

## Differences in Adhesion Markers, Activation Markers, and TcR in Islet Infiltrating vs. Peripheral Lymphocytes in the NOD Mouse

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Insulin dependent diabetes mellitus (IDDM) in the non-obese diabetic (NOD) mouse is the result of a cellular mediated autoimmune event that destroys pancreatic islet  $\beta$  cells. This destruction is characterized by a progressive lymphocytic infiltration into the islets as well as circulating autoantibodies and T cells reactive with islet antigens. To gain a better understanding of the cells responsible for islet destruction we isolated lymphocytes from the islets of prediabetic NOD mice and conducted a comparative phenotypic analysis with the analogous subpopulations of lymphocytes isolated from peripheral blood and lymph node (LN) of the same mice. CD3<sup>+</sup> cells were analysed for T cell receptor (TcR); cell bearing  $\gamma\delta$  TcR were consistently observed at a higher frequency in the infiltrating T cells than in the periphery. Lymphocytes were also characterized for the expression of CD4 and CD8 T cell markers and, within each population, for the expression of activation markers (CD25, CD69) and adhesion markers (CD51, CD54, CD11b, CD49e, L-selectin). Significantly increased levels of CD4<sup>+</sup>CD8<sup>+</sup> double-positive and CD4<sup>-</sup>CD8<sup>-</sup> double-negative T cell populations were observed in the infiltrating lymphocytes as compared with peripheral lymphocytes. In addition, within both CD4 and CD8 subpopulations isolated from islet infiltrates, CD11b<sup>+</sup> and CD49e<sup>+</sup> cells were increased with respect to the same subset of cells isolated from the periphery. In contrast, the level of cells that expressed L-selectin was significantly higher in the periphery for both CD4<sup>+</sup> and CD8<sup>+</sup> cells than for infiltrating cells. These data describe the phenotype of islet reactive T cells in the NOD mouse and identify possible targets for therapeutic intervention.

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## Introduction

IDDM is a chronic organ-specific autoimmune disease in which the insulin-producing  $\beta$  cells within the pancreatic islets are destroyed [1, 2]. The destruction of  $\beta$  cells is a cell mediated immunological event [3]; however, which antigens and cells are responsible for the initiation of this autoimmune response remains unanswered.

To gain insight into this question, several groups have looked at cell surface marker expression on peripheral blood lymphocytes (PBL) from IDDM patients. These studies show an increase in cells bearing the activation markers CD25 (IL-2 receptor) and HLA-DR (MHC class II) in the blood of recent onset IDDM patients [4–6]. Additional studies point out differences in populations of naive and memory cells in association with IDDM. For example, CD4<sup>+</sup>CD45RA<sup>+</sup> cells, described alternatively as naive T helper cells or suppressor/inducer cells, are increased in prediabetic subjects in direct correlation with insulin autoantibody titer [7], as well as in recent onset IDDM patients [5, 8, 9]. Following this initial increase in cells expressing the naive phenotype, there is an overall decrease in CD45RA expression as the disease progresses [8]. Prediabetic and recent onset IDDM subjects also show a decrease in CD4<sup>+</sup>CD29<sup>+</sup> helper/inducer cells [7, 9] and CD8<sup>+</sup>CD11b<sup>+</sup> suppressor/effector cells [5] relative to healthy controls. Taken together, these data support the idea that alterations in T cell activation and maturation are important in IDDM.

In as much as it is prohibitive to look at cell surface marker expression on infiltrating lymphocytes *in situ* from the pancreas of IDDM patients, few such studies have been performed [10–12]. However, animal models such as the NOD mouse [13, 14] and BB rat [15] are now well-established models of human type I diabetes and provide a tool for identifying the islet-specific cells responsible for the disease. The course of disease in both models closely resembles the disease in humans and includes insulinitis, the infiltration of leukocytes into the islet periphery, followed by islet destruction, and overt diabetes.

Characterization of the cells which are  $\beta$  cell reactive can help in the development of potential new strategies for treatment or prevention of IDDM. In this study we characterize the phenotype of infiltrating lymphocytes isolated from the islets of NOD mice for the expression of adhesion molecules (CD51, CD54, CD11b, CD49e, L-selectin), activation molecules (CD25, CD69), and TcR (see Table 1), and compare these phenotypes to that of peripheral lymphocytes isolated from the same mice.

## Materials and methods

### *Animals*

Female NOD mice were obtained from Taconic Farm (Germantown, NY) at 4 weeks of age. Animals were housed in standard conditions and, following a 10-day resting period, animals were available for experiments. For each experiment, pancreas, peripheral blood, and LN samples were obtained from each animal. Samples from 10–15 NOD mice were then pooled, processed, and analysed. First, peripheral blood was obtained by retro-orbital bleeding. Animals were

Table 1. Description of cell surface markers analysed

Marker	Name	Distribution	Function
CD11b	CR3	macrophages, granulocytes,	adhesion to ICAM-1,
	Mac-1	CD5, B, NK, T cell subset	fibrinogen, C3bi
CD25	IL-2R $\alpha$	NK, activated T and B cells	cell proliferation, activation
	p55		and differentiation
CD49e	Fibronectin receptor	thymocytes, T cells	extracellular adhesion
CD51	Vitronectin receptor	platelets, activated T cells, B subset	intra/extracellular adhesion
CD54	ICAM-1	endothelial, epithelial, B, T,	ligand for LFA-1 and MAC-1,
	MALA-2	monocytes, dendritic cells	adhesion of lymphocytes
			and monocytes to
			endothelial cells
CD69	Very early activation	expressed on T cells within hours of activation	activation and regulation?
L-selectin	MEL-14	T and B cells, neutrophils, monocytes	adhesion to peripheral LN, activated endothelial cells

then killed by asphyxiation and a cervical LN and pancreas were harvested from each animal.

#### *Isolation of islet infiltrating cells*

Islets were isolated from pooled pancreata of NOD mice as described previously [16]. Briefly, the bile duct was cannulated and the pancreas was perfused *in situ* with 0.625 mg/mL collagenase P (Boehringer Mannheim, Indianapolis, IN) dissolved in HBSS medium (HBSS, 1% P/S; Whittaker Bioproducts, Inc., Walkersville, MD; 4 mM sodium bicarbonate, 0.22 g/L bovine serum albumin; Sigma, St. Louis, MO) and removed from the peritoneal cavity. LN were removed from pancreatic tissue and the pancreata were incubated at 37°C for 15 min. The tissue was washed twice in HBSS and passed through a wire mesh. Islets were separated from digested tissue by means of a discontinuous gradient of Ficoll (Sigma) at 27%, 25%, 23%, and 11%. After centrifugation at 800  $\times$  *g* for 10 min, islets were removed from the 23–11% interface and washed in HBSS. Islets were homogenized in a tissue grinder and infiltrating lymphocytes from the homogenate were isolated from islet cells over Lympholyte-M (Cedar Lane, Hornby, Ontario, Canada).

#### *Isolation of lymphocytes from peripheral blood and LN*

Peripheral blood was pooled, diluted 1:2 with PBS, underlayered with Lympholyte-M, and spun for 20 min at 800  $\times$  *g*. Cells at the interface were collected and washed. LN were pooled, homogenized, and washed.

#### *Staining for FACS analysis*

All antibodies were obtained from Pharmingen (San Diego, CA). CD4, CD45, and  $\gamma\delta$  TcR were FITC conjugated. CD8,  $\alpha\beta$  TcR, and B220 were PE conjugated.

CD3, CD11b, CD49e, CD25, CD69, CD51, CD54, and L-selectin were biotinylated. A total of  $5 \times 10^5$  cells was stained with 0.5  $\mu\text{g}$  of each stain in the appropriate combination, for 30 min at 4°C. Cells were washed and 10  $\mu\text{L}$  of streptavidin Per-CP (Becton Dickenson, San Jose, CA) was added for 30 min at 4°C. Cells were washed and immediately run on a Becton Dickenson FACScan.

#### *Statistical analysis*

Student's paired, one-tailed *t*-test was used to analyse all data.

### **Results**

#### *B and T cell markers*

Three color FACS analysis was performed on infiltrating lymphocytes, peripheral blood lymphocytes, and LN lymphocytes isolated from pooled samples of female NOD mice at 6 weeks of age. When gated on total lymphocytes (CD45<sup>+</sup> cells) the number of B220<sup>+</sup> (B) cells was 35% in the infiltrating lymphocytes and 5–10% in the lymphocytes isolated from peripheral blood or LN. The number of CD3<sup>+</sup> (T) cells was 50% in the infiltrating lymphocytes and 75–80% in cells from peripheral blood and LN (Figure 1).

#### *TcR*

We next wanted to examine TcR expression on the CD3<sup>+</sup> population. Lymphocytes from the three populations were stained with antibodies to CD3,  $\alpha\beta$  TcR and  $\gamma\delta$  TcR. The number of CD3<sup>+</sup> cells expressing  $\gamma\delta$  TcR was consistently increased in the infiltrating lymphocyte population as compared to cell from peripheral blood and LN (Table 2).

#### *CD4, CD8 Cell surface markers*

Cells were stained with antibodies to CD3, CD4, and CD8 and the level of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was assessed by gating on CD3<sup>+</sup> cells. The number of CD4<sup>+</sup> cells was 70–80% in all three lymphocyte populations. However, the number of CD8<sup>+</sup> cells was about 10% in the infiltrating T cell population compared to 20–25% in T cells isolated from peripheral blood or LN ( $P < 0.005$ ). This decrease may be due to the differences seen in the CD4<sup>+</sup>CD8<sup>+</sup> double-positive and CD4<sup>-</sup>CD8<sup>-</sup> double-negative populations. Both the numbers of CD4<sup>+</sup>CD8<sup>+</sup> double-positive and CD4<sup>-</sup>CD8<sup>-</sup> double-negative cells were significantly increased ( $P < 0.005$ ) in the infiltrating T cells compared to T cells isolated from peripheral blood or LN (Table 2).

#### *Activation and adhesion markers*

Next we looked at the expression of a variety of activation and adhesion markers (see Table 1) on the three lymphocyte populations. The data for these markers are

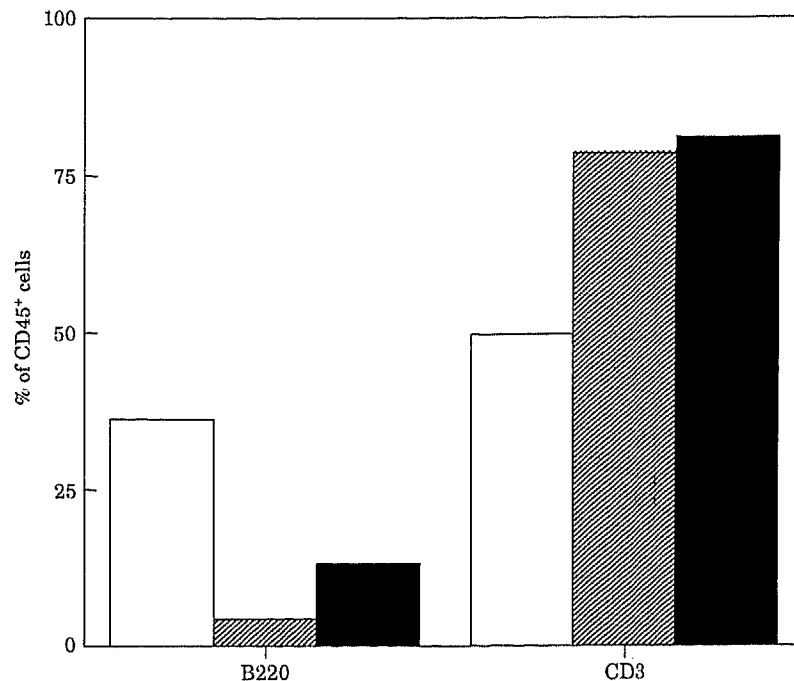


Figure 1. Percentage of B cells (B220<sup>+</sup>) and T cells (CD3<sup>+</sup>) gated on total lymphocytes (CD45<sup>+</sup>). Data are representative of one of four experiments. (□=infiltrating; ▨=peripheral blood lymphocytes; ■=lymph node.)

analysed by gating on CD4<sup>+</sup> or CD8<sup>+</sup> cells. We did not detect a consistent difference in the number of CD25<sup>+</sup> cells (IL-2 receptor) or CD69<sup>+</sup> cells (a marker of very early activation) in the different cell populations. However, we did see a significant increase in cells expressing these markers on LN lymphocytes from diabetic (>12 weeks) compared to NOD mice at 6 and 10 weeks of age (Figure 2).

With respect to adhesion markers, the level of infiltrating cells expressing CD11b, the adhesion promoting complement receptor type 3, ranged from 12–21% for CD4<sup>+</sup> cells and 8–14% for CD8<sup>+</sup> cells. The corresponding populations ranged between 1–4% and 0–2% for both peripheral blood and LN lymphocytes ( $P < 0.05$ ; Table 2). Similarly, the number of infiltrating lymphocytes expressing CD49e, the fibronectin receptor, was between 17–28% for both CD4<sup>+</sup> and CD8<sup>+</sup> cells. The number of peripheral blood or LN lymphocytes expressing CD49e ranged between 0 and 4% ( $P < 0.05$ ; Table 2).

In contrast to the results seen with adhesion markers CD11b and CD49e, the number of cells expressing L-selectin was significantly decreased ( $P < 0.05$ ) for both CD4<sup>+</sup> and CD8<sup>+</sup> infiltrating lymphocytes compared with lymphocytes isolated from the peripheral blood and LN (Table 2). L-selectin (also called MEL-14, LECAM-1, LAM-1) mediates leukocyte adhesion to peripheral LN. Two additional adhesion markers, CD51, the vitronectin receptor, and CD54 (also known as ICAM-1), the ligand for LFA-1 and MAC-1, show an inconsistent

**Table 2.** Percent of cell surface markers on infiltrating, PBL, and LN lymphocytes

Experiment	Marker	Infiltrating (%)	PBL (%)	LN (%)
1	CD3 <sup>+</sup> γδTcR <sup>+</sup> *	1.6	1.4	0.8
	CD4 <sup>+</sup> CD8 <sup>+</sup> *	6.3	0.9	2.0
	CD4 <sup>-</sup> CD8 <sup>-</sup> *	11.4	1.9	1.0
	CD4 <sup>+</sup> CD11b <sup>+</sup> †	21.0	1.8	3.4
	CD8 <sup>+</sup> CD11b <sup>+</sup> ‡	8.5	0.6	0.6
	CD4 <sup>+</sup> CD49e <sup>+</sup> †	22.1	2.4	3.6
	CD8 <sup>+</sup> CD49e <sup>+</sup> ‡	24.6	1.0	0.8
	CD4 <sup>+</sup> selectin <sup>+</sup> †	42.6	85.3	79.3
	CD8 <sup>+</sup> selectin <sup>+</sup> ‡	51.5	83.8	79.1
2	CD3 <sup>+</sup> γδTcR <sup>+</sup> *	1.5	1.0	0.7
	CD4 <sup>+</sup> CD8 <sup>+</sup> *	3.4	1.1	2.0
	CD4 <sup>-</sup> CD8 <sup>-</sup> *	16.7	2.3	0.9
	CD4 <sup>+</sup> CD11b <sup>+</sup> †	17.8	2.2	3.5
	CD8 <sup>+</sup> CD11b <sup>+</sup> ‡	13.6	1.7	0.7
	CD4 <sup>+</sup> CD49e <sup>+</sup> †	27.8	2.4	3.1
	CD8 <sup>+</sup> CD49e <sup>+</sup> ‡	19.5	1.0	1.1
	CD4 <sup>+</sup> selectin <sup>+</sup> †	6.4	84.1	75.8
	CD8 <sup>+</sup> selectin <sup>+</sup> ‡	7.4	56.5	48.2
3	CD3 <sup>+</sup> γδTcR <sup>+</sup> *	1.8	1.4	1.0
	CD4 <sup>+</sup> CD8 <sup>+</sup> *	4.8	1.6	0.9
	CD4 <sup>-</sup> CD8 <sup>-</sup> *	16.4	4.6	1.3
	CD4 <sup>+</sup> CD11b <sup>+</sup> †	12.1	1.1	1.1
	CD8 <sup>+</sup> CD11b <sup>+</sup> ‡	11.6	0.2	0.2
	CD4 <sup>+</sup> CD49e <sup>+</sup> †	17.6	3.1	3.7
	CD8 <sup>+</sup> CD49e <sup>+</sup> ‡	17.0	1.4	2.1
	CD4 <sup>+</sup> selectin <sup>+</sup> †	21.8	88.8	72.2
	CD8 <sup>+</sup> selectin <sup>+</sup> ‡	26.1	93.6	51.6
4	CD3 <sup>+</sup> γδTcR <sup>+</sup> *	2.7	1.0	0.8
	CD4 <sup>+</sup> CD8 <sup>+</sup> *	4.5	1.4	1.1
	CD4 <sup>-</sup> CD8 <sup>-</sup> *	15.8	2.8	0.8
	CD4 <sup>+</sup> CD49e <sup>+</sup> †	22.6	2.5	2.3
	CD8 <sup>+</sup> CD49e <sup>+</sup> ‡	17.1	2.1	1.2

\*Gated on CD3<sup>+</sup> cells.†Gated on CD4<sup>+</sup> cells.‡Gated on CD8<sup>+</sup> cells.

staining pattern when evaluated on the three different lymphocyte populations (data not shown).

### Discussion

Diabetes has been shown to be prevented in the NOD mouse by the treatment with attenuated T cells [17], several antibodies directed against T cells [18–20], and, more recently, antibodies directed against adhesion molecules such as L-selectin and VLA-4 [21–23]. We designed this study to characterize the phenotype of the cells involved in β cell destruction because these cells are logical targets for

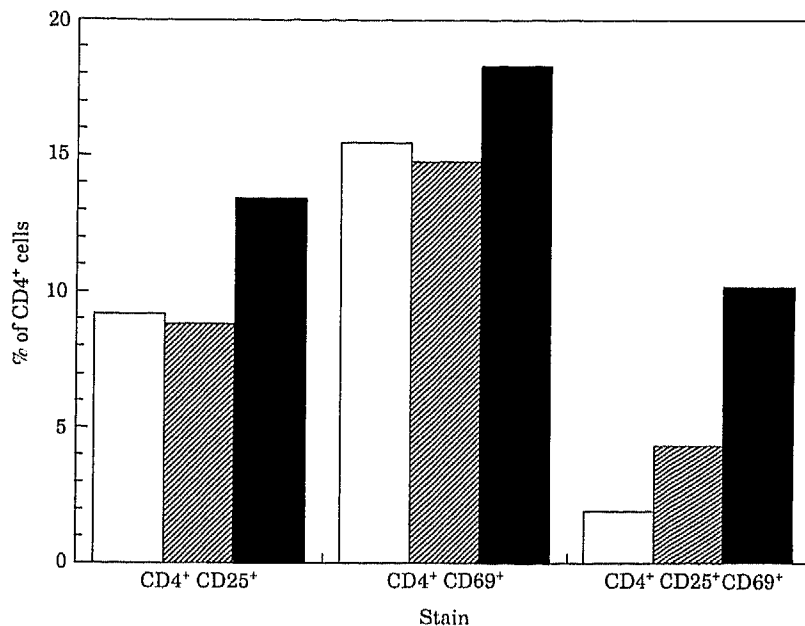


Figure 2. Percentage of CD4<sup>+</sup> cells bearing CD25 and CD69 on LN T cells from NOD mice at 6 weeks, 10 weeks, and following onset of diabetes. CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>CD69<sup>+</sup>, and CD4<sup>+</sup>CD25<sup>+</sup>CD69<sup>+</sup> cells are increased in diabetic (■) animals ( $P < 0.05$ ) compared with 6 weeks (□) and 10 weeks (▨).

therapeutic intervention. We looked at lymphocytes isolated from the islets, LN, and peripheral blood, from 6-week-old female NOD mice. In our colony over 90% of these mice will proceed to overt diabetes within 7 months. Our results demonstrate a difference in the expression of several cell surface markers on infiltrating as compared with peripheral T cells.

Looking at the number of CD4<sup>+</sup> and CD8<sup>+</sup> cells gating on CD3<sup>+</sup> cells, we find an increase in both CD4<sup>+</sup>CD8<sup>+</sup> and CD4<sup>-</sup>CD8<sup>-</sup> cells on infiltrating as compared with peripheral CD3<sup>+</sup> cells. Co-expression of CD4 and CD8 is found on intrathymic T cells during differentiation, but usually the CD4 or the CD8 marker is lost before the cells enter the circulation. These CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells may result from premature entry of thymocytes into the circulation before differentiation into mature CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> or CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> cells [24]. Although the function of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells is unknown they have been identified in the peripheral blood of patients with certain autoimmune conditions including myasthenia gravis [25] and multiple sclerosis [26]. These cells have also been identified during rejection of renal transplants and have been hypothesized to have suppressor activity [27]. The observation that CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells are increased in early islet infiltrates compared with peripheral T cells of NOD mice may indicate an increase in suppressor cells coincident with the onset of the autoimmune response in the islets of NOD mice. Involvement of suppressor cells in NOD diabetes has been previously suggested [28].

In this study we also find a significant increase in the number of CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> double-negative cells in the infiltrating as compared with the peripheral T cell population (11–17% vs. less than 5% in the periphery). This is consistent with the results of several other groups looking at CD4<sup>-</sup>CD8<sup>-</sup> double-negative cells in the spleen or islets of prediabetic or diabetic rats or mice [29, 30]. These double-negative cells have also been implicated in the pathogenesis of other autoimmune diseases [31–33] although their importance in the autoimmune process is unclear. Functional studies will help to determine the role of this T cell population in the pathogenesis of autoimmunity.

The level of  $\gamma\delta^+$  TcR and  $\alpha\beta^+$  TcR cells was examined as a subset of CD3<sup>+</sup> cells. We have observed a consistent, although not significant, increase in  $\gamma\delta^+$  CD3<sup>+</sup> cells in infiltrating T cell populations as compared with cells from LN or peripheral blood (Table 2). Interestingly, a distinct feature of  $\gamma\delta^+$  cells is their reactivity towards mycobacterial antigens [34], one of which has been implicated in IDDM [35]. An increased level of cells expressing  $\gamma\delta$  TCR is also observed in peripheral blood of prediabetic patients with high islet cell antibody levels [36]. In this study a detailed analysis reveals that the  $\gamma\delta$  TCR expressing T lymphocytes are associated with normal  $\beta$  cell function while lower percentages of  $\gamma\delta^+$  cells are associated with the diminished insulin response associated with islet destruction. The authors suggest that the  $\gamma\delta$ -bearing T lymphocytes are implicated in the autoimmune process leading to IDDM and may have a regulatory role. Our observation that  $\gamma\delta^+$  T cells are increased in islet lesions is in agreement with this hypothesis.

A recent paper looking at T cells from pancreas graft biopsies of patients shortly after the onset of recurrent IDDM shows that activated T cells are CD4<sup>-</sup>CD8<sup>-</sup> $\gamma\delta^+$  [12]. Although we have detected an increase in both CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> cells and CD3<sup>+</sup> $\gamma\delta^+$  cells, we are unable to determine if these are the same cell populations due to the stain combinations used.

In contrast to the increase in CD25- and HLADR-expressing cells seen in the peripheral blood of IDDM patients at diagnosis [4–6], we do not see an increase in the level of cells expressing activation markers (CD25, CD69) in the infiltrating compared with the peripheral lymphocytes of NOD mice. Since this was at first surprising, we looked at the expression of these activation markers on LN lymphocytes from NOD mice at 10 weeks of age and at the onset of diabetes. There was a significant ( $P < 0.05$ ) increase in cells expressing CD25 and CD69 in diabetic mice compared to that seen at 6 or 10 weeks (Figure 2). This may indicate that the activation of these cells is coincident with disease but that these cells are not involved in the initial islet infiltrate.

Migration and adhesion of leukocytes to the endothelial venules in the islets is one of the first steps in the development of IDDM. To address the role of adhesion molecules in the pathogenesis of IDDM we looked at the expression of several adhesion markers on lymphocytes isolated from the islets, LN and peripheral blood of NOD mice. The involvement of these molecules in IDDM has been documented by previous studies [21–23, 37–43]; however, their actual function is not known due to the complexity of the adhesion pathways. We observe that CD11b<sup>+</sup> (adhesion promoting complement receptor type 3) cells are found in both CD4 (12–21%) and CD8 (8–14%) subpopulations of infiltrating lymphocytes (Table 2). The number of the corresponding cell populations from LN and peripheral blood is



significantly lower (1–4% for CD4, 0–2% for CD8;  $P < 0.05$ ). Antigen-primed T cells may express CD11b and recently it has been shown that expression of CD11b on activated CD8<sup>+</sup> T cells facilitates homing to inflammatory sites [44]. These results are consistent with a previous study showing that treatment with a monoclonal antibody directed against the CD11b marker prevented diabetes transfer in NOD mice by delaying macrophage and T cell migration into the pancreas [45].

CD49e, the fibronectin receptor, is also involved in extracellular adhesion. Our results looking at the expression of CD49e were similar to those with CD11b. CD49e<sup>+</sup> cells are found in both CD4 and CD8 subpopulations of infiltrating lymphocytes (Table 2) with the percentage of the corresponding cell populations from LN and peripheral blood being significantly lower (17–28% vs. 0–4%).

Inhibition of insulinitis and prevention of diabetes in NOD mice has been achieved by blocking L-selectin [22] and very late antigen 4 adhesion receptors [21–23]. Antibodies directed against these markers blocked leukocyte attachment to the inflamed vessels within the pancreas. In our studies we looked at the expression of L-selectin on infiltrating, LN, and peripheral blood lymphocytes. L-selectin (also called MEL-14, LECAM-1, LAM-1) is expressed on a subset of T cells and mediates leukocyte adhesion to peripheral lymph nodes. L-selectin also appears to be involved in lymphocyte adhesion to activated endothelial cells [46]. We have shown that L-selectin was present on infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> cells; however, the percentage of cells expressing L-selectin in the periphery was significantly higher ( $P < 0.05$ ; Table 2) in 6-week-old NOD mice. This result is in agreement with a previous study [42] which also shows that the proportion of CD4<sup>+</sup> and CD8<sup>+</sup> cells expressing L-selectin is decreased in lymphocytes from infiltrated islets as compared with peripheral lymphocytes.

As leukocyte homing is a multi-step process with different receptors acting at different stages of the adhesion process, it will be of interest to extend these studies looking at these and additional adhesion markers at different stages of the disease process.

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