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# The Subpopulation of $CD4^+CD25^+$ Splenocytes That Delays Adoptive Transfer of Diabetes Expresses L-Selectin and High Levels of CCR7[1]

**Veronika Szanya, Joerg Ermann, Cariel Taylor, Claire Holness, and C. Garrison Fathman[2]**

**Recently, $CD4^+CD25^+$ T cells have been implicated in the control of diabetes, suggesting that the inflamed islets of Langerhans in prediabetic NOD mice are under peripheral immune surveillance. Here we show that $CD4^+CD25^+$ splenocytes inhibit diabetes in cotransfer with islet-infiltrating cells. Furthermore, CD62L expression is necessary for this disease-delaying effect of $CD4^+CD25^+$ cells in vivo, but not for their suppressor function in vitro. We demonstrate that the $CD4^+CD25^+CD62L^+$ splenocytes express CCR7 at high levels and migrate toward secondary lymphoid tissue chemokine and ELC (macrophage-inflammatory protein-3$\beta$), lymphoid chemokines, whereas $CD4^+CD25^+CD62L^-$ splenocytes preferentially express CCR2, CCR4, and CXCR3 and migrate toward the corresponding inflammatory chemokines. These data demonstrate that $CD4^+CD25^+CD62L^+$, but not $CD4^+CD25^+CD62L^-$, splenocytes delay diabetes transfer, and that $CD4^+CD25^+$ suppressor T cells are comprised of at least two subpopulations that behave differently in cotransfer in vivo and express distinct chemokine receptor and chemotactic response profiles despite demonstrating equivalent suppressor functions in vitro.** ***The Journal of Immunology*****, 2002, 169: 2461–2465.**

The NOD mouse represents an animal model of human type I diabetes (1). Inflammation of the pancreatic islets of Langerhans in NOD mice starts before 3 wk of age with infiltration initially by dendritic cells (DC)[3] and macrophages, which is soon followed by the recruitment of lymphocytes. Despite extensive infiltration of the pancreatic islets, called insulitis, the disease remains clinically silent for many weeks (2). In our colony, NOD mice only start to become hyperglycemic beyond 15 wk of age. The considerable lag between the beginning of insulitis and the onset of overt diabetes suggests that regulatory mechanisms that delay disease progression are present in prediabetic NOD mice. However, diabetes can be rapidly transferred into immune-incompetent NOD recipients with islet-infiltrating cells (IIC) isolated from inflamed pancreata of prediabetic NOD mice (3). Both $CD4^+$ and $CD8^+$ T cells are necessary for efficient transfer of disease (4). Splenocytes from prediabetic NOD mice transfer diabetes less rapidly than IIC and, in cotransfer with IIC, delay diabetes transfer (3, 4). These data suggest that peripheral (as opposed to intraislet) regulation is critical for maintaining NOD insulitis in a nondestructive state. Several lines of evidence suggest that $CD4^+$ splenocytes contain the regulatory function in prediabetic NOD mice (5). Both of the cell surface markers, CD45RB and CD62L, have been successfully used to define these regulatory $CD4^+$ T cells as subpopulations expressing $CD4^+CD45RB^{low}$ or $CD4^+CD62L^+$ (6–8). More recently, it was demonstrated that CD25-depleted splenocytes from prediabetic NOD mice more rapidly transferred diabetes into immune-incompetent NOD mice than did total splenocytes, suggesting that $CD4^+CD25^+$ T cells play a role in immune regulation in the NOD mouse (9). Based on these studies, some recent reviews have defined the regulatory T cell population in prediabetic NOD mice as $CD4^+CD25^+CD62L^+$ (5, 10). However, experimental evidence for this functional phenotype has been lacking to date.

$CD4^+CD25^+$ T cells have been shown to be potent regulatory cells in a number of mouse models as well as in rats and humans (9, 11–15). The mechanism of action by which $CD4^+CD25^+$ T cells prevent the development of autoimmune disease is not completely understood. $CD4^+CD25^+$ T cells, activated in vitro, suppress the proliferation of responder $CD4^+CD25^-$ T cells in a cell contact-dependent manner (16). Their effect in vivo appears (in some, but not all, systems) to depend on IL-10 and/or TGF-$\beta$ expression (17). Very little is known about the trafficking behavior of $CD4^+CD25^+$ T cells or the site where they exert their regulatory activity in vivo. $CD4^+CD25^+$ T cells can be easily found in secondary lymphoid organs. $CD4^+CD25^+$ splenocytes express high levels of CCR5 and are attracted by activated APCs that express macrophage inflammatory protein-1$\beta$ (ligand for CCR5). This may be essential for maintaining normal humoral immune responses in the secondary lymphoid tissues (18). At the same time, $CD4^+CD25^+$ T cells from human peripheral blood selectively express CCR8 and CCR4 and show a strong chemotactic response to ligands for CCR4, monocyte-derived chemokine (MDC), and thymus- and activation-regulated chemokine (TARC). These chemokines are produced at high levels by activated APC and attract activated T cells, suggesting that $CD4^+CD25^+$ T cells may be attracted to inflamed tissues to regulate or prevent autoimmune disease (19).

In this manuscript we demonstrate that $CD4^+CD25^+CD62L^+$, and not $CD4^+CD25^+CD62L^-$, splenocytes inhibit diabetes transfer into immune-compromised NOD mice, whereas both subsets are equally effective in suppression assays in vitro. We address whether differential trafficking of these two subsets may explain these seemingly contradictory results.

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[3] Abbreviations used in this paper: DC, dendritic cell; HEV, high endothelial venule; IIC, islet-infiltrating cell; MDC, monocyte-derived chemokine; SLC, secondary lymphoid tissue chemokine; TARC, thymus- and activation-regulated chemokine.



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## Materials and Methods

### *Mice*

Female NOD mice were obtained from Taconic Farms (Germantown, NY). Female NOD.SCID mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were used as recipients between 4–6 wk of age. All animals were maintained under specific pathogen-free conditions at the Department of Comparative Medicine, Stanford University School of Medicine (Stanford, CA).

### *Antibodies*

Purified anti-CD3 (145-2C11) and anti-CD28 (37.51), FITC-labeled anti-CD4 (GK1.5), anti-CD62L (MEL-14), PE-labeled anti-CD25 (PC61), biotinylated anti-CD25 (7D4), and CyChrome-labeled anti-CD4 (RM4-5) were purchased from BD PharMingen (San Diego, CA). PE-conjugated streptavidin was purchased from Caltag Laboratories (Burlingame, CA).

### *Lymphocyte preparation*

Single cells containing IIC were prepared from the pancreata of 11-wk-old NOD mice using a procedure previously described (20). $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were prepared from single-cell suspensions by CD4 enrichment using anti-CD4 magnetic Microbeads (Miltenyi Biotec, Auburn, CA), followed by FACS sorting. $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ cells were prepared as single-cell suspensions from splenocytes stained with PE-labeled anti-CD25, enriched with anti-PE magnetic Microbeads (Miltenyi Biotec), and then sorted by FACS as $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ cells. The purity of these sorted cell populations was routinely >95%.

### *FACS analysis and FACS sorting*

Cell preparation was performed in PBS (Life Technologies, Gaithersburg, MD) plus 5% heat-inactivated FBS (HyClone, Logan, UT). Cells were sorted aseptically on a FACStar cell sorter (BD Biosciences, Mountain View, CA) in the Shared FACS Facility, Center for Molecular and Genetic Medicine, Stanford University. The data were analyzed using the Herzenberg desk facility (Stanford University), FloJo 2.7.8 (TreeStar, San Carlos, CA).

### *Cell transfer procedure*

Cells were resuspended in PBS and were injected i.p. at the numbers indicated. NOD.SCID recipients were checked for glucosurea using Glucostix (Roche, Indianapolis, IN) twice a week. If glucosurea was observed, blood glucose was measured with a One Touch Basic glucometer (Johnson & Johnson, Milpitas, CA). Mice with glucosurea and blood glucose 250 mg/dl were considered diabetic. Glucosurea always coincided with high blood glucose levels.

### *In vitro proliferation assays*

RPMI-C, i.e., RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 1% nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin plus 100 $\mu$g/ml streptomycin, 2 mM L-glutamine (all obtained from Life Technologies), and 50 $\mu$M 2-ME (Sigma, St. Louis, MO), was used for in vitro cultures. Cells were incubated with equal number of anti-CD3- and anti-CD28-coated beads in RPMI-C in 96-well U-bottom plates (BD Biosciences). Beads were coated as described previously (21) using 2.5 $\mu$g/ml anti-CD3 and 2.5 $\mu$g/ml anti-CD28 mAbs. Cells were pulsed with 1 $\mu$Ci [$^3$H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ)/well for the last 15 h of the 72-h culture. Cells were then harvested onto filter membranes using a Wallac harvester (PerkinElmer, Gaithersburg, MD), and the amount of incorporated [$^3$H]thymidine was measured with a Wallac Betaplate counter (PerkinElmer).

### *Analysis of chemokine receptor mRNA by RNase protection assay*

Sorted cells were washed in PBS, pelleted, and frozen. RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). Probes were labeled with [$\alpha$-$^{32}$-P]UTP and hybridized with the isolated RNA. Two custom-designed probe sets (BD PharMingen) were used. One set detects CCR1, CXCR4, CCR5, BLR-1, CCR8, CCR6, L32, and GAPDH. The other set detects CXCR2, CCR3, CCR4, CCR2, CCR7, CXCR3, L32, and GAPDH. After digestion of ssRNA, the protected fragments were separated by PAGE. Controls included the probe set hybridized to tRNA only and to tRNA plus a pool of synthetic sense RNAs complementary to the probe set. For quantification, autoradiographs were scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and band density was assessed with ImageQuant image software (Molecular Dynamics, Sunnyvale, CA). The arbitrary units of expression levels for the chemokine receptors are the values obtained from the densitometer and normalized against GAPDH.

### *Chemotaxis assays*

Sorted cells were allowed to incubate in RPMI-C for 1 h at 37°C. Migration assays were performed in Costar 24-well plate tissue culture inserts with 5-$\mu$m pore size polycarbonate filters (Corning, Corning, NY). All recombinant mouse chemokines were purchased from R&D Systems (Minneapolis, MN) and were used at the following final concentrations: ELC (macrophage-inflammatory protein-3$\beta$), 100 nM; secondary lymphoid tissue chemokine (SLC), 100 nM; monokine induced by IFN-$\gamma$, 100 nM; IFN-$\gamma$-inducible protein 10, 25 nM; MDC 100 nM; and TARC, 100 nM. Six hundred microliters of the diluted chemokine solution was placed in the lower well, and 100 $\mu$l medium containing cells ($1 \times 10^5$) was placed in the upper chamber. Migration was conducted in equilibrated RPMI-C at 37°C with 6% $CO_2$ for 90 min. After removal of the Transwell insert, 400 $\mu$l of the medium in the lower well containing the migrated cells was mixed with $5 \times 10^4$ PE-streptavidin (Caltag Laboratories)-labeled latex beads (Interfacial Dynamics, Portland, OR) and analyzed by FACS. The percentage of cells that had migrated through the filter was determined by counting the proportion of cells vs PE-positive beads, taking into account the volumes. Background/nonspecific migration was assessed by migration to plain medium.

### *Statistical analysis*

For comparison of disease onset times, the Mann-Whitney *U* test was used.

## Results and Discussion

### *$CD4^+CD25^+$ splenocytes delay diabetes onset, induced by islet-infiltrating lymphocytes, in coadoptive transfer studies into immune-compromised NOD mice*

Prompted by the previous observations that IIC from prediabetic NOD mice rapidly transferred diabetes into NOD.SCID recipients (3) and that total splenocytes from prediabetic NOD mice inhibited this transfer of disease (4), we attempted to identify the splenocyte subset that mediated this protective effect. We tested the effects of $CD4^+$, $CD4^+CD25^+$, or $CD4^+CD25^-$ splenocytes on diabetes onset following cotransfer with IIC from inflamed pancreatic islets (Fig. 1). Seven of eight NOD.SCID recipients that received 500,000 IIC from 11-wk-old NOD mice became diabetic by 36 days post-transfer. Cotransfer of 5 million $CD4^+$ splenocytes from 6-wk-old NOD mice significantly delayed the onset of diabetes

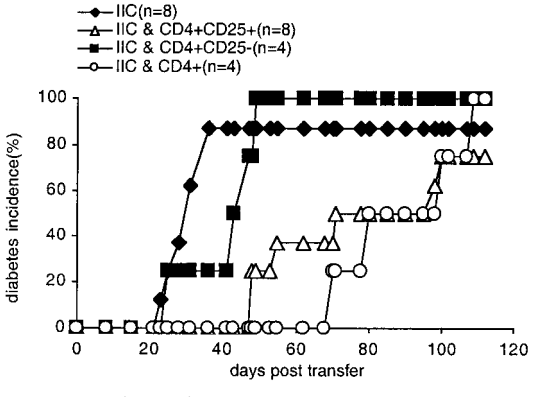


**FIGURE 1.** $CD4^+CD25^+$ splenocytes from prediabetic NOD mice delay diabetes transfer with IIC. Single-cell suspensions were prepared from inflamed pancreatic islets of 11-wk-old NOD mice. A total of 500,000 IIC were transferred into each NOD.SCID recipient either alone or together with 5 million $CD4^+$ splenocytes or 500,000 $CD4^+CD25^+$ splenocytes or 5 million $CD4^+CD25^-$ splenocytes from 6-wk-old NOD mice. The onset of diabetes was monitored through urinary glucose measurements twice weekly. The data displayed are pooled from two independent experiments with similar results.

($p = 0.0485$). This regulatory capacity appeared to be contained primarily within the $CD4^+CD25^+$ subpopulation, as cotransfer of 500,000 $CD4^+CD25^+$ splenocytes, i.e., the fraction of $CD4^+CD25^+$ cells within 5 million $CD4^+$ cells, similarly delayed the onset of disease ($p = 0.0070$). Five million cotransferred $CD4^+CD25^-$ splenocytes did not have a significant impact on disease ($p = 0.2828$). Our results confirm the regulatory function of $CD4^+CD25^+$ T cells in this particular disease model (9). Interestingly, neither $CD4^+CD25^+$ nor total $CD4^+$ splenocytes blocked the transfer of diabetes completely. Six of eight mice receiving IIC together with $CD4^+CD25^+$ cells, and four of four mice receiving IIC together with $CD4^+$ total cells became diabetic by 110 days after transfer. This temporary effect may be due to a limited regulatory capacity of $CD4^+CD25^+$ T cells to inhibit the pathogenic IIC or to a loss of $CD4^+CD25^+$ regulatory T cell function with time. Our studies do not exclude the possibility that a statistically significant delay in the onset of diabetes might be achieved with a larger number of $CD4^+CD25^-$ splenocytes (>5 million) or a much larger number of recipient mice. However, this delaying effect would be modest in comparison with that seen with only 500,000 $CD4^+CD25^+$ cells. As previously reported $CD4^+CD25^-$ peripheral lymphocytes depleted of recent thymic emigrants could protect against the development of diabetes in rats, suggesting that some regulatory activity is present in the $CD4^+CD25^-$ compartment (14). Similarly, in the BDC2.5 transgenic system the abrupt onset of diabetes in BDC2.5/RAG2$^{-/-}$ mice could be delayed with both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from NOD mice (22). Whether these putative $CD4^+CD25^-$ regulatory T cells are related to $CD4^+CD25^+$ T cells or represent a separate lineage is not known.

### *$CD4^+CD25^+$ splenocytes bearing the CD62L marker delay adoptive transfer of diabetes*

It has previously been reported that $CD4^+CD62L^+$ and $CD4^+CD45RB^{low}$ splenocytes from prediabetic NOD mice could inhibit diabetes transfer using splenocytes from diabetic NOD mice in cotransfer into immune-incompetent NOD.SCID recipients (6–8), and a possible overlap between $CD4^+CD25^+$, $CD4^+CD62L^+$, and $CD4^+CD45RB^{low}$ regulatory cells in the NOD mouse has been suggested (5, 10). We therefore analyzed the surface expression of activation/memory markers on $CD4^+CD25^+$ splenocytes in 6-wk-old NOD mice. NOD $CD4^+CD25^+$ T cells were uniformly $CD45RB^{low}$ and $CD44^{medium}$, but heterogeneous for the expression of CD62L (25% $CD62L^-$ and 75% $CD62L^+$) and CD69 (85% $CD69^-$ and 15% $CD69^+$; data not shown).

We then tested whether CD62L expression on $CD4^+CD25^+$ T cells would allow a more precise definition of the regulatory splenocyte population in our adoptive cotransfer model of diabetes. To this end we transferred 500,000 IIC alone or together with 500,000 $CD4^+CD25^+CD62L^+$, $CD4^+CD25^+CD62L^-$, or $CD4^+CD25^+$ splenocytes into NOD.SCID recipients (Fig. 2*A*). $CD4^+CD25^+$ splenocytes significantly delayed the onset of diabetes ($p = 0.0021$). $CD4^+CD25^+CD62L^+$ cells similarly delayed the onset of diabetes ($p = 0.0147$), whereas $CD4^+CD25^+CD62L^-$ splenocytes showed a slight, but nonsignificant, delay ($p = 0.0892$). This demonstrates that in NOD mice $CD4^+CD25^+$ T cells can be separated into two functionally distinct subpopulation based upon the expression of a third surface marker.

### *Both the $CD62L^-$ and $CD62L^+$ subpopulations of the $CD4^+CD25^+$ splenocytes are suppressive in vitro*

The idea of distinguishing regulatory from activated nonregulatory $CD4^+CD25^+$ T cells based on the expression of other surface markers has been tested in normal mice. $CD4^+CD25^+CD62L^-$ and $CD4^+CD25^+CD62L^+$ T cells were found to be equally suppressive in vitro (23, 24). However, it remained possible that in NOD mice there exists a large population of autoantigen-activated conventional $CD4^+CD25^+$ T cells within the $CD62L^-$ subpopulation that might not have suppressor qualities. Thus, we sorted $CD4^+CD25^+CD62L^+$ or $CD4^+CD25^+CD62L^-$ NOD splenocytes and cultured them with anti-CD3- and anti-CD28 mAb-coated beads as surrogate APC (21) either alone or together with $CD4^+CD25^-$ splenocytes at decreasing ratios to test their function in vitro. Data presented in Fig. 2*B* show that in contrast to the findings in vivo, both populations were equally anergic and suppressive in vitro.

It is possible that this assay is not sensitive enough to detect a mild contamination with nonregulatory T cells that nonetheless may be significant in vivo. In addition, the polyclonal stimulus neglects potential Ag-specific effects. However, an alternative explanation takes into account the specific nature of the CD62L molecule. CD62L (L-selectin) is an adhesion molecule that mediates lymphocyte extravasation through high endothelial venules (HEV) into lymph nodes and Peyer's patches as well as into chronically inflamed tissues through HEV-like structures (25). We reasoned

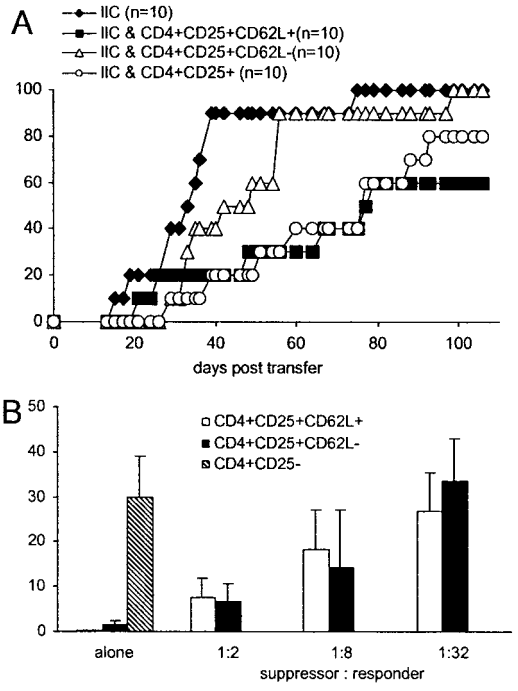


**FIGURE 2.** $CD4^+CD25^+$ splenocytes that delay diabetes transfer with IIC are $CD62L^+$. However, $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ cells are equally suppressive in vitro. *A*, IIC were isolated from pancreata of 11-wk-old NOD mice. A total of 500,000 cells were transferred into each NOD.SCID recipients either alone or together with 500,000 $CD4^+CD25^+CD62L^+$ splenocytes, 500,000 $CD4^+CD25^+CD62L^+$, or 500,000 $CD4^+CD25^+$ splenocytes from prediabetic NOD mice. The data displayed are pooled from two independent experiments with similar results. *B*, $CD4^+CD25^+CD62L^+$ or $CD4^+CD25^+CD62L^-$ splenocytes from prediabetic NOD mice were incubated with anti-CD3 and anti-CD28 mAb-coated beads either alone or together with $CD4^+CD25^-$ splenocytes from the same mice. As a positive control, $CD4^+CD25^-$ splenocytes were incubated alone with beads. A total of 12,500 cells per well were used when cells were stimulated alone. In coincubation, decreasing numbers of $CD4^+CD25^+CD62L^+$ or $CD4^+CD25^+CD62L^-$ cells were added to a constant ($n = 12,5000$) number of $CD4^+CD25^-$ cells at the ratios indicated. The proliferation of cells was assessed by [$^3$H]thymidine incorporation. The results are representative of three independent experiments.

that the functional difference between $CD4^+CD25^+CD62L^-$ and $CD4^+CD25^+CD62L^+$ T cells in their capacity to delay diabetes transfer might be due to differential trafficking patterns of the two subpopulations.

### *$CD4^+CD25^+CD62L^+$, but not $CD62L^-$, cells express high levels of CCR7*

Lymphocyte trafficking through the endothelium is a sequence of events involving adhesion molecules (such as CD62L), chemokine receptors, and integrins. Lymphoid chemokines are critical for trafficking into lymph nodes and within lymphoid compartments, whereas inflammatory chemokines attract lymphocytes into inflamed peripheral tissue (26).

To test whether $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ splenocytes differ in their chemotactic properties, we analyzed the expression of chemokine receptors in these subsets using RNase protection assays (Fig. 3*A*). Different expression levels between the two subpopulations were apparent for CCR7 expression (higher in $CD62L^+$ T cells) and CCR2, CCR4, and CXCR3 expression (higher in $CD62L^-$ T cells). These bands were quantified by densitometry (Fig. 3*B*); $CD4^+CD25^+CD62L^+$ cells expressed 3.1-fold higher levels of CCR7 than $CD4^+CD25^+CD62L^-$ cells. CCR4, CCR2, and CXCR3 expression levels in $CD4^+CD25^+CD62L^-$ cells were 1.9-, 2.8-, and 4.4-fold higher, respectively, than that expressed on $CD4^+CD25^+CD62L^+$ cells. There was no significant difference in the expression levels of the other chemokine receptors examined. CCR4, CCR8, and CCR5 have been suggested to be specific for $CD4^+CD25^+$ cells (18, 19). We found that $CD4^+CD25^+$ NOD splenocytes expressed all these chemokine receptors. While there was no difference between the expression levels of CCR8 and CCR5 in $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ cells, the latter subpopulations expressed 1.9-fold higher levels of CCR4. This suggests that $CD4^+CD25^+$ cells may not be homogenous in terms of their trafficking properties.

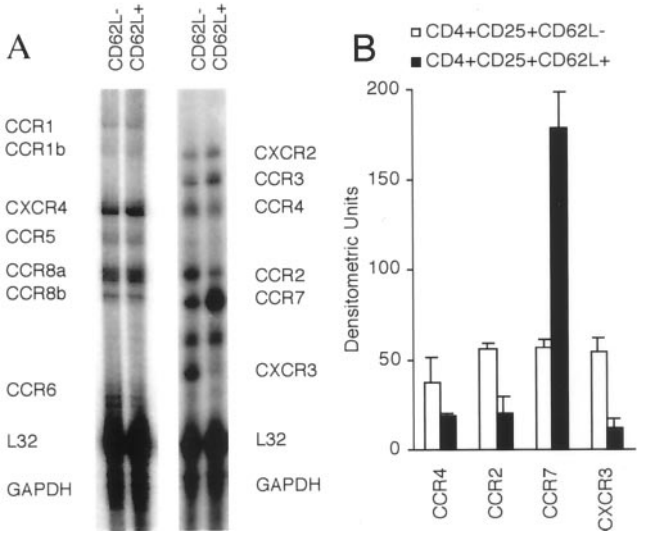


**FIGURE 3.** Differential chemokine receptor mRNA expression of $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ splenocytes. *A*, $CD4^+CD25^+CD62L^+$ (labeled $CD62L^+$) and $CD4^+CD25^+CD62L^-$ (labeled $CD62L^-$) splenocytes from prediabetic NOD mice were FACS sorted. RNA was prepared, and RNase protection assay was performed using the indicated chemokine probes. *B*, The polyacrylamide gel (shown in *A*) was scanned, and densitometry was performed. The values obtained for the chemokine receptors were normalized against GAPDH. The results are expressed as the mean ± SD of values from two independent experiments.

### *$CD4^+CD25^+CD62L^+$ splenocytes preferentially migrate to ELC and SCL*

We then tested whether the differences observed in chemokine receptor mRNA expression of $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ splenocytes were reflected in the differential chemotactic activity of these cells in response to the corresponding chemokine ligands in a cell migration assay in vitro. In agreement with the chemokine receptor expression data, migration of $CD4^+CD25^+CD62L^+$ cells toward ELC and SLC (ligands for CCR7) was 3.6- and 1.9-fold higher, respectively, than was migration of $CD4^+CD25^+CD62L^-$ cells. $CD4^+CD25^+CD62L^+$ cells migrated preferentially to monokine induced by IFN-$\gamma$ and IFN-$\gamma$-inducible protein 10 (ligands for CXCR3), and MDC and TARC (ligands for CCR4; Fig. 4).

Interestingly, a combinational code of CD62L and CCR7 expression is essential for entry into peripheral lymph nodes (27). CD62L binding to peripheral node addressin on HEV initiates rolling (25), which allows the engagement of CCR7 on lymphocytes to its ligand, SLC, on HEV. These steps are essential for the following integrin activation, firm adhesion, and transmigration (28). Once in the lymph nodes, CCR7 expression directs the movement of T cells into the T cell area in response to SLC and ELC, where their interaction with APC and other T cells may take place (26). In contrast, CCR2, CXCR3, and CCR4 have been found on activated/memory lymphocytes from a variety of noninflamed and inflamed tertiary tissues. Their chemokine ligands are expressed on APC and/or endothelial cells at tertiary sites (29–31).

How do these findings relate to the differential outcome in diabetes development following cotransfer of $CD4^+CD25^+CD62L^-$ or $CD4^+CD25^+CD62^+$ splenocytes and IIC into immune-incompetent NOD mice? The events following adoptive transfer of IIC into immune-incompetent NOD mice are complex. Islet-reactive lymphocytes are believed to be primed in the pancreatic lymph nodes by APC that present islet Ags (32). Following activation, islet-reactive lymphocytes home to the pancreas and initiate $\beta$-cell destruction. We hypothesize that the specific adhesion molecule and chemokine receptor expression profile of the $CD4^+CD25^+CD62L^+$ cells allows them to more efficiently enter the pancreatic lymph nodes where they can productively interact with and potentially inhibit the activation of IIC.

Our data are suggestive, but not conclusive. We have tried to follow the early migration of the transferred cell population using CFSE-labeled cells. However, the nature of the system (difficulties

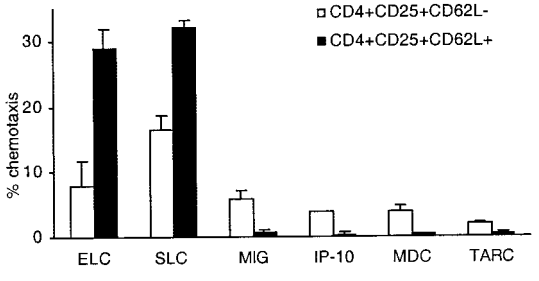


**FIGURE 4.** Differential migratory response of $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ splenocytes to lymphoid and inflammatory chemokines. The chemotaxis of sorted $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ splenocytes from prediabetic NOD mice was examined in response to lymphoid chemokines ELC and SLC (ligands for CCR7) and inflammatory chemokines MIG and IP-10 (ligands for CXCR3) and MDC and TARC (ligands for CCR4). Nonspecific migration of cells to medium alone was deducted from the specific migration. Chemotaxis to all chemokines was performed in triplicate. The results are representative of two independent experiments.

to generate requisite cell numbers) makes it hard to generate appropriate and controlled data. Chemokine receptor knockout mice on an NOD background are not available. Finally, Abs and small molecule inhibitors of chemokine receptors (even if they were available) would not be helpful, as they would unselectively affect both IIC as well as $CD4^+CD25^+$ T cells.

*Conclusion*

$CD4^+CD25^+$ splenocytes are composed of two subsets based upon differential expression of CD62L and CCR7. $CD4^+CD25^+$ cells that express CD62L and high levels of CCR7 preferentially migrate to lymphoid chemokines, while those that are $CD62L^-$ express CCR4, CCR2, and CXCR3 and preferentially migrate toward inflammatory chemokines. $CD4^+$ splenocytes that most efficiently inhibit adoptive transfer of diabetes in cotransfer with IIC are $CD4^+CD25^+CD62L^+$. This differential chemokine receptor expression and migration pattern of $CD4^+CD25^+CD62L^+$ compared with $CD4^+CD25^+CD62L^-$ splenocytes might explain why the $CD4^+CD25^+CD62L^+$ NOD splenocytes delay diabetes upon cotransfer with IIC despite the fact that both subsets are equally suppressive in vitro.

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## References

1. Makino, S., K. Kunimoto, Y. Muraoka, Y. Mizushima, K. Katagiri, and Y. Tochino. 1980. Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu 29:1.*
2. Delovitch, T. L., and B. Singh. 1997. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity 7:727.*
3. Rohane, P. W., A. Shimada, D. T. Kim, C. T. Edwards, B. Charlton, L. D. Shultz, and C. G. Fathman. 1995. Islet-infiltrating lymphocytes from prediabetic NOD mice rapidly transfer diabetes to NOD-*scid/scid* mice. *Diabetes 44:550.*
4. Ablamunits, V., D. Elias, and I. R. Cohen. 1999. The pathogenicity of islet-infiltrating lymphocytes in the non-obese diabetic (NOD) mouse. *Clin. Exp. Immunol. 115:260.*
5. Chatenoud, L., B. Salomon, and J. A. Bluestone. 2001. Suppressor T cells: they're back and critical for regulation of autoimmunity! *Immunol. Rev. 182:149.*
6. Shimada, A., B. Charlton, P. Rohane, C. Taylor-Edwards, and C. G. Fathman. 1996. Immune regulation in type 1 diabetes. *J. Autoimmun. 9:263.*
7. Shimada, A., P. Rohane, C. G. Fathman, and B. Charlton. 1996. Pathogenic and protective roles of $CD45RB^{low}$ $CD4^+$ cells correlate with cytokine profiles in the spontaneously autoimmune diabetic mouse. *Diabetes 45:71.*
8. Lepault, F., and M. C. Gagnerault. 2000. Characterization of peripheral regulatory $CD4^+$ T cells that prevent diabetes onset in nonobese diabetic mice. *J. Immunol. 164:240.*
9. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity 12:431.*
10. Bach, J. F., and L. Chatenoud. 2001. Tolerance to islet autoantigens in type 1 diabetes. *Annu. Rev. Immunol. 19:131.*
11. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med. 184:387.*
12. Suri-Payer, E., A. Z. Amar, A. M. Thornton, and E. M. Shevach. 1998. CD4+$CD25^+$ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol. 160:1212.*
13. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. *J. Exp. Med. 192:295.*
14. Stephens, L. A., and D. Mason. 2000. 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. *J. Immunol. 165:3105.*
15. Shevach, E. M. 2001. Certified professionals: $CD4^+CD25^+$ suppressor T cells. *J. Exp. Med. 193:F41.*
16. Thornton, A. M., and E. M. Shevach. 1998. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med. 188:287.*
17. Read, S., and F. Powrie. 2001. CD4+ regulatory T cells. *Curr. Opin. Immunol. 13:644.*
18. Bystry, R. S., V. Aluvihare, K. A. Welch, M. Kallikourdis, and A. G. Betz. 2001. B cells and professional APCs recruit regulatory T cells via CCL4. *Nat. Immunol. 2:1126.*
19. Iellem, A., M. Mariani, R. Lang, H. Recalde, P. Panina-Bordignon, F. Sinigaglia, and D. D'Ambrosio. 2001. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by $CD4^+CD25^+$ regulatory T cells. *J. Exp. Med. 194:847.*
20. Lejon, K., and C. G. Fathman. 1999. Isolation of self antigen-reactive cells from inflamed islets of nonobese diabetic mice using $CD4^{high}$ expression as a marker. *J. Immunol. 163:5708.*
21. Ermann, J., V. Szanya, G. S. Ford, V. Paragas, C. G. Fathman, and K. Lejon. 2001. $CD4^+CD25^+$ T cells facilitate the induction of T cell anergy. *J. Immunol. 167:4271.*
22. Gonzalez, A., I. Andre-Schmutz, C. Carnaud, D. Mathis, and C. Benoist. 2001. Damage control, rather than unresponsiveness, effected by protective $DX5^+$ T cells in autoimmune diabetes. *Nat. Immunol. 2:1117.*
23. Kuniyasu, Y., T. Takahashi, M. Itoh, J. Shimizu, G. Toda, and S. Sakaguchi. 2000. Naturally anergic and suppressive $CD25^+CD4^+$ T cells as a functionally and phenotypically distinct immunoregulatory T cell subpopulation. *Int. Immunol. 12:1145.*
24. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T cells is antigen nonspecific. *J. Immunol. 164:183.*
25. Rosen, S. D. 1999. Endothelial ligands for L-selectin: from lymphocyte recirculation to allograft rejection. *Am. J. Pathol. 155:1013.*
26. Cyster, J. G. 1999. Chemokines and cell migration in secondary lymphoid organs. *Science 286:2098.*
27. Mackay, C. R. 1999. Dual personality of memory T cells. *Nature 401:659.*
28. Campbell, J. J., and E. C. Butcher. 2000. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. *Curr. Opin. Immunol. 12:336.*
29. Luther, S. A., and J. G. Cyster. 2001. Chemokines as regulators of T cell differentiation. *Nat. Immunol. 2:102.*
30. Kim, C. H., L. Rott, E. J. Kunkel, M. C. Genovese, D. P. Andrew, L. Wu, and E. C. Butcher. 2001. Rules of chemokine receptor association with T cell polarization in vivo. *J. Clin. Invest. 108:1331.*
31. Kunkel, E. J., J. Boisvert, K. Murphy, M. A. Vierra, M. C. Genovese, A. J. Wardlaw, H. B. Greenberg, M. R. Hodge, L. Wu, E. C. Butcher, et al. 2002. Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. *Am. J. Pathol. 160:347.*
32. Hoglund, P., J. Mintern, C. Waltzinger, W. Heath, C. Benoist, and D. Mathis. 1999. Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. *J. Exp. Med. 189:331.*