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Differential Effects of IL-2 and IL-15 on the Death and Survival of Activated TCRγδ+ Intestinal Intraepithelial Lymphocytes

Ching-Liang Chu,*† Shih-Shun Chen,*† Tzong-Shoon Wu, † Szu-Cheng Kuo, † and Nan-Shih Liao*‡†

TCRγδ+ cells are enriched in the intestine mucosa and constitute approximately half of the intestinal intraepithelial lymphocytes (iIEL) in mice. They are likely activated by self and foreign Ags in situ, but little is known about how the activated γδ iIEL are regulated. In the iIEL compartment, IL-2 is produced by activated TCRαβ+ iIEL, and IL-15 message is detected in iIEL and in the epithelial cells. We found surface expression of IL-2 as well as IL-15Rs on activated γδ iIEL, and examined the effects of IL-2 and IL-15 on the survival and death of γδ iIEL during secondary stimulation through TCR. We found that both cytokines supported growth of the restimulated γδ iIEL, but exerted different effects on their survival. A significant higher number of live cells were recovered from the γδ iIEL cultures restimulated in IL-15 than in IL-2. Quantitation of apoptotic cells showed more cell death in the IL-2 group than in the IL-15 group. The cell death was associated with restimulation through TCR and was not caused by insufficient growth factor, thus representing activation-induced cell death. Western blot analyses found no difference in the levels of Bel-2 and Bax proteins between the two groups. However, the level of Bel-1 protein diminished with time in the IL-2 group whereas the level was sustained in the IL-15 group, which may contribute to the pro-survival effect of IL-15. These results demonstrated that the survival of activated γδ iIEL is differentially regulated by IL-2 and IL-15. The Journal of Immunology, 1999, 162: 1896–1903.

Intestinal intraepithelial lymphocytes (iIEL) are T cells that reside in between the epithelial cells that line the intestine lumen. These T cells are phenotypically and functionally distinct from T cells of the central immune system (1). Approximately half of the iIEL express TCRγδ (γδ iIEL). Among them, most are CD8αα+ cells with the rest being CD8−CD8−, a phenotype that resembles γδ T cells in the central immune system (2–4). Other unique features of γδ iIEL include their extrathymic origin (5) and usage of the FceR γ-chain in the TCR signaling module (6–8). γδ iIEL are actively involved in the normal biology of the intestine mucosa. They have been shown to proliferate, produce cytokines, and exert cytotoxicity in response to TCR stimulation or infection (4, 9, 10). Studies using mice deficient in TCRγδ suggest the involvement of γδ iIEL in turnover of the intestinal epithelial cells (IEC) (11), in generation of IgA response to Ags delivered orally (12), and in down-regulation of αβ T cell-mediated immune responses in the intestine (13). Many of these activities were observed when cells or animals were stimulated with either TCR-specific Abs or Ags/pathogens. However, little is known about the regulation of γδ iIEL along the course of their activation.

IL-15 is a recently identified T cell growth factor that shares some activities with IL-2 (14). Despite the lack of significant sequence homology to IL-2, IL-15 binds to IL-2R β- and γ-chains and results in signal transduction (15, 16). A novel IL-15R α-chain was also identified (17). In contrast to the T cell-restricted expression of IL-2, IL-15 mRNA is detected in various tissues and cell types (14), including primary IEC and iIEL (Refs. 18 and 19 and our unpublished observations). The IL-15 protein was also detected in human (h) IEC lines by Western blot analysis (19). Because IL-15 supports proliferation of epidermal and intestinal γδ IEL in vitro (18, 20), it is likely used as a local cytokine by γδ iIEL.

Activated T cells undergo apoptosis upon repeated stimulation. This activation-induced cell death (AICD) is an important mechanism underlying peripheral tolerance (21) and may also contribute to T cell homeostasis after an immune response (22). In addition to repeated stimulation through TCR, the prototype T cell growth factor, IL-2, was also shown to promote AICD at the late stage of T cell activation (23–25). Although most studies on AICD of normal T cells were performed on TCRαβ+ T cells, especially the CD4+ subset, little is known about AICD of TCRγδ+ cells. In this study, we compared the effects between IL-15 and IL-2 on the survival and death of γδ iIEL receiving secondary stimulation through TCR.

Materials and Methods

Animals

C57BL/6 mice were purchased from Cheng-Kung University (Tainan, Taiwan) and bred at the animal facility at the Institute of Molecular Biology.
Academia Sinica (Nankang, Taipei) under specific pathogen-free conditions. Ten- to 14-wk-old males were starved overnight before being used for experiments.

**Antibodies**

Abs used include anti-TCRγδ FITC (clone GL3) (26), anti-TCRαβ FITC (clone H57-597) (27), anti-CD86 PE (clone 53-6.7; Caltag Laboratories, San Francisco, CA), anti-CD25 PE (clone 7D4; Caltag Laboratories), anti-CD22 FITC (clone 5H4; Pharmingen, San Diego, CA), anti-CD132 biotin (clone 4G3; Pharmingen), anti-IL-2 neutralizing mAb (clone S4B6; Ref. 28), goat-anti-mouse IL-15Rα Ab (Santa Cruz Biotechnology, Santa Cruz, CA), and donkey-anti-goat IgG FITC (Jackson Immunoresearch Laboratories, West Grove, PA). Monoclonal Ab specific for Bcl-2, Bcl-x, and Bax were purchased from Santa Cruz Biotechnology.

**Primary activation of γδ iIEL**

Total iIEL were isolated as described (29) with some modifications. Briefly, iIEL were dissociated from small intestine pieces in Ca2+-, Mg2+-free PBS (Life Technologies, Grand Island, NY) containing 1 mM DTT and 1 mM EDTA by stirring at 37°C for 40 min, and then enriched by filtration through nylon wool columns and by centrifugation in a discontinuous Percoll gradient (40%/70%). Total iIEL were panned on tissue culture dishes (100 mm diameter) precoated with GL3 mAb (10 μg) for 1 h. After removal of the nonadherent cells, the adherent cells were cultured in RPMI 1640 (Life Technologies) supplemented with 2 mM l-glutamine, 20 mM HEPES, 2000 U/liter penicillin/streptomycin, 5 × 10−5 M 2-ME, 10% FCS, and 10 ng/ml mouse (m) rIL-2 (R&D Systems, Minneapolis, MN) for 7 days. Activated γδ iIEL were then transferred to new dishes without GL3 coating to rest for 1 day before restimulation.

**Restimulation of activated γδ iIEL**

Cells harvested from the rested primary culture were centrifuged through Ficoll (density, 1.09) to remove dead cells. Live cells (2 × 105 cells/well) were restimulated in 96-well half-area tissue culture plates (Corning, Corning, NY) precoated with GL3 (0.2 μg/well) in 200 μl of RPMI/10% FCS containing rmIL-2, rIL-15 (R&D Systems, Minneapolis, MN) for 7 days. Activated γδ iIEL were then transferred to new dishes without GL3 coating to rest for 1 day before restimulation.

**Measurement of cell growth**

Cell proliferation was determined by [3H]TdR incorporation in which 1 μCi/well of [3H]TdR (Amersham, Buckinghamshire, U.K.) was added to each well 12 h before harvesting or by counting the number of live cells defined by trypan blue exclusion under microscope.

**Measurement of cell death**

Apoptosis was determined by two methods. One was to stain suspension cells with propidium iodide (PI) and then analyze by FACScan (Becton Dickinson, Mountain View, CA) as previously described (30). Cells with subdiploid DNA content were taken as apoptotic cells. Another method was to assess nuclear morphology. Parafomaldehyde-fixed cells were air dried onto slides and then stained with PI in PBS containing 0.3% Triton X-100. The cells were then observed using a laser scanning confocal microscope (LSM 510; Zeiss, Oberkochen, Germany).

**Western blot analysis**

Restimulated γδ iIEL were harvested at indicated time points. After removal of dead cells by centrifugation through Ficoll, cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 2 mM EDTA, 1% Triton X-100, 50 mM NaF, 0.1 mM Na3VO4, and 0.3 mM PMSF for 30 min on ice. Fifty micrograms of protein from each sample were boiled in sample buffer for 5 min and analyzed by 12% SDS-PAGE. Proteins were transferred from gel to polyvinylidene difluoride membranes (Millipore, Bedford, MA) that were then blocked with 5% skim milk in PBS and reacted with various Abs. The binding of Ab was detected by using the horseradish peroxidase-conjugated goat anti-rabbit IgG (Caltag Laboratories). The blots were detected using enhanced chemiluminescence (Amersham).

**Results**

**Activated γδ iIEL expressed all components of IL-2R and IL-15R**

Freshly isolated iIEL that adhered to anti-TCRγδ mAb-coated plates were cultured in IL-2 for 7 days and then examined for expression of surface markers by staining with specific Abs. Flow cytometry analyses showed that all cells were TCRγδ+ with most expressing CD8α (Fig. 1, A–C). They also expressed all components of the IL-2R (Fig. 1, D–F). To determine whether IL-15Rα-chain is expressed on the surface of activated γδ iIEL, cells were stained with a polyclonal Ab and observed under confocal microscope (Fig. 2). As shown in Fig. 2C, a weak but clear surface staining was demonstrated. These results suggest that the activated γδ iIEL could be affected by IL-2 and IL-15 upon restimulation.

**IL-2 and IL-15 supported the growth of restimulated γδ iIEL**

We then determined the optimal concentrations of IL-2 and IL-15 to use in restimulation of γδ iIEL. As shown in Fig. 3, exogenous IL-2 or IL-15 supported proliferation of the restimulated γδ iIEL in a dose-dependent manner in which a plateau was reached at 20 ng/ml of IL-2 or 200 ng/ml of IL-15. These concentrations were used in all later experiments unless otherwise specified. It is worth noting that without exogenous cytokine, the restimulated γδ iIEL did not proliferate, but underwent apoptosis even in the presence of CD28 costimulation (data not shown), suggesting that γδ iIEL produced little growth factor(s) under the given activation condition. To determine whether the growth-supporting effect of IL-15...
was mediated through induction of IL-2. IL-2-specific neutralizing mAb was used. As shown in Fig. 4, although 50 μg/ml of neutralizing mAb completely blocked the proliferation of γδ iIEL restimulated in the presence of exogenous IL-2 and caused cell death, it had little effect on the proliferation and survival of cells restimulated in the presence of exogenous IL-15. The amount of IL-2 in the supernatant of the IL-15 culture was below the detection sensitivity of ELISA (data not shown). These results indicate that the growth promoting effect of IL-15 on restimulated γδ iIEL is independent of IL-2.

Differential effects of IL-15 and IL-2 on the restimulated γδ iIEL

To examine the effects of IL-2 vs IL-15 on restimulation of γδ iIEL, we followed the growth kinetics of restimulated γδ iIEL up to 8 days and found significantly higher numbers of live cells and [3H]TdR incorporation in the IL-15 group than in the IL-2 group starting from day 4 (Fig. 5, A and B). As the difference in viable cell counts was more distinct than in [3H]TdR incorporation between the two groups, mechanisms other than proliferation might contribute to the difference in the live cell numbers. We then examined cell death along the course of restimulation by quantitating cells with subdiploid DNA content using flow cytometry (Fig. 5C). We found that the percentage of dead cells was significantly higher in the IL-2 group than in the IL-15 group. On the other hand, cells cultured in IL-2 or in IL-15 without restimulation through TCR showed only low levels of apoptosis (Fig. 5C). Therefore, the observed cell death was induced by activation through TCR, which agrees with the definition of AICD. We also examined the nuclear morphology of the γδ iIEL before and after restimulation (Fig. 5D and Table I). After primary activation for 7 days, most cells showed healthy nuclear morphology with ~6% of the cells displaying apoptosis features. After restimulation through TCR, the percentages of apoptotic cells in the IL-2 group were significantly higher than those in the IL-15 group at various time points. This method detected earlier and higher numbers of apoptotic events than measurement of subdiploid DNA content in cells, probably because nuclear morphology reveals the earlier apoptosis feature, i.e., DNA condensation, as well as the later DNA fragmentation and formation of apoptotic bodies. Both methods demonstrated higher cell death in the IL-2 group than in the IL-15 group. These results suggest that IL-15 is more efficient than IL-2 in supporting the growth of restimulated γδ iIEL via protecting cells from AICD.

FIGURE 2. Activated γδ iIEL showed surface expression of IL-15Rα-chain. Activated γδ iIEL as described in Fig. 1 were stained with donkey anti-goat IgG FITC alone (A), or with goat-anti-mIL-15Rα Ab and followed with donkey-anti-goat IgG FITC (C). The cells were then mounted onto slides and observed with the confocal microscope. The bright field images of A and C are displayed in B and D, respectively, to reveal the location of the cells. The arrows in C and D point to the same cells. Similar results were obtained from three independent experiments.

FIGURE 3. IL-2 and IL-15 supported the growth of restimulated γδ iIEL. Activated γδ iIEL were restimulated through TCR as described in Materials and Methods in medium alone or plus various amounts of mrIL-2 (A) or hrIL-15 (B) for 2 days. Cell proliferation and apoptosis were determined by measurement of [3H]TdR incorporation and by staining permeated cells with PI, respectively, as described in Materials and Methods. The percent apoptosis represents the percentage of cells with subdiploid DNA content. All data points were the average of triplicate samples, and the error bars represent the sample SD. Similar results were obtained from three independent experiments.

FIGURE 4. IL-15 promoted cell growth independent of IL-2. Activated γδ iIEL were restimulated through TCR as described in Materials and Methods in the presence of 20 ng/ml of IL-2 or 200 ng/ml of IL-15 with or without 50 μg/ml of IL-2-specific neutralizing mAb for 2 days. Cell proliferation and apoptosis were determined as described in the legend of Fig. 3. All data points were the average of triplicate samples and the error bars represent the sample SD. Similar results were obtained from two independent experiments.
The death of γδ iIEL restimulated in IL-2 was not due to insufficient cytokine

Because IL-2 is also used as a growth factor by the restimulated γδ iIEL, cell death may result from insufficient amounts of cytokine present during later periods of culturing even though γδ iIEL were fed with fresh medium containing IL-2 every 2 days. To test this possibility, the growth and death kinetics of γδ iIEL restimulated at various concentrations of IL-2 were examined (Fig. 6). Cells activated in 5 ng/ml of exogenous IL-2, a suboptimal concentration (Fig. 3A), showed the lowest viable cell counts and the highest level of apoptosis, reflecting an insufficient presence of growth factor. When 50 and 100 ng/ml of IL-2 were used, which represented 2.5- and 5-fold of the optimal concentration, respectively, the numbers of viable cells and the percentage of apoptotic cells...
were similar to those of cells activated in 20 ng/ml of IL-2. These results indicate that the death of γδ iIEL restimulated in 20 ng/ml of IL-2 was not due to insufficient cytokine.

The pro-apoptosis effect of IL-2 was dominant over the anti-apoptosis effect of IL-15

Because IL-2 and IL-15 both bind to the IL-2R β- and γ-chains but exert different effects on restimulated γδ iIEL, we examined the growth and death kinetics of γδ iIEL restimulated in the presence of both cytokines (Fig. 7). Addition of 5 ng/ml of IL-2 into the IL-15 culture resulted in a significant drop of viable cell numbers and an increment of cell death. Addition of 20 or 50 ng/ml of IL-2 further decreased the number of live cells to a level similar to that exhibited when 20 ng/ml of IL-2 alone was used. These results showed that the pro-apoptotic effect of IL-2 was dominant over the anti-apoptotic effect of IL-15 on restimulated γδ iIEL under the given conditions. The addition of IL-2 into the IL-15 culture promoted death rather than life of the restimulated cells further ruling out the possibility that death was caused by insufficient growth factor.

The level of Bcl-xL was maintained in γδ iIEL restimulated in IL-15

As the members of the Bcl gene family are critical in determination of the life and death of T cells (31), we analyzed the amounts of various Bcl gene family members in γδ iIEL in the presence of IL-2 or IL-15. As shown in Fig. 8, the level of Bcl-2 and Bax proteins did not change along the course of restimulation in either the IL-2 or the IL-15 group and was quite similar in both groups. In the case of Bcl-xL, the protein level between the IL-2 and IL-15 groups was similar at 2, 4, and 5 days after restimulation. This level was maintained in the IL-15 group, but reduced in the IL-2 group, starting ~6 days after restimulation, when significant difference in viable cell numbers between the two groups was observed (Fig. 5A). These results demonstrate a correlation between cell death and the reduction of Bcl-xL in γδ iIEL restimulated in IL-2, which suggests that IL-15 protects γδ iIEL from AICD by sustaining the expression of Bcl-xL or that IL-2 promotes AICD by down-regulating the expression of Bcl-xL.

Discussion

Activation of γδ iIEL through TCR by foreign and self Ags is likely to constantly occur in the intestine mucosa (32–34). In the iIEL compartment, IL-2 is produced by activated CD4+ CD45+CD8−, and CD8αβ+ TCRαβ+ cells (35, 36), and the IL-15 mRNA was detected in both αβ and γδ iIEL and in IEC. In the present study, we demonstrated surface expression of all the components of the IL-15R and IL-2R on activated γδ iIEL. We also demonstrated AICD of γδ iIEL that received secondary stimulation through TCR and examined the role of IL-2 and IL-15 in the survival and death of the restimulated γδ iIEL. We found that exogenous IL-2 or IL-15 supported the growth of the restimulated γδ iIEL that would otherwise not have proliferated but would have die. However, only minimal increment in viable cell numbers was observed in the IL-2 group in comparison with the significant increase in the IL-15 group along the course of restimulation. Quantitation of apoptotic cells revealed higher AICD in the IL-2 group than in the IL-15 group, indicating a protective role of IL-15 in AICD of γδ iIEL. Similar protective effect of IL-15 on TCR-triggered apoptosis was observed in CD8αβ− αβ iIEL, but not in CD8αβ+ αβ iIEL, during primary activation (our unpublished data), which suggests that the rescue by IL-15 is not specific for γδ T cells in the gut but may be specific for cells of CD8αβ− phenotype. We are currently examining the effect of IL-15 on AICD of peripheral γδ T cells which are mainly comprised of CD4−CD8−. The pro-survival effect of IL-15 observed in this study is consistent with previous observations in activated human peripheral blood B and T cells in which IL-15 promoted their survival upon cross-linking of Fas or antigenic receptors (37).

Table I. Apoptosis of γδ iIEL restimulated in IL-2 or IL-15

<table>
<thead>
<tr>
<th>Activation Day</th>
<th>IL-2</th>
<th>IL-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>6 (20/338)</td>
<td>5 (16/345)</td>
</tr>
<tr>
<td>Second</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16 (44/274)</td>
<td>17 (47/285)</td>
</tr>
<tr>
<td>4</td>
<td>39 (86/218)</td>
<td>20 (47/285)</td>
</tr>
<tr>
<td>6</td>
<td>77 (200/260)</td>
<td>37 (118/320)</td>
</tr>
</tbody>
</table>

* Freshly isolated γδ iIEL were activated in IL-2 and then restimulated in IL-2 or IL-15 as described in Materials and Methods. Cells at various activation stages were analyzed for apoptosis by assessment of nuclear morphology as described in Materials and Methods. Cells with DNA condensation or apoptotic bodies (Fig. 5D) were scored as dead cells.
expression of Bcl-xL, Bcl-2, and Bax by γδ iIEL in the presence of IL-15. Activated γδ iIEL collected from primary culture were restimulated through TCR as described in Materials and Methods in the presence of 20 ng/ml of IL-2 (solid square), 200 ng/ml of IL-15 (solid triangle), or IL-15 plus 5 ng/ml (diamond), 20 ng/ml (square), or 50 ng/ml (circle) of IL-2 for 8 days. Viable cell counts and cell death were determined as described in the legend of Fig. 6. All data points were the average of triplicate samples, and the error bars represent the sample SD. Similar results were obtained from two independent experiments.

Similar results were obtained from two independent experiments.

Materials and Methods

Expression of Bcl-xL, Bcl-2, and Bax by γδ iIEL restimulated in IL-2 or in IL-15. Activated γδ iIEL collected from primary culture were restimulated through TCR as described in Materials and Methods in the presence of 20 ng/ml of IL-2 or in 200 ng/ml of IL-15 for 8 days. Viable cells were harvested at indicated time points and analyzed for expression of Bcl-xL, Bcl-2, and Bax by Western blotting as described in Materials and Methods. Similar results were obtained from two independent experiments.

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murine T cell hybridoma 2B4 triggered by anti-CD3 mAb (57). In this study, γδ iIEL reconstituted in either IL-2 or IL-15 expressed the same level of Bcl-2 during the 8-day restimulation period. This is consistent with the notions that Bcl-2 does not block AICD of peripheral T cells, and that Bcl-2 expression is induced by signaling through the IL-2R β-chain (58). On the other hand, the level of Bcl-γδ diminished with time in the IL-2 group, but was maintained in the IL-15 group. This result suggest that Bcl-γδ may contribute to the protective role of IL-15 in AICD of murine γδ iIEL.

The distinct effect of IL-2 and IL-15 on AICD of γδ iIEL suggests that the survival of activated γδ iIEL is well regulated. The presence of IL-15 in the microenvironment, e.g., through production by IEC, would allow the activated γδ iIEL to live and to carry out their function. When IL-2 comes into the picture, e.g., through production by activated αβ iIEL, activated γδ iIEL would die by AICD. The dynamic interactions among the stimuli that modulate cytokine production, the cytokine producers, and the iIEL as the effectors could ultimately determine the fate of the activated γδ iIEL. This possibility may provide a basis for further studies into the regulation of iIEL function and mucosal immunity.

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References


