Recirculation and Homing of Lymphocyte Subsets: Dual Homing Specificity of β7-Integrin^high^-Lymphocytes in Nonobese Diabetic Mice

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The β7-integrin subunit can pair with two α-chains, α4 and αE, and is expressed mainly on lymphocytes. As an αE-heterodimer it binds to the mucosal addressin MadCAM-1, thus acting as a mucosal homing receptor. As an αE-heterodimer it binds to E-cadherin and is mainly found on intestinal intraepithelial lymphocytes. Consequently, β7 is mostly expressed on lymphocytes of the mucosal immune system. To study the compartmentalization of these cells further we compared the distribution of such lymphocytes in two strains of mice (BALB/c and NOD) and found that the distribution of β7-positive lymphocytes among various lymphoid tissues in these strains was very different. In NOD mice a conspicuous population of β7-integrin^high^-lymphocytes expressing either α4, αE, or both, was found in nonmucosal lymphoid tissues such as peripheral lymph nodes (PLNs). They mostly expressed the PLN homing receptor L-selectin, and included both naive and memory cells on the basis of their expression of CD44 and CD45RB, as did the few β7^high^-lymphocytes in BALB/c PLNs. Their homing to Peyer's patches (PPs) and PLNs was equally effective and the cells homing to PPs and PLNs were equal in their level of L-selectin and α4/β7 expression. However, functional studies indicated that their homing to PPs mostly depended on α4/β7-integrin, whereas they mainly used L-selectin to home to PLNs. β7^high^-lymphocytes were found also in circulating blood of unmanipulated NOD mice, and their L-selectin expression was higher than in BALB/c mice. These results show that lymphocytes of the mucosal immune system may also express the peripheral node homing receptor L-selectin during their recirculation and that in NOD mice they frequently retain a dual homing specificity, which leads to their accumulation in nonmucosal tissues.

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NOD mice also expressed the peripheral node homing receptor L-selectin. In recirculation experiments in vivo, their homing to peripheral tissues was mainly dependent on L-selectin, showing that these lymphocytes are able to interact with vascular endothelium by both the mucosal and peripheral node homing receptors. This duality may explain the distribution of $\beta^7$-integrin$^+$ cells not only in mucosal but also in peripheral tissues in NOD mice.

MATERIALS AND METHODS

Mice. Local colonies of NOD and BALB/c mice were bred and housed in the animal facilities at Turku University. Both strains of mice were kept under specific pathogen-free conditions. Microbiological tests were done regularly (Microbiology Laboratories, North Harrow, UK) and samples were found to be specific pathogen-free.

Monoclonal antibodies (MoAbs). Of the MoAbs used in this study (Table 1), MoAb M290 reacts with an integrin $\alpha$-chain (aE, CD103) preferentially expressed on lymphocytes in intestinal epithelium and MoAb DATK-32 recognizes a combinatorial epitope only expressed on $\alpha$4/87-heterodimers. American Type Culture Collection (ATCC, Rockville, MD) antibodies were purified from supernatants of hybridoma cells. In dl experiments, control MoAbs were provided by Dr E.C. Butcher (Stanford University, Stanford, CA).

Isolation of lymphocytes from lymphoid organs, thymus, and the blood. Lymphocytes were isolated from peripheral (inguinal, axillary, brachial and cervical) and mesenteric lymph nodes, PPs, and from the thymus by gently squeezing the organs in a glass-homogenizer. After filtering the homogenate the suspension containing the lymphoid cells was washed in phosphate-buffered saline (PBS) and cells were thereafter used for stainings or prepared for in vivo homing experiments. For isolation of blood lymphocytes RPMI-medium was injected into the left ventricle and, simultaneously, blood was drawn into a heparinized syringe from the right ventricle, or alternatively, a blood sample was simply drawn into a heparinized syringe after single intracardial puncture immediately after killing. Mononuclear cells including lymphocytes were isolated from these samples by Ficoll-gradient (Pharmacia, Uppsala, Sweden) centrifugation.

Immunohistochemistry and flow cytometry. To compare the expression of endothelial adhesion molecules on postcapillary venules, cryocut sections of lymph nodes from age- and sex-matched BALB/c and NOD mice were stained by an indirect immunoperoxidase method using peroxidase-conjugated goat Igs to rat IgG (Dako, Glostrup, Denmark) as the detecting antibody. Sections were slightly counterstained with hematoxylin. All comparisons between BALB/c and NOD mice were based on simultaneous stainings including samples from both strains, and photographs of the stained lymph node sections were taken in succession with similar exposure times to circumvent the possibility of obtaining any differences in staining intensity merely because of technical reasons. For detection of lymphocyte adhesion molecules, isolated lymphocytes were first incubated with MoAbs and thereafter with a second-step fluorescein isothiocyanate (FITC)-conjugated antibody (goat anti-rat IgG; Sigma, St Louis, MO) as described. For three-color stainings to determine the phenotype of $\beta^7$-integrin$^+$ lymphocytes the anti-$\beta^7$ MoAb was FITC-conjugated in our laboratory and the anti-4E MoAb, or the anti CD45RB MoAb, was used as a phycoerythrin (PE)-conjugate (PharMingen, San Diego, CA). For these stainings, anti-4E, anti-$\alpha$4/37, anti-L-selectin and anti-CD44/ppg-1 MoAbs were biotinylated using NHS-biotin (Calbiochem, La Jolla, CA). In these stainings lymphocytes were simultaneously incubated with a mixture of the FITC-conjugated anti-$\beta^7$ MoAb, phycoerythrin-conjugated anti-$\alpha$E MoAb (or PE-conjugated anti-CD45RB) and biotinylated anti-4E, anti-L-selectin, or anti-$\alpha$4/37 MoAb (or anti-CD44/
pgp-l), or with a mixture of one or two of these and a control MoAb in all combinations necessary to measure background staining intensity. After two washings the cells were incubated with the fluorochrome PerCP-conjugated streptavidin (SA-PerCP; Becton Dickinson, San Jose, CA) to detect the biotinylated MoAb, and washed. Blood-derived lymphocytes were analyzed by one-, two- and three-color stainings as follows: lymphocytes were simultaneously stained with FITC-conjugated MoAb Fib 504 and PE-conjugated anti-CD45RB, with PE-conjugated anti-εε alone, or with FITC-conjugated MoAb Fib 504 and biotinylated anti-L-selectin, washed, and finally stained with SA-PerCP. To analyze β7-integrin+ lymphocytes from thymocyte subsets expressing either CD4, CD8, or both, thymocytes were stained simultaneously with FITC-conjugated anti-Lyt2.2 (CD8) and PE-conjugated L3T4 (CD4; both purchased from PharMingen) and biotinylated anti-β7 MoAb Fib 504, or with any one or two of these and (a) negative control MoAb(s), then washed twice and finally incubated with SA-PerCP. Similarly, to analyze the expression of CD8αβ heterodimers on PLN β7high cells, lymphocytes were stained with FITC-conjugated anti-Lyt2.2 (reacts with CD8α), phycoerythrin-conjugated anti-CD8β (PharMingen) and biotinylated MoAb Fib 504, washed, and finally stained with SA-PerCP. After staining procedures, stained cells were analyzed with flow cytometry (FACScan and Lyssys Software; Becton Dickinson).

In vivo homing experiments. To determine the homing behavior of β7high lymphocytes and to compare them to L-selectinhigh lymphocytes, fluorescently labeled cells were injected into recipient mice that were killed 30 minutes later and their lymphoid organs collected. After an in vitro staining of lymphoid cells with corresponding MoAbs and phycoerythrin-conjugated second-step antibody the cells were analyzed by flow cytometry (Fig 1). To determine how different subsets of β7-integrinhigh lymphocytes (positive for α4, α4β7, and/or L-selectin) redistribute and home they were analyzed from PLNs and PPs of recipient mice after a 30-minute recirculation period for their homing receptor expression. To this end, NOD lymph node lymphocytes were first labeled with a fluorescent dye (5-chloromethylfluorescein diacetate, CMFDA; Molecular Probes, Eugene, OR) according to manufacturer's instructions and then, 20 × 10⁶ labeled lymphocytes were injected intravenously (IV) into naive recipients which were killed 30 minutes later and their PLNs and PPs collected. Cell suspensions of these PLNs and PPs were then stained with an MoAb against the corresponding homing receptor determinant (see Table 1), then with PE-conjugated anti-rat κ light chains (mouse anti-rat κ light chains; Sigma), followed by biotinylated Fib504 (anti-β7-integrin MoAb), and finally with SA-PerCP (Becton Dickinson). When the cells were analyzed by flow cytometry only cells that were both CMFDA-labeled (IV injected) and β7-integrinhigh (stained with biotinylated anti-β7 and SA-PerCP) were included in the analysis and their homing receptor expression was determined as the level of phycoerythrin staining (which detected the MoAbs against α4/β7-integrins and L-selectin). To determine the degree to which the β7-integrinhigh lymphocyte population in NOD lymph nodes used the mucosal and peripheral node homing receptor, α4/β7 and L-selectin, in their homing, NOD lymph node lymphocytes were, again, labeled with CMFDA. After labeling, lymphocytes were incubated for 30 minutes at 7°C with saturating concentrations of Fib504 (anti-β7-integrin), MEL-14 (anti-L-selectin), or an isotype matched control antibody 281.2 (anti-syndecan 1), washed once and

![Fig 1. Design of the method which was used for analyzing the recirculation and homing of a particular lymphocyte subset (β7high lymphocytes). Lymphocytes were isolated from PLNs, labeled with 5-chloromethylfluorescein diacetate and injected IV to recipient mice. After 30 minutes, recipients were killed and their lymphoid organs were collected. Lymphocytes were isolated, stained with the desired MoAb (anti-β7 or control MoAb), PE-conjugated second-step reagent, and analyzed by flow cytometry. Expression of β7 was then analyzed on labeled (donor) lymphocytes only.](image-url)
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Fig 2. Expression of homing-associated molecules on lymphocytes isolated from (A) Peyefsky's patches (PPs), (B) mesenteric lymph nodes (MLNs), and (C) peripheral lymph nodes (PLNs) of NOD and BALB/c mice. Lymphocytes from 7-week-old NOD (upper panel) and BALB/c (lower panel) mice were stained with MoAbs to the indicated molecules or with a control MoAb and analyzed by flow cytometry. Arrows indicate the population of lymph node lymphocytes in NOD mice which expressed high levels of \( \beta^{'\text{high}} \)-integrin (mean percentage of total cells in NOD PLNs = 19.8%; range, 15.6% to 21.2%; in BALB/c PLNs = 6.7%; range, 3.9% to 8.2%; in NOD MLNs = 19.4%, range, 17.1% to 22.4%; and in BALB/c MLNs = 9.8%, range 7.4% to 13.0%, n = 7).

RESULTS

Expression of homing-associated molecules on BALB/c and NOD lymphocytes. To screen for possible differences in lymphocyte recirculation capacity in NOD and BALB/c mice, lymphocytes were isolated from various lymph nodes (inquinal, axillary, brachial, cervical, and mesenteric) and from PPs, which represent mucosal lymphoid tissue (from 10 NOD and BALB/c mice, aged 6 to 10 weeks), and their expression of homing-associated molecules was studied. The expression of L-selectin, \( \alpha^4 \)-, and \( \beta^1 \)-integrin and LFA-1 was equal in NOD and BALB/c lymphocytes (Fig 2A through C). In contrast to the comparable expression of \( \beta^7 \)-integrin among PP lymphocytes in both strains, a major difference in the expression of \( \beta^7 \)-integrin among lymph node lymphocytes of these strains existed. A population of lymphocytes which express high levels of \( \beta^7 \)-integrin was prominent in all peripheral and mesenteric lymph nodes of NOD mice but was almost absent in the lymph nodes of BALB/c mice (Fig 2B and C).

\( \beta^7 \)-integrin\(^{\text{high}} \) lymphocytes in NOD and BALB/c lymph nodes are phenotypically similar. To characterize the \( \beta^7 \)-integrin\(^{\text{high}} \) lymphocytes of NOD mice and to compare them to those few of BALB/c mice, we took lymph node lymphocytes from both strains and examined them by two- and three-color immunofluorescence (Fig 3). The majority of \( \beta^7 \)-integrin\(^{\text{high}} \) lymphocytes in both strains were \( \text{CD}^8^+ \) T cells (73% to 78%) whereas a minority of them were \( \text{CD}^4^+ \) T cells (11% to 14%; Fig 3A). None of them reacted with the B-lymphocyte marker B220 (CD45) (not shown). For comparison, NOD and BALB/c PP lymphocytes were stained similarly, which showed that most \( \beta^7 \)-integrin\(^{\text{low}} \) lymphocytes in PPs of both strains were also \( \text{CD}^8^+ \) T cells (not shown). The majority of \( \beta^7 \)-integrin\(^{\text{high}} \) lymphocytes in both strains expressed high levels of L-selectin (75% to 82%) whereas a minority was L-selectin\(^{\text{low}} \) (18% to 25%). A distinct population (22% to 38%) of these lymphocytes expressed low levels of CD45-RB and a small population (5% to 10%) expressed high levels of CD44, suggesting that they represent memory lymphocytes. To define the expression of the two alternative \( \alpha \)-chains known to pair with \( \beta^7 \), i.e., \( \alpha^4 \) and \( \alpha^E \), NOD, and BALB/c lymph node lymphocytes were stained with three-color immunofluorescence to analyze the relative numbers of \( \beta^7 \)-integrin\(^{\text{high}} \) lymphocytes expressing \( \alpha^4 \) and/or \( \alpha^E \) (Fig 3B). In lymph node lymphocytes of both strains 70% to 90% of \( \beta^7 \)-integrin\(^{\text{high}} \) lymphocytes expressed \( \alpha^E \) and 35% to 50% of these also coexpressed low levels of \( \alpha^4 \). In contrast, 10% to 30% of \( \beta^7 \)-integrin\(^{\text{high}} \) lymphocytes expressed only \( \alpha^4 \) and no \( \alpha^E \). The \( \beta^7 \)-integrin\(^{\text{high}} \) lymphocytes were further characterized to determine whether they were of donor origin and had homed to the lymphoid organ during the 30-minute recirculation period.
Fig 3. Phenotype of NOD and BALB/c lymph node $\beta^7_{\text{int}}$-high lymphocytes. (A) Distribution of $\beta^7_{\text{int}}$-high lymphocytes among CD4$^+$ and CD8$^+$ T lymphocytes and their expression of L-selectin, CD45RB, and CD44/pgp-1. (B) Distribution of $\beta^7_{\text{int}}$-high lymphocytes between cells that express $\alpha_4$ and/or $\alpha E$, their expression of L-selectin and CD44, and expression of CD45RB on the $\alpha_4^+/\beta^7_{\text{int}}^-$ and on the CD44$^+/\beta^7_{\text{int}}$ lymphocytes. In (A), lymphocytes were stained for two-color fluorescence-activated cell sorting (FACS) and, in (B), for three-color FACS and, in each case, only $\beta^7_{\text{int}}$-high lymphocytes were analyzed for their expression of the indicated molecules. In (B), cursors were positioned according to stainings with isotype-matched control MoAbs except for the CD45RB dot-plots, in which the vertical cursor was positioned between CD45RB$^-$ and CD45RB$^+$ lymphocytes.

Phenotypically, lymphocytes that expressed $\alpha E$ were often L-selectin$^+$ (65% to 75%) whereas $\beta^7_{\text{int}}$-high lymphocytes that expressed only $\alpha 4$ and no $\alpha E$ also included a more prominent subpopulation of L-selectin$^-$ lymphocytes (32% to 47%). The $\alpha E^+$ subpopulation expressed mostly intermediate levels of CD44 whereas the $\alpha 4^+/\alpha E^-$ cells mostly expressed relatively high levels of CD44. The $\alpha 4^+$ cells often (≥50%) expressed intermediate-to-low levels of CD45RB (Fig 3B).

Because a proportion of lymph node $\beta^7_{\text{int}}$-high lymphocytes were phenotypically like naive lymphocytes (CD45RB$^-$/CD44$^-$), we sought for differences in the expression of $\beta^7_{\text{int}}$-high on maturing thymocytes in NOD and BALB/c mice. In both strains the $\beta^7_{\text{int}}$-high lymphocytes were mainly CD8 single positive cells and they had a similar distribution of $\beta^7_{\text{int}}$-int expression (not shown), suggesting that the increased number of $\beta^7_{\text{int}}$-high lymphocytes in NOD PLNs does not result from differential regulation of $\beta^7$ expression on developing thymocytes. To test the possibility that the $\alpha E/\beta^7_{\text{int}}$ cells would be derived from the thymus-independent pool of intraepithelial lymphocytes, many of which express CD8 as $\alpha/\alpha$ homodimers instead of $\alpha/\beta$ heterodimers, we examined the expression of CD8$\beta$ on the $\beta^7_{\text{int}}$ cells. However, with respect to their expression of CD8$\beta$, the $\beta^7_{\text{int}}$ cells did not differ from the rest of NOD PLN CD8$^+$ T cells, suggesting that there are no more thymus-independent cells among the $\beta^7_{\text{int}}$ than among other CD8$^+$ T cells (not shown).

Postcapillary venules of NOD PLNs frequently express MadCAM-1. We examined the possibility that postcapillary high endothelial venules (HEVs) in NOD PLNs would differ from PLN venules in BALB/c mice sufficiently to give rise to persistent homing of $\beta^7_{\text{int}}$ lymphocytes. To this end, we stained PLN HEVs for the expression of vascular adhesion molecules that interact with lymphocytes' homing-associated molecules. The expression of the non–tissue-specific endothelial adhesion molecules ICAM-1, VCAM-1, and CD31/PECAM-1 was equal in NOD and BALB/c PLN venules (Fig 4A through C) as was the expression of the peripheral lymph node vascular addressin (PNAd; Fig 4D). In contrast, the mucosal vascular
Fig 4. Expression of endothelial adhesion molecules on postcapillary venules of peripheral lymph nodes (PLN HEVs). In 7-week-old NOD (left) and BALB/c (right) mice, (A) ICAM-1 and (B) CD31 are expressed with low intensity on PLN HEVs and on many lymphocytes, whereas (C) VCAM-1 is not detected on NOD and BALB/c PLN HEVs (arrowheads). (D) PNAd expression is equal on both NOD and BALB/c PLN HEVs, but (E) MAdCAM-1 is expressed more frequently on PLN HEVs of NOD than of BALB/c mice. (F) The intensity of MAdCAM-1 staining on NOD PLN venules is also stronger (two HEVs of NOD PLN with high PNAd expression are numbered 1 and 2). Cryocut sections of lymph nodes were stained using immunoperoxidase method (original magnifications: A through E: ×100; F: ×400).
addressin MAdCAM-1 was expressed more often and with stronger intensity in PLN HEVs of NOD than of BALB/c mice (Fig 4E and F).

**β7**<sup>high</sup> lymphocytes isolated from peripheral lymph nodes home both to peripheral and mucosal lymphoid tissues. Homing of β7<sup>high</sup> lymphocytes was analyzed by injecting fluorescently labeled cells to recipient mice. The labeling of lymphocytes enabled their analysis separately from host cells because they appeared as a distinct FL-1-positive population in flow cytometry (Fig 5A). β7<sup>high</sup> lymphocytes homed to PLNs, mesenteric lymph nodes (MLNs), and PPs with an almost equal efficiency, as did cells which expressed L-selectin (Fig 5B). After the 30-minute recirculation period β7-integrin<sup>high</sup> lymphocytes became slightly overrepresented in the blood whereas the relative number of L-selectin<sup>+</sup> lymphocytes was diminished. To examine whether the homing behavior of different subsets of β7-integrin<sup>high</sup> lymphocytes varied according to their expression of homing receptors α4, α4/β7, and L-selectin, the β7<sup>high</sup> lymphocytes that had homed to PLNs or PPs after a 30-minute recirculation period were analyzed for their expression of α4, α4/β7, and L-selectin (see Materials and Methods). The relative numbers of β7<sup>high</sup> lymphocytes expressing L-selectin, α4, or α4/β7 did not vary markedly between PLN and PP samples (L-selectin: 89% v 74%; α4: 61% v 62%; α4/β7: 24% v 27% of β7<sup>high</sup> lymphocytes), indicating that, in their recirculation and homing, β7<sup>high</sup> lymphocytes behaved as a rather homogeneous population of cells (Fig 5C).

**NOD PLN β7**<sup>high</sup> lymphocytes use both L-selectin and α4/β7 for their homing. To compare homing receptor usage of β7<sup>high</sup> lymphocytes to that of other NOD PLN lymphocytes we first assessed the extent to which the homing of all PLN lymphocytes (Fig 6; first column of each column pair) depended on L-selectin, α4, α4/β7, and MAdCAM-1. The inhibition of homing that was achieved with MoAbs against these molecules is in accordance with the previous studies using different methods of labeling cells. Next, we assessed the extent to which the homing of β7<sup>high</sup> lymphocytes,
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Calculated by dividing the percentage of lymphocytes expressing various levels of L-selectin, $\alpha 4/\beta 7$ and MAdCAM-1. This was done by measuring the percentage of $\beta^{7\text{high}}$ lymphocytes among IV injected lymphocytes that had homed to PLNs, to MLNs, and to PPs (see Figs 1 and 5A). The homing of $\beta^{7\text{high}}$ lymphocytes to PLNs was efficiently blocked by anti-L-selectin MoAb MEL-14 because MEL-14 pretreatment inhibited their PLN-homing by 91% while the PLN-homing of the total cell population was inhibited by 74% (Fig 6). Consequently, $\beta^{7\text{high}}$ lymphocytes are even more dependent on L-selectin than other lymphocytes in their PLN-homing. The anti-$\alpha 4/\beta 7$ MoAb Fib504 had no apparent effect on the PLN-homing of lymphocytes as a whole population and the percentage of $\beta^{7\text{high}}$ lymphocytes among lymphocytes that had homed to PLNs was reduced by 15% after Fib 504 treatment. This implies that $\alpha 4/\beta 7$ is unimportant for the PLN-homing of all lymphocytes and only of marginal importance in the PLN homing of $\beta^{7\text{high}}$ lymphocytes. In contrast, anti-$\beta 7$ MoAb Fib504 treatment inhibited homing of all lymphocytes to MLNs and to PPs considerably (Fig 6). This treatment also resulted in a pronounced decrease in the homing of $\beta^{7\text{high}}$ lymphocytes to PPs and to MLNs, the inhibitory effect that MoAb Fib 504 had on their homing being 69% to MLNs and 87% to PPs compared with 40% and 72% inhibition for the total lymphocyte population, respectively. When donor lymphocytes were pretreated with MEL-14 the homing of $\beta^{7\text{high}}$ lymphocytes to PPs was, in turn, less affected than the homing of all lymphocytes (51% v 60% inhibition). These data show that $\alpha 4/\beta 7$-integrin is important in the homing of $\beta^{7\text{high}}$ lymphocytes to mucosal lymphoid tissues and that in their mucosal homing, $\beta^{7\text{high}}$ lymphocytes are less dependent than other cells on L-selectin. We also assessed whether the prominent expression of MAdCAM-1 ($\alpha 4/\beta 7$ ligand) on PLN HEVs had any effect on the homing of $\beta^{7\text{high}}$-lymphocytes (Fig 6). Blocking endothelial MAdCAM-1 with MoAb MECA-367 inhibited the homing of all lymphocytes to PLNs by 37% and the homing of $\beta^{7\text{high}}$-lymphocytes by 45%. MECA-367 inhibited the homing of all and of $\beta^{7\text{high}}$-lymphocytes to MLNs by 86% and 87%, respectively. MECA-367 inhibited lymphocyte homing to PPs almost completely (99% to 100%) and neither $\beta^{7\text{high}}$ lymphocytes nor other donor lymphocytes were recovered from PPs after this treatment. Thus, $\beta^{7\text{high}}$ lymphocytes preferentially use L-selectin to home to peripheral lymphoid tissues and $\alpha 4/\beta 7$ to home to mucosal lymphoid tissues.

Circulating $\beta^{7\text{high}}$ lymphocytes express various levels of L-selectin. To test the possibility that $\beta^{7\text{high}}$ lymphocytes during the course of their natural recirculation could home to peripheral lymph nodes, we isolated blood lymphocytes from unmanipulated NOD and BALB/c mice and stained them for the expression of $\beta 7$, $\alpha E$, CD45RB, and L-selectin. The difference between NOD and BALB/c blood lymphocytes was similar to that observed in PLN lymphocytes: the population of circulating $\beta^{7\text{high}}$ and circulating $\alpha E^+$ lymphocytes was clearly more prominent in NOD than in BALB/c mice (Fig 7A). With respect to the expression of CD45RB, circulating NOD and BALB/c $\beta^{7\text{high}}$ lymphocytes were similar, indicating that their naive versus memory cell distribution was equal (55% to 60% were of the memory phenotype, data not shown). In both strains, the $\beta^{7\text{high}}$ lymphocytes expressed various levels of L-selectin, but in NOD mice, L-selectin expression was slightly more pronounced, and a small fraction of $\beta^{7\text{high}}$ lymphocytes in NOD mice were even L-selectin$h^h$ (Fig 7B).
DISCUSSION

The $\beta_7$-integrins on lymphocytes are associated with the mucosal immune system. To study the compartmentalization of cells expressing high levels of $\beta_7$-integrins (as $\alpha_4/\beta_7$ and/or $\alpha E/\beta_7$) further, we compared the distribution of such lymphocytes in two strains of mice (BALB/c and NOD) and found that, between these two strains, major differences in their distribution among various lymphoid tissues exist. Unlike in BALB/c mice, in NOD mice a conspicuous population of $\beta_7$-integrin-high lymphocytes consisting of three subpopulations, according to their expression of the $\alpha$-chain ($\alpha_4/\beta_7^{+/}$ $\alpha E/\beta_7^{+/}$ cells; $\alpha_4/\beta_7^{+/}\alpha E/\beta_7^{+/}$ cells; and $\alpha_4/\beta_7^{+/}/\alpha E/\beta_7^{+/}$ cells), was found in nonmucosal lymphoid tissues. Most of these lymphocytes, including the $\alpha E/\beta_7^{+/}$ cells, were CD8$^+$ T cells like IELs but unlike IELs, many of these cells were also L-selectin$^+$. The $\beta_7$-integrin-high lymphocytes contained lymphocytes of both naive and memory/effector subtypes.

Although at much lower frequency, $\beta_7$-high lymphocytes were also found in PLNs of BALB/c mice in this as well as in a previous study. Lefrancois et al found that a population of PLN lymphocytes in C57/BL mice express high levels of $\beta_7$-integrin, suggesting that the number of these cells in PLNs varies between mouse strains. We have tested several strains (DBA, C57/BL, SJL, SWR, NON, NOR) for numbers of $\beta_7$-high cells in PLNs and found that although C57/BL mice have a relatively large population of lymphocytes expressing $\beta_7$-integrin, the level of $\beta_7$-integrin expression on these cells in C57/BL mice is clearly below that in NOD mice. The closely related nondiabetic strains NOR and NON mice have a population of $\beta_7$-high cells comparable to that in NOD mice, suggesting that the presence of increased numbers of $\beta_7$-high cells in PLNs represents a genetic trait common to mouse strains with a potentially autoimmune-prone background.

In both NOD and BALB/c mice, $\beta_7$-high lymphocytes found in PLNs were phenotypically very similar: the majority expressed $\alpha E$, either with or without $\alpha_4$, and a smaller fraction expressed $\alpha_4$ alone. In both subsets of $\beta_7$-high lymphocytes, there were those which expressed higher than average levels of CD44/pgp-1 and intermediate-to-low levels of CD45RB (especially the $\alpha_4^+$ cells). Therefore, it is likely that a substantial part of the $\beta_7$-high lymphocytes harbored in PLNs are memory cells. These cells are likely to represent lymphocytes that were previously activated in mucosal lymphoid tissues and, during their recirculation, spread to nonmucosal lymphoid tissues with an increased tendency in NOD mice. Consistently, a population of $\beta_7$-high lymphocytes was identified in the blood of unmanipulated NOD mice, which indicates that these cells recirculate and not merely represent a population that can be found in lymphoid tissues. Furthermore, the frequency of circulating $\beta_7$-high lymphocytes that express L-selectin in NOD mice could be connected to their frequent homing to PLNs.

Interestingly, a conspicuous proportion of BALB/c lymph node lymphocytes can also be induced to express $\alpha E/\beta_7$-integrin by in vitro treatment of these cells with transforming growth factor-$\beta$ (TGF-$\beta$) and an antibody against the Tcr-complex. Therefore, we considered the possibility that the presence of $\beta_7$-high lymphocytes would merely be caused by environmental factors in NOD PLNs. To this end we depleted NOD PLN lymphocytes of $\beta_7$-cells, labeled the $\beta_7$-depleted cells, and injected them into normal NOD recipients. In this experiment (data not shown), during 4 days of follow-up, no $\beta_7$ expression developed in $\beta_7$-depleted donor lymphocytes that were recovered from recipient lymph nodes. This favors the hypothesis that $\beta_7$-high lymphocytes in NOD PLNs have homed aberrantly to these organs and not developed from lymphocytes already residing there. Although NOD mice are prone to develop autoimmune diabetes and may therefore harbor lymphocytes undergoing immune

![Fig 7. Circulating $\beta_7$-high lymphocytes in NOD and BALB/c mice. (A) $\beta_7$-high lymphocytes, and to a lesser extent, $\alpha E$-lymphocytes, appear as a distinct population in the circulation of NOD mice. (B) Circulating $\beta_7$-high lymphocytes express variable levels of L-selectin in both strains, but the percentage of $\beta_7$-high lymphocytes which express intermediate and high levels of L-selectin is higher in NOD mice. Lymphocytes were isolated from unmanipulated 15-week-old female mice and stained as described in Materials and Methods.](image-url)
stimulation in their lymphoid organs, we find it unlikely that a general immune stimulation would be the reason for finding many $\beta^{+\text{high}}$ lymphocytes in PLNs of NOD mice. This is based on the fact that the percentage of such lymphocytes did not increase with age in lymph nodes, irrespective of an increasing level of inflammation in the pancreas. Further, a study of human mucosal lymphocytes derived from bronchoalveolar lavages found no increase in the number of $\alpha E/\beta^{+}$ lymphocytes during asthma and berylliosis, which suggests that expression of this integrin would not be subject to much upregulation during inflammation. On the other hand, lymphocytes expressing high levels of $\beta$ are very abundant during the development of inflammatory infiltrates in the pancreas of NOD mice, suggesting that they are involved in the autoimmune pathology of these mice.

To study the recirculation and homing receptor usage of $\beta^{+\text{high}}$ lymphocytes that were accumulating in nonmucosal lymphoid tissues in NOD mice, NOD lymph node cells were isolated, fluorescently labeled, and, in some experiments, treated with antibodies against homing receptors, and then injected into nonmanipulated recipients. These studies indicated that $\beta^{+\text{high}}$ lymphocytes homed equally well to peripheral and mucosal lymphoid tissues. Because the $\beta^{+\text{high}}$ lymphocytes were divided into subpopulations according to their expression of $\alpha 4/\beta$, $\alpha E/\beta$, L-selectin, and $\alpha 4$, it was important to determine if these subsets dispersed in a different fashion during their recirculation and homing in the recipient mice. However, different $\beta$-integrin$^{+\text{high}}$ lymphocyte subsets appeared to behave in a similar fashion during their recirculation, since the proportions of $\beta^{+\text{high}}$ lymphocytes expressing $\alpha 4$, $\alpha 4/\beta$, or L-selectin were essentially the same in PLNs and in PPs. Therefore, they were studied as one subpopulation of lymphocytes also when determining their homing receptor usage by antibody treatments of injected cells. These treatments indicated that their homing to PLNs depended mainly on L-selectin, whereas their homing to mucosal lymphoid tissue depended mostly on $\alpha 4/\beta$. Given the fact that $\beta^{+\text{high}}$ lymphocytes contained a subpopulation of cells that were phenotypically effector/memory cells (they expressed high levels of CD44 and low levels of CD45RB), it appears that at least a part of $\beta^{+\text{high}}$ lymphocytes can be viewed as mucosa-derived lymphocytes that normally home preferentially to mucosal tissues but appear to retain a dual homing-specificity in NOD mice. On the contrary, it is likely that the rest of the $\beta^{+\text{high}}$ lymphocytes are naive lymphocytes, given the fact that in the thymus, $\beta$-integrin was also expressed on a subset of mature CD8$^+$ thymocytes (and data not shown).

Because whole MoAbs were used for the inhibition of lymphocyte homing it is theoretically possible that injected lymphocytes which were coated with MoAbs would activate an immune response, resulting in their destruction. However, the observation time of the homing experiments was relatively short (30 minutes), meaning that there would be very little time for Fc-mediated removal of the injected cells. Furthermore, anti-L-selectin, anti-$\beta$-integrin, and the control MoAb used were all of the same Ig isotype (IgG2a), which means that all cells irrespective of the MoAb treatment applied should be comparable. Thirdly, it is unlikely that Fc-mediated killing of injected, MoAb-coated cells could explain the differences in the percentages of $\beta^{+\text{high}}$ lymphocytes which, for example, were found in PLNs or MLNs, respectively.

Postcapillary venules in lymphoid tissues are lined by high endothelial cells that specialize in interacting with lymphocytes and in mediating lymphocyte extravasation and homing into the tissue. Therefore, we addressed the possibility that aberrant homing of $\beta^{+\text{high}}$ lymphocytes to all lymph nodes in NOD mice might be caused by inappropriate expression or function of vascular addressins or other endothelial adhesion molecules on these venules. Interestingly, we found that in adult mice, the mucosal vascular addressin MadCAM-1 was expressed more frequently and more intensely on PLN HEVs of NOD mice than of BALB/c mice. In the recirculation assays, the anti-MadCAM-1 MoAb MECA-367 inhibited PLN homing of all lymphocytes in NOD mice by 37%, whereas in another study, MECA-367 inhibited the homing of BALB/c lymphocytes to PLNs by 25%. This implies that aberrant MadCAM-1 expression in NOD PLNs affects lymphocyte homing. However, the homing of $\beta^{+\text{high}}$-lymphocytes to PLNs was inhibited most effectively by anti-L-selectin MoAb, this inhibition being much more pronounced for these lymphocytes than for lymphocytes in general. Therefore, despite their high-level expression of $\beta$-integrin, their homing to PLNs is mediated by L-selectin rather than by an $\alpha 4/\beta$-MadCAM-1 interaction. At a young age, when $\beta^{+\text{high}}$ lymphocytes are numerous in PLNs of BALB/c (and other) mice, MadCAM-1 is expressed at moderate levels on PLN HEVs. During the first weeks of life, however, MadCAM-1 expression rapidly declines which coincides with the disappearance of $\beta^{+\text{high}}$ lymphocytes from PLNs. This natural association supports a role for MadCAM-1 in the homing of $\beta^{+\text{high}}$ lymphocytes to PLNs at an early age, and may therefore also affect their sustained homing in NOD mice. Given the fact that the anti-L-selectin MoAb effectively inhibited the homing of $\beta^{+\text{high}}$ lymphocytes to PLNs but the anti-$\beta$ MoAb failed to do so, MadCAM-1 could perhaps be involved, together with the peripheral vascular addressins, in the presentation of carbohydrate ligands to L-selectin.

In conclusion, our results show that lymphocytes which, according to their surface phenotype, can be viewed as mucosa-associated effector/memory cells have a dual homing specificity and may also home to nonmucosal (peripheral) lymphoid tissues in NOD mice. A substantial proportion of these cells also express the peripheral node homing receptor L-selectin which selectively mediates their homing to peripheral lymphoid tissues. Although the consequences of this dual homing specificity of these cells remains elusive, they could be implicated in the immune pathology of NOD mice by enhancing the spreading of antigen-experienced lymphocytes to unrelated tissues where potential autoantigens could hence become exposed.

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