Interleukin 4 (IL-4) or IL-7 Prevents the Death of Resting T Cells: Stat6 Is Probably Not Required for the Effect of IL-4

By Anthony Vella,* T. Kent Teague,* James Ihle,§ John Kappler,*§ and Philippa Marrack*‡§

Summary

Although much is known about the activation, proliferation, and function of CD4+ T cells, little is known about how they survive as resting T cells in animals. Resting T cells have a half-life in animals of more than a week; however, when they are removed from animals and placed in tissue culture their half-life falls to ~24 h. In this paper, we show that the survival of resting T cells in vitro is promoted by two cytokines, interleukins 4 and 7 (IL-4, IL-7). They may do this in part by maintaining levels of survival-promoting proteins such as Bcl-2 in the cells, because the levels of Bcl-2 and Bcl-X_l in resting T cells fall rapidly after the cells are isolated from animals, and are maintained by culture in IL-4. Because the IL-4 receptor is known to signal through the Jak1 and Jak3/Stat6 pathway, we tested whether Stat6 was required for IL-4–dependent T cell survival. Surprisingly, we found that IL-4 rescued T cells from apoptosis in what appeared to be a Stat6-independent manner. These results demonstrate that the survival of resting T cells is an active process that can be affected by signals delivered by cytokines and also suggest that the IL-4 receptor on resting T cells may use a novel signaling pathway to facilitate T cell viability.
cells came from a Stat6-deficient animal, although slightly higher amounts of IL-4 were required to achieve the rescue. Stat6 is a signaling protein often associated with IL-4 driven effects. Thus, IL-4 and IL-7, which share the γc in their receptors (9), may affect T cell survival via some common signaling pathway that does not involve Stat6.

Materials and Methods

Mice. The AD10 TCR reacts with a peptide from pigeon cytochrome c bound to I-E\textsuperscript{a} (16). Mice transgenic for this TCR were the gift of Dr. S. Hedrick. They were bred in the Animal Care Facility at the National Jewish Medical and Research Center (Denver, CO). Normal C57BL/10 animals were purchased from T he Jackson Laboratory (Bar Harbor, ME). Stat6\textsuperscript{-/-} mice (17, 18) were bred at the Animals Care Facility, St. Jude Children’s Hospital (Memphis, TN). All mice were maintained under specific pathogen-free conditions.

Preparation and Culture of T Cells. T cells were isolated by nylon wool purification as previously reported (19), and resuspended at 5 × 10\textsuperscript{6} cells/ml in DMEM supplemented with 10% of a batch of fetal bovine serum previously tested for its ability to maintain hybridoma cells at excellent viability. The medium also contained essential and nonessential amino acids (GIBCO BRL, Gaithersburg, MD), 5 × 10\textsuperscript{-5} M 2-mercaptoethanol, sodium pyruvate, glutamine, and antibiotics (20). Cultures were set up in 96-well tissue culture plates at 100,000 cells/well. Cytokines were added at the beginning of the culture period at the indicated concentrations. IL-4 was obtained from Genzyme (Cambridge, MA). The other cytokines were purchased from R & D Systems, (Minneapolis, MN).

Analysis of Cell Death. At the time that the T cells were isolated and 24 and 48 h thereafter, samples of the cells were stained to measure their DNA content as previously described (21). In brief, the 96-well plates were spun, and supernatant removed from the wells by gentle dumping. A DNA staining mixture, containing 180 μg/ml RNAse (Boehringer Mannheim, Indianapolis, IN) was added at the beginning of the culture period at the indicated concentrations. IL-4 was obtained from Genzyme (Cambridge, MA). The other cytokines were purchased from R & D Systems, (Minneapolis, MN).

Immunoprecipitation and Western Blots. Lymph node T cells were isolated and cultured at 5 × 10\textsuperscript{6} cells/ml as described above. At 0, 4, 24, and 48 h T cells were lysed in 0.5% NP-40, 20 mM Hepes, pH 7.2, 100 mM NaCl, 2 mM DTT, 1 mM EDTA, 1.0 mM PMSF, 1 μg/ml aprotinin (all reagents purchased from Sigma). The nuclei were pelleted for 10 min at 14,000 rpm in the cold in a microfuge. Supernatants were removed and 2 × 10\textsuperscript{6} cell equivalents were fractionated by SDS-PAGE, using 10% acrylamide, and blotted onto Immobilon PVDF membranes (Millipore, Bedford, MA) using a Trans-Blot apparatus (BioRad, Freeport, CA) for 30 min at 15 V. Blots were blocked with 5% powdered milk in Tris-buffered saline pH 7.5, Bcl-2 and Bcl-X\textsubscript{s}, were detected with rabbit polyclonal antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A goat anti–rabbit Ig peroxidase-conjugated antibody (Boehringer Mannheim) was used as the secondary reagent. Bands were detected using Amersham ECL reagents (Arlington Heights, IL).

For analysis of Stat6 phosphorylation, T cells were cultured with and without IL-4 as described above. Nuclear lysates were generated by resuspending T cells in 20 mM Hepes, pH 7.9, 300 mM sucrose, 10 mM KC\textsubscript{l}, 1.5 mM Mg\textsubscript{2}\textsuperscript{+}, with protease inhibitors. The cells were allowed to swell for 5 min and then spun. Cells were Dounce homogenized and spun. The nuclei were resuspended in 20 mM Hepes, pH 7.9, 300 mM sucrose, 420 mM Na\textsubscript{2}C\textsubscript{6}H\textsubscript{4}O\textsubscript{7}, 1.5 mM Mg\textsubscript{2}\textsuperscript{+}, with protease inhibitors. After a 30-min incubation on ice, exuded nuclear proteins were immunoprecipitated with anti-phosphotyrosine antibody (Santa Cruz Biotechnology), followed by precipitation with protein G PLUS-agarose (Santa Cruz Biotechnology). Immunoprecipitates were fractionated by SDS-PAGE and immunoblotted for the presence of Stat6 using rabbit polyclonal anti-Stat6 (Santa Cruz Biotechnology). Bands bound to the rabbit antibody were detected as described above.

Results and Discussion

Two Cytokines, IL-4 and IL-7, Prevent the Death In Vitro of Resting T Cells. T cells bearing the transgenic TCR, AD10, were purified from the lymph node cells of young mice and set up in culture. Most of these cells were resting cells that had not been exposed to antigen, as judged by their small size, low frequency (<10%) of CD69 expression, and maintenance of a TCR, AD10, not expected to engage environmental antigens. At 0 time and 24 or 48 h later, viability of the cells was assessed by measuring the amount of DNA they contained, using PI (21). Cells that have died by apoptosis, the primary mechanism of death in these cul-
features, degrade their DNA into small fragments, consequently the nuclei of such cells contain less than 2 N DNA.

Sample data from such an experiment are shown in Fig. 1. Before culture less than 5% of the cells were dead. However, 24 and 48 h later 50 and 77%, of the cells had died respectively.

Using this assay, we measured the effects of various cytokines on the appearance of dead cells in vitro. To do this, T cells were cultured for 24 or 48 h in the presence or absence of the cytokines, used at concentrations above those known to be active in vitro as judged by publications on the subject and the manufacturer’s recommendations. Many cytokines had no effect on the death of resting T cells in culture. Such cytokines included IL-1β, IL-3, IL-5, huIL-8, IL-9, IL-10, IL-12, IFN-γ, TNF-α, TNF-β, RANTES, MIP-1α, MIP-1β, MIP-2, vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PDGF), and TGF-β. As we have previously reported, IL-6 proved to be an excellent survival factor (22; data not shown). However, we were surprised to find that two other cytokines, IL-4 and IL-7, also rescued resting T cells from death in culture very effectively. For example, as shown in Fig. 1 a, addition of IL-4 or IL-7 reduced the percent of dead cells after 24-h culture from 50% to 15 or 20%, respectively.

Titrations were performed to compare the relative efficacy of the two cytokines (Fig. 2). On a nanograms per milliliter basis, IL-4 was somewhat more effective than IL-7, with a half-maximal effect at about 2 ng/ml (~10^{-10} M) versus 50 ng/ml (~3 × 10^{-9} M) for IL-7. The effective concentration of IL-4 matched well with the dissociation constant of its high affinity receptor, 10^{-10} M (23). However, the dissociation constant of the high affinity IL-7 receptor is reported to be only slightly greater, at 2 × 10^{-10} M (24). Collectively, these data suggest that IL-4 may be acting via its high affinity receptor, whereas IL-7 may act via some combination of high and low affinity receptors (the latter have K_d of 10^{-9} M).

Two other cytokines, IL-2 and IL-15, also had, at very high concentrations, some effect on the viability of the cultured cells (Fig. 2). Their effects were small but reproducible in several assays. We believe that these small effects may have been due to engagement of these cytokines by low affinity forms of their receptors. Resting T cells are thought not to bear high affinity receptors for IL-2 and perhaps also IL-15.

IL-4 and IL-7 did not inhibit death by inducing cell cycle progression, because at 24 and 48 h T cells cultured with these cytokines were still in G0/G1 and did not contain more than 2-N amounts of DNA (see Fig. 1; data not shown). Thus, these two cytokines acted as true survival factors in this assay.

Culture with IL-4 Maintains Bcl-2 Levels in Resting T Cells. In many cases, lymphocyte survival is affected by proteins in the Bcl-2 family (11–15). Therefore, we tested whether culture in IL-4 had any effect on the levels of Bcl-2 and/or Bcl-X_l in the T cells. Purified resting T cells were cultured with or without IL-4 for 4, 24, and 48 h. At these times, T cells were lysed and proteins fractionated by SDS-PAGE. After transfer onto immobilon, BCL-2 (a) and BCL-X_l (b) were detected by immunoblotting. (c) T cells were incubated with or without IL-4 for 0, 4, and 24 h. Nuclear lysates were immunoprecipitated with anti-IL-4 and immunoblotted for the presence of Stat6.

IL-4 prevents decay of Bcl-2 and Bcl-X_l but not phosphorylated Stat6. Lymph node T cells from CD10 TCR transgenic mice were cultured in the presence or absence of 25–50 U/ml of IL-4 for 0, 4, 24, and 48 h (IL-4 was added at 0 and 24 h). At these times, T cells were lysed and proteins fractionated by SDS-PAGE. After transfer onto immunoblotting, BCL-2 (a) and BCL-X_l (b) were detected by immunoblotting. (c) T cells were incubated with or without IL-4 for 0, 4, and 24 h. Nuclear lysates were immunoprecipitated with anti-phosphotyrosine and immunoblotted for the presence of Stat6.

Figure 2. Related cytokines, IL-4 and IL-7, rescue resting T cells from apoptosis. T cells were purified from the lymph nodes of CD10 TCR transgenic mice and 2 × 10^6 cells were cultured in triplicate for 24 h with IL-2 (□), IL-4 (■), IL-7 (○), IL-9 (○), IL-13 (○), and IL-15 (△) at the concentrations shown. At 24 h, the T cells were stained with PI and the mean percent ± SD of apoptotic cells was determined by flow cytometry.

Figure 3. IL-4 prevents decay of Bcl-2 and Bcl-X_l but not phosphorylated Stat6. Lymph node T cells from CD10 TCR transgenic mice were cultured in the presence or absence of 25–50 U/ml of IL-4 for 0, 4, 24, and 48 h (IL-4 was added at 0 and 24 h). At these times, the T cells were lysed and proteins fractionated by SDS-PAGE. After transfer onto immobilon, BCL-2 (a) and BCL-X_l (b) were detected by immunoblotting. (c) T cells were incubated with or without IL-4 for 0, 4, and 24 h. Nuclear lysates were immunoprecipitated with anti-phosphotyrosine and immunoblotted for the presence of Stat6.

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regulated the levels of Bcl-2–related proteins in T cells.

Fore culture. These data support the idea that IL-4 acts by

event 48 h. In fact, levels of Bcl-2 appeared higher in cells

is made up of the IL-2R common

JAK/Stat pathway (17, 19, 25–28). The IL-4 receptor (IL-4R)

express Bcl-2 and the small amounts of Bcl-X

Pear at 4 h and were even lower at 24 and 48 h (Fig. 3

contained moderate levels of Bcl-2, which began to disap-

contrast, lysates of cells cultured with IL-4 continued to

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By contrast, lysates of cells cultured with IL-4 continued to

express Bcl-2 and the small amounts of Bcl-X, at 4, 24, and

even 48 h. In fact, levels of Bcl-2 appeared higher in cells

cultured for 24 or 48 h in IL-4 than they were in cells be-

fore culture. These data support the idea that IL-4 acts by

regulating the levels of Bcl-2–related proteins in T cells.

It is well known that IL-4 transduces signals through a

JAK/Stat pathway (17, 19, 25–28). The IL-4 receptor (IL-4R)

is made up of the IL-2R common γ chain and a ligand-

specific α chain (9, 23). The common γ chain associates with

JAK3 and the α chain with JAK1 (25, 26). Upon ligation,

Stat6 associates with the IL-4R complex and is tyrosine

phosphorylated by JAK1 (27-31). Activated Stat6 forms

homodimers and translocates to the nucleus (27). Because

Stat6 is activated by IL-4R ligation, we hypothesized that

Stat6 may be responsible for transducing the T cell survival

signal delivered by IL-4.

To test this, we compared the effects of IL-4 on the sur-

vival of resting T cells from Stat6–deficient (Stat6−/−) and

normal mice (17, 18). The results are shown in Fig. 4 a. In

the absence of added cytokines, T cells from Stat6−/− mice,

like those from normal animals, died in culture. Increasing

doses of IL-4 prevented the death of T cells from both

types of animals. The sensitivity of the two types of cells to

IL-4 was slightly different, with the Stat6−/− cells requiring

between two- and fivefold more IL-4 than normal T cells

for half-maximal rescue.

As a control we compared the sensitivity to IL-4 of B

cells from the two types of animals. B cell responses to IL-4

were measured by the ability of the cytokine to increase

MHC class II levels on the surfaces of the cells during 24 h

of culture. As shown in Fig. 4 b, high concentrations of IL-4
did induce increased amounts of class II on Stat6−/−B cells.

Even so, at optimum IL-4 concentrations less class II ap-

peared on the knockout cells. Also, half-maximal induction

of class II by cells from Stat6−/− mice required ~20 times

more IL-4 than normal B cells did. It has previously been

reported that IL-4 could not induce class II expression on

Stat6−/− B cells (17, 18). However, the previous experi-

ments were done with low concentrations of IL-4, concen-

trations near the threshold required to observe the induc-

tion shown in Fig. 4 b.

These results suggested that the ability of IL-4 to rescue

resting T cells from death did not require the presence in T

cells of the transcription factor Stat6, which is usually asso-

ciated with IL-4 signal transduction (27–31). However, the

presence of Stat6 caused the rescuing signal to be delivered

slightly more efficiently, suggesting that the Stat6 pathway

might act to amplify the rescuing signal. This conclusion

was supported by the effects on Stat6−/− B cells of IL-4.

As in T cells, response to IL-4 occurred in Stat6−/−B cells,

although the response was considerably amplified in the

presence of Stat6. Alternatively, it was possible that rescue

required Stat6 and that the Stat6−/− cells contained some

very small residual Stat6 activity due to expression of a

modified Stat6 protein from the knock out Stat6 gene

(18). If this latter explanation was correct, resting T cell

responses to IL-4 were driven by lower amounts of signal

than B cell responses were.

However, cells from genetically deficient animals are not

always equivalent to those from normal mice. Sometimes

they use intracellular signaling pathways that are not em-

ployed by normal cells. Therefore, we did a second exper-

iment to find out whether or not Stat6 signaling was a com-

ponent of IL-4 rescue of normal resting T cells from death.
Nuclear lysates were prepared from resting T cells from normal mice, after various times of culture with or without IL-4. The lysates were immunoprecipitated with anti-phosphotyrosine polyclonal sera, the precipitate was electrophoresed, blotted onto immobilon, and probed with anti-Stat6. As shown in Fig. 3, phosphorylated Stat6 was present in resting T cells at the time they were harvested from the animals. This material rapidly disappeared from resting T cells cultured without cytokines and was not apparent after as little as 4 h in culture. Phosphorylated Stat6 increased somewhat in amount during the first 4 h of culture of resting T cells with IL-4 but then decreased to levels that were not detectable in T cells after 24 h in culture, whether or not IL-4 was present.

These data indicated that an early event in the signaling pathway whereby IL-4 rescued resting T cells from death might involve Stat6. However, activated Stat6 did not have to be in the cells permanently for rescue to occur. IL-4 prevented the death of normal cultured T cells 24 and 48 h after the beginning of the culture, at a time when levels of phosphorylated Stat6 were undetectable.

Collectively, these data suggest that Stat6 is not an essential component of the intracellular signaling pathway whereby IL-4 prevents the death of normal resting T cells after 24 and 48 h.

Do either of the cytokines studied in these experiments actually affect the life expectancy of mature resting T cells in vivo? In an uninfected animal it is unlikely that IL-4 levels are high enough to manage this. Resting T cells also bear high affinity IL-4R, yet in animals they do not have elevated class II MHC protein levels that would be expected of B cells exposed to inducing concentrations of IL-4 (32). However, during infections, IL-4 levels in lymphoid tissues are certainly high enough to bind to high affinity IL-4R and hence increase the half-lives of bystander resting T cells. Under these circumstances, IL-4 may affect the life expectancy of bystander resting T cells.

On the other hand, experiments suggest that IL-7 may be at effective concentrations in lymphoid tissues, even in uninfected animals, and IL-7 has already been reported to affect T cell turnover time (7, 33). Future experiments will determine whether IL-7 does affect T cell life expectancy in vivo.

Finally, it is worth noting that cytokines are known to increase the half-lives of activated T cells as well and that the cytokines that do this (IL-2, IL-4, IL-7; references 34, 35) are either the same as or related to the two cytokines identified here. Perhaps this is not a coincidence.

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