TNF-α Is the Critical Mediator of the Cyclic AMP-Induced Apoptosis of CD8⁺4⁺ Double-Positive Thymocytes

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Apoptosis is one of the key regulatory mechanisms in tissue modeling and development. In the thymus, 95–98% of all thymocytes die by apoptosis because they failed to express a TCR with an optimal affinity for the selecting intrathymic peptide-MHC complexes. We studied the possible role of two prominent nerve growth factor (NGF-TNF) family member systems, Fas ligand (Fasl)-Fas receptor (FasR) and TNF-α-TNFR, in apoptosis of murine CD8⁺4⁺ double-positive (DP) thymocytes induced via TCR-CD3- and cAMP-mediated signaling. TCR-CD3e-mediated apoptosis of DP thymocytes was found not to be dependent on either of the two systems. The Fasl-FasR system was also found to be dispensable for the cAMP-mediated apoptosis. By contrast, cAMP agonists (dibutyryl-cAMP and forskolin) induced apoptosis via TNF-α, as evidenced by 1) the ability of anti-TNF-α mAbs to abrogate cAMP analogue-induced DP apoptosis in a dose-dependent manner; and 2) increased resistance of DP thymocytes from TNF-α−/− and TNFR1−/−II−/− animals to cAMP agonist-mediated apoptosis. cAMP agonists induced DP thymocyte death by a combination of two mechanisms: first, they induced selective up-regulation of TNF-α production, and, second, they sensitized DP thymocytes to TNF-α. The latter effect may be due to the down-regulation of TNFR-associated factor 2 protein. These results identify TNF-α as the critical mediator of cAMP-induced apoptosis in thymocytes and provide a molecular explanation for how the cAMP stimulators, including the sex steroids, may modulate T cell production output, as observed under physiological and pharmacological conditions. The Journal of Immunology, 2000, 164: 1689–1694.

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poptosis plays a critical role at several points in the life of a T cell. First, apoptosis weeds out useless and harmful thymocytes during T cell development. Second, apoptosis is in charge of maintaining mature T cell homeostasis in the course of an immune response by eliminating the majority of activated lymphocytes after the pathogen has been cleared. Finally, apoptosis is likely to be involved in maintaining peripheral T cell tolerance. Molecular events during the activation-induced cell death (AICD) of mature T lymphocytes occur in two phases. The first is initiated by primary TCR-mediated signaling pathways that activate preexisting transcription factors or induce the expression of new transcription factors. These factors regulate expression of many genes, including the genes encoding ligand-receptor pairs belonging to the nerve growth factor-TNF (NGF-TNF) and the corresponding receptor (NGFR-TNFR) families, many of which (e.g., Fas ligand (Fasl):Fas receptor (FasR)-CD95: TNF-α:TNFR1 and II), and CD30 ligand:CD30) have been implicated in the regulation of T cell death (reviewed in Refs. 1–4). The second phase of AICD begins with the interaction of these secondary ligands with their receptors, and culminates in the activation of preexisting proteins, including the caspase family of proteases (reviewed in Refs. 5–7) and endonucleases (8), that execute cell death.

Molecular details of thymocyte apoptosis are, by contrast, less well defined (3). Developing thymocytes (particularly CD8⁺4⁺ double-positive, or DP, cells) readily undergo apoptosis in response to not only TCR stimulation, but also when stimulated via cAMP, corticosteroid, and other pathways. These pathways may or may not share downstream signaling cascades (reviewed in Refs. 6 and 9). Although thymocyte apoptosis remains one of the favorite models to study apoptosis, neither the involvement nor the identity of secondary apoptosis-effecting signaling cascades has been defined for any of the apoptotic pathways. Inhibition of thymocyte apoptosis by transcription and translation blockers argues in favor of the primary-secondary scenario. But definitive evidence for the obligatory involvement of the TNF-TNFR family members and their identification is missing.

Here, we studied TCR-CD3e-mediated and cAMP-mediated apoptosis of DP thymocytes. The TCR-CD3e-mediated apoptosis is an accepted model for negative selection by AICD, and cAMP-mediated apoptosis operates physiologically in the thymus in vivo, where it can be induced, among other stimuli, by sex steroids and β-adrenergic stimulation. We focused on the TNF-TNFR family member systems: TNF-TNFR and Fasl-FasR. Although both TNF-α and Fasl, as well as their cognate receptors, are expressed in the thymus (10–14), thymic selection and apoptosis were not perturbed in mice with deficient expression-function of Fasl-CD95, Fasl, nor in any of the two TNFR knockouts or their combination.
Thymocytes express high levels of cAMP (19–21), and a number of molecules (e.g., PGs, PE, and PGE2; reviewed in Ref. 22) and receptors (e.g., the β2 adrenergic, glucagon, and estrogen receptors; Refs. 22–26) that directly induce cAMP signaling are expressed in the thymus. The neurohumoral axis that operates via the cAMP pathway, and in particular the sex steroids, have long been known to negatively modulate thymic cellularity and T cell production output, as documented by transient thymic involution in physiological (e.g., pregnancy and menstrual cycle–estrus) and pharmacological or experimental (sex hormone therapy; castration, that leads to hypercellularity of the thymus) situations (reviewed in Ref. 27). cAMP signaling was also shown to block thymocyte maturation (28) and to regulate thymocyte adhesion (29). These changes affect immature cortical thymocytes and have fewer, if any, effects on mature T cells (27). In this study, we provide compelling evidence that TNF-α plays a fundamental role in apoptosis induced via the cAMP pathway.

Materials and Methods

Mice

C57BL/6, fas+/+, C57BL/6, fas+/-, and lpr, respectively, in the text), C57BL/6-Tnf-/- (17) and C57BL/6-Tnf-/- (18) (TNFRI and TNFRII, respectively, in the text) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). TNFR I and II mice were obtained from the F2 offspring of the (TNFRI x TNFRII) F1, mice by breeding in the Memorial Sloan-Kettering Cancer Center Core Animal Facility. TNF-α mice were described previously (30). All mice were used at 6–10 wk of age.

Thymocyte preparation, activation, and apoptosis detection

All experiments were performed in RPMI 1640 medium supplemented with pyruvate, 2-ME, glutamine, antibiotics, and 7.5% FBS (RP 7.5). CD4 CD8 DP thymocytes were enriched from total thymocytes by "pan-cytometric analysis was performed on a FACScan flow cytometer using wide scatter gates, to in- (i.e., late apoptotic or necrotic). Mean values and SD of at least three samples per group are reported.

ELISA for the detection of secreted TNF-α

A TNF-α capture ELISA was used to detect secreted TNF-α in cell culture supernatants from DP thymocytes incubated in the presence or the absence of 10 μM forskolin and 10 μg/ml of plate-bound mAb 2C11 (18 h at 37°C), respectively. Wells of polystyrene plates (Immulon II or CellQuest 3.1 software (Becton Dickinson, Mountain View, CA), by analyzing 5 x 10³ cells/sample, using wide scatter gates, to include late apoptotic cells. For the sake of simplicity, the results are reported as a percentage of total annexin + cells, of which <23% (range, 9.3–22.7%) was also PI+ (i.e., late apoptotic or necrotic). Mean values ± SD of at least three samples per group are reported.

ELISA for the detection of secreted TNF-α

A TNF-α capture ELISA was used to detect secreted TNF-α in cell culture supernatants from DP thymocytes incubated in the presence or the absence of 10 μM forskolin and 10 μg/ml of plate-bound mAb 2C11 (18 h at 37°C), respectively. Wells of polystyrene plates (Immulon II or Fisher Biotech, Pittsburgh, PA) coated with 10 μg/ml of mAb anti-TNF-α (PharMingen) in carbonate coating buffer (pH 9.6) and blocked with 2% FCS-PBS were incubated with 200 μl of cell culture supernatant. The plates were then washed and an optimal concentration of biotinylated anti-TNF-α-polyclonal Ab (PharMingen) was added and incubated for 1 h at 37°C. Bound TNF-α was detected using avidin-conjugated HRP (Pierce, Rockford, IL), developed using the substrate o-phenylenediamine dihydrochloride. The developing reaction was stopped by adding 50 μl of 3 M H2SO4, and the OD at 490 nm was analyzed by a MCC/340 Multiskan microplate reader (Fisher Biotech). The assay was conducted in quadruplicate. Results are reported as mean values (±SD).

Northern blot analysis

Total RNA (~10 μg) extracted from DP B6 thymocytes after an 18-h incubation in the presence of complete medium, 10 μM forskolin, and plate-bound anti-CD3ε mAb at 10 μg ml-1, respectively, was electrophoretically fractionated in a 1% agarose formaldehyde gel and then transferred to a nylon membrane (Ambion, Austin, TX). A 260-bp [32P]dCTP probe was generated using PCR primers specific for the partial length cDNA templates of TNF-α. Probe was applied to the blot, with hybridization occurring at 65°C for 16 h. RNA loading was confirmed by the intensity of the 18S RNA bands. Autoradiography of the blot was performed at ~70°C for 72 h on Kodak X-OMAT-AR (Eastman Kodak, Rochester, NY). Optic densitometric analysis of TNF-α mRNA was standardized according to the RNA 18S OD value using the GS-700 Imaging densitometer (Bio-Rad, Hercules, CA) and the accompanying bimolecular analysis software.

Western blot analysis

B6 DP thymocytes were harvested after an 18-h incubation with increasing concentrations of forskolin and db-cAMP. Total cellular extracts were subjected to electrophoresis under nonreducing conditions on a 12.5% polyacrylamide gel (Pharmacia Biotech, Piscataway, NJ) before being electro- phosphorylase transferred to a nitrocellulose (0.2-mm pore size; Schleicher & Schuell, Dassel, Germany). Blots were blocked in PBS containing 5% (w/v) BSA and probed with a 1/1000 dilution of rat anti-TNF-associated factor 2 (TRAF-2) or anti-p34 antisera (Santa Cruz Biotechnology, Santa Cruz, CA). After washing three times in PBS containing 0.05% (v/v) Tween 20, bound Ab was incubated with goat anti-rat IgG conjugated to HRP (1/4000 dilution; Amersham Pharmacia Biotech, Piscataway, NJ), developed, and visualized using the enhanced chemiluminescence technology (enhanced chemiluminescence system; Amersham Pharmacia Biotech).

Results and Discussion

CD3-TCR and cAMP stimulation induce DP thymocytes apoptosis in normal and lpr mice

In this study, we investigated the role of FasL-FasR and TNF- TNFR in the induction of apoptosis by TCR and cAMP pathways as models of Ag-mediated negative intrathymic selection and of neurohumorally induced thymocyte death, respectively. The ability of the TCR agonist anti-CD3ε mAb and the cAMP agonist forskolin to induce apoptosis in overnight-cultured DP thymocytes is shown in Fig. 1A. This time point was elected because it allows an accurate assessment of apoptosis and a relatively acceptable spontaneous background. At later time points, in both normal and knockout mice used in this study, spontaneous apoptosis increases to 40–60%, disallowing the observation of specific effects of stimulators. Both stimuli induced profound DP apoptosis. Moreover, either forskolin or db-cAMP (cAMP analogue) induced apoptosis in a dose-dependent manner (Fig. 1B), demonstrating the equipo- tentcy of these two cAMP-agonists. (Although all experiments in this study were performed with both compounds with indistinguishable results, for the sake of brevity we elected to show the db-cAMP experiments only at certain critical points.) The fact that cAMP agonists caused substantial apoptosis of DP thymocytes was consistent with the effect of cAMP stimulators on thymic cellularity (33), but stood in contrast to the protective role of these two cAMP-agonists. (Although all experiments in this study were performed with both compounds with indistinguishable results, for the sake of brevity we elected to show the db-cAMP experiments only at certain critical points.) The fact that cAMP agonists caused substantial apoptosis of DP thymocytes was consistent with the effect of cAMP stimulators on thymic cellularity (33), but stood in contrast to the protective role of these compounds against the AICD of peripheral T cells (34, 35). As expected, cAMP-mediated apoptosis occurred in a caspase-depen- dent manner, as this, like many other types of DP thymocyte apo- ptosis (6), could be inhibited by the specific caspase inhibitor 13 ×VAD-fmk (Fig. 2).

We next investigated the involvement of Fas-dependent signal- ing in the two types of apoptotic death by stimulating DP thymo- cytes from normal and Fas-defective lpr/lpr B6 mice. Overall,
stimulation with dexamethasone, PMA, ionomycin, anti-CD3ε, forskolin, and their combination induced comparable apoptosis in both normal and lpr/lpr thymocytes (data not shown), corroborating previous results (15, 37) and excluding the obligatory role of Fas signaling in these types of apoptosis in vitro.

**cAMP agonists induce DP thymocyte apoptosis via TNF-α**

To investigate the role of TNF-TNFR in cAMP- or TCR-dependent apoptosis, we initially used a neutralizing anti-TNF-α mAb (MP6-XT22) that was introduced to the DP thymocytes simultaneously with primary stimuli. Fig. 3 shows that this Ab abrogated, in a dose-dependent manner, either db-cAMP or forskolin-mediated apoptosis in DP thymocytes, whereas the control rat IgG1 had no effects (data not shown). By contrast, TNF-α neutralization had no effect on DP apoptosis induced by dexamethasone (data not shown, but see Fig. 4). Furthermore, TNF-α neutralization had variable and inconclusive effects on apoptosis induced by TCR agonists (data not shown), consistent with the findings that this pathway may be dispensable for the induction of TCR-mediated apoptosis in vitro.
apoptosis of thymocytes (17, 18, 37). The above data were confirmed in six separate experiments and using two different anti-TNF-α Abs (MP6-XT22 in Fig. 3; catalogue no. 05-168, Upstate Biotechnology; data not shown). These results strongly suggested that TNF-α is the key mediator of cAMP-mediated apoptosis.

To test the relevance of the above observations by a genetic approach, we took advantage of the TNF-α2/2 and TNFR I2/2 II2/2 knockout mice that have normal thymocyte numbers and phenotype, and are otherwise indistinguishable from normal counterparts (17, 18, 30). Of note, in DP thymocytes of these animals, db-cAMP- (data not shown) and forskolin-mediated (Fig. 4) apoptosis was inhibited by >70%. In contrast, as expected, dexamethasone-mediated apoptosis was not affected (Fig. 4). Although sex steroids (such as testosterone and estrogens, produced in the gonads) invariably induce the activation of cAMP signaling, corticosteroids (produced in the adrenal gland cortex) are known to induce apoptosis by a different mechanism, possibly via their nuclear receptor (8). A similar level of inhibition (62–81%) was observed in a dose titration experiment over a range of cAMP agonist concentrations (2–50 μM). In contrast, DP AICD induced by TCR agonists in DP TNFRI2/2 II2/2 thymocytes was indistinguishable from that observed in the wild-type controls (V. N. Ivanov, unpublished observations), corroborating the redundant role of the TNF-TNFR pathway in negative selection. These results definitively demonstrate that TNF-α is the central player in the induction of cAMP-mediated DP thymocyte death.

Mechanism of action of cAMP agonists

Mechanistically, the most straightforward explanation for the effect of cAMP agonists would be that they stimulate DP thymocytes to produce TNF-α that then kills them by fratricide or suicide. To test this hypothesis, we investigated TNF-α mRNA and protein production in stimulated DP cells. Northern blot analysis was conducted to investigate whether specific cAMP agonists could induce mRNA TNF-α up-regulation. The results reveal a 5- and 9-fold increase of TNF-α mRNA following cAMP agonist and anti-CD3ε stimulation, respectively (Fig. 5, A and B). These results speak to the effect of TNF-α mRNA induction by cAMP agonists. The remaining question, however, was whether this was translated into protein production. Several reports have shown that TNF-α is processed from the membrane-bound precursors into soluble effector molecules by a metalloproteinase (38–42). A TNF-α capture ELISA was conducted to assess whether such processing of TNF-α into a soluble form occurred following stimulation of DP thymocytes. Stimulation with either cAMP agonist or immobilized anti-CD3ε mAb of DP thymocytes induced processing of the membrane TNF-α form and releasing of TNF-α (Fig. 5C). We therefore conclude that cAMP agonist apoptotic activity is likely to be due to an increase of TNF-α protein. However, given the relatively unremarkable effect of TNF-α on unstimulated thymocytes...
(24, 43) and given that αTCR-induced apoptosis is not TNF-dependent despite large induction of TNF-α, it was likely that cAMP agonists may also facilitate DP apoptosis by other means.

Recent studies have shown that inhibition or targeted deletion of the TNFR-associated factor TRAF-2 enhanced TNF-α-induced apoptosis of murine lymphocytes or fibroblast, suggesting that TRAF-2 may be responsible for recruitment of antiapoptotic proteins (44–46). To determine the relationship between TRAF-2 and cAMP analogue-induced apoptosis, we studied TRAF-2 protein expression levels in DP thymocytes treated with cAMP agonists. Fig. 6 shows that the expression of TRAF-2 in DP thymocytes from wild-type mice incubated with increasing amounts of forskolin and db-cAMP was impaired in a dose-dependent manner. These data suggest that cAMP agonists in DP thymocytes not only induce an increase of the TNF-α production, but also a decrease of TRAF-2 protein. The reduction of TRAF-2 protein was observed at cAMP agonist levels slightly higher than those inducing apoptosis in our assays. This may mean that the protein is functionally disabled by cAMP agonist treatment before its disappearance or that longer time periods are required for the decay to be evident (we measured TRAF-2 after 12 h in this experiment). Alternatively, it is possible that we are observing an unrelated epiphenomenon, and additional experiments will be required to address this issue. Regardless, there is an inversely proportional association between TRAF-2 and DP thymocyte cAMP-mediated apoptosis. These findings are in agreement with previous studies in which TRAF-2 has been determined to mediate antiapoptotic signaling (44–46).

It is well established that the mere presence of murine TNF-α has rather marginal effects on DP thymocyte apoptosis. How, then, do cAMP agonists exert apoptotic activity? The above data were consistent with the possibility that cAMP agonists both induce TNF-α secretion and sensitize DP thymocytes to its apoptotic effects, possibly by down-regulating TRAF-2. To address this possibility directly, recombinant murine TNF-α (rTNF-α) was added to DP thymocytes from TNF-α−/− mice in the presence or the absence of forskolin. DP cells from TNF-α−/− did not undergo apoptosis in the presence of forskolin or rTNF-α alone. Remarkably, DP thymocytes from TNF-α−/− underwent significant apoptosis in the presence of rTNF-α when incubated simultaneously with cAMP agonists (Fig. 7). These data indicate that cAMP agonists induce DP apoptosis by a two-pronged action: by inducing TNF-α secretion and by sensitizing DP thymocytes to the apoptotic effect of TNF-α.

The above results, like the previous reports (17, 18, 30), show that TCR-mediated apoptosis of DP thymocytes does not depend on the TNF-TNFR pathway. However, the increased levels of mRNA and soluble TNF-α detected following anti-CD3ε stimulation (Fig. 5) suggest that this pathway might still be one of the many redundant pathways that induces DP apoptosis in vivo. The other possibility is that the TNF-α activity on DP thymocytes is countered by a concomitant TNF-α-mediated activation of NF-κB. In several recent publications, an essential role of NF-κB in preventing TNF-α-induced death was established in RelA−/− cells or by inhibition of RelA in normal cells (47–50). Consistent with these results, we observed that Bcl-2 protection against cAMP-induced apoptosis was always accompanied by RelA-p50 up-regulation (V. N. Ivanov, unpublished results). Of note, anti-CD3ε stimulation was accompanied by both TNF-α expression and NF-κB induction (data not shown), perhaps providing an explanation for protection against TNF-α-induced death.

The major conclusion from this study is that TNF-α acts as one of the main mediators of cAMP apoptosis in normal DP thymocytes. The fact that TNF-α−/− mice do not have enlarged thymi most likely reflects the action of compensatory mechanisms that frequently operate in knockout animals. In the normal mouse, where such mechanisms are not operative, TNF-α neutralization completely abrogates cAMP-mediated apoptosis (Fig. 3), whereas the gene disruption in TNF-α−/− DP cells inhibits this death by only 70%. Regardless of the existence and the importance of the compensatory mechanisms, the main conclusion of this work is that TNF-α plays a very prominent, and probably critical, role in mediating cAMP-induced apoptosis of DP cells. In the context of the extensive sympathetic innervation of the thymus, the presence of neurohumoral receptors and high thymic cAMP cellular content (20–26), and the recent findings on the effects of cAMP on thymocyte development (28), our results elucidate perhaps the key molecular mechanism explaining the negative influence of the cAMP axis on thymocyte production-output, including the well-known phenomena of thymocyte depletion following stimulation with the sex steroids in pregnancy and menstrual cycle-estrus and thymocyte hypercellularity following experimental and pharmacological castration (Ref. 33; reviewed in Ref. 27). Physiological relevance of our findings is further confirmed by the demonstration that testosterone can mediate all of the effects ascribed to cAMP agonists in this study, and that it does so in a TNF-α-dependent way.
manner (J. A. Guevara, M. W. Marino, V. N. Ivanov, and J. Nikolić-Zugić, manuscript in preparation).

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References