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CD73-Generated Adenosine Restricts Lymphocyte Migration into Draining Lymph Nodes

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After an inflammatory stimulus, lymphocyte migration into draining lymph nodes increases dramatically to facilitate the encounter of naive T cells with Ag-loaded dendritic cells. In this study, we show that CD73 (ecto-5′-nucleotidase) plays an important role in regulating this process. CD73 produces adenosine from AMP and is expressed on high endothelial venules (HEV) and subsets of lymphocytes. Cd73−/− mice have normal sized lymphoid organs in the steady state, but ∼1.5-fold larger draining lymph nodes and 2.5-fold increased rates of L-selectin-dependent lymphocyte migration from the blood through HEV compared with wild-type mice 24 h after LPS administration. Migration rates of cd73+/+ and cd73−/− lymphocytes into lymph nodes of wild-type mice are equal, suggesting that it is CD73 on HEV that regulates lymphocyte migration into draining lymph nodes. The A2B receptor is a likely target of CD73-generated adenosine, because it is the only adenosine receptor expressed on the HEV-like cell line KOP.2.16 and it is up-regulated by TNF-α. Furthermore, increased lymphocyte migration into draining lymph nodes of cd73−/− mice is largely normalized by pretreatment with the selective A2B receptor agonist BAY 60-6583. Adenosine receptor signaling to restrict lymphocyte migration across HEV may be an important mechanism to control the magnitude of an inflammatory response. The Journal of Immunology, 2008, 180: 6288–6296.

Lymphocyte circulation from the bloodstream to lymph nodes is necessary for immune homeostasis (reconnaissance) under normal physiological conditions and for immune responses against exogenous Ags. This trafficking requires coordinated action of adhesion molecules, chemokines, and chemokine receptors expressed on lymphocytes and high endothelial venules (HEV) (reviewed in Refs. 1 and 2). The interaction of L-selectin with peripheral lymph node addressins (PNAd) initiates lymphocyte tethering and rolling on HEV (3). Chemokine receptor signaling activates the integrin LFA-1 on lymphocytes and induces stable adhesion via binding to ICAM-1 on HEV (4, 5), which is followed by transmigration. The importance of each molecule associated with the entrance of lymphocytes into lymph nodes through HEV has been shown by decreases in lymph node cellularity and defective immune responses in gene-targeted mice (6–10).

TLR signaling activates innate immune responses (reviewed in Ref. 11) in part by inducing APC maturation and recruitment to lymphoid organs via the afferent lymphatics (12, 13). Furthermore, recent reports showed that inflammation induced by a TLR4 or TLR9 agonist controlled naive lymphocyte recirculation in an Ag-independent manner, resulting in an increase in the number of naive lymphocytes in the draining lymph node and an increase in the efficiency of lymphocyte–APC encounters (14). TLR-dependent lymph node hypertrophy was proposed to require vascular growth and arteriole thickening. Although these changes needed at least a few days before they were detectible (14, 15), lymph node growth began within 24 h after a stimulus, implying the existence of other mechanisms that contribute to lymph node swelling. In this study, we present data to show that adenosine (Ado) receptor (AR) signaling, mediated by CD73-generated Ado, plays an important role in regulating early migration of lymphocytes to draining lymph nodes.

CD73 is a 70-kDa GPI-anchored protein with ecto-5′-nucleotidase enzyme activity that catalyzes the dephosphorylation of extracellular nucleoside monophosphates such as AMP to nucleosides such as Ado (16). Extracellular Ado can engage four types of ubiquitously expressed AR (A1AR, A2AAR, A2BAR, and A3AR) to modulate a wide array of physiological responses, including vascular tone, neurotransmission, cytokine production, heart rate, and adaptation to hypoxia (reviewed in Ref. 17). In addition to being generated by CD73, Ado can also be generated...
intracellularly through the action of cytoplasmic nucleotidases and then exported via nucleoside transporters. Extracellular Ado has a very short 1/2 because it is efficiently taken up into the cytoplasm, where it can be phosphorylated to AMP or degraded to inosine by Ado deaminase. In humans, Ado deaminase can be localized to the cell surface via binding to CD26 (18, 19), giving it the potential to inhibit AR signaling through deamination of extracellular Ado (20). Mice deficient in the expression of each AR have been engineered and characterized (21–25). Each strain has a variety of interesting phenotypes, revealing the diverse consequences of AR signaling. However, the mechanism by which extracellular Ado levels are regulated to modulate AR engagement in vivo is not fully understood.

Ado is a well-known anti-inflammatory mediator (26). Recent studies clearly showed that CD73 makes a major contribution to the generation of extracellular Ado in a number of physiologically relevant experimental models and plays a critical role in host defense systems. For example, CD73 attenuates hypoxia-induced vascular leakage, FMLP-stimulated neutrophil adhesion to endothelial cells, and neutrophil accumulation in tissues (27–29). Furthermore, cd73-deficient mice are susceptible to vascular inflammation and neointima formation due to decreased concentrations of endogenous Ado (30). CD73 deficiency increases VCAM-1 expression on endothelial cells isolated from carotid arteries through NF-κB activation; however, ICAM-1 expression is unchanged. This proinflammatory phenotype of cd73-deficient endothelium causes the arrest of monocytes and exacerbates wire-induced injury. These observations demonstrated a crucial role for CD73-generated Ado in the interaction of myeloid cells with vascular endothelium. However, the in vivo function of this molecule in lymphocyte-endothelium cross-talk remains unclear. In addition to its enzymatic role in the production of extracellular Ado, CD73 has also been characterized as a signaling molecule (31) and an adhesion molecule (32). Engagement of lymphocyte CD73 with anti-CD73 mAbs has been shown to stimulate proliferation, IL-2 secretion, and IL-2R expression (33, 34). Furthermore, blocking this molecule on lymphocytes with an Ab appears to inhibit adhesion of lymphocytes to cultured endothelial cells (35). Thus, there are multiple mechanisms by which CD73 could impact lymphocyte migration across HEV. We show in this study that cd73-deficient mice have increased rates of lymphocyte homing to draining lymph nodes, and propose that CD73-generated Ado regulates the ability of lymphocytes to migrate across HEV, thus limiting their access to inflamed lymph nodes.

Materials and Methods

**Mice**

Cd73-deficient mice developed in our laboratory (27) were backcrossed onto C57BL/6J for 14 generations. Genotyping by PCR, using primers that differentiate between the wild-type cd73 allele and the mutated cd73 allele containing a neomycin resistance cassette, was performed, as previously described. A2BAR/−/− mice were obtained from DeltaGen and have also been backcrossed onto the C57BL/6 background. All mice were bred and maintained in our animal facility under specific pathogen-free conditions. All protocols were approved by the Oklahoma Medical Research Foundation Institutional Animal Care and Use Committee.

**Cell culture**

The cell line KOP2.16 was derived from stromal cells taken from pooled mouse lymph nodes and has been described previously (36). It was cultured in DMEM supplemented with 20% FCS (HyClone), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM l-glutamine, 5 × 10−4 M 2-ME, nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. In some experiments, 20 ng/ml TNF-α (R&D Systems) was added for 3–6 h.

**Cd73 and AR gene expression**

Cd73 and AR expression were analyzed by PCR in a full-length cDNA library derived from MACs (Miltenyi Biotec)-isolated PNA+/− endothelial cells from lymph nodes (37) using previously described primers (38). In other experiments, RNA was prepared from KOP2.16, and RT-PCR was performed as described using β-actin as an internal control (38).

**Digestion of lymph nodes for characterization of HEV or enumeration of CD11c+/dendritic cells (DC) by flow cytometry**

Lymph nodes were dissected from mice, minced with scissors, and digested in RPMI 1640 containing 10% FCS, 1 mg/ml collagenase B (Roche), and 2 μg/ml DNase I (Roche) for 30 min at 37°C with shaking at 50 rpm. The cell suspension was passed through a Pasteur pipette 40 times, followed by digestion with 0.2% trypsin (Mediatech) and 0.5 mM EDTA at 37°C for 10 min. Cells were then passed through a 70-μm filter, washed, and stained.

**Immunofluorescence**

Lymphoid cells or PNA+/− cells were stained with the following mAbs: FITC anti-CD4, FITC anti-CD8, FITC anti-MHC class II, PE anti-CD11c, PE Cy5.5 anti-CD19, and allophycocyanin anti-CD45R (Caltag Laboratories); PE anti-TCRβ (BD Pharmingen); allophycocyanin anti-CD45 (Southern Biotechnology Associates); and biotinylated anti-CD73 (TY/23) (39), according to standard methods. PE-streptavidin was from BD Pharmingen. Data were collected with a FACS Calibur (BD Biosciences) and analyzed with CellQuest software. For lymphocyte migration experiments and experiments to enumerate DC, data were collected on 750,000 and 350,000 cells, respectively.

**Immunohistochemistry**

Frozen sections (7 μm) of lymph nodes were fixed with cold acetone and blocked with 3% BSA in PBS. Sections were stained with TY/23 (anti-CD73; IgG2a), followed by Alexa Fluor 488-conjugated donkey anti-rat IgG (Molecular Probes), and then blocked with purified mouse IgG at 500 μg/ml. After washing, they were then stained with Alexa Fluor 546-conjugated anti-PNA mAb MECA-79 (IgM; BD Pharmingen). Other slides were stained with a combination of TY/23 and rabbit anti-collagen IV (Chemicon International), followed by a combination of Alexa Fluor 488-conjugated donkey anti-rat IgG plus Alexa Fluor 546-conjugated donkey anti-rabbit IgG (Molecular Probes).

**Inflammatory stimuli**

Anesthetized mice were injected with 1 μg of Escherichia coli LPS (055: B5; Sigma-Aldrich) or 5 μg of poly(I:C) (Sigma-Aldrich) in 30 μl of PBS in the left front footpad using an insulin syringe. The right footpad was injected with same volume of PBS. Twenty-four hours later, brachial lymph nodes were examined as draining lymph nodes. In other experiments, mice were injected in either the rear footpad or thigh, and popliteal or inguinal lymph nodes, respectively, were studied as draining lymph nodes.

**Lymphocyte homing assay**

Total splenocytes were labeled with 0.25 μM 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes) for 30 min at 37°C. Ten million labeled cells were injected i.v. into mice, and 1 h later, spleen and lymph nodes were harvested. In some experiments, cd73-deficient and wild-type splenocytes were labeled with 0.25 μM CMFDA and 2 μM 5(4′)-(4′-chloromethylbenzoyl)amino)tetramethylrhodamine (CMTMR; Molecular Probes), respectively, for 30 min at 37°C. Equal numbers of labeled cells were coinjected i.v. into both strains of mice. Harvested lymph nodes were pushed through 70-μm filters to make single-cell suspensions. Cells were then counted, and the percentages of labeled cells were determined by flow cytometry. In selected experiments, anti-L-selectin Ab (MEL-14; Southern Biotechnology Associates) was given to mice (50 μg/mouse i.v.) simultaneously with LPS. In other experiments, mice were pretreated with the A2BAR agonist BAY 60-6583 (40) at 0.32 mg/kg (Bayer HealthCare; dissolved in polyethylene glycol (PEG) 400 and diluted to 80 μg/ml in PBS for i.v. injection) 30 min before the injection of labeled splenocytes.

**Results**

**CD73 and AR expression in lymphoid tissues and HEV**

We previously reported the expression pattern of CD73 in lymphoid tissues of BALB/c mice (39); however, experiments indicating that CD73 expression was strain dependent prompted us to
investigate CD73 expression in lymphoid cells of C57BL/6 mice before using this strain for the experiments described in this study. Staining with mAb TY/23 revealed that ~50% of CD4+ , 85% of CD8+ , and 2% of CD19+ lymphocytes derived from lymph nodes of wild-type mice expressed CD73 (Fig. 1A). We confirmed the findings of Kobie et al. (41) that CD73 is expressed on CD4+CD25+Foxp3+ regulatory T cells. Nevertheless, cd73+/+ and cd73−/− mice had similar proportions of T cells with this phenotype (data not shown). Lymphocytes from cd73−/− mice expressed no detectable CD73, confirming the deletion. We also analyzed CD73 expression on HEV by flow cytometry, gating on the rare population of CD45−PNAd+ cells (0.05–0.15% of cells from enzyme-digested whole lymph nodes). Relatively high CD73 expression was observed on HEV compared with lymphocytes (Fig. 1B). To substantiate the results, immunohistochemistry was performed (Fig. 1C). Sections stained with anti-PNAd Ab and 24 h later, the brachial (draining) lymph node cellularity was increased (30). Taking this information into account, and considering whether CD73 plays a role in lymphocyte homing to secondary lymphoid tissue in the steady state. The sizes of spleen, peripheral lymph nodes, Peyer’s patches, and mesenteric lymph nodes in lymphoid tissue in the steady state. The sizes of spleen, peripheral lymph nodes, Peyer’s patches, and mesenteric lymph nodes of unmanipulated wild-type mice does not have an obvious function in lymphocyte homing under steady-state conditions.

Steady-state mRNA levels of cd73 and the AR were measured in HEV by PCR, using a cDNA library derived from PNAd+ endothelial cells (Fig. 1D). Cd73 and A1AR were detected, but A2AR, A2bAR, and A3AR were not expressed at detectable levels. Steady-state levels of AR mRNA in murine splenocytes are shown in Fig. 1E. All AR except the A1AR were easily detected.

**CD73-deficient mice have large draining lymph nodes**

CD73 has been proposed to modulate lymphocyte-endothelial cell interactions as an adhesion molecule (32, 35). Therefore, we asked whether CD73 plays a role in lymphocyte homing to secondary lymphoid tissue in the steady state. The sizes of spleen, peripheral lymph nodes, Peyer’s patches, and mesenteric lymph nodes in cd73-deficient mice were normal (27 and our unpublished data). Furthermore, the migration of CMFDA-labeled cd73−/− splenocytes to lymphoid tissues (spleen and lymph node) of unmanipulated cd73−/− mice was also comparable to that of cd73+/+ splenocytes to lymphoid organs of unmanipulated wild-type mice (our unpublished data). These observations suggested that CD73 does not have an obvious function in lymphocyte homing under steady-state conditions.

CD73 plays important roles in vivo in maintaining the integrity of the vascular endothelium during hypoxia (27–29) and in regulating endothelial adhesion molecule expression after wire-induced injury (30). Taking this information into account, and considering the well-known anti-inflammatory properties of Ado, we hypothesized that CD73 might also regulate lymphocyte-HEV interactions after an inflammatory stimulus. To address this issue, LPS was injected into front left footpads of cd73+/+ and cd73−/− mice, and 24 h later, the brachial (draining) lymph node cellularity was examined (Fig. 2A). The same volume of PBS was administered to the contralateral side as a control. As expected from previous studies (14, 15), the draining lymph nodes were dramatically enlarged compared with those on the control side in wild-type mice. Consistent with our hypothesis, there was a further increase in the size of the draining lymph nodes from cd73-deficient mice, which were ~1.5-fold larger than those of wild-type mice. To examine whether lymphocyte migration from the bloodstream to lymph nodes is also accelerated in cd73−/− mice, CMFDA-labeled wild-type splenocytes were injected i.v. 24 h after LPS injection and the accumulation of labeled cells in the lymph nodes was measured after 1 h by flow cytometry (Fig. 2B). Although no differences were observed between cd73+/+ and cd73−/− mice on the control side, the number of lymphocytes that migrated into the draining lymph nodes of cd73-deficient mice was 2.7-fold greater than in

**FIGURE 1.** CD73 expression on lymphocytes and HEV. A. Single-cell suspensions of lymph node cells from cd73+/+ and cd73−/− mice were stained with FITC anti-CD4, FITC anti-CD8, or PE Cy5.5 anti-CD19 plus allophycocyanin anti-CD45, purified anti-PNAd, or PE Cy5.5 anti-CD19 plus biotinylated anti-CD73 (TY/23) and PE-streptavidin or the relevant isotype-matched control Abs. CD73 expression in cd73−/− mice is shown in the shaded histograms, and that in cd73+/+ mice is shown with solid lines. Staining with isotype control Abs is shown with dotted lines. The dotted lines and solid lines are virtually overlapping. B. Other lymph node cells from wild-type mice were digested with collagenase, DNase I, and trypsin, as described in Materials and Methods, and cells were stained with allophycocyanin anti-CD45, purified anti-PNAd plus Alexa Fluor 488 anti-IgM, and biotinylated anti-CD73 plus PE-streptavidin. HEV were identified as PNAd+ cells. Frozen sections of lymph nodes were stained with anti-CD73 (TY/23) and either anti-PNAd or anti-collagen IV, as described in Materials and Methods. D. Cd73 and AR expression were assessed by RT-PCR in a cDNA library derived from PNAd+ endothelial cells. Representative results are shown from more than three experiments. E. AR expression was assessed by RT-PCR in total wild-type splenocytes. Representative results from more than three experiments are shown.
wild-type mice. These results suggest that it is CD73 on HEV (rather than on lymphocytes) that is responsible for the larger sizes of draining lymph nodes in cd73-deficient mice. Similar results were seen when poly(I:C), a TLR3 ligand, was used instead of LPS as the inflammatory stimulus (Fig. 2, C and D). Furthermore, staining with TCRβ (Fig. 2E) and B220 (Fig. 2F) Abs revealed that migration of both T and B lymphocytes was increased in cd73-deficient draining lymph nodes. Similar results were observed when LPS or poly(I:C) was injected into the rear footpad or thigh, as revealed by examination of popliteal or inguinal lymph nodes, respectively (our unpublished data).

Next, we asked whether the increased lymphocyte migration and enlarged lymph nodes seen in cd73-deficient mice were the result of increased migration across HEV. To address this question, mice were pretreated with L-selectin Ab. This Ab was chosen because lymphocyte-expressed L-selectin is known to initiate rolling on HEV (through its interaction with PNAd) and because administration of L-selectin Ab has been shown to diminish lymphocyte migration to peripheral lymph nodes under steady-state conditions in vivo (3, 42). We observed that treatment of mice with L-selectin Ab i.v. at the same time as LPS abrogated CMFDA-labeled lymphocyte migration even after 24 h in both strains of mice (Fig. 2H) and abolished the hallmark increased size of cd73-deficient draining lymph nodes (Fig. 2G).

Contribution of lymphocyte CD73 expression to migration across HEV

Because lymphocyte CD73 has been reported to be a signaling molecule, an adhesion molecule, and a maturation and subpopulation marker (16, 39), we next evaluated its role in lymphocyte migration into draining lymph nodes. We first examined the percentage of CD4+, CD8+, and CD19+ lymphocytes that coexpressed CD73 in wild-type draining lymph nodes by flow cytometry 24 h after stimulation. LPS-induced lymph node hypertrophy did not affect the CD73 expression pattern compared with that in lymph nodes from the PBS-treated side (Fig. 3A). The CD73 expression pattern was also equivalent to that in naive lymph nodes and spleen (our unpublished data). We next evaluated migration of cd73-deficient lymphocytes compared with wild-type lymphocytes in both wild-type and cd73-deficient mice. This was done by injecting mice with a 1:1 mixture of splenocytes from cd73+/+ and cd73−/− mice labeled with either CMFDA or CMTMR. Virtually identical ratios of cd73−/−:cd73+/+ lymphocytes were observed in both wild-type and cd73-deficient draining lymph nodes (Fig. 3B). These results suggest no bias between CD73-positive and -negative lymphocytes in their ability to migrate after an inflammatory stimulus. They further suggest that it is a lack of CD73 expression on HEV that is responsible for the larger sizes of draining lymph nodes in cd73-deficient mice.

Contribution of DC to increased draining lymph node size in cd73−/− mice

The accumulation of activated DC in draining lymph nodes is critical for the regulation of proinflammatory cytokine production and induction of vascular growth (15). Local injection of LPS is known...
to induce DC migration to draining lymph nodes through the lymphatics and also their maturation and cytokine production (13). Because previous reports showed that Ado is one of the key regulators of DC function (43, 44), we speculated that the hypertrophied draining lymph nodes in cd73−/− mice might be due to increased migration of DC though the lymphatics. Therefore, we measured the absolute numbers of MHC class IIhigh CD11c+ DC in the draining lymph nodes of wild-type and cd73−/− mice 6 h after the injection of LPS (Fig. 4A). As expected, the numbers of DC were increased in the draining lymph nodes of both strains of mice compared with those on the contralateral side. There was a trend toward higher numbers of DC in the draining lymph nodes of cd73−/− mice, because the average number was almost 50% higher than for wild-type mice; however, this difference was not statistically significant ($p = 0.14$). Nevertheless, these data suggest that increased cytokine production by DC could contribute to the larger size of draining lymph nodes in cd73−/− mice. It is interesting to note that the draining lymph nodes in the cd73−/− mice were significantly larger than those of wild-type mice ($p = 0.034$) even at this early time point (Fig. 4B), suggesting that the kinetics of the inflammatory response are accelerated when CD73 is absent.

**Up-regulation of CD73 and A$_{2B}$AR on HEV after an inflammatory stimulus**

Previous studies showed CD73 expression can be regulated on HUVEC by mediators that are released during an inflammatory response, such as TNF-α (45), IFN-α (46), and Ado (47). Flow cytometry revealed a slight up-regulation of cell surface CD73 on CD45+ cells in draining lymph nodes relative to its level on HEV from control lymph nodes (Fig. 5A). Due to the lack of specific AR Abs suitable for flow cytometry, we used KOP2.16, a cell line derived from lymph node endothelial cells, and semiquantitative RT-PCR to examine the regulation of AR expression. Similar to what we observed in the HEV cDNA library (Fig. 1D), KOP2.16 expressed only the A$_{2B}$AR, suggesting that the HEV cDNA library represents a specific subset of HEV. The expression of A$_{2B}$AR increased 3- to 5-fold 3 h after TNF-α stimulation in two independent experiments (Fig. 5B). These results suggest that elevated Ado, known to occur at sites of inflammation, could trigger the A$_{2B}$AR on HEV in draining lymph nodes, and that this could play a role in regulating lymphocyte migration into these nodes.

**AR stimulation inhibits the increased lymphocyte migration into draining lymph nodes of CD73-deficient mice**

Our previous findings and those of others suggest that Ado generated extracellularly by CD73 can modulate endothelial cell...
adhesion molecule expression and permeability via the A2B AR and A2BAR. Based on these findings, and our own observation that the A2B AR was the only AR expressed on HEV, we asked whether the A2B AR-specific agonist BAY 60-6583 could influence lymphocyte homing after an inflammatory stimulus. As shown in Fig. 6A, treatment with BAY 60-6583 markedly decreased (p = 0.0034) the number of lymphocytes that migrated into the draining lymph nodes of CD73-deficient mice. There was no impact on lymphocyte migration into lymph nodes on the contralateral side. The effect of BAY 60-6583 on lymphocyte migration into draining lymph nodes of wild-type mice was much more modest and did not reach statistical significance (data not shown), perhaps because of higher concentrations of endogenous Ado in these mice. All of these results support our hypothesis that CD73-generated Ado serves to regulate lymphocyte migration into draining lymph nodes after an inflammatory stimulus at least in part by triggering A2BAR signaling on HEV. Consistent with this hypothesis, A2B AR +/− and A2BAR −/− lymphocytes showed equivalent rates of lymphocyte migration into draining lymph nodes of LPS-treated wild-type and cd73 −/− mice (Fig. 6B).

Discussion

Lymphocyte homing to peripheral lymph nodes depends on interactions with HEV, specialized blood vessels that express chemokines and adhesion molecules required for lymphocyte transmigration. Although numerous reports have demonstrated the importance of L-selectin, PNAd, LFA-1, ICAM-1, and specific chemokines and chemokine receptors in the steady state, the way in which lymphocyte migration across HEV is regulated during an inflammatory response is not fully understood. The goal of this investigation was to determine whether CD73, which is expressed on basal lamina is not known. However, CD73 is a GPI-anchored protein, and previous studies by Mehul et al. (48) showed that CD73 can bind to laminin, one of the components of the basal lamina. Therefore, we hypothesize that CD73 may be synthesized in cells such as endothelial cells, cleaved from the cell surface by

FIGURE 5. CD73 and the A2B AR are up-regulated on HEV after an inflammatory stimulus. A, LPS (1 μg) was injected into the left front footpad of cd73 +/− mice, and an equivalent volume of PBS was injected into the right front footpad. Twenty-four hours later, lymph nodes were digested with collagenase, DNase I, and trypsin, as described in Materials and Methods, and cells were stained with allophycocyanin anti-CD45, purified anti-PNAd plus Alexa Fluor 488 anti-Ly5.1, and biotinylated anti-CD73 plus PE-streptavidin. HEV were identified as CD45− PNAd− cells. The relative mean (MFI) and median fluorescence intensities for CD73 staining are shown (mean ± SD; total n = 8 from three independent experiments, p < 0.01 for MFI, and p < 0.02 for median fluorescence intensities in paired t tests comparing PNAd− cells from inflamed and control lymph nodes). B, KOP2.16 cells were cultured ± TNF-α for 3–6 h. RNA was isolated and A2B AR expression was determined by semiquantitative RT-PCR on 5-fold serial dilutions of cDNA using β-actin expression as an internal standard. Data are representative of one of two experiments. Quantitation of the band intensities revealed a 5-fold increase in A2B AR mRNA at 3 h and a 2.5-fold increase at 6 h relative to β-actin expression.

FIGURE 6. The A2B AR agonist, BAY 60-6583, inhibits the increased lymphocyte migration into draining lymph nodes of CD73-deficient mice. A, LPS (1 μg) was injected into the left front footpad of cd73 +/− and cd73 −/− mice, and an equivalent volume of PBS was injected into the right front footpad. Twenty-two hours and 30 min after the LPS injection, the mice were injected i.v. with BAY 60-6583 (320 μg/kg) or an equivalent volume of diluted PEG 400 carrier, and 30 min later with 107 CFMDFAd stained cd73 +/− splenocytes. One hour after the injection of labeled splenocytes, lymph nodes were harvested, the total numbers of cells in each lymph node were counted, and the percentages of CFMDFAd + cells were determined by flow cytometry (n = 20–26; p = 0.0034 for diluted PEG 400 vs BAY 60-6583 in draining lymph nodes; data combined from four separate experiments). B, LPS (1 μg) was injected into the left front footpad of wild-type and cd73 −/− mice, and an equivalent volume of PBS was injected into the right footpad (five mice/group). Twenty-three hours later, the mice were injected i.v. with an equal mixture of 107 wild-type and A2B AR −/− splenocytes labeled with CMFDA or CMTMR, respectively. One hour later, brachial lymph nodes were harvested, and the percentages of CMFDA + and CMTMR + lymphocytes were determined by flow cytometry. Data are representative of one of two experiments.
a phospholipase, and then bind to a component of the basal lamina such as laminin.

The contribution of CD73 to the formation of extracellular Ado, a well-known anti-inflammatory mediator, has been revealed in several experimental models. For example, the anti-inflammatory action of methotrexate in the carrageenan-treated air pouch model of inflammation is dependent upon CD73 (49). Similarly, CD73-generated Ado is necessary for ischemic preconditioning in both the heart (40) and kidney (50), and protects mice from bleomycin-induced lung injury (51). Furthermore, cd73-deficient mice exhibit a vascular leak syndrome characterized by neutrophil infiltration into tissues when exposed to normobaric hypoxia, suggesting a critical role for CD73-generated Ado in vascular barrier function (27–29). In contrast, several in vitro studies suggested that CD73 functions as a costimulatory molecule on T lymphocytes (33, 34) and an adhesion molecule that is important for lymphocyte binding to endothelium (35). The possibility that CD73 could impact lymphocyte interactions with HEV by multiple mechanisms prompted us to examine the role of this molecule in lymphocyte homing to lymph nodes. We showed in this study that Ado generated by CD73 on HEV negatively regulates lymphocyte migration from the bloodstream into LPS-induced draining lymph nodes. Because cd73+/− and cd73−/− splenocytes showed equivalent rates of migration into draining lymph nodes, it is unlikely that any signaling or adhesive function of CD73 on lymphocytes plays a role in regulating migration of lymphocytes across HEV.

Although no abnormalities were observed in the cellularity of lymphoid organs of cd73−/− mice or in the migratory capacity of cd73+/− lymphocytes under steady-state conditions, cd73−/− mice had larger draining lymph nodes when LPS, a TLR4 agonist, was injected into a local site. Short-term assays with CMFDA-labeled lymphocytes administered i.v. showed increased rates of migration into the draining lymph nodes of cd73−/− mice. This observation, coupled with the forward vs side scatter profile of the lymphocytes (our unpublished data), suggested that the lymph node hypertrophy induced by LPS was not due to the proliferation of lymphocytes, but rather to the accumulation of nondividing lymphocytes. Furthermore, anti-L-selectin Ab treatment demonstrated that CD73 modulates lymphocyte migration into draining lymph nodes by an L-selectin-dependent pathway. Interestingly, both T and B lymphocyte entrance was promoted in cd73−/− mice, suggesting that a common pathway for both cell types is modulated by CD73. In addition, the percentages of CD73+ and CD73− lymphocytes did not change when splenocytes from cd73+/− mice were used as donors in migration experiments in cd73+/+ mice, indicating that the ability to migrate across HEV was not influenced by the CD73 expression status of lymphocytes. Furthermore, the migration of splenocytes from cd73+/+ and cd73−/− mice into draining lymph nodes of cd73−/− mice was comparable, demonstrating that CD73 expression on lymphocytes cannot compensate for a lack of CD73 on HEV.

Information in the literature concerning the regulation of endothelial CD73 expression by proinflammatory cytokines is conflicting. For example, Kalsi et al. (45) showed a decrease in its expression and in its enzyme activity after TNF-α treatment of HUVEC. In contrast, Niemela et al. (46) demonstrated that IFN-α and IFN-γ, but not other inflammatory cytokines such as IL-1β, IL-4, or TNF-α, could increase CD73 expression on HUVEC. Furthermore, Ado has been implicated in an increase in CD73 expression in microvascular endothelial cells that is mediated by a paracrine pathway (47). Our experiments revealed that the expression of CD73 on HEV in draining lymph nodes is up-regulated compared with HEV in the contralateral side. Although our analysis has the advantage of evaluating changes in CD73 expression in vivo, the mechanism by which CD73 expression is modulated is still unknown.

To determine whether the enhanced lymphocyte migration in cd73-deficient mice was caused by a lack of AR signaling, we treated mice with the A2AR agonist BAY 60-6583. This approach was taken because of the four known subtypes of AR; only the A2AR is found in a cDNA library derived from PNAd+ cells or in cDNA from the HEV-like cell line KOP2.16 (36). We also observed an up-regulation of A2AR expression in KOP2.16 cells after exposure to TNF-α. Indeed, BAY 60-6583 treatment markedly reduced the rate of migration of labeled splenocytes into draining lymph nodes of cd73−/− mice. These data are consistent with the hypothesis that the A2AR is at least partially responsible for the regulation of lymphocyte migration across HEV by CD73-generated Ado. Furthermore, the Ado is most likely derived from CD73 on HEV, because lymphocytes from cd73+/+ and cd73−/− mice show similar increased rates of migration across HEV in draining lymph nodes of cd73−/− mice (i.e., Ado produced by cd73+/+ lymphocytes does not appear able to trigger AR on HEV to regulate lymphocyte migration). Similarly, lymphocytes from A2ARR+/+ and A2ARR−/− mice showed similar rates of migration into draining lymph nodes of wild-type mice, suggesting that it is triggering of the A2AR on HEV that is relevant.

The expression of the adhesion molecules ICAM-1 and VCAM-1 is normal on cd73−/− HEV in the steady state (our unpublished data). Our findings differ from those in a previous report (30), which concluded that CD73 deficiency resulted in increased VCAM-1 expression and decreased ICAM-1 expression on carotid arteries due to the lack of A2AR signaling. This discrepancy could be explained by the fact that different cell types were being examined and that the A2AR, rather than the A3AR, seems to be the predominant AR on HEV. We did find that VCAM-1, but not ICAM-1, expression was up-regulated on HEV in draining lymph nodes after LPS administration and this effect was more pronounced in cd73−/− mice. However, neither anti-VLA-4 nor anti-VCAM-1 Ab treatment reversed the increased rates of lymphocyte migration into draining lymph nodes of cd73−/− mice after LPS treatment (our unpublished data), suggesting that the increase in VCAM-1 expression did not augment cell adhesion between lymphocytes and HEV. Furthermore, although the migration of CMFDA-labeled lymphocytes to draining lymph nodes was inhibited by treatment with an anti-LFA-1 Ab, the effect was the same in both cd73+/+ and cd73−/− mice (our unpublished data). Taken together, these data support the conclusion that increased lymphocyte migration into draining lymph nodes of cd73-deficient mice is not mediated by increases in cell adhesion.

We propose instead that Ado generated by endothelial cell (and/or basal lamina) CD73 regulates lymphocyte migration across HEV through A2AR signaling. The A2AR is a seven-transmembrane-spanning G protein-coupled receptor that is coupled to Gi and uses cAMP as a second messenger (52). It has been firmly established that cAMP can modulate endothelial cell-cell junctions through the protein kinase A and/or Epac-Rap1 pathways (53, 54). Other reports suggest that the A2AR can also be coupled to Go (55). In the 1970s, several studies concluded that lymph node vasculature changed within 24 h after an inflammatory stimulus, and that this was associated with changes in vascular integrity (56, 57). Our studies do not address the mechanisms by which rates of lymphocyte migration are increased after an inflammatory stimulus, but do suggest that CD73-generated Ado may trigger a feedback mechanism to keep increases in permeability under control. Additional experiments with endothelial cell lines will be required to determine whether AR signaling modulates the ability of lymphocytes to migrate across HEV through changes in myosin L chain...
phosphorylation and decreased formation of stress fibers and/or through Rapi/Epac-mediated increases in VE-cadherin-based cell-cell contacts. Future work will also address the consequences of increased lymphocyte migration into cd73−/− draining lymph nodes during an immune response.

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Disclosures

Thomas Krahn is an employee of Bayer HealthCare, the manufacturer of BAY 60-6583. All other authors have no conflicting financial interests.

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