Role of L-selectin in the development of autoimmune diabetes in non-obese diabetic mice

Conchi Mora1,3, Iqbal S. Grewal1,4, F. Susan Wong1,5 and Richard A. Flavell1,2

1Section of Immunobiology, Yale University School of Medicine, and 2Howard Hughes Medical Institute, New Haven, CT 06520, USA

3Present address: Clinic Foundation for Biomedical Research, IDIPAPS, Clinic and University Hospital of Barcelona, University of Barcelona, Barcelona 08036, Spain
4Present address: Department of Immunology, Genentech, Inc., South San Francisco, CA 94080, USA
5Present address: Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, Bristol BS8 ITD, UK

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Abstract

Autoimmune diabetes is characterized by an early mononuclear infiltration of pancreatic islets and later selective autoimmune destruction of insulin-producing β cells. Lymphocyte homing receptors have been considered candidate targets to prevent autoimmune diabetes. L-selectin (CD62L) is an adhesion molecule highly expressed in naive T and B cells. It has been reported that blocking L-selectin in vivo with a specific antibody (Mel-14) partially impairs insulitis and diabetes in autoimmune diabetes-prone non-obese diabetic (NOD) mice. In the present study we aimed to elucidate whether genetic blockade of leukocyte homing into peripheral lymph nodes would prevent the development of diabetes. We backcrossed L-selectin-deficient mice onto the NOD genetic background. Surprisingly NOD/L-selectin-deficient mice exhibited unaltered islet mononuclear infiltration, timing of diabetes onset and cumulative incidence of spontaneous diabetes when compared to L-selectin-sufficient animals. CD4, CD8 T cells and B cells were present in islet infiltrates from 9-week-old L-selectin-sufficient and -deficient littermates. Moreover, total splenocytes from wild-type, heterozygous or NOD/L-selectin-deficient donor mice showed similar capability to adoptively transfer diabetes into NOD/SCID recipients. On the other hand, homing of activated, cloned insulin-specific autoaggressive CD8 T cells (TGNFC8 clone) is not affected in NOD/L-selectin-deficient recipients. We conclude that L-selectin plays a small role in the homing of autoreactive lymphocytes to regional (pancreatic) lymph nodes in NOD mice.

Introduction

The non-obese diabetic (NOD) mouse constitutes one of the best models for the study of autoimmune diabetes or insulin-dependent diabetes mellitus (IDDM) (1,2). The β cell destruction in NOD mice is T cell dependent (3–5), and leads to impaired insulin production and, as a result, overt diabetes. Mononuclear infiltration of pancreatic tissue, including perivascular and peri-islet infiltrates, consists of T cells, B cells, macrophages and dendritic cells, and begins early in life of NOD female mice (at 3–5 weeks of age). With age, this infiltration becomes more prevalent and invasive, exhibited as intra-islet infiltration or insulitis, but diabetes is not observed before 12 weeks of age (2,6). Since autoaggressive lymphocytes must home to the islets in order to cause the autoimmune destruction of β cells, they need to extravasate from blood vessels surrounding the islets before reaching the target cells. Multiple adhesion molecules are involved in the interaction of lymphocytes with inflamed endothelium. These consist of ligand–receptor pairs constituted by the adhesion molecules and their receptors. Lymphocyte recruitment to tissues is regulated and restricted to physiological situations in which

Correspondence to: R. A. Flavell, Section of Immunobiology, Yale University School of Medicine, 310 Cedar Street, FMB 412, New Haven, CT 06520, USA. E-mail: richard.flavell@yale.edu

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Moreover, it could be argued that prolonged diabetogenic lymphocytes in adoptive transfer experiments derived from naive lymphocytes, but used instead primed the role of L-selectin in the development of spontaneous diabetes (17). This previous work (17) did not, however, address development of peri-insulitis and adoptively transferred diabetes into NOD females from week 5 of age to week 12 impaired the in vivo administration of anti-L-selectin mAb antibody (Mel-14) into NOD females from week 5 of age to week 12 impaired the development of peri-insulitis and adoptively transferred diabetes (17). This previous work (17) did not, however, address the role of L-selectin in the development of spontaneous diabetes from naive lymphocytes, but used instead primed diabetogenic lymphocytes in adoptive transfer experiments. Moreover, it could be argued that prolonged in vivo administration of anti-L-selectin-blocking antibody might exert a depleting effect on the L-selectin-expressing lymphocyte population (17,18,19,23). We therefore decided to assess whether L-selectin is a key factor required to prime autoaggressive lymphocytes in peripheral/pancreatic lymph nodes by using L-selectin KO NOD mice.

Methods

Generation and genotyping of NOD/L-selectin-deficient mice

L-selectin-deficient [L-selectin knockout (KO)] mice were previously generated in our laboratory by gene-targeted disruption and homozygosity for the mutation was obtained by intercrossing on the C57BL/6 background (11). L-selectin KO mice were backcrossed 6, 10 and 13 times onto the NOD background (N7, N11 and N14 generations respectively). Mice were selected at N6 to be homozygous for the 15 Idd (IDDM described susceptibility loci in mice) NOD alleles tested (Idd1–15) (20) for further backcrossing and intercrossing to provide experimental mice. Screening for the L-selectin mutation was performed by PCR analysis using primers: Neo1: 5′-CAT TGA AGA TGG ATT GCA CGC-3′; Neo2: 5′-CTC GAT GGC ATG TTT CGC TTG GTG-3′ [PCR product: 450 bp, present in KO mice or HTZ littermates at the N7 or N14 generation were monitored weekly for the manifestation of glycosuria using Diastix (Ames, Elkhart, IN), starting at 7 weeks of age. Diabetes was confirmed by measuring glycemia using One Touch test strips (Lifescan; Johnson & Johnson, Palmitas, CA) and values >250 mg/dl (>13.9 mM) were considered positive for diabetes. Pancreata were either fixed in 10% buffered formalin or processed for immunohistochemistry by fixation in paraformaldehyde–lysine–periodate buffer. In the former case the tissue was embedded in paraffin, sectioned and stained with hematoxylin & eosin to examine the presence of mononuclear infiltration in pancreatic islets (islets). For immunohistochemistry, after 16 h in paraformaldehyde–lysine–periodate buffer at 4°C the tissue was incubated in increasing concentrations of sucrose dissolved in phosphate buffer (10, 20 and 30%), embedded in Tissue-Tek OCT (Miles, Elkhart, IL) and frozen in 2-methylbutane (isopentane). Frozen sections (7 μm) were taken, and stained for insulin and glucagon (alkaline phosphatase kit; BioGenex, San Ramon, CA); for mouse CD4, CD8 and CD3 (PharMingen, San Diego, CA); and F4/80, as a marker of macrophages (Serotec, Raleigh, NC). The sections were incubated in the presence of alkaline phosphatase-conjugated streptavidin and subsequently in the presence of the HistoMark Red substrate (Kirkegaard & Perry, Gaithersburg, MD) for color development.

Adoptive transfer experiments

Immunocompromised NOD/SCID female mice (>4 weeks) were used as recipients of total splenocytes from NOD/L-selectin KO, wild-type or HTZ littermates used as donors, depending on the experiment. Total splenocytes were isolated under sterile conditions by physical disruption of the spleen using frosted glass slides, then red blood cells were lysed by hypotonic shock with distilled water. Cells were transferred in 200 μl of physiological saline (0.9% NaCl) by i.v. injection.

Sublethally irradiated NOD/L-selectin-deficient (KO) or -sufficient (HTZ) female mice between 6 and 8 weeks of age (725 rad/mouse) were used as recipients of in vitro activated TGNFC8 CD8 T cells (107 cells/mouse). Cells were transferred in 200 μl of physiological saline (0.9% NaCl) by i.v. injection.
Flow cytometric analysis of spleen cells
Splenocytes were isolated as described above and stained following standard procedures in PBS supplemented with 1% FBS. The antibodies used for flow cytometric analysis [CD4, CD8 and CD62L (L-selectin)] were directly conjugated to one of the following fluorochromes: FITC, phycoerythrin or CyChrome (PharMingen). Splenocytes were incubated with

Fig. 1. (A) Splenocytes from NOD/N7 L-selectin-deficient mice transfer diabetes into NOD/SCID recipients with lower incidence and delayed kinetics compared to those from L-selectin-sufficient littermates. Total splenocytes from 7- to 8-week-old pre-diabetic NOD/N7 L-selectin KO (−/−; open circles), wild-type (+/+; filled triangles) or HTZ (+/−; open triangles) female donors were isolated and adoptively transferred (15–20 × 10⁶ splenocytes per recipient mouse depending on the experiment) into 5- to 8-week-old NOD/SCID recipients. Recipient mice were monitored for the development of diabetes every second day. The graph shown corresponds to data from three independent experiments. For statistical analysis, we applied the log-rank/Kaplan–Meier test (P < 0.05). (B) NOD/N11–12 wild-type and L-selectin KO mice splenocytes adoptively transfer disease into NOD/SCID recipients similarly. NOD/SCID recipient females (5–6 weeks old) were adoptively transferred with either 15 × 10⁶ total splenocytes from either NOD/N11–12 wild-type (+/+; filled rhomboids; n = 4) or KO (−/−; filled squares; n = 4) 11-week-old female donors. Diabetes was monitored every other day until day 115 after the adoptive transfer was performed. For statistical analysis, we applied the log-rank/Kaplan–Meier test (P < 0.05).

Fig. 2. NOD L-selectin-deficient mice exhibit unaltered development of diabetes compared to wild-type littermates. (A) NOD/N7 L-selectin wild-type (+/+; filled squares; n = 10), HTZ (+/−; filled triangles; n = 18) and KO (−/−; filled circles; n = 13) females were monitored for the development of diabetes on a weekly basis starting at week 7 of age until week 35 of age. Once glycosuria was found, diabetes mellitus was confirmed by determining the glycemia. The autoimmune condition was confirmed by histological analysis of the pancreata. For statistical analysis, we applied the log-rank/Kaplan–Meier test (P < 0.05). (B) NOD/N7 L-selectin-deficient mice were backcrossed up the N14 generation, intercrossed and females [wild-type (+/+; filled squares; n = 22), HTZ (+/−; filled triangles; n = 14) and KO (−/−; filled circles; n = 9)] were observed up to 27 weeks of age for the development of diabetes. For statistical analysis, we applied the log-rank/Kaplan–Meier test (P < 0.05).
the corresponding antibody at 4°C for 30 min. After staining, the cells were analyzed on a FACS IV (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA).

Statistical analysis
A log-rank/Kaplan–Meier test was applied to compare the cumulative incidence of diabetes (survival curve) between the different experimental groups studied. Differences were considered statistically significant when $P < 0.05$. Statistical analyses were performed using SPSSwin software.

Results

Generation of NOD/L-selectin KO mice at N7, N11 and N14
L-selectin KO mice were generated by targeted disruption of the L-selectin (CD62L) locus and were backcrossed to the C57BL/6 background (11). We then backcrossed these mice onto the murine NOD autoimmune diabetes-prone genetic background. Mice which were selected to be homozygous for the 15 NOD Idd susceptibility alleles tested (Idd1–15) (20) were further backcrossed onto the NOD background to the six (N7), 10 (N11) and 13 (N14) generations respectively, and intercrossed at each of these three generations to obtain experimental mice. Only females were monitored for diabetes development. The L-selectin gene is located on mouse chromosome 1, relatively far (20–40 cM) from the Idd5 markers tested (D1Mit24, D1Mit26), and Idd5, as stated previously, was included in the set of the 15 Idd tested for NOD homozygosity. Total splenocytes from 8-week-old NOD/L-selectin wild-type or KO N7 female mice were analyzed for the levels of L-selectin+ cells, CD4 T cells and CD8 T cells. Spleen cells were stained for flow cytometry analysis of the CD4, CD8 and CD62L markers respectively. The levels of CD4 T cells and CD8 T cells in the spleen were not affected in the NOD/L-selectin KO mice, but expression of L-selectin was undetectable, as expected (45% of splenocytes in wild-type mice compared to 0.5% background levels in KO mice; data not shown).

Splenocytes from NOD/L-selectin KO mice transfer diabetes into NOD/SCID recipients
Since L-selectin enables naive lymphocytes to extravasate thorough HEV into peripheral lymph nodes and thus be primed, we assumed that peripheral naive autoaggressive
lymphocytes in NOD/L-selectin-deficient mice would contact pancreatic antigens for the first time in the spleen rather than pancreatic lymph nodes. Therefore, at the age of 9 weeks, primed diabetogenic lymphocytes should be found in the spleen of both L-selectin-deficient and -sufficient NOD/N7 mice, since by this age we already observe insulitis in NOD/N7 L-selectin KO mice (see Fig. 3). To test this hypothesis, we performed adoptive transfer experiments using total splenocytes from 7- to 8-week-old NOD/N7 L-selectin wild-type, KO or HTZ female donors, transferred them into NOD/SCID female recipients and monitored the development of diabetes (Fig. 1A). Interestingly, splenocytes from KO donors were able to transfer diabetes into immuno-compromised NOD/SCID recipients with a significant delay and lower diabetes incidence than that induced by wild-type donors (30% by the KO versus 90% wild-type and 100% HTZ). Since HTZ showed a significant delay in transferring diabetes compared to wild-type donor splenocytes, there could be either a L-selectin gene dosage effect or incomplete NOD genetic background at the N7 backcross, which could also explain that the KO significantly impaired transfer of diabetes. In agreement with the insufficient backcrossing hypothesis proposed above, when total spleen cells from pre-diabetic N11–12 wild-type donor were transferred into NOD/SCID recipients we observed that 100% of the recipient mice developed diabetes by day 110 following adoptive transfer. When splenocytes from a pre-diabetic NOD/N11KO donor were used, the incidence was also 100% at day 115 without exhibiting any significant delay in disease transfer (see Fig. 1B). This result is consistent with the hypothesis that the spleen constitutes an alternate

![Fig. 3. NOD/N7 L-selectin-deficient mice exhibit insulitis. Pancrata from either 9-week-old NOD/N7 L-selectin HTZ+/– or KO+/– females were fixed in paraformaldehyde-lysine-periodate buffer and processed to obtain frozen sections. (A, C, E, G, I and K) Sections from a HTZ female; (B, D, F, H, J and L) sections from a KO female. The sections were stained for insulin (A and B), glucagons (C and D), CD8 (E and F), CD4 (G and H), CD3 (I and J) and F4/80 (K and L). Hematoxylin counterstaining was applied after developing the HistoMark red staining reaction.](image-url)
route for priming of autoaggressive naive lymphocytes in the absence of a homing factor like L-selectin, although we did not observe the expected delayed kinetics compared to the natural peripheral lymphocyte route. These observations led us to study the spontaneous development of diabetes in NOD/N7 L-selectin-deficient mice, since a delay in autoaggressive lymphocyte priming via recruitment to the spleen should result in retardation in the onset of spontaneous rather than transferred disease.

NOD/L-selectin KO mice exhibit spontaneous development of IDDM and insulin

NOD/L-selectin KO, HTZ and wild-type females were monitored for the development of diabetes up to 35 weeks of age at the N7 generation and for 27 weeks at the N14 generation. While in the wild-type female group the cumulative incidence of diabetes was 80% by N7, the incidence was 39% in both the HTZ and KO groups respectively (Fig. 2A) by 40 weeks of age. This difference in the cumulative incidence of diabetes was statistically significant.

![Image](image_url)

**Table 1.** Rough genetic mapping of a putative resistance gene close to the L-selectin locus in mouse chromosome 1

<table>
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<th>N14</th>
<th>D1Mit30</th>
<th>D1Mit445</th>
<th>D1Mit396</th>
<th>D1Mit108</th>
<th>D1Mit143</th>
<th>D1Mit144</th>
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<td>NOD</td>
<td>NOD</td>
<td>HTZ</td>
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<tr>
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Mouse tail DNA was isolated according to a standard procedure (22), and using the above-mentioned set of primers we PCR amplified fragments that are polymorphic for the strains 129/SV and NOD (Mouse Genome Informatics; Jackson Laboratory, Bar Harbor, ME). The PCR products were run in 4% agarose gels. The L-selectin gene is estimated to be located ~86.6 cM from the acrocenter in mouse chromosome 1.

aThe marker D1Mit30 is not polymorphic for the NOD and C57BL/6 strains respectively. Mice #4 and 5 developed diabetes by 30 weeks of age.

We examined pancreata from HTZ and KO N7 females by immunohistochemistry (Fig. 3). Both NOD/N7 L-selectin KO and HTZ mice exhibited insulitis at 9 weeks of age. The infiltrates observed included CD8+ cells, CD4+ T cells and some macrophages (Fig. 3).

After monitoring the development of diabetes for almost 30 weeks in the N14 generation, no significant difference either in the incidence or kinetics of spontaneous disease was observed between L-selectin-deficient and -sufficient in this generation. The cumulative incidence of diabetes in wild-type females remained unchanged after increasing the number of backcrosses (81% at N14 versus 80% at N7), KO females from the N14 generation reached 55% of individuals in the experimental group (compared to 39% at N7) and this was also the case for HTZ females (64% at N14 versus 39% at N7) (Fig. 2B).

**Discussion**

L-selectin has been considered a target molecule for in the therapy of autoimmune diabetes, not only as a lymphocyte adhesion molecule susceptible to antagonism (17,18), but also as a marker of regulatory T cells (23). Since the existing published data regarding the requirement of L-selectin for the induction of autoimmune diabetes relied on blocking specific anti-L-selectin antibodies in adoptively transferred diabetes models (17–19,24), we decided to study the role of L-selectin in autoimmune diabetes in NOD mice by abating its expression via gene targeting. This method is capable of providing less equivocal results than blockade with specific antibodies.
since prolonged in vivo treatment could promote depletion of L-selectin^+ T or B cells. Our results reveal that the role of L-selectin in the initial homing of lymphocytes into the islets is not crucial for the development of autoimmune diabetes in NOD mice. Our results, including additional immunohistochemical and TGNFC8 clone information, are supported by those recently published by Friedline et al. (25). In later phases, it is possible that the requirement for L-selectin may be overridden by other adhesion factors, very probably VLA4-VCAM (19), since young pre-diabetic NOD/L-selectin-deficient female mice exhibit islet infiltrates similar to those in L-selectin-sufficient (HTZ) females. The fact that the diabetogenic capabilities of splenocytes from L-selectin-deficient and -sufficient mice are similar could be interpreted as an enrichment in fully diabetogenic T cells in the spleen from pre-diabetic donors. This observation is consistent with what has been previously observed using different lymphoid organs as a source of diabetogenic lymphocytes: bone marrow and spleen recruit higher frequencies of diabetogenic T cells than the blood (27).

A statistically significant difference in diabetes onset was observed between NOD/N7 wild-type (80%) and KO/HTZ (39%). This impairment in diabetes development was not observed at the N14 generation in KO (55%) or HTZ (64%) compared to wild-type (81%). Further backcrossing into the NOD genetic background rescued the diabetic phenotype in NOD/N14 KO and NOD/N14 HTZ mice. Surprisingly it seems that the resistance allele(s) map outside the L-selectin genetic interval comprehended between 86.6 and 87.8 cM (L-selectin maps to 86.6 cM in chromosome 1), since NOD/N14 HTZ mice also heterozygous for the NOD markers in this region developed diabetes (preliminary results; see Table 1). Therefore, L-selectin plays a minor role in the development of autoimmune diabetes in NOD mice and this role may be shared with a resistance locus, probably not in the close vicinity of the L-selectin gene, that persists even after thorough backcrossing.

In summary, we propose a scenario in which the presence of L-selectin on the surface of naive lymphocytes would facilitate the rolling and extravasation of diabetogenic lymphocytes in early phases of diabetes. Inflammation signals would up-regulate the expression of addressins on islet vessels (26) which would augment the lymphocyte recruitment process. In the absence of L-selectin, naive T cells would be primed elsewhere (likely in the spleen) or in the pancreatic lymph node via entry using alternative adhesion molecules (such as a4 integrins). The results presented in our work suggest the necessity to study lymphocytic adhesion molecules other than L-selectin such as VLA4 in order to effectively prevent the development of diabetes.

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Abbreviations

IDDM insulin-dependent diabetes mellitus
KO knockout
NOD non-obese diabetic
HTZ heterozygous
HEV high endothelial venules
PLN pancreatic lymph nodes

References