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The Subpopulation of CD4⁺CD25⁺ Splenocytes That Delays Adoptive Transfer of Diabetes Expresses L-Selectin and High Levels of CCR7¹

Veronika Szanya, Joerg Ermann, Cariel Taylor, Claire Holness, and C. Garrison Fathman²

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-3β), 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 insulinitis, 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 insulinitis 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 insulinitis 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-β 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-1β (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.

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 glucosuria using Glucostix (Roche, Indianapolis, IN) twice a week. If glucosuria was observed, blood glucose was measured with a One Touch Basic glucometer (Johnson & Johnson, Milpitas, CA). Mice with glucosuria and blood glucose 250 mg/dl were considered diabetic. Glucosuria 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 µg/ml streptomycin, 2 mM L-glutamine (all obtained from Life Technologies), and 50 µM 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 µg/ml anti-CD3 and 2.5 µg/ml anti-CD28 mAbs. Cells were pulsed with 1 µCi [³H]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 [³H]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 [α -³²P]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-µm 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-3β), 100 nM; secondary lymphoid tissue chemokine (SLC), 100 nM; monokine induced by IFN-γ, 100 nM; IFN-γ-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 µl medium containing cells (1 × 10⁵) was placed in the upper chamber. Migration was conducted in equilibrated RPMI-C at 37°C with 6% CO₂ for 90 min. After removal of the Transwell insert, 400 µl of the medium in the lower well containing the migrated cells was mixed with 5 × 10⁴ 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

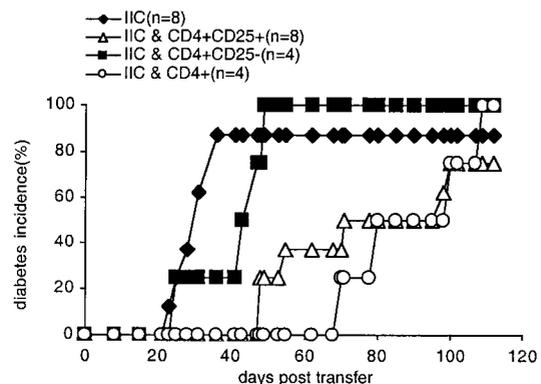


FIGURE 1. CD4⁺CD25⁺ splenocytes from prediabetic NOD mice delay diabetes transfer with IIC. Single-cell suspensions were prepared from inflamed pancreatic islets of 11-wk-old NOD mice. A total of 500,000 IIC were transferred into each NOD.SCID recipient either alone or together with 5 million CD4⁺ splenocytes 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 $CD4^+CD45RB^{\text{low}}$ 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 $CD4^+CD45RB^{\text{low}}$ 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 $CD45RB^{\text{low}}$ and $CD44^{\text{medium}}$, 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. 2A). $CD4^+CD25^+$ 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. $CD4^+CD25^+CD62L^-$

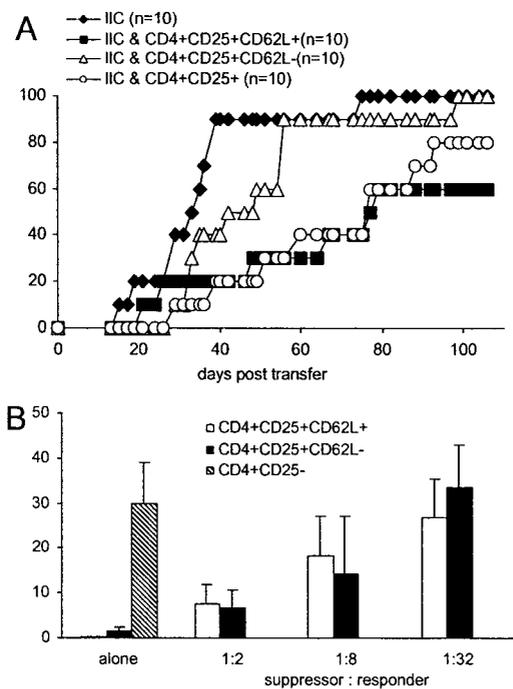


FIGURE 2. $CD4^+CD25^+$ splenocytes that delay diabetes transfer with IIC are $CD62L^+$. However, $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ cells are equally suppressive in vitro. *A*, IIC were isolated from pancreata of 11-wk-old NOD mice. A total of 500,000 cells were transferred into each NOD.SCID recipient 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 cocultivation, decreasing numbers of $CD4^+CD25^+CD62L^+$ or $CD4^+CD25^+CD62L^-$ cells were added to a constant ($n = 12,500$) number of $CD4^+CD25^-$ cells at the ratios indicated. The proliferation of cells was assessed by [³H]thymidine incorporation. The results are representative of three independent experiments.

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. 2B 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

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. 3A). 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. 3B); 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.

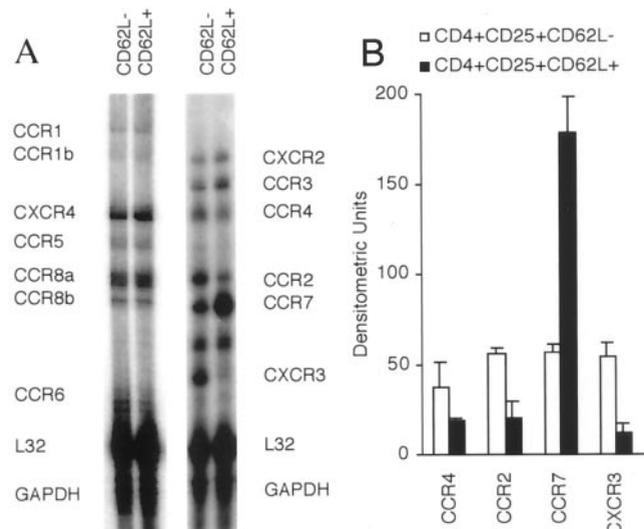


FIGURE 3. Differential chemokine receptor mRNA expression of CD4⁺CD25⁺CD62L⁺ and CD4⁺CD25⁺CD62L⁻ splenocytes. *A*, CD4⁺CD25⁺CD62L⁺ (labeled CD62L⁺) and CD4⁺CD25⁺CD62L⁻ (labeled CD62L⁻) splenocytes from prediabetic NOD mice were FACS sorted. RNA was prepared, and RNase protection assay was performed using 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 \pm 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- γ and IFN- γ -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⁺CD62L⁺ 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 β -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

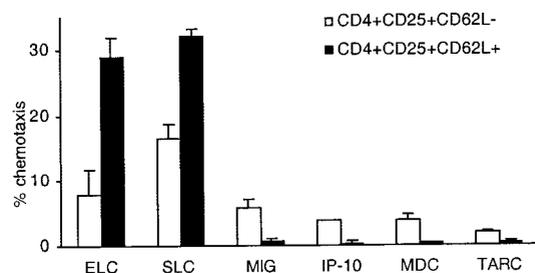


FIGURE 4. Differential migratory response of CD4⁺CD25⁺CD62L⁺ and CD4⁺CD25⁺CD62L⁻ splenocytes to lymphoid and inflammatory chemokines. The chemotaxis of sorted CD4⁺CD25⁺CD62L⁺ and CD4⁺CD25⁺CD62L⁻ splenocytes from prediabetic NOD mice was examined in response to lymphoid chemokines ELC and SLC (ligands 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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