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Inhibition of TCR-Induced CD8 T Cell Death by IL-12: Regulation of Fas Ligand and Cellular FLIP Expression and Caspase Activation by IL-12

Seung Woo Lee, Yunji Park, Jae Kwang Yoo, So Young Choi, and Young Chul Sung

In this study we demonstrate the anti-apoptotic effect of IL-12 and its underlying mechanism in CD8 T cells. The prolonged stimulation of CD8 T cells with anti-CD3 alone caused apoptosis mediated by Fas and the caspase signaling pathway. However, costimulation with IL-12 significantly prevented anti-CD3-induced apoptosis of CD8 T cells. IL-12 decreased the number of Fas ligand-positive CD8 T cells and inhibited the activation of caspase-8 and caspase-3. In addition, IL-12 up-regulated cellular FLIPs but not Bcl-2 family proteins or cellular inhibitor of apoptosis proteins. These data suggest that IL-12 provides survival signals to CD8 T cells by down-regulating Fas ligand and up-regulating cellular FLIPs, followed by inhibiting caspase activation, which implies a role for IL-12 in peripheral responses of CD8 T cells in vivo. The Journal of Immunology, 2003, 170: 2456–2460.

Materials and Methods

Purification and stimulation of CD8 T cells

Lymph node cells from 6 to 8-wk-old female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used with microbead-conjugated Abs (Miltenyi Biotec, Auburn, CA) such as anti-CD4, -CD11b, -CD11c, -CD45R, and -MHC class II and then were passed over a mini-MACS column (Miltenyi Biotec). The negatively selected cell population (~95% CD3\(^+\) and CD8\(^+\)) was used in this study. A total of 10\(^5\) of CD8 T cells were stimulated with plate-coated anti-CD3 (8 \(\mu\)g/ml) plus 20 U/ml human recombinant IL-2 (BD PharMingen, San Diego, CA). Where indicated, either mouse recombinant IL-12 (10 ng/ml; R&D Systems, Minneapolis, MN) or plate-coated anti-CD28 (4 \(\mu\)g/ml) was added to CD8 T cells at the start of cultures.

Abs, reagents, and flow cytometry

Abs to caspase-8 (H-134), caspase-3 (H-277), c-FLIPs (H-202), Bcl-2 (C-2), Bcl-x\(_L\) (H-5), Bax (P19), cellular inhibitors of apoptosis protein (c-IAP1 (H-83), c-IAP2 (H-85), and actin for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The following mAbs (BD Pharmingen) were used for immunofluorescent staining: FITC-conjugated anti-CD8, biotinylated anti-Fas (Jo2), biotinylated anti-FasL (MFL3), anti-TNFR-1 (55R-170), anti-TNFR-2 (TR75-32), and biotinylated hamster IgG. For the secondary staining, PerCP-conjugated streptavidin or PE-conjugated anti-hamster IgG was used. Anti-mouse TNF-\(\alpha\) (G281-2626; BD Pharmingen) and anti-mouse IFN-\(\gamma\) (XMG1.2; BD Pharmingen) were used for neutralization of TNF-\(\alpha\) and IFN-\(\gamma\), respectively, and the mouse Fas/Fc fusion protein and human IgG were purchased from R&D Systems and Calbiochem (La Jolla, CA), respectively. The caspase inhibitors, such as z-VAD-fmk and z-IETD-fmk, were purchased from Calbiochem and dissolved in DMSO.

Evaluation of apoptosis and Western blot analysis

Apoptosis was detected by dual staining with FITC-conjugated annexin V (BD Pharmingen) and propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) or PI staining of subdiploid DNA. A total of 50 or 100 \(\mu\)g of cell extracts from CD8 T cells were resolved on SDS-PAGE and transferred onto nitrocellulose membranes. After blocking of membrane, the blots were probed with specific Abs, and then visualized with the appropriate HRP-conjugated secondary Abs (Santa Cruz Biotechnology) and an ECL detection system (Pierce, Rockford, IL).

Results

IL-12 provides survival signals to CD8 T cells

To determine whether IL-12 provides survival signals to CD8 T cells, we examined the apoptosis of CD8 T cells activated with plate-coated anti-CD3 in the presence or absence of recombinant
IL-12. The apoptosis was determined either by PI staining of subdiploid DNA or by dual staining with annexin V and PI. As a positive control, CD8 T cells were costimulated with anti-CD28 in addition to anti-CD3, because anti-CD28 was known to provide survival signals to CD8 T cells (8, 9). As expected, CD8 T cells cultured with medium alone survived poorly at 48 h (Table I). Stimulation with anti-CD3 appeared to give survival signals to CD8 T cells within 48 h, but apoptosis rapidly increased at 72 h. This is consistent with a previous report that TCR signaling alone is sufficient for the early activation, proliferation, and survival of CD8 T cells, but it does not give late survival signals (9). In contrast, costimulation with IL-12 or anti-CD28 significantly prevented apoptosis at 72 h, compared with anti-CD3 stimulation alone, suggesting that like anti-CD28, IL-12 provides survival signals to CD8 T cells.

Inhibition of Fas-FasL interaction or caspase activation prevents anti-CD3-induced apoptosis of CD8 T cells

AICD of CD8 T cells was shown to be mediated through TNFR or Fas signaling (10–12). To determine whether IL-12 prevents CD8 T cell apoptosis through modulating these death-related receptors, we examined the expression levels of Fas, Fasl, TNFR-1 (p55), and TNFR-2 (p75) (Fig. 1A). Compared with naive CD8 T cells, the anti-CD3 stimulation alone induced Fas and TNFR-2 expressions, but costimulation with IL-12 or anti-CD28 had no additional effect. The expression of TNFR-1 was not detected in all conditions. Interestingly, costimulation with IL-12 or anti-CD28 was shown to decrease the number of Fasl-positive CD8 T cells compared with anti-CD3 stimulation alone. To determine the role of Fas and TNFR signaling in anti-CD3-induced apoptosis of CD8 T cells, we blocked the ligation of Fas or TNFR. The interruption of Fas-FasL interaction with a Fas-Fc protein reduced apoptosis of CD8 T cells stimulated with anti-CD3 alone, but not with anti-CD3 plus IL-12 (Fig. 1B). However, the neutralization of TNF-α did not affect the apoptosis of CD8 T cells stimulated either with anti-CD3 alone or with anti-CD3 plus IL-12. These results suggest that anti-CD3-induced CD8 T cell apoptosis is mediated by Fas, but not by TNFR, signaling pathway, which is inhibited by IL-12 costimulation. Because a large amount of IFN-γ is produced from activated CD8 T cells by IL-12 (1), we also examined the effect of IFN-γ on the IL-12-mediated CD8 T cell survival by neutralizing IFN-γ (Fig. 1B). Any inhibitory effects were observed in this condition, suggesting that IL-12-induced CD8 T cell survival is independent of IFN-γ. The treatment of either human IgG or rat IgG as a negative control of Fas-Fc or anti-TNF-α and anti-IFN-γ, respectively, had no effects on apoptosis of CD8 T cells. Several cellular molecules are involved in the Fas-mediated apoptosis (13). Upon Fas activation and trimerization, a set of effector proteins is recruited to this receptor, forming a death-inducing signaling complex (DISC). Fas-associated death domain protein, the first protein that binds to Fas, recruits procaspase-8, thereby resulting in the activation of caspase-8. Active caspase-8 initiates

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** IL-12 decreases Fasl expression, and the blockade of Fas-Fasl inhibits the anti-CD3-induced apoptosis of CD8 T cells. A, CD8 T cells were stimulated as indicated for 72 h, and the expression of death receptors in live cells was analyzed. Live CD8 T cells were gated by PI staining or forward light scatter/side light scatter profile. The expression level of each receptor was determined by staining with biotinylated anti-Fas, biotinylated anti-Fasl, hamster anti-TNF-R1, and hamster anti-TNF-R2, followed by streptavidin-PerCP or streptavidin-PE and anti-hamster IgG-PE, respectively (solid line). The dashed lines represent isotype control curves, and the expression level of each receptor in naive CD8 T cells is shown in the shaded curve. B, CD8 T cells were stimulated as indicated for 48 h, and FasFcel protein (10 µg/ml), human IgG (10 µg/ml), anti-TNF-α mAb (25 µg/ml), anti-IFN-γ mAb (25 µg/ml), or anti-rat IgG (25 µg/ml) was added to cultures for an additional 30 h. The results from annexin V-PI dual staining are shown. Representative data of three independent experiments are shown, and each bar indicates the mean value ± SEM.

<table>
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<th>Stimulation</th>
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<tr>
<td>Anti-CD3</td>
<td>18.3 ± 4.2</td>
<td>57.3 ± 6.2</td>
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<td>Anti-CD3 + IL-12</td>
<td>15.7 ± 2.1</td>
<td>25.7 ± 4.9</td>
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<td>Anti-CD3 + anti-CD28</td>
<td>12.3 ± 3.5</td>
<td>21.1 ± 3.2</td>
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*CD8 T cells were stimulated with plate-coated anti-CD3 ± IL-12 or plate-coated anti-CD28. Apoptosis was analyzed either by PI DNA staining or by annexin V/PI dual staining. The results are representatives of five independent experiments, and data are shown by the mean value ± SEM.  
 a Percentage of CD8 T cells containing sub-diploid (<2 N) DNA.  
 b Percentage of CD8 T cells showing annexin V+PI+ plus annexin V+PI-.
a cascade of effector caspase activation, including caspase-3, that eventually leads to the cleavage of cellular death substrates (13). Thus, we next addressed the involvement of caspases in the anti-CD3-induced CD8 T cell apoptosis. As shown in Fig. 2, incubation of z-IETD-fmk, a specific inhibitor of caspase-8, or z-VAD-fmk, a broad spectrum inhibitor of caspases including caspase-3 (14), increased the survival of CD8 T cells stimulated with anti-CD3 alone in a dose-dependent manner. These results suggest that Fas and caspase signaling pathways are involved in the anti-CD3-induced apoptosis of CD8 T cells.

**IL-12 inhibits the activation of caspase-8 and caspase-3 and up-regulates c-FLIPs**

To directly answer whether IL-12 negatively regulates caspase activation, we investigated the processing of caspase-8 and caspase-3 in anti-CD3-stimulated CD8 T cells at different time points (Fig. 3). Caspases are initially produced as inactive proenzymes that require processing and heterodimerization for their activity (15). As expected, we could not detect active cleavage products of either caspase-8 or caspase-3 from nonstimulated naive CD8 T cells. A 40-h stimulation of CD8 T cells with anti-CD3 alone generated active caspase-8 (p20), which was further increased at 72 h. In contrast, the active caspase-8 was not detected at 40 h and greatly decreased at 72 h in anti-CD3-stimulated CD8 T cells costimulated with either IL-12 or anti-CD28. The activation of caspase-3 was likely to show a delayed kinetics compared with that of caspase-8, because the active caspase-3 (p20) was not detectable at 40 h in all stimulation conditions. At 72 h, however, the anti-CD3 stimulation alone produced a large amount of active caspase-3 (p20), which was significantly inhibited by the addition of either IL-12 or anti-CD28. Consistently, the costimulation with either IL-12 or anti-CD28 showed higher expression levels of procaspase-8 and procaspase-3 at 72 h than did anti-CD3 stimulation alone. These results suggest that like anti-CD28, costimulation with IL-12 inhibits the activation of caspase-8 and caspase-3, probably through inhibiting the processing of caspases.

It has been shown that the activation of caspase-8 and caspase-3 is regulated by various cellular anti-apoptotic molecules. Cellular homologues of viral FLICE inhibitory protein, so called c-FLIPs, were shown to prevent Fas-mediated apoptosis by inhibiting caspase-8 activation at the DISC (16, 17). In addition, Bcl-2 family proteins eventually regulate caspase-3 activation through modifying the mitochondrial membrane potential (18), and cellular inhibitors of apoptosis proteins (c-IAPs) are potent inhibitors of caspase-3 (19). Thus, we next addressed whether IL-12 up-regulates the expression of these anti-apoptotic molecules. The anti-CD3 stimulation alone induced both splicing variants of c-FLIPs, c-FLIPlong and c-FLIPshort, at 40 h compared with nonstimulated cells, which was slightly decreased at 72 h (Fig. 4A). This result suggests that the induction of c-FLIPs at the first 40 h of stimulation might be involved in the early survival signal provided by anti-CD3 stimulation alone, but the decreased levels of c-FLIPs by anti-CD3 stimulation alone at 72 h might be correlated with the increase of anti-CD3-induced CD8 T cell apoptosis. Importantly, the induction of c-FLIPs is more prominent in the IL-12-costimulated CD8 T cells at 40 h, which was further enhanced at 72 h, suggesting that the up-regulation of c-FLIPs might play an important role in IL-12-induced CD8 T cell survival. It is possible that c-FLIPs induction might be caused by a lack of Fas engagement because the level of FasL-positive CD8 T cells was decreased by IL-12. However, the increased level of c-FLIPs by IL-12 is also observed in the presence of Fas-Fc protein, which interrupts Fas-FasL interaction (data not shown), suggesting that the up-regulation of c-FLIPs is directly dependent on IL-12. As a positive control, the addition of anti-CD28 also increased c-FLIPs, which...
FIGURE 4. IL-12 up-regulates c-FLIPs, but not Bcl-2 family or c-IAP proteins, in anti-CD3-induced CD8 T cells. A, CD8 T cells were stimulated as indicated for 40 or 72 h, and the expression of c-FLIPs and actin was determined by Western blot analysis. B, CD8 T cells were stimulated as indicated for 72 h, and the expression of Bcl-2, Bcl-xL, Bax, c-IAP1, c-IAP2, and actin was determined. The results shown here are representatives of five and three independent experiments for A and B, respectively.

agrees well with a previous result in human CD4 T cells (20). However, costimulation with IL-12 or anti-CD28 did not up-regulate c-IAP1/2 molecules, Bcl-2, or Bax, compared with the anti-CD3 stimulation alone (Fig. 4B). It is interesting to note that costimulation with anti-CD28, but not IL-12, slightly increased Bcl-xL. Collectively, these results suggest that IL-12 may inhibit the processing of an initiator caspase, caspase-8, through the up-regulation of c-FLIPs that subsequently suppresses the processing of an effector caspase, caspase-3.

Discussion

A primary CD8 T cell response to a newly encountered Ag requires massive proliferation and expansion of naive CD8 T cells, resulting in a large population of effector CD8 T cells. This clonal expansion of Ag-specific CD8 T cells is critical for eliminating pathogens and the development of subsequent immunological memory. However, activation of CD8 T cells through TCR signaling alone results in AICD, which might prevent effective clonal expansion. This AICD can be rescued by a second signal from costimulatory molecules, adhesion molecules, and cytokine receptors (13). In this study, we demonstrate that IL-12 provides survival signals to CD8 T cells through regulation of the Fas-mediated death signaling pathway, including FasL and c-FLIPs expression and caspase activation.

It is well-known that AICD of CD8 T cells is mediated by Fas or the TNFR signaling pathway in different experimental systems (10–12), and caspase activation might be involved in the CD8 T cell apoptosis as a downstream pathway of these death receptors, although the dependence of caspase cascade in CD8 T cell death has not been extensively studied yet. Based on our data, the anti-CD3-induced CD8 T cell apoptosis is mediated by activation of caspase-8 and caspase-3. In addition, the activation of caspases appeared to be triggered by Fas, but not by the TNFR signaling pathway, because only the interruption of Fas-FasL interaction, but not the neutralization of TNF-α, reduced the anti-CD3-induced CD8 T cell apoptosis. This is consistent with a report by Zaks et al. (21) showing that the AICD of tumor-reactive CD8 T cells is mediated by Fas interaction and caspase activation. Nevertheless, we did not rule out the possible involvement of other members of the TNFR family, such as TNFR-related apoptosis-mediating protein and TRAIL receptor, in CD8 T cell apoptosis (22, 23). The anti-apoptotic effects of IL-12 on CD8 T cells possibly occur on at least three levels: 1) the down-regulation of FasL+CD8 T cells, 2) the up-regulation of c-FLIPs, and 3) the down-regulation of active cleavage products of caspase-8 and caspase-3. Therefore, it is likely that costimulation with IL-12 may decrease the Fas-mediated death signals by down-regulating the expression of FasL and the susceptibility against Fas-mediated apoptosis through the up-regulation of c-FLIPs in the anti-CD3-induced CD8 T cells. Meanwhile, costimulation with IL-12 did not up-regulate expression of c-IAPs or Bcl-2 family proteins in CD8 T cells. Interestingly, anti-CD28 costimulation appeared to up-regulate Bcl-xL, but not Bcl-2 and Bax, in CD8 T cells, consistent with a previous report that anti-CD28 increases expression of Bcl-xL in CD4 and CD8 T cells (8, 9). These results suggest that Bcl-xL may play a role in the anti-apoptotic effect of anti-CD28 costimulation in CD8 T cells.

In our study, the up-regulation of both c-FLIPlong and c-FLIPshort by IL-12 costimulation may play an important role in the IL-12-induced CD8 T cell survival. Because c-FLIPlong is structurally homologous to procaspase-8, it can be cleaved at the DISC upon Fas triggering (20). According to this, it can be speculated that the increase of c-FLIPlong expression by IL-12 costimulation in this study might be a consequence of reduced proteolytic processing rather than the result of up-regulation induced by IL-12. However, this increased level of c-FLIPlong was also observed at 40 h of IL-12 costimulation, at which anti-CD3-induced CD8 T cell death was less apparent (data not shown), and thus, less Fas triggering might occur. This suggests that IL-12 actually up-regulates c-FLIPlong as well as c-FLIPshort. To our knowledge, it is the first report to demonstrate that IL-12 up-regulates c-FLIPs that might be involved in the inhibition of caspase activation and subsequent apoptosis of CD8 T cells.

Several reports have shown that the level of c-FLIPs expression is correlated with the sensitivity of Fas-mediated apoptosis in CD4 T cells. The anti-CD28 costimulation reduces AICD of human CD4 T cells through the up-regulation of c-FLIPshort (20), whereas IL-2 enhanced AICD of CD4 T cells via suppression of transcription and expression of c-FLIPlong (24). Despite the importance of c-FLIPs in T cell apoptosis, very little is known about the signaling pathways that control the expression of c-FLIPs. Recent reports showed that mitogen-activated protein kinase kinase 1 induces c-FLIP in activated T cells (25) and that the phosphatidylinositol 3-kinase/Akt pathway is a predominant regulator of c-FLIP expression in tumor cells (26). In this respect, the up-regulation of c-FLIPs by IL-12 in CD8 T cells shown in our findings suggests a possible signaling pathway in c-FLIPs expression, which might be mediated by IL-12R signaling, including a Janus kinase-STAT pathway (1).

Although CD8 T cells play an important role in the host defense system, the mechanism of CD8 T cell apoptosis is not extensively studied compared with that of CD4 T cells. It was suggested that some cytokines may compensate or synergize with costimulatory signals for survival of CD8 T cells. IL-6 and TNF-α were shown to provide costimulatory pathways for proliferation and survival of CD8 T cells independently of CD28 and IL-2 costimulation (27).
Our data also provide evidence that IL-12 can give the survival signals to CD8 T cells in the absence of CD28 costimulation. It can be speculated that the survival signal provided by IL-12 may contribute to the clonal expansion of effector CD8 T cells, which is important for the killing and the clearance of virus-infected cells or tumor cells. Furthermore, this survival signal by IL-12 can also contribute to the generation and maintenance of memory CD8 T cells. Consistently, the immunization of peptide along with IL-12 was shown to prevent the induction of tolerance while supporting the establishment of memory CD8 T cells (28). It is interesting to note that the neutralization of IL-12 showed a marked increase in apoptosis of alloreactive CD8 T cells within the liver grafts from H-2m-like tyrosine kinase 3 ligand-treated donors, leading to the enhanced graft acceptance (29). These results suggest that the local expression of IL-12 might play a key role in determining the balance between liver transplant tolerance and rejection, presumably caused by the anti-apoptotic effects of IL-12. In summary, our data suggest that IL-12 increases survival of CD8 T cells in TCR-induced cell death, which might provide the important role of IL-12 in peripheral responses of CD8 T cells in autoimmunity, transplantation, and memory generation in vivo.

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