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*J Immunol* 2002; 168:597-603; doi: 10.4049/jimmunol.168.2.597
http://www.jimmunol.org/content/168/2/597

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
IL-2 and Related Cytokines Can Promote T Cell Survival by Activating AKT

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The regulated elimination of T cells serves to maintain normal immune function and prevents autoimmune responses. IL-2 family cytokines play an important role in controlling the survival of immature and mature T cells. These molecules activate the protein kinase, AKT/PKB. AKT has been shown to transduce an antiapoptotic signal in numerous cell types. In this study, we show that an active form of AKT can protect T cells from apoptosis following growth factor withdrawal and that IL-2 family cytokines can promote T cell survival by activating this kinase. We also provide evidence that AKT does not block death receptor-mediated killing of lymphocytes. These data suggest that AKT may serve as a common signaling element by which members of the IL-2 family of cytokines promote T cell survival. The Journal of Immunology, 2002, 168: 597–603.

The immune system maintains an optimal number of lymphocytes and prevents autoimmune responses by eliminating nonfunctional, excess, and autoreactive T cells (1). At the same time, it supports the survival of T cells that respond to pathogens, thus promoting protective immune responses and immunological memory (2). This balance is achieved by a complex network of lymphocyte survival and cell death signals. Much recent work has focused on identifying the receptors, ligands, and signaling molecules that mediate these signals.

Members of the IL-2 family of cytokines have been shown to play an important role in regulating T cell apoptosis. These cytokines are produced both by lymphocytes and stromal cells and signal via multimeric receptors that are comprised of the common receptor γ-chain and one or two cytokine-specific chains (3–6). Besides their effects on apoptosis, they are also involved in many different facets of T cell maturation and function. Our current understanding of the role of these cytokines in T cell survival comes largely from the analysis of knockout mice or from experiments where specific family members were inhibited in vivo (reviewed in Ref. 7). These studies suggest that IL-7 is required to maintain normal numbers of immature and naive T cells (Refs. 8–12; it may share this latter function with IL-4; Refs. 13 and 14). IL-2 prevents the spontaneous accumulation of activated T cells (12, 15–17), and IL-15 promotes the survival of CD8+ memory cells (12, 18, 19).

How IL-2 family cytokines provide a survival signal to primary T cells remains incompletely understood. Natural occurring mutations in humans and gene-targeting experiments in mice suggest that the common receptor γ-chain is required to initiate this signal (4, 20, 21). This surface molecule is thought to promote T cell survival by recruiting and activating the tyrosine kinase, Jak3 (22–27). Activation of this kinase ultimately leads to up-regulation of Bcl-2 and the inhibition of apoptosis (28). Little is known about how this receptor-associated tyrosine kinase is coupled to T cell survival.

One potential downstream mediator of the IL-2 cytokine family survival signal is the protein kinase, AKT/PKB (29–31). AKT is highly expressed in T cells and is activated in response to cytokines and costimulation (32–34). In many cell types, including T cells, activated AKT has been shown to provide an antiapoptotic signal (35–37). Mutations in the IL-2 or IL-4 receptors that abrogate AKT activation also prevent these cytokines from inducing a survival signal (32, 33, 38), indicating that AKT may be a critical component of the IL-2 family cytokine survival signal. It is not clear whether Jak3 is responsible for activating AKT, or whether other signaling molecules are required to activate this kinase.

AKT promotes cell survival by increasing the expression and activity of antiapoptotic molecules while inhibiting those that induce cell death. The downstream targets of AKT in T cells have not yet been fully defined, but may include antiapoptotic molecules, such as Bcl-2, Bcl-x, and cellular c-Fas-associated death domain-like IL-1β-converting enzyme inhibitory protein (c-FLIP), as well as proapoptotic molecules, including Fas ligand (FasL), Bad, and caspase 9 (37, 39–43). This suggests that AKT might also be able to regulate the activity of Fas and other death receptors. Death receptors play an important role in maintaining self-tolerance by eliminating autoreactive lymphocytes (44). They induce apoptosis via a signaling pathway that is not blocked by IL-2 family cytokines or Bcl-2 (45, 46). Studies with mice that are deficient in PTEN, a phosphatase that inactivates AKT, provide indirect evidence that AKT blocks death receptor function. Lymphocytes from these mice have high constitutive AKT activity and defects in Fas-mediated apoptosis (47, 48). With age, PTEN-deficient mice develop tumors and autoimmune disease.

In this study, we examined the role of AKT in regulating apoptosis in Ag-primed CD4+ T cells. Our results suggest that this kinase may play an important role in mediating the survival effect of IL-2 family cytokines, but does not directly interfere with death receptor-induced apoptosis.

References

Received for publication June 11, 2001. Accepted for publication November 1, 2001.

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1 This work was funded by a Career Development Award from the Juvenile Diabetes Research Foundation, a Career Development Award from the Arthritis Foundation, and an award from the David Koch Research Fund (to L.V.P.). Y.R. is a Merck fellow of the Life Sciences Research Foundation.

2 Address correspondence and reprint requests to Dr. Luk Van Parijs, Center for Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139. E-mail address: lukvp@mit.edu

3 Abbreviations used in this paper: c-FLIP, cellular Fas-associated death domain-like IL-1β-converting enzyme inhibitory protein; FasL, Fas ligand; Myr AKT, myristoylated AKT; DN AKT, dominant negative AKT; AKT-ER, AKT fused to the ligand binding domain of the estrogen receptor; estrogen receptor; GFP, green fluorescent protein; MIG, murine stem cell virus IRES GFP; AICD, activation-induced cell death.
Materials and Methods

Mice

DO11.10 TCR-transgenic mice were a generous gift from Dr. C. London (University of California, Davis, CA). IL-2 knockout mice (C57BL/6 strain) were purchased from The Jackson Laboratory (Bar Harbor, ME) and genotyped by PCR. The generation of IL-2-deficient 3A9 TCR-transgenic mice has been described before (49).

Construction and production of retroviruses

The cDNAs encoding myristoylated AKT (Myr AKT) or dominant negative AKT (DN AKT) (50), kind gifts from Dr. Z. Songyang (Baylor College of Medicine, Houston, TX), or AKT fused to the ligand binding domain of the estrogen receptor (AKT-ER; Ref. 51), a kind gift from Dr. R. Roth (Stanford University, Palo Alto, CA) were cloned into the murine stem cell virus IRES green fluorescent protein (MIG) retroviral expression vector (33). High titer retrovirus was obtained by transfecting 293T cells with retroviral plasmid DNA and the pCL-Eco packaging plasmid (52). Spin infections were performed at 2500 rpm for 1 h at 30°C as described (33). Cells were infected twice within a 24-h period. Infection efficiency was determined by quantifying green fluorescent protein (GFP) expression by flow cytometry and, for both B and T cells, was between 30 and 70% in all experiments.

Purification and activation of B and T cells

In some experiments, spleen and lymph node cells were first depleted of CD8 T cells by staining with CD8α microbeads and elution on an autoMACS column (Miltenyi Biotec, Auburn, CA). To obtain activated B cells, spleen and lymph node cells were activated with anti-CD40 (1 μg/ml; BD PharMingen, San Diego, CA) for 3 days. To obtain activated T cells, spleen and lymph node cells from wild type (C57BL/6) and IL-2−/− mice were activated with 1 μg/ml anti-CD3 Ab (BD PharMingen). To obtain activated DO11.10 T cells or 3A9 T cells, spleen and lymph node cells from TCR-transgenic mice were activated with 1 μg/ml OVA peptide (residues 323–339) or 1 μg/ml hen egg lysozyme peptide (residues 54–61).

Survival assays

Activated DO11.10 cells that were left uninfected or infected with MIG, MIG Myr AKT, MIG DN AKT, or MIG AKT-ER were cultured in triplicate in a 96-well plate with or without 100 ng/ml IL-2, IL-4, IL-7, or IL-15 (BioSource International) for 30 min. T cells infected with MIG, MIG Myr AKT, or MIG AKT-ER were harvested for Western blot analysis 24 or 72 h after the last infection. In experiments with DN AKT, GFP+ populations were isolated by high-speed cell sorting. In experiments with AKT-ER, T cells were cultured with or without 100 nM of 4-OH tamoxifen. All cells were lysed as previously described (49), run on a 12% SDS-polyacrylamide gel and blotted on a polyvinylidene difluoride membrane. Blots were blocked overnight (TBST + 5% milk) and probed with Ab to AKT or phospho-AKT (S473; Cell Signaling Technology, Beverly, MA), α-tubulin (kind gift from Dr. J. Tschopp, University of Lausanne, Vaud, Switzerland), Bcl-2 (AC21; Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (Sigma-Aldrich) and, subsequently, with a goat anti-rabbit or mouse HRP-conjugated Ab. Cell survival was determined by flow cytometry for FasL expression. Activated D011.10 cells that were left uninfected or infected with MIG, MIG Myr AKT were stimulated overnight with 1 μg/ml anti-CD3, FasL levels were determined by staining with a biotinylated Ab to FasL (Alexis Biochemicals, San Diego, CA) followed by PE-conjugated streptavidin (BD PharMingen) and analysis by flow cytometry.

Results

AKT is activated by IL-2 family cytokines in Ag-primed CD4+ T cells

IL-2 family cytokines can promote the survival of T cells (Ref. 7; Fig. 1A). AKT is a candidate to mediate this effect. To test whether IL-2 family cytokines activate AKT in primary T cells, we exposed Ag-primed DO11.10 cells to IL-2, IL-4, IL-7, and IL-15 for 30 min. As shown in Fig. 1B, all of these cytokines led to the phosphorylation of Ser73, a modification that is involved in the activation of AKT (Ref. 54 and data not shown). This appears to be a major pathway of AKT activation, because we could not detect phosphorylated AKT in IL-2-deficient TCR-transgenic T cells that were stimulated by APC and cognate peptide, or in nontransgenic IL-2-deficient T cells activated with anti-CD3 and anti-CD28, unless we added back IL-2 or other IL-2 family cytokines (Fig. 1C, data not shown).

AKT is required for CD4+ T cell survival mediated by IL-2 family cytokines

We next tested whether AKT promotes the survival of CD4+ T cells by expressing a constitutively active form of this kinase (Myr AKT) in Ag-primed DO11.10 cells using a retrovirus-based gene transduction approach (33). In these experiments, retrovirally transduced cells were cultured for 24–72 h, in the absence of Ag or growth factors. As shown in Fig. 2A, expression of Myr AKT alone led to the survival and expansion of Ag-primed T cells following growth factor withdrawal. The expansion of T cells driven by AKT is dependent on the protooncogene c-myc and can be

Flow cytometry for FasL expression

Activated DO11.10 T cells infected with MIG or MIG Myr AKT were stimulated overnight with 1 μg/ml anti-CD3. FasL levels were determined by staining with a biotinylated Ab to FasL (Alexis Biochemicals, San Diego, CA) followed by PE-conjugated streptavidin (BD PharMingen) and analysis by flow cytometry.
uncoupled from the survival function of this signaling molecule (Y. Refaeli and L. Van Parijs, manuscript in preparation).

We also used a retrovirus to express a conditional allele of AKT (AKT-ER) in our Ag-primed DO11.10 cells. This reagent was generated by fusing a constitutively active form of AKT to a mutated version of the ligand-binding domain of the estrogen receptor (51). AKT-ER is inactive, unless the estrogen analog, 4-OH-tamoxifen, is added to the culture medium (Fig. 2C). The levels of AKT, phospho-AKT, and Bcl-2 in these cells were then assayed by Western blotting.

To test directly whether AKT is required for the survival effect of IL-2 family cytokines, we used a retrovirus to introduce DN AKT into Ag-primed DO11.10 cells. These were then sorted for GFP expression and cultured for 48 h in the presence of IL-2, IL-4, IL-7, or IL-15. In all cases, the expression of DN AKT led to a decrease in T cell survival (Fig. 3, data not shown), suggesting that AKT is required for the survival effect of these cytokines.

**FIGURE 1.** IL-2 family cytokines promote the survival of Ag-primed CD4+ T cells and activate AKT. A, Activated DO11.10 T cells were treated with 100 ng/ml IL-2, IL-4, IL-7, or IL-15 for 24 h. The percentage of viable cells present in culture was determined by flow cytometry (see Materials and Methods). B, Activated DO11.10 T cells were starved overnight in medium lacking growth factors and then stimulated with 100 ng/ml IL-2, IL-4, IL-7, or IL-15 for 30 min. The levels of AKT and phospho-AKT in these cells were assayed by Western blotting. C, Naive 3A9 IL-2/IL-4/IL-7 T cells were activated with 1 μg/ml hen egg lysozyme peptide for 3 days in the presence of increasing concentrations of IL-2. The levels of AKT, phospho-AKT, and Bcl-2 in these cells were then assayed by Western blotting.

**FIGURE 2.** An active form of AKT promotes the survival of Ag-primed CD4+ T cells. A, Activated DO11.10 T cells were infected either with a retrovirus expressing a constitutively active form of AKT (Myr AKT) or a control retrovirus (MIG) and cultured for 24–72 h in the absence of growth factors. The percentage of viable cells present in culture at these different time points was determined by flow cytometry (see Materials and Methods). B, Activated DO11.10 T cells were infected with a retrovirus expressing an inducible form of AKT (AKT-ER) or a control retrovirus (MIG). Infected T cells were cultured for 24–72 h in the presence or absence of 4-OH tamoxifen (TMX). The percentage of viable cells present in culture at these different time points was determined by flow cytometry (see Materials and Methods). C, The levels of phospho-AKT and β-actin present in the cells described in B were determined by Western blotting. Note that the AKT-ER fusion protein is larger than endogenous AKT and runs at ~73 kDa.
block AICD (47, 48, 57), possibly by regulating the expression of FasL or inhibiting caspases that function downstream of Fas (41, 42). To test directly whether AKT interferes with AICD, we expressed Myr AKT in Ag-primed DO11.10 cells and then stimulated these cells with IL-2 and increasing concentrations of plate-bound anti-CD3 (58). As shown in Fig. 4A, expression of a constitutively active form of AKT did not protect T cells from AICD. As a positive control, c-FLIP, a known inhibitor of Fas signaling, was able to protect T cells from AICD under the same conditions (Fig. 4A; Ref. 53). Consistent with these results, FasL expression was identical on T cells transduced with a Myr AKT-expressing virus or a control virus (Fig. 5A).

Fas-mediated apoptosis also serves to eliminate autoreactive B cells (59). To test whether AKT blocks Fas-mediated killing of B cells, we introduced Myr AKT into anti-CD40-activated B cells. These were then cultured with an Ab to Fas or Ag-primed DO11.10 cells in the presence of increasing concentrations of OVA peptide. Under these conditions, the B cells present Ag to, and stimulate, the OVA-specific T cells. These then up-regulate FasL and kill the B cells by engaging Fas (53). Expression of a

FIGURE 3. DN AKT inhibits the survival effect of IL-2 family cytokines in Ag-primed CD4+ T cells. Activated DO11 T cells were infected with a retrovirus expressing DN AKT or a control retrovirus (MIG). Infected cells were cultured for 48 h in the presence of 100 ng/ml IL-2, IL-4, IL-7, or IL-15. The percentage of viable cells present in culture was determined by flow cytometry (see Materials and Methods).

FIGURE 4. An active form of AKT fails to block Fas-mediated apoptosis in lymphocytes. A. Activated DO11.10 T cells were infected with a retrovirus expressing Myr AKT, DN AKT, or c-FLIP, or a control retrovirus (MIG). Infected cells were cultured in the presence of increasing concentrations of anti-CD3 and 10 ng/ml IL-2 for 24 h to induce AICD. The percentage of apoptotic cells present in culture was determined by flow cytometry (see Materials and Methods). B, B cells were infected as in A. Infected cells were cultured in the presence of increasing concentrations of anti-Fas for 24 h. The percentage of apoptotic cells present in culture was determined by flow cytometry (see Materials and Methods). C, B cells were infected as in A and cultured with activated DO11.10 T cells in the presence of increasing concentrations of OVA peptide. The percentage of apoptotic cells present in culture was determined by flow cytometry (see Materials and Methods).
constitutively active form of AKT did not protect B cells from Fas-dependent apoptosis induced by Abs (Fig. 4B) or by Ag-specific T cells (Fig. 4C). In contrast, expression of c-FLIP was able to inhibit B cell killing under both conditions (Fig. 4B, data not shown, and Ref. 53).

**AKT is required for the induction of Bcl-2 by IL-2 family cytokines**

The results of our genetic analysis suggest that AKT plays an important role in regulating T cell survival, but not in death receptor-mediated apoptosis. The downstream targets of AKT that might be responsible for this function in T cells remain poorly defined. We tested whether AKT regulates the expression of two well-defined antiapoptotic molecules, Bcl-2 and c-FLIP (60, 61). As shown in Fig. 5B, expression of Myr AKT in Ag-primed DO11.10 cells led to an up-regulation of Bcl-2, but not c-FLIP. These results are consistent with our observations that AKT blocks cell death following growth factor withdrawal but not death receptor-mediated apoptosis.

**Discussion**

We show in this study that AKT can prevent apoptosis of Ag-primed T cells following the removal of growth factors and that this kinase may be responsible for the survival effect of IL-2 family cytokines. We correlate the activation of AKT with the expression of Bcl-2 and provide evidence that this kinase couples IL-2 and related cytokines to the expression of this antiapoptotic molecule. Our experiments do not provide support for a role of AKT in blocking death receptor-mediated apoptosis. In agreement with this, we did not see any effect of AKT activation on the expression of c-FLIP or FasL in Ag-primed T cells.

The IL-2 family of cytokines plays an important role in T cell survival. Although each IL-2 family cytokine has a number of unique functions, circumstantial evidence suggests that the survival effect of these molecules might be mediated by a conserved mechanism. Probably due to differences in the expression patterns of these cytokines and their receptors, different family members regulate the turnover of T cells at different maturation stages (7). Our results suggest that the activation of AKT may serve as a universal relay of survival signals induced by IL-2 family cytokines.

How AKT promotes T cell survival remains unclear. A number of possible mechanisms have been proposed, however most of these have only been validated in nonlymphoid cells (62). Our results, and those from other groups, provide indirect evidence that, in lymphocytes, AKT provides a survival signal by increasing the expression of Bcl-2 and Bcl-xL (32, 37). No clear evidence has been obtained yet that AKT inhibits proapoptotic molecules, such as Bad or FasL, in T cells (37). The effects of AKT activation in T cells on the expression and activity of other members of the Bcl-2 family still have to be investigated.

Studies in knockout and transgenic mice that have increased AKT activity in T cells have also implicated this kinase in the regulation of self-tolerance (47, 48, 57). T cells from these mice show decreased sensitivity to Fas-mediated killing, which raises the possibility that AKT plays a part in controlling the elimination of autoreactive T cells by this death receptor. Our results suggest that AKT does not directly interfere with Fas-mediated apoptosis in lymphocytes. Furthermore, work from a number of laboratories indicates that Fas prevents autoimmunity primarily by eliminating autoreactive B cells (53, 59, 63–65). Experimentally, it has been shown that inhibiting Fas killing in T cells alone has no effect on tolerance (66). In contrast, activating AKT in T cells leads to autoimmune disease (57). These observations suggest that AKT may regulate tolerance through a novel mechanism, possibly by regulating the elimination of autoreactive T cells in the thymus or by extending the lifespan of mature self-reactive T cells (48).

IL-2 family cytokines are not the only molecules that regulate T cell survival. A diverse array of costimulatory and inflammatory proteins also protect T cells from apoptosis (67, 68). How they function to promote T cell survival remains unclear, but recent studies have implicated the NF-κB signaling cascade in this effect (69). It remains to be determined whether the signals induced by
these molecules intersect or function in parallel to those activated by the IL-2 family of cytokines.

Acknowledgments

We thank Dr. Pamela Ohashi for sharing the results of her laboratories studies on AKT transgenic mice before publication (57).

References


In Fig. 5, no indication was given that bands with “smiles” were straightened in panels A and B (bottom). In both the figure and the legend, the time points in A are 70 min, not 60 min. The gel photograph in B (bottom row labeled “CaM”) was separated unnecessarily. The control labeled “Co1” in A and B should be “Co” because the samples were not from the same control. In B, the labels “P1” and “P2” designating patient sources for “CaM” were reversed.

In Results, under the heading No inherent defect of NFAT or CN in the SCID patients’ T cells, the reference to “60 min” in the last sentence of the first paragraph is incorrect. The corrected sentence should read: “Cytoplasmic extracts from patient and control lines were incubated with CN plus calmodulin for 20 and 70 min at 30°C, and NFAT1 was detected by Western blotting (Fig. 5A), revealing the same amount and kinetics of dephosphorylation in control and patient T cells.”


Erin Kelly, Angela Won, and Yosef Refaeli wish to retract Fig. 5B. The retraction involves the part of the paper claiming to show that expression of AKT in Ag-primed T cells leads to up-regulation of Bcl-2 but not cFLIP. This result was used to support the idea that Akt blocks apoptosis of Ag-primed T cells following growth factor withdrawal but not following death receptor activation. Kelly, Won, and Refaeli have no reason to believe that the other results and interpretations in this paper need to be corrected or retracted. This retraction follows an investigation by the Massachusetts Institute of Technology into scientific misconduct by Dr. Luk Van Parijs, the corresponding author of the paper, that found the retracted figure had been falsified or fabricated. The investigation also found that Dr. Van Parijs was solely responsible for the scientific misconduct that resulted in the falsified or fabricated data or conclusions in this paper.


The authors incorrectly stated that the truncated IL-17RA FRET constructs extend from amino acids 1–441 of murine IL-17RA. In fact, these truncated receptors encode residues 1–526 and also incorporate some additional amino acids before the commencement of the CFP or YFP moieties (introduced from cloning). Thus, the final amino acid sequence of the junction is IL-17RA: . . . SRYP-HAY-RL . . . CFP/YFP, where double underlining indicates IL-17RA sequence, single underlining indicates residues introduced from the vector, and dashed underlining indicates CFP or YFP sequence. This error does not affect any of the conclusions in the paper.


In Fig. 1 and the figure legend there are errors regarding the number of amino acids in a protein. The label “ΔC Splice Variant: 364 aa” should be “ΔC Splice Variant: 442 aa” in Fig. 1. In the legend to Fig. 1, the third sentence should read: “The truncated form lacks almost the entire COOH tail (171 aa, green, red, and short black traits) but bears an extra 18 aa (light blue trait) due to inclusion of the intron between exons 10 and 11 (94)”.


In the Introduction, Materials and Methods, Discussion, Fig. 4D, and the Fig. 4 legend, all but one reference made to IRGC should be to IRGM. The sentence on page 7191, repeated on page 7194, “There has been no investigation of the human homolog of LRG-47 (IRGC) in humans” is incorrect. There are two human homologs; the one under investigation in our publication was in fact IRGM. Materials and Methods correctly describe primers to detect the transcript of IRGM,