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Most Murine CD8⁺ Intestinal Intraepithelial Lymphocytes Are Partially But Not Fully Activated T Cells¹

Heuy-Ching Wang, Qin Zhou, Jolene Dragoo, and John R. Klein²

Murine small intestine intraepithelial lymphocytes (IELs) bear properties of both activated and nonactivated T cells, although the significance of that dichotomy remains unclear. In this study, we show that although IELs express CD69 in situ and ex vivo, and have cytotoxic activity ex vivo, most CD8⁺ IELs from normal mice are phenotypically similar to naive T cells in that they are CD45RB<sup>high</sup>, CD44<sup>low/int</sup>, and lack or have low levels of expression of CD25, Ly-6C, OX40, Fas ligand (FasL), and intracellular IFN-γ synthesis. Unlike CD8⁺ lymph node cells, IELs express high levels of the FasL gene, but do not express surface FasL until after CD3-mediated stimulation has occurred. Additionally, anti-CD3 stimulation of IELs in the presence of actinomycin-D did not inhibit FasL expression, suggesting that regulation FasL expression on IELs is controlled at least partially at the posttranscriptional level. Following CD3-mediated stimulation, IELs synthesize and secrete IFN-γ more rapidly and to greater levels than CD8⁺ lymph node cells, and they acquire the phenotype of fully activated effector cells as seen by an up-regulation of CD44, Ly-6C, OX40, FasL, and CD25 with the kinetics of memory T cells, with down-regulation of CD45RB expression. These findings indicate that contrary to previous interpretations, most small intestine IELs are not fully activated T cells, but rather that they are semiautivated T cells ready to shift to a fully activated state once a CD3-mediated signal has been received. These data also imply that under appropriate conditions it is possible for T cells to be sustained in a state of partial activation. The Journal of Immunology, 2002, 169: 4717–4722.

Understanding the activational properties of intestinal intraepithelial lymphocytes (IELs)¹ is essential for gaining insight into mechanisms of local immunity, and for elucidating events associated with autoimmunity and immunopathology in the intestinal mucosa. Recent studies indicate that the activation state of intestinal IELs is considerably more complex than previously realized. For example, DNA microarray or serial analysis of gene expression of αβ and/or γδ IELs revealed the striking yet paradoxical finding of constitutive expression of genes of activated cytotoxic T cells (granzyme A, granzyme B, and the cytotoxic-associated proteins serglycin, Fas ligand (FasL), and cryptidin) while concomitantly expressing genes involved in immune down-regulation, including CTLA-4, Ly-49E-G, the NK receptor gp95B, and PD-1/programmed cell death 1 genes (1, 2).

Similarly, an important unresolved question is whether IELs are activated naive effector cells or whether they are memory CD8⁺ T cells poised for reactivation. Studies using transgenic mice expressing TCR for an OVA peptide (OT-1 mouse) indicate that T cells can be activated in situ in the gut epithelium, and that cytotoxic activity to nominal Ags increases with immunization (3). Moreover, naive and memory CD8⁺ T cells can migrate to or be recruited into the intestinal epithelium (3, 4). However, because nearly all T cells in those experimental systems consist of TCRαβ cells, a subset that makes up only about half of the total IELs (see Results), those studies provide little information about the other types of T cells present among the IELs, including populations that originate from precursors within the intestinal mucosa and may not recirculate (5). Additionally, if in fact most IELs are memory T cells, it is hard to reconcile why IELs from OT-1 mice not primed with OVA are cytotoxic ex vivo (3) unless some type of regional preactivation event has occurred in vivo. In an effort to resolve those differences, murine IELs were studied using freshly isolated cell preparations with a panel of markers associated with T cell activation and/or memory, and by following functional and phenotypic changes that occur shortly after CD3-mediated stimulation. Our findings indicate that most CD8⁺ IELs are partially activated T cells that are phenotypically similar to naive T cells yet can proceed into a state of full activation with the kinetics of memory cells.

Materials and Methods

Mice

Adult female BALB/c mice, 8–12 wk of age were purchased from Harlan Sprague Dawley (Houston, TX) and were maintained at the University of Texas (Houston, TX) vivarium.

Cell isolation, purification, and culture

Small intestine tissues were removed and Peyer’s patches were dissected out. Tissues were flushed of fecal material, opened longitudinally, and cut into 3- to 4-mm pieces in RPMI 1640 supplemented with FCS (10% v/v), 100 U/ml penicillin-streptomycin, 2 mM t-glutamine, and 5 × 10⁻⁵ M 2-ME (all reagents; Sigma–Aldrich, St. Louis, MO). Tissue fragments were rinsed several times in Ca²⁺/Mg²⁺-free PBS and stirred at 37°C for 30 min in Ca²⁺/Mg²⁺-free PBS containing 5 mM EDTA and 2 mM DTT (Sigma–Aldrich). Cells were filtered successively through three 10-ml syringe bars containing wetted nylon wool, centrifuged, suspended in 3 ml of 40% isotonic Percoll, layered on top of 70% isotonic Percoll, and centrifuged for 20 min at 600 × g. IELs were recovered from the Percoll interface. Lymph node cells (LNCs) were isolated by pressing lymph node tissues through a 60-mesh stainless steel screen into supplemented RPMI 1640 for autoMACS cell sorting.

¹ Abbreviations used in this paper: IEL, intraepithelial lymphocyte; FasL, Fas ligand; LNC, lymph node cell; int, intermediate.

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Purification of CD8+ IELs and LNCs by MACS was done using an autoMACS cell sorter (Miltenyi Biotec, Auburn, CA). For IELs, 15–20 × 10^6 freshly isolated IELs were reacted with 1 ml of anti-CD16 tissue culture supernatant for 10 min at 4°C. Cells were centrifuged and washed with labeling buffer (PBS (pH 7.2) supplemented with 2 mM EDTA) and reacted with labeled anti-CD4 mAb plus 1 ml of unlabeled CR6 mAb (6) for 20 min at 4°C. G8.8 mAb was used to deplete epithelial cells from IEL preparations as previously described (7). Our studies using G8.8 mAb (6) for 20 min at 4°C acted with 1 ml of unlabeled anti-CD4 mAb and 1 ml of unlabeled G8.8 labeling buffer (PBS (pH 7.2) supplemented with 2 mM EDTA) and reacted with 20 μl of anti-rat microbeads (Miltenyi Biotec) in 180 μl of labeling buffer for 15 min at 4°C. Cells were washed, suspended in 1 ml of separation buffer (PBS (pH 7.2) supplemented with 0.5% BSA) and applied to autoMACS. For CD8+ LNC enrichment, autoMACS sorting was done using 15–20 × 10^6 cells reacted with anti-B220 and anti-CD4 mAbs, followed by treatment with anti-rat microbeads to remove B cells and CD4+ T cells similar to that described for IELs. Flow cytometric cell sorting of CD8+ IELs was done at the Baylor College of Medicine Department of Immunology flow cytometry core facility (Houston, TX) using an EPICS cell sorter (Coulter Scientific, Hialeah, FL).

Microtitre plates (24- or 96-well) were coated overnight with 10 μg/ml of anti-hamster mAb in PBS. Wells were washed with PBS and reacted with 5 μg/ml of hamster anti-mouse CD3 or control hamster mAb. IELs or LNCs were cultured in coated 24-well plates at a density of 1.0–2.0 × 10^6 cells/ml, or in coated 96-well plates at a density of 5 × 10^5 cells/ml in 200 μl in supplemented RPMI 1640 (Cell isolation, purification, and culture) containing 4 ng/ml rIL-2 and 100 ng/ml IL-15 (Sigma-Aldrich). Cells were cultured after 24 h, stained, and analyzed by flow cytometry. Cell-free supernatants were collected at the designated intervals for IFN-γ assays. Actinomycin-D (Sigma-Aldrich) was used at 2 μg/ml, a concentration previously reported to inhibit transcription in T cells (8).

**Antibodies**

Abs used were: purified NA/LE anti-CD3 (145-2C11), FITC-anti-CD8α (53-6.7), PC5-CD8β (53-5.8), FITC-anti-TCRβ (H57), FITC-anti-TCRα (GL3), biotin-anti-CD45RB (16A), CyChrome anti-CD44 (IM7), PE-anti-CD25 (PC61), PE-anti-CD69 (H1.2F3), biotin-anti-OX40 (OX86), biotin-anti-Ly-6C (AL-21), biotin-anti-FasL (MFL-3), FITC-, PE-, and Cy-isotype control Abs, purified anti-hamster Ig (G94-56), purified hamster IgG, propidium iodide, streptavidin-CyChrome, anti-CD16/32 Fc block (2A42; all reagents; BD Pharmingen, San Diego, CA). Anti-CD4 (GK1.5) and anti-B220 (RA3-6B2) cells were purchased from American Type Culture Collection (Manassas, VA). G8.8 cells were generously provided by Dr. A. Farr (University of Washington, Seattle, WA). Intracellular IFN-γ staining was done using a commercial cell staining kit (BD Pharmingen) with the manufacturer’s reagents, protocols, and controls. Stained cells were analyzed on a FACScan (BD Biosciences, Mountain View, CA). In two color histograms, background staining by species-matched control reagents is demarcated by the position of cells in the lower left quadrant.

**Immunocytochemistry**

Immunocytochemistry was done using small intestine tissues frozen in liquid N₂. Acetone-fixed 5-μm sections were treated for 15 min at room temperature with avidin block (DAKO, Carpinteria, CA), washed, and treated for 15 min at room temperature with biotin block (DAKO). Tissues were washed and treated for 15 min at room temperature with anti-CD16/32 (BD Pharmingen). Tissues were washed and reacted with PE-anti-CD69 for 3 h at 4°C. Tissues were washed and examined with an Olympus BH-2 immunofluorescence microscope (Olympus, Lake Success, NY).

**RT-PCR analyses**

CD8+ IELs and LNCs were enriched by autoMACS cell sorting. RNAs were extracted from 1000 cells from each group using an RNasequeous-4PCR kit (Ambion, Austin, TX). A total of 0.5 μg of RNAs were converted to cDNAs with an Advantage RT-for-PCR kit (Clontech Laboratories, Palo Alto, CA). PCR amplification was done using the following primers: FasL, forward 5’-CAGCTTCCTCACCCTGAGAAGG-3’, and reverse 5’-ATGGATGAGCAGTATCGTCTG-3’. FasL, reverse 5’-ATGGAGTTAGCTGCTGCTG-3’. FasL, reverse 5’-ATGGAGTTAGCTGCTGCTG-3’. Nitrigen, Carlsbad, CA). Semiquantitative RT-PCR was done by adding 3-fold serially diluted cDNAs from LNC or IEL FasL or Fas-L beta to tubes with PCR buffers (Applied Biosystems, Foster City, CA) and Taq polymerase (Promega, Madison, WI). Amplification of FasL consisted of 35 cycles at 95°C for 1 min, 55°C for 2 min, 72°C for 3 min; for actin consisted of 30 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min using a Biometra T-Gradient thermocycler (Whatman Biometra, Gottingen, Germany).

**Enzyme-linked immunoassay and redirected cytotoxicity assay**

Cell-free supernatants were collected from IEL and LNC cultures and frozen at −70°C. Samples were analyzed together to reduce variation between assays. Secreted IFN-γ was measured with a commercial cytokine assay kit (eBioscience, San Diego, CA) using the manufacturer’s protocols and standards. Redirected cytotoxicity assays were done as previously reported (9).

**Results**

**IELs express CD69 and are cytotoxic but lack other markers of activated T cells**

Consistent with other reports (9, 10), murine IELs were primarily CD8+ T cells of which most were CD8αα cells (Fig. 1A) that consist of TCRαβ (Fig. 1B) or TCRγδ cells (Fig. 1C). Nearly all CD8+ IELs expressed CD69 ex vivo (Fig. 1D) and in situ (Fig. 1E), and had lytic activity when tested in redirected cytotoxicity assays (Fig. 1F), thus demonstrating a classic functional property of activated CTL.

However, IELs were uniformly low in expression of OX40 (Fig. 2A), a marker expressed on activated CD8 T cells (11) and on IELs after anti-CD3 stimulation (9). Moreover, only a small proportion of CD8+ IELs expressed Ly-6C (Fig. 2B), FasL (Fig. 2C), or CD25 (Fig. 2D), all of which have been linked to activated peripheral CTL (12–15). CD8+ IELs were CD45RB<sup>high</sup> (Fig. 2E) and CD44<sup>low/med</sup> (Fig. 2F). Because memory T cells express low levels of CD45RB (16), high levels of CD44 (17) and Ly-6C (18), and are low-to-negative for CD69 expression (18), this is not a phenotype compatible with a population of memory T cells. Conversely, because IELs express some properties of activated naive T cells, although they lack other distinguishing features of fully activated T cells such as OX40 and CD25 expression and high levels of FasL expression, IELs cannot be readily defined as either naive or memory T cells using conventional standards. It should be noted that because down-regulation of CD62 ligand is needed for homing of lymphocytes and lymphocyte precursors to mucosal tissues (3), the level of CD62 ligand expression on IELs is not a reliable marker of memory T cells in the intestine.

IELs do not spontaneously produce IFN-γ but rapidly up-regulate IFN-γ synthesis in a manner typical of memory T cells following CD3 triggering

To more precisely define the activational state of CD8 IELs, IFN-γ synthesis was studied for CD8+ IELs. This was selected because IFN-γ is produced by activated but not resting CD8+ T cells (19), and is secreted more rapidly by memory T cells than naive T cells upon immune stimulation (20). Based on intracellular IFN-γ staining, CD8+ IELs did not synthesize IFN-γ (Fig. 3, A and B) even though those cells were cytotoxic and expressed CD69 (Fig. 1, D and F).

If CD8 IELs are in fact partially activated T cells, this should be evident by an accelerated immune response following immune stimulation. To test this, IELs and LNCs were cultured with immobilized anti-CD3 mAb in the presence of 4 ng/ml of IL-2 and 100 ng/ml of IL-15 according to procedures previously reported (9, 21, 22). After 18 h, cells were collected and stained for expression of intracellular IFN-γ. Several new findings emerged from these experiments. First, following anti-CD3 stimulation, IELs consisted of a CD4<sup>high</sup> population (Fig. 3C, R4) typical of freshly isolated IELs (Fig. 3A), and a population of CD8<sup>low</sup> cells (Fig. 3C, R3) that was not present in freshly isolated IELs (Fig. 3A). Within both of those groups, approximately half of all cells...
expressed intracellular IFN-γ after short-term anti-CD3 stimulation (Fig. 3, E and F), implying that those IELs had rapidly proceeded to a state of full activation. Second, after 18 h of anti-CD3 stimulation under conditions identical with IELs, LNCs retained the basic LNC CD8 phenotype consisting of CD8<sup>high</sup> cells (Fig. 3G, R4), and did not express significant levels of intracellular IFN-γ/H9253 (Fig. 3, H–J). Interestingly, when an equivalent number of MACS-purified CD8<sup>+</sup>/H11001 IELs and LNCs were analyzed for expression of the FasL gene, IELs expressed 9-fold higher levels of FasL gene compared with LNCs (Fig. 4), yet they expressed minimal surface FasL (see Fig. 2C). Moreover, when IELs were cultured for 24 h with immobilized anti-CD3 mAb, with or without 2 μg/ml actinomycin-D, an equivalent proportion of cells in both groups expressed surface FasL (Fig. 5). Taken together, these findings indicate that the expression of FasL on IELs is regulated post-transcriptionally, and they support the notion that resident IELs are partially activated non-memory T cells.

The accelerated capacity of IELs to produce IFN-γ was further demonstrated using equivalent numbers of MACS-purified CD8<sup>+</sup> IELs and LNCs cultured in microplates coated with anti-CD3 or control mAbs. Supernatants were collected from individual wells at intervals poststimulation and assayed for secreted IFN-γ by enzyme-linked immunoassay. Shown in Fig. 6, throughout the time of analyses the kinetics of IFN-γ production differed significantly between IELs and LNCs in that CD8<sup>+</sup> IELs produced significantly higher levels of IFN-γ sooner than that of an equivalent number of LNCs under the same stimulatory conditions. Without stimulation, IFN-γ production was negligible (<25 pg/ml) for both IELs and LNCs.

FIGURE 1. Expression of A, CD8α and CD8β; B, TCRαβ; C, TCRγδ; and D, CD8 and CD69 on freshly isolated IELs. D, Immunocytochemical staining of murine small intestine frozen sections demonstrating in vivo expression of CD69 (Ep, epithelium; LP, lamina propria). F, Ex vivo cytotoxic activity of murine IELs in redirected cytotoxic assay. Data are representative of two to eight experiments.

FIGURE 2. Most murine CD8<sup>+</sup> IELs lack or have low levels of expression of A, OX40; B, Ly-6C; C, FasL; and D, CD25, and express high levels of CD45RB (E) and low-intermediate levels of CD44 (F). Data are representative of six to eight experiments.

FIGURE 3. A and B, Freshly isolated CD8<sup>+</sup> IELs do not synthesize intracellular IFN-γ. After overnight anti-CD3 stimulation, a subset of CD8<sup>high</sup> (F) and CD8<sup>low</sup> (E) IELs (C, R4 and R3, respectively) express intracellular IFN-γ. In contrast, intracellular IFN-γ is negligible in CD8<sup>+</sup> LNCs (G, R4, I) stimulated with anti-CD3 under the same conditions as IELs, nor was IFN-γ detected for CD8<sup>+</sup> (G, R2; H) or for CD8<sup>low</sup> (G, R3; I) LNCs. Data are representative of two to three experiments.
Short-term CD3-mediated stimulation of IELs results in a rapid shift to an activated memory phenotype

Finally, to identify the changes in activation and memory phenotypic markers on IELs after short-term CD3-mediated activation, CD8\(^+\) IELs purified by flow cytometric cell sorting were cultured for 24 h with or without anti-CD3 stimulation. Cells were collected and stained for expression of CD44, CD45RB, Ly-6C, OX40, and FasL using propidium iodide to discriminate dead and viable cells. In the absence of anti-CD3 stimulation, little change occurred in the expression of CD44, CD45RB, Ly-6C, OX40, or FasL, (Fig. 7, control Ab) compared with freshly isolated IELs (Figs. 1 and 2). However, of particular interest was the dramatic change in the expression of all five markers as a consequence of CD3-mediated stimulation (Fig. 7, anti-CD3 Ab). This included an up-regulation of Ly-6C, OX40, and FasL expression, high level of expression of CD44, and a decrease in CD45RB expression.

Moreover, CD25 expression was up-regulated by 3–6 h post-stimulation (Fig. 8) in a manner similar to that which occurs for memory T cells (18). Changes in CD25 expression did not occur in 24-h IEL cultures in the presence of control Ab stimulation (data not shown). These findings, coupled with the studies of IFN-\(\gamma\) synthesis by fresh IELs, strongly suggest that although IELs bear many phenotypic properties of non-memory T cells, CD3-mediated signaling leads to activation in a manner kinetically typical of memory T cells.

Discussion

Studies from our laboratory have shown that murine IELs exist in two activational states based on expression of CD69 and OX40. The data reported in this study, demonstrating that IELs consist of partially activated T cells that can rapidly shift to a fully activated state but only after a CD3-mediated signal has been received, extend those observations and raise new questions about how T cells proceed through activation. The reasons for the split in effector activities (i.e., ex vivo cytotoxic activity in the absence of IFN-\(\gamma\) synthesis) by IELs that have not received a CD3-mediated signal is curious. However, clearly IELs can be induced to secrete IFN-\(\gamma\) as reported in this study and as seen in studies of mice infected orally with reovirus in which there was an increase in IFN-\(\gamma\) message in IELs following infection (23). Similarly, IFN-\(\gamma\) mRNA and secreted IFN-\(\gamma\)-have been reported for IELs following oral Listeria monocytogenes infection (24). IFN-\(\gamma\) production by IELs also is increased in mice following injection of nonlethal doses of LPS.
and synthesis of IFN-γ by IELs may be influenced at least in part by intestinal-derived cytokines, possibly stem cell factor (26) and IL-15 (27). Moreover, because IFN-γ synthesis has been linked to tissue destruction of intestinal epithelial cells as seen in an IL-12 model of inflammatory bowel disease (28), under normal conditions IFN-γ may have a protective role in the removal of diseased or infected epithelial cells, although continual synthesis of IFN-γ by fully activated IELs could have severe biological consequences if left unchecked. However, IFN-γ also may be involved in oral tolerance (29, 30), suggesting a complex role for IFN-γ at the level of the intestinal mucosa. In that vein also, our observation that the FasL gene is expressed in IELs even though only low levels of surface FasL protein are present, and the ability of IELs to express FasL when stimulated in the presence of concentrations of actinomycin-D that inhibit T cell transcription in other studies (8) suggest that FasL protein expression on IELs is controlled through posttranscriptional mechanisms. Therefore, this would provide a rapid mechanism for further up-regulating the lytic activity of IELs along with IFN-γ synthesis. In short, the ability to dissociate cytolytic activity from IFN-γ activity in the partially activated state would permit rapid full activation of IELs after cognate Ag recognition and CD3 signaling have occurred.

Taken together, these findings indicate that the process of T cell activation in the gut epithelium (and possibly elsewhere) is not a cascade of activational steps, one automatically following the other until full activation has taken place, but rather that multiple consequential signals are needed to drive the activational process to completion. Furthermore, it appears that this process can be suspended and then resumed after the initial activation signal has been initiated. In that context, it will be of interest to determine whether some T cells in extraintestinal peripheral lymphoid tissues also exist as partially activated cells. Interestingly, some evidence for this comes from studies that have identified a population of peripheral “resting primed” CD8+ T cells in lymphocytic choriomeningitis virus-infected mice that have properties similar to those reported in this study, with a potential for hyperresponsive Ag-induced activation (31). Questions that remain to be addressed center on the types of signals that prompt the first stage of IEL activation, as well as mechanisms that halt the activational process in the semiactivated state. Both issues are central to an understanding of how IELs respond to foreign Ag, and will be needed to elucidate mechanisms that prevent broad-spectrum tissue destruction by IELs as occurs in situations of chronic intestinal inflammation and immunopathology. The findings described in this study should enable further exploration into those areas by more accurately defining the stages of IEL activation.

Finally, the differences between CD8+ IELs and CD8+ T cells in extraintestinal lymphoid compartments are notable in many ways, in part due to the high degree of heterogeneity of murine IELs (32), and the potential for some IELs to develop along an extrathymic, intraintestinal pathway (5). Moreover, recent studies have linked the recognition of MHC class I thymus leukemia Ag to CD80+ IELs, and have demonstrated that those interactions are involved in the homeostatic maintenance of the intestinal epithelium by IELs (33). A dynamic role for IELs, both in terms of a classical immune defense response and for the elimination of damaged or senescent epithelial cells, may require rapidly generated effector activity similar to that described in this study. Experiments are underway to explore the events involved in IEL activation in the context of both health and disease at the level of the intestinal epithelium.

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