CD73 Mediates Adhesion of B Cells to Follicular Dendritic Cells

By Laura Airas and Sirpa Jalkanen

Lymphocyte-vascular adhesion protein-2 was recently identified as CD73. The CD73 molecule, otherwise known as ecto-5'-nucleotidase, is a lymphocyte maturation marker that is involved in intracellular signaling, and lymphocyte proliferation and activation. We now show that CD73, in addition to mediating lymphocyte binding to endothelial cells, also mediates adhesion between B cells and follicular dendritic cells (FDC), as a monoclonal antibody (MoAb) against CD73 inhibited the aggregation of isolated germinal center B cells and FDC in vitro. Cytocentrifuge preparations of isolated germinal center cells and two-color immunofluorescence stainings of different tonsillar B-cell populations show that CD73 is expressed on FDC and on small, recirculating IgD+ B cells, but only on a few B cells inside the germinal center. Thus, we propose that CD73 on FDC has an important role in controlling B cell-FDC interactions and B-cell maturation in germinal centers.

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MATERIALS AND METHODS

MoAbs and other reagents. MoAb 4G4 (IgG,) is reactive with CD73, CD5 MoAb against complement receptor 1 (CR1, CD35) and phycocerythrin (PE)-conjugated anti-CD19, anti-CD3, anti-CD4, anti-CD8 MoAbs, and streptavidin-PE (SA-PE) were from Becton Dickinson (Mountain View, CA). PE-conjugated anti-CD38, anti-CD45RA, and anti-CD45RO MoAbs were from Pharmingen (San Diego, CA), and MoAb against VLA-4 (HP2/1) was purchased from Immunotech (Marseille, France). Hybridoma cell lines producing MoAbs against CD18 (HB203), IgD (HB70), CD38 (CRL8022), CD20, CD3 (CRL8001), CD4 (CRL8002), and CD8 (CRL8014) were obtained from the American Type Culture Collection (Rockville, MD). A hybridoma cell line producing MoAb against VCAM-1 (P8B1) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). Anti-ICAM-1 MoAb was a kind gift from Dr Timothy Springer (Dana-Farber Institute, Boston, MA). Production of SC3 MoAb recognizing ICAM-1 has been described earlier.

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MoAb IB2 recognizes Vascular Adhesion Protein-1 (VAP-1) and Hermes-3 is reactive with CD44. The characterization of both of these MoAbs has been described earlier.\textsuperscript{24,25} MoAbs against CR1 and VCAM-1 were used as markers for FDC.\textsuperscript{21,24} An MoAb against IgD was used as a marker for small mantle zone primary follicle B lymphocytes\textsuperscript{7} and an MoAb against CD38 was used for detection of germinal center B cells (CD38\textsuperscript{+} cells\textsuperscript{7}). Irrelevant antibodies against chicken T cells (3G6, IgG), and 10D3, IgM) were used as negative controls. For two-color immunofluorescence staining, MoAbs 10D3 and 4G4 were biotinylated with Biotin-NHS (Calbiochem, La Jolla, CA), according to manufacturer’s instructions.

**Cells and cell lines.** Human PBL from healthy, voluntary donors were isolated using Ficoll-Hypaque (Histopaque-1077; Pharmacia, Uppsala, Sweden). Tonsillar B lymphocytes were isolated by mincing fresh tonsillar tissue, filtering the released cells through a metal screen and finally doing a Ficoll-Hypaque centrifugation. CD3\textsuperscript{+}, CD8\textsuperscript{+}, and CD4\textsuperscript{+} cells were depleted from the total lymphocyte population using magnetic beads (Dynal, Oslo, Norway) following the manufacturer’s instructions. The resulting B-cell-enriched population contained more than 95% CD19\textsuperscript{+} cells and less than 3% CD3\textsuperscript{+} cells. To obtain a B-cell population enriched for germinal center cells, lymphocytes were layered on Percoll gradients (Pharmacia), consisting of five density layers (1.085/1.077/1.067/1.056/1.043), and centrifuged for 20 minutes at 1,200g. Low-density cells with densities less than 1.067 were collected and used in further experiments, and T cells and IgD\textsuperscript{+} cells were depleted as described above.

For germinal-center binding assays, lymphocytes from the total tonsillar lymphocyte population were cultured in complete medium (RPMI 1640 containing 10% fetal calf serum [FCS], 100 U/mL penicillin, 100 mg/mL streptomycin, 4 mmol/L L-glutamine, 25 mmol/L HEPES) containing fungizone, 90 mg/mL gentamycin, and Staphylococcus aureus Cowan Strain 1 (SAC) at 1:5,000 dilution for 3 days.

**FDC isolation from tonsillar tissue was performed as described earlier.**\textsuperscript{21,26} Briefly, fresh tonsillar tissue was cut into small pieces which were then incubated under continuous rotation in 10 mL of Iscove’s modified Dulbecco’s medium (IMDM) with 15 U/mL type X1 collagenase (Sigma, St Louis, MO), 10 U/mL DNAse I (Boehringer Mannheim GmbH, Mannheim, Germany), 5 mmol/L EDTA, 0.5% bovine serum albumin (BSA; Bovine Albumin Fraction V), 0.05% streptomycin (ICN Biomedicals, Bucks, CA) and 90 mmol/L HEPES at room temperature for 30 minutes. Next, the cells were centrifuged for 600g at 4°C, resuspended in 3 mL of IMDM, and subjected to 1g sedimentation (0°C, 30 minutes) in discontinuous BSA gradients consisting of layers of 1.5%, 2.5%, 5.0%, and 7.5% BSA in phosphate-buffered saline (PBS). Cells at the interface between 5.0% and 2.5% BSA were collected and layered on Percoll gradients (Pharmacia), consisting of layers with densities of 1.070, 1.060, and 1.030, and centrifuged for 20 minutes at 1,200g. Cells with densities less than 1.060 were collected and used for further experiments.

The U266B1 myeloma cell line was a kind gift from Dr J.-Y. Bonnefoy (Glaxo Institute for Molecular Biology, Geneva, Switzerland).

**Immunoperoxidase staining.** Immunoperoxidase staining of aceto-fixed cytocentrifuge preparations (50,000 FDC per microscope slide) and tonsillar frozen sections was performed as described earlier.\textsuperscript{4} Briefly, the first-stage MoAb was used as a hybridoma supernatant or purified MoAb at a saturating concentration. The second-stage MoAb was a peroxidase-conjugated rabbit-antimouse IgG (Dakopatt, Glostrup, Denmark), at 1:40 dilution, containing 5% normal human AB-scrum (Finnish Red Cross, Helsinki, Finland).

**Immunofluorescence staining of cells.** Immunofluorescence staining of PBL and freshly isolated tonsillar lymphocytes was performed as described earlier.\textsuperscript{7} Briefly, for single-color immunofluorescence staining, cells were incubated either with an unconjugated first-stage antibody and a fluorescein isothiocyanate (FITC)-conjugated sheep-antimouse IgG (Sigma), or with a biotinylated MoAb followed by SA-PE at 1:40 dilution, or only with a PE-conjugated first-stage antibody. For two-color analyses, the cells were first incubated with an unconjugated first-stage antibody, followed by an FITC-conjugated sheep-antimouse IgG. After three washes, biotinylated 4G4 MoAb or biotinylated negative control antibody containing 2% normal mouse serum was incubated with the cells at saturating concentrations for 15 minutes and, after two washes, SA-PE at 1:1 dilution was added and incubation was continued for another 15 minutes. Alternatively, directly conjugated PE-CD19 or PE-CD38 were used. After two final washes, detection of different surface antigens was performed using fluorescence activated cell sorter (FACSScan; Becton Dickinson).

**Germinal center binding assay.** The binding of U266B1 cells or SAC-stimulated tonsillar lymphocytes to tonsillar germinal centers on frozen tissue sections was assessed using a modification of a previously reported method.\textsuperscript{29} Briefly, U266B1 cells and lymphocytes were pretreated with a given MoAb for 25 minutes at room temperature. Eight-micrometer tonsillar frozen sections on glass slides inside a 2.2-cm-diameter well were incubated with the indicated MoAb at room temperature. Before the binding assay, the MoAb from tissue sections was carefully removed by tilting the glass slide, and the pretreated U266B1 cells and lymphocytes were washed twice, resuspended in RPMI medium containing 5% FCS and 25 mmol/L HEPES buffer at a concentration of 30 × 10\textsuperscript{6} cells/mL, and filtered through a nylon mesh to remove cellular aggregates. One hundred microliters of this cell suspension was placed onto the tissue section. Slides were rotated 70 rpm for 25 minutes at 25°C in a humidified chamber, the nonadherent cell suspension was removed by tilting the slides, and the remaining cells were fixed in cold 3% glutaraldehyde in PBS overnight. The adherence of U266B1 cells or lymphocytes to germinal centers was examined on serial tissue sections by dark field microscopy or by light microscopy after staining. The number of cells bound to germinal centers on serial tissue sections in the presence of different MoAbs was counted. For presenting the data, the level of binding was expressed as follows: on average ≥50 cells/germinal center = +++, representing extensive binding (see Fig 5a, c, and d); 30 to 50 cells/germinal center = +++, representing intermediate binding; 10 to 30 cells/germinal center = +, representing weak binding; and ≤10 cells/germinal center = −, representing rare cells or no cells bound to germinal centers (Fig 5b and e).

**FDC–B-cell aggregation assay.** The binding of B cells to FDC was assessed using a modification of a previously reported method.\textsuperscript{13} Briefly, isolated FDC preparations consisting of 5% to 15% FDC, 80% to 90% B cells, and 2% to 5% T cells, as analyzed by FACS (not shown), were seeded in 96-well flat-bottom microtiter plates, 2 × 10\textsuperscript{3} cells per well in 50 μL of complete medium. One hundred
Fig 1. Expression of different antigens on FDC and coprecipitated lymphocytes. Cytocentrifuge preparations of isolated FDC were immunoperoxidase stained for CD73 (a and b), CR1 (c and d), VCAM-1 (e), and CD44 (f), as described in Materials and Methods. (Original magnifications: a through d and f, ×400; e, ×250). In (b) and (f), arrows point to single, large, binucleated cells typical of FDC morphology. In (d), a large in vivo formed cluster consisting of FDC and B cells strongly stained with anti-CR1 MoAb can be seen. In (e), arrowheads point to VCAM-1-positive FDC. In (f), single, positively stained lymphocytes can also be seen (arrowheads). In (g), single CD8 positive T cells can be seen, and (h) shows a CD20 B cell/FDC cluster.
microliters of MoAb was added to the wells where indicated, either as tissue culture hybridoma supernatant or as purified antibody diluted in RPMI at 20 µg/mL. After careful mixing the plates were incubated for the indicated times at 37°C, after which the cells were fixed by addition of 100 µL of ice-cold 1% formaldehyde in PBS.

To study the bivalent cation requirement of the aggregation formation, cells were plated in Hanks’ balanced salt solution (HBS) containing 2 mg/mL D-glucose (without Ca²⁺ and Mg²⁺), and incubated as described above. For studying the metabolic requirement of aggregate formation, cells were plated in complete medium and incubated for the indicated time periods at 4°C. The numbers of cell aggregates (>4 cells in a cluster) in six different microscopic fields in two duplicate wells were counted in a blind assay by two independent readers in half of the experiments and by one reader in the rest. For presenting the data, adhesion was scored as follows: 0, mean of 0-5 aggregates/field counted from six different microscopic fields (magnification 200×); 1, 6-10 aggregates/field; 2, 11-15 aggregates/field; 3, 16-25 aggregates/field; 4, 26-35 aggregates/field; 5, 36-50 aggregates/field; 6, >50 aggregates/field. Data shown are the mean score from two to six independent experiments ± SEM.

Statistical analysis. The results are expressed as mean values ± SEM. Statistical significance was evaluated by paired Student’s t-test.

RESULTS

Expression of CD73 in tonsil. In tonsil, CD73 is expressed on venules, on the basal layer of the surface epithelium, and in the germinal centers.1,4 CD73 expression in mature secondary follicles has been well reported: mantle zone lymphocytes surrounding the germinal center are CD73⁺ and the FDC in the dark zone and in the basal light zone of the germinal center express CD73.1,4 CD73 expression of lymphocytes has been shown to depend on the maturational stage of the cell.5-9 The expression of CD73 inside germinal centers also seems to be tightly controlled because only certain germinal centers display CD73 reactivity. Based on the histologic appearance of the follicles, we believe that the CD73 expression on FDC depends on the developmental stage of the germinal center reaction. Large, mature germinal centers strongly express CD73,1,3,4 but in the early secondary follicles there are no CD73⁺ FDC in the germinal center (Fig IA and B). Primary follicles are entirely CD73⁺ (Fig 1B). For comparison, IgD expression on small B cells of a mantle zone and a primary follicle and CD38 expression on activated germinal center B cells are shown (Fig 1C and D).

Expression of CD73 on tonsillar lymphocytes versus PBL. To study the expression of CD73 on different functional subpopulations of lymphocytes in tonsil, tonsillar lymphocytes were isolated and the expression of CD73 on the isolated cells was studied using two-color immunofluorescence staining. As shown in Table 1, the percentage of CD73⁺ cells in different tonsillar lymphocyte subpopulations is similar to that seen in PBL subpopulations, with a large proportion of B lymphocytes expressing CD73. There is also a high percentage of CD73⁺ cells among CD8⁺ lymphocytes (Table 1). Only a few percent of the CD4⁺ cells in tonsil express CD73, whereas the percentage of CD73⁺ CD4⁺ cells among the PBL is in the range of 7% to 19%, with the average of 13% (Table 1). Twenty-five percent ± 3% of all tonsillar lymphocytes (n = 12; range, 8% to 46%) and 15% ± 2% of PBL (n = 12; range, 9% to 30%) were found to be CD73⁺ (Table 1). Next, we studied the relative expression

<table>
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<th>Expression of CD73 in Tonsillar Lymphocytes and PBL</th>
<th>CD73⁺ Cells</th>
<th>n</th>
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<tr>
<td><strong>Tonsillar lymphocytes</strong></td>
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<tr>
<td>Total population</td>
<td>25 ± 3*</td>
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</tr>
<tr>
<td>CD3⁺</td>
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</tr>
<tr>
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<td>6</td>
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<tr>
<td><strong>PBL</strong></td>
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<td></td>
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<tr>
<td>Total population</td>
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<td>12</td>
</tr>
<tr>
<td>CD3⁺</td>
<td>16 ± 3</td>
<td>7</td>
</tr>
<tr>
<td>CD4⁺</td>
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<td>7</td>
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<tr>
<td>CD8⁺</td>
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<td>7</td>
</tr>
<tr>
<td>CD19⁺</td>
<td>70 ± 7</td>
<td>7</td>
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One-color or two-color immunofluorescence staining was performed on isolated tonsillar lymphocytes and PBL as described in Materials and Methods and cells were analyzed using FACS.

* Results are expressed as mean ± SEM of the number of indicated experiments.

Fig 2. Expression of CD45RA⁺ and CD45RO⁺ lymphocytes in peripheral blood (panels on the left) and tonsil (panels on the right) was studied using two-color immunofluorescence staining. The percentage of positive cells in each quadrant is indicated. In these samples, after reducing the background staining seen in presence of the negative control antibody, 69% of CD73⁺ lymphocytes were CD45RA⁺ and 43% of CD73⁺ lymphocytes were CD45RO⁺ in the peripheral blood, and −100% of CD73⁺ lymphocytes were CD45RA⁺ and 4% of CD73⁺ lymphocytes were CD45RO⁺ in the tonsil. Division into quadrants was established on the basis of the fluorescence of the negative control (shown in panels 1 and 2).
expression of CD73 on the lymphocyte surface is not known. Only ∼4% of CD73+ cells express CD45RO (Fig. 2). In the staining of germinal center B cells; isolation and staining procedures were performed as described in Materials and Methods.

Levels of CD45RA, CD45RO, and CD73 molecules in tonsillar lymphocytes versus PBL. This showed that almost all CD73+ cells in tonsil belong to the CD45RA+ population, whereas only ∼4% of CD73+ cells express CD45RO (Fig. 2). In the peripheral blood, a much higher percentage of CD73+ cells express CD45RO (43%; Fig. 2). In both B cells and T cells the expression of CD73 has been connected to the maturational stage of the cell, but what exactly regulates the expression of CD73 on the lymphocyte surface is not known at present.

Expression of CD73 on tonsillar B cells. To examine the expression of CD73 on different populations of tonsillar B lymphocytes we isolated tonsillar B cells and performed two-color immunofluorescence stainings on these. Data from these experiments showed that within the IgD+ population originating from the mantle zone and the primary follicles, ∼75% of cells were CD73+. The IgD- B cells originate either from the germinal center (IgD-CD38+ cells) or from the mantle zone (IgD-CD38- memory cells). Within the IgD+ population ∼20% of cells were CD73+. Staining of a subpopulation of tonsillar B cells depleted of IgD+ and CD38- cells confirmed that the CD73+ IgD+ cells belong to the resting IgD+CD38- memory B-cell population; a large proportion of the cells in the IgD+CD38- population were CD73+ (data not shown). In addition, practically none of the CD38high germinal center B lymphocytes were CD73+ when stained by two-color immunofluorescence staining (Fig. 3), confirming previous data showing that germinal center B cells do not express CD73 to any significant degree.1,2 Germinatal center B cells were also isolated by gradient centrifugation and depletion of IgD+ cells. Staining of these low-density IgD+ cells showed that ∼95% of them were CD38high and only ∼2% of them were CD73+. Thus, germinal center expression of CD73 is mainly concentrated on the FDC.

Expression of CD73 on isolated FDC. Cytocentrifuge preparations of isolated tonsillar FDC are shown in Fig. 4 (see page 1757). The isolation procedure described here results in a mixed population of FDC and B lymphocytes with a few T lymphocytes. Clusters of cells consisting of B cells bound to FDC in vivo can be seen (Fig. 4a, d, e, and h), but also singular, large, binucleated cells possessing antigens characteristic of FDC are found (Fig. 4c). Isolated FDC were expressing CR1 and VCAM-1 (Fig. 4c, d, and e) and CD73+ staining could also be detected in these cytocentrifuge preparations of isolated FDC (Fig. 4a and b); about 50% of the VCAM-1+ cells/aggregates were CD73+ (data not shown). However, B cells clustered with FDC were CD73- (Fig. 4a, and data not shown). As expected, CD4 and CD8 were not expressed on FDC but could be detected on individual lymphocytes (Fig. 4g, and data not shown). CD44 expression on isolated FDC as well as on tonsillar lymphocytes could be detected (Fig. 4f). Thus, these data obtained from freshly isolated cell preparations confirm that CD73 is expressed on FDC but not significantly on germinal center B lymphocytes.

CD73 mediates adhesion of B cells to FDC. To study whether CD73 has a role in mediating cellular adhesion in germinal centers, a modification of an assay described by Freedman et al.20 was used. In some of the experiments we used a myeloma cell line, U266B1, which expresses CD73 and VLA-4 but not other molecules shown to be involved in lymphocyte adhesion, such as LFA-1 or L-selectin.22 U266B1 cells adhere to germinal centers in a highly specific manner when the cells are incubated on tonsillar sections at 25°C under conontinue rotation. MoAb 4G4 against CD73 on either U266B1 cells or tonsillar sections had no inhibitory effect in this frozen section binding assay (Fig. 5a) when compared with adhesion in the presence of an irrelevant MoAb 3G6 (Fig. 5c), whereas antibodies against VCAM-1 on tonsillar sections and against VLA-4 on U266B1 cells were able to nearly completely block the adhesion (Fig. 5b). Lymphocytes isolated from tonsil and cultured for 3 days with SAC bind to germinal centers on tonsillar tissue sections in a highly specific way, comparable to that described for cells of the U266B1 cell line (Fig. 5d). We used the in vitro SAC-activated lymphocytes to study the involvement of a number of adhesion molecules in lymphocyte binding to germinal centers. Again, the VLA-4/VCAM-1 receptor-ligand pair was seen to be completely responsible for the binding, whereas antibodies against CD73, LFA-1, ICAM-1, ICAM-2, CD38, and VAP-1 had no inhibitory effect (Fig. 5d and e, and Table 2).

Next, we studied the effect of different antibodies on aggregation of FDC and B lymphocytes isolated from tonsillar germinal centers. An MoAb against CD73 was able to inhibit...
the formation of FDC-B cell clusters (Fig 6c and f). An anti-LFA-1 MoAb completely inhibited cluster formation (Fig 6b and f), whereas the inhibition obtained with a VLA-4 MoAb was also only partial (Fig 6f). An anti-CD44 MoAb induced formation of large aggregates (Fig 6d and f). The aggregation in question was both temperature and divalent cation dependent, as no aggregates were seen if cells were incubated either at low temperature, or without divalent cations (Fig 6g). Adding 1 mmol/L CaCl₂ restored the aggregation capability of the cells (data not shown). These aggregation data suggest that CD73, in mediating the adhesion of B cells to the FDC, might contribute significantly to the regulation of B-cell–FDC interactions and the selection of B cells.

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**Table 2. Binding of SAC-Stimulated Lymphocytes to Germinal Centers on Frozen Tissue Sections in the Presence of Different Antibodies**

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Binding</th>
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<tr>
<td>3G6*</td>
<td>+++</td>
</tr>
<tr>
<td>CD73</td>
<td>+++</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>+++</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>+++</td>
</tr>
<tr>
<td>VAP-1</td>
<td>+++</td>
</tr>
<tr>
<td>VLA-4 + VCAM-1</td>
<td>-</td>
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</tbody>
</table>

Binding to germinal centers on frozen tissue sections was assessed as described in Materials and Methods.  
* Irrelevant MoAb used as negative control.

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**Fig 5.** Binding of U266B1 cells and SAC-stimulated tonsillar lymphocytes to germinal centers on tonsillar frozen sections. The binding of U266B1 cells is shown in the panels on the left. Here, pretreatment of cells and tissue sections was done before the binding assay, either with (a) MoAb 4G4 against CD73, (b) MoAbs against VLA-4 and VCAM-1, or (c) negative control MoAb 3G6. The binding of SAC-stimulated tonsillar lymphocytes is shown in the panels on the right. Pretreatment of cells and tissue sections was done before the binding assay with either MoAb 4G4 (d) or with MoAb against VLA-4 and VCAM-1 (e). MZ, mantle zone; gc, germinal center. Adherence of U266B1 cells or lymphocytes to germinal centers was examined by light microscopy after staining.
CD73 IN SECONDARY LYMPHOID TISSUES

Fig. 6. Antibodies against CD73, LFA-1, and VLA-4 inhibit aggregation of B cells and FDC. A tonsillar cell preparation enriched for FDC was isolated and an aggregation experiment performed as described in Materials and Methods. (a through d) Photomicrograph of cells after a 3-hour incubation in the presence of different MoAbs. In (a), incubation was in the presence of an isotype-matched negative control antibody 3G6; in (b), in the presence of an anti-LFA-1 MoAb; in (c), in the presence of an anti-CD73 MoAb 4G4; and in (d), in the presence of an anti-CD44 MoAb. In this particular experiment the aggregation indexes (AI) in presence of the different MoAbs were the following: 3G6: AI = 5; anti-CD73: AI = 2; anti-LFA-1: AI = 6; anti-CD44: AI = 6. Panel (e) shows an area of a well immediately after addition of cells at time point 0 hours. Note that the area shown in each photomicrograph is only a small portion of a microscopic field. (f) Numbers of cellular aggregates at 1-hour, 2-hour, and 3-hour time points were counted and an AI value representing the number of aggregates was given as described in Materials and Methods. The values shown represent the mean AI ± SEM and the number of individual experiments is shown in parentheses. P values (v negative control MoAb) are indicated. (g) Aggregate formation is both temperature and divalent cation dependent. The number of individual experiments is shown in parentheses.

DISCUSSION

CD73 is expressed on the majority of peripheral blood B cells. Its expression is tightly connected to lymphocyte maturation and it has been shown to have importance in lymphocyte adhesion.1,2,4,6,8,9 We have now performed a detailed analysis on the different lymphocytic cell populations expressing CD73 in tonsil. We also show that an MoAb against CD73 inhibits aggregation of isolated germinal center FDC and B cells. This is the first report with evidence for a functional role for CD73 in the microenvironment of secondary lymphoid tissue, the site where B-cell maturation and proliferation takes place in vivo.

In immunohistochemical stainings of tonsillar frozen sections, CD73 is detected on small, recirculating mantle zone
B cells, on the B cells of a primary follicle, and on a subpopulation of FDC inside germinal centers of some secondary follicles. This FDC subpopulation has earlier been described to localize to the dark zone and basal light zone of the germinal center. Interestingly, not all germinal centers are positive in respect of CD73, but the expression of CD73 on the FDC seems to be finely regulated, perhaps depending on the developmental stage of the follicular reaction. The lymphocytes expressing CD73 in the primary follicle and in the mantle zone are resting, IgD+ recirculating B cells and small IgD- memory B cells.

When isolated tonsillar lymphocytes were analyzed in respect of different surface markers, it was noted that only 2% of CD4+ T cells, and practically none of the CD45RO- cells representing the memory T-cell subgroup, were positive for CD73. There is a clear difference compared with the PBL in this respect, as in the peripheral blood ~10% of the CD4+ T cells express CD73, and almost half of the CD45RO memory T cells are CD73+. It is possible that the CD73 molecule is downregulated from the CD4+ lymphocyte surface as they enter the tonsillar tissue. Interestingly, this seems specific for the CD4+ subtype, because the CD73 expression on CD8+ T cells and B cells in tonsil is comparable to that in the peripheral blood.

Cytocentrifuge preparations of isolated germinal center FDC and B cells confirmed the CD73 expression on FDC and lack of CD73 expression on B cells. Large, binucleated cells of typical FDC morphology were visible, as well as large aggregates consisting of several lymphocytes and an underlying FDC. The cells with FDC morphology were positively stained with FDC markers such as anti-CR1 and anti-VCAM-1 MoAbs and the lymphocytes in aggregates were CD20+, thus confirming that they were B cells. A staining with an anti-CD44 MoAb showed CD44 expression on FDC and on some lymphocytes. In previous work it has been reported that CD44 expression on germinal center lymphocytes is either low or nonexistent.21,28

Two-color immunofluorescence staining of isolated tonsillar B cells confirmed that only a few of the CD38+IgD- low-density germinal center B cells are CD73+. On the other hand, the majority of the mantle zone and primary follicle B lymphocytes were found to express CD73+. These B cells belong to the recirculating lymphocyte population that is in a continuous search for its specific antigen and periodically leaves the secondary lymphatic tissues to enter the blood circulation. Accordingly, these cells also express the adhesion molecules CD44 and L-selectin on their surface.23,27

Different combinations of adhesion molecules and signaling molecules are known to direct the homing of lymphocytes to their distinct target tissues, ie, the peripheral lymphoid tissues, the mucosa-associated lymphoid tissues, and the various tertiary lymphoid tissues.29 Our unpublished results suggest that CD73 might be specifically involved in controlling the homing of lymphocytes into inflamed skin, as an MoAb against CD73 inhibits the binding of lymphocytes to high endothelial venules (HEV) of frozen sections of inflamed skin, but has no effect on the binding of lymphocytes to tonsillar HEV (Arvilommi et al, submitted).

Clark et al20 have proposed that CD73 expressed on small B cells in secondary lymphoid tissues might have a role of an accessory molecule mediating signals that drive a distinct subset of resting B cells into the cell cycle. They hypothesize that the signals created by different accessory molecules (eg, CD73 or CD20) lead to activation of a characteristic set of adhesion molecules and thus determine the subsequent localization of this cell within the secondary lymphoid tissue.15,23 At present the molecular basis of microenvironmental homing is poorly understood and thus the potential involvement of CD73 in controlling the microenvironmental homing of lymphocytes within the lymphoid tissue, ie, in directing migration of lymphocytes from one cellular compartment to another within the tissue, is an interesting hypothesis.29

Isolation of germinal center FDC and B cells allowed us to study the functional role of CD73 expressed on FDC. A number of molecules have been reported to be essential for the generation and maintenance of a germinal center response. Among these molecules are adhesion molecules expressed on B cells and FDC. Adhesion molecules allow close contact between FDC and B cells and, in particular, the receptor-ligand pairs VLA-4-VCAM-1 and LFA-1-ICAM-1 have been thought important in this respect.16,20-23 By in vitro aggregation studies of isolated FDC and B cells we have shown that also CD73 is involved in mediating binding of B cells to FDC. Because B cells adhering to FDC are CD73+, we conclude that the CD73 molecule expressed on FDC is interacting with an as-yet-unknown ligand expressed on the B-cell surface, and so we propose an important role for CD73 in controlling B-cell-FDC interactions. Whether proliferation of germinal center B cells is halted by blocking the function of the CD73 molecule on FDC, as has been described for MoAb blocking of ICAM-1 expressed on FDC,30 is not known at present.

Our results from binding experiments with tonsillar frozen sections and U266B1 cells and in vitro SAC-stimulated tonsillar lymphocytes indicate how different binding assays can give varying results about the function of different adhesion molecules. In the frozen section assay the VLA-4-VCAM-1 receptor-ligand pair seemed fully responsible for lymphocyte adhesion to germinal centers. However, using another type of assay that measures the function of live, active FDC (v the dead cells of a frozen tissue section), we have now shown for the first time the importance of CD73 for the adhesion of B cells to FDC. Previously it has been shown that the ICAM-1-LFA-1 receptor-ligand pair mediates adhesion between isolated FDC and B cells.21,30 But again, in the frozen section assay, no inhibition could be obtained in B-cell binding to germinal centers when tonsillar sections were incubated with anti-ICAM-1 or anti-ICAM-2 antibodies20,22 (and Table 2). Therefore, it seems that the involvement of CD73 and LFA-1/ICAM-1 receptors in the regulation of B-cell/FDC binding requires functionally active cells and the frozen tissue section assay does not meet these requirements. We also show that the aggregation of isolated cells, which can be inhibited by anti-CD73 MoAb, is energy and divalent cation-dependent.

CD73 functions also as an ecto-5'-NT.1 Whether the enzyme activity is necessary for the adhesive function of the CD73 molecule is not known at present, but it seems unlikely. In ecto-enzymes that have been studied, the enzyme...
activity has not been shown to be necessary for the other functions of these molecules, i.e., the ecto-5'-NT activity of CD73 and the proteolytic dipeptidylpeptidase IV activity of CD26 were not necessary for the signaling capacity of these molecules when tested by mutagenesis studies with transformed cells. Another ecto-enzyme, namely CD38, has also been described to be involved in regulating lymphocyte adhesion. It is possible that many of these cell-surface molecules have a dual function, one in mediating cell adhesion and/or signal transduction, and the other an enzymatic function. In the case of CD38 and CD73 it is possible that the intracellular signaling mediated through these molecules alters the activation status of other adhesion molecules, and thus adhesion is regulated in that way. Interestingly, low levels of lymphocyte ecto-5'-NT activity have been measured in several immunodeficiency syndromes, and lymphocytes of patients suffering from familial reticuloendotheliosis (also called Omenn’s syndrome), an immunodeficiency syndrome characterized by high levels of serum IgE, repeated infections, and lymphadenopathy, among other symptoms, are completely devoid of ecto-5'-NT activity. Unfortu-

nately, as MoAbs against the ecto-5'-NT (CD73) molecule were not available at the time of those studies it is not known if the molecule is absent from the surface of lymphocytes (and on FDC in lymphoid tissues) or if the enzyme is present but inactive. It remains to be determined whether a link exists between the reduced ecto-5'-NT activity of lymphoid cells in the immunodeficiency diseases and the adhesive function of the CD73 molecule in secondary lymphoid tissues described in this report.

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CD73 mediates adhesion of B cells to follicular dendritic cells

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