Damage control, rather than unresponsiveness, effected by protective DX5+ T cells in autoimmune diabetes

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The progression of autoimmune diabetes is regulated. We examined here the cellular controls exerted on disease that developed in the BDC2.5 T cell receptor–transgenic model. We found that all BDC2.5 mice with a monoclonal, β cell–reactive, T cell repertoire developed diabetes before 4 weeks of age; transfer of splenocytes from young standard NOD (nonobese diabetic) mice into perinatal monoclonal BDC2.5 animals protected them from diabetes. The protective activity was generated by CD4+ αβ T cells, which operated for a short time at disease initiation, could be partitioned according to DX5 cell surface marker expression and split into two components. Protection did not involve clonal deletion or anergy of the autoreactive BDC2.5 cells, permitting their full activation and attack of pancreatic islets; rather, it tempered the aggressiveness of the insulitic lesion and the extent of β cell destruction.

Autoimmune diabetes reflects widespread destruction of the insulin-producing pancreatic islet β cells. At the core of the disease lies the recognition of β cell–derived antigens by autoreactive T lymphocytes, but a number of processes come into play before the catastrophic endpoint. These include the generation of a self-reactive T cell repertoire through the failure of central mechanisms of tolerance induction, or the dysfunction of peripheral control mechanisms that normally prevent immune pathology directed against normal organs.

The BDC2.5 T cell receptor (TCR)–transgenic (Tg) mouse line has been useful for studying the development and control of the autoimmune attack that underlies diabetes[1]. These mice display, on a large fraction of their T cells, a clonotypic TCR derived from a T cell clone that was isolated from a nonobese diabetic (NOD) mouse. This TCR is specific for an antigen made by pancreatic islet β cells bound to major histocompatibility complex (MHC) class II A^{g7} (Ref. 2). When propagated on the NOD genetic background, BDC2.5 Tg animals (designated hereafter BDC2.5/N mice) show a generalized and very extensive islet infiltration after a few weeks of age, but most remain free of overt diabetes for long periods of time. This prediabetic state reflects a relatively innocuous ("respectful") form of insulitis that does not cause β cell destruction. The lesion is an active one: infiltrating lymphocytes express activation markers and are actively cycling, and yet a clear demarcation between the infiltrate and the residual foci of healthy β cells is evident[3–5]. Thus, although other Tg models with more severe forms of diabetes may be more appropriate for investigating the mechanisms of organ destruction[6], the BDC2.5/N mouse provides a useful model for dissecting disease regulatory mechanisms. The state of innocuous insulitis is under genetic control: C57BL/6 (B6) background genes promote a form of diabetes that is more rapid and penetrant than in the presence of NOD genes[3]. The establishment of a state of metastable insulitis involves the action of CTLA-4 and ICOS costimulatory family members[4,7] (and A. Hermann *et al*, unpublished data). Once set, cyclophosphamide, lipopolysaccharide (LPS) or viral infection of the pancreas[5,8,9] can perturb this equilibrium with the result that diabetes ensues rapidly.

It is possible that the regulatory mechanisms responsible for maintaining an innocuous infiltrate are intrinsic to the autoreactive T cells themselves. It may also be that such regulation involves interactions between the autoreactive and other cells, perhaps analogs of the "regulatory" T cells uncovered in several models of autoimmune disease, including diabetes[10–14]. The BDC2.5/N line is a powerful system with which to address this issue, both because of the relative synchrony and simplicity of the disease that develops and the possibility it affords to directly monitor the behavior of the pathogenic cells. Here, we provide evidence for the existence of T cells that regulate the progression of spontaneous diabetes. We define them and describe their influence on the phenotype of the autoaggressive T cells.

Results

Loneliness breeds aggressiveness

To generate mice with a T cell repertoire that was even more restricted than in standard BDC2.5 animals[1], we introduced deletions into the line. The first was a mutation of the TCRα locus ($C\alpha^{-/-}$), which prevented

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expression of any endogenously encoded TCRα chains and thereby blocked differentiation of αβ T cells. The second was a mutation of recombination-activating gene 1 (RAG-1$^{-/-}$)[15], which prevented rearrangement of all of the genes encoding antigen-specific lymphocyte receptors, thus precluding maturation of αβ T, γδ T and B lymphocytes. We found that diabetes progression was greatly affected by prevention of rearrangements of genes encoding non-BDC2.5 antigen-specific receptors (**Fig. 1a**). By several months of age, overt diabetes appeared in only 12% of BDC2.5 mice. BDC2.5 RAG-1$^{-/-}$ (referred to hereafter as B/R^{o}) animals all succumbed to marked insulin deficiency by 20–31 days, with peak incidence at 25 days. BDC2.5 Cα$^{-/-}$ (referred to hereafter as B/Cα^{o}) animals showed an intermediate phenotype with respect to both the penetrance and rapidity of diabetes. Heterozygous BDC2.5 RAG-1$^{+/-}$ or BDC2.5 Cα$^{+/-}$ control littermates showed no greater disease susceptibility than BDC2.5/N mice did.

The autoimmune attack is manifested in BDC2.5 mice only after a silent latent period. The first sign of a response is the accumulation of activated T cells in the draining pancreatic lymph nodes (PLNs), this is soon followed by infiltration of the islets. Insulitis occurs in a synchronous manner at 15–18 days of age, an event that is termed "checkpoint-1"[16]. One possible explanation for the accelerated course of diabetes in B/R^{o} and B/Cα^{o} mice was that autoimmunity begins earlier in these animals, perhaps during the neonatal stage. However, histological analysis ruled out this interpretation (**Fig. 1b**): 15-day-old mice of all three phenotypes had intact islets that were essentially devoid of infiltrating cells; infiltration was first evident on day 18 or later. In fact, insulitis was slightly delayed in B/R^{o} mice, with most animals becoming insulitic only at day 23. This perhaps reflected some role for CD8^{+} T cells in initiating insulitis, even in the context of the BDC2.5 transgenes[17], or for B lymphocytes. Once infiltration began in B/R^{o} mice, it quickly affected almost all the islets and was of an aggressive nature. Instead of the "respectful" insulitis typical of standard BDC2.5/N mice, in which the infiltrate is in direct contact with β cells but leaves large islands intact and functional (see below), insulitis in B/R^{o} animals involved invasion of the entire islet area by lymphoid cells, with unrestricted intermingling with the β cells. The β cells showed obvious signs of cellular damage and apoptosis: edema in the connective and exocrine tissue surrounding the islets was also observed. This histological picture closely resembled those reported for BDC2.5/N mice in which controls on insulitis had been disrupted (for example, by blockade of regulatory molecules, in the presence of disease-accelerating *Aid* genes or after destabilization of the regulatory balance by viral infection, cyclophosphamide or LPS[3–5,8,9]).

Control of BDC2.5 T cell aggressiveness

T cells that were displaying the BDC2.5 clonotype seemed to be far more aggressive in the absence of other lymphocytes. There could be several explanations. First, the relatively lymphopenic environment of the B/R^{o} mouse might provoke homeostatic expansion and, as a consequence, increased T cell reactivity[18,19]. Second, the greater number of clonotype^{+} cells that mature in B/R^{o} mice might cause an amplification of the anti-self response *via* the higher amounts of cytokine produced, the more aggressive autoimmune attack resulting from a positive-feedback "chain reaction". Third, "regulatory" cells expressing non-BDC2.5 clonotypes, which are absent from B/R^{o} mice, might dampen the reactivity of BDC2.5 T cells.

The first possibility was ruled out by flow cytometric analysis of cells from the lymphoid organs of prediabetic (20-day-old) BDC2.5/N and B/R^{o} mice. As shown by the surface expression of several activation markers, there was no evidence for a generalized stimulation of T cells in B/R^{o} mice: all CD4^{+} T cells (uniformly V$_{\beta}$4^{+}, as expected) in spleen and peripheral lymph nodes had a completely naïve phenotype. If anything, there were even fewer activated cells than in BDC2.5/N mice, most likely because nonclonotypic receptors in the latter would be capable of recognizing environmental antigens (data not shown). Signs of activation (CD69 expression and CD62 ligand (CD62L) down-modulation) could only be detected in the pancreatic lymph nodes and in pancreas-infiltrating cells. Thus, there was no evidence of antigen-independent sensitization of T cells that resided in the relatively lymphopenic environment of the B/R^{o} mouse.

To address the other two possibilities, we set up a transfer system and introduced splenocytes from young NOD donors into 10-day-old B/R^{o} recipients. This protocol was chosen—rather than the cotransfer strategies more frequently used for the analysis of putative regulatory cells—to allow us to assess the effects on B/R^{o} T cells that matured normally, unperturbed by isolation and transfer. Unless indicated otherwise, 10-day-old recipients were chosen for all experiments so that the potentially protective cells would be in place before initiation of the autoimmune response between days 15 and 18. Donors were 4-week-old NOD mice, thus, before any substantial autoimmune manifestations. Transfer of 5.0×10^{7} splenocytes from NOD mice into B/R^{o} animals had a marked effect, preventing early diabetes in all recipients (**Fig. 2a**). For the maximum protective effect, spleen cells had to be introduced at the very beginning of the process (**Fig. 2b**): transfer on day 15, as on day 10, protected all mice; transfer on days 17 and 19 was less effective, although some recipients were still protected. These data implied that protective cells must be present at the start of the autoimmune reaction; in addition, the short lag between their infusion and their effect suggested that they

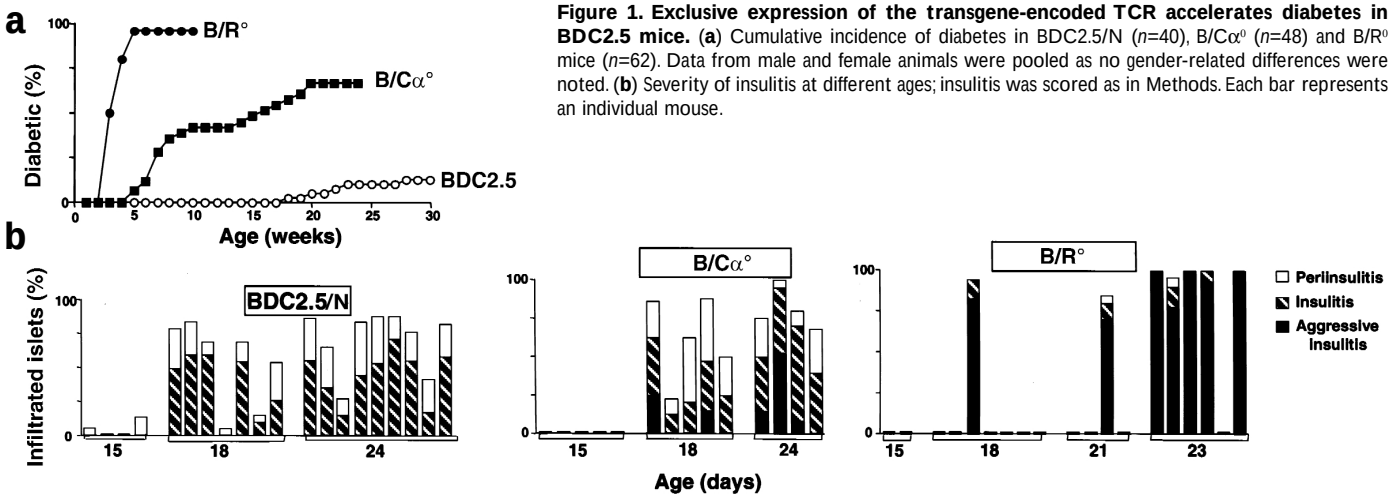

Figure 1. Exclusive expression of the transgene-encoded TCR accelerates diabetes in BDC2.5 mice. (**a**) Cumulative incidence of diabetes in BDC2.5/N (*n*=40), B/Cα^{o} (*n*=48) and B/R^{o} mice (*n*=62). Data from male and female animals were pooled as no gender-related differences were noted. (**b**) Severity of insulitis at different ages; insulitis was scored as in Methods. Each bar represents an individual mouse.

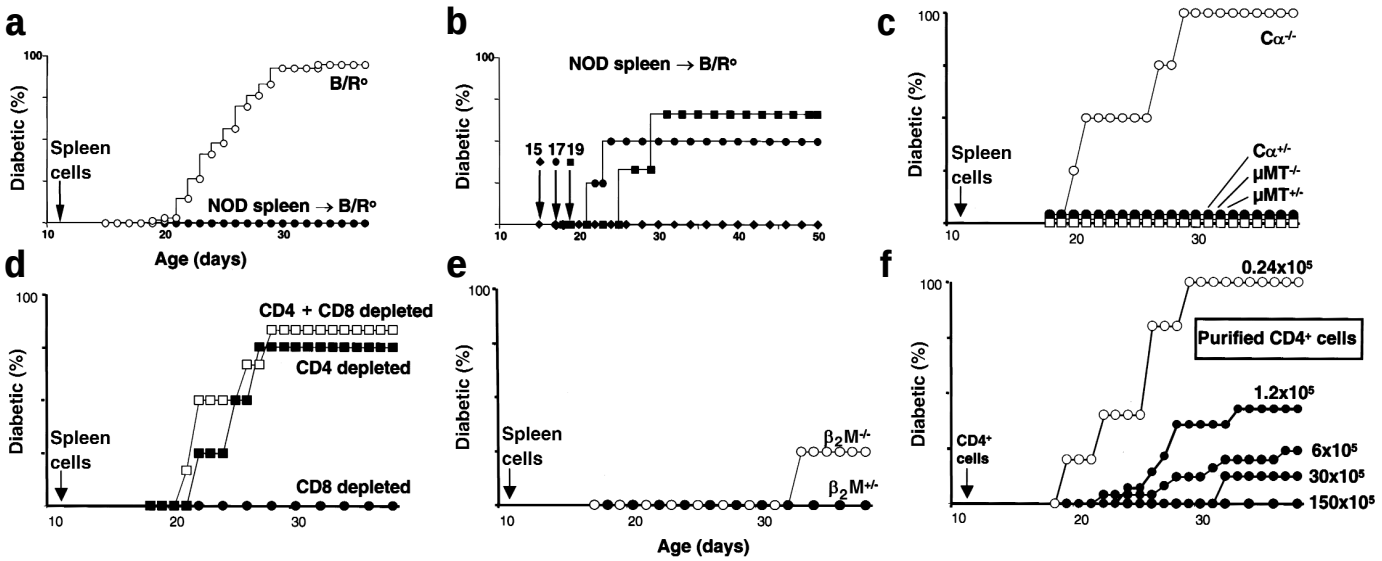

Figure 2. CD4⁺ αβ T cells protect B/R° mice from diabetes. (**a**) B/R° mice were injected with 5.0×10^7 NOD spleen cells at 10 days of age and the occurrence of diabetes assessed. Unmanipulated B/R° mice, n=21; injected B/R° mice, n=6. (**b**) Diabetes incidence in B/R° mice injected at 15, 17 or 19 days of age with 5.0×10^7 NOD splenocytes. Three to four mice were analyzed at each time-point. (Diabetes appeared much later in some recipients, mainly at 60–80 days of age; this late-onset diabetes also occurred in non-Tg RAG-1$^{-/-}$ recipients. Thus, it is most likely induced by the transferred population itself; it was not observed with purified CD4⁺ T cells (see below), consistent with the fact that transfer of diabetes requires both CD4⁺ and CD8⁺ T cells.) (**c**) B/R° mice were injected with 5×10^7 spleen cells from αβ T cell–deficient Cα$^{-/-}$ or B cell–deficient μMT$^{-/-}$ mice or their phenotypically normal Cα$^{+/-}$ and μMT$^{+/-}$ littermates. Three or four mice were used per group. (**d**) B/R° mice with injected with 5×10^7 NOD splenocytes depleted of CD4⁺or CD8⁺ T cells or both. Four to six mice were used per group. (**e**) B/R° mice were injected with 5×10^7 NOD splenocytes from β_2M$^{-/-}$ mice or control littermates. Four and three mice were analyzed, respectively. (**f**) Different doses of magnetically purified CD4⁺ spleen cells from NOD mice were transferred into 10-day-old B/R° mice. Data are from at least five mice per dose.

do not act by inducing a cascade of indirect effects. Transfer of NOD splenocytes into B/R° mice recapitulated the BDC2.5/N situation, without visibly reducing the number of transgene-expressing $V_\beta4^+$ cells (below). This indicated that it was not the absolute number of clonotype⁺ cells that was important but, rather, that the presence of nonclonotypic protective cells prevented uncontrolled autoimmunity when present at the onset of the response.

The BDC2.5 system also provided a manageable approach with which to define the nature of the protective cells. We did a series of transfer experiments using cells from donors that carried null mutations on the NOD genetic background or purified populations of cells from NOD mice (**Figs. 2** and **3**). Transfer of splenocytes from B cell–deficient μMT$^{-/-}$ NOD animals was fully protective, whereas transfer of splenocytes from αβ T cell–deficient Cα$^{-/-}$ NOD animals was not, which pointed to a critical role for αβ T cells (**Fig. 2c**). Antibody-mediated depletion of CD4⁺, but not CD8⁺, T cells abolished protection (**Fig. 2d**). That the protective T cells were CD4⁺ was confirmed by the strong dampening effect of splenocytes derived from β_2-microglobulin–deficient (β_2M$^{-/-}$) donors, which contain almost no CD8⁺ T cells (**Fig. 2e**); note that in pilot experiments we found that these donor cells were not targets of natural

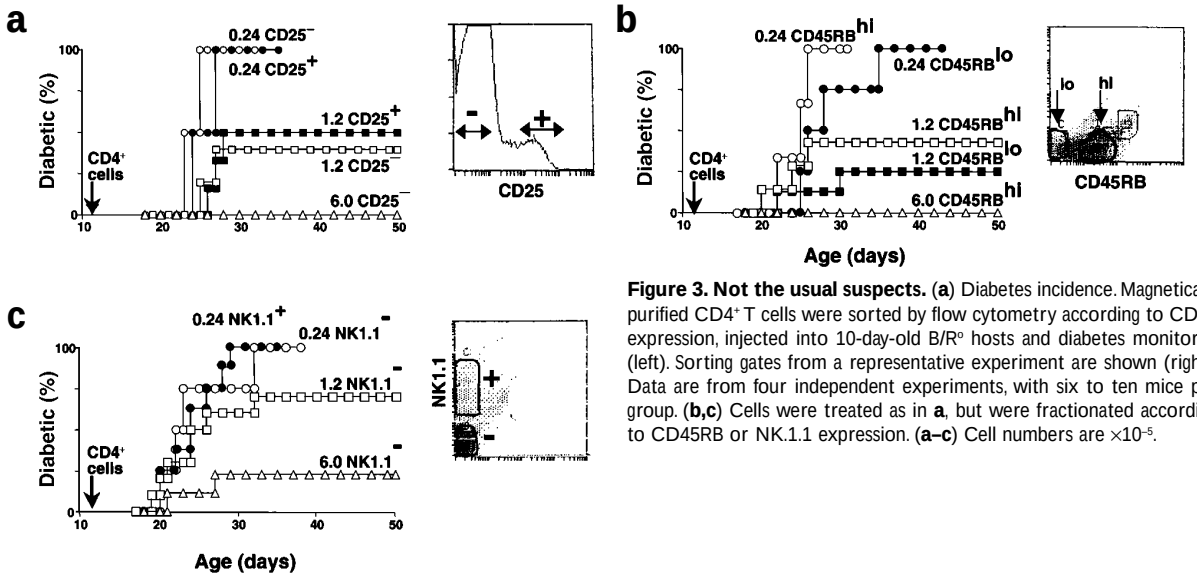

Figure 3. Not the usual suspects. (**a**) Diabetes incidence. Magnetically purified CD4⁺ T cells were sorted by flow cytometry according to CD25 expression, injected into 10-day-old B/R° hosts and diabetes monitored (left). Sorting gates from a representative experiment are shown (right). Data are from four independent experiments, with six to ten mice per group. (**b,c**) Cells were treated as in **a**, but were fractionated according to CD45RB or NK.1.1 expression. (**a–c**) Cell numbers are $\times10^{-5}$.

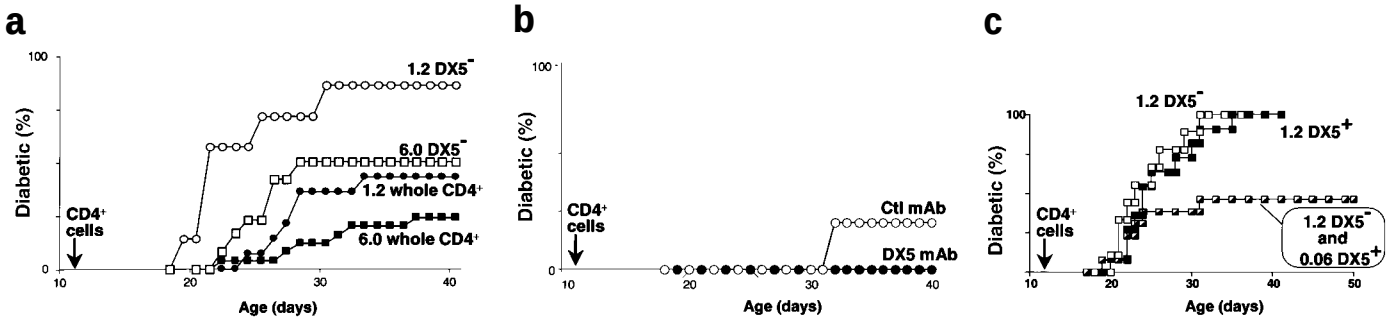

Figure 4. Surface expression of the DX5 distinguishes two populations required for optimal protection. Diabetes incidence was monitored in B/R^o mice injected with the following. (**a**) 1.2×10^5 or 6.0×10^5 NOD CD4$^+$ T cells, either unfractionated or depleted of DX5$^+$ cells by magnetic sorting. (**b**) 6.0×10^5 NOD CD4$^+$ T cells blocked by preincubation with DX5 mAb. (**c**) 1.2×10^5 DX5$^+$ or DX5$^-$ NOD CD4$^+$ T cells or 1.2×10^5 DX5$^-$ cotransferred with 0.06×10^5 DX5$^+$ cells.

killer (NK) cells in young NOD mice (A. Gonzalez and P. Hoglund, unpublished data). Transfer of CD4$^+$ T cells that had been purified by magnetic sorting conferred protection, and a low number of these cells (1.2×10^5) was sufficient to induce a marked effect (**Fig. 2f**). Once protected by transferred CD4$^+$ T cells, B/R^o mice remained free of diabetes for at least 1 year. Thus, CD4$^+\alpha\beta$ T cells were necessary and sufficient to protect mice against the BDC2.5 T cells, at least when introduced at disease initiation.

A number of regulatory phenomena have been attributed to CD4$^+$ T cells over the past several years[10–14]. Although the exact relationships between subsets of cells that show protective effects in an autoimmune setting are not always well understood, two major cell types have been described. NKT cells are a subset of CD4$^+$ $\alpha\beta$ T cells that are restricted by nonclassical MHC class I, rather than MHC class II. They express surface molecules that are typical of NK cells, produce copious amounts of cytokines early in an immune response and exert regulatory activity in several systems[12]. Another subset of CD4$^+$ T cells, defined as CD25$^+$ and CD45RB^{lo}, interferes with organ-specific autoimmunity in transfer experiments[10,11,13]. Whether such populations might mediate the protective effects observed in our transfer system was tested by fractionating CD4$^+$ T cells by flow cytometry before transfer into 10-day-old B/R^o hosts. Fractionation according to CD25 or CD45RB expression had no effect (**Fig. 3a,b**). CD4$^+$ T cells depleted of the CD25$^+$ or CD45RB^{lo} subsets could protect with the same dose-response profiles as unfractionated cells; conversely, enrichment of these minor fractions did not lead to enhanced protection.

To evaluate the activity of NKT cells, we used congenic NOD/NK1.1 mice as donors, as the NOD strain does not naturally express the epitope recognized by anti-NK1.1[20]. Once again, CD4$^+$ T cells depleted of NK1.1$^+$ cells had the same protective ability as unfractionated CD4$^+$ T cells; this was corroborated by the inability of purified NK1.1$^+$CD4$^+$ T cells to protect (although the latter result must be interpreted with caution, as antibody blockade of NK1.1 could potentially inhibit their function).

Thus, the CD4$^+$ $\alpha\beta$ T cells that had a protective effect in the B/R^o transfer system were different from the major populations of regulatory cells that have been identified in other systems. This may be because our model assessed regulatory mechanisms that impinged on the earliest stage of autoimmune activation, rather than mechanisms that inhibited the activity of the effector cells, as usually tested (see below).

Novel regulatory subsets identified by DX5

Although attempts to enrich for protective CD4$^+$ T cells using the usual markers failed, expression of DX5 was found to partition with the protective CD4$^+$ T cells. We uncovered a role for cells characterized by this marker serendipidously, while trying to use it as a surrogate marker for NK1.1 in NOD mice. DX5 recognizes a cell-surface molecule expressed

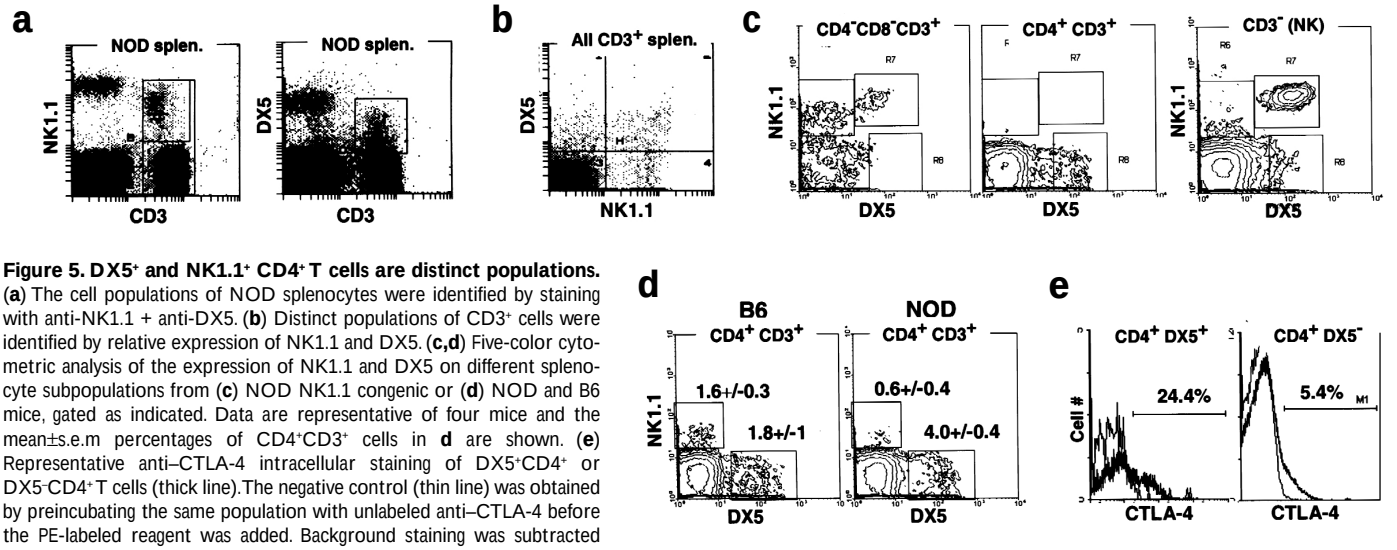

Figure 5. DX5$^+$ and NK1.1$^+$ CD4$^+$ T cells are distinct populations. (**a**) The cell populations of NOD splenocytes were identified by staining with anti-NK1.1 + anti-DX5. (**b**) Distinct populations of CD3$^+$ cells were identified by relative expression of NK1.1 and DX5. (**c,d**) Five-color cytometric analysis of the expression of NK1.1 and DX5 on different splenocyte subpopulations from (**c**) NOD NK1.1 congenic or (**d**) NOD and B6 mice, gated as indicated. Data are representative of four mice and the mean±s.e.m percentages of CD4$^+$CD3$^+$ cells in **d** are shown. (**e**) Representative anti–CTLA-4 intracellular staining of DX5$^+$CD4$^+$ or DX5$^-$CD4$^+$ T cells (thick line). The negative control (thin line) was obtained by preincubating the same population with unlabeled anti–CTLA-4 before the PE-labeled reagent was added. Background staining was subtracted from the values shown.

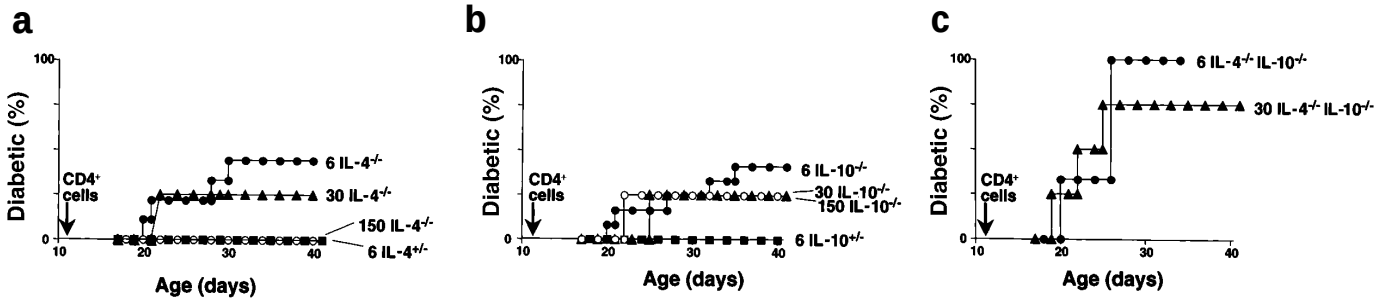

Figure 6. IL-4 and IL-10 are required by protective cells, but cross-complement. Diabetes incidence in B/R° mice injected with purified CD4+ splenocytes from (**a**) IL-4–deficient (**b**) IL-10–deficient or (**c**) IL-4 $^{-/-}$IL-10$^{-/-}$ mice or their control littermates. Cell numbers are $\times 10^{-5}$. Nine to twelve mice were used per group, except the 150×10^5 dose, in which two mice were used.

on conventional NK cells, identified as CD49b (α_2 integrin)[21]. This mAb also stains 2–4% of CD4+ T cells, which were initially thought to correspond to NKT cells, as identified with the NK1.1 marker[22,23]. Removal of DX5+ cells dampened the ability of CD4+ T cells to protect B/R° mice from diabetes (**Fig. 4a**). This reduction, which averaged ~80% over several experiments, was not complete; some protection was afforded at higher cell concentrations. This was likely because staining of CD4+ T cells by DX5 was not sharply bimodal, so that the fractionation cut-off may have allowed contamination of the DX5– fraction with some DX5+ cells. No protection was provided by the DX5+ fraction of CD4+ T cells, however, even when concentrated so that 1.2×10^5 (equivalent to 50×10^5 total CD4+ T cells) were transferred. The inactivity of the DX5+ and DX5– subsets probably did not stem from a blockade of DX5 itself, as simply incubating the cell preparation with DX5 left the protective ability of unfractionated CD4+ T cells intact (**Fig. 4b**). However, mixing both fractions before transfer substantially restored protection. Just 6×10^3 DX5+CD4+ T cells cotransferred with 1.2×10^5 DX5– T cells were sufficient to restore the protection to the same degree seen with 1.2×10^5 unfractionated CD4+ T cells (**Fig. 4c**); we did not attempt to titrate down the number of DX5– cells. It appeared, therefore, that protection from diabetes in the B/R° transfer system required concomitant input from two distinct cell populations, one DX5+ and one DX5–, the former necessary in only minute numbers.

Given these results, we decided to characterize in greater detail the DX5+ splenocyte subset of NOD mice, and in particular to re-explore its relationship with the NKT cell subset. DX5+CD3+ T cells constituted a population that was reminiscent of NK1.1+CD3+ cells, and expressed slightly less CD3 than mature αβ T cells and less DX5 than actual NK cells (**Fig. 5a**). The population of CD3+ T cells included a constituent that displayed both DX5 and NK1.1, but also substantial components that expressed only one or the other (**Fig. 5b**). The single marker–expressing cells partitioned sharply within the standard CD4 or CD8 subsets (**Fig. 5c**). NK1.1+DX5– T cells predominated in CD4–CD8– T cells along with a few cells that were NK1.1+DX5+, whereas the CD4+ population was dominated by NK1.1–DX5+ T cells. We found few or no cells of either type among CD8+ T cells. In contrast, conventional CD3– NK cells expressed both NK1.1 and DX5 (**Fig. 5c**, right panel). NOD mice seemed to compensate for a reduced number of NK1.1+CD4+ T cells by a higher prevalence of DX5+ cells (**Fig. 5d**). Although their CD25 and CD45RB expression was unremarkable, DX5+ CD4+ T cells expressed unusually high amounts of intracellular CTLA-4 (**Fig. 5e**).

Role of cytokines

To examine the mechanism of action of protective CD4+ T cells in the B/R° transfer system, we assessed the importance of the cytokines interleukin 4 (IL-4) and IL-10, which have immunomodulatory activity. We introduced CD4+ splenocytes from various cytokine-deficient mouse lines (all backcrossed for more than eight generations with the NOD mice[24], data not shown) or inoculated CD4+ splenocytes in the presence of mAb blockade. IL-10–deficient donors were used before the onset of the wasting that results from inflammatory bowel disease[25] (data not shown). A protective effect was observed with splenocytes that lacked either IL-4 or IL-10, although the effect was reduced when compared to the same number of wild-type cells (**Fig. 6a,b**). This result was confirmed by mAb blockade experiments: administration of anti–IL-4 still permitted full protection, whereas blockade of IL-10 only partially

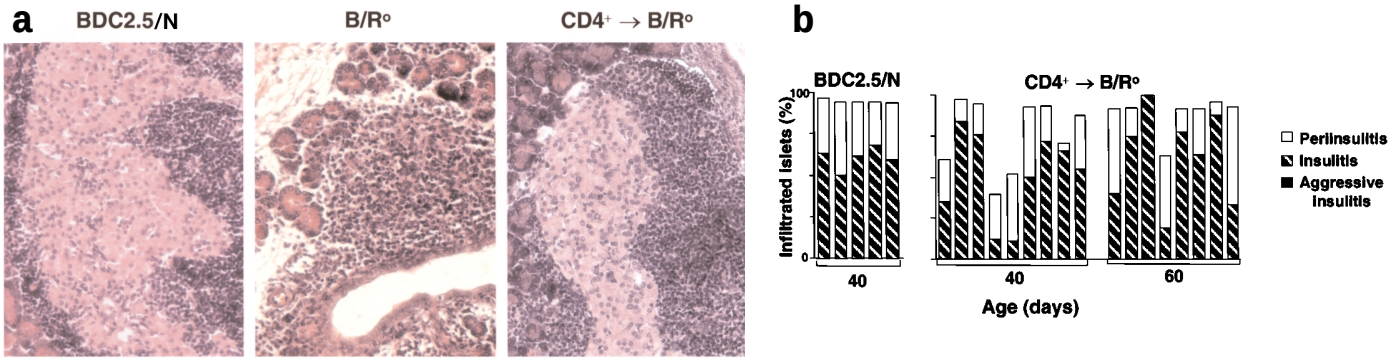

Figure 7. Protective cells do not prevent insulitis; they merely control it. (**a**) Representative hematoxylin-eosin–stained pancreas sections from 25-day-old BDC2.5/N, B/R° or B/R° mice injected at 10 days of age with 6×10^5 CD4+ T cells from young NOD donors. Original magnification: ×100. (**b**) Insulitis severity in BDC2.5/N mice or B/R° mice protected by transfer of 6.0×10^5 CD4+ spleen cells from NOD animals. For insulitis scoring see Methods. Each bar represents an individual mouse.

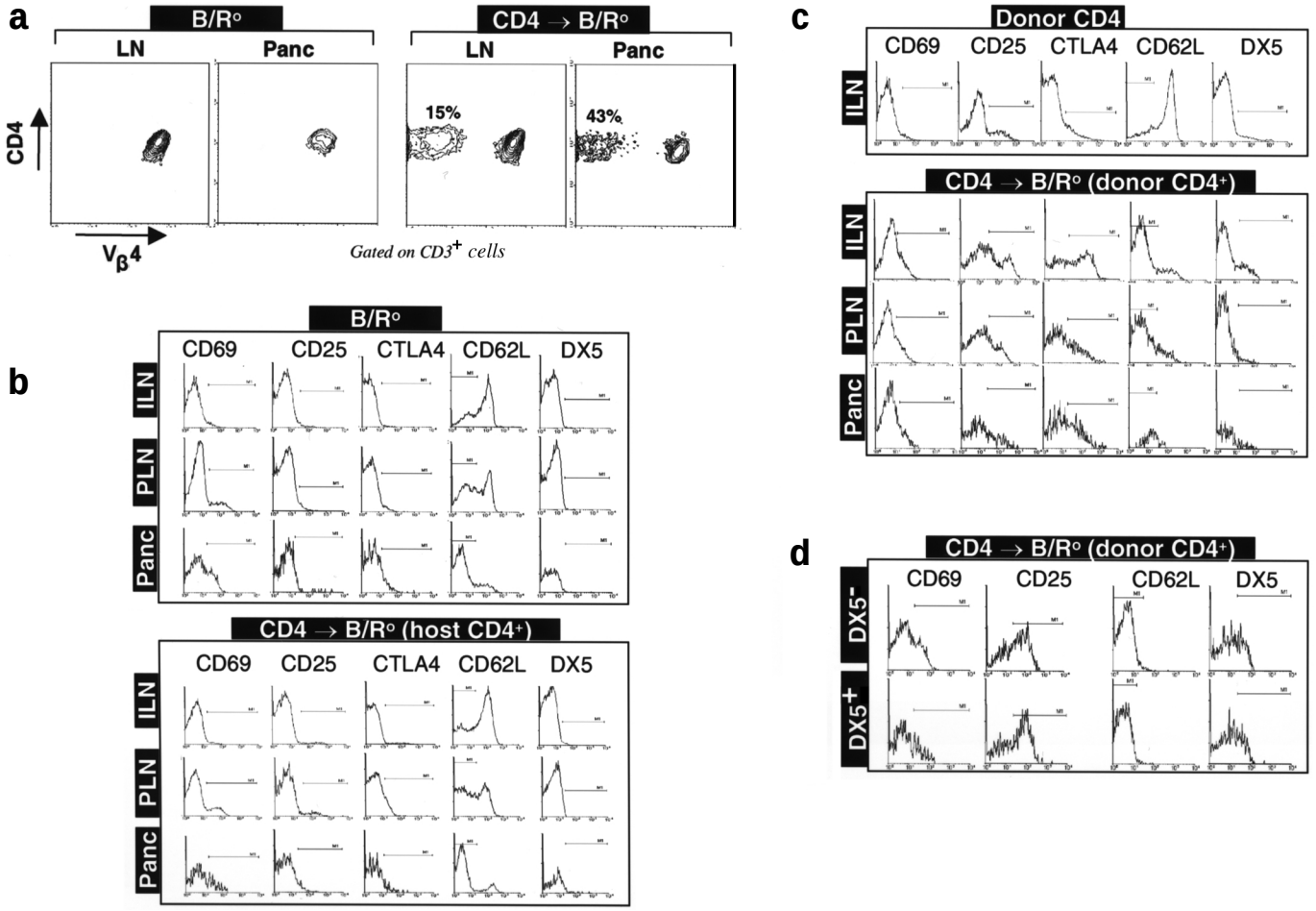

Figure 8. Phenotypic alterations in aggressive and protective populations. (**a**) Donor and recipient populations were identified by anti-CD4 + $V_\beta4$ staining of the inguinal lymph nodes (ILN) and pancreases (Panc) of 25-day-old B/R° mice injected at 10 days of age with 6×10^5 $CD4^+$ T cells from young NOD donors. Panels were gated on $CD3^+$ cells. Recipient cells were uniformly $V_\beta4^+$, whereas donor cells (missing from nontransferred B/R° mice, left panel) were mostly $V_\beta4^-$ ($V_\beta4^+$ donor cells made a minor ~5% contribution to the $V_\beta4^+$ pool of recipient origin that was disregarded in subsequent analyses). (**b**) Flow cytometric analyses of activation and/or memory markers on host cells (gated as $V_\beta4^-CD4^+CD3^+$cells as in **a**) from 25 day-old protected B/R° mice (lower panel) or nontransferred control littermates (top panel). Cells were from irrelevant ILNs, draining pancreatic lymph nodes (PLNs) or the pancreatic infiltrate. Profiles are representative of at least four mice. (**c**) Parallel analyses of donor-derived cells (gated as $V_\beta4^-CD4^+CD3^+$ cells as in **a**) from 25-day-old protected B/R° mice (lower panel) or of donor NOD splenocytes at the time of transfer (top panel). Tissues were stained as in **b**. Profiles are representative of at least four mice. (**d**) Analyses were done as in **c**, except that B/R° mice were injected at 10 days of age with 1.2×10^5 purified $DX5^+CD4^+$ or $DX5^-CD4^+$ NOD splenocytes. ILNs were stained in 25-day-old mice. Data are representative of 2–12 mice.

hampered protection, as did anti–TGF-β (data not shown). However, splenocytes from IL-$4^{-/-}$IL-$10^{-/-}$ mice had a much reduced protective ability. They were only partially effective when 30×10^5 $CD4^+$ T cells were transferred and had no effect when 6×10^5 cells, a dose that normally gave full protection, were transferred (**Fig. 6c**). Thus, IL-4 and IL-10 seemed to have partially redundant roles in the differentiation or action of protective $CD4^+$ T cells.

BDC2.5 T cell fate in "protected" contexts

One of the major benefits of studying TCR-Tg mice is that they allow examination of the fate of transgene-expressing cells. Therefore we next asked what happened to both the recipient and donor cells when B/R° mice were protected from diabetes by the transfer of limited numbers of $CD4^+$ T cells from young NOD mice. Did the protective cells eliminate autoreactive T cells, allow them to survive but reduce their reactivity, or was autoimmunity controlled at some later point?

Histological analysis showed that transfer of NOD splenocytes did not prevent BDC2.5 cells from invading the islets; rather, they converted insulitis to the "respectful" state typical of BDC2.5/N mice, instead of the aggressive state found in unmanipulated B/R° recipients (**Fig. 7a**). This phenotype remained stable over time (**Fig. 7b**).

Flow cytometric analysis showed that the donor cells did not substantially displace recipient cells, either from the lymphoid organs or the target tissue. Similar numbers of $V_\beta4^+CD4^+$ T cells remained in protected mice ($1.2\pm0.7\times10^6$ $V_\beta4^+CD4^+$ T cells in 25-day-old B/R° mice, n=19; $1.6\pm0.9\times10^6$ $V_\beta4^+CD4^+$ T cells in protected mice, n=18) and coexisted with the donor $V_\beta4^-CD4^+$ population. Thus, it seems that neither clonal deletion of autoreactive cells in the B/R° recipients nor their exclusion from the autoimmune target occurred.

The phenotypes of both the autoaggressive recipient cells and the protective donor cells were analyzed in parallel by flow cytometry, with gating on the $V_\beta4^+$ *versus* the $V_\beta4^-$ $CD4^+$ populations (**Fig. 8a**). The phenotype of the autoreactive BDC2.5 T cells in the B/R° recipient was unaffected by the transfer of protective cells (**Fig. 8b**): cells in the spleen and peripheral lymph nodes remain naïve ($CD69^-CD62L^{hi}$); activation markers were expressed only on cells from the pancreatic lymph nodes

or the pancreatic infiltrate. Thus, the protective cells did not act through prevention of antigen-specific activation of BDC2.5 cells.

In contrast, the phenotype of the transferred $V_\beta4^-CD4^+$ population changed markedly. Donor $CD4^+$ T cells expanded in the 2 weeks after transfer, which resulted in a progressive increase in their numbers. In mice that had been injected with 6×10^5 $CD4^+$ T cells at 25 days of age, ~10×10^5 and 5×10^5 donor $CD4^+$ T cells were found in the spleen and peripheral lymph nodes, respectively; there were equivalent numbers in mice injected with only 1.2×10^5 $CD4^+$ T cells. Donor T cells were enriched in the pancreas (43% of all $CD4^+$ T cells at 25 days) compared to in the peripheral or pancreatic lymph nodes (19 and 16%, respectively). This preferential accumulation in the pancreas might have been related to the migration characteristics of T memory cells; proliferation of the donor $CD4^+$ T cells was indicated by the phenotypic changes that occurred over this 2-week period (**Fig. 8c**). Starting with an essentially naïve phenotype (negative or low CD69, CD25, DX5, CD44 and intracellular CTLA-4; high CD45RB and CD62L) (**Fig. 8c**, top panel), the donor $CD4^+$ T cells acquired characteristics of memory or activated cells (high surface CD25 and intracellular CTLA-4; CD69 also increased, although less so; down-regulated CD62L and CD45RB). An increase in DX5 expression was also seen, especially in lymph nodes that did not drain the pancreas. This activation pattern was dependent on the extent of expansion, as the percentages of activated cells ($CD69^+$ and $CD25^+$) increased with lower doses of injected cells (data not shown). These changes were widespread and were not confined to the pancreas (**Fig. 7c**, B/R^0; similar profiles were obtained with spleen cells, data not shown). This phenotypic alteration was not a reaction to the presence of autoreactive T cells in the recipient: when transferred into non-Tg RAG-$1^{-/-}$ hosts, donor cells underwent the same expansion and phenotypic changes (data not shown). This result suggested that homeostatic proliferation[18,19] or the expansion of cells that recognized a particular self-ligand underlie the observed changes.

We also compared the evolution of the phenotype of purified $DX5^+$ and $DX5^-$ cells when they were injected separately. Both populations reacted in the same way (**Fig. 8d**; note that 1.2×10^5 cells were injected in this experiment, so the increase in CD69 and CD25 was a more marked than in **Fig. 8c**). In particular, DX5 expression increased in both. The lack of stable surface expression of DX5 prevented more extensive analysis of the fate of the $DX5^+$ fraction in mice injected with total $CD4^+$ splenocytes.

Discussion

Despite displaying diabetogenic TCR on many of their T cells, and the activation of these T cells in the pancreatic lymph nodes[1,16], BDC2.5/N mice do not develop diabetes early on in life. Instead they maintain, over long periods of time, a metastable balance between an extensive lymphocyte infiltrate and surviving β cells. We show here that the establishment of this balance requires $CD4^+$ αβ T cells that express receptors other than the transgene-encoded clonotype. This was first indicated by the differences between B/R^o mice, in which no other antigen-specific receptor genes can be rearranged, and conventional BDC2.5/N animals. In B/R^o mice, the islet attack was highly aggressive, never settled into a balanced state and immediately progressed to diabetes. These observations were in agreement with results from a parallel analysis of the same transgenes carried on the NOD-SCID (severe–combined immunodeficient) background[26]. There were several possible explanations for the more aggressive disease in B/R^o mice. It seemed unlikely, however, that the severe diabetes in B/R^o mice was due merely to a higher absolute number of BDC2.5 $clonotype^+$ cells; if this were the case, the introduction of a modest number of $clonotype^-$ NOD splenocytes should not have had such a marked protective effect. Similarly, the naïve phenotype and fairly normal numbers of T cells in B/R^o lymphoid organs ruled out the possibility of an artefact that reflected "space-filling" homeostatic proliferation, as was seen after cell transfer into lymphopenic hosts. Thus, it appears that the BDC2.5/N mouse has lymphocytes with regulatory properties, and this activity could be recapitulated by transfer of splenocytes from NOD mice into B/R^o animals.

In contrast to the accelerated of BDC2.5-promoted diabetes described here, little effect was noted when diabetogenic 4.1 TCR transgenes were introduced onto a RAG-deficient background[6]. This might indicate a lack of, or a different mode of, regulation of cells with this specificity. Alternatively, and more likely given that diabetes already developed rapidly on a RAG-sufficient background, it simply reflects effective allelic exclusion induced by these particular transgenes, which allowed few other specificities to escape. On the other hand, our results parallel analyses showing that encephalomyelitis was more intense in mice that expressed a myelin basic protein–reactive TCR–Tg when crossed onto a RAG-deficient background[27,28]. This effect was prevented by a single injection of $CD4^+$ αβ T cells from a normal donor[27,28], which suggested that similar mechanisms modulate autoimmune destruction in diabetes and experimental autoimmune encephalomyelitis.

It is not clear whether the intermediate phenotype of $B/C\alpha^o$ mice corresponded to incomplete allelic exclusion at the β locus that allowed the appearance of a few nonclonotypic $CD4^+$ αβ T cells, or to a dampening role of γδ T or B lymphocytes, both of which were missing in B/R^o mice. Cells that expressed nonclonotypic TCRβ chains were found in $B/C\alpha^o$ mice (I. Andre-Schmutz, unpublished data). This makes the former a likely explanation, and supports the interpretation from experiments with the encephaletogenic TCR-Tg system[27].

The accelerated diabetes of B/R^o mice was similar to that seen when BDC2.5 transgenes were crossed onto a B6.H-2^{g7} background. Diabetes was precocious, with aggressive and highly inflammatory histology[3]. It is possible that the genes that underlie this genetic difference (which are primarily located on chromosome 7) condition protective T cell activity. It may also be that the dampening effects we have described are related to those observed when protective MHC alleles (primarily $I\text{-}A^b$) were crossed onto the BDC2.5 TCR–Tg background[29]; in this case there was also extensive, albeit slightly delayed, insulitis—which did not lead to β cell destruction. This led to the hypothesis that the non-A^{g7} MHC class II molecule promoted the positive selection and maintenance of regulatory T cells that were expressing non-BDC2.5 TCRs. These cells may be related to those that were absent from B/R^o mice. Finally, the phenotype of B/R^o mice and the time-frame for infusion of protective cells were similar to those observed when diabetes was promoted in BDC2.5/N mice *via* interference with costimulatory molecules[4,7] (and A. Hermann *et al.*, unpublished data). In this case too, blocking antibodies were only effective when administered at or before the onset of insulitis. This implies that signaling through costimulatory receptors may be necessary to promote the differentiation of, or mediate the inhibitory potential of, protective cells.

Several immunoregulatory cells have been described in a variety of experimental systems[10–14]. Several features distinguish them from the cells we observed here. Some of the characteristics are likely linked to the experimental context. Rather than influencing the pathogenicity of diabetogenic cells transferred into an irradiated host, which focuses more on late effector stages, the protective cells were introduced into an unmanipulated animal before autoimmunity had developed.

The first distinguishing feature was that the number of $CD4^+$ T cells required for substantial protection was modest (1×10^5–3×10^5) and marked effects could be observed with small numbers (6,000) of purified $DX5^+$ cells. The second was that the time-window of the transferred cells' efficacy was narrow. In the standard protocol, cells were injected into 10-day-old B/R^o mice. Inoculation could be delayed until day 15, but not much

beyond. This timing coincided with the abrupt, highly synchronous, onset of insulitis in BDC2.5 mice. It appears, then, that the protective cells need to be present at the time of initial attack: they cannot reverse an aggressive insulitis once initiated. The third feature was that the cell population that elicited protection did not seem to be any of those described previously. Purification of $CD25^+$ or $CD45RB^{lo}$ cells did not enrich for protective cells, as was reported for other autoimmune or inflammatory model systems. In particular, our results differed from those that argue for a protective influence of $CD25^+CD45RB^{lo}$ [30,31] or NKT cells[32–34] on the development of diabetes in NOD mice. The key to the difference may be in the disease stage examined. The earlier studies used analysis systems focused on the late effector phases of diabetes in non-Tg animals; in contrast, we focused on disease initiation, when protective cells conditioned the phenotype of autoreactive T cells during lesion establishment. The fourth feature was that complete protection appeared to require input from two distinct subsets of cells. Both were $CD4^+$ because, according to titration experiments, the $CD4^+$ fraction accounted for total protective effect seen with whole splenocytes. Separation according to DX5 expression seemed to partition the $CD4^+$ T cells, and both subsets were required for optimal protection. Apparently, two different cell types collaborate to effectively modulate immune destruction. Rather than invoking the suppressor cell cascades that were modeled in the early 1980s, we propose that the different cells may contribute different, synergistic, dampening signals to the autoaggressive cells, in the form of cytokines or signals through cell surface receptors and their ligands. It is also possible that the $DX5^+$ and $DX5^-$ fractions provide trophic signals to one another.

We do not know whether the protective cells in the $DX5^-$ fraction represented the bulk of the MHC class II–restricted αβ T cell population, nor whether any particular TCR specificity was required. Their partners, $DX5^+$ T cells, are an intriguing population. Not surprisingly, they were greatly reduced in B/R^o mice compared to NOD mice (data not shown). Like NK1.1, DX5 is primarily a marker of conventional NK cells[21]. It is also expressed on some T cells; $DX5^+CD3^+$ cells are often equated with the more classical NKT cells identified by NK1.1 expression[22,23]. That this is not the case can be inferred from the flow cytometry data we have presented as well as published data[35]. It was particularly evident when one splits the $CD4^+$ and $CD4^-CD8^-$ subsets of αβ T cells. Yet $DX5^+$ T cells share with $NK1.1^+$ T cells a slightly lower expression of αβ TCR and also display inhibitory Ly49 family receptors[36]. Hence, if it is defined broadly as containing cells that express one of several of the markers of classical NK cells, they are probably members of the NKT cell family. This further emphasizes the heterogeneity of this group αβ T cells[35,37,38].

It remains to be determined whether the various subsets identified by NK1.1, DX5 or FcγR expression correspond to independent populations, or rather to different stages of maturation or activation within the same lineage. Our preliminary data suggest that there is substantial TCRβ repertoire diversity among $DX5^+$ T cells, but it will be of interest to determine the full breadth of their repertoire and the nature of their restriction element. $CD4^+DX5^+$ cells appear to compensate for the relative NKT cell deficit reported for NOD mice[20,32,39,40]; they are present in higher proportions in NOD than in age-matched B6 mice. They do not express particularly high amounts of CD25 and have a heterogeneous distribution of CD45RB (data not shown); however, they do share with $CD45RB^{lo}CD25^+$ cells high amounts of intracellular CTLA-4[41,42]. It is not clear whether $DX5^+CD4^+$ T cells correspond to a particular lineage (and are thus true "regulatory cells") or to cells deviated by ineffective recognition of a self-ligand, endowing them with an "infectious" capacity to dampen the reactivity of other cells[14].

The high reproducibility of disease progression in BDC2.5 mice, and the ability to trace both recipient (autoreactive) and donor (protective) T cells in transferred B/R^o mice, allowed a unique view of the mechanism by which protective cells exerted their influence in detail. It is unlikely that it involves an anti-idiotypic response against the host transgenic TCR, as host T cells appeared largely unaffected and their numbers unchanged. The protection did not entail a blockade of the initial phase of BDC2.5 priming in the pancreatic lymph nodes. Expression of activation markers was normal, as were the kinetics and extent of migration and retention in the target organ. The behavior of autoreactive cells in the pancreas was the only discernible consequence of the presence of protective cells: instead of the highly invasive and destructive insulitis of B/R^o mice, the protective cells somehow promoted the "respectful" infiltrate typical of BDC2.5/N mice.

It is often thought that regulatory T cells prevent the activation and expansion of self-reactive T cells[43]. This is an extrapolation from *in vitro* observations, in which $CD25^+$ cells strongly dampen the activation of bystander T cells[44,45]. Our data show that this may not always be the case, and that regulatory cells can effect "damage control", permitting self-reactive activation and invasion of the target organ, but preventing its most noxious consequences. Such a possibility has often been overlooked in the interpretation of protection experiments *in vivo*, where the absence of disease is often assumed to imply the absence of autoreactivity. It will be useful to know whether our findings also apply to other protected situations, in particular whether disease prevention by $CD45RB^{lo}$, $CD25^+$ or $NK1.1^+$ cells also operates at this level.

Although the autoreactive recipient T cells showed little change in surface phenotype, the protective donor cells underwent marked alterations and acquired the characteristics of activated cells, including high CTLA-4 expression. These changes were not specifically elicited by the activity of the recipient's autoreactive cells, as they were detected in all lymph nodes and not just in the PLNs where BDC2.5 cells were locally activated; they were also seen upon transfer into non-Tg RAG-$1^{-/-}$ recipients. The protective donor cell population also expanded, from 1.2×10^5 input cells to ~20×10^5 cells in pooled spleen and LNs; this expansion continued to up to 50×10^5 cells by day 60. Proliferation appeared to correspond to some form of homeostatic expansion. Yet it was paradoxical because the B/R^o recipients were not lymphopenic and the bulk of their T cells had a resting phenotype. This expansion may have reflected the filling of a particular niche or the manifestation of a particular reactivity, one that could not be filled or manifested by conventional BDC2.5 αβ T cells. For example, cells that engaged nonclassical MHC or responded to a particular trophic cytokine might have expanded, and their niche might have been inaccessible to the monoclonal A^{g7}-restricted $CD4^+$ T cells of the B/R° mouse.

Given the profound changes that occurred in the protective donor cell population, the relevance of our experimental model might be questioned. Did the protection observed in a day 10–reconstituted B/R^o mouse have a true bearing on physiological immunoregulation, or was it merely an artefact of a massive cellular expansion? (This caveat might also be applied to many experiments purporting to show the existence of "regulatory" T cells[46,47].) One argument that supports relevance is that autoimmune lesions in reconstituted B/R^o mice are similar to the innocuous lesions in standard BDC2.5/N mice. Thus, although the protective cells may overamplify in this transfer context, their features likely reflect those of cells that regulate self-destructiveness in unmanipulated animals.

It will be important to determine the specificity and molecular targets of these protective cells, as for other regulatory pathways. Particular cell surface molecules may mediate T cell–T cell interactions, or protective cells may act by competing for trophic factors required by activated cells (a form of subset-specific homeostatic control). These will be key to understanding the complex phenomena that underlie control of autoimmune diabetes.

Methods

Mice. NOD/Lt (referred to as "NOD" throughout) mice were bred at our animal facility. BDC2.5 TCR–Tg mice, as described[1], were backcrossed onto the NOD background for >16 generations. Mutant mice were crossed onto a NOD/Lt background as follows: C$\alpha^{-/-}$ mice[48] >12 generations; RAG-1$^{-/-}$ mice[15] >9 generations; μMT B cell–deficient mice[49] 11 generations; NOD β_2M$^{-/-}$ mice[50] >12 generations; and IL-4$^{-/-}$ mice[24] 12 generations. IL-10$^{-/-}$ mice[25] were from W. Muller (Institute for Genetics, Köln, Germany) and were backcrossed for eight generations onto a NOD/Lt background. Mice were kept under microisolators in virus-free conditions.

For adoptive transfer experiments, recipient BDC2.5 RAG-1$^{-/-}$ mice were identified, at 7–9 days of age, by flow cytometric analyses on blood lymphocytes. Staining was done with fluorescein isothiocyanate (FITC)–anti-V$_\beta$4 (PharMingen, San Diego, CA), phycoerythrin (PE)–anti-CD4 and tricolor-immunoglobulin M (tricolor-IgM Caltag, Palo Alto, CA) and analyzed on a Profile II cytometer. C$\alpha^{-/-}$[48], RAG-1$^{-/-}$ [15], μMT[49], β_2M$^{-/-}$[50] IL-4$^{-/-}$[24] and IL-10$^{-/-}$[25] were typed as described.

Diabetes was assessed by measurement of blood glucose with Glucofilm strips and a Glucometer 3 reader (Bayer Diagnostics, Heidelberg, Germany). After preliminary experiments, animals were tested twice-weekly for BDC2.5 mice, weekly for BDC2.5 C$\alpha^{-/-}$ mice and every 2 days for BDC2.5 RAG-1$^{-/-}$ mice. Animals were considered diabetic after two sequential measurements of glucose above 300 mg/dl. Diabetes incidence after adoptive transfer was tested by urine measurements of glucose with Uristix (Bayer Diagnostics). Two consecutive measurements above 10 g/l were considered indicative of diabetes, which was then confirmed by a blood test.

Histology. Thin sections from Bouin's-fixed pancreata embedded in paraffin were examined for the presence of insulitis after hematoxylin-eosin staining. Multiple sections were taken from six different levels that were selected as being representative of the whole organ. All the islets in these sections were analyzed and evaluated.

Insulitis scoring. Peri-insulitis refers to the presence of a few inflammatory cells outside or in the immediate vicinity of the islets. Insulitis refers to typical BDC2.5 lesions, with a clear and often extensive islet infiltrate that shows direct lymphocyte–β cell contact, but with clear demarcation of infiltrate and endocrine areas and relatively healthy β cells. Aggressive insulitis refers to extensive infiltrate, where lymphoid cells invade the entire islet, intermingling with endocrine cells, with extensive signs of β cell damage.

Adoptive transfer. Total spleen cells from 4-week-old donor mice were dispersed and injected intraperitoneally into 10-day-old B/R^o recipient mice. Subset selection was done after erythrocyte lysis in hypotonic NH_4Cl. CD4 and CD8 depletion was done by incubation with RL172 and 31M mAbs, respectively[51], followed by incubation at 37 °C with low-tox rabbit complement (Cedarlane, Oakville, CA); this was repeated twice. CD4$^+$ selection was done with anti–CD4 beads (Miltenyi Biotec, München, Germany), following the manufacturer's protocol. CD25, CD45RB and NK1.1 fractionation of MACS-selected CD4$^+$ T cells was done on a Coulter Elite cytometer (Coulter, Hialeah, FL). DX5 selections were done by enrichment of either CD4$^+$ or DX5$^+$ T cells on MACS beads followed by cell sorting for the second marker (Elite, Coultor, Hialeah, FL). Sorted cells were washed and counted before injection. For anti-cytokine treatment experiments, 10-day-old B/R^o mice were transferred with 6.0×10^5 CD4$^+$ NOD spleen cells, then injected with cytokine mAbs (every other day from 14 to 22 days of age, 0.5 mg/dose of protein G–purified mAbs from ascites). The mAbs used were 11B11 (anti–IL-4), JES-05 (anti–IL-10, from M. Goldman, Brussels, Belgium) and 1D11.16.8 (anti–TGF-β).

Flow cytometry. Flow cytometric analyses were done with standard staining protocols. For anti–CTLA-4, intracellular stainings were done without *in vitro* stimulation after permeabilization[52]. Briefly, surface staining with other antibodies was followed by formaldehyde fixation and saponin permeabilization. PE-conjugated anti–CTLA-4 or an isotype-matched control antibody were incubated with cells on the presence of saponin; this was followed by washing and analysis of the cells.

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