestingly, our studies in chimeric F1 hosts suggest that not only the split tolerance phenotype, but also the general tolerance resistance, may be demonstrated to be a recessive trait. This contrasts with existing data showing the resistance of diabetes-free (NOD × B6)F1 mice to tolerance induction by a donor-specific transfusion and MR1 treatment (22). The difference in outcomes could be due to the greater potency of our tolerance induction protocol, in combination with chimerism induction, in overriding any inherent tolerance resistance of F1 hosts.

Treg cells are increasingly thought to play a critical role in both self-tolerance and acquired tolerance (36). Therefore, the increased split tolerance in NOD chimeras could alternatively be due to deficient or defective regulatory activity that operates through the tolerance resistance conferred by non-MHC NOD genes. However, our analysis of the frequency of potential Treg cells in chimeras with or without split tolerance (Fig. 6D) suggests that any association between altered Treg cell activity and split tolerance would more likely be related to function rather than frequency, given the lack of differences in the frequency of FoxP3-expressing CD4 T cells. This would be consistent with the idea that the loss of self-tolerance leading to autoimmune diabetes induction is related to a temporal, functional defect in Treg cells rather than their reduced frequency (37). However, at present, we cannot rule out a difference in the frequency of donor-specific Treg cells. Furthermore, the possibility that the known thymic defect in NOD mice (38–40) could have contributed to split tolerance in chimeras also warrants future investigation.

Various protocols have been tested to generate long-term mixed chimerism (defined here as ≥0.1% donor cells without full chimerism) in NOD mice, but few attempts (27, 41) were made with fully mismatched combinations and none achieved adequate longevity of mixed chimerism induced by practical means. From past studies, chimerism induction either led to initial mixed chimerism that eventually became full chimerism (42), or had an unknown fate because it was not monitored long term (41), or was sustained but required mega-dose bone marrow and was generated by potentially more risky infusion of large numbers of donor CD8 T cells (27). Many studies reported the ability of chimerism induction to promote long-term islet acceptance in NOD mice that already manifested autoimmune diabetes (31, 32, 41, 43–46). The strain combinations used in these studies in most cases were not fully mismatched, having either partial (31, 32, 44, 45) or complete MHC matches (46). Difficulty in sustaining mixed chimerism long term was encountered even in studies that used partially matched donor bone marrow (32, 44). However, in one study, fully mismatched islets were accepted by diabetic NOD mice made chimeric, although full chimerism may have explained the tolerance (43). The lack of success in some instances may reflect a competitive developmental advantage of resident NOD hematopoietic stem cells over the exogenously introduced stem cells from non-autoimmune donors (47). We developed a relatively mild conditioning regimen that would generate long-term mixed chimerism in NOD mice and extensively characterized the chimeric state and the effects of chimerism on the host immune system. We found that split tolerance can indeed occur in NOD mixed chimeras and that the split tolerance extended to many more donor tissues (skin, islets, and some hematopoietic cells) in NOD chimeras than in B6 chimeras (only donor skin grafts given late). In contrast to our study, Liang et al. (27) generated stable multilineage chimerism in nondiabetic NOD mice and found a lack of split tolerance with skin grafts (islet transplants were not assessed). The differing outcomes might be explained by the induction protocols used, the strain combinations, the multilineage nature of the chimerism, or simply the level of chimerism achieved.

With regard to the type of protocol used and its association with split tolerance induction in chimeras, we and others (11, 12, 28) have shown that the timing of tissue transplantation in relation to the initiation of a chimerism induction protocol that is based on costimulation blockade could influence whether split tolerance is observed. This, however, did not apply to NOD chimeras. Moreover, we tried a number of induction protocols to generate chimerism in NOD mice (our unpublished observations), but with the exception of the protocol reported in this study, we were unable to achieve stable mixed chimerism, thus preventing us from assessing split tolerance. This is clearly consistent with the known tolerance resistance of NOD mice (21–23) and indicates that greater requirements must be met for chimerism induction in the NOD model. However, it does not appear that the level of chimerism by itself explains the rejection of donor islets in NOD but not B6 chimeras, as even a very low-level chimerism (1–3%) was sufficient to prevent rejection of fully mismatched donor islets in B6 mice (Figs. 1D and 3C and our unpublished observations). Given these and other data (32), it seems likely that the threshold level of chimerism required to prevent islet rejection is increased in the autoimmune-prone NOD background.

We were able to detect alloantibodies in NOD chimeras but not B6 chimeras, suggesting more complete B cell tolerance in the latter hosts. It was surprising to find that this partial or full humoral tolerance extended to a third-party strain fully mismatched with the recipient. In most other studies of humoral immunity in mixed chimeras, alloantibody production to third-party cells was not investigated (48–50). The lack of alloantibody responses to third-party cells is unlikely to be due to the previously described general immunodeficiencies in mixed chimeras (51), attributed to MHC mismatches between the host thymus and peripheral APCs; the alloantibody-producing B cells in NOD chimeras are of the host type, expressing the appropriate MHC that is also present in the host thymus. In addition, Serreze et al. (49) examined Ab responses after generation of mixed chimerism and observed an intact response to nominal Ag in adjuvant. Further supporting the conclusion that the absence of alloantibodies was due to tolerance rather than immunodeficiency, NOD mixed chimeras were often able to make alloantibodies to donor (and third-party) non-T cells,
while maintaining specific tolerance in terms of the reduced allografts specific to Ags on donor T cells. Instead, the extension of alloantibody tolerance to third-party cells is explained by the cross-reactive nature of the alloantibody response. Such cross-reactivity has been observed previously (52–57). However, humoral tolerance to third-party cells was surprising in that an alloantibody response to private specificities might have been expected. This suggests that humoral tolerance may involve a dominant tolerance mechanism (e.g., Ab feedback through FcR (58) or Treg cells to public specificities on MHC or additional Ags (57, 59) and not simply deletion of donor-specific B cells (60). Although cross-reactive alloimmunity has been well demonstrated, we are not aware of any published data showing that humoral tolerance to one donor can extend to additional donors. One previous study did find that in xenogeneic chimeras, xenoreactive Abs cross-reactive to third-party cells were absent; however, it was not determined whether these recipients were humorally tolerant or simply ignorant of third-party cells (recipients were not immunized with third-party cells before assessment of humoral immunity) (57). An important implication of our finding is in the setting of transplantation from multiple consecutive tissue donors, as occurs in clinical islet transplantation (61), where tolerance to many donors could be of substantial benefit (62). In addition, the related donor-nonspecific humoral tolerance could play a role in third-party heart graft acceptance found in a neonatal tolerant model (63).

Collectively, our data indicate that in addition to the recognized role of tissue-specific Ags, an inherent tolerance-resistant genotype may contribute to the development of split tolerance. This suggests that split tolerance is likely to be a more important obstacle to the success of chimerism approaches than previously considered. In contrast, the potential for multiple donor humoral tolerance appears to be a distinct advantage of the mixed chimerism approach.

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Disclosures

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References


