The Trophic Action of IL-7 on Pro-T Cells: Inhibition of Apoptosis of Pro-T1, -T2, and -T3 Cells Correlates with Bcl-2 and Bax Levels and Is Independent of Fas and p53 Pathways

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The Trophic Action of IL-7 on Pro-T Cells: Inhibition of Apoptosis of Pro-T1, -T2, and -T3 Cells Correlates with Bcl-2 and Bax Levels and Is Independent of Fas and p53 Pathways

Kyungjae Kim,* Chong-kil Lee,* Thomas J. Sayers,† Kathrin Muegge,† and Scott K. Durum*

Signals from the IL-7R are essential for normal thymocyte development. We isolated thymocytes from early developmental stages and observed that suspensions of pro-T1, -T2, and -T3 cells rapidly died in culture. Addition of IL-7 promoted their survival, but did not induce cell division. Pro-T4 cells did not undergo rapid cell death, and their survival was therefore independent of IL-7. Death in the absence of IL-7 showed the hallmarks of apoptosis, including DNA fragmentation and annexin V binding; however, caspase inhibitors blocked DNA fragmentation, but did not block cell death. The trophic effect of IL-7 was partially inhibited by blocking protein synthesis. The p53 pathway was not involved in this death pathway, since pro-T cells from p53−/− mice also underwent cell death in the absence of IL-7. The Fas/Fas ligand pathway was not involved in cell death, since Fas-deficient pro-T cells died normally in the absence of IL-7, anti-Fas Abs did not protect cells from death in the absence of IL-7, and Fas expression was undetectable on cells at these stages. The IL-7 trophic affect correlated with increased intracellular levels of Bcl-2 and decreased levels of Bax, whereas no Bcl-XL, Bcl-w, or Bad was detectable. Thus, maintaining a favorable Bcl-2/Bax ratio may account for the trophic action of IL-7.


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ormal T cell development in the mouse and in man has been shown to depend on signals from the IL-7R (reviewed in Ref. 1). This conclusion is based on the phenotypes of mice deficient for IL-7Rα (2, 3), IL-7 itself (4, 5), mice treated with Abs against IL-7 (6, 7), and deficiencies of the γc component of the IL-7R in humans (8) and mice (9, 10).

Some αβ T cell development occurs in about 40% of IL-7R−/− mice, suggesting that alternative pathways can also support T cell development; however, the peripheral T cells that eventually accumulate in these leaky mice do not proliferate in response to stimuli (11). The γδ T cell lineage is completely unresponsive in all IL-7R−/− mice (3, 12) based on a failure to rearrange the TCRγ locus (13, 14) or a failure to express these genes (15). The development of B lymphocytes is also severely impaired in IL-7R−/− mice and γc−/− mice, but not in γc-deficient humans reflecting the IL-7 independence of human B cells (16). NK development is normal in IL-7R−/− mice (12).

The earliest stages in murine T cell development (before expression of CD4, CD8, and CD3) have been distinguished based on CD44 and CD25 expression (reviewed in Ref. 17): pro-T1 (CD44+CD25−), pro-T2 (CD44+CD25+), pro-T3 (CD44−CD25−), and pro-T4 (CD44−CD25−). Expression of c-Kit, the receptor for stem cell factor, corresponds with expression of CD44 at these pro-T cell stages. The CD25+ stages are deficient in IL-7R−/− mice and in mice treated with anti-IL-7. This suggests that one requirement for IL-7R signals occurs before or during the pro-T2 stage. IL-7 has been reported to induce proliferation of early T cells (18) and could therefore play a role in the expansion as well as differentiation or survival of these cells. To clarify the nature of the IL-7R signal requirement, pro-T cells at different stages were isolated from the thymus and examined for the effects of IL-7 on survival and cell cycle, and the cell death process that occurred in the absence of IL-7 was characterized.

Materials and Methods

Mice

C57BL/6 and MRL-lpr/lpr mice were housed in a specific pathogen-free environment. Mice were mated overnight and checked for plugs the following day, which was designated day 1 of gestation. On the indicated day of gestation, mothers were killed by CO2 asphyxiation, and embryos were killed by chilling on ice. Thymi were removed from embryos using a dissecting microscope. Rag-2−/− (19) and p53−/− (20) mice were bred in our facility from breeders purchased from The Jackson Laboratory (Bar Harbor, ME).

Preparation and culture of fetal thymocytes

Fetal thymus lobes were obtained from embryos on day 14, 15, 16, 17, or 18 of gestation. Cell suspensions were prepared by gentle disruption with a micropipette after treatment with 0.2% collagenase (Sigma, St. Louis, MO) dissolved in PBS containing 20% heat-inactivated FCS (HyClone, Logan, UT) for 1 h at 37°C. The cells were cultured in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME, and 10% heat-inactivated FCS. Fetal thymocytes (4 × 105/200 µl/well) were cultured in 96-well U-bottom plates in the presence or the absence of IL-7 (50 ng/ml; PeproTech, Rocky Hill, NJ) for the indicated length of time. Hamster anti-Fas (Joz; 10 µg/ml; PharMingen, San Diego, CA) was added to some cultures. Caspase inhibitors, z-VAD-FMK and z-DEVD-FMK (20 µM; Enzyme Systems Products, Dublin, CA), were preloaded into thymocytes at 0°C for 30 min before placing cells in culture together with inhibitors at 37°C.

Anti-Fas control treatment

Rena cells were stimulated with IFN-γ (100 U/ml) for 18 h to increase their susceptibility to fas-mediated killing. Rena cells were then labeled with 51Cr and incubated with d11S cells that express FasL and hamster anti-Fas (10 µg/ml) for 16 h, and the release of 51Cr was determined.

Abbreviations used in this paper: FasL, Fas ligand.
Flow cytometric analysis and cell sorting with Abs

For Ab staining, cells were harvested, washed in a staining solution of PBS containing 5% FCS and 0.1% NaN₃, and resuspended in 50 μl of staining solution containing 0.5 μg of rat mAb 2.4G2 (anti-mouse FcγRII, Pharmingen) and 10% normal mouse serum to reduce nonspecific binding of Abs to Fc receptors. Cell suspensions were stained with mAbs (1/200 dilution) for 20 min at 4°C. Abs were purchased from PharMingen, including R-phycocerythrin-conjugated anti-CD44 (clone IM7), FITC-conjugated anti-CD25 (clone 7D4), FITC-anti-CD4 (RM4-5), and R-phycocerythrin-conjugated anti-mouse CD8α (clone 53-6.7). Cells were washed and fixed in 1% paraformaldehyde in PBS, and analyzed on a FACStar Plus (Becton Dickinson Immunocytometry System, Mountain View, CA), gating out dead cells by forward low angle scatter and low right angle scatter. For sorting cells, the same staining method was used without fixation, and cells were sorted on a FACStar Plus or a modified FACSSII (Becton Dickinson). Pro-T1 (CD44+CD25−) cells were sorted from day 14 thymocytes. Pro-T2 (CD44+CD25+) cells were sorted from day 14 of 15 thymocytes. Pro-T3 (CD44−CD25+) cells were sorted from day 16 thymocytes or from Rag2−/− adult thymocytes. Pro-T4 (CD4−CD8+CD44−CD25+) cells were sorted from day 17 thymocytes. From p53−/− mice a mixture of pro-T2 and pro-T3 cells (CD4+CD8−CD25+) was sorted from thymocytes from adult mice. The purity of sorted populations was generally >98%. Sorted cells were then cultured overnight with or without IL-7.

Intracellular staining of Bcl-2, Bcl-XL, Bax, and Bad

Single-cell suspensions were permeabilized with saponin buffer (PBS containing 1% BSA (Sigma) and 0.04% saponin) for 20 min, then incubated with monoclonal anti-Bcl-2 (clone 3F11, PharMingen), anti-mouse Bcl-XL (clone 4, Transduction Laboratory, Lexington, KY), anti-mouse Bax (clone 2G11, PharMingen), or a polyclonal anti-mouse Bax (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min on ice. Isotype-matched control Abs were purified mouse IgG2b (clone 49.2, PharMingen), purified polyclonal hamster IgG (PharMingen), or purified rabbit IgG. Cells were washed twice with saponin buffer, incubated with FITC anti-hamster IgG (clone G94-56, PharMingen), FITC anti-mouse IgG2b (clone R12-3 PharMingen), or FITC anti-rabbit IgG (Santa Cruz Biotechnology) for 30 min at 4°C, and washed once with saponin buffer and once with PBS containing 1% BSA.

Annexin V staining and viability

Annexin V binding was performed with a commercial kit (Clontech, Palo Alto, CA). Cells were collected, then resuspended in annexin V binding buffer containing annexin V-FITC (1 μg/ml) and propidium iodide (2 μg/ml) for 10 min at room temperature in the dark, washed, and analyzed by flow microfluorometry. To analyze for viability, cells were incubated with propidium iodide, then the percentage of cells excluding the dye was determined by flow microfluorometry.

Cell cycle analysis

Cells were placed in a detergent buffer (21) and an equal volume of staining buffer (propidium iodide, 50 μg/ml). Cells were mixed by inversion, incubated at room temperature in the dark for 1 h, and analyzed by flow microfluorometry.

Results

Thymocytes from mouse embryos on day 14 of gestation were cultured with or without IL-7 for various times. The recovered cells were examined for viability by exclusion of propidium iodide, which stains the nuclei of either necrotic or apoptotic cells. Apoptotic cells were detected by binding annexin V to their plasma membranes. As shown in Figure 1, these freshly explanted thymocytes began to die by apoptosis within 2 h, and by 20 h, 90% had undergone apoptotic death. IL-7 protected about half the cells from apoptotic death at 20 h, and its survival effects could be seen as early as 6 h. In >20 experiments, IL-7 protected 50 to 80% of the cells from death at the 20 h point. Further culture in IL-7 beyond 20 h (not shown) showed that with each additional day, about half of the cells died. We noted that culture density had an effect on viability, with IL-7 being less effective as culture density was lowered.

The thymocyte population on day 14 of gestation is comprised of the first two stages of pro-T cell development, pro-T1 and pro-T2, in approximately equal proportions. We sorted the cells into pro-T1 and pro-T2 based on the CD25 and CD44 markers and tested each population for the effect of IL-7 using cell cycle analysis. In this analysis, apoptotic cells were visualized based on DNA fragmentation to subG1 quantities of DNA. As shown in Figure 2, both pro-T1 and pro-T2 populations were protected from apoptotic death by IL-7. We also visualized DNA fragmentation by gel electrophoresis and observed a comparable IL-7 effect (not shown). It is clear from this analysis that IL-7 did not promote cycling of pro-T cells, as shown by the decline in the proportion of cells in S and G2-M during culture in IL-7 compared with that at the start of the culture (see also Fig. 6). The percentages of pro-T2 cells in G1, S, and G2-M phases when initially isolated were 36.5, 37.5, and 6.1%, respectively. After IL-7 culture, they were 81.5, 16.5, and 1.9%, respectively. Thus, cell division declined during culture in IL-7, and its activity is best described as “trophic” rather than “growth” for pro-T cells.

The later stages of pro-T cell development were then compared with earlier stages for responsiveness to IL-7. Pro-T3 cells were isolated by sorting from day 16 embryos using CD25 and CD44 markers. As shown in Figure 3, pro-T3 cells were protected by IL-7 from cell death during culture. Of the first three stages (pro-T1, -T2, and -T3), the most dramatic IL-7 trophic effects were consistently observed at pro-T2.

Pro-T4 cells were sorted from day 17 embryos by selecting for CD4−, CD8−, CD25−, and CD44− cells. As shown in Figure 3, these cells survived independently of IL-7 in culture, and addition of IL-7 produced only a slight increase in survival that was not observed consistently. Pro-T4 cells also rapidly differentiated in culture (not shown), expressing CD4 and CD8 after overnight culture, and this, too, was unaffected by IL-7. Pro-T4 cells were also cycling much more rapidly than pro-T1, -T2, and -T3 cells (not shown), and this cycling was unaffected by IL-7.
After the pro-T4 stage, thymocytes express CD4 and CD8. We examined CD4$^+$CD8$^+$ cells for IL-7 trophic effects (not shown) and observed that, like pro-T4 cells, these cells survived independently of IL-7 for several days in vitro. We conclude that the trophic effects of IL-7 end with the pro-T3 stage.

The role of p53 in the apoptotic response to IL-7 deprivation was tested for two reasons. First, p53 mediates one type of thymocyte apoptosis, that induced by dsDNA breaks (20, 22, 23). Second, p53$^{−/−}$ mice develop thymic lymphomas at a very high frequency (24, 25), suggesting that they evade the normal death mechanisms. Although the phenotype of such p53$^{−/−}$ thymomas is primarily CD4$^+$CD8$^−$, an IL-7-independent stage, we nevertheless considered it possible that the actually transformed cell was a pro-T cell because such thymic lymphomas also arise in rag$^{−/−}$ p53$^{−/−}$ mice (24) (S. Candèias and S. Durum, unpublished observation) that do not develop beyond the IL-7-dependent pro-T3 stage. However, a role for p53 was ruled out by using pro-T cells from p53$^{−/−}$ mice, as shown in Figure 4. Thus, a similar dependency on IL-7 was observed comparing p53$^{−/−}$ thymocytes to their heterozygous littermates. Note that cell death (in the absence of IL-7) was not as extensive in this experiment as that shown in Figure 3; this is because adult pro-T cells die more slowly in culture than their fetal counterparts. We also tested fetal pro-T cells from p53$^{−/−}$ mice (not shown) and similarly showed that they are IL-7 dependent. It can be noted (Fig. 4) that p53$^{−/−}$ thymocytes survive better in culture than do their heterozygous counterparts. This improved survival of cultured p53$^{−/−}$ cells was not observed in studies using unfractionated thymocytes or pre-B cells (20, 22, 23); however, we consistently observed it in pro-T cells and activated mature T cells (S. Candèias and S. Durum, unpublished observations), and it has been noted in embryonic fibroblasts (26).

A cascade of caspases is a feature of the apoptotic response, and inhibiting this cascade has been shown to block cell death in many studies. Caspase inhibitors were added to thymocyte cultures, and the effect on IL-7 deprivation was studied. Two different inhibitors were used: z-VAD, which inhibits caspases 1 and 4, and z-DEVD, which inhibits caspases 3, 6, 7, 8, and 10. As shown in Figure 5 (left panel), neither caspase inhibitor prolonged life, as assessed by propidium iodide exclusion. However, both caspase inhibitors notably reduced DNA fragmentation (right panel). Thus, IL-7 deprivation activates caspases (recognized by these inhibitors), leading to DNA fragmentation; however, an additional mechanism must also be involved in cell killing when IL-7 is withdrawn; this mechanism could involve caspases not blocked by these inhibitors or may be caspase independent.
FasL signals through Fas, inducing apoptotic death in peripheral T cells, and this pathway also appears to operate in late pro-T4 and early double-positive cells (27). Two methods were used to test whether Fas is involved in death from IL-7 deprivation. First, anti-Fas Ab was added to cultures of thymocytes to determine whether it blocked cell death in the absence of IL-7. Second, pro-T cells from MRL-<i>lpr</i>/<i>lpr</i> mice, which lack functional Fas, were also tested. As shown in Figure 6 (<i>left</i>), anti-Fas did not block the death of pro-T cells, nor did the MRL-<i>lpr</i>/<i>lpr</i> thymocytes fail to die in the absence of IL-7. As a positive control for anti-Fas blocking, the same Ab was shown to inhibit the FasL-Fas killing pathway of FasL, expressing d11S killers on the Fas-expressing Renca target cells (Fig. 6, <i>right</i>). Although this Ab can have agonist activity and kill Fas targets, we do not observe this unless it is secondarily cross-linked (T. Sayers unpublished observation). Further evidence against a Fas mechanism is our failure to detect Fas expression by flow microfluorometry before or after culture of pro-T cells (not shown). Thus, Fas is not involved in the death of pro-thymocytes deprived of IL-7.

The relative concentrations of antiapoptotic (e.g., Bcl-2) and proapoptotic members (e.g., Bax) of the Bcl-2 family determine whether a cell will live or die. Intracellular staining was performed for the antiapoptotic factors Bcl-2 and Bcl-X<sub>L</sub> and for the proapoptotic factors Bax and Bad. As shown in Figure 7 (<i>left</i>), freshly isolated cells expressed high levels of Bcl-2 and Bax, whereas no Bcl-X<sub>L</sub> or Bad was detected. During culture (Fig. 7, <i>right panels</i>), Bcl-2 levels declined sharply, and IL-7 reduced this decline. Bax levels increased during culture, and IL-7 prevented this increase. Thus, IL-7 preserved the ratio of Bcl-2 to Bax at a level intermediate between that at initiation and that of cultures deprived of IL-7. Therefore, the trophic action of IL-7 on day 14 thymocytes correlated with its maintaining a favorable Bcl-2/Bax ratio.

Discussion

We analyzed the effect of IL-7 on survival of pro-T cells. IL-7 protected pro-T1, -T2, and -T3 cells from rapid apoptotic death, whereas pro-T4 cells survived independently of IL-7. IL-7 did not induce cell cycling of pro-T cells. There was no evidence for the involvement of p53 or Fas pathways in cell death. Inhibiting caspases inhibited DNA fragmentation, but did not protect cells from death. IL-7 protected pro-T cells from a decline in Bcl-2 and a rise in Bax, suggesting that the Bcl-2/Bax ratio could be the basis of the trophic action of IL-7.

These findings complement the recent reports that a bcl-2 transgene restores αβ T cell development in IL-7R<sup>−/−</sup> mice (28, 29) and in γc<sup>−/−</sup> mice (30), demonstrating that Bcl-2 can substitute for some IL-7 activities. It has also recently been reported that IL-7 regulates Bcl-2 levels in pro-T cells, and that this regulation is...
deficient in IL-7−/− mice (31). Taken together with our observation that IL-7 influences the Bcl-2/Bax ratio in vitro, it suggests that these regulators of apoptosis could mediate the IL-7 response. It has previously been shown that IL-7 enhanced the Bcl-2 levels in cell lines (32, 33), and our study extends this to pro-T cells, which have a physiologic requirement for IL-7Rα signals. However, Bcl-2 induction may not completely explain the trophic effects of IL-7 on pro-T cells in embryonic life. Mice lacking Bcl-2 have relatively normal numbers of thymocytes at birth; however, these numbers rapidly decline by 1 mo (34). The decline in thymocytes after birth in these mice has been attributed to differences in stem cells that seed the thymus before and after birth (35). Thus, it was proposed that Bcl-2 was not required for embryonic thymopoiesis generated by stem cells derived from fetal liver. At birth, thymopoiesis is subsumed by a stem cell arriving from bone marrow, and it or its progeny are dependent on Bcl-2. Perhaps another Bcl-2 family member is also induced by IL-7 in fetal cells. We sought two other Bcl-2 family members; Bcl-XL protein was not detected in pro-T cells (Fig. 7), nor was bcl-w (36) mRNA detectable by Northern blotting (not shown). Remaining family members that have not been evaluated are Mcl-1 (37) and A1 (38).

The trophic activity of IL-3 on a cell line was attributable to phosphorylation of Bad (39); this mechanism would be resistant to cycloheximide, as the IL-7 trophic effect is in part; however, we did not detect Bad in pro-T cells (Fig. 7).

Blocking caspase activity is reported to inhibit apoptosis in many cell types (reviewed in Ref. 40). It is therefore surprising that caspase inhibitors, although inhibiting DNA fragmentation, did not preserve the viability of these pro-T cells. Several lines of evidence suggest that these cells die by apoptosis, including the phenotype of the cells (Figs. 1 and 2) and the observation that Bcl-2 protects them (28–30). One possibility is that death is mediated by caspases that are insensitive to the effects of these inhibitors. Another possibility is that the death pathway does not involve caspases at all. This is reminiscent of cytotoxic T cell killing, which is primarily mediated by perforin; caspase inhibitors block DNA fragmentation, but not cell death, which in that case is caused by pores in the plasma membrane (41).

It was observed that wortmannin inhibited the trophic action of IL-7 on a pre-B cell line, suggesting that phosphatidylinositol 3-kinase is required (42). The same study noted that a tyrosine site on IL-7Rα that mediated activation of phosphatidylinositol 3-kinase was required for the trophic activity in pre-B cells. However, we did not observe that wortmannin inhibited the trophic activity of IL-7 on pro-T cells (data not shown). Hence, the seemingly similar activities of IL-7 on survival of early T and B cells may involve different signaling pathways and, by extension, different survival and death pathways.

The Fas pathway did not appear to mediate death from IL-7 deprivation. It has been reported that Fas expression does not begin until the CD4+CD8+ stage. We have confirmed this and also observed that its expression is not induced during IL-7 deprivation (not shown). A recent report implicates the Fas system in the death of cells that fail to productively rearrange their TCRβ genes (27). This death presumably occurs in cells at stage pro-T3, which, according to our findings, also die when deprived of IL-7. The signal for a successful β gene rearrangement is thought to emanate from the pre-TCR, which incorporates the successfully produced β-chain. Thus, the trophic signals from pre-TCR may be different from those of IL-7R, and this is substantiated by the failure of a bcl-2 transgene to rescue T cells in rag−/− mice (which cannot generate β gene rearrangements), whereas it protects IL-7R−/− thymocytes (28). Consistent with this interpretation, it has been reported that Bcl-2 cannot protect cells from Fas-mediated killing (43, 44). We also found no evidence for IL-7 serving as a cofactor together with the pre-TCR signal, since pro-T4 cells survived independently of IL-7; pro-T4 cells also rapidly proliferated and differentiated into CD4+CD8+ cells within 18 h in the absence of IL-7 (not shown). We recently observed that the IL-7R activates the α4β1 integrin, increasing its affinity for the extracellular matrix protein fibronectin (45). Integrins, in turn, are known to provide viability signals to some types of cells. However, we tested whether fibronectin could augment the trophic effect of IL-7 on pro-T cells and could not detect such an effect (data not shown). IL-7 produced by thymic epithelial cells appears to be bound to extracellular matrix in the thymus (45), which raises questions about the molecular form, solubility, and concentration of the IL-7 actually encountered by pro-T cells. Thus, we do not know whether the concentrations of IL-7 used in our experiments fall into the physiologic or the pharmacologic range.

We did not observe an induction of cell cycle progression by IL-7, but, rather, found a completion of the cycle and accumulation of cells in G1. There are reports that IL-7 induced the growth of pro-T cells (for example, Ref. 46), but these effects may be attributable to the presence of other stimuli that in some studies were
intentionally added or in other cases may have been produced endogenously; it is also possible that in long term cultures, a subpopulation of cells that grows in response to IL-7 eventually dominates; however, it can be seen from our results (and inferred from the bcl-2 transgenic mice noted above (28–30)) that this is not the prevailing response. Stem cell factor has been identified as a potent cofactor that, together with IL-7, induces rapid growth (47). Other cytokines may be produced by the thymocytes during culture in our studies. The evidence for such autocrine factors is that culturing the cells at high density in U-bottom wells promoted the trophic effects of IL-7, and conditioned medium from high cell density cultures promoted the survival of low cell density cultures (not shown).

Differentiation of thymocytes has also been reported to be induced by IL-7. VDJ recombination is promoted by the IL-7R in thymocytes (13, 14, 48, 49) and pro-B cells (reviewed in Ref. 1; 42). This effect is partly attributable to inducing rag1 and rag2 expression (48, 50, 51) and perhaps also to enhancing locus accessibility, especially of the TCRy locus. We examined whether IL-7 induced expression of CD25 on pro-T1 cells, since CD25 activity.

mechanisms that remain to be identified for this type of biologic effects of IL-7, and conditioned medium from high cell density cultures promoted the survival of low cell density cultures (not shown).

In conclusion, IL-7 has a trophic effect on several early stages of pro-T cells, promoting survival without growth. Bcl-2 and Bax levels are associated with these effects, but there may be other mechanisms that remain to be identified for this type of biologic activity.

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References