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CD25 Is a Marker for CD4$^+$ Thymocytes That Prevent Autoimmune Diabetes in Rats, But Peripheral T Cells with This Function Are Found in Both CD25$^+$ and CD25$^-$ Subpopulations[1]

Leigh Andrea Stephens[2] and Don Mason

Previously we have shown that autoimmune diabetes, induced in rats by a protocol of adult thymectomy and split-dose gamma irradiation, can be prevented by the transfer of a subset of CD4$^+$ T cells with a memory phenotype (CD45RC$^-$), as well as by CD4$^+$CD8$^-$ thymocytes, from syngeneic donors. Further studies now reveal that in the thymus the regulatory cells are observed in the CD25$^+$ subset of CD4$^+$CD8$^-$ cells, whereas transfer of the corresponding CD25$^-$ thymocyte subset leads to acceleration of disease onset in prediabetic recipients. However, in the periphery, not all regulatory T cells were found to be CD25$^+$. In thoracic duct lymph, cells that could prevent diabetes were found in both CD25$^-$ and CD25$^+$ subsets of CD4$^+$CD45RC$^-$ cells. Further, CD25$^-$ regulatory T cells were also present within the CD4$^+$CD45RC$^-$ cell subset from spleen and lymph nodes, but were effective in preventing diabetes only after the removal of CD25$^-$ recent thymic emigrants. Phenotypic analysis of human thymocytes showed the presence of CD25$^+$ cells in the same proportions as in rat thymus. The possible developmental relationship between CD25$^+$ and CD25$^-$ regulatory T cells is discussed. *The Journal of Immunology*, **2000, 165: 3105–3110.**

Insulin-dependent diabetes mellitus (IDDM)[3] results from the inappropriate destruction of the insulin-producing β-cells of the pancreas via a T cell-mediated autoimmune response. Although many self-reactive T cells are deleted during their development in the thymus, some autoreactive cells remain but do not usually lead to autoimmune disease. A variety of immunological mechanisms have been shown to have a role in the maintenance of peripheral self-tolerance, including the ignorance, anergy, and deletion of autoaggressive T cells, as well as active suppression of their activation/expansion by regulatory T cells. The existence of the latter subset of cells has now gained convincing experimental support in a variety of autoimmune disease models (1).

Previous work in this laboratory has demonstrated that a subset of CD4$^+$ T cells exists in normal PVG.RT1^u rats that is capable of preventing the onset of IDDM that otherwise develops in syngeneic recipient rats with experimentally induced lymphopenia (TxX rats) (2). Fractionation of CD4$^+$ T cells according to expression of CD45RC revealed that the regulatory T cells were contained in the CD45RC^{low} fraction, which is the phenotype associated with resting memory cells. The full phenotype of these protective cells was CD4$^+$TCR$\alpha\beta^+$CD45RC^{low}RT6$^+$Thy1$^-$ (2). Despite the fact that according to their surface phenotype the regulatory cells were Ag experienced, the thymus was also found to contain a potent source of regulatory cells among the mature CD4$^+$CD8$^-$ subset (2, 3). Later experiments demonstrated that these intrathymic regulatory cells had differentiated in situ rather than homed back to the thymus from the periphery (4).

More recently, CD25 has emerged as a marker for T cells with regulatory activity in mouse models of autoimmune gastritis. This autoimmune disease, among others, develops in BALB/c mice rendered lymphopenic by thymectomy at 3 days of age (5) or in nude recipients of CD25$^-$ T cells (5, 6). In both models transfer of a minority population of CD4$^+$ T cells expressing CD25 prevented autoimmune disease. The majority of these CD25$^+$ cells were CD45RB^{low} (equivalent to CD45RC^{low} cells in rats), and hence corresponded to a subset of the regulatory cells previously described in this laboratory. CD25 (IL-2Rα) expression is induced on T cell activation to form part of the trimeric, high affinity IL-2R complex in combination with CD122 (IL-2Rβ) and CD132 (IL-2Rγ) and has commonly been used as a marker for activated T cells. However, it was reported that the majority of CD25$^+$ T cells in mice lacked coexpression of IL-2Rβ ex vivo and were anergic in vitro, as shown by nonproliferation to antigenic stimulation except in the presence of high levels of exogenous IL-2 (7–9). Thus, unlike activated cells, these regulatory cells may be expressing homodimers of CD25, which have been reported to bind IL-2 with low affinity and lack signaling capacity (10).

The observation in the mouse that the T cells that prevent organ-specific autoimmunity are CD25$^+$ appeared to be contrary to earlier work from this laboratory, which established that at least some regulatory cells in the rat are CD25$^-$ (2). To further explore this apparent discrepancy in the data from the two species, work has been conducted on the phenotype of regulatory T cells in rats. The findings form the subject of this report.

Consistent with the published studies of T cells that prevent autoimmune gastritis in mice, we have now shown that CD25 is a phenotypic marker for CD4$^+$ T cells in the thymus and peripheral lymphoid tissues of normal rats that prevent autoimmune diabetes. However, we have also shown that, compatible with earlier data in

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[3] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; TDL, thoracic duct lymph; TxX, thymectomized and irradiated rats; RTE, recent thymic emigrants; DAM, donkey anti-mouse IgG.

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the rat, there is a population of peripheral $CD4^+CD45RC^-CD25^-$ T cells that can prevent diabetes. The protective activity of these cells from spleen and lymph nodes is revealed only after the removal of recent thymic emigrants (RTE) from this subset, suggesting the presence of diabetogenic cells in $CD25^-$ RTE that are insufficiently controlled by $CD25^-$ regulatory cells alone. Given that CD25 is expressed on both activated T cells as well as some regulatory T cells, and that, as we show here, not all regulatory T cells express CD25, it is evident that currently there is no marker that uniquely defines T cells that prevent organ-specific autoimmunity. Nevertheless, CD25 appears to be a useful marker for cells with regulatory activity in the thymus.

Materials and Methods

Antibodies

The following mouse IgG1 Abs were tissue culture supernatants produced from hybridomas in the Medical Research Council Cellular Immunology Unit (Oxford, U.K.) by Mike Puklavec: OX8 (anti-rat CD8α) (11), OX22 (anti-rat CD45RC) (12, 13), OX32 (anti-rat CD45RC, noncompetitive with OX22) (13, 14), OX39 (anti-rat CD25) (15), NDS62 (anti-rat CD25, noncompetitive with OX39) (16), W3/25 (anti-rat CD4) (17), OX21 (isotype control mouse IgG1, reactive with human factor I) (18), OKT4 (anti-human CD4) (19), and OKT8 (anti-human CD8) (20). Some of these Abs were purified and biotinylated using standard methods.

Abs and fluorescent reagents obtained from commercial sources include W3/25-FITC, anti-human CD25-FITC, anti-human CD8-PECy5, and conjugated isotype control mAbs (all obtained from Serotec, Oxford, U.K.). Donkey anti-mouse IgG (DAM)-PE (Chemicon, Temecula, CA) was used to detect staining of the unconjugated mouse mAbs, and streptavidin-QR (Sigma, St. Louis, MO) was used to detect staining of biotinylated Abs.

Flow cytometry

For flow cytometric analysis, typically 10^6 cells were stained with 50 μl of the appropriately diluted Ab for 30 min on ice, washed twice with PBS/0.2% BSA, and analyzed on the FACScan (Becton Dickinson, Mountain View, CA). For triple-color analysis, cells were incubated first with the unconjugated mAb and washed, followed by detection with DAM-PE. After 20-min incubation with 5% normal mouse serum in PBS, the cells were coincubated with the FITC and biotin-conjugated Abs, washed, and incubated for an additional 30 min with streptavidin-QR.

Induction and monitoring of IDDM in PVG.RT1^u rats

PVG.RT1^u rats were bred and housed in specific pathogen-free conditions at the Sir William Dunn School of Pathology (Oxford, U.K.). IDDM was induced in female PVG.RT1^u rats by a protocol involving thymectomy at 6 wk of age, followed by four doses of 250 rad gamma irradiation at 2-wk intervals, beginning 2 wk after thymectomy. This protocol was described in more detail previously (2, 21). Cells to be tested for regulatory activity were injected i.v. immediately following the last irradiation. Animals were subsequently weighed twice weekly for 12–15 wk, and those exhibiting weight loss were tested for the development of diabetes, as assessed by measurement of blood glucose levels using Glucostix (Bayer Diagnostics, Newburg, OK), and confirmed by the presence of glycosuria with Clinistix (Bayer Diagnostics). Animals were considered diabetic if their blood glucose levels were >16 mM and they also tested positive for glycosuria.

Purification of peripheral T cell subsets

Donor cells were obtained from pooled single-cell suspensions of cervical and mesenteric lymph nodes and spleen (after lysis of RBCs) or from the thoracic duct lymph (TDL) of 8- to 16-wk-old female PVG.RT1^u rats. Cells from TDL were obtained by cannulation of the thoracic duct (22) and were collected at 4°C overnight into PBS containing heparin (2 U/ml final concentration). Enrichment for $CD4^+CD45RC^-$ cells involved incubation for 50 min on ice with the mouse mAbs OX8, OX22, and OX32, followed by washing and two or three consecutive rounds of depletion with goat anti-mouse IgG-coated magnetic beads (Dynal, Oslo, Norway). At this stage, the efficiency of the depletion was tested by labeling cells pre- and postdepletion with DAM-PE for 20 min for flow cytometric analysis. The percentage of contaminating cells was consistently <5%. Fractionation of the enriched $CD4^+CD45RC^-$ cells according to expression of CD25 was then performed by incubation of cells for 50 min on ice with OX39 and NDS62 (noncompeting Abs to CD25), followed by two washes, incubation with anti-mouse IgG-coated magnetic beads (Miltenyi Biotec, Auburn, CA), a further wash, and separation on a LS^+ column (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the fractionated cells was again analyzed on the FACScan by labeling pre- and postdepletion samples with DAM-PE. The percentage of $CD4^+$ cells in the fractionated cells was also determined by analysis on the FACScan after incubation of cells for 20 min with W3/25-FITC. It may be noted that although in principle the W3/25-FITC could be bound by the anti-mouse IgG-coated MACS beads on the surface of the positively selected cells, control experiments indicated that this potential problem did not detectably alter the pattern of staining (data not shown).

Purification of thymocyte subsets

Enrichment of $CD4^+CD8^-$ thymocytes was performed by negative selection of CD8-expressing thymocytes from 5- to 7-wk-old female PVG.RT1^u rats, by rosette depletion with OX8-coated SRBCs (23). The resulting population of cells typically contained 80% single-positive $CD4^+CD8^-$ thymocytes, 20% $CD4^-CD8^-$ thymocytes, and <2% contaminating $CD8^+$ cells. CD8-depleted rat thymocytes were further fractionated on the basis of CD25 expression as described above for peripheral $CD4^+$ T cells. Human thymocyte tissue, necessarily removed during cardiac surgery, was obtained from Dr. Katja Simon, Institute of Molecular Medicine (Oxford, U.K.). $CD8^+$ cells were depleted from human thymocytes using OKT8-coated SRBCs.

Results

CD25 is expressed on a peripheral CD4$^+$ T cell subset that can prevent IDDM in rats

In these experiments $CD4^+CD45RC^-$ T cells, which were shown previously to prevent diabetes in TxX PVG.RT1^u rats, were further subdivided on the basis of CD25 expression. The proportion of $CD4^+$ cells coexpressing CD25 in young adult rats varies slightly between peripheral tissues, with consistently lower frequencies found in TDL (Fig. 1*A*). Cells expressing CD25 were predominantly $CD45RC^{low/int}$, in contrast with $CD25^-$ cells, which were ~70% $CD45RC^{high}$ (Fig. 1*B*). In initial experiments $CD4^+CD45RC^-$ cells, enriched from the spleen and lymph nodes of normal PVG.RT1^u rats and fractionated according to expression of CD25 (Fig. 2*A*), were tested for their ability to protect TxX rats from diabetes. Depletion of $CD25^+$ cells from the previously defined $CD4^+CD45RC^-$ regulatory T cell population resulted in a loss of the ability of the remaining cells to protect TxX recipients from diabetes (Fig. 2*B*). Conversely, positive enrichment of $CD25^+$ cells from $CD4^+CD45RC^-$ cells resulted in a population of cells able to protect against diabetes at a reduced cell dose. Thus diabetes-preventing cells were present in the $CD25^+$ subset of $CD4^+CD45RC^-$ T cells, but the $CD25^-$ fraction did not protect. However, subsequent experiments revealed that this conclusion required important qualification (below).

CD25 expression on thoracic duct lymphocytes does not functionally divide regulatory T cells

The observation that $CD4^+CD45RC^-CD25^-$ T cells from spleen and lymph nodes did not prevent IDDM in prediabetic recipients (Fig. 2*B*) contrasted with previous findings in this laboratory, in which cells with this phenotype, when isolated from TDL, did prevent IDDM (2). To reconfirm the previous result, we repeated the in vivo functional experiments using TDL as a source of donor T cells and fractionation of cells according to CD25 expression exactly as described for spleen and lymph node cells. In concordance with the published data, but at variance with the results obtained using donor cells from spleen and lymph node, $CD4^+CD45RC^-$ cells from TDL protected TxX rats from diabetes even after depletion of $CD25^+$ cells, indicating that there are regulatory cells in TDL that are $CD25^-$ (Fig. 3). However, $CD4^+$ T cells enriched for CD25 expression also prevented the development of diabetes (Fig. 3) at a cell dose lower than that needed for protection by unfractionated $CD4^+CD45RC^-$ cells (3). It is notable, in the light of these results, that CD25 is expressed on fewer

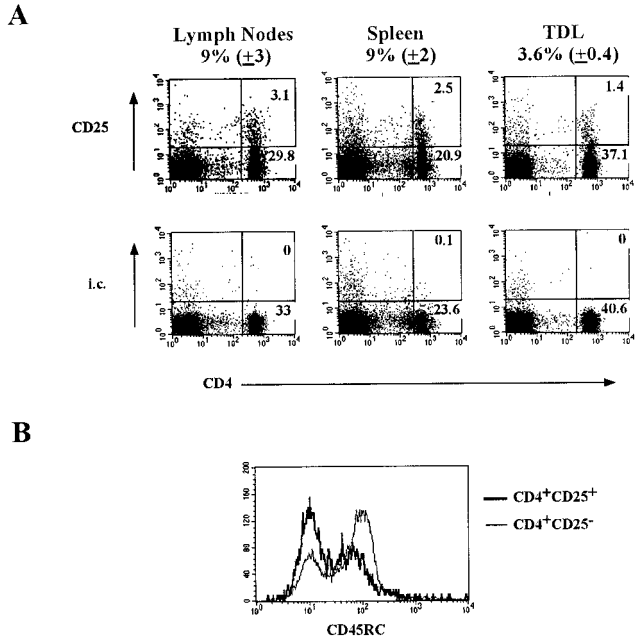

FIGURE 1. Expression of CD25 on peripheral $CD4^+$ T cells in adult rats. A, Cells recovered from different lymphoid organs of 8- to 12-wk-old rats were double stained with Abs to CD4 (biotinylated W3/25 and streptavidin-QR) and CD25 (OX39 and DAM-PE) or an isotype control Ab (OX21 and DAM-PE) and were analyzed on the flow cytometer. The plots shown are representative, and the numbers at the top of the profiles refer to the mean percentage of $CD4^+$ cells expressing CD25 ± SD ($n \geq 4$). No differences were noted between male and female rats. *B*, Expression of CD45RC on $CD4^+CD25^+$ cells. Shown is the profile of CD45RC expression on lymph node cells after gating on $CD4^+CD25^+$ cells (bold line) or $CD4^+CD25^-$ cells (normal line). This profile is also representative of cell surface analysis of cells from spleen and TDL.

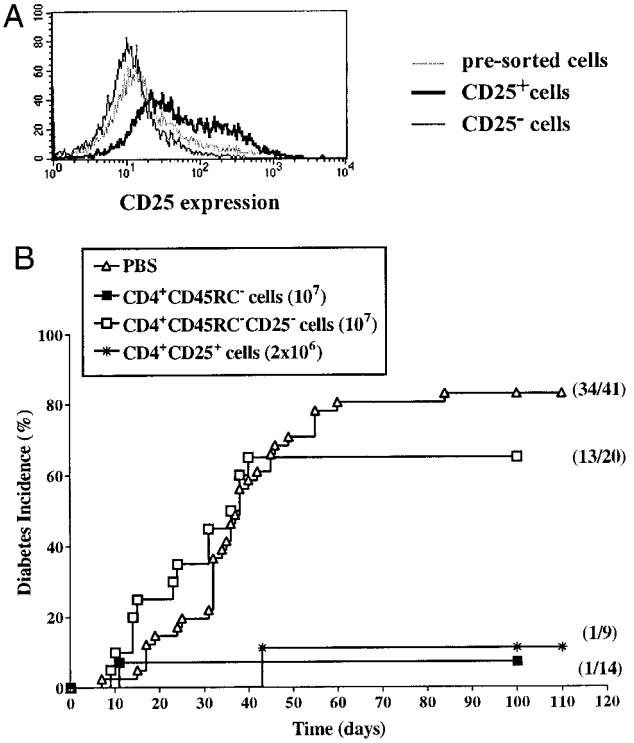

FIGURE 2. CD25 is a marker for regulatory $CD4^+$ T cells, present in the spleen and lymph nodes of normal rats, that can transfer protection from diabetes. *A*, $CD4^+CD45RC^-$ cells were enriched by negative selection with the Abs OX8, OX22, and OX32 and magnetic beads (Dynal; purity of depletion, >98%). Cells were then fractionated on the basis of CD25 expression by staining with the Abs OX39 and NDS62 and separation with magnetic beads (Miltenyi Biotec). Shown is a typical CD25 profile of cells after sorting. *B*, Female PVG. $RT1^u$ rats were taken through the TxX protocol, and on the day of the final irradiation, recipient rats were injected with the indicated cells from normal syngeneic donors or with PBS alone. The lines represent the cumulative incidence of diabetes, and the numbers in parentheses indicate the final number of diabetic rats per total number of rats. The results are pooled from six experiments.

$CD4^+$ T cells from TDL compared with spleen and lymph nodes (Fig. 1*A*).

Mature $CD4^+CD8^-$ thymocytes that prevent IDDM coexpress CD25

It was shown previously in this laboratory that mature $CD4^+CD8^-$ thymocytes are a potent source of regulatory cells capable of transferring protection against diabetes to TxX rats (3, 24); however, it had not been determined whether the regulatory cells were a phenotypically distinct subset in the thymus or were derived from uncommitted precursors that had been educated in the periphery to become regulatory. Because CD25 expression had been found to be a feature of many of the regulatory cells in the periphery, especially in spleen and lymph nodes, experiments were conducted to determine whether expression of CD25 on $CD4^+$ thymocytes could similarly be used as a marker to distinguish thymocytes capable of preventing autoimmune disease from those mediating other functions.

CD25 expression was examined on thymocytes from 6-wk-old rats and was found on ~6% of $CD4^+CD8^-$ thymocytes (±1.5; $n = 4$) and ~8% of recent thymic emigrants. Fig. 4 shows this result for thymocytes together with comparable data on human thymus, in which the CD25 expression profile is very similar (9 ± 1.7%; $n = 3$). In the standard adoptive transfer assay, only thymocytes expressing CD25 were able to protect against the development of diabetes in TxX rats (Fig. 5). In contrast, the transfer of CD25-depleted $CD8^-$ thymocytes actually led to acceleration of disease onset ($p < 0.02$, by Mann-Whitney two-tailed test), in confirmation of our earlier determination that some mature $CD4^+$ thymocytes were diabetogenic (3). The $CD25^+$ cells responsible for protection were $CD4^+CD8^-$, because although a minority of $CD4^-CD8^-$ cells were also present in the $CD25^+$ protective inoculum, <1% of these coexpressed the TCR, and depletion of $CD4^+$ cells from the $CD8^-CD25^+$ thymocytes resulted in a population of cells that no longer protected against diabetes (data not shown). Thus, the $CD4^+CD8^-$ thymocytes capable of preventing autoimmune disease were already phenotypically and functionally distinct before leaving the thymus. The significance of this result with regard to the lineage relationship between $CD25^+$ and $CD25^-$ peripheral regulatory cells will be discussed below.

The removal of RTE reveals the presence of a subset of memory $CD25^-$ cells from lymph nodes and spleen that can prevent diabetes in TxX rats

The presence of autoreactive cells in the peripheral T cell repertoire of rats, which is revealed by the TxX protocol, raised the question of whether these cells played any useful part in protective immune responses to pathogens or whether their presence simply reflected inefficient clonal deletion in the thymus. In an attempt to examine this question experiments were conducted to determine whether autoreactive T cells could be found in the memory T cell pool. A positive result would be presumptive evidence that they played a useful role.

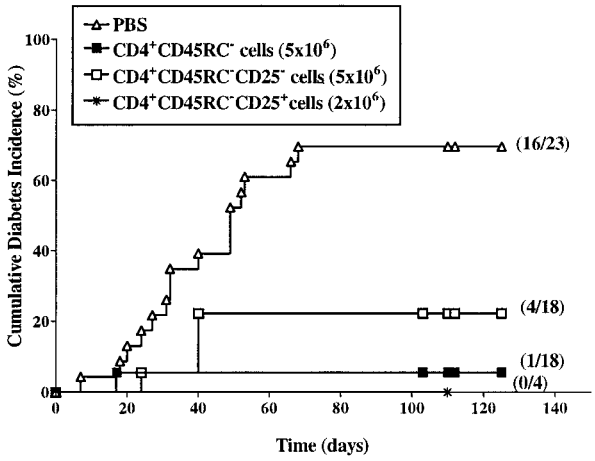

FIGURE 3. Regulatory T cells from TDL are found in both $CD25^+$ and $CD25^-$ T cell subsets of $CD4^+CD45RC^-$ cells. Female PVG. $RT1^u$ rats were taken through the TxX protocol, and on the day of the final irradiation, recipient rats were injected with the indicated dose of cells purified from thoracic duct lymph. The lines represent the cumulative incidence of diabetes, and the number in parentheses indicate the final number of diabetic rats per total number of rats. The results are pooled from four experiments.

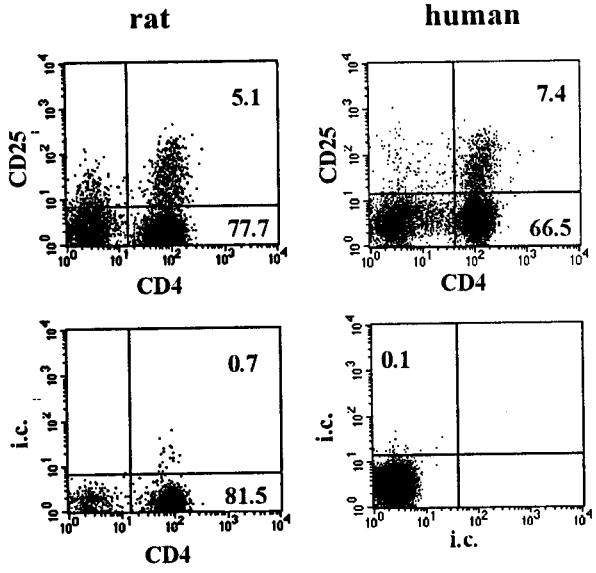

FIGURE 4. Expression of CD25 on $CD4^+$ thymocytes in rats and humans. Single-cell thymocyte suspensions were depleted of CD8-expressing cells by a rosetting technique using anti-CD8-coated SRBCs and then were double stained for CD4 and CD25 for analysis on the flow cytometer. Shown are representative profiles of at least three individuals.

The $CD45RC^-$ subset of $CD4^+$ T cells in rats contains both memory cells and RTE, and early experiments had provided an indication that the $CD25^-$ subset of these cells led to an acceleration of diabetes onset. Because RTE would be expected, like their intrathymic precursors, to contain autoreactive T cells, $CD4^+CD45RC^-CD25^-$ T cells were isolated from rats that had been thymectomized 4 wk previously. As RTE mature to become $CD45RC^+$ within 3–7 days of leaving the thymus (25), the $CD45RC^-$ subset of $CD4^+$ T cells from donors thymectomized 4 wk previously contains only memory T cells. It was anticipated that if the memory T cell pool contained autoreactive T cells, the injection of $CD4^+CD45RC^-CD25^-$ T cells from the spleen and lymph nodes of thymectomized donors into prediabetic recipients would accelerate the onset of diabetes, as $CD4^+CD25^-$ thymocytes had been found to do. Contrary to expectation, the transfer of these $CD25^-$ memory cells from thymectomized rats prevented diabetes (Fig. 6). This protection could be attributed to the elimination of RTE from the $CD25^-CD45RC^-$ subset of thymectomized donors, because the same subset purified from the spleen and lymph nodes of sham-thymectomized control donors was not protective. In a separate experiment to support these data, $CD45RC^-CD25^-$ cells from normal donors were fractionated into memory cells and RTE by sorting cells according to expression of Thy1, which in rats is expressed on RTE and is lost as the cells mature (25). $CD25^-Thy1^-CD45RC^-$ (memory) cells (10^7) from normal donors prevented diabetes (one in four rats developed diabetes compared with all seven recipients given no cells; $p < 0.01$, by one-tailed log-rank test). The regulatory $CD45RC^-CD25^-$ cells from thymectomized donors were $CD4^+$, because protection from diabetes was lost upon removal of $CD4^+$ cells from this subset (data not shown). Taking these data together, it follows that in peripheral lymphoid organs the expression of CD25 on $CD4^+$ memory cells does not identify all cells with regulatory activity. These data also provide evidence that diabetogenic cells can be found within the $CD4^+CD25^-$ RTE population.

Discussion

The current data show that the transfer of $CD4^+$ T cells that express CD25 (IL-2Rα) from syngeneic donors can prevent the development of diabetes in TxX PVG.$RT1^u$ recipients. This is true for $CD4^+$ T cells isolated from both the thymus and the peripheral lymphoid tissue of rats and is compatible with an increasing body of data on the phenotype of T cells that prevent autoimmunity in other disease models in mice (5, 6, 26, 27). Although no functional data for the human thymus are yet available, the very similar frequency of $CD25^+$ cells in the $CD4^+CD8^-$ thymocyte subset of human and rat (Fig. 4) as well as mouse (27) suggests that the control of autoimmunity in the three species depends on identical mechanisms.

In the thymus there was no evidence of any protective cells among the $CD25^-CD4^+$ subset. On the contrary, transfer of this population led to disease acceleration in prediabetic recipients, supporting the earlier inference that diabetogenic cells are present in the $CD4^+$ thymocyte subset (3). With peripheral $CD4^+$ cells the situation is more complex. In confirmation of earlier experiments (2), $CD25^-CD45RC^-CD4^+$ donor cells from TDL could protect TxX recipients from diabetes (Fig. 3). However, as the present experiments show, the transfer of similar cells from lymph node and spleen did not protect, even at a high cell dose (Fig. 2*B*). This latter result is in agreement with published data in the mouse (5), but appeared paradoxical, in that TDL is composed of cells that migrate into the lymph from lymph nodes. The presence of protective $CD25^-$ T cells in lymph node and spleen was revealed by the removal of RTE from the transferred $CD25^-CD45RC^-$ subpopulation either by prior thymectomy of donor animals or by the removal of $Thy1^+$ cells from the donor inoculum (Fig. 6). These data indicated that TDL differed from lymph node and spleen only in the relative ratios of $CD25^-$ regulatory and diabetogenic cells. The reason for this difference is not known, but it may reflect different recirculation kinetics for autoreactive T cells and regulatory ones. Alternatively, as discussed below, the level of expression of CD25 on regulatory T cells may be down-regulated as the cells migrate from lymph nodes into lymph.

The requirement to remove RTE to demonstrate regulatory T cell activity among $CD4^+CD45RC^-CD25^-$ lymph node and spleen cells (which contain a mixture of RTE and memory T cells)

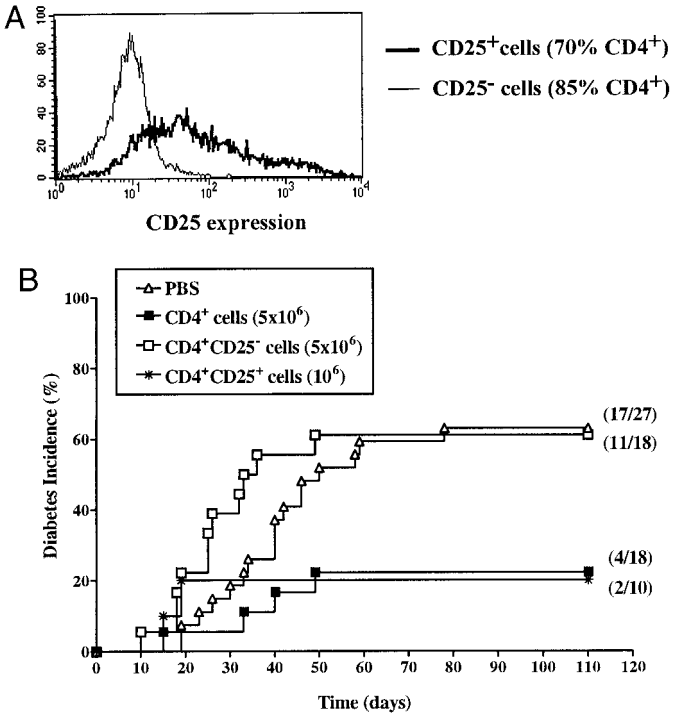

FIGURE 5. CD25 is a marker for regulatory $CD4^+CD8^-$ thymocytes. *A*, Representative profile of CD25 expression of cells after fractionation. $CD4^-CD8^+$ and $CD4^+CD8^+$ T cells were depleted from thymocytes by a rosetting technique using OX8-coated SRBCs. Separation of the remaining $CD4^+CD8^-$ thymocytes on the basis of CD25 expression was performed using Abs OX39 and NDS62 and anti-mouse IgG-coated MACs beads. Shown is a representative profile of CD25 expression of the thymocytes after fractionation. *B*, Prediabetic female TxX PVG.RT1^u rats were reconstituted with the indicated dose of thymocytes from 5- to 7-wk-old normal syngeneic donors or with PBS alone. The number of cells refers to the number of $CD4^+$ cells injected, as determined by flow cytometric analysis of the sorted subsets after staining with W3/25-FITC. The lines represent the cumulative incidence of diabetes, and the numbers in parentheses indicate the final number of diabetic rats per total number of rats. The results are pooled from five experiments.

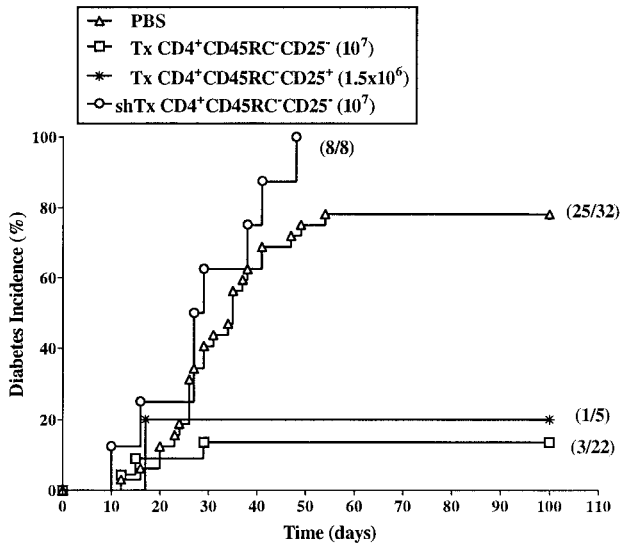

FIGURE 6. Removal of RTE from $CD45RC^-$ spleen and lymph node cells reveals a regulatory $CD25^-$ T cell population. Prediabetic male and female TxX PVG.RT1^u rats were reconstituted with the indicated dose of peripheral T cells from 10-wk-old syngeneic donors that had undergone thymectomy or sham thymectomy 4 wk previously. The lines represent the cumulative incidence of diabetes, and the numbers in parentheses indicate the final number of diabetic rats per total number of rats. The results are pooled from four experiments.

indicates that the balance between autoimmunity and regulation is different in these two T cell populations. This conclusion raises the question of the origin of the $CD25^-$ regulatory cells in the memory T cell pool. It follows that any difference in potency among mature peripheral cells with regard to the prevention of autoimmunity can be properly evaluated only when both $CD25^+$ and $CD25^-$ regulatory T cells are obtained from peripheral $CD4^+$ T cells depleted of RTE. Nonetheless, the data support the conclusion that the thymus does not export $CD25^-$ cells precommitted to a regulatory role and that these cells either acquire their regulatory function in the periphery or derive from $CD25^+$ precursors. If the latter interpretation is the correct one, then CD25 expression is not a stable marker for regulatory T cells in the periphery. While our data do not rule out this conclusion, there are a number of examples in other studies in which regulatory T cells were generated in the periphery by a process of recruitment or infectious tolerance (28). In the mouse, peripheral T cells that prevent skin allograft rejection can be induced by the in vivo coculture of their precursors with established $CD4^+$ regulatory cells (28, 29). While it remains to be determined whether $CD25^-$ T cells that prevent diabetes in TxX rats, are recruited from uncommitted precursors, it is probable that the recruitment mechanism, demonstrated in skin allograft tolerance, is an illustration of a process that normally plays a physiological role in the maintenance of self-tolerance.

These allograft tolerance experiments suggest that regulatory T cells generated by infectious tolerance have specificity for the target alloantigens. In this context we have shown that the regulatory $CD4^+$ T cells that prevent autoimmune thyroiditis, while present in the thymus of athyroid rats, are absent in the periphery (4). This result strongly suggests that the readily demonstrable regulatory T cells that prevent thyroiditis in these experiments are specific for thyroid autoantigens. Preliminary data indicate that, as expected, this population is $CD25^+$ (L. Stephens, unpublished observations). No data are yet available regarding the Ag specificity of the $CD25^-$ regulatory cells.

Our experiments gave no evidence for the presence of autoreactive T cells among $CD4^+CD45RC^-$ memory cells. Previous studies from this laboratory have revealed that naive peripheral $CD4^+CD45RC^+$ T cells can cause a range of autoimmune diseases (30), and the current experiments demonstrate similarly autoreactive cells among $CD4^+$ thymocytes and RTE. These data together with earlier results on diabetes in TxX rats (2) indicate that the autoimmune diseases that develop in these animals represent at least in part the activation of naive cells. The presence of regulatory T cells in the $CD25^-$ memory pool of $CD4^+$ cells prevented determination of the coexistence of any autoreactive T cells in this subset. However, if the $CD25^-$ regulatory subset is recruited from uncommitted precursors, as the $CD4^+$ cells that prevent skin allograft rejection in the mouse appear to be (29), then the fate of at least some of the naive autoreactive T cells in normal animals may be to become, after activation, T cells that prevent disease rather than induce it. Currently, the developmental lineage of $CD25^-$ regulatory T cells is being studied.

In summary, these studies show that CD25 is a useful marker for regulatory T cells present in the thymus, which are likely to be the precursors of at least some of the $CD25^+$ peripheral regulatory cells. However, our results also demonstrate that CD25 expression in the periphery is not a characteristic of all cells that can control organ-specific autoimmunity. It remains to be determined whether

$CD25^-$ regulatory cells are induced in the periphery rather than being precommitted to a regulatory role in the thymus. There are possible therapeutic implications in the answer to this question.

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