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*J Immunol* 1998; 160:5790-5796; ;
http://www.jimmunol.org/content/160/12/5790

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Differential Involvement of a Fas-CPP32-Like Protease Pathway in Apoptosis of TCR/CD9-Costimulated, Naive T Cells and TCR-Restimulated, Activated T Cells¹

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Our previous study showed that CD9 costimulation of TCR-triggered naive T cells elicits activation ([³H]TdR incorporation) that is similar to CD28 costimulation; however, unlike CD28 costimulation, CD9 costimulation results in apoptosis of these previously activated T cells. Here, we investigated whether the apoptosis occurring after TCR/CD9 stimulation is associated with a death pathway involving Fas stimulation and Fas-mediated caspase activation as observed in activation-induced cell death (AICD). In contrast to AICD, the apoptosis resulting from TCR/CD9 stimulation in C57BL/6 T cells was independent of Fas, because this form of apoptosis was not prevented by anti-Fas ligand mAb and was also induced in MRL/lpr T cells. AICD was observed at 12 h after the restimulation of activated T cells with anti-CD3 and reached a peak level at 24 h after this restimulation. CPP32-like protease activity was detected during AICD. Although TCR/CD9 stimulation-associated apoptosis was observed at 24 h after the stimulation of naive T cells and reached a peak level at 36 h after this stimulation, CPP32-like protease activity in these T cells was only marginal at all time points. Nevertheless, both forms of apoptosis were prevented similarly by two different peptide-based caspase inhibitors. These results indicate that the apoptosis that follows the T cell activation which is induced as a result of CD9 costimulation does not involve a Fas-CPP32-like protease pathway, but suggest that different caspase members are likely to be critical in this form of apoptosis. The Journal of Immunology, 1998, 160: 5790–5796.

Lymphocyte death plays an important role in controlling immune responses. Because such cell deaths are associated with apoptotic features, it has been commonly assumed that they share a common core of intracellular biochemical steps which lead to death, even though the triggering receptors may differ. Numerous studies have implicated members of the caspase family as key participants in apoptotic cell death (1–3). Evidence that this family of proteases are part of a core apoptotic pathway comes largely from studies showing that the inhibitors of these proteases block a wide range of apoptotic death systems (1). Among the caspase family members, CPP32 (caspase-3) appears to play a major role in apoptosis, for the following reasons: 1) CPP32 is processed into active subunits in cells receiving various apoptosis-inducing stimuli (4–8), and 2) CPP32 accounts for the proteolytic activity that is responsible for the cleavage of potential substrates at the onset of apoptosis (9–12).

Apoptosis due to the absence of necessary survival signals is a universal property of almost all cells (13). However, T cells are uniquely capable of undergoing another form of apoptotic death, called activation-induced cell death (AICD).² AICD occurs as a result of repeated TCR stimulation (14–16) and is due to the co-expression of Fas and Fas ligand (FasL) (17–19). Moreover, the caspase family members, especially CPP32-like protease, are thought to be involved in Fas-mediated apoptosis, implying that AICD uses a common apoptotic pathway which leads to CPP32 activation (5, 6, 20). In addition to AICD, various forms of apoptosis occur in T cells, including a Fas-independent form of apoptosis (21, 22). We have recently shown that CD9 costimulation of TCR-triggered naive T cells induces potent an activation (measured by [³H]TdR incorporation) as CD28 costimulation (23); however, the TCR/CD9 stimulation-induced activation is followed immediately by the apoptosis of previously activated T cells (24). It remains to be determined whether this form of apoptosis involves a Fas-CPP32 apoptotic pathway.

In this study, we investigated whether Fas stimulation and/or CPP32 activation are involved in the apoptosis of naive T cells that occurs when TCR is stimulated in combination with the costimulation of CD9 as a non-CD28 costimulatory molecule. The results indicated that in contrast to AICD, TCR/CD9 costimulation-associated apoptosis is independent of Fas, and CPP32 activation is hardly observed even at the time point when high proportions of cells are rendered apoptotic. Nevertheless, this form of apoptosis was prevented by two different peptide-fluoromethyl ketone (FMK) reagents. These FMK reagents were capable of blocking the activity of the caspase family, including the IL-1β-converting

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Received for publication November 24, 1997. Accepted for publication February 12, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan.

3 Abbreviations used in this paper: AICD, activation-induced cell death; FasL, Fas ligand; ICE, IL-1β-converting enzyme; FMK, fluoromethyl ketone; NAC, N-acetyl-l-cysteine; MCA, (7-methoxycoumarin-4-yl)-acetyl; PI, propidium iodide; PCD, programmed cell death.
enzyme (ICE) and CPP32. These results indicate that unlike apoptosis in AICD, neither Fas nor CPP32-like protease(s) are involved in the apoptosis following T cell activation which is induced upon inappropriate costimulation; however different caspase members are likely to be activated in such T cells.

Materials and Methods

Mice

Female C57BL/6 (B6), MRL/Mp-lpr/lpr (MRL/lpr) and control MRL/Mp-lpr/+ (MRL/lpr+) mice were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and used at 5 to 7 wk of age.

Reagents

The following reagents were used: Anti-CD3 (145-2C11) (25), anti-CD9 (9D3) (23), anti-FasL (K10) (26), and anti-I-A\(^\alpha\) (34-5-35) (27) mAbs were purified from culture supernatants or from the ascitic fluids of hybridomas producing the relevant mAb. Anti-Fas mAb (RMF6) was purchased from MBL International (Wataertown, MA), and anti-TNF mAb and anti-TNF antisera were obtained from Genzyme (Cambridge, MA). Rat and mouse control IgG were purchased from Biomedica (Foster City, CA) and Jackson ImmunoResearch Labs (West Grove, PA), respectively. Normal rabbit serum was obtained from the Shizuoka Laboratory Animal Center. The ICE family protease (caspase) inhibitors Cbz-Val-Ala-Asp-(Ome)-FMK (Z-VAD-FMK) and Boc-Asp-(Ome)-FMK (BD-FMK) as well as the control reagent Cbz-Phe-Ala-FMK (ZFA-FMK) were purchased from Enzyme System Products (Dublin, CA). These reagents were dissolved as stock solutions of 50 mM in DMSO and stored at –80°C. The CPP32 fluorochrome substrate (7-methoxycoumarin-4-yl)-acetyl (MCA)-Asp-Glu-Val-Asp-Ala-Pro-Lys-DNP (MCA-DEVDAPK-DNP) was purchased from Peptide Institute (Osaka, Japan). N-acetyl-l-cysteine (NAC) and Con A were purchased from Sigma (St. Louis, Mo).

Preparation of a purified T cell population

Lymph node cells were depleted of B cells and Ia\(^+\) APCs by immunomagnetic negative selection as previously described (28). Briefly, Ia\(^+\) APCs in a lymph node cell population were allowed to react with the anti-I-A\(^\alpha\) mAb. The lymph node cells containing these labeled cells and surface Ig\(^+\) cells (B cells) were incubated with magnetic particles that had been conjugated to goat anti-mouse IgG (Advanced Magnetic, Cambridge, MA). Surface Ig\(^+\) and Ia\(^+\) cells (B cell- and APC-depleted populations) were obtained by removing cell-bound magnetic particles with a rare earth magnet (Advanced Magnetic). The purity of the resulting population was verified by flow cytometry with anti-I-A\(^\alpha\). Purified T cells were consistently >98% CD3\(^+\).

Primary T cell stimulation culture system and culture system of AICD

mAbs were diluted to 1 \(\mu\)g/ml (anti-CD3) or 10 \(\mu\)g/ml (anti-CD9 or anti-CD28) in PBS unless otherwise indicated and then immobilized to the individual wells of 24-well culture plates (Corning 25860, Corning Glass Works, Corning, NY) in a final volume of 0.5 ml. After 3 h, solutions were discarded, and plates were washed twice with PBS. Purified T cells were cultured in 1 ml of RPMI 1640 medium supplemented with 10% FBS and 5 \(\times\) 10\(^{-5}\) 2-ME at a concentration of 2.0 \(\times\) 10\(^5\) cells/well in mAb-immobilized 24-well culture plates in a humidified atmosphere at 5% CO\(_2\) at 37°C for various days. For the AICD system, lymph node cells (2 \(\times\) 10\(^5\)) were cultured in 100 \(\mu\)l of reaction buffer containing 50 mM Pipes-NaOH (pH 7.0), 50 mM KCl, 5 mM EDTA, 2 mM MgCl\(_2\), 1 mM DTT, 1 mM PMSF, 10 \(\mu\)g/ml leupeptin, and 10 \(\mu\)g/ml aprotinin. Cell lysates (5–10 \(\mu\)g) were diluted with reaction buffer (100 mM HEPES-KOH buffer (pH 7.5), 10% sucrose, 0.1% 3-[3-cholamidopropyl]dimethylaminomino]-1-propane-sulfonate, and 10 mM DTT), and incubated at 30°C for 60 min with 1 \(\mu\)M fluorescent substrate. The fluorescence of the cleaved substrates was determined using a spectrofluorometer that had been set at an excitation wavelength of 328 nm and an emission wavelength of 393 nm, and the enzyme activity was expressed in units: 1 U corresponds to the enzyme activity that cleaves 100 pmol of the fluorescent substrate at 30°C in 60 min.

Measurement of apoptosis

Apoptosis was examined as follows: cells were fixed and stained with propidium iodide (PI), and apoptotic cells were quantitated by the determination of the hypodiploid areas in the PI-staining profiles which were used for cell cycle analysis (30). Apoptosis was also determined by DNA fragmentation analysis after agarose gel electrophoresis. T cells (5 \(\times\) 10\(^4\) sample) that had been harvested after cultures were washed in PBS and lysed, and their DNA was isolated as previously described (31). DNA was electrophoresed in 3% agarose gels and stained with 0.1 \(\mu\)g/ml ethidium bromide.

Immunofluorescence staining and flow cytometry

Cells (1 \(\times\) 10\(^6\)) were incubated with anti-Fas (RMF6) mAb. Anti-Fas-labeled cells were incubated with biotinylated mouse anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) followed by RED670-conjugated streptavidin (Life Technologies, Gaithersburg, MD). Cells were analyzed on a FACScalibur (Becton Dickinson, San Jose, CA).

Results

CD9-mediated costimulation of TCR-triggered naive T cells leads to activation followed by apoptosis

Our previous studies have shown that CD9 costimulation of TCR-triggered naive T cells elicits a potent activation of the T cells that is similar to CD28 costimulation (23); however, this costimulation, unlike CD28 costimulation, results in the apoptosis of these previously activated T cells instead of proliferation (24). This finding is indicated in Figure 1. Purified B6 T cells were cultured for 1 to 3 days in wells containing anti-CD9 or anti-CD28 mAb that had been coimmobilized with a suboptimal dose (0.25 \(\mu\)g/ml) of anti-CD3 mAb in the absence of APCs (Fig. 1A). The anti-CD9 mAb strikingly increased the \([\text{H}]\text{TdR}\) uptake of T cells. The magnitude of CD9 costimulation was significantly greater than CD28 costimulation when evaluated on days 1 or 2. While CD28 costimulation exhibited a progressive increase in \([\text{H}]\text{TdR}\) incorporation up to day 3 in culture, CD9 costimulation reached a peak response on day 2 and exhibited a substantial decrease in \([\text{H}]\text{TdR}\) uptake on day 3.

A cell cycle analysis of T cells from the above three groups (Fig. 1B) revealed that the content of the hypodiploid DNA was high in T cells stimulated with anti-CD3 alone; in contrast, T cells stimulated with anti-CD9 plus anti-CD28 displayed low hypodiploid DNA content, and a significant proportion of these cells were at the S/G2/M phases. Despite the induction of potent \([\text{H}]\text{TdR}\) uptake, CD9 costimulation elicited only a slight increase in the proportion of cells at the S/G2/M phases compared with the increase observed in the costimulation-free group. Instead, CD9 costimulation induced levels of apoptosis (induction of cells with hypodiploid DNA) that were comparable with those in the anti-CD3 alone group. Thus, the levels of proliferation did not match with the cell cycle analysis data that we described previously (24). Consistent with this, our previous study (24) showed that CD9 costimulation induced a decrease in viable cell recovery and an increase in the dead cell number.

Apoptosis associated with TCR/CD9 costimulation is independent of Fas

It is known that AICD is dependent upon the interaction between Fas and FasL, both of which are expressed on activated T cells.
and that a Fas-mediated death signal is generated upon the re-
stimulation of these T cells with anti-CD3 (17–19). We first con-
firmed the expression of Fas in TCR/CD9-costimulated T cells.
The activated T cells used as responders in AICD were prepared as
previously described (29): unfractionated B6 spleen cells were
stimulated with 2 μg/ml Con A for 2 days, and cells were subse-
quently maintained with 50 U/ml rIL-2 for an additional 2 days.
These activated T cells and TCR/CD9-costimulated T cells were
stained with anti-Fas mAb. As shown in Figure 2, both types of T
cells express Fas. However, although resting T cells expressed Fas,
the levels of Fas on resting T cells were slightly lower than those
on activated T cells (data not shown).

We examined the effect of NAC, a thiol that is known to down-
regulate Fas expression, on the induction of Fas in TCR/CD9-costimulated T cells. The activated T cells used as responders in AICD were prepared as previously described (29): unfractionated B6 spleen cells were stimulated with 2 μg/ml Con A for 2 days, and cells were subsequently maintained with 50 U/ml rIL-2 for an additional 2 days. These activated T cells and TCR/CD9-costimulated T cells were stained with anti-Fas mAb. As shown in Figure 2, both types of T cells express Fas. However, although resting T cells expressed Fas, the levels of Fas on resting T cells were slightly lower than those on activated T cells (data not shown).

We examined the effect of NAC, a thiol that is known to down-
regulate Fas expression, on the induction of Fas in TCR/CD9-costimulated or activated T cells. When NAC was included during a 2-day maintenance culture with rIL-2, Fas expression was down-regulated (data not shown). The addition of NAC to a 1-day re-
stimulation culture with immobilized anti-CD3 also resulted in the
down-regulation of Fas expression in activated T cells (Fig. 2), which is consistent with previous results (32). Activated T cells restimulated with anti-CD3 in the absence of NAC were rendered apoptotic (AICD), whereas AICD was largely prevented when ac-
tivated T cells were restimulated in the presence of NAC (Fig. 3).

Fas expression was also down-regulated in TCR/CD9-stimulated T
cells by the addition of NAC (Fig. 2). Nevertheless, apoptosis in
these T cells was only marginally prevented, suggesting that this
form of apoptosis is independent of Fas.

To investigate the involvement of the Fas-FasL interaction in
two types of apoptosis more directly, we examined the effect of
anti-FasL mAb on the prevention of apoptosis. AICD was largely
inhibited by anti-FasL mAb, whereas anti-FasL mAb failed to in-
hibit apoptosis in TCR/CD9-stimulated T cells (Fig. 4). This was
also the case with T cells from MRL/+ mice (Fig. 5). In T cells
from MRL/lpr mice, AICD was not induced, due to a lack of Fas
expression (Ref. 21 and Fig. 5). However, TCR/CD9 stimulation-
associated apoptosis occurred in MRL/lpr T cells irrespective of
the presence of anti-FasL mAb (Fig. 5). Taken together, these
results indicate that apoptosis in TCR/CD9-costimulated T cells is
induced independently of Fas.
Our previous study demonstrated that TCR/CD9 costimulation-induced apoptosis is due to a defect in IL-2 production and is corrected by the addition of exogenous IL-2 (24). This finding is shown in Figure 6, Exp. 2. Together, TCR/CD9 costimulation-induced apoptosis is independent of Fas and TNF pathways.

CPP32-like protease activity in TCR/CD9-stimulated, naive T cells or TCR-restimulated, activated T cells

We measured CPP32-like protease activity in naive T cells that were harvested at various hours after primary TCR/CD9 stimulation or in activated T cells that were obtained at various hours after TCR restimulation. Measurements were done in parallel to the detection of the levels of apoptosis as measured by cell cycle analysis. Figure 7 shows that AICD reaches a peak as early as 24 h after restimulation with anti-CD3, while apoptosis after TCR/CD9 costimulation reaches maximal levels around 36 h after primary stimulation of TCR and CD9. The CPP32-like protease activity in both types of T cells harvested 0 to 36 h after restimulation or primary stimulation is summarized in Figure 8. In the AICD model, the CPP32-like protease activity became detectable at 12 h after anti-CD3 restimulation and reached a peak level after 24 h. In contrast, CPP32 activity was undetectable throughout the entire period (0–36 h) of TCR/CD9 stimulation. The results suggest that the apoptosis due to TCR/CD9 stimulation is independent of CPP32 activation.

Peptide-based caspase inhibitors prevent both forms of apoptosis

Finally, we determined whether the peptide-based inhibitors that are capable of blocking caspase-1 (ICE) and/or caspase-3 (CPP32) activities affect the apoptosis induced in TCR/CD9-stimulated T cells and TCR-restimulated T cells. Figure 9 shows that both types of apoptosis are largely prevented by two different peptide-FMK caspase inhibitors. When the doses of these inhibitors were titrated, we found that their efficacy to prevent TCR/CD9 stimulation-associated apoptosis was comparable with their efficacy to prevent AICD (Fig. 10). Taken together, TCR/CD9 stimulation-associated apoptosis requires some members in the caspase family that are different from the caspase-3 (CPP32) subfamily proteases.

Discussion

The results obtained in this study demonstrate that the costimulation of CD9 as a non-CD28 costimulatory molecule during TCR stimulation results in a potent activation of naive T cells, as measured by [3H]TdR uptake, which is followed by apoptosis instead of full activation (cellular proliferation). In contrast to AICD, apoptosis in T cells that are activated based on such an inappropriate costimulation is neither dependent upon Fas nor associated with...
the activation of CPP32-like protease. Nevertheless, this form of apoptosis is also prevented by two different peptide-based caspase inhibitors. These observations indicate that two types of T cell activation-associated apoptosis (AICD vs TCR/CD9 stimulation-induced cell death) differ in the involvement of the pathway from Fas stimulation to CPP32 activation. The results also suggest that TCR/CD9 stimulation-associated apoptosis involves the participation of caspase family member(s) that are different from CPP32 but still sensitive to two representative caspase inhibitors, Z-VAD-FMK and BD-FMK.

It has been established that T cells undergo apoptotic death as a consequence of repeated stimulation (14–16). This form of apoptosis, called AICD, occurs due to the interaction of Fas with FasL, both of which are expressed on activated T cells (17–19). AICD is thought to represent a homeostatic mechanism for controlling the number of Ag-stimulated T cells. A recent study by Van Parijs et al. (21) has shown that TCR-stimulated naive T cells undergo apoptotic death when they are not given a CD28-mediated co-stimulus simultaneously. Namely, the stimulation of T cells from TCR-transgenic mice with the relevant peptide Ag plus paraformaldehyde-fixed APCs which lack costimulatory activity fails to induce activation as measured by [3H]TdR incorporation; rather, this type of stimulation results in the induction of apoptosis. This type of apoptosis, termed programmed cell death (PCD), was found to be independent of Fas and to be corrected by the introduction CD28 costimulation (21).

The present form of apoptosis resulting from TCR/CD9 co-stimulation differs from AICD but appears to resemble the aforementioned PCD. However, there are substantial differences between PCD and apoptosis in our model. PCD occurs as a failure of the activation of naive T cells; in our model, naive T cells are strongly activated by TCR/CD9 stimulation alone, whereas most of the TCR/CD9-stimulated T cells express high levels of IL-2R (24). This finding is consistent with the idea that naive T cells are previously activated by TCR/CD9 costimulation. Therefore, the present form of apoptosis is regarded as an inappropriate co-stimulation-induced cell death. Our results also show that high levels of Fas are expressed on TCR/CD9-costimulated naive T cells. Despite the expression of Fas, our results indicate that, unlike AICD but similar to PCD, the present form of apoptosis is independent of Fas. This conclusion is based on the finding that TCR/CD9 stimulation-associated apoptosis is observed in MRL/lpr T cells, is not blocked by anti-FasL mAb, and is induced even when Fas expression is completely down-regulated by NAC.

Inhibitors of either caspase-1 (ICE) or caspase-3 (CPP32) block Fas-induced apoptosis, which suggests that both subfamilies of caspases are involved in Fas-mediated apoptosis (20, 34, 35). The activation of caspase-3 is dependent upon the activation of a caspase-1 (5), indicating that these proteases are sequentially activated. Thus, a representative death-pathway is formed from Fas through caspase-1 to caspase-3. AICD uses this Fas-CPP32 (caspase-3) protease pathway (5, 6, 20). In contrast, neither Fas nor CPP32 appears to be involved in the present form of apoptosis. While Fas-independent apoptosis appears to occur in the aforementioned PCD (21), little is known regarding CPP32 activation-independent apoptosis.

Recent studies have revealed the complexity regarding the relationship between CPP32 activation and apoptosis induction (34–36). Miossec et al. (36) observed that CPP32 activation through precursor processing can occur in situations in which T cells are activated but are not undergoing apoptosis. This processing was associated with the detection of CPP32-like protease activity. CPP32 activation that is not associated with apoptosis may be explained by more recent observations that apoptosis is inhibited by guest on April 13, 2017 http://www.jimmunol.org/ Downloaded from
by suppressing the activation of proteases that function downstream of CPP32 (37, 38). Thus, CPP32 activation and apoptosis induction are not necessarily associated. Our present results show the induction of apoptosis in T cells that are not expressing CPP32. These results may provide evidence for another line of dissociation between CPP32 activity and apoptosis induction.

Although it is unlikely that CPP32-like protease activity is involved in the apoptosis of TCR/CD9-costimulated naive T cells, this form of apoptosis is blocked by two different peptide-based caspase inhibitors, Z-VAD-FMK and BD-FMK. Z-VAD-FMK and BD-FMK are potent and weak inhibitors, respectively, for caspase-1 (ICE) (22). CPP32 activity was found to be blocked by these two inhibitors in the previous study (22) and in the present in vitro assay for CPP32-like protease activity (our unpublished observations), although the efficacy of these reagents to block CPP32 appeared to be significantly lower compared with that of DEVD-FMK (22). The fact that the apoptosis of T cell lines due to IL-2 withdrawal can be prevented by BD-FMK but not by Z-VAD-FMK (22) suggests that this type of apoptosis does not use a caspase cascade from caspase-1 (ICE) to caspase-3 (CPP32). Likewise, it is possible that Z-VAD-FMK and BD-FMK, especially the latter inhibitor, prevent the present form of apoptosis through an inhibition of the activity of caspase(s) other than CPP32. This postulation is based on the observations of others: The disruption of the CPP32 gene prevented apoptosis in some but not many cell types (39), indicating the dispensable role of CPP32 in most cell types. Consistent with this, caspses other than CPP32 can also cleave poly(ADP-ribose) polymerase and are sensitive to CPP32 inhibitors (40).

Our results illustrate that the apoptosis of naive T cells which is induced as a result of inappropriate costimulation during primary T cell activation exhibits different molecular features from those observed in AICD. This form of apoptosis is not dependent on Fas stimulation or on CPP32 activation. Nevertheless, the apoptosis is prevented by peptide-based inhibitors that are capable of blocking the caspase family members, including caspase-1 and caspase-3. Considering that caspases other than CPP32 can also be blocked by CPP32 inhibitors (40), these observations strongly suggest the involvement of the thus far unidentified caspase cascade in the present form of apoptosis. Thus, the current study could provide a model for investigating the molecular mechanisms underlying the induction of apoptosis by a pathway different from the Fas-CPP32 death pathway.

Acknowledgments
We thank Tomoko Katsuta and Mari Yoneyama for secretarial assistance.

References


