Transpl Int (2004) 17: 261–269
DOI 10.1007/s00147-004-0698-3

ORIGINAL ARTICLE

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Resistance to anti-CD45RB-induced tolerance in NOD mice: mechanisms involved

Received: 2 May 2003
Revised: 24 July 2003
Accepted: 24 July 2003
Published online: 11 May 2004

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Abstract While great advances have been made in the success of islet transplantation to cure autoimmune diabetes, this protocol remains limited by our inability to induce donor-specific tolerance within the recipient. The profound resistance of the NOD mouse to tolerance-inducing regimens that are routinely successful in other strains further defines the imposing barriers that must be surmounted. Herein, we have assessed the utility of anti-CD45RB therapy to induce tolerance to allografts in C57BL/6 and NOD-strain mice. We find that, as with other therapies, NOD mice are also resistant to this manipulation, despite robust tolerance induction in the comparison strain. Analysis of cell surface markers revealed a number of changes within the B lymphocyte compartment following contact with antibody and alloantigen in the B6 strain. The absence of reciprocal changes within the NOD lymphocyte compartment suggests that B cells might contribute to the mechanism of action of this therapy and to the resistance to immunological tolerance noted in the NOD strain.

Keywords Tolerance · Transplantation · NOD mouse · Anti-CD45RB · T lymphocyte

Introduction

While understanding the pathogenesis of autoimmune diabetes might afford its prevention, the correction of the diabetic state is currently accomplished most readily by the replacement of the destroyed islet tissue. Exciting advances have been made in immunosuppressive therapies that permit the transplantation and survival of islet tissue in diabetic patients; nonetheless, the long-term sequelae of these pharmacological agents detract from the benefit gained by the restoration of euglycemia [1]. The ultimate goal in transplantation biology remains the induction of donor-specific tolerance and the discontinuation of these reagents. However, it is unclear how this goal will be achieved in the autoimmune patient in whom the endogenous mechanisms promoting self-tolerance have already failed. This failure in the maintenance of peripheral tolerance might foreshadow complications for ongoing efforts to cure autoimmune diabetes.

Normal murine strains can be modified quite easily by a number of strategies that permit the induction of donor-specific tolerance [2, 3, 4, 5, 6, 7, 8, 9]. Although these therapies have not been transferred directly to human application, the future still appears bright in this regard. However, the NOD mouse has proved a particularly difficult system in which to induce tolerance, a condition which seems logically implicated by the initial failure of the strain's own immune system to produce tolerance to self antigens

[10]. Currently, no pharmacological therapy that promotes tolerance in the presence of an intact immune system has translated from normal murine strains into this background, even when the transplanted tissue is other than islets and, hence, not subject to autoimmune recurrence. The difficulty of inducing tolerance in this model suggests that autoimmunity might alter the immune system in ways that are not reversible by current strategies.

Given the critical importance of this issue, we sought to evaluate the utility of anti-CD45RB antibody therapy and, by applying a comparative strategy between NOD and B6 strain mice, to begin to elucidate the mechanism of action. This therapy afforded a number of advantages over previous efforts; in particular, it was not known to be globally immunosuppressive but has been thought to combine both deletion and the induction of regulatory T lymphocytes [2, 11, 12]. Moreover, the therapy does not require the introduction of donor-specific lymphocytes to mediate its effect, a manipulation that could confuse efforts to study recipient immune cell function. If the therapy were successful, it would aid in revealing changes in the NOD immune system that can be utilized to promote peripheral tolerance and further our understanding of NOD immunobiology. On the other hand, if the NOD mouse cannot be tolerized, it can be used to assess those affects of the therapy that might be necessary to promote tolerance in normal mice.

Materials and methods

Mice

Mice (NOD/LtJ, C57BL/6, C3H, and Balb/c strain) were purchased from the Jackson Laboratories (Bar Harbor, Me., USA). All mice were housed under specific pathogen-free barrier conditions. All procedures detailed below were performed under the principles of laboratory animal care.

Islet isolation and transplantation

C57BL/6 (B6) and NOD mice were used as recipients, and C3H mice were used as islet donors. Prior to islet transplantation, diabetes was established in all recipient mice. Female NOD mice developed diabetes spontaneously, but for B6 mice, diabetes was induced by a single intraperitoneal injection of 250 mg/kg freshly reconstituted STZ (Sigma Chemical, St. Louis, Mo., USA). Diabetes was defined as blood glucose levels > 300 mg/dl for at least 3 consecutive days. Islets from C3H mice were isolated by the standard technique of collagenase digestion and Ficoll purification. Following isolation, 500 fresh islets were transplanted under the kidney capsule of diabetic mice (C57BL/6 and NOD). Euglycemia was defined as a non-fasting blood glucose level < 200 mg/dl. Rejection was diagnosed when animals became hyperglycemic again with BG > 200 mg/dl for at least 2 consecutive days.

Heart grafting

Experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Transplantation was performed in accordance with the Ono–Lindsey model as adapted for mice [13, 14]. Mice were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (10 mg/kg), after which a midline abdominal incision was made in the donor mouse, which was then heparinized through the inferior vena cava (50 U). The incision was extended cephalad to open the chest through a median sternotomy. The heart was rapidly harvested after arrest with potassium cardioplegia solution given via the inferior vena cava (1 ml, 20 mEq/l), and the coronary arteries were flushed (0.5 ml of preservation solution) and placed into lactated Ringer's solution for 2 h at 4°C. Transplantation was accomplished by our exposing the recipient's abdominal aorta and inferior vena cava by a similar abdominal incision. The donor aorta and pulmonary artery were anastomosed, end to side, to the recipient's abdominal aorta and inferior vena cava, respectively, with 10-0 nylon suture. To test tolerance, we transplanted a second heart graft, from the same C3H or Balb/c strain, in the neck of B6 mice bearing long-term functioning cardiac allograft without any additional treatment.

Anti-CD45RB therapy

Animals were treated with intraperitoneal injection of 100 μg of rat anti-mouse CD45RB antibody (clone: MB23G2, ATCC, Rockville, Md., USA) on days 0, 1, 3, 5, and 7 following transplantation. Control animals were left untreated.

In vitro allo-stimulation

Lymph nodes from C57BL/6 or NOD mice were harvested, and single-cell suspensions were prepared by passage of tissue through a cell strainer (70 μm; Falcon, Franklin Lakes, N.J., USA). Cells were re-suspended at a density of 1×10^7 cells per ml in IMDM. An equal volume of 5 mmol/l CFSE (Molecular Probes, Eugene, Ore., USA) in IMDM was added, and cells were incu-

bated at 37°C for 5 min. The reaction was quenched through the addition of an equal volume of heat-inactivated FCS (Gibco-BRL). Labeled cells were washed twice and re-suspended in PBS for in vitro mixed lymphocyte reaction (MLR) assay, in which 5×10^5 lymph node cells from B6 or NOD mice were mixed with the irradiated C3H splenocytes in a total of 1 ml culture media (IMDM +10% FCS), in the presence or absence of anti-CD45RB antibodies. After a 4-day incubation at 37°C with 5% CO_2, the cells were harvested, stained with anti-CD4 antibodies and analyzed by flow cytometry in order for us to determine the effect of anti-CD45RB on T cell proliferation in response to allo-stimulation.

In vivo allo-stimulation

C57BL/6 or NOD mice were injected with ten million irradiated C3H splenocytes as a source of alloantigens. The mice were divided into two groups; one group was treated with anti-CD45RB as per the treatment protocol, and the second group was left unmanipulated. The mice were killed, the spleen and lymph nodes were harvested, and the cells were analyzed by flow cytometry for alterations in cell surface markers. We also used cells from allo-stimulated mice in in vitro MLR to assess alterations in sensitivity resulting from the therapy.

Flow cytometry

One million cells were suspended in biotin-free RPMI containing 0.1% azide and 3% FCS and surface-stained in 96-well plates with the appropriate mAbs: RA3-6B2 biotin (anti-CD45R/B220), RM4-5 allophycocyanin (anti-CD4), 10-3.6 (anti-I-Ag7), AF6-120.1 PE (anti-I-Ab) (BD PharMingen). The biotin-conjugated mAbs were subsequently stained with streptavidin RED670 (Life Technologies); cells were washed twice prior to the addition of the secondary reagent. All samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif., USA) using CellQuest software.

Results

Anti-CD45RB therapy prevents allograft rejection in B6 but not NOD mice

Anti-CD45RB had previously proven to be effective in inducing tolerance to both renal and islet allografts [15, 16, 17, 18]. As previous success had been demonstrated in islet transplantation models, we wished to investigate whether anti-CD45RB therapy possessed sufficient potency to promote tolerance in the presence of vigorous autoimmune recurrence, as is experienced by islet transplants in diabetic NOD mice. For comparison, we transplanted C3H islet tissue into C57BL/6 (B6) mice rendered chemically diabetic by streptozotocin treatment. Untreated B6 mice rejected islet allografts rapidly, with no animal remaining euglycemic for longer than 11 days (Table 1). However, anti-CD45RB therapy (100 μg on days 0, 1, 3, 5, and 7) resulted in significant prolongation of all transplants and in permanent acceptance for 4/7 grafts ($P<0.01$). These results could not be extended to the NOD strain. Even in the presence of treatment, all allografts in diabetic NOD mice were rejected, with only a modest prolongation in survival (MST = 7.0 days vs 16.0 days, respectively).

As islet allografts in the NOD strain are subject to both rejection and recurrence, we sought a less immunogenic transplant to assess the ability of anti-CD45RB to promote tolerance in the NOD background. Thus, cardiac C3H allografts were placed in the abdominal cavity of recipient mice, and anti-CD45RB administered intraperitoneally (100 μg on days 0, 1, 3, 5, and 7). Graft function was monitored by daily palpation, and rejection was noted at the cessation of beating. As shown in Table 2, B6 animals rapidly rejected C3H hearts within 12 days. However, treatment with anti-CD45RB led consistently to the indefinite acceptance of these grafts (> 100 days). The specificity of the tolerance-inducing regimen was confirmed by the acceptance of a second heart from the same B6 strain by tolerant animals (MST > 60 days, $n=3$) while a third-party graft from Balb/c mice was rejected normally in these animals (MST = 7.8 days, $n=4$). However, when we examined the efficacy of this therapy in pre-diabetic NOD mice, permanent

Table 1 Survival of islet allografts in B6 and NOD recipients

Group	No.	Treatment	Survival (days)	MST ± SD
1. C3H → B6	6	None	8, 8, 9, 9, 10, 11	9.2 ± 1.2
2. C3H → B6	7	Anti-CD45	34, 45, 87, > 100×4	> 80.9[a]
3. C3H → NOD	6	None	5, 5, 7, 8, 8, 9	7.0 ± 1.7
4. C3H → NOD	6	Anti-CD45	8, 9, 11, 18, 21, 29	16.0 ± 8.2[b]

[a] $P<0.01$ compared with group 1
[b] $P<0.05$ compared with group 3

Table 2 Survival of cardiac allografts in B6 and NOD recipients

Group	N°	Treatment	Survival (days)	MST ± SD
1. C3H → B6	9	None	6, 7, 7, 7, 8, 8, 9, 10, 12	8.3 ± 1.9
2. C3H → B6	9	Anti-CD45	28, 46, 62, 84, 90, > 100×4	> 78.9[a]
3. C3H → NOD	6	None	7, 8, 8, 8, 12, 12	9.2 ± 2.2
4. C3H → NOD	9	Anti-CD45	21, 22, 22, 23, 24, 24, 29, 29	24.3 ± 3.1[b]

[a] $P < 0.01$ compared with group 1
[b] $P < 0.05$ compared with group 3

acceptance was not established, although a moderate prolongation in graft survival was noted (MST = 9.2 days vs 24.3 days, respectively). These data indicate that, even in the absence of autoimmune recurrence, the NOD murine strain is resistant to the tolerance-induction mechanism of this therapy.

Histological information was also obtained on all graft specimens, either at the time of rejection or after long-term acceptance had been established (survival > 100 days). Rejecting tissues are characterized by dense cellular infiltration and disruption of tissue architecture (Fig. 1A, C). Interestingly, tolerated graft tissues also show evidence of immune surveillance, as the sectioned tissues reveal the presence of lymphocytes in the cardiac graft (Fig. 1B) or surrounding the islet graft but without penetration into the islet tissues (Fig. 1D). The persisting lymphocytes present in these grafts may play an important role in the establishment or maintenance of the tolerant state.

Contact with alloantigens in the presence of anti-CD45RB leads to enhanced T cell activation and distinct alterations in B lymphocytes

As the development of T cell effector function is intimately linked to cell cycle progression, we next determined the effect of anti-CD45RB therapy on cellular proliferation in in vitro MLR assays [10, 19]. T lymphocytes from both B6 and NOD mice were able to proliferate in response to alloantigen stimulation, but both the precursor frequency and the percentage of dividing cells were significantly smaller for NOD CD4 T cells than for B6 CD4 T cells. In the presence of anti-CD45RB antibodies this alloantigen-specific T cell response was significantly enhanced for both B6 and NOD T cells, but the effect appeared more profound on the B6 T cells than on the NOD T cells, as shown by flow cytometry (Fig. 2). These data indicate that anti-CD45RB therapy is unable to relieve the proliferative

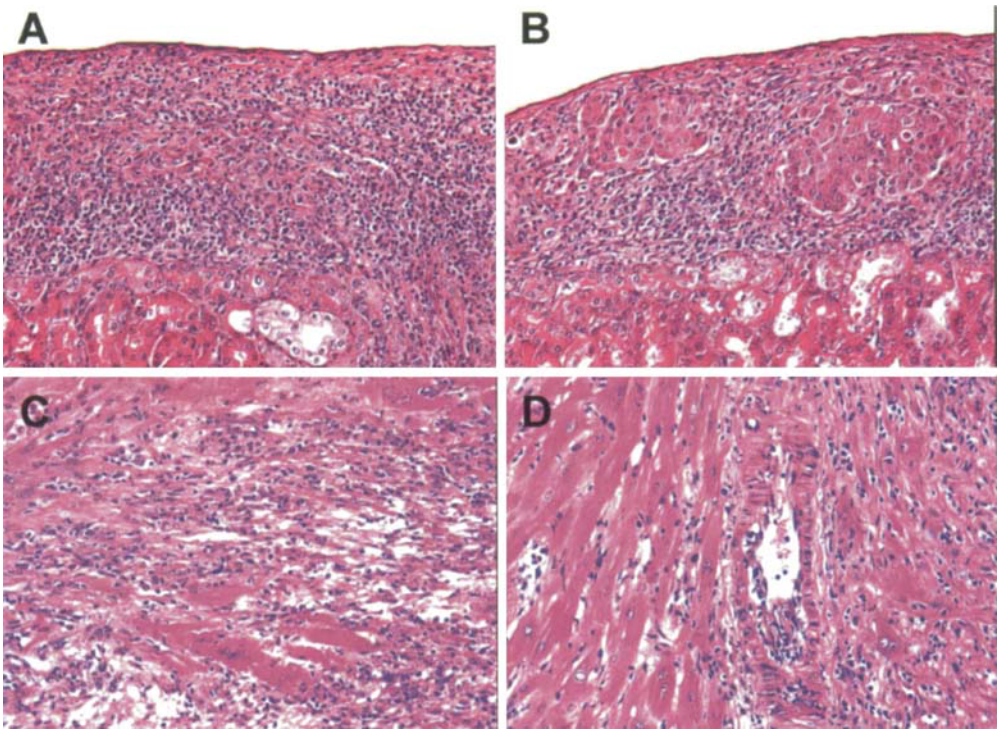

Fig. 1A–D Comparative histology of transplanted islet and heart grafts. *The upper panels* illustrate islet allografts under the kidney capsule and *the lower panels* contain sections from cardiac allografts harvested from NOD and B6 mice treated with anti-CD45RB antibody. Rejected tissues of islet (**A**) and heart (**C**) grafts in NOD mice are characterized by dense cellular infiltration and disruption of tissue architecture; Accepted islet (**B**) and heart (**D**) graft in B6 mice are healthy and viable with some lymphocytes surrounding the grafts. All sections: hematoxylin and eosin, ×20

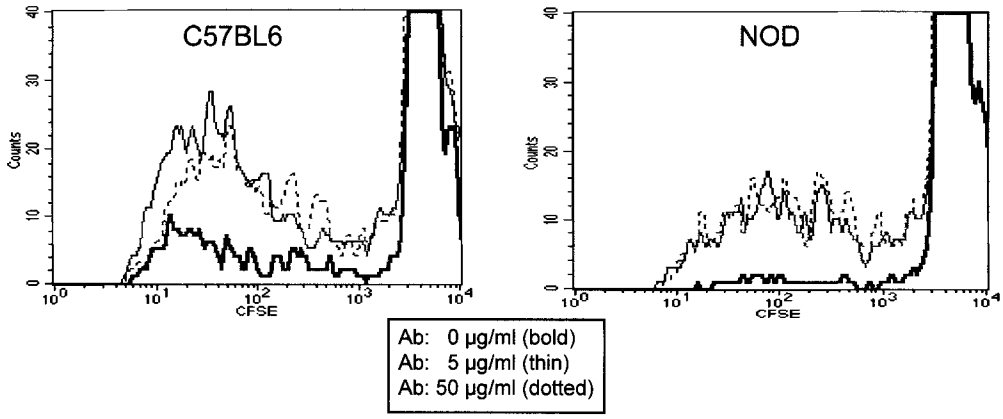

Fig. 2 Proliferative response of CD4 T cells to allo-stimulation in the presence of anti-CD45RB antibodies. B6 and NOD Lymphocytes were labeled with CFSE and were stimulated with irradiated C3H splenocytes in the absence or presence of anti-CD45RB antibodies at two different concentrations. After 4 days of incubation the cells were harvested and stained with anti-CD4 antibodies to determine the proliferative response of CD4 T cells to alloantigens. Representative data demonstrate changes in the precursor frequency and percentage of dividing CD4 T cells in the presence of anti-CD45RB antibodies

hypo-responsiveness that characterizes the NOD mouse and distinguishes its cellular function from that of non-autoimmune strains [20].

Previous dissection of this proliferative phenotype within the NOD immune system had indicated that the CD4 T cell compartment was intrinsically normal when confronted with stimuli that did not require interaction with other cell types [21, 22, 23]. In light of these data, we considered the possibility that this antibody therapy might not mediate its effect solely by action on the T cell compartment, for were that the complete mechanism, NOD mice might be receptive to its effects. We therefore suspected that there might be contributions from other cell lineages. To assess this hypothesis, we employed FACS screening following contact with alloantigen during antibody therapy to characterize changes in cell surface molecules indicative of altered cell function. Alloantigen was provided by i.v. injection of irradiated C3H splenocytes; antibody therapy was administered as described. On day 5 and day 10, splenocytes were harvested and analyzed for a number of cell surface markers. Of note, we detected no significant changes in surface markers within the CD4 cell compartment (data not shown), except the expected down-regulation of CD45RB expression (Fig. 3A). While other groups have reported increases in CTLA-4 expression, we did not note this alteration in our system [16, 24]. It will be important for us to determine whether we can detect this change in mice receiving heart grafts and, if not, whether it is restricted to the systems used by prior investigators and is not a requisite for tolerance induction.

However, a number of functional changes were noted within the B cell compartment. Most noticeable among these alterations were an increase in CD54 and MHC class II expression and a decrease in the levels of CD19, but no significant changes were noted in levels of B7-1, B7-2, or CD40 (Fig. 3B–G). Interestingly, upregulation of CD54 has also been reported following treatment with the immunomodulatory agent linomide [25, 26]. To gain insight into the relevance of these alterations, we performed the same analysis within the NOD system where tolerance was not induced. Treated NOD mice also exhibited an increase in CD54 and a decrease in CD19 levels; however, NOD mice exhibited no change in the expression of the class II molecule on the B cell compartment (Fig. 4). These changes were transient and had resolved within 2 weeks. As antigen presentation by B cells is critical for T cell activation in NOD mice, the absence of class II up-regulation might be critical to the failure of this therapeutic regimen in the NOD mouse [20, 21, 27, 28, 29]. It is also important to note that alterations in other cell surface molecules might be necessary but not sufficient for the efficient function of this modality.

Discussion

The resistance of the NOD immune system to tolerance-inducing stratagems portends future limitations for the success of islet transplantation in the autoimmune diabetic patient. Investigation into NOD immunobiology continues to provide new insights that may assist in the design of rational strategies to alter the phenotype and function of cells in this background toward tolerance. A number of recent studies have elucidated that feature of the NOD CD4 T cell compartment [21, 23, 29, 30, 31, 32, 33, 34, 35]. These studies have demonstrated that this lymphocyte subset is poorly responsive to TCR-mediated stimuli in that fewer divisions are achieved by activated NOD CD4 T cells than by CD4 T cells from non-autoimmune comparison strains. In addition, the

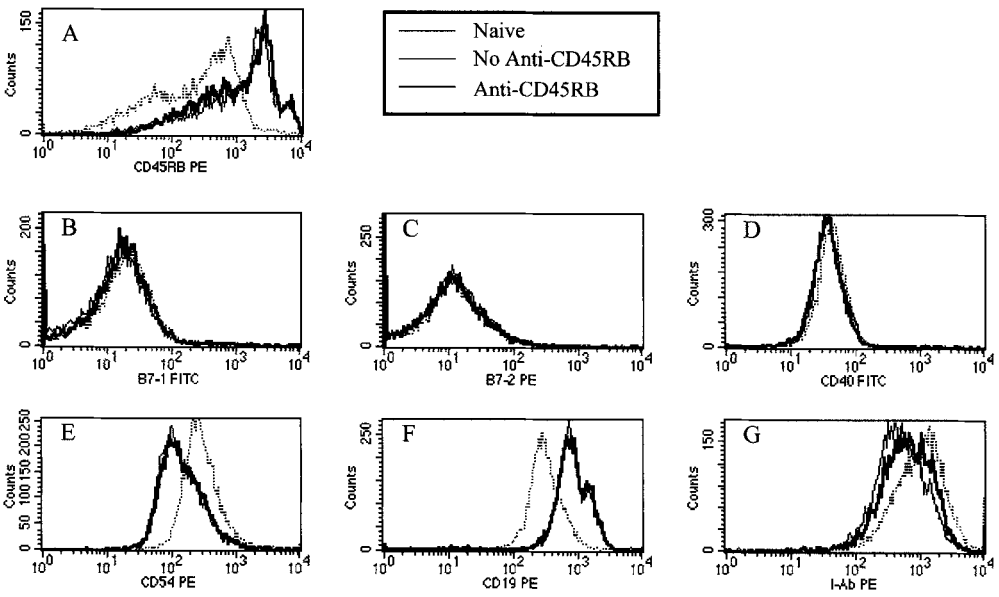

Fig. 3A–G Analysis of cell surface phenotypes in B6 recipients following anti-CD45RB and allo-stimulation. The effect of anti-CD45RB was assessed by analysis of expression of cell surface markers in both T and B cell compartments of B6 mice. The presence of anti-CD45RB in treated animals was verified by the diminution in CD45RB levels detected on T lymphocytes (*upper left, dotted line*). Within the B cell compartment, no significant changes were noted in levels of B7–1 (**B**), B7–2 (**C**) or CD40 (**D**), but several alterations were noted, including a decrease in CD19 levels (**E**), an increase in CD54 level (**F**) and up-regulation in I-A levels (**G**). In each figure, cells from naive animals are indicated by *the thin solid line*, cells from animals exposed to allogeneic cells in the absence of anti-CD45RB are indicated by *the thick solid line*, and cells from animals treated with allogeneic cells and anti-CD45RB are indicated by *the dotted line*

dose responsiveness of these NOD cells is also markedly attenuated. As several studies have linked progression through the cell cycle to the development of Th2 cells and to activation-induced cell death, early exit from cell proliferation may prevent the adoption of these differentiated states in which graft cells may be protected [36, 37, 38, 39]. If this limitation in TCR responsiveness impairs the ability of the NOD mouse to develop a tolerant peripheral immune system, the improvement of this sensitivity might augment tolerance induction. CD45RB is known as a critical modulator of T and B cell receptor sensitivity [40, 41, 42, 43, 44]. Sequestration of this molecule from the receptor complex is critically important in promoting the development of a robust cellular response. Given the deficiencies in NOD T cell signaling and the potential involvement of CD45RB in cell receptor sensitivity, anti-CD45RB monoclonal antibody therapy represented a rational choice for tolerance induction in the resistant NOD background.

However, despite the theoretical foundation for the utility of this approach, NOD mice were resistant to tolerance induction following anti-CD45RB antibody therapy in an allogeneic cardiac transplantation model. Nonetheless, it is well known that the CD4 lymphocyte compartment is not the only dysfunctional cell type within the NOD lineage. Critical alterations have been reported in the development and action of dendritic cells, macrophages, and B cells. In fact, studies have suggested that this compartment possesses no intrinsic dysfunction but rather that these alterations in responsiveness can be traced to the requirement for T cells to interact with other cell lineages for their stimulation [21, 23, 24, 45, 46, 47, 48]. The participation of B-lymphocytes as APCs is an absolute requirement in the development of diabetes [19, 20, 28, 29]. In addition, T cell activation in NOD mice cannot proceed in the absence of B cells, a limitation that suggests B cells are critical regulators of T cell activation in this strain [21, 23]. These data demanded investigation of other cell subsets in the function of anti-CD45RB, as derangements in these compartments might also hinder its function.

Further characterization of lymphocyte function following treatment has allowed us to use this resistance to gain insight into the mechanism of action of this therapy. When we carried out analysis, we noted a number of changes in cell surface molecules on B lymphocytes induced by interaction with anti-CD45RB. That these changes were not completely observed in the NOD background opens the possibility that B cells play a critical role in this process. Our study represents the first indication that modifications of the APC compartment, particularly notable changes in B lymphocyte function, in addition to direct effects on T cell function, might be required to promote long-term tolerance in transplantation models.

Whether the B cell is a requisite participant in the tolerogenic cascade elicited by this antibody is a subject

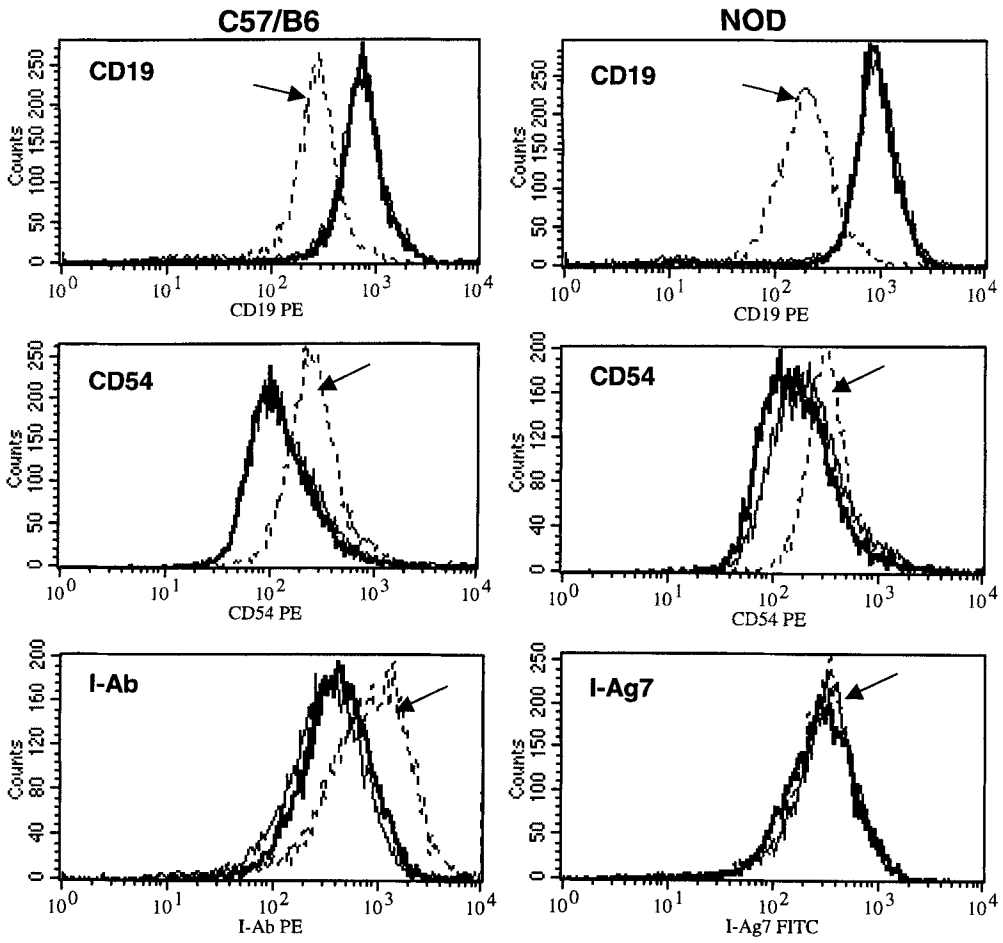

Fig. 4 Comparative analysis of cell surface phenotypes between B6 and NOD recipients following anti-CD45RB and allostimulation. The expression of cell surface markers on lymphocytes of NOD mice was assessed, as these animals were resistant to anti-CD45RB-mediated tolerance induction. In comparison with B6 mice, while the up-regulation in CD54 and down-regulation in CD19 were similar to that observed in B6 animals, the alterations seen in MHC class II were not detected in this background, which suggests a role for this alteration in the mechanism of tolerance induction. *Thin lines* represents the naive control; *thick lines* represents mice receiving cells in the absence of anti-CD45RB; *dotted lines* (marked with *arrow*) represent mice exposed to allogeneic cells in the presence of anti-CD45RB

of ongoing investigation in our laboratory. Of particular interest is the up-regulation of MHC class II molecules, a phenomenon not seen in tolerance-resistant NOD mice. This alteration in antigen-presenting function might alter the effective dose of presented antigen and, thereby, contribute to changes within the functional outcomes of T cells encountering alloantigen. As T cells are sensitive to antigen dose, the augmentation of the provided stimulus may produce a number of different outcomes [21, 49, 50]. The susceptibility to AICD is likely to be enhanced by an increase in T cell activation [38, 39]. Alternatively, this change might also promote the recruitment of additional specificities that were unstimulated at lower antigen densities; regulatory cells or cells with differentiated functions that are not harmful to the graft may be included among this subset. Further analysis may pinpoint those alterations in APC function that are requisite for tolerance induction. This avenue of exploration might provide a number of new strategies for the induction of long-term tolerance against target antigens.

Acknowledgements This research was supported by grants DK54215 and P01-DK-49814 from the National Institutes of Health. D.J.M. was supported by the American Diabetes Association Physician Scientist Training Grant.

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