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A Role for Caspases in Controlling IL-4 Expression in T Cells


Although caspase activation is critical for T cell proliferation following activation, the role of caspases in T cell differentiation is unclear. In this study, we have examined the effect of inhibition of caspases on the process of Th1/Th2 differentiation. Naïve CD4⁺ T cells activated under neutral differentiation conditions in the presence of the pan caspase inhibitor benzoylloxy carbonyl-Val-Ala-Asp (Z-VAD) fluoromethylketone showed increased Th2 cell differentiation concomitant with an up-regulation of GATA-3. Z-VAD induced optimal Th2 differentiation when T cells were stimulated under strong primary activation conditions. Treatment of naïve CD4⁺ T cells with Z-VAD under strong activation conditions led to a 6-fold increase in IL-4 mRNA compared with control-treated T cells. The Z-VAD-induced increase in IL-4 transcription occurred within 24 h of activation and was independent of Stat6. IFN-γ mRNA expression was not affected by Z-VAD at the 24-h time point. Z-VAD did not augment IL-4 expression from a committed Th2 cell, suggesting that caspases regulate IL-4 expression specifically during primary T cell activation. Z-VAD did not augment IL-12-driven Th1 differentiation. Activation of T cells in the presence of Z-VAD led to a specific increase in the expression of the transcription factor c-fos. Lastly, retrovirus-mediated expression of the antiapoptotic protein Bcl-2 resulted in an enhancement of Th2 cytokine expression, suggesting that inhibition of caspase activation by Bcl-2 can also modulate IL-4 expression. These findings reveal a novel regulatory mechanism of cytokine expression by caspases, and may explain how signaling pathways that inhibit apoptosis tend to promote Th2 differentiation.


Materials and Methods

Mice

Animals were maintained under specific pathogen-free conditions in animal facilities certified by the American Association of Laboratory Animal
Care. Four- to 8-wk-old wild-type (WT) mice and STAT6+/− mice were maintained on a mixed C57BL/6 129 background, and TCR transgenic mice have been previously described (23).

**T cell isolation and culture**

Naive (CD62 ligand-positive) spleen and lymph node CD4+ T cells were isolated by FACS as described elsewhere (24). Sorted naive CD4+ T cells were activated with plate-bound anti-CD3 Ab (10 μg/ml) (BD Pharmingen) in the presence of pan caspase inhibitor Z-VAD (R&D Systems) or the vehicle control (DMSO). Z-VAD was used at a final concentration of 100 or 50 μM. Lymph node cells from AND TCR transgenic were stimulated with irradiated autologous splenocytes at a ratio of 1:5 with various concentrations of pigeon cytochrome c (Sigma-Aldrich). Activated T cell cultures were expanded with human IL-2 (100 U/ml; Biological Resources Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute-Frederick Cancer Research and Development Center) for 3–5 days. These cells were then washed with PBS and restimulated with anti-CD3 Ab (10 μg/ml) alone. Supernatants were collected after 24 h and levels of IL-4, IL-5, IL-13, IL-10, and IFN-γ were measured by ELISA. Reagents for these ELISAs were obtained from BD Pharmingen.

**Western blot analysis**

Whole cell lysates were prepared from naive CD4+ T cells with standard techniques. Abs to GATA-3 (clone H9262) and c-fos (clone H-125) were obtained from Santa Cruz Biotechnology. Proteins were detected with HRP-conjugated secondary Abs and developed by ECL (Amersham Pharmacia Biotech). Blots were also probed with Ab to β-actin (Sigma-Aldrich) to demonstrate equivalent protein loading.

**Relative quantitation of mRNAs by RT-PCR and real-time quantitative RT-PCR**

Total RNA was isolated from the naive CD4+ T cells stimulated with anti-CD3 Ab with and without Z-VAD after 24, 48, or 72 h with TRIzol reagent (Invitrogen Life Technologies). Reverse-transcriptase reactions were done using the SuperScript First-Strand cDNA Synthesis System (Invitrogen Life Technologies). Empirically determined concentrations of first-strand cDNA were used in RT-PCR to ensure linear amplification of sequences. Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems) and following manufacturer’s protocols. Primers and concentrations used were: IL-4 sense (300 nM) TAAGGTGGATAGATGTGGAAGAA, IL-4 antisense (300 nM) TCAAGTGGCATAGATGTGGAAGAA, GAPDH antisense (50 nM) ATACCAGGAAATGAGTGGCT, GAPDH sense (50 nM) CCAGGTTGCTCTGCAGGATTTTCATG; GAPDH antisense (300 nM) GAAGCCCTACAGACGAGCTCA; IFN-γ antisense (500 nM) TGGGCTCTGAGACTGGCTCTGCAGGATTTTCATG; GAPDH sense (50 nM) CCAGGTTGCTCTGCAGGACT, GAPDH antisense (50 nM) ATACACGAAATGAGCITTGACAAAGTG. Each of these primer sets gave a unique product. PCR assays were performed in triplicate and the data was pooled. Values obtained for levels of mRNAs were normalized to the levels of GAPDH mRNA.

**Retroviral infection of T cells**

The pMIEG3 plasmid was described earlier (25). A plasmid containing the Bcl-2 cDNA was removed from the plasmid by SalI digestion and cloned into pMIEG3 via XhoI. The pMIEG3 plasmid was described earlier (25, 26). One day after transfection the phoenix retroviral supernatants were harvested and used to infect lymph node T cells by spin infection after polybrene (8 μg/ml; Sigma-Aldrich) addition. Lymph node cells isolated from WT mice were activated with concanavalin A (Con A) (2 μg/ml) at a concentration of 2 million cells/ml for 24 h before infection. After spin infection, the T cells were cultured at 32°C for 24 h, then stimulated with human IL-2 (100 U/ml) and moved to the 37°C incubator for 48 h before sorting the GFP-positive cells by FACS. Sorted cells were stimulated with plate-bound anti-CD3 Ab for 24 and 48 h after which the supernatants were analyzed for the production of IL-4, IL-10, and IFN-γ by ELISA.

**Cell proliferation and apoptosis assay**

Sorted naive CD4+ T cells were washed twice in PBS, resuspended at a concentration of 107 cells/ml and incubated with 5 μM of CFSE (Molecular Probes) at 37°C for 10 min. Cells were then washed twice with cold DMEM supplemented with 10% FCS. Cell division status of cells was determined by measuring CFSE fluorescence on day 5. Apoptosis was measured by annexin V staining according to the manufacturer’s protocol.
the undivided population in the cells treated with Z-VAD was much higher (37%) as compared with control cells (2%). Thus these findings are in accordance with earlier reports that examined the effects of caspase inhibitors on T cell proliferation (12–14). Next, we determined whether Z-VAD could rescue CD3-activated primary T cells from apoptosis. Thus, we activated naive CD4⁺ T cells with anti-CD3 Ab for 24 h with and without Z-VAD and analyzed the proportion of apoptotic cells by annexin V staining (Fig. 2). Annexin V binds to phosphatidylserine that presents on the outer membrane in cells undergoing apoptosis. The results demonstrate that Z-VAD treatment resulted in a slightly lower proportion of cells undergoing apoptosis: 18% with Z-VAD vs 29% in the control (Fig. 2). Thus, this data indicates that Z-VAD treatment does not dramatically inhibit apoptosis of primary T cells activated with anti-CD3 Abs.

**Increased Th2 differentiation when caspase activity is inhibited**

To investigate the role of caspases in Th1/Th2 differentiation, naive CD4⁺ cells from WT mice were again activated with plate-bound anti-CD3 in the presence of Z-VAD or appropriate control (DMSO). After 48 h of stimulation with anti-CD3 Abs, IL-2 (100 U/ml) was added to the cells to promote cell expansion. After 3 or 4 days of growth in IL-2-containing medium, the cells were restimulated with anti-CD3 and supernatants were taken after 24 h for analysis of cytokine production by ELISA. We found that Z-VAD led to a marked increase in the production of the Th2-specific cytokines IL-4, IL-5, and IL-13; at the same time, there was a significant reduction in IFN-γ production (Fig. 3). Similar results were also obtained when naive CD4⁺ T cells were stimulated with Con A in the presence of Z-VAD (data not shown). We have also examined the percentages of IL-4 and IFN-γ-producing T cells by intracellular cytokine staining, and we have found that although the percentage of IFN-γ-producing T cells decreases dramatically with Z-VAD treatment, the percentage of IL-4-producing T cells does not change significantly with Z-VAD treatment (data not shown). The reason for this may relate to the relative sensitivity of detection of IL-4 vs IFN-γ by intracellular staining. The cathepsin-B inhibitor peptide benzoxycarbonyl-Phe-Ala-fluoromethyl-ketone (Z-FA-fmk) did not increase Th2 differentiation, and in some experiments inhibited Th2 differentiation (data not shown). Our results with Z-VAD were therefore specific and reproducible, indicating that caspases play an important and previously unrecognized role in Th cell differentiation.

**Strong primary T cell activation is required for the increased Th2 differentiation induced by caspase inhibition**

We next analyzed whether the increased Th2 differentiation induced with Z-VAD was dependent upon the initial T cell priming conditions. We used two different systems to test this idea: first, by titrating the amount of anti-CD3 Ab used to activate naive CD4⁺ T cells (Fig. 4A), and second, by using T cells from the “AND” TCR transgenic mouse (23), and activating the T cells with varying doses of Ag (Fig. 4B). After the initial T cell activation in the presence of either DMSO (control) or Z-VAD, we again grew the cells for 3 or 4 days in IL-2-containing medium, and then the cells were restimulated with anti-CD3 and supernatants were taken after 24 h for analysis of cytokine production by ELISA. With either system, we observed a similar pattern. Under the lowest amount of anti-CD3 Ab tested or the lowest amount of Ag tested, Z-VAD actually inhibited Th2 differentiation (shown by the ratio of IL-4 to IFN-γ in Fig. 4). In contrast, under stronger activation conditions, with higher amounts of anti-CD3 Ab or higher amounts of Ag, Z-VAD enhanced Th2 differentiation markedly similar to what was observed in Fig. 3. Consistent with other reports (27–30), we
observed that Th2 differentiation decreases and Th1 differentiation increases as the strength of T cell activation increases (Fig. 4, control bars). Caspase inhibition with Z-VAD appears to alter this normal relationship between the strength of TCR signaling and T cell differentiation.

Caspase inhibition up-regulates GATA-3 expression

To determine whether the increased Th2 differentiation observed with Z-VAD correlates with the induction of GATA-3 expression, we measured GATA-3 protein levels in naive CD4+ T cells treated with Z-VAD and activated under strong activation conditions. Thus naive CD4+ T cells from WT mice were stimulated with anti-CD3 with or without Z-VAD or Con A with and without Z-VAD for 3 days, and whole cell lysates were assessed for protein levels of GATA-3. The increased Th2 differentiation induced by Z-VAD clearly resulted in a significant up-regulation of GATA-3 expression (Fig. 5).

Caspase inhibition induces IL-4 but not IFN-γ mRNA in WT naive CD4+ T cells

We next addressed whether Z-VAD can affect cytokine expression at the mRNA level. For this purpose, naive CD4+ T cells from WT mice were activated by anti-CD3 Abs under strong activation conditions with and without Z-VAD for 24, 48, or 72 h. IL-4 and IFN-γ mRNA levels at these time points were carefully quantified by real time RT-PCR, and levels of these mRNAs were normalized to GAPDH mRNA levels also obtained by real time RT-PCR. We found that Z-VAD induced 6-fold higher IL-4 mRNA levels at 24 h compared with control, whereas Z-VAD did not alter the IFN-γ mRNA levels (Fig. 6). At 48 h there was a 2-fold increase in IL-4 levels and a slight reduction in IFN-γ levels, and at 72 h there was only a slight increase in IL-4 levels and a significant reduction in IFN-γ levels. These results strongly suggest that caspases can regulate IL-4 transcription during the early stages of primary T cell activation.

Caspases can regulate early IL-4 expression independently of Stat6, however Stat6 is required for normal Th2 differentiation

We next examined whether Stat6 signaling is necessary for the increased IL-4 expression seen with Z-VAD treatment. For this purpose, naive CD4+ T cells from Stat6−/− mice were stimulated under similar conditions as WT T cells, and the mRNA levels for IL-4 and IFN-γ were quantified by real time RT-PCR. Stat6−/− naive T cells stimulated under strong activation conditions in the presence of Z-VAD showed a 4-fold increase in IL-4 mRNA at 24 h compared with control-treated cells, while IFN-γ mRNA levels were not affected (Fig. 7A). These results indicate that the early burst in IL-4 expression induced by Z-VAD is independent of Stat6. We next wanted to test whether Z-VAD could also promote Th2 differentiation in Stat6−/− T cells. Thus naive CD4+ T cells from Stat6−/− mice were stimulated with anti-CD3 Abs in the presence of Z-VAD or DMSO as a control and then grown in IL-2 media as with the WT T cells treated with Z-VAD. However, Z-VAD-treated Stat6−/− T cells produced similar amounts of IL-4 as control-treated Stat6−/− cells (Fig. 7B). These data show that although Z-VAD increases the early production of IL-4 independent of Stat6 activity, subsequent Th2 differentiation occurs via the conventional Stat6 dependent pathway.

Caspase inhibition does not affect IL-4 production by committed Th2 cells

To determine whether the effect of IL-4 up-regulation by Z-VAD extends to cells that are already committed to producing IL-4, or fully differentiated Th2 cells, we stimulated the well-characterized D10 Th2 cell line with varying concentrations of anti-CD3 in the presence of Z-VAD or appropriate control for 24 h. IL-4 production was determined by ELISA. We found that IL-4 expression was not affected by Z-VAD in D10 cells, over a very broad range of CD3 stimulation (Fig. 8). Our data suggest that Z-VAD does not alter TCR signaling leading to IL-4 expression, but rather Z-VAD affects a pathway regulating IL-4 expression that is unique to naive T cells.

Caspase inhibition does not augment Th1 differentiation response to IL-12

We next explored the effect of Z-VAD in naive CD4+ T cells under Th1-inducing conditions. We therefore activated naive CD4+ T cells from WT mice with anti-CD3 Abs and cytokine levels in the supernatants taken after 24 h were determined by ELISA.
CD4⁺ T cells with anti-CD3 under strong activation conditions with and without Z-VAD plus varied concentrations of IL-12 ranging from 0.1 to 20 ng/ml. Following the primary stimulation, the cells were grown in IL-2-containing medium for 4 days. The cells were restimulated with anti-CD3, and IFN-γ secretion was analyzed. We found that as measured by the level of IFN-γ secretion, caspase inhibition with Z-VAD did not significantly augment Th1 differentiation induced by IL-12 (Fig. 9). This experiment suggests that caspases do not regulate the general differentiation of primary naive T cells, but rather that caspases act specifically by regulating the transcription of IL-4 during primary T cell activation.

Caspase inhibition leads to an increase in c-fos expression following primary T cell activation

Because Z-VAD affects the early IL-4 transcription, we decided to analyze signaling pathways and transcription factors known to be involved in the regulation of IL-4 expression and T cell differentiation. To analyze whether Z-VAD affects these signaling molecules and transcription factors, we stimulated naive WT CD4⁺ T cells with anti-CD3 under strong activation conditions with and without Z-VAD for 24 h. As shown in Table I, Z-VAD treatment for 24 h does not affect a large number of regulatory proteins such as transcription factors and signaling kinases. Notably with respect to IL-4 expression, GATA-3 and NFAT levels are not affected after 24 h of activation in the presence of Z-VAD. In contrast, the level of c-fos protein was increased dramatically by Z-VAD treatment, as assessed by Western blot analysis (Fig. 10). Because c-fos has been connected to IL-4 transcription (31, 32), and AP-1 factors in general are strongly implicated in IL-4 transcription (33, 34), the up-regulation of c-fos after Z-VAD treatment may be responsible for the increased IL-4 expression also observed with Z-VAD.

Bcl-2 specifically increases expression of Th2 cytokines

We next tested whether Bcl-2, an antiapoptotic protein that can inhibit caspase activation (35), can regulate Th2 cytokine expression. A retroviral system was used to introduce Bcl-2 into primary T cells. WT lymph node T cells were stimulated with Con A and infected with control (GFP) or Bcl-2 expressing retrovirus. Infected T cells were isolated by GFP expression. The sorted GFP-positive cells were restimulated with anti-CD3 Ab for analysis of cytokine secretion (Fig. 11). Overexpression of Bcl-2 in CD4⁺ T cells led to an enhancement of both IL-4 and IL-10 at 24 and 48 h, although the effect was more pronounced at 48 h. In contrast, IFN-γ levels were not altered by Bcl-2, which indicates that Bcl-2 specifically promotes the expression of Th2 cytokines in T cells.

Discussion

Our current study reveals that caspases play an important role in Th cell differentiation by controlling cytokine expression during primary T cell activation. In this report we demonstrate that strong T cell activation conditions together with the pan caspase inhibitor Z-VAD preferentially promotes Th2 differentiation. The mechanism for Z-VAD action appears to be that, within 24 h of naive T cell activation, Z-VAD induces a large increase of IL-4 transcription in the T cell that is independent of Stat6 function. This early burst of IL-4 induced by Z-VAD then can feed back to the activated T cell and promote Th2 differentiation via the conventional Stat6-dependent mechanism. Z-VAD does not alter IFN-γ transcription in this early activation period, indicating the effect of Z-VAD is specific for IL-4.

We have found that Z-VAD is ineffective at inducing Th2 differentiation, and may even inhibit Th2 differentiation, when naive CD4⁺ T cells are activated under weak primary activation conditions (Fig. 4). In the absence of Z-VAD, we found that weaker activating conditions with primary T cells favor Th2 differentiation (Fig. 4), and this is consistent with published data (27, 28, 30). Caspases are necessary for normal T cell proliferation, and caspase inhibition also leads to defective transcription of IL-2 by T cells (12–14, 36). Our data can therefore be interpreted as supporting the idea that caspase inhibition weakens T cell activation. Thus, Z-VAD may convert a strong TCR signal, which normally favors Th1 differentiation, into a weaker TCR signal, which favors Th2 differentiation (27–30). When naive T cells are activated with weak primary signals, Z-VAD weakens this signal even more, leading to poor T cell activation, weak IL-4 transcription and poor Th2 differentiation. However, we do not detect the same pattern of NFATp, NFATc, Erk1, and AP-1 regulation with Z-VAD treatment as seen with the weak TCR signaling system (28–30). One
way to reconcile these results is that weak TCR signaling is indeed critical for initial IL-4 secretion by CD4+ T cells and their subsequent Th2 differentiation, but this effect can also be mediated by other signaling pathways than NFATp, NFATc, Erk1, and AP-1.

An alternative mechanism for how Z-VAD increases IL-4 transcription and Th2 differentiation involves the AP-1 protein c-fos. For example, c-fos has been shown to bind to the P2 NFAT element in the IL-4 promoter in T cells (31). In addition, c-fos has been shown to strongly activate transcription of a multimerized NFAT/AP-1 site from the IL-4 promoter (32). In general, the AP-1 transcriptional complex, of which c-fos is a strongly activating component, has been linked to IL-4 transcription (33, 34). Although Jorritsma et al. (28) have reported that increased IL-4 transcription in activated naïve CD4+ T cells is associated with an AP-1 complex that is devoid of c-fos protein and is composed of Jun-Jun dimers, our results with Z-VAD clearly diverge from the Jorritsma et al. (28) system in a number of respects, as pointed out earlier. Of course, we cannot rule out that the inhibition of caspases during primary T cell activation may affect another transcriptional pathway, besides c-fos, that impinges upon the IL-4 gene. Nonetheless, because we do not observe significant changes in several key regulatory proteins known to be involved directly in IL-4 transcription, we think the up-regulation of c-fos with Z-VAD treatment is particularly critical in our system. One possibility is that c-fos up-regulates the expression of a transcription factor (yet to be characterized) that can promote IL-4 transcription.

He et al. (37) have shown that c-fos undergoes rapid breakdown following the induction of apoptosis and caspase activation, and moreover that Bcl-2 can inhibit c-fos degradation. These data therefore strongly support our current results showing up-regulation of c-fos by caspase inhibition. This finding may also explain the fact that overexpression of Bcl-2 can also promote Th2 differentiation.

Interestingly, we found that although Z-VAD promotes Th2 differentiation under strong activation conditions, Z-VAD also inhibits the proliferation of naïve CD4+ T cells under these same conditions. This finding is in disagreement with the hypothesis that IL-4 transcription and Th2 differentiation requires induction of cell cycle progression (38). Our data therefore strongly support the data from Ben-Sasson et al. (39) that cell division is not a clock for cytokine production, and in particular our data support the idea that IL-4 can be produced from CD4+ T cells that have not entered the cell cycle.

A recent report from Wu et al. (40) evaluated the effect of cellular FLIP long isoform (c-FLIPL), a caspase-defective homologue of caspase 8 that can inhibit apoptosis, in a transgenic system in which c-FLIPL is expressed in T cells. This group showed that activated CD4+ T cells from c-FLIPL transgenic mice manifest a strong Th2 cytokine bias and enhanced allergic airway inflammation. The Th2 bias in their study correlated with decreased levels of NF-KB and increased levels of GATA-3 expression. In our study, we observed an up-regulation of GATA-3 at later stages of Th2 differentiation, which is consistent with the findings of Wu et al. (40). We observed a slight decrease in NF-KB activation after 24 h of Z-VAD treatment (data not shown), but it is not clear that this decrease in NF-KB can explain the striking increase in IL-4 expression we observe at this same time point. Wu et al. did not examine whether there was an up-regulation of IL-4 transcription 24 h after activation in the transgenic T cells nor did they assess the dependence of the Th2 differentiation upon Stat6. Moreover, they did not analyze specifically c-fos levels, although they did observe increased AP-1 transcriptional activity with the c-FLIPL transgene, which would be consistent with increased c-fos levels as in our study. The c-FLIPL transgene promoted ERK activity, which we did not observe in our system. Additionally, overexpression of c-FLIPL led to hyperproliferation of T cells as opposed to the decreased proliferation seen with Z-VAD. Although there are clear differences, this study has striking similarities to our current study, in that we also find that caspase inhibition and decreased apoptosis can promote Th2 differentiation. Thus, it is tempting to speculate that the increased Th2 bias in Wu et al. (40) is due to a similar molecular mechanism as the Th2 bias induced by Z-VAD because both systems involve an interference in caspase activity. This mechanism may be due to up-regulation of c-fos, or via some other mechanism that is as yet uncharacterized.

Our current study showing increased Th2 differentiation with both the caspase inhibitor Z-VAD as well as with overexpression of Bcl-2, helps highlight an emerging connection between factors that regulate apoptosis and Th2 differentiation. The trend from several studies is that decreased apoptosis promotes Th2 differentiation. Thus, up-regulation of apoptosis inhibitory factors such as c-FLIPL (40), Gfi-1 (41), and CD28 (42, 43), can all promote increased Th2 differentiation. This idea is reinforced by the increased Th2 differentiation seen with loss of regulatory proteins.
that can promote apoptosis, such as CTLA-4 (44), Bcl-6 (26), and p53 (17). In our study, when we inhibit caspase activity, we observe an early burst of IL-4 production that can promote further Th2 differentiation. Many of these other studies that show increased Th2 differentiation could conceivably be explained by a similar mechanism involving increased IL-4 transcription early after Th cell activation, due to alterations in caspase activity during Th cell activation.

The data presented in this study show the ability of Z-VAD to promote Th2 differentiation in primary T cells through increased production of IL-4. Our studies point to a novel role of caspases in cytokine expression, as well as a novel control of IL-4 production and Th2 differentiation by caspases. Additionally, our results provide a context for understanding how cellular pathways that promote cell survival can also promote Th2 differentiation. Further work will be required to investigate the relationship among caspases, apoptosis, and IL-4 transcription.

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Disclosures
The authors have no financial conflict of interest.

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