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CD4⁺ CD45RB Low-Density Cells from Untreated Mice Prevent Acute Allograft Rejection

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In the absence of therapy that suppresses the action of the immune system, the immune response to transplantation Ags results in rapid rejection of the transplant. The most successful mechanism so far described that achieves organ-specific immunological tolerance is that which controls peripheral tolerance to self-tissue. Until now, no similarities have been documented between the peripheral response to self-Ags and the response to transplantation Ags. CD4⁺ cells that express a high density of CD45RB (in the mouse) and CD45RC (in the rat) on their surface have been shown to cause a number of autoimmune disorders. In contrast, autoimmunity caused by the CD45RB high-density cells is inhibited by CD4⁺ CD45RB low-density cells that express a low density of CD45RB (CD45RC in the rat). In this paper we show that CD4⁺ CD45RB high-density cells are sufficient to cause rejection of a MHC-mismatched pancreas allograft, whereas CD4⁺ CD45RB low-density cells are not. Unexpectedly, the CD45RB low-density cells prevent the CD45RB⁺ expressing cells from causing rejection. These data suggest that the response to foreign tissue can be controlled in the same way as the response to self-tissue. The Journal of Immunology, 1999, 163: 5353–5357.

CD45RB is a transmembrane protein tyrosine phosphatase expressed on leukocytes and involved in T lymphocyte activation (23). Its density on T lymphocytes has been used to separate T cells into functionally distinct subsets that have either pathological or nonpathological activity (10–16). In addition, although not an absolute marker for the distinction of T cells into Ag-experienced (memory, CD45RBlow) and Ag-inexperienced (naive, CD45RBhigh) cells, in combination with two other cell surface molecules, CD62L and CD44, it has been used to identify such subsets (24–28). We have investigated the possibility that CD4⁺ CD45RBhigh cells are sufficient to cause allograft rejection and that CD4⁺ C45RBlow cells are capable of inhibiting that rejection process.

Materials and Methods

Mice
C57BL/6J (C57BL/6, H-2b) adult mice and BALB/cByJ (BALB/c, H-2d) neonates were obtained from the Scripps Breeding Colony (La Jolla, CA). C57BL/6J-SCID/SjJ (C57BL/6-SCID, H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Grafting of neonatal pancreata
Pancreata of BALB/c (H-2d) neonates were grafted under the kidney capsule of C57BL/6-SCID males (H-2b).

Histological assessment
Grafts were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin for histological examination or were tested for the presence of insulin by immunohistochemistry as described (29). The area encompassed by islets and cellular infiltration was measured using a Leica Image Processing and Analyzing System (Q500 MC; Leica, Cambridge, U.K.). Statistical analysis was performed using InStat version 2.0 for Macintosh. Data were analyzed using ANOVA, then the Bonferroni Multiple Comparison Test (30). A p value ≤0.05 is considered significant.

Cellular infiltration
In some experiments mice will receive two grafts. The first is given at the time of the cell infusion; the second is given 6 wk later. For first grafts, those that were scored positive for cellular infiltrates were infiltrated in 75–100% of the tissue. Those grafts that were scored negative for cellular infiltrates showed 0% cellular infiltration. For second grafts, those that...
were scored positive for cellular infiltrates showed infiltration in 12–100% of the graft. Grafts which scored negative for cellular infiltrates showed 0% infiltration.

Cell purification

Spleen cells from 2- to 4-mo-old C57BL/6 male mice were prepared for single cell suspensions. RBC were removed with lysing buffer (Sigma, St. Louis, MO), and the remaining spleen cells were resuspended in PBS with 1% FBS (Intergen, Purchase, NY). Splenocytes were labeled with a PE-conjugated CD4-specific mAb, GK1.5 (Becton Dickinson, Mountain View, CA) (31), and a FITC-conjugated CD45RB-specific mAb, MB23G2 (PharMingen, La Jolla, CA) (32), and sorted under high speed on a FACS-Vantage SE with TurboSort (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The CD45RBhigh and CD45RBlow populations are defined as the brightest-staining 30% and dullest-staining 15% of the CD4+ cells, respectively. All cell populations were sampled and analyzed using FACSCalibur with CELLQuest version 3.2 software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The CD45RBhigh and CD45RBlow populations are defined as the brightest-staining 30% and dullest-staining 15% of the CD4+ cells, respectively. All cell populations were sampled and analyzed using FACSCalibur with CELLQuest version 3.2 software (Becton Dickinson Immunocytometry Systems) to determine the purity of the sorted populations (Fig. 1). CD4+ CD45RBhigh and CD4+ CD45RBlow populations used were >98% CD4+ (data not shown). Cell subsets were washed in PBS once after sorting but before i.v. injection into recipients.

Results

CD4+ CD45RBhigh but not CD4+ CD45RBlow cells reject a pancreas allograft

To determine whether CD45RBhigh cells can cause the rejection of a pancreas allograft, C57BL/6J-SCID mice were grafted with a pancreas from a 1-day-old BALB/c donor and infused with 0.25 \( \times \) 10^6 CD4+ CD45RBhigh cells, 0.25 \( \times \) 10^6 CD4+ CD45RBlow cells, or no cells. The grafts were assessed histologically, and the area encompassed by islets and cellular infiltration was determined. C57BL/6-SCID mice that were infused with CD4+ CD45RBhigh cells rejected 70% of the allografts within 3 wk after transplantation (Fig. 2a). The grafts contained massive cellular infiltration (Fig. 2e) and a reduction in islet area compared with the group that received either CD45RBlow cells (\( p < 0.01 \)) or no cells (\( p < 0.05 \), Fig. 2f). In contrast, only 1 of 10 of the grafts harvested from mice that received an equal number of CD4+ CD45RBlow cells displayed any signs of rejection (Fig. 2b). As expected, none of the grafts (0 of 7) from mice that did not receive a cell infusion showed signs of rejection (Fig. 2c). The infusion of fewer CD4+ CD45RBhigh cells (0.125 \( \times \) 10^6) resulted in a much lower incidence of allograft rejection (data not shown). In contrast, the infusion of 0.5 \( \times \) 10^6 CD45RBhigh cells resulted in a similar incidence of rejection as with 0.25 \( \times \) 10^6 cells (data not shown).

Because we were interested in determining whether rejection could be inhibited by CD4+ CD45RBlow cells, we have decided to use the minimum number of cells required to give a consistently high incidence of rejection. This number is 0.25 \( \times \) 10^6. Therefore, in subsequent experiments 0.25 \( \times \) 10^6 CD4+ CD45RBhigh cells will be used.

CD4+ CD45RBlow cells prevent CD4+ CD45RBhigh cells from rejecting a pancreas allograft

Having established that CD4+ CD45RBlow cells were substantially less efficient at causing allograft rejection than CD45RBhigh...
cells, we determined whether the CD45RB<sup>low</sup> population had the capacity to prevent the CD45RB<sup>high</sup> population from rejecting foreign tissue. The experiment was set up in the same way as above but with an additional group infused with both CD45RB<sup>high</sup> cells and CD45RB<sup>low</sup> cells. A 1:1 ratio of high \((0.25 \times 10^6)\) to low \((0.25 \times 10^6)\), and a 2:1 ratio of high \((0.25 \times 10^6)\) to low \((0.125 \times 10^6)\) were used with similar results. Only the data for the 2:1 ratio are shown. Pancreas allografts in mice that had received a mixture of CD45RB<sup>high</sup> and CD45RB<sup>low</sup> cells (Fig. 2<i>d</i>) were morphologically similar to those grafts in the groups that received CD45RB<sup>low</sup> cells only (Fig. 2<i>b</i>) or no cells (Fig. 2<i>c</i>); that is, no signs of rejection were evident. Pancreas grafts from mice that had received CD45RB<sup>high</sup> and CD45RB<sup>low</sup> cells displayed a significant increase in islet area compared with grafts from mice that had received CD45RB<sup>high</sup> cells only \((p < 0.05)\). Therefore, these data suggest that CD4<sup>+</sup> cells expressing low levels of CD45RB are able to prevent allograft rejection mediated by CD45RB<sup>high</sup> cells.

**Pancreas allografts in recipients of a CD4<sup>+</sup> CD45RB<sup>high</sup> and CD45RB<sup>low</sup> cells are tolerant of graft Ags at 10 wk after grafting**

The data shown thus far are taken at 3 wk after grafting. Therefore, it was important to determine whether allograft protection mediated by CD45RB<sup>low</sup> cells was maintained for longer periods and whether the recipient was then rendered tolerant of graft Ags. Tolerance was tested using the classical method of placing a second graft into the recipient. Specifically, C57BL/6-SCID recipients were infused with a 1:1 ratio of CD45RB<sup>low</sup> and CD45RB<sup>high</sup> cells, low-density cells only, or no cells. The first graft was placed 1 day before the cell infusion. The second graft, also a pancreas graft from a BALB/c donor, was placed under the contralateral kidney 6 wk later, and subsequently all grafts were removed for histologic examination after an additional 4 wk (see Fig. 3 for time course of treatment).

The first pancreas allografts from mice that received no cells showed no signs of rejection 10 wk after grafting (Fig. 4<i>a</i>). This was also true of the grafts taken from mice that received CD45RB<sup>low</sup> cells only (Fig. 4<i>b</i>) or CD45RB<sup>low</sup> and CD45RB<sup>high</sup> cells (Fig. 4<i>c</i>). Specifically, cellular infiltration was absent in all grafts from those three groups. In addition, islet area was not significantly different between groups and was comparable to the islet area seen in the single grafts described in Fig. 2. These data suggest that allograft survival was maintained long term.

The islet area in the second grafts from mice that received no cells (Fig. 4<i>d</i>) or CD45RB<sup>low</sup> cells only (Fig. 4<i>e</i>) were similar to grafts in mice in the groups that received both CD45RB<sup>low</sup> and CD45RB<sup>high</sup> cells (Fig. 4<i>f</i>). Therefore, the protective effect of the CD45RB<sup>low</sup> cells on the response of the CD45RB<sup>high</sup> cells seen in the long-term appears to be mediated by inducing functional tolerance at the whole animal level. Despite the fact that the islet area

**FIGURE 3.** A diagrammatic view of the protocol used for experiments described in Fig. 4. The mice used in the experiments described in Fig. 4 were grafted twice and given a cell infusion. This diagram shows the chronological order of the treatment given.

**FIGURE 4.** Pancreas allografts in recipients of a mixture of CD4<sup>+</sup> CD45RB<sup>low</sup> and CD45RB<sup>high</sup> cells are tolerant to the allograft. C57BL/6-J-SCID mice were grafted with pancreas allografts from 1- to 3-day-old BALB/c donors. The following day they were divided into groups and injected with no cells, \(n = 3\) (a); \(0.25 \times 10^6\) CD45RB<sup>low</sup> cells, \(n = 3\) (b); or \(0.25 \times 10^6\) CD45RB<sup>low</sup> cells and \(0.25 \times 10^6\) CD45RB<sup>high</sup> cells, \(n = 3\) (c). Six weeks after placement of the first graft, all mice were grafted with a second 1- to 2-day-old BALB/c pancreas (d–f). Four weeks later the mice were culled and the grafts were prepared for histological examination. Original magnification, \(\times 100\).
in the second grafts was maintained, cellular infiltration was observed in 100% of second grafts (Fig. 4f) but not first grafts (Fig. 4c) from the mice that had received the mixture of CD45RB<sup>high</sup> and CD45RB<sup>low</sup> cells.

**Discussion**

We have shown that the CD<sup>+</sup> CD45RB<sup>high</sup> cell subset is sufficient for pancreas allograft rejection. In contrast, those CD<sup>+</sup> cells that express a low density of the CD45RB molecule do not reject a pancreas allograft and protect such allografts from rejection by CD<sup>+</sup> CD45RB<sup>low</sup> cells. This protection lasts for at least 10 wk and functional tolerance is shown in the protected recipients by the finding that second grafts in these recipients are also not rejected.

Our data confirm the finding that the CD<sup>+</sup> CD45RB<sup>low</sup> cell subset is a regulatory cell subset capable of preventing pathological responses. It also extends these findings to show that the regulatory cells do not distinguish between self-Ag and foreign MHC Ag. This is in contrast to the published data which have suggested that regulatory cells do not distinguish between self-Ag and foreign MHC Ags. Alternatively, a specific Ag might not be necessary for the CD45RB<sup>low</sup> cells to function in a regulatory manner in this transplantation model. It is also possible that the CD45RB<sup>low</sup> cells develop their regulatory function after contact with transplantation Ag in the grafted recipient.

There are a number of possibilities as to the mechanism by which the CD45RB<sup>low</sup> cells prevent the CD45RB<sup>high</sup> cells from rejecting the allograft. They may prevent homing of the high cells to the graft. In this regard, we found that second grafts of recipients of CD45RB<sup>high</sup> and CD45RB<sup>low</sup> cells display cellular infiltration within 4 wk of grafting, even though they contained an islet area equivalent to those of grafts from mice that received CD45RB<sup>low</sup> cells only. This observation might indicate that the CD45RB<sup>low</sup> cells are able to prevent naive cells, but not primed cells, from infiltrating the graft. Cellular infiltration was not observed in the first (established) grafts. Alternatively, the CD45RB<sup>low</sup> cells might prevent priming of the high cells to the graft Ags, or they might affect the effector capacity of the graft-Ag-specific CD45RB<sup>high</sup> cells so that they are no longer pathogenic. Finally, the CD45RB<sup>low</sup> cells might render the graft less immunogenic. Such effects might occur through cell-mediated events or through soluble mediators (16, 34–35).

In summary, CD<sup>+</sup> CD45RB<sup>high</sup> cells are sufficient to induce pancreas allograft rejection. In contrast, CD<sup>+</sup> CD45RB<sup>low</sup> cells prevent CD<sup>+</sup> CD45RB<sup>high</sup> cells from causing rejection. In the model described here the CD45RB<sup>low</sup> cells are purified from a mouse that has not been previously immunized with the transplantation Ags in question. In addition, the ratio of CD45RB<sup>high</sup> to CD45RB<sup>low</sup> cells used in the experiments described here was 2:1, which is the same as the ratio normally present in the spleen of untreated mice and the same as the ratio used to prevent autoimmune disorders (10–13); this suggests that in this model the mechanism that prevents autoimmunity also prevents allograft rejection. In the context that fully immunocompetent mice do reject pancreas allografts whereas they do not normally show signs of autoimmunity, we have drawn the following series of possible conclusions: 1) in a fully immunocompetent animal, CD<sup>+</sup> CD45RB<sup>low</sup> cells do not inhibit the ability of a greater number of CD<sup>+</sup> CD45RB<sup>high</sup> cells from rejecting an allograft; 2) CD<sup>+</sup> CD45RB<sup>low</sup> cells do not inhibit the ability of other T and B cell subsets from causing rejection of an allograft; 3) in an immunocompetent mouse, a cell subset other than, or as well as, the CD<sup>+</sup> CD45RB<sup>low</sup> cell subset prevents autoimmune disorders but not allograft rejection; or 4) the immunocompromised recipient model is unusually susceptible to the effect of CD<sup>+</sup> CD45RB<sup>low</sup> cells. Further studies will indicate which, if any, of these possibilities is true.

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