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Cutting Edge: Bcl-3 Up-Regulation by Signal 3 Cytokine (IL-12) Prolongs Survival of Antigen-Activated CD8 T Cells

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Clonal expansion of T cells requires cell division and survival during the proliferative phase of the response. Naive murine CD8 T cells responding to Ag and costimulation undergo an abortive response characterized by impaired clonal expansion, failure to develop effector functions, and long-term tolerance. A third signal provided by IL-12 is required for full expansion, activation, and establishment of memory. The enhanced survival, and thus clonal expansion, supported by IL-12 is not due to increased Bcl-2 or Bcl-xL expression; both are maximally activated by signals 1 and 2. In contrast, Bcl-3, recently shown to enhance survival when ectopically expressed in T cells, is increased only when IL-12 is present. Furthermore, examination of Bcl-3-deficient CD8 T cells demonstrates that the increased survival caused by IL-12 depends upon Bcl-3. The time courses of expression suggest that Bcl-2 and Bcl-xL promote survival early in the response, whereas Bcl-3 acts later in the response. The Journal of Immunology, 2005, 174: 600–604.

Survival of activated T cells is tightly regulated to insure that adequate clonal expansion occurs to control infections, but that sufficient death occurs to prevent autoimmune responses and shrink expanded populations following Ag elimination. Bcl-2 and Bcl-xL, antiapoptotic members of the Bcl-2 family of proteins, promote survival of T cells, and are up-regulated in response to Ag and costimulation. Adjuvants can also play a critical role in regulating survival of T cells (1, 2), but do not so by increasing expression of the Bcl-2 family members (3, 4). Mitchell et al. (3) have recently shown that expression of Bcl-3 is up-regulated in Ag-stimulated T cells in an adjuvant-dependent manner and that ectopic expression of Bcl-3 could increase survival of activated T cells, suggesting that this might be a mechanism by which adjuvants increase clonal expansion. Their studies did not define how Bcl-3 gene expression is induced by adjuvants, but they suggested that this might involve the inflammatory environment induced by the adjuvants (3).

Curtisinger et al. (5) demonstrated that Ag and B7-1-dependent costimulation (signals 1 and 2) of naive CD8 T cells is not sufficient to stimulate optimal clonal expansion or development of effector functions, but that both occur if IL-12 is present to provide a third signal. Production of IL-12 by APC is one of the components of the inflammatory response induced by many adjuvants, and IL-12 could replace adjuvant in supporting a strong in vivo response by CD8 T cells to peptide Ag (6, 7). Administration of Ag in the absence of adjuvant results in some cell division but clonal expansion is limited, and the cells develop little if any effector function and become tolerant to re-stimulation (1, 6–8). When IL-12 is provided along with Ag, strong clonal expansion occurs, effector function develops, and a responsive memory population results (6, 9). Clonal expansion is promoted, in part, by IL-12 enhancing survival of the cells (6). These effects of IL-12 suggested that it might act in part by up-regulating Bcl-3 expression and the results described here demonstrate that this is the case.

Materials and Methods
Stimulation and survival of naive CD8 T cells

OT-1 mice (10) were housed in a specific pathogen-free environment. C57Bl/6 mice were from National Cancer Institute (Frederick, MD). FVB;129P2-BCL-3tm1Ver Bcl-3-deficient (11) and control FVB mice were from The Jackson Laboratory. Naive CD8 T cells were purified by negative selection (12). Artificial APC were prepared by coating microspheres with 2C11 anti-CD3ε mAb (BD Pharmingen) at 1.0 μg/10⁶ beads, or with DimerX H-2Kb-g fusion protein (BD Pharmingen) at 1.0 μg/10⁶ beads. Peptide was loaded onto the H-2Kb by incubating microspheres with 0.1 μM OVA257–264 for 2 h at 37°C. Murine B7-1-Fc (R&D Systems) was coimmobilized at 0.2 μg/10⁶ beads. Ag and B7-1 densities were in the range shown to be effective for T cell stimulation (13).

Responder CD8⁺ T cells (5 × 10⁵) and Ag-coated microspheres (10⁶) were placed in flat-bottom microtiter wells and cultured at 37°C (5). Where indicated, human rIL-2 (2.5 U/ml; R&D Systems), mouse rIL-12 (2.0 U/ml; Genetics Institute, Cambridge, MA), and/or mouse rIL-1 (5 ng/ml; R&D Systems) were added. Proliferation was determined by adding [³H]Tdr during the final 8 h of culture. For in vitro survival experiments, equal numbers of live CD8⁺ T cells that had been cultured for 3 days in wells coated with Ag (0.1 μg/well) and B7 (0.05 μg/well) were washed, labeled with CFSE, and returned to culture in the absence of Ag or cytokines. Survival was measured by counting trypan blue-negative live cells.

To measure survival in vivo, OT-1 CD8⁺ T cells were activated for 3 days in vitro and CFSE labeled (above). Cells were then adoptively transferred by i.v.
injection (tail vein) into Thy 1.2+ C57BL/6 recipient mice. OT-1 cells remaining 2–3 days later were identified and quantitated by gating on Thy1 or CD45 congenic markers on the OT-I cells.

Western blot and RT-PCR

Expression of mRNA for Bcl-2, Bcl-3, Bcl-xL, and β-actin was determined using a semiquantitative two-step RT-PCR assay. PCR amplification was done using primers designed with Primer3 software (14). Experiments showed that 27 cycles allowed for detection, and normalization to β-actin, within the linear range of amplification. For detection of Bcl-3 protein by Western blotting, whole-cell lysates from 3 x 10^6 cells were run on SDS-PAGE gels, transferred to a nitrocellulose membrane and blotted using anti-Bcl-3 Ab (Santa Cruz Biotechnology), and detected using an HRP-conjugated secondary Ab.

Results and Discussion

To determine the requirements for up-regulation of Bcl-3, naive CD8 T cells were purified from C57BL/6 mice and stimulated in vitro with microspheres having immobilized 2C11 anti-CD3ε mAb, with IL-2 alone or along with IL-12 or IL-1. IL-1 was compared with IL-12, because previous work had shown that IL-1 could replace the need for adjuvant in stimulating CD4 T cells, whereas IL-12, but not IL-1, could replace adjuvant for CD8 T cell responses (5). After 72 h, Bcl-3 mRNA and protein levels were examined. A low level of Bcl-3 mRNA was detected in unstimulated cells, and increased little if at all upon stimulation with anti-TCR and either IL-2 or IL-2 and IL-1 (Fig. 1A). In contrast, there was a large increase when IL-12 was present. In three independent experiments, addition of IL-12 caused a 5.0 ± 0.8-fold change in mRNA expression level, whereas IL-1 caused no significant change. Similar results were obtained when Bcl-3 protein levels were examined by Western blotting (Fig. 1, B and C). In five independent experiments, IL-12 caused a similar increase in Bcl-3 protein, whereas IL-1 caused no significant increase in the four experiments where it was examined. Based on densitometric scans, with background subtracted, the average increase in Bcl-3 protein expression in cells stimulated in the presence of IL-2 and IL-12 was 3.2 ± 0.1-fold in comparison to cells stimulated with only IL-2, and the increase was 3.3-fold in the experiment shown in Fig. 1B.

B7 costimulation and IL-2 stimulate increased production of Bcl-2 and Bcl-xL in T cells (15). Therefore, we tested whether B7-1 had a similar effect on Bcl-3 mRNA, and whether IL-12 could up-regulate Bcl-2 or Bcl-xL. Naive OT-I T cells specific for H-2Kb/OVA257–264 (10) were stimulated with either 2C11 mAb or Kb/OVAp on microspheres, either alone or along with B7-1. The efficacy of the coimmobilized B7-1 was confirmed by the fact that substantial proliferation occurred without cytokines when B7-1 was present (Fig. 2A). Similar results were obtained when DimerX Kb/OVAp was used as the stimulus (not shown). 

FIGURE 1. IL-12 up-regulates Bcl-3 expression in activated naive CD8 T cells. A. Purified naive CD8 T cells were stimulated with microspheres coated with 2C11 anti-CD3ε mAb. IL-2 was added alone, or along with IL-1 or IL-12, as indicated. Cells were harvested after 72 h and analyzed for Bcl-3 and β-actin mRNA expression by RT-PCR. B and C. Cells were stimulated as in A, and Bcl-3 protein expression was analyzed after 72 h by Western blotting. The specificity of the anti-Bcl-3 polyclonal Ab was confirmed by preincubating the Ab with a blocking peptide for 30 min. Numbers indicate intensity of bands relative to that of naive cells without peptide blocking.

FIGURE 2. Effects of B7.1 costimulation and IL-12 on Bcl-3, Bcl-2, and Bcl-xL mRNA expression. Microspheres coated with 2C11 anti-CD3ε mAb or Kb/OVAp either alone or with B7.1-Ig were used to stimulate naive OT-1 cells in the presence of IL-2 and IL-12, as indicated. A. Proliferation of CD8 T cells responding to 2C11 mAb. Identical results were obtained when DimerX Kb/OVAp was used as the stimulus (not shown). B. Cells were harvested at 72 h and analyzed for Bcl-3, Bcl-2, and Bcl-xL mRNA expression by RT-PCR. C. Cells were harvested every 24 h for a period of 3 days and analyzed as in B. D. Expression of Bcl-3, Bcl-2, and Bcl-xL mRNA was analyzed by RT-PCR as in C, and the bands were quantitated by densitometry. mRNA abundance is expressed relative to actin (∗10).

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obtained using K\textsuperscript{b}/OVAp vs K\textsuperscript{b}/OVAp/B7 microspheres (data not shown).

Naive cells had low but detectable levels of Bcl-3, Bcl-2, and Bcl-x\textsubscript{L} mRNA (Fig. 2B). Stimulation for 72 h with 2C11 and IL-2 up-regulated Bcl-x\textsubscript{L} and Bcl-2 mRNA expression and B7-1 did not further increase these levels, whereas Bcl-3 mRNA expression did not increase in response to these stimuli. In contrast, addition of IL-12 resulted in strong up-regulation of Bcl-3 mRNA, by 2.4-fold for 2C11 stimulation and 3.0-fold for 2C11/B7 stimulation based on densitometric scans. Addition of IL-12 did not increase Bcl-2 or Bcl-x\textsubscript{L} mRNA levels and, in most experiments, caused a small decrease.

The experiment shown in Fig. 2B examined mRNA expression levels at 72 h, late in the response when proliferation is maximal and lytic effector function has developed. To determine how expression is regulated during the response, naive OT-I cells were stimulated with Kb/OVAp and examined at 24, 48, and 72 h (Fig. 2C). Bcl-2 and Bcl-x\textsubscript{L} mRNA levels increased strongly by 24 h and then declined, and IL-12 had little or no effect. Some up-regulation of Bcl-3 mRNA occurred early in the absence of IL-12, but declined by 72 h. In contrast, IL-12 stimulated strong and sustained up-regulation of Bcl-3 mRNA levels. Fig. 2D shows the time course for mRNA expression levels for the survival proteins in response to Kb/OVAp/B7 and IL-12, the condition that leads to maximal clonal expansion and effector function. Thus, Bcl-2 and Bcl-x\textsubscript{L} mRNAs are up-regulated early in the response by signals 1 and 2 and then decline, whereas Bcl-3 mRNA expression is strongly up-regulated only by IL-12, and is sustained late in the response.

Ectopic expression of Bcl-3 enhances survival of activated T cells (3), suggesting that IL-12 might sustain survival at later times to support maximal clonal expansion by up-regulating Bcl-3 expression. To determine whether IL-12 does provide a survival advantage, we examined survival of OT-I T cells following activation with K\textsuperscript{b}/OVAp/B7 for 72 h, when expansion has peaked and the cells begin to die (data not shown). To examine survival in the absence of continued exposure to Ag, cells were activated for 72 h in microtiter wells having immobilized K\textsuperscript{b}/OVAp/B7. Cells were then harvested, washed to remove cytokines, and placed in secondary cultures in fresh medium, and the number of live cells remaining was determined over 2 days. We consistently found that cells stimulated in the presence of IL-12 for the first 72 h survived better during the subsequent 2-day period in culture (Fig. 3A). To determine whether greater cell recovery in the secondary cultures might be due to more proliferation during this 2-day period, cells were labeled with CFSE at the end of the 72-h primary culture. Dilution of CFSE showed that the cells undergo one to two additional rounds of division following transfer to secondary cultures, but this did not differ for cells initially grown in the presence or absence of IL-12 (Fig. 3B). Thus, differential recovery during the secondary culture reflects differences in the ability of the cells to survive.

OT-I cells stimulated in the presence of IL-12 for 72 h also survived better when they were then adoptively transferred into mice and recovered from the lymph nodes, spleens, blood, lungs, and livers 48 h later (Fig. 3C). Again, CFSE dilution indicated that there was no significant difference in the extent of cell division that could account for the differential recovery of the two populations (Fig. 3D). These results suggest that signals derived from IL-12 confer a survival advantage to activated cells that allows them to survive longer in an in vivo environment.

To determine whether the survival advantage provided by IL-12 is due to up-regulation of Bcl-3, CD8 T cells from wild-type and Bcl-3-deficient mice were compared. For these experiments, immobilized 2C11 anti-CD3\textsuperscript{e} mAb was used to provide signal 1 for polyclonal activation of the naive CD8 T cells. As expected, activated T cells from FVB wild-type mice survived better in secondary cultures when they received an IL-12 stimulus during the primary culture (Fig. 4A), and this was not due to differential cell division (B). In contrast, the IL-12-dependent survival advantage was lost in cells that lacked Bcl-3.

IL-12 acts as a required third signal along with TCR engagement and costimulation to activate naive CD8 T cells to
differences in clonal expansion at early times, and the Fas pathway might not contribute substantially to the death of activated T cells (19). Later in the response, cytokine withdrawal leads to activation of death pathways, which can be countered by the action of the antiapoptotic Bcl-2 family members (17, 19). Bcl-2 is up-regulated by IL-2, and its down-regulation toward the end of the response (Fig. 2) may coincide with IL-2 depletion, consistent with decreased levels of Bcl-2 in T cells at their peak of activation (20, 21). It appears that Bcl-3 becomes a critical survival factor at this time (Figs. 3 and 4).

Unlike Bcl-2 and Bcl-xL, Bcl-3 is unlikely to act directly to inhibit apoptosis. Rather, Bcl-3 is a member of the IκB protein family and can interact with p50 and p52 NF-κB complexes to repress or induce gene transcription (22, 23). Thus, Bcl-3 may regulate expression of one or more genes involved in survival, which could include up-regulation of antiapoptotic proteins or down-regulation of proapoptotic proteins such as Bim (19) or other members of the Bcl-2-related family of proteins. Most studies examining the role of NF-κB complexes in T cell survival have focused on NF-κB heterodimers containing the canonical p65 (reA) subunit (24, 25). Our results and those of Mitchell et al. (3, 4) suggest that NF-κB complexes that depend upon Bcl-3 transactivation may also play critical roles. In addition to regulating survival genes, Bcl-3 may regulate genes required for other signal 3 effects on CD8 T cells including effector functions, avoidance of tolerance, and acquisition of memory. Experiments are in progress to examine genes whose expression is regulated by Bcl-3.

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References


