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CD73 and Ly-6A/E Distinguish In Vivo Primed but Uncommitted Mouse CD4 T Cells from Type 1 or Type 2 Effector Cells

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Primed CD4 T cells may develop into effector T cells such as Th1 and Th2, or remain uncommitted as Th primed precursor (Thpp) cells that can subsequently differentiate into Th1 and Th2 cells. Although mouse Thpp-like cells have also been identified among spleen and particularly lymph node cells, further characterization of these cells has been difficult without a defining cell surface marker. Using Affymetrix GeneChips followed by FACS analysis, we found that in vitro-derived Thpp cells expressed CD73 but not Ly-6A/E, whereas Th1 and Th2 cells showed the reciprocal pattern. CD73+ Ly6A/E+ memory CD4 T cells were identified in normal C57BL/6 mice, and the proportion of these cells was highest in lymph nodes, lower in spleens, and lowest in the lungs. These cells produced IL-2 and MIP-1α, but much less IL-4 and IFN-γ than CD73− Ly6A/E− cells. Similar results were obtained with additional Ly-6.2 mouse strains, but not Ly-6.1 strains. Restimulation of Thpp-like CD73+ Ly-6A/E− cells in Th1- or Th2-polarizing conditions induced differentiation into populations producing mainly IFN-γ or mainly IL-4, respectively. In contrast, the effector-like CD73− Ly-6A/E+ population was more committed, and continued to produce both IL-4 and IFN-γ in both conditions. CD73 and Ly-6A/E expression therefore identify a population of Thpp-like cells in C57BL/6 mice and at least some other Ly-6.2 mice. The Journal of Immunology, 2005, 175: 6458–6464.

Depending mainly on the cytokine environment, Ag-stimulated naive CD4 T cells can differentiate in vitro into effector Th1 or Th2 cells (1, 2) or remain as uncommitted, Th primed precursor (Thpp)3 CD4 T cells (3). These nonpolarized Thpp cells mainly produce IL-2 and lymphotakin, but not Th1, Th2, or common effector cytokines such as IFN-γ, IL-4, IL-5, IL-10 or GM-CSF. However, in contrast to naive CD4 T cells, in vitro-derived Thpp cells produce biologically active CC and C chemokines, such as MIP-1α, MIP-1β, RANTES, TCA3, and lymphotoxin (4). The nonpolarized Thpp cells can further differentiate into either Th1 or Th2 effector CD4 T cells (3). Similar cells exist in vivo. Mouse CD4 T cells in lymphoid organs express a higher ratio of IL-2 to IFN-γ than cells in peripheral tissues (5). A Thpp-like population of mouse CD44highCD4− Ag-specific T cells was identified by cytokine production and split cloning in lymph nodes: these cells produced IL-2 but not IFN-γ or IL-4; and individual cells could subsequently become either Th1 or Th2 cells (6). These cells were enriched in lymph nodes relative to spleen (X. Wang and T. R. Mosmann, unpublished observations) and in vitro-derived Thpp cells homed preferentially to lymph nodes rather than spleen, lungs, or peritoneal cavity (7).

It is critical for the immune system to choose the right effector function for particular infections, otherwise the reciprocal inhibition of Th1 and Th2 responses (8) may hinder subsequent induction of the correct response. Thus the uncommitted Thpp cells might represent an expanded pool of memory cells with the flexibility to become either Th1 or Th2 cells later in the same or subsequent responses. The chemokines produced by Thpp cells may attract other immune cells into the site of a Thpp reaction, without biasing the type of response.

Similar uncommitted CD4 T cells may exist among human memory (CD45RA−) T cells. Central memory T cells expressed higher levels of CCR7 (which should enhance lymph node homing) and produced mainly IL-2 on activation (9). The effector memory cell population expressed lower levels of CCR7 and produced IL-2, IFN-γ, IL-4, and IL-5. Central memory T cell populations contained precursors of cells that could differentiate into effector cells that produce IL-4, IL-5, or IFN-γ. Although in vitro-derived mouse Thpp cells selectively express CCR7 (4), CCR7 expression is not always associated with nonpolarized memory CD4 T cells (10, 11).

Thus the further characterization of the mouse and human Thpp/central memory T cell subsets has been held back by the lack of well-defined surface markers. During the analysis of T cell gene expression (4) we found that in vitro-derived Thpp expressed CD73 but not Ly-6A/E, in contrast to the reciprocal pattern expressed by Th1 and Th2 effector subtypes. These two markers also define a Thpp-like subpopulation of in vivo memory CD4 T cells: CD73− Ly6A/E− cells are found preferentially in lymph nodes, secrete IL-2 and MIP-1α but not IL-4 or IFN-γ, and show flexibility of differentiation. Although the Ly-6A/E marker may be useful only in Ly-6.2 mouse strains, nevertheless the combination of CD73 and Ly-6A/E should have considerable utility for ex vivo isolation and further analysis of the primed, uncommitted CD4 T cell subset.

Materials and Methods

Mice

Female C57BL/6, C57L, PL, BALB/c, and C3H mice, 6- to 8-wk-old, were obtained from The Jackson Laboratory. All animal experiments were approved by the University of Rochester University Committee on Animal Resources.

Footnotes

1. This work was supported by National Institutes of Health Grant AI48604.
2. Address correspondence and reprint requests to Dr. Tim R. Mosmann, David H. Smith Center for Vaccine Biology and Immunology, University of Rochester Medical Center, Rochester, NY 14642.
3. Abbreviations used in this paper: Thpp, Th primed precursor; KLH, keyhole limpet hemocyanin; CD62L, CD62 ligand.
Abs and cell lines

Purified anti-mouse CD3e (145-2C11), CD28 (37.51), IL-2 (JES6-1A12), IFN-γ (R4-6A2), biotinylated rat anti-mouse IL-2 (JES6-5H4), IL-4 (BVDV-24G2), and IFN-γ (XMG1.2) were obtained from BD Pharmingen. Purified anti-mouse MIP-1α (39624.11) and biotinylated anti-mouse MIP-1α were from R&D Systems. Additional rat anti-mouse cytokine Abs were purified on a protein G column (Pharmacia) from the supernatants of 11B11 (IL-4), XMG1.2 (IFN-γ), and AN18 (IFN-γ) hybridoma cell lines. The following FITC-, R-PE-, CyChrome-, PerCP-, allophycocyanin-, and biotin-conjugated Abs were also purchased from BD Pharmingen: anti-CD45R/B220 (RA3-6B2), anti-CD3e (53-6.7), anti-CD3e (145-2C11), and anti-CD62 ligand (CD62L) (C6D2L, Mel-14). The cell line TA3, which expresses H-2Id/k (12) on the surface, is a hybrid between a B cell (145-2C11), and anti-CD62 ligand (CD62L) (C6D2L, Mel-14). The cell line TA3, which expresses H-2Id/k (12) on the surface, is a hybrid between a B cell (145-2C11), and anti-CD62 ligand (CD62L) (C6D2L, Mel-14).

T cell purification and differentiation

Thp, Th1, and Th2 cell populations were prepared and characterized as previously described (4). Briefly, C57BL/6 CD4 T cells were enriched by magnetic bead depletion, and naive cells (CD44low, CD4high, CD62Lhigh) were analyzed by flow cytometry, and subpopulations were sorted as indicated in the protocol. Differentiation of sorted populations was analyzed by flow cytometry, and subpopulations were sorted as indicated in the protocol. Additional genes are shown. The TCR subunit CD3 expression, we examined surface expression on in vitro-derived Thp, Th1, and Th2 cells by flow cytometry (Fig. 2). Unstimulated Thp cells expressed high levels of CD73, but very low levels of Ly-6A/E. In contrast, Th1 and Th2 cells expressed low levels of CD73, but uniformly high levels of Ly-6A/E. Activation with anti-CD3 Abs did not alter the expression of CD73 on any of the three T cell populations, but induced slightly higher levels of Ly-6A/E on Thp cells. The FACS analysis therefore confirmed significant (p < 0.05) expressed by Thp cells (Fig. 1), but not naive, Th1, or Th2 cells. Conversely, Ly-6A/E was strongly expressed in both Th1 and Th2 cells as expected (14), but not in Thp cells (Fig. 1). As controls for the fidelity of the Genechip analysis, two additional genes are shown. The TCR subunit CD3 was expressed in all nine T cell samples and T1/ST2L (15) was expressed only in resting and activated Th2 cells (Fig. 1).

In vitro CFSE labeling

Naive cells (CD44low, CD4high, CD62Lhigh) were labeled with CFSE (Molecular Probes) by incubation with 2 μM CFSE in PBS containing 5% FBS for 5 min at room temperature. Cells were then washed twice with PBS.

Isolation and analysis of lymphocytes from mouse lymphoid and nonlymphoid (lung) tissues

C57BL/6 mice were sacrificed, and after cardiac perfusion with PBS, lymphocytes were prepared from lymph nodes (a pool of cervical, axillary, brachial, inguinal, and popliteal nodes), spleen, and lungs. Single-cell suspensions of the tissues were prepared by passage through 40-μm nylon mesh, followed by depleting erythrocytes by Ficoll/Lite-LM (Atlanta Biologicals) for lymph node and spleen, or Histopaque 1083 (Sigma-Aldrich) for lung. Lymphocytes at the interface were collected and washed before staining with PerCP anti-CD4, allophycocyanin anti-CD44 (IM7), anti-CD45R/B220 (RA3-6B2), anti-CD62ligand (CD62L, Mel-14). The cell line TA3, which expresses H-2Id/k (12) on the surface, is a hybrid between a B cell (145-2C11), and anti-CD62 ligand (CD62L) (C6D2L, Mel-14). The cell line TA3, which expresses H-2Id/k (12) on the surface, is a hybrid between a B cell (145-2C11), and anti-CD62 ligand (CD62L) (C6D2L, Mel-14). The cell line TA3, which expresses H-2Id/k (12) on the surface, is a hybrid between a B cell (145-2C11), and anti-CD62 ligand (CD62L) (C6D2L, Mel-14).

Immunization and T cell subset analysis

Mice were immunized i.p. with 100 μg of keyhole limpet hemocyanin (KLH, endotoxin-free; Calbiochem) in 100 μl of PBS. After 14 days, cells from both spleen and lymph nodes (cervical, axillary, brachial, inguinal, and popliteal) were enriched for CD4 T cells by depleting CD8 and B cells on MACS columns. After staining with PerCP anti-CD4, allophycocyanin anti-CD44, FITC anti-Ly-6A/E, and PE anti-CD73. The stained cells were analyzed by flow cytometry to obtain the frequency of CD73+ Ly-6A/E− and CD73− Ly-6A/E+ memory CD4 cells.

T cell subset gene expression evaluation by GeneChip

Gene expression analysis of Thp, Th1, and Th2 cells has been previously described (4). Briefly, mRNA was extracted from stimulated or unstimulated naive, Thp, Th1, and Th2 populations, and these mRNA samples were analyzed on Affymetrix mouse U74 GeneChips. The Affymetrix Microarray Suite software was used to assess significant expression levels.

Cytokine assays

Differentiated cell populations were washed and restimulated (1 × 106/ml, 100 μl/well) for 24 h with plate-bound anti-CD3 (2 μg/ml) plus anti-CD28 (1 μg/ml), or alloantigens, or KLH (80 μg/ml) plus 5 × 105/well syngeneic splenocytes. Cytokines and chemokines produced in the supernatant were assayed by ELISA and ELISPOT as previously described (4, 6). ELISPOTs were enumerated using the ImmunoSpot reader (CTL Analyzers).

Results

In vitro-derived Thp cells express CD73 mRNA, whereas Th1 and Th2 cells express Ly-6A/E mRNA

In a previous Affymetrix Genechip analysis of cytokine and chemokine expression in activated and resting Thp, naive, Th1, and Th2 cells (4), we also examined these data sets for selective expression of cell surface proteins, to identify markers that might be used to isolate Thp cells from mixed in vivo populations.

Two genes encoding cell surface proteins were selectively expressed in Thp vs naive, Th1, or Th2 cells. CD73, a GPI-linked surface protein with ecto-5'-nucleotidase activity (13), was significantly (p < 0.05) expressed by Thp cells (Fig. 1), but not naive, Th1, or Th2 cells. Conversely, Ly-6A/E was strongly expressed in both Th1 and Th2 cells as expected (14), but not in Thp cells (Fig. 1). As controls for the fidelity of the Genechip analysis, two additional genes are shown. The TCR subunit CD3 was expressed in all nine T cell samples and T1/ST2L (15) was expressed only in resting and activated Th2 cells (Fig. 1).

In vitro-derived Thp cells express higher levels of CD73, and lower levels of Ly-6A/E proteins

To test whether these mRNA differences resulted in selective protein expression, we examined surface expression on in vitro-derived Thp, Th1, and Th2 cells by flow cytometry (Fig. 2). Unstimulated Thp cells expressed high levels of CD73, but very low levels of Ly-6A/E. In contrast, Th1 and Th2 cells expressed low levels of CD73, but uniformly high levels of Ly-6A/E. Activation with anti-CD3 Abs did not alter the expression of CD73 on any of the three T cell populations, but induced slightly higher levels of Ly-6A/E on Thp cells. The FACS analysis therefore confirmed significant (p < 0.05) expressed by Thp cells (Fig. 1), but not naive, Th1, or Th2 cells. Conversely, Ly-6A/E was strongly expressed in both Th1 and Th2 cells as expected (14), but not in Thp cells (Fig. 1). As controls for the fidelity of the Genechip analysis, two additional genes are shown. The TCR subunit CD3 was expressed in all nine T cell samples and T1/ST2L (15) was expressed only in resting and activated Th2 cells (Fig. 1).
that in vitro Thpp cells express higher CD73 and lower Ly-6A/E levels than Th1 or Th2 effector cells.

The kinetics of the differentiation of naive cells in Thpp-, Th1-, or Th2-promoting conditions were analyzed by labeling sorted naive (CD4^+CD44^{low}CD62L^{high}) cells with CFSE, stimulating with alloantigen and analyzing cell surface marker expression at different time points. Results were gated on the CD4^+CFSE^{low} 7-aminoactinomycin D^- population to analyze only live cells that had divided in response to the alloantigen. Expression of CD73 was limited to cells differentiating to the Thpp phenotype (Fig. 2B). From 5 days onward, the majority of the cells in Thpp conditions expressed CD73, whereas the frequency of CD73-expressing cells in Th1 or Th2 cultures remained low at all time points.

Ly6A/E was expressed at moderate levels under all three differentiation conditions, consistent with previous data showing that Ly-6A/E is induced on activation of T cells. However, only in the Th1 and Th2 cultures did the expression of Ly-6A/E increase to high levels (Fig. 2B). Almost all of the proliferating cells in the Th1 cultures expressed high Ly-6A/E levels by day 4, the earliest point at which proliferating cells were detected, whereas Th2 cultures showed similarly high levels of expression only after 9 days.

**CD73 and Ly-6A/E expression identify subsets of CD4^+CD44^+ spleen and lymph node T cells in C57BL/6 mice**

As CD73 and Ly-6A/E were reciprocally expressed in in vitro-derived Thpp vs Th1 and Th2 cells, we next measured CD73 and Ly-6A/E expression on CD4 T cells taken directly from normal C57BL/6 mice. Naive CD4 T cells (CD4^+CD44^{low/med}) expressed low levels of either CD73 or Ly-6A/E. Memory/activated CD4 T cells (CD4^+CD44^{high}) could be separated into two populations, CD73^+ Ly-6A/E^- and CD73^- Ly-6A/E^+ (Fig. 3A). The proportion of CD4^+CD44^+ T cells expressing Thpp-like surface markers (CD73^+ Ly-6A/E^-) was highest in lymph nodes (47%), moderate in spleen (22%), and lowest among lung lymphocytes (14%) (Fig. 3B). Conversely, the CD73^- Ly-6A/E^+ effector-like cells were most abundant in lung tissue (60%). As Thpp cells selectively home to lymph nodes rather than peripheral tissues or spleen (X. Wang and T. R. Mosmann, unpublished observations) (7), it reinforces the possibility that the CD73^- Ly-6A/E^- cells are in vivo Thpp-like cells.

**CD73 and Ly-6A/E expression identify primed, non-Th1, non-Th2 cells in vivo in C57BL/6 mice**

To test whether CD73^- Ly-6A/E^- cells expressed the cytokine pattern of in vitro-defined Thpp cells, Thpp-like (CD4^+, CD44^+, CD73^+, Ly-6A/E^-) and effector-like (CD4^+, CD44^+, CD73^-, Ly-6A/E^+) cell populations were purified by cell sorting from C57BL/6 mice that had been immunized for 2 wk with KLH. After restimulation with KLH and allophycocyanin, Thpp-like (CD73^+, Ly-6A/E^-) T cells from both spleen and lymph nodes contained a high frequency of KLH-specific cells that produced IL-2 and/or MIP-1, and not IL-4 or IFN- (Fig. 4). In contrast, substantial numbers of the effector-like cells (CD73^- Ly-6A/E^+) secreted IL-4 and IFN-γ, in addition to IL-2 and MIP-1α. CD4^+CD44^+ memory T cells of C57BL/6 mice also contained low numbers of CD73^- Ly-6A/E^- cells, which may have been contaminating naive cells because the separation of naive and memory cells by CD44 expression is not complete. More substantial numbers of CD73^- Ly-6A/E^- cells were present. When analyzed by cell sorting followed by ELISpot assay, these cells were more similar to the CD73^- Ly-6A/E^- effector cells than Thpp-like cells, as they produced IL-2, IL-4, IFN-γ, and MIP-1α (data not shown). Although we cannot exclude the possibility that there could be additional Thpp-like cells in the double-positive population, the in vivo CD73^- Ly-6A/E^- population expressed both the cell surface markers and cytokine patterns of the in vitro-defined Thpp cells.

These in vivo Thpp-like cells are present in a range of conditions. In addition to the cytokine patterns produced after KLH immunization, we also tested the cytokines produced by these two populations in response to alloantigens, which should stimulate a representative sampling of memory CD4 T cell responses, and

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**FIGURE 2.** CD73 and Ly-6A/E are reciprocally expressed on the surface of Thpp vs Th1 and Th2 cells. Thpp, Th1, and Th2 cells were derived by differentiation from naive CD4 cells in vitro. A, Resting (0 h) and anti-CD3/CD28-stimulated cells (9 h) were stained with FITC anti-Ly-6A/E and PE anti-CD73 and analyzed by flow cytometry. These results are representative of more than three experiments. B, At various times during the differentiation of naive CFSE-labeled cells, aliquots were stained and analyzed by flow cytometry. The expression patterns of CD73 and Ly-6A/E on cells within the CD4^+CFSE^{low} 7-aminoactinomycin D^- population are shown (mean ± SE of quadruplicate differentiation cultures).
anti-CD3 plus anti-CD28, to stimulate all T cells. Analyzing populations from both KLH-primed or unprimed mice, these two additional stimuli also induced mainly IL-2 and MIP-1α responses from CD73^+ Ly-6A/E^− cells, but higher levels of the effector cytokines IFN-γ and IL-4 from CD73^+ Ly-6A/E^− cells (data not shown). Also, following administration of Ag in CFA, the CD73^+ Ly-6A/E^− cells still produced a substantially higher ratio of IL-2 to IFN-γ than the reciprocal CD73^− Ly-6A/E^+ population (data not shown).

**FIGURE 3.** Expression of CD73 and Ly-6A/E on CD4 T cells from lymph nodes, spleen, and lungs. A, Lymph node cell populations (depleted of B220^+ and CD8^+ cells) from KLH-immunized C57BL/6 mice were stained with PerCP anti-CD4, allophycocyanin anti-CD44, FITC anti-Ly-6A/E, and PE anti-CD73 and analyzed by four-color flow cytometry. The panels shown were gated on CD4^+ CD44^high lymphocytes. B, Lymphocytes from lymph nodes, spleen, and lungs were analyzed by flow cytometry as in A. The proportions of CD73^+ Ly-6A/E^− and CD73^− Ly-6A/E^+ cells are shown as percentages of total CD4^+ CD44^+ T cells from each of these tissues. These results are representative of more than two experiments.

**FIGURE 4.** CD73 and Ly-6A/E expression identify in vivo Thpp-like cells. Spleen and lymph node cell populations from KLH-immunized C57BL/6 mice were enriched for CD4 T cells and sorted into populations with the cell surface markers of Thpp cells (CD4^+ CD44^+ CD73^+ Ly-6A/E^−) and effector-like cells (CD4^+ CD44^− CD73^− Ly-6A/E^+). Sorted cells were restimulated with KLH plus syngeneic splenocytes in ELISPOT assays for IL-2, IL-4, IFN-γ, and MIP-1α. Background ELISPOT values (spleen APC + KLH) were <75, 25, 50, and 200 (IL-2, IL-4, IFN-γ, and MIP-1α, respectively) spots per million sorted cells. These results are representative of more than three experiments.

**Relationship to other cell surface markers**

We compared CD62L expression on the CD73^+ Ly-6A/E^+ and CD73^− Ly-6A/E^− cell populations, and found that CD62L expression was heterogeneous on both populations (data not shown), in agreement with previous results (6, 7). We also found that CCR7 mRNA was expressed by both populations, and that there was no striking difference in expression between Thpp-like (CD73^+ Ly-6A/E^−) and effector-like (CD73^− Ly-6A/E^+) CD4 memory cells (data not shown). This is consistent with reports (10, 11) that CCR7 expression alone does not distinguish uncommitted (non-polarized) CD4 memory cells from effector cells.

**CD73^+ Ly-6A/E^− CD44^+ CD4^+ T cells of C57BL/6 mice contain uncommitted precursor cells**

One of the important characteristics of in vitro Thpp cells is that they can further differentiate into either Th1 or Th2 cells. To test the differentiation flexibility of the in vivo populations, we stimulated sorted cell populations with irradiated TA3 cells (allograft) in the presence of IL-12 (Th1 conditions) or IL-4 (Th2 conditions). Six days later, the T cells were washed and restimulated with anti-CD3 plus anti-CD28 Abs and tested for cytokine and chemokine secretion. Thpp-like CD73^+ Ly-6A/E^− cells, from either spleen or lymph nodes, showed flexibility in their differentiation patterns: they produced more IFN-γ or more IL-4 under Th1 or Th2 conditions, respectively (Fig. 5). In contrast, the spleen and lymph node CD73^− Ly-6A/E^+ effector-like populations produced...
both IL-4 and IFN-γ before in vitro differentiation, and the ratios of IFN-γ to IL-4 were not substantially changed after culture in either Th1- or Th2-polarizing conditions. This suggests that the CD73<sup>+</sup> Ly-6A/E<sup>+</sup> cell population comprised mainly uncommitted precursor cells, whereas the CD73<sup>+</sup> Ly-6A/E<sup>+</sup> population contained many committed effector cells. Thus the CD73<sup>+</sup> Ly-6A/E<sup>+</sup> population isolated from C57BL/6 mice were in vivo Thpp-like cells based on their preferential localization in lymph nodes, production of IL-2 and MIP-1<sup>+</sup> but not IL-4 or IFN-γ, and flexible in vitro differentiation.

CD73 and Ly-6A/E also identify Thpp-like cells in C57L and PL mice, but not in BALB/c mice

In Ly-6.2 (Ly-6A.2) mouse strains, Ly-6A/E is expressed on a subpopulation of resting memory T cells, as well as activated T cells (16), whereas Ly-6.1 (Ly-6E.1) mouse strains express Ly-6A/E mainly on activated, not resting T cells. We therefore tested whether the CD73<sup>+</sup> Ly-6A/E<sup>+</sup> population could be identified in additional mouse strains.

Subpopulations of CD73<sup>+</sup> Ly-6A/E<sup>+</sup> CD4<sup>+</sup>CD44<sup>+</sup> cells were present in additional Ly-6.2 strains (C57L, PL, C58, SJL, and AKR), particularly in lymph nodes (Fig. 6A and data not shown). Sorted CD73<sup>+</sup> Ly-6A/E<sup>+</sup> spleen or lymph node cells from C57L and PL mice produced high levels of IL-2 and MIP-1α, but low levels of IL-4 or IFN-γ (Fig. 6B). However, CD4<sup>+</sup> T cells from Ly-6.1 mice (BALB/c and C3H/He) expressed low levels of Ly-6A/E (Fig. 6A and data not shown). Sorting BALB/c lymph node cells using CD73 alone did not allow separation into Thpp-like and effector populations (data not shown), even though Thpp-like cells are present in BALB/c mice (3, 6). Thus reciprocal expression of

**FIGURE 5.** CD73<sup>+</sup> Ly-6A/E<sup>+</sup> CD4<sup>+</sup>CD44<sup>+</sup> cells of C57BL/6 mice contain precursor cells that can further differentiate into Th1 or Th2 effector cells. CD4<sup>+</sup> T cell-enriched spleen and lymph node cell populations from C57BL/6 mice were stained and sorted as in Fig. 4, and the CD73<sup>+</sup> Ly6A/E<sup>+</sup> and CD73<sup>+</sup> Ly6A/E<sup>+</sup> populations were stimulated with irradiated allogeneic TA3 cells in Th1 or Th2 conditions. Six days later, cells were restimulated with anti-CD3 plus anti-CD28 for 24 h. The supernatants were tested for IL-4 and IFN-γ production by ELISA. These results are representative of three experiments.

**FIGURE 6.** CD73 and Ly-6A/E expression identify Thpp-like cells in Ly-6.2 but not Ly-6.1 mouse strains. A, Lymph node and spleen cell populations from C57BL/6, C57L, PL, and BALB/c mice were stained and analyzed as Fig. 3A. B, Thpp-like (CD73<sup>+</sup> Ly-6A/E<sup>+</sup>) and effector-like cells (CD73<sup>+</sup> Ly-6A/E<sup>+</sup>) from C57L and PL lymph nodes were isolated and analyzed as described in Fig. 4. These results are representative of three experiments.
CD73 and Ly-6A/E resolves a population of resting Thpp from effector memory cells in some but not all mouse strains.

Discussion

CD73 is a 69-kd GPI-linked protein with four N-terminal glycosylation sites (13). CD73 functions as an ecto-5′-nucleotidase, which converts extracellular pyrimidine and particularly purine monophosphates to the corresponding nucleosides, which can pass through the plasma membrane and are thus available for cell metabolism. Dephosphorylation of AMP by CD73 produces adenosine, which binds to and activates adenosine receptors (17). There are also suggestions that CD73 could costimulate T cell activation (18), or mediate lymphocyte adhesion to cultured endothelial cells (19).

By expressing CD73, Thpp cells might contribute to the extracellular pool of adenosine, which regulates a number of physiologic processes including inflammation. Adenosine inhibited IL-12 production but increased IL-10 production by dendritic cells and macrophages and selectively decreased some cytokotive functions of neutrophils (20). A protective role for adenosine in Con A- or oxotxin-induced inflammatory liver damage was indicated by treatment with adenosine agonists and antagonists, or in mice deficient for A2aR, the major adenosine receptor expressed on lymphocytes, dendritic cells, and neutrophils (21). Although Thpp cells may induce a mild inflammatory response by secreting chemokines (4), at least in some mouse strains this response may be dampened by the concomitant action of adenosine produced by CD73 on the Thpp cell surface.

Ly-6A/E (Sca-1) is a small (10–18 kD) GPI-linked surface protein, mainly expressed on hematopoietic stem cells, subsets of lymphocytes (T, B, NK) and some nonlymphoid tissues (16). Ly-6E.1 and Ly-6A.2 are two alleles of the same gene that differ by only one amino acid in the mature proteins, but more significantly, these two alleles show striking differences in their patterns of expression (22). In Ly-6.1 (Ly-6E.1) mouse strains, such as BALB/c, C3H, CBA, and DBA/1, New Zealand B, and New Zealand A, few resting peripheral lymphocytes express high levels of Ly-6A/E; whereas in Ly-6.2 (Ly-6A.2) mouse strains, including C57BL/6, C57L, C58, PL, SJL, DBA/2, SWR, AKR, 129, and C57BR, Ly-6A/E molecules are expressed on substantial numbers of resting lymphocytes (>5-fold higher than Ly-6.1 strains). The nature of this regulation may be related to the extra requirement for TNF signaling to induce Ly-6A/E expression on Ly-6.1 strains (23). Thus, even though Thpp-like cells are present in BALB/c mice (3, 6), we found that Ly-6A/E could not be used as a marker to separate Thpp from effector memory cells in Ly-6.1 mouse strains. In Ly-6.2 mice, the Ly-6A.2 marker distinguishes uncommitted from effector T cells in resting T cell populations, but both Ly-6.1 and Ly-6.2 strains express higher Ly-6A/E levels on all CD4 T cells after activation (16) (Fig. 2).

Ly-6A/E may play roles in the immune responses of T cells (24) and B cells (25) but the major function remains unknown. In T cells, anti-Ly-6A/E induces an intracellular Ca2+ flux and enhances IL-2 secretion (26). This effect requires expression of the TCR-CD3 complex, but anti-Ly-6A/E can also inhibit anti-CD3e-induced IL-2 production (27), possibly due to differences in the extent of cross-linking or the expression levels of Ly-6A/E. The lack of expression of Ly-6A/E on all subtypes of resting T cells in Ly-6.1 mice suggests that this molecule does not have an important role in resting T cells, and so the significance of the selective lack of Ly-6A/E on Thpp of Ly-6.2 strains is not clear.

The in vivo Thpp-like CD73+ Ly-6A/E+ population may be similar or identical with the CD4+ “central memory” T cells described in human PBMC populations (9, 28). Both cell types produce IL-2 but not IL-4 or IFN-γ, both are found preferentially in lymph nodes rather than nonlymphoid tissue, and both show plasticity of differentiation. It should be noted, however, that the term central memory cells has also been applied to cells with lymphoid homing patterns, even if they show committed cytokine patterns (29). Both mouse and human Thpp-like (i.e., IL-2-secreting, uncommitted) cells are a substantial part of the response to immunization with protein Ags, with or without adjuvants such as alum or Ribi adjuvant (6, 9) (D. M. Zais, A. Divekar, and T. R. Mosmann, unpublished observations), but to a lesser extent by infections (A. Divekar, D. M. Zais, and T. R. Mosmann, unpublished observations). The data we present, showing that Th1 or Th2 cells do not express CD73 transiently during early differentiation, suggests that Thpp are not just cells that have been arrested at an intermediate stage of differentiation down either the Th1 or Th2 pathways, but may represent a distinct differentiation state.

Independently from the functional roles of CD73 and Ly-6A/E expression on different T cell subsets, these two markers provide valuable tools for identifying and enriching T cell populations from normal Ly-6.2 lymphocyte populations. For example, we have used these markers to confirm that Thpp-like CD73+ Ly-6A/E+ cells are mainly localized in lymphoid tissue rather than peripheral tissue (lung). The ability to separate Thpp from Th1- and Th2-like effectors should greatly aid the analysis of the induction, distribution and roles of the uncommitted, nonpolarized Thpp cells during in vivo immune responses.

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References


