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Differential Susceptibility of Allogeneic Targets to Indirect CD4 Immunity Generates Split Tolerance

William F. N. Chan,2* Haide Razavy,† and Colin C. Anderson3,4*†

CD4 T cells frequently help to activate CD8 T and B cells that effect transplant rejection. However, CD4 T cells alone can reject transplants, either directly or indirectly. The relative effectiveness of indirect CD4 immunity in rejecting different types of allogeneic grafts is unknown. To address this, we used a TCR transgenic mouse model in which indirect CD4 alloimmunity alone can be studied. We challenged transgenic recipients with hematopoietic cells and shortly thereafter skin transplants that could only be rejected indirectly, and observed Ag-specific indirect donor B cell and skin rejection, but not T cell elimination, reflecting a state of split tolerance. Deficiency of indirect CD4 alloimmunity in donor T cell rejection was also apparent when acute indirect rejection of donor islets occurred despite generation and maintenance of mixed T cell chimerism, due to migration of the few passenger T cells into recipient circulation. Although passenger lymphocytes delayed indirect islet rejection, they enhanced rejection by a full repertoire capable of both direct and indirect reactivity. Interestingly, the persistence of chimerism was associated with the eventual development of tolerance, as demonstrated by acceptance of donor skin grafts given late to hematopoietic cell recipients, and hyporesponsiveness of transgenic T cells from islet recipients in vitro. Mechanistically, tolerance was recessive and associated with progressive down-regulation of CD4. Collectively, our data indicate that indirect CD4 immunity is not equally destructive toward different types of allogeneic grafts, the deficiency of which generates split tolerance. The futility of these responses can convert immunity into tolerance. The Journal of Immunology, 2008, 181: 4603–4612.

CD4 T cells help to activate CD8 T and B cells that respond to various immunological challenges, including the undesirable outcome of transplant rejection. The provision of T cell help is not the only role played by CD4 T cells, however, as it has been demonstrated that they are alone sufficient to induce transplant rejection (1–7) or tumor clearance (8). As proposed by the passenger leukocyte hypothesis (9, 10), graft rejection can occur following T cell activation by the direct pathway of allore cognition, and it is generally agreed that acute rejection depends initially on recipient T cell recognition of alloantigens presented on donor MHC molecules (11). However, indirect allore cognition involving the presentation of donor MHC (12) or non-MHC (13–15) peptides by recipient MHC molecules is now viewed to be relevant to the overall immunity toward donor grafts. Certain alloantigens may even be preferentially presented to recipient T cells via the indirect pathway (16). Importantly, CD4 T cells with known antigenic specificities have been shown to reject transplants following direct (7, 17, 18) or indirect (7, 17) activation, in the absence of immunity from CD8 T or B cells, and in the absence of any demonstrable antigenic crossreactivity (7). Moreover, indirect CD4 responses may be particularly important in xenograft rejection (19), and alloimmunity and/or autoimmunity generated by NOD mice toward islet transplants (6, 20, 21). In contrast, tolerance induction in certain transplant situations may depend on the indirect pathway (22). A largely unexplored issue relating to indirect CD4 immunity is the determination of whether it is equally destructive toward different types of allogeneic grafts. In this study, we provide evidence that this is not the case. The indirect response can, in fact, be futile in its efforts to eliminate some allogeneic targets but not others, thereby generating split tolerance. Surprisingly, the deficiency of indirect CD4 immunity could lead to tolerance induction.

Materials and Methods

Animals

Adult C57BL/6 (B6; H-2b), CD45.1-expressing B6, BALB/c (H-2d), and BALB/c-SCID mice were purchased from National Cancer Institute, Frederick. MHC class II-deficient B6.129-H2-Ab1tm1Gru mice were purchased from Taconic Farms. (C57BL/6j × C57BL/10scSnJ)F1[KO]yc–[KO]Rag2 mice (23) that are double knockouts (KO) devoid of T, B, and NK cells (RAG2/yc-KO) were obtained through the National Institute of Allergy and Infectious Diseases Exchange Program. B6.C-H2-Ab1tm12 (bm12; H-2bKO) and B6.C-H2-Ab1tm12/LilMcD (BALB.B; H-2b) mice were purchased from The Jackson Laboratory. B6.129S7-Rag1tm1Mom (RAG-KO) mice and TCR transgenic Marilyn (24) mice on the B6 background were bred at the University of Alberta. Fetuses harvested from pregnant CD45.1-expressing B6 mice or bm12 mice were used at days 14–15 of gestation. All care and handling of animals was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

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The Journal of Immunology

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Fetal liver cell (FLC) transplantation

Fetal livers were harvested and homogenized into single cell suspensions. Sex determination of each fetal liver was performed as previously described (25). Fetal livers were pooled according to sex, and 10 million male or female CD45.1-expressing B6, or bm12 FLCs were injected i.v.

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). Mice were made diabetic by a single i.p. injection of streptozotocin (STZ; Sigma-Aldrich Canada) at 200 mg/kg. Diabetes was confirmed by a blood glucose of >20.0 mmol/L. Five hundred islets were transplanted into the renal subcapsular space of diabetic recipients. Recipient blood glucose was monitored to detect rejection (>15.0 mmol/L on two consecutive readings on different days). Some recipients that rejected the islets were given a second, syngeneic transplant to the contralateral kidney, or into naive female Marilyn recipients. In other experiments testing for dominant tolerance, male B6 and male BALB.B splenocytes (6 million nonirradiated cells of each type) were injected i.p. into naive female Marilyn mice. Some recipients that rejected the islets were immunized 10 days earlier, as well as immunized male and female B6 mice and unimmunized female B6 mice, all received a mixture of male and female B6 and BALB.B CFSE-labeled target cells i.v. (3 million cells of
each type). Forty-four hours later, the recipients were sacrificed, and their splenocytes were harvested for flow cytometric analysis.

**In vitro proliferation assay**

In vitro Marilyn responses were tested by a standard MLR. In brief, $5 \times 10^4$ female Marilyn responder splenocytes were cocultured with titrated numbers (starting at $1 \times 10^6$) of irradiated (1500 rads) stimulator splenocytes for 72 h and then pulsed with methyl-[^3H]thymidine and incubated for an additional 16 h before harvesting and counting. In the indicated experiments, responders were magnetically sorted for Vβ6 expression before culture (negative selection by anti-CD8, CD19, CD49b, and B220 microbeads, followed by positive selection using biotinylated anti-Vβ6 Ab and streptavidin microbeads; 89.8 ± 3.9% of T cells were CD4^+ Vβ6^+ after enrichment).

**Statistical analysis**

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

**Results**

*Indirect CD4 alloimmunity alone rejects islet transplants but not their passenger cells; passenger cells play opposing roles in transplantation immunity*

Marilyn, a TCR transgenic mouse that contains a monoclonal population of CD4 T cells specific for the male histocompatibility Ag, H-Y, presented in I-Ab (24), has been used to demonstrate that indirect CD4 immunity to a defined alloantigen, alone, can be sufficient to acutely reject skin transplants (7) and tumor cells (8) but not heart (18) or thymus (27) transplants. In these recipients, only those tissues expressing the male Ag evoke T cell immunity, the activation of which is MHC-
restricted and does not occur by crossreactivity (7). Thus, manipulation of the donor/recipient combination permits the indirect pathway of T cell activation alone to be studied. To assess the relative effectiveness of indirect CD4 immunity in rejecting different allogeneic grafts, we began by testing the ability of Marilyn mice to indirectly reject male islet transplants and their few passenger lymphocytes, the latter cells being readily detectable in vivo in immunodeficient hosts bearing healed in grafts from immunocompetent donors (primarily donor T cells) (25), as well as ex vivo in islets harvested from immunocompetent (BALB/c, B6, and FVB) but not immunodeficient (NOD-RAG-KO) mice (our unpublished observations). We gave Marilyn, male or female fully MHC-mismatched BALB/c islets and found that the indirect CD4 response alone was sufficient to induce alloantigen-specific islet rejection (Fig. 1A). At the time of male islet rejection, however, we did not detect, in blood and spleen, passenger lymphocytes that would have migrated out from the donor graft (five recipients analyzed; our unpublished observations). As Marilyn mice contained not only monoclonal CD4 T cells but also NK cells, the inability to detect passenger lymphocytes could be due to their rejection by recipient NK cells (28). We confirmed this by transplanting BALB/c islets into NK cell-replete RAG-KO or NK cell-deficient RAG/y-KO mice and detected passenger lymphocytes, consisting of T cells but not B cells, only in RAG/y-KO recipients (Fig. 1B).

Because of the confounding host NK cell rejection of fully MHC-mismatched passenger lymphocytes, we repeated the study using male or female bm12 islet transplants (bm12 mice carry an MHC class II molecule mutated from I-Ab but are fully class I-matched) (29) and again observed alloantigen-specific indirect islet rejection (Fig. 2A). Rejection was associated with a predominance of Th1 cytokines, including IL-12, TNF-α, IFN-γ and its associated chemokine, monokine induced by γ IFN, as detected in recipient serum (our unpublished observations). After rejection of male islets, we gave the recipients “syngeneic” female RAG-KO islets to maintain normoglycemia (Fig. 2B) and then analyzed their peripheral blood for the presence of passenger lymphocytes (i.e., non-Vβ-expressing CD4 and/or CD8 T cells; transgenic TCR uses Vβ6). Importantly, Marilyn mice that indirectly rejected male bm12 islets were found to be chimeric for passenger T cells (Fig. 2C) that we were also able to detect directly ex vivo in islet preparations (Fig. 2D). This suggested that the establishment of chimerism was due to the migration of passenger cells from donor islets into host systemic circulation. Moreover, there was clearly an inability of the host immune response to eliminate donor T cells but not the islet grafts in which they resided (Fig. 2, A and C). To determine whether the islet passenger cell-derived chimerism would induce male-specific tolerance in Marilyn, we performed MLR assays in one cohort of recipients after male bm12 islet rejection (rejection at days 16 × 2, 18, and 29) and syngeneic transplantation (28–41 days later), using Vβ6-enriched Marilyn responders. Responders from Marilyn recipients of male islets proliferated at least 35-fold less than those from recipients of female islets, after culture with male stimulators in vitro (Fig. 2E).

Consistent with the passenger leukocyte hypothesis, removal of passenger cells from transplants usually results in improved graft survival (30–36), which is reversible when donor leukocytes are reintroduced (37, 38). To determine what effect the absence of passenger lymphocytes has on the indirect CD4 response toward allogeneic islet grafts, we challenged Marilyn with male islets provided by donor mice genetically deficient in T and B cells and compared the survival of these transplants to those from WT, immunocompetent donors. Marilyn showed significantly faster rejection of fully MHC-mismatched BALB/c-SCID islets that lack passenger lymphocytes than WT BALB/c islets (Fig. 1A). This difference was not simply due to a peculiarity of the BALB/c background or the scid mutation. Marilyn rejected male B6-RAG-KO islets that also lack passenger lymphocytes, significantly faster than WT B6 islets (Fig. 3A), thus confirming the ability of passenger lymphocytes to delay islet rejection. Together, these data indicate that the vigor of indirect islet rejection by CD4 T cells could be diminished by the presence of passenger lymphocytes. Initially, this seemed contradictory to the concept that passenger cell depletions enhances allograft acceptance, as described above. This difference could potentially be due to the removal of passenger dendritic cells in previous studies, while our study instead involved removal of passenger lymphocytes. However, we had previously shown that passenger T cells could be immunogenic within the context of a full recipient T cell repertoire containing both CD4 and CD8 T cells (28). We therefore tested whether genetic elimination of passenger lymphocytes in islet transplants would enhance or delay islet rejection in hosts containing polyclonal CD4 and CD8 T cells. Fig. 3B shows that passenger lymphocyte-replete WT islet transplants were rejected significantly faster than islets from lymphocyte-deficient donors. Collectively, these data indicate that passenger T cells (but not islets) are able to withstand indirect CD4 alloimmunity, and that they contribute to more rapid graft rejection, except under the conditions where the response is restricted to the CD4-mediated indirect pathway, in which case delayed rejection occurs.

FIGURE 3. Passenger lymphocytes delay indirect islet rejection by CD4 T cells, but enhance rejection in the presence of a full repertoire. A, STZ-induced diabetic female Marilyn mice were given male B6-RAG-KO (n = 7), or male (n = 11) or control female (n = 7) WT B6 islet transplants and monitored by blood glucose for rejection. Survival curves are shown; p = 0.0015 between WT male and female B6 islets, p = 0.0002 between male RAG-KO and control female islets, and p = 0.0001 between male WT vs RAG-KO islets. B, STZ-induced diabetic BALB/c mice given WT B6 (n = 7) vs B6-RAG-KO (n = 8) islet transplants were monitored for rejection; p = 0.0001.
Indirect CD4 alloimmunity is highly destructive for donor skin and B cells but the inability to eliminate donor T cells switches the response from immunity to tolerance

To further address whether indirect CD4 immunity would be equally destructive toward different types of allogeneic grafts, we determined the sensitivity of male hematopoietic cells vs male skin grafts to rejection. We challenged Marilyn with male or control female congenic (CD45.1) B6 FLCs (a source of hematopoietic stem cells that can generate T cells and B cells de novo, and lack MHC class II expression; Fig. 4A) and examined the survival and differentiation of the donor cells (i.e., establishment of chimerism). Marilyn demonstrated a significant but transient rejection of male FLCs (Fig. 4B). The inability to completely eliminate the FLCs was not due to a general inability of CD4 T cells to kill hematopoietic cells. Marilyn fully rejected the male B cells that developed from the fetal liver precursors (Fig. 4C). In contrast, following a transient rejection, the level of donor T cells steadily increased. Thus, indirect CD4 alloimmunity may not be equally destructive toward donor T cells and B cells (or their precursors). However, rejection of male B6 B cells did not prove that B cells were cleared efficiently by an indirect CD4 response. B cells, but not T cells, express MHC class II in mice and this might have made the B6 B cells targets of direct rejection by Marilyn T cells. To stringently test whether an indirect CD4 response can efficiently eliminate donor B cells, we challenged Marilyn mice with male or control female bm12 FLCs. We confirmed in vitro that male cells expressing I-A<sup>b</sup>m12 were unable to directly trigger proliferation of Marilyn T cells, unlike I-A<sup>b</sup> expressing male cells (Fig. 4D). Strikingly, in Marilyn...
given male or female bm12 FLCs, male bm12 B cells were efficiently rejected (female bm12 B cells persisted; Fig. 4C), indicating that indirect CD4 alloimmunity was effective in B cell but not T cell rejection. To determine whether differential expression of the male Ag between T and B cells could explain our findings, we immunized Marilyn mice with purified male T or B cells that can only be recognized indirectly and found a similar frequency of Marilyn T cells with up-regulated CD44 expression, a marker for Ag encounter (Fig. 5). Thus, differential Ag expression between T and B cells is unlikely to explain the differences in their survival.

Having established that indirect CD4 alloimmunity was effective for elimination of donor B but not T cells, we next asked whether this “split tolerance” extended to donor skin grafts. We gave Marilyn mice that received male or control female B6 FLCs 3 days previously, a male and a female MHC class II-deficient B cells (Fig. 6A), despite the long-lasting presence of donor male hematopoietic cells that consisted of T but not B cells (Fig. 4C). Thus, Marilyn T cells mounted an effective response against male B cells and skin transplants, but the same response ongoing within the same animal was relatively inefficient in eliminating male T cells.

To assess whether the split tolerant state could persist long-term, we tested whether Marilyn mice that received male or female B6 FLCs ~12 wk previously (and that subsequently became mixed hematopoietic chimeras; Fig. 6B), would accept male and female class II-deficient B6 skin grafts. Surprisingly, we found that the previous outcome of split tolerance toward donor skin transplants (Fig. 6A) was no longer present in Marilyn recipients of male FLCs, as specific tolerance toward male skin transplants given late was achieved (Fig. 6C). At the time of skin transplantation, however, the frequency of Marilyn T cells was similar between recipients of male vs female FLCs (Fig. 6D), suggesting that a nondeletional mechanism of tolerance was responsible for skin graft acceptance in Marilyn given male FLCs. We found that, unlike Marilyn chimeras that were generated with female FLCs, those given male FLCs contained Marilyn T cells that down-regulated CD44 expression (Fig. 7). This began as early as 10–12 wk after FLC injection (our unpublished observations). Donor T cells, however, were unaffected in their expression of the CD4 or CD8 coreceptor (Fig. 7). To determine whether Marilyn mice given male FLCs contained regulatory T cells that were responsible for male-specific tolerance, we tested, in an in vivo killing assay, for linked suppression of the immune response to male cells that additionally express multiple minor Ags of the BALB background. After immunization, Marilyn given female FLCs efficiently killed CFSE-labeled target cells expressing the male Ag only, male Ag plus multiple minor Ags derived from the BALB background, or BALB minor Ags only, but not syngeneic female B6 targets (Fig. 8). In contrast, Marilyn given male FLCs did not kill male or female B6 targets, but efficiently cleared targets expressing BALB minor Ags, with or without their additional expression of the male Ag (Fig. 8). Together these data indicate that the inability of indirect CD44 alloimmunity to eliminate certain allogeneic cells leads to an intrinsic T cell unresponsive state of tolerance.

Discussion

CD4 T cells are important in adaptive immunity, either acting as helpers or effectors. The latter function is not traditionally associated with CD4 T cells but is gradually becoming better appreciated especially in transplant immunity. CD4 T cells alone can be sufficient in skin rejection (1, 2) and can be necessary and sufficient in allogeneic (3) or xenogeneic (4) islet rejection. Moreover, CD4 T cells may preferentially respond by the indirect pathway (6, 19–22). However, whether indirect CD4 alloimmunity is equally destructive toward different types of allogeneic grafts has not been extensively assessed. In this study, we used Marilyn (24), a TCR transgenic mouse model specific for the male Ag, H-Y, to demonstrate that indirect CD4 responses are highly destructive toward allogeneic B cells, skin and islet transplants but are ineffective at eliminating allogeneic T cells within the same animal.

We found that Marilyn mice undergoing indirect rejection of islet transplants simultaneously developed mixed T cell chimerism (predominately of CD4+ T cells, even when female islets were transplanted; unpublished observations) due to passenger lymphocyte migration. This suggested that the same indirect CD4 response that was sufficient for allogeneic islet rejection was ineffective in eliminating donor T cells. We also observed that Marilyn given male FLCs became mixed chimeras of T cells but efficiently rejected donor B cells (or their precursors) by the indirect pathway (Fig. 4C). The reason for the reduced effectiveness of the indirect Marilyn response in male T cell but not B cell elimination is currently unknown. It does not appear to involve differential expression of H-Y between T cells and B cells (Fig. 5). Moreover, when we challenged Marilyn mice with male bm12 FLCs, neither donor T nor B cells would be able to directly present the male Ag in a cognate fashion to Marilyn T cells (i.e., T cells lack MHC class II while B cells express I-A<sup>bm12</sup> instead of I-A<sup>e</sup>; our MLR findings support this.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Marilyn T cells up-regulate CD44 at a similar frequency after indirect priming by male T or B cells. Marilyn mice were left unimmunized, or immunized either by male MHC class II-deficient T or B cells at the indicated doses, after which flow cytometric analysis of CD44 expression on Marilyn T cells was performed (n = 3–4). 

A. Peripheral blood analysis on days 3, 7, 14, and 21 after immunization. B. Frequency of CD44<sup>high</sup> Marilyn T cells in spleen on day 21 after immunization. There was no statistical difference in the frequency when comparing immunized mice to mice immunized with 1 million T or B cells. Mean and SEM are shown for all data.
Potentially, T cells are intrinsically more refractory than B cells to indirect CD4 immunity. In support of this hypothesis, Marilyn mice contain CD4 T cells of a single specificity and lack CD8 T cells and B cells, rendering them immunodeficient both qualitatively and quantitatively. Given the ability of T cells to homeostatically proliferate due to a deficiency in T cell number (39), the few donor T cells that were initially generated from FLCs could have expanded upon entering the periphery and acquired a phenotype that conferred further resistance to indirect CD4 immunity. In contrast, B cells, while also capable of homeostatic proliferation, appear to do so at a slower rate than T cells and retain a quiescent phenotype (40, 41) (i.e., do not acquire memory-like properties as detected in T cells; Ref. 42). Therefore, these phenotypic differences may account for the resistance of T cells but not B cells to indirect CD4 alloimmunity.

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Previously, the susceptibility of different types of allografts to T cell-mediated rejection has been examined in relation to CD8 T cells responding to either an intact allogeneic class I molecule (49) or a minor Ag presented on recipient MHC class I (50). In terms of CD8-mediated rejection, graft size may be an important factor that dictates susceptibility to rejection.
However, graft size seems an unlikely explanation for the hierarchy of susceptibility toward indirect CD4 alloimmunity that we observed. Instead, a clear difference between tissues and hematopoietic cells as targets of elimination is the distribution of their Ags (local vs systemic). The systemic nature of hematopoietic cells may make them a more difficult target to be completely eliminated. Also, certain mechanisms of rejection, such as attack on the vasculature, could be irrelevant to rejection of hematopoietic cells. However, these latter considerations would not explain the differential susceptibility to indirect rejection of B cells vs T cells.

Interestingly, the inability to clear donor hematopoietic cells caused split tolerance toward donor skin grafts to turn into full tolerance, as male skin transplants given late were accepted longer, as compared with acute rejection when given early. Mechanistically, this was associated with down-regulated CD4 expression, which, to our knowledge, has not been reported in allogeneic tolerance induction. However, down-regulation of CD4 was previously reported in a rat model of experimental allergic encephalomyelitis (51). Similarly, down-regulation of CD8 has been observed as a mechanism for self-tolerance (52, 53). Tolerance was also observed in vitro in Marilyn recipients of male islet transplant that became mixed hematopoietic chimeras despite islet rejection. The deficiency of indirect CD4 alloimmunity may, therefore, have the potential to be exploited in developing new tolerance induction protocols.

To begin to understand the function of Marilyn T cells that down-regulated CD4 expression, we used an in vivo killing assay to test for linked suppression. Although dominant regulatory tolerance can develop in certain transplantation settings (54–56), our data clearly indicated that in Marilyn mice that became chimeric after male FLC injection and showed tolerance to the male Ag, this tolerance was not manifested in a dominant fashion. However, we cannot currently rule out the possibility that a weaker form of dominant tolerance, not detectable in our assay, exists in these chimeras.

We observed that passenger T cells delayed indirect CD4-mediated rejection. However, within the context of a complete host T cell repertoire capable of both direct and indirect immunity, passenger T cells enhanced rejection. In our previous studies suggesting that passenger T cells could provide an immunogenic source of Ag (28), we could not rule out the possibility that the immunity observed was dependent on a lymphopenic environment and consequent homeostatic effects. The current data (Fig. 3B), in non-lymphopenic recipients, clearly substantiate the immunogenic nature of passenger lymphocyte-derived Ags when they are encountered by a complete repertoire. Thus, in terms of immunogenic passenger cells within a graft, our data indicate that it is not just donor dendritic cells that are important, but also donor lymphocytes.

Finally, the split tolerance that we observed in our model is novel in relation to transplant immunity. Split tolerance can be generally defined as the simultaneous presence of immunity toward one type of donor graft but tolerance toward a second type of graft of the same donor origin (57–61). It is most frequently manifested as skin rejection by hematopoietic chimeras due to immunity toward skin-specific Ags (57–65). However, because we studied immunity toward the well-defined male Ag that is not tissue-specific, our data would suggest that a form of split tolerance that occurs independently of tissue-specific Ags may

![Figure 7](http://www.jimmunol.org/)
be possible and mediated through the variable effectiveness of the indirect CD4 response in eliminating different types of allogeneic grafts. In this regard, diabetes-prone NOD mice demonstrate potent indirect CD4 responses (6, 20), and we have recently found that generation of mixed chimerism in NOD recipients can result in a split tolerance characterized by rejection of donor B cells, skin, and islets and survival of donor T cells (66), paralleling the split tolerance data shown here in a TCR transgenic model. Secondly, the susceptibility of B cells to indirect rejection that we have shown here has implications for understanding B cell deletion induced by T cell killing of B cells (67, 68). Because Marilyn T cells rejected male bm12 B cells that were unable to present the relevant Ag directly, our data bring into question the previous conclusion that CD4 T cell targets; (primed) male and female B6 mice (n = 2–3) were assayed. A, A representative dot plot from each of the groups is shown to illustrate the pattern of target killing. Lymphoid-gated cells positive for CFSE were analyzed. The upper left gray box depicts where each type of target cell is located in the dot plot. Numbers in the quadrants are calculated ratios between the number of events within a quadrant and that within the quadrant for syngeneic female B6 targets. B, The ability of individual mice within a group to kill male B6 (top), male BALB.B (middle), or female BALB.B (bottom) targets is shown in the scatter plot (data are reported as ratios to syngeneic female B6 targets; p values are provided when comparing between Marilyn given male vs female FLCs). The lower the ratio, the higher is the killing of test targets compared with syngeneic control targets.

Collectively, our data provide evidence that indirect CD4 alloimmunity can either be highly destructive or relatively futile depending on its target of elimination. Serendipitously, the “natural” deficiency of the indirect CD4 response may benefit our attempts to induce transplantation tolerance.

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Disclosures

The authors have no financial conflict of interest.

References


