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Akt inhibition upregulates FasL, downregulates c-FLIP$_s$ and induces caspase-8-dependent cell death in Jurkat T lymphocytes

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Abstract

In T lymphocytes, the role of Akt in regulating Fas/Fas ligand (FasL)-mediated apoptotic signaling and death is not clearly understood. In this study, we observed that inhibition of Akt causes enhanced expression of FasL mRNA and protein and increased death-inducing signaling complex (DISC) formation with Fas-associated death domain (FADD) and procaspase-8 recruitment. Also, caspase-8 was activated at the DISC with accompanying decrease in c-FLIP$_s$ expression. FasL neutralizing antibody significantly decreased apoptotic death in the Akt-inhibited T cells. Additionally, Akt inhibition-induced Fas signaling was observed to link to the mitochondrial pathway via Bid cleavage. Further, inhibition of caspase-8 activity effectively blocked the loss of mitochondrial membrane potential and DNA fragmentation, suggesting that DISC formation and subsequent caspase-8 activation are critical initiating events in Akt inhibition-induced apoptotic death in T lymphocytes. These data demonstrate yet another important survival function governed by Akt kinase in T lymphocytes, which involves the regulation of FasL expression and consequent apoptotic signaling.
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Abbreviations: FasL, Fas ligand; Akt, Akt/protein kinase B; PI3K, phosphatidylinositol-3-kinase; NOK-2, anti-human Fas ligand monoclonal antibody; IgG_{2a}, monoclonal immunoglobulin isotype; FADD, Fas-associated death domain; FLICE, FADD-like interleukin 1 β-converting enzyme; DISC, death-inducing signaling complex; FLIP, FLICE inhibitory protein; PDK1, a 3-phosphoinositide-dependent protein kinase 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; t-Bid, truncated-Bid

Introduction

The role of the serine–threonine kinase protein kinase B (PKB/Akt) in T lymphocyte survival is well documented.[1–4] Akt is activated through the direct binding of phosphatidylinositol (3, 4, 5)-triphosphate (PIP_3), a lipid second messenger generated by phosphatidylinositol-3-kinase (PI3K).[5,6] PIP_3 generated by the action of PI3K binds Akt via the pleckstrin homology (PH) domain of Akt, followed by 3-phosphoinositide-dependent protein kinase 1 (PDK1)-mediated phosphorylation at Thr-308 and Ser-473.[5,6] Upon activation Akt promotes cell survival by phosphorylating and inactivating a variety of key proapoptotic targets in T lymphocytes.[1]

Fas/FasL-mediated activation-induced cell death (AICD) plays a pivotal role in the physiological elimination and turnover of lymphocytic cells. Induction of FasL expression has been implicated in drug- and stress-induced apoptotic death of T lymphocytes.[7] Upon stimulation, Fas undergoes trimerization and recruits the proapoptotic adapter protein Fas-associated death domain (FADD) and pro-caspase-8, also called FADD-like interleukin 1 β-converting enzyme (FLICE), to form a death-inducing signaling complex (DISC).[8–11] Recruitment of pro-caspase-8 to the DISC leads to its proteolytic activation to caspase-8, followed by initiation of a caspase cascade leading to apoptosis.[11] FLICE inhibitory protein (c-FLIP) is an antiapoptotic cytoplasmic protein that has sequence homology to caspase-8. c-FLIP is unable to undergo cleavage to an active caspase due to substitution of a tyrosine for an active site cysteine, and hence can function as a dominant-negative inhibitor of caspase-8, thereby preventing Fas-induced apoptosis.[12–14] Alternative splicing yields multiple isoforms of c-FLIP; two commonly occurring isoforms are designated c-FLIP$_L$ (long isoform) and c-FLIP$_s$ (short isoform).[15,16] c-FLIP is known to play an antiapoptotic role in a number of cell types and elevated c-FLIP expression renders cells resistant to Fas-induced apoptosis.[17] In fact, the susceptibility of T lymphocytes to Fas-mediated apoptosis correlates with c-FLIP levels, which are elevated during the early stages of activation but are significantly reduced upon re-stimulation when the T cells undergo AICD.[7,18,19] The molecular mechanisms involved in the regulation of expression of c-FLIP in T lymphocytes are not completely understood.

Analysis of the Fas-mediated pathway has shown that formation of DISC and subsequent caspase-8 activation can link to the mitochondrial apoptotic pathway through the activation of Bid, a proapoptotic member of the Bcl-2 family.[20,21] Bid-mediated mitochondrial changes include the

opening of the mitochondrial pores regulating permeability transition, decrease in the membrane potential ($\Delta\Psi_m$) and release of cytochrome *C* into the cytoplasm, which further complexes with Apaf-1 and activates caspase-9.[22–24] Eventually, these apoptotic events culminate in the activation of executioner caspases, for example, caspase-3 leading to DNA fragmentation and cell death.[25]

Although several antiapoptotic functions of Akt have been identified in T lymphocytes, the potential role of Akt in regulating FasL and c-FLIP expression, and the impact on subsequent apoptotic signaling, has not been examined. The results showed that inhibition of Akt, using either PI3 kinase inhibitor or a selective Akt inhibitor, upregulates FasL gene expression. Moreover, inhibition of Akt resulted in a correspondent decrease in c-FLIP$_s$ expression and an increase in caspase-8 activity leading to Fas-mediated apoptosis. Role of FasL-dependent cell death in Akt-inhibited cells was further supported by the observations using FasL antagonistic (neutralizing) antibody and caspase-8 inhibitor, both of which significantly attenuated apoptosis. Taken together, these data demonstrate a critical survival function of Akt in T lymphocytes, that involves downregulation of FasL and control of DISC formation by regulating the expression and/or function of c-FLIP and caspase-8.

Results

To inhibit activation of Akt, T cells were treated with LY-294002 (LY), a known pharmacological inhibitor of PI3 kinase (PI3K), which is the upstream activator of Akt. Although LY has been extensively used to examine the role of PI3K/Akt in several cellular systems, it is also known to inhibit casein kinase 2 (CK2) with potency similar to PI3 kinase.[26] Hence, in order to specifically evaluate the role of Akt kinase, a selective Akt inhibitor (AI–III) was also employed in these studies. AI–III is a 2-modified, 3-deoxy phosphatidylinositol analog with phosphate linkers, which specifically inhibits Akt phosphorylation and activity without affecting any upstream kinases such as PI3K or PDK-1 as well as extracellular signal-regulated kinase (ERK).[27,28]

Effect of Akt inhibition on cell viability and apoptotic cell death

Both LY and AI–III blocked Akt activation in a dose-dependent manner as measured by Akt enzymatic activity (Figure 1a) and level of phosphorylated Akt (Figure 1b). Additionally, AI–III did not have any effect on PI3 kinase activity (data not shown).

The effect of Akt inhibition using LY and AI–III on cell viability of Jurkat T cells was examined by trypan blue dye exclusion. Both LY and AI–III treatments led to a dramatic decrease in cell viability after 24 h as compared to untreated cells (Figure 2). To determine if the cell death due to inhibition of Akt was by the induction of apoptosis, we measured DNA fragmentation, which is a well-established marker of apoptosis. The effect of Akt inhibition on DNA fragmentation was quantified in cytoplasmic extracts using the Cell Death detection ELISA assay. LY induced concentration-dependent DNA fragmentation within 6 h, with a three-fold increase at 50 μM compared to untreated cells (Figure 3a, $P<0.01$). Similar results were obtained using AI–III. Treatment of Jurkat T cells with different concentrations of AI–III (10 and 20 μM)

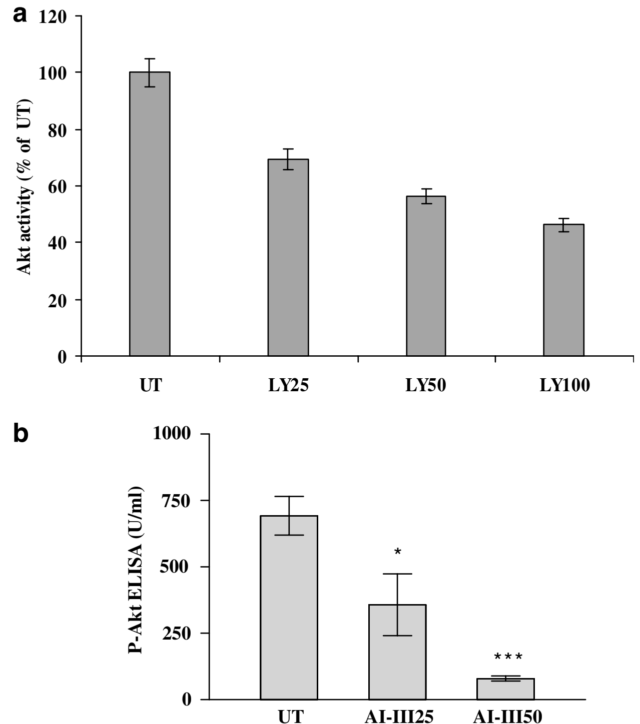

Figure 1 LY and AI–III block Akt activity in a dose-dependent manner. (**a**) Jurkat T cells were untreated (UT) or treated with different concentrations of LY (25, 50, 100 μM) for 6 h. Enzymatic activity of Akt was measured in cytoplasmic extracts as described in Materials and Methods. (**b**) Jurkat T cells were untreated (UT) or treated with different concentrations of AI–III (25 and 50 μM). Cells were harvested after 6 h and phosphorylation of Akt was analyzed by ELISA as described in Materials and Methods. Data represented as the mean ± S.E.M. of triplicate assays and are normalized to untreated control in (**b**). $^{*}P<0.05$, $^{***}P<0.001$ compared to UT

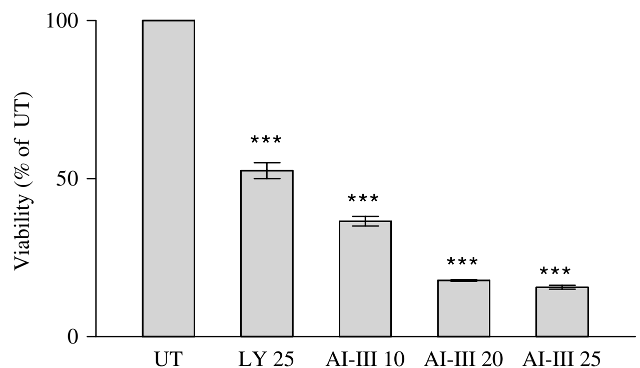

Figure 2 Inhibition of Akt induces cell death in Jurkat T cells. Trypan blue exclusion method was used to quantitate survival in Jurkat T cells that were untreated (UT) or treated with LY (25 μM) or AI–III (10, 20, 25 μM) for 24 h. Data are normalized to the untreated control (which is set to 100%) and represented as the mean ± S.E.M. of two assays. $^{***}P<0.001$ compared to UT

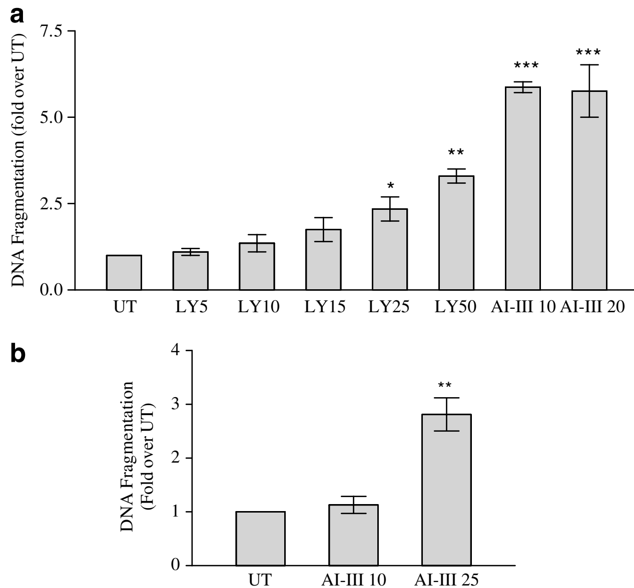

Figure 3 Inactivation of Akt induces DNA fragmentation in a dose dependent fashion. (**a**) Jurkat T cells were untreated (UT), treated with increasing concentrations of LY (5, 10, 15, 25, 50 μM) or AI–III (10, 20 μM) for 6 h. (**b**) MOLT-4 T cells were untreated (UT), or treated with increasing concentrations of AI–III (10, 25 μM) for 6 h. Cytoplasmic extracts were prepared and analyzed for DNA fragmentation by Cell Death ELISA kit as described in Materials and Methods. Data are normalized to untreated control (which is set to 1) and represented as the mean $\pm$ S.E.M. of three different experiments. Each experiment had duplicate wells assayed separately for each treatment. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to UT

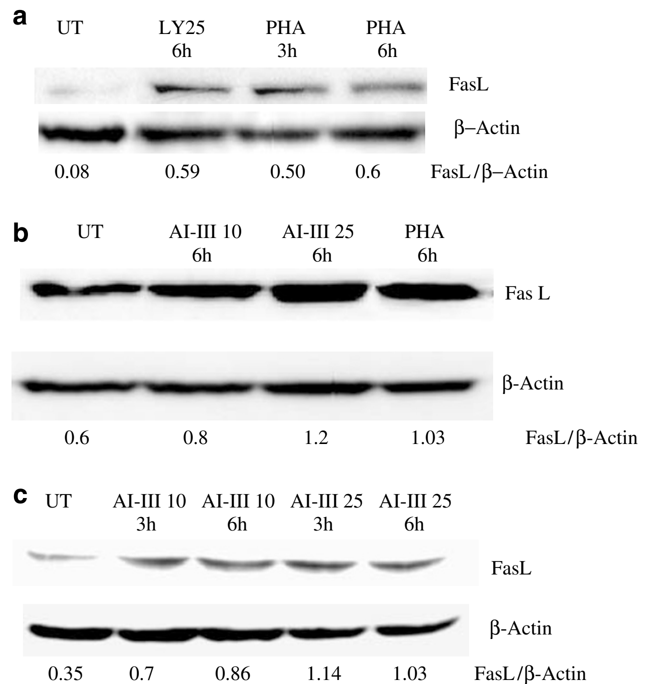

Figure 4 Inhibition of Akt up regulates FasL expression. (**a**, **b**) Jurkat T cells were treated with LY (25 μM), AI–III (10, 25 μM) or PHA (5 μg/ml). (**c**) MOLT-4 T cells were treated with AI–III (10, 25 μM). Total cell lysates were collected at 3 or 6 h for Western blot analysis as described in Materials and Methods. Untreated cells (UT) were used as a negative control. FasL expression was assayed by immunoblotting using an anti-FasL-specific antibody; blots were stripped and reprobed with antibody to β-actin to ensure equivalent loading. A representative gel out of three experiments is shown

for 6 h resulted in a six-fold increase in DNA fragmentation at both concentrations as compared to untreated cells (Figure 3a, $P<0.001$). To ensure that the induction of apoptosis by Akt inhibition was not cell line specific (i.e. restricted to Jurkat T cells), we tested the effect of Akt inhibition on another human leukemic T-cell line, namely, MOLT-4. We obtained similar results with MOLT-4 cells, wherein treatment of MOLT-4 cells with AI–III (10 and 25 μM) for 6 h resulted in a 2.8-fold increase in DNA fragmentation as compared to untreated cells (Figure 3b, $P<0.01$). These data confirm previous observations that Akt is critical for T lymphocyte survival and that inhibition of Akt leads to apoptotic T-cell death.[29–31]

Effect of Akt inhibition on FasL expression

The above data demonstrate that inhibition of Akt induces apoptotic T-cell death. Since FasL is a well-established apoptotic stimulus for T lymphocytes, we examined FasL expression in Akt-inhibited Jurkat T cells. FasL protein expression was analyzed by Western blotting. Inhibition of Akt by LY led to a significant increase in the expression of FasL protein (Figure 4a). Similarly, treatment of Jurkat T cells with AI–III increased FasL expression and this increase was dose-dependent (Figure 4b). Importantly, FasL expression in response to both LY and AI–III was equal to or greater than the level induced by the T-cell mitogen phytohemoagglutinin (PHA), which is known to induce FasL and AICD in T cells. To further extend the observations in Jurkats, the effect of AI–III treatment was analyzed in MOLT-4 T cells. Similar to Jurkat T cells, MOLT-4 T cells also showed induction of FasL expression in response to Akt kinase inhibition (Figure 4c).

To determine whether the upregulation of FasL protein in response to Akt inhibition was at the transcriptional level, we analyzed FasL mRNA levels by real-time PCR as well as reverse transcriptase polymerase chain reaction (RT-PCR). The data showed that, in correlation with FasL protein, FasL mRNA was dose dependently induced two- to 20-fold after 6 h treatment with AI–III as documented by real-time PCR (Figure 5a). Similar results were obtained by semi-quantitative RT-PCR (Figure 5b). Jurkat T cells treated with Akt-specific inhibitor AI–III for 3 and 6 h showed a time-dependent increase in FasL mRNA expression (Figure 5b) compared to untreated cells which had a low basal level of FasL mRNA. Treatment with LY also resulted in induction of FasL mRNA that peaked at 3 h and was slightly reduced by 6 h. The level of FasL mRNA induction by Akt inhibition equaled or exceeded that induced by PHA, which is known to upregulate FasL mRNA in these cells. Thus, inhibition of Akt up regulates both protein and mRNA levels of FasL, indicating that Akt functions as a negative regulator of FasL expression in T cells.

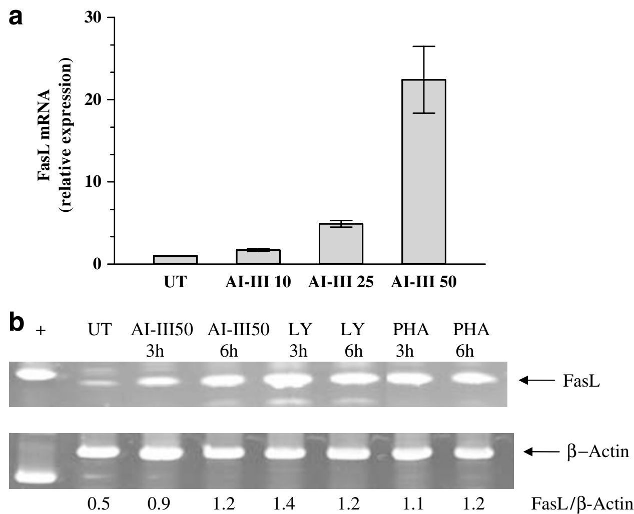

Figure 5 FasL mRNA expression is induced in response to inhibition of Akt. (**a**) Real-time PCR: Total RNA was isolated at 6 h from Jurkat T cells untreated (UT) or treated with AI–III (10, 25 and 50 μM), FasL mRNA levels were determined by real-time PCR. Data are represented as mean$\pm$S.E.M. of three different experiments. (**b**) RT-PCR: Total RNA was isolated at 3 and 6 h from Jurkat T cells untreated (UT) or treated with AI–III (50 μM), LY (25 μM) or PHA (5 μg/ml). FasL mRNA levels were determined by RT-PCR and visualized by 10% polyacrylamide gel electrophoresis. A representative gel out of three experiments is shown. The arrows point to the position of FasL and β-actin mRNA. The RT-PCR product size for the positive control (+) for FasL is 275 bp and the β-actin is 340 bp. In experimental samples, the RT-PCR product size for FasL is 239 bp and β-actin is 528 bp

Effect of Akt inhibition on FADD recruitment, c-FLIP$_S$ expression and caspase-8 activation

To further elucidate the effect of Akt kinase inhibition, components of the Fas/FasL apoptotic pathway were examined in Akt kinase-inhibited cells. FasL binding to the Fas receptor (Fas) is known to induce the formation of DISC involving the recruitment of FADD and subsequent recruitment and activation of caspase-8. Hence, the effect of Akt kinase inhibition on FADD recruitment to Fas was examined. A Fas antibody was used to immunoprecipitate the DISC from lysates (normalized for protein) of cells treated with AI–III. Immunoprecipitates were resolved using SDS-PAGE and examined by Western blot analysis using antibodies against FADD and Fas. Increased recruitment of FADD was seen in Akt kinase-inhibited cells as compared to the control cells (Figure 6a). Further analysis of the immunoprecipitates showed that the increase in FADD recruitment was accompanied by increase in procaspase-8 recruitment into the DISC (Figure 6a). The 56 kDa procaspase-8 undergoes several processing steps to generate cleaved fragments of p42–44, p28, p18 and p10–12 kDa in different cells undergoing apoptosis. Under the experimental conditions examined in these studies, we could predominantly detect the presence of the p28 kDa cleavage product of caspase-8 in the DISC assembly (Figure 6a). Although increased FADD and procaspase-8 recruitment was observed, equivalent amounts of immunoprecipitated Fas were seen in both AI–III-treated and untreated cells. The increase in DISC assembly and recruitment of FADD, procaspase-8, cleaved caspase-8 correlated with the dose-dependent inhibition of Akt kinase by AI–III. In keeping with cleavage/activation of procaspase-8 at the DISC, we observed a significant induction of caspase-8 enzymatic activity after 8 h of treatment with both LY and AI–III as compared to untreated cells (Figure 6b).

Since it has been demonstrated that c-FLIP$_s$ is a dominant-negative inhibitor of caspase-8, we examined the level of c-FLIP$_s$ under Akt-inhibited conditions. Western analysis was performed on Jurkat T cells treated with LY or AI–III to examine the effect of Akt kinase inhibition on c-FLIP$_s$. Inhibition of Akt activation by LY led to a substantive decrease in the c-FLIP$_s$ levels (Figure 6c). Similarly, selective Akt inhibition by AI–III decreased c-FLIP$_s$ levels in a dose dependent fashion (Figure 6c). Thus, Akt inhibition down-regulated c-FLIP$_s$, thereby allowing recruitment and activation of caspase-8. Additionally, pretreatment of Jurkat cells with a caspase-8-specific inhibitor resulted in protection from apoptotic DNA fragmentation induced by LY and AI–III (Figure 6d).

Taken together, these data demonstrate that inhibition of Akt leads to the upregulation of FasL expression, DISC formation and accompanying loss of c-FLIP$_s$ resulting in caspase-8-dependent apoptotic death.

Effect of Akt inhibition on Bid truncation, mitochondrial transmembrane potential ($\Delta\Psi_m$) and caspase-3 maturation

Fas mediated caspase-8 activation can initiate the mitochondrial apoptotic pathway by cleaving Bid, a proapoptotic member of the Bcl-2 family.[20,21] After activation, the truncated Bid (t-Bid) translocates to the mitochondrial membrane and causes a decrease in the mitochondrial transmembrane potential ($\Delta\Psi_m$) initiating mitochondrial apoptotic signaling, leading ultimately to the activation of the effector caspases (e.g. caspase-3). Hence, to evaluate the involvement of the mitochondrial pathway in the Fas-mediated apoptotic death induced in Akt kinase-inhibited cells, Bid cleavage, decrease in $\Delta\Psi_m$, and maturation of caspase-3 were examined.

In correspondence with caspase-8 activation, treatment of cells with AI–III induced significant Bid cleavage (t-Bid formation) as compared to the untreated cells (Figure 7a). Consistent with the known effect of Bid cleavage on mitochondrial $\Delta\Psi_m$, treatment of cells with AI–III led to a dose-dependent dissipation of mitochondrial $\Delta\Psi_m$ as indicated by an increase in the percentage of cells showing green fluorescence from 4.94% in untreated cells to 19.86% in cells treated with 25 μM AI–III (Figure 7b). To determine whether mitochondrial depolarization was a direct result of caspase-8 activation, we examined the effect of caspase-8 inhibitor on AI–III-induced dissipation of mitochondrial $\Delta\Psi_m$. Treatment with caspase-8 inhibitor significantly blocked the dissipation of mitochondrial $\Delta\Psi_m$ in Akt kinase-inhibited cells (Figure 7c), as well as DNA fragmentation (Figure 6d). These data suggest that caspase-8 activation triggered by Akt kinase inhibition is not secondary to the mitochondrial amplification loop, and is critical for initiating mitochondrial depolarization leading to apoptotic death.

The effect of the above events on the activation of the effector caspase-3 was further determined. In correspondence

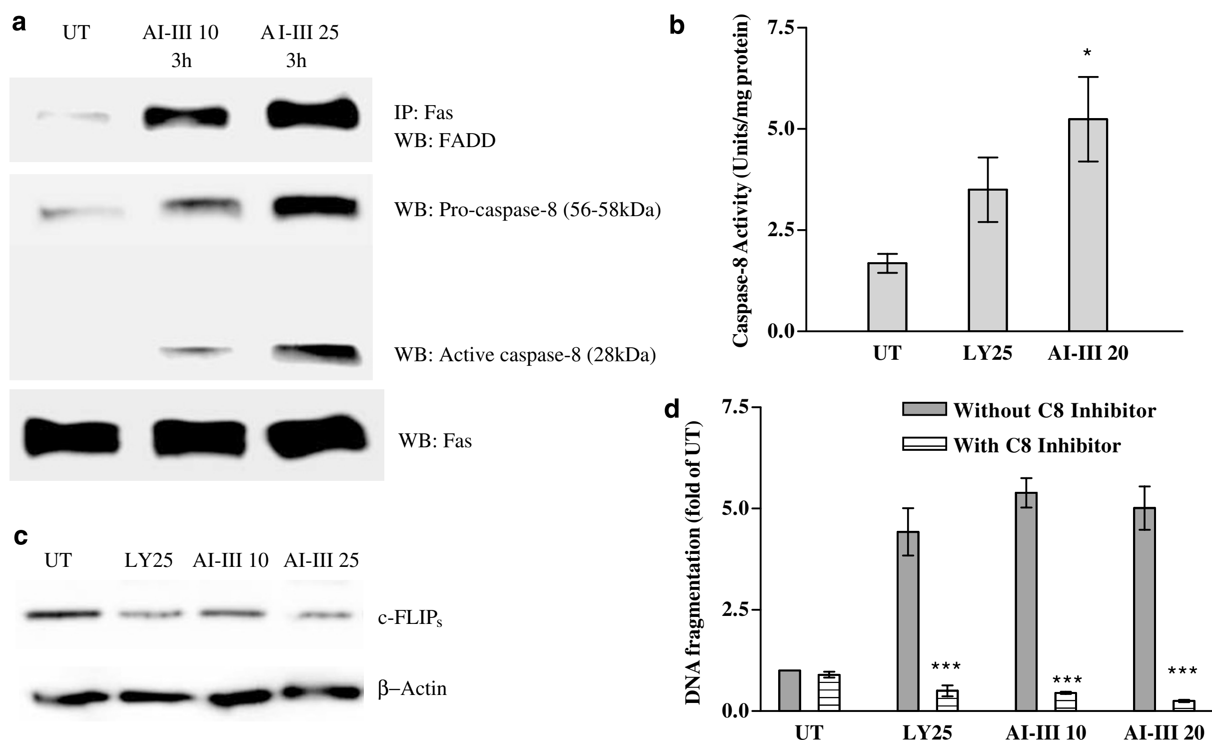

Figure 6 Akt inhibition induces DISC formation, caspase-8 activation, down regulation of c-FLIP$_s$ expression and caspase-8 dependent apoptosis. (**a**) Jurkat T cells were untreated (UT), or treated with AI–III (10 and 25 μM); total cell lysates were collected at 3 h for immunoprecipitation with Fas receptor antibody and then a Western blot analysis with FADD or caspase-8 antibodies were performed as described in Materials and Methods. Untreated cells (UT) were used as a negative control; blots were stripped and reprobed with Fas receptor antibody (Fas B-10) to ensure equivalent loading. A representative gel out of three experiments is shown. (**b**) Jurkat T cells were untreated (UT), treated with LY (25 μM) or AI–III (20 μM) for 8 h. Cells were harvested and cytosolic extracts were prepared for caspase-8 activity assay. *$P<0.05$ compared to UT. (**c**) Jurkat T cells were treated with LY (25 μM) or AI–III (10 and 25 μM); total cell lysates were collected at 6 h for Western blot analysis as described in Materials and Methods. Untreated cells (UT) were used as a negative control. c-FLIP$_s$ was detected by Western blot analysis; blots were stripped and reprobed with antibody to β-actin to ensure equivalent loading. A representative gel out of three experiments is shown. (**d**) Jurkat T cells were untreated (UT), treated with LY (25 μM) or AI–III (10 and 20 μM) for 8 h. In one group of cells, caspase-8 Inhibitor (C8, 100 μM) was added 1 h prior to the addition of LY or AI–III. Cytoplasmic extracts were prepared and analyzed for DNA fragmentation using the cell death ELISA kit. Data are normalized to the untreated control (**d**) and each column represents the mean $\pm$ S.E.M. of triplicate assays. ***$P<0.001$ compared to LY25, AI–III 10 and AI–III 20, respectively

with the upregulation in FasL expression, DISC formation and associated apoptotic signaling, treatment of cells with AI–III led to significant activation of caspase-3 in a dose-dependent manner (Figure 7d) and correlated with the induction of DNA fragmentation. These results indicated that the increase in upstream apoptotic events caused by enhanced FasL expression channel through the mitochondrial apoptotic signaling, ultimately leading to the activation of the effector caspase-3 and apoptotic death in Akt kinase-inhibited cells.

Relevance of FasL expression to cell death induced by Akt inhibition

To test the functional relevance of FasL expression in Akt inhibition-mediated cell death, we examined whether blocking Fas/FasL interaction would protect Jurkat T cells from undergoing apoptosis. For this purpose, we used the anti-FasL IgG antibody (NOK-2) which recognizes and neutralizes both membrane-bound and soluble forms of human FasL, interferes with Fas/FasL interactions and inhibits Fas signaling. Incubation of Jurkat T cells with NOK-2 antibody after 1 h treatment with LY or AI–III significantly attenuated apoptosis by approximately 50 and 65%, respectively (Figure 8). Treatment with the IgG_{2a} isotype antibody (negative control) did not protect cells from undergoing apoptosis (Figure 8), showing a direct and specific association between Akt inhibition and FasL-dependent apoptosis.

Discussion

Apoptotic death induced in activated thymocytes or previously stimulated mature T cells plays a critical role in the normal functioning of the immune system.[25] FasL expression plays a major role in the AICD of activated mature T cells and T cell hybridomas.[32] The crucial role of FasL in the regulation of immune homeostasis strongly indicates that its expression must be tightly regulated. Identification of the pathways regulating FasL expression is highly relevant in order to understand the function and survival of T lymphocytes and consequently the immune response.

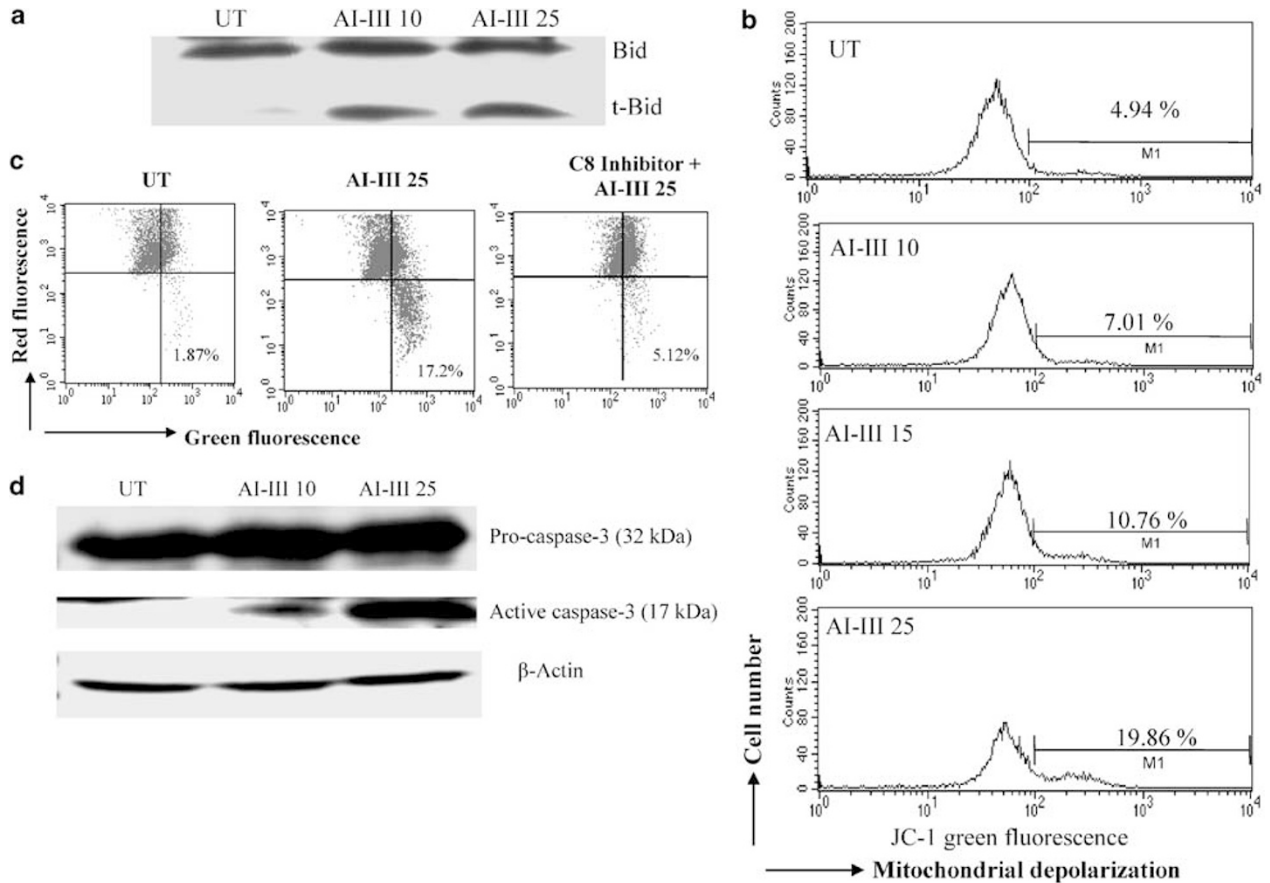

Figure 7 Akt inhibition induces Bid cleavage, mitochondrial depolarization and caspase-3 activation. (**a**) Jurkat T cells were treated with AI–III (10 and 25 μM); total cell lysates were collected at 6 h for Western blot analysis as described in Materials and Methods. Untreated cells (UT) were used as a negative control. Bid and the cleaved form t-Bid were detected by Western blot analysis; blots were stripped and reprobed with antibody to β-actin to ensure equivalent loading. A representative gel out of three experiments is shown. (**b**) To monitor changes in mitochondrial membrane potential, Jurkat T cells were untreated (UT) or treated with increasing concentrations of AI–III (10–15 and 25 μM) for 6h; after treatment cells were incubated with JC-1 dye (5 μg/ml) as described in Materials and Methods. Mitochondrial depolarization was analyzed by flow cytometry. Dose-dependent changes in mitochondrial $\Delta\Psi_m$ were observed in Akt-inhibited conditions (gate window M1) compared to UT cells. (**c**) Jurkat cells were untreated (UT), treated with AI–III (25 μM) for 6 h or pretreated with caspase-8 inhibitor (C8 I, 100 μM) for 1 h prior to the addition of AI–III. After treatment, cells were incubated with JC-1 dye (5 μg/ml) as described in Materials and Methods. Mitochondrial depolarization was analyzed by flow cytometry. The lower right quadrant represents the cells showing changes in mitochondrial $\Delta\Psi_m$ (green fluorescence). (**d**) Jurkat T cells were treated with AI–III (10 and 25 μM); total cell lysates were collected at 6 h for Western blot analysis as described in Materials and Methods. Untreated cells (UT) were used as a negative control. Pro and active caspase-3 was detected by Western blot analysis; blots were stripped and reprobed with antibody to β-actin to ensure equivalent loading. A representative gel out of three experiments is shown

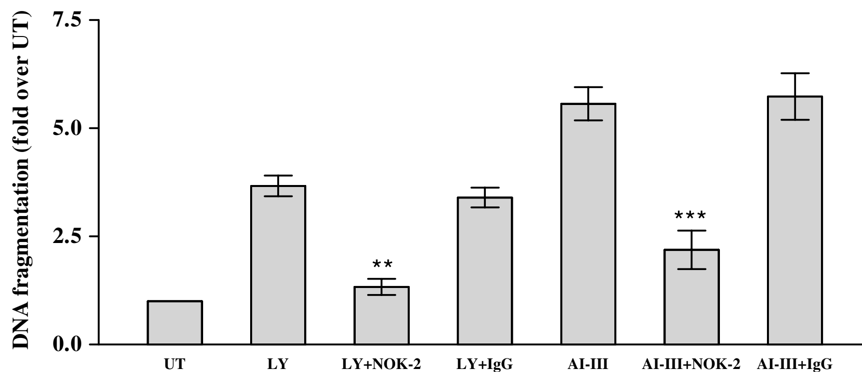

Figure 8 Neutralization of FasL protects Jurkat T cells from apoptosis induced by inhibition of Akt. Jurkat T cells were untreated (UT) or treated with LY (25 μM) or AI–III (20 μM). In one group, NOK-2 antibody (50 μg/ml) or the isotype IgG_{2a} (50 μg/ml) control was added 1 h after treating the cells with LY or AI–III, and then the cells were harvested after 6 h to measure apoptosis. Cytoplasmic extracts were prepared and analyzed for DNA fragmentation using the Cell Death ELISA kit. Data are normalized to the untreated control and represented as mean $\pm$ S.E.M. of three different experiments. $^{**}P<0.01$, $^{***}P<0.001$ compared to LY or AI–III, respectively

Akt is involved in the lymphokine and co-stimulatory molecule activated survival pathways that inhibit Fas-mediated apoptosis.[30,33] Recent work in vascular smooth muscle cells has shown that Akt kinase regulates FasL expression,[34] and our work shows that Akt inhibition leads to the upregulation of FasL expression and DNA fragmentation in HeLa cells (data not shown). Hence, although Akt kinase is implicated in other cell systems, its role in the regulation of FasL expression and subsequent apoptotic pathway has not been examined in T lymphocytes. In the present work, we examined the role of Akt in the regulation of FasL expression, Fas-mediated apoptotic signaling and cell survival in Jurkat T lymphocytes that have been extensively used as a model system to detail molecular mechanisms involved in T cell activation, function and death.[35] Additionally, the effect of Akt inhibition was also examined in a different human T cell line – MOLT 4.

Inhibition of Akt by both a PI3 kinase inhibitor as well as a selective Akt inhibitor led to an upregulation in FasL mRNA and protein. The upregulation in FasL mRNA expression could be due to the effect of Akt-mediated phosphorylation affecting transcription factors like FKHRL1, which have been proposed to regulate FasL gene promoter activity.[29] Studies to investigate the mechanisms of FasL upregulation in Akt-inhibited T lymphocytes are currently underway. In contrast to our findings, data obtained from T cells treated with glucocorticoids indicate that Akt kinase under the influence of Ft1 via GSK3 and NF-AT$_c$ signaling induces the production of FasL and causes apoptotic death.[36] These findings indicate that Akt kinase plays a differential role in regulating FasL expression and apoptotic signaling and is dependent on the cellular context and the type of stimulus and signaling components involved.

Apoptosis induced by Akt inhibition was significantly blocked by incubation with FasL neutralizing antibody, demonstrating the functional relevance of FasL expression under these (Akt inhibited) conditions. Although neutralization of FasL significantly protected Jurkat cells from undergoing apoptosis, the NOK-2 antibody did not completely abrogate DNA fragmentation, suggesting the possible involvement of both Fas-dependent and Fas-independent pathways leading to T cell death.

Early events in the FasL-initiated signaling pathway have been well characterized. Triggering of Fas by FasL leads to its trimerization followed by recruitment of the adaptor molecule FADD, and procaspase 8, forming DISC (reviewed in Peter and Krammer[17]). Our data show that Akt kinase inhibition induces FasL expression leading to Fas-FasL-dependent clustering of Fas and formation of DISC involving recruitment of FADD and procaspase-8. Also, the presence of cleaved caspase-8 together with FADD and procaspase-8 in the DISC assembly shows that caspase-8 is activated at the DISC. Moreover, AI–III treatment enhanced the recruitment of FADD and procaspase-8 in a dose-dependent manner, suggesting that the enhancement in the DISC formation is correspondent to the extent of Akt inhibition occurring in T lymphocytes.

Apoptosis of activated T cells mediated by Fas is known to be regulated by c-FLIP expression. There are two isoforms of c-FLIP, c-FLIP$_s$ and c-FLIP$_L$, both of which can be recruited to the DISC.[17] Although the role of c-FLIP$_L$ is controversial, in T cells c-FLIP$_s$ is demonstrated to be antiapoptotic and confers resistance to Fas-mediated apoptosis by blocking proteolytic activation of caspase-8 at the DISC.[17,18] Regulation of c-FLIP expression in T lymphocytes is not completely understood. In cells stimulated with the T cell mitogen concanavalin A (conA), MAP kinase kinase (MKK-1) is implicated in the induction of c-FLIP expression.[37] Studies using PI3 kinase inhibitors have implicated the PI3 kinase pathway as an important regulator of c-FLIP expression in nonlymphoid tumor cells;[38,39] however, the specific role of Akt in regulating c-FLIP expression in T lymphocytes has not been elucidated. Our study shows that inhibition of Akt downregulates c-FLIP$_s$ expression, and specifically implicates Akt as the key regulator of c-FLIP$_s$ and Fas-mediated apoptosis in T cells. Similar to our observations in T lymphocytes, other studies have also found a link between Akt inhibition and consequent c-FLIP down-regulation in different cell types.[40–42] It is possible that, in addition to suppressing Akt, the Akt inhibitors used in this study may affect Fas-mediated apoptosis through modulation of other relevant signaling molecules besides c-FLIP. Additionally, it should be noted that the requirement of Akt kinase to support c-FLIP$_s$ expression may differ depending on the cell type and its activation state. Recently, Kuenzi *et al*[43] reported that the PI3K inhibitor LY did not downregulate c-FLIP expression in T cells of bovine origin transformed by Theileria parva parasite infection. These results indicate that Akt kinase can regulate Fas-mediated apoptotic signaling in a differential and cell/signaling context-dependent manner.

In AI–III-treated cells, the resultant increase in FasL expression, DISC assembly and caspase-8 activation was accompanied by Bid cleavage, decrease in $\Delta\Psi_m$ and activation of the executioner caspase-3. These data suggest that the Fas-mediated apoptotic pathway induced by Akt inhibition via FasL expression is linked to the mitochondrial pathway. Consistent with this notion, we found that caspase-8 inhibitor treatment significantly blocked the decrease in $\Delta\Psi_m$ and downstream DNA fragmentation caused by Akt inhibition. Additionally, activation of caspase-8 at the DISC and the ability of caspase-8 inhibitor to prevent the loss of $\Delta\Psi_m$ as well as DNA fragmentation strongly suggest that caspase-8 activation triggered by Akt inhibition is not secondary to the mitochondrial amplification loop. Importantly, these data also suggest that in T lymphocytes, formation of DISC leading to increased caspase-8 activation is the critical initiator of apoptotic death induced in response to Akt inhibition.

Overall, our study indicates that, in addition to other known antiapoptotic functions, Akt regulates Fas-mediated as well as mitochondrial apoptotic pathways in T lymphocytes and hence plays a critical role in physiological as well as pathophysiological conditions that involve Fas-mediated apoptosis of T lymphocytes.

Materials and Methods

Cell culture

Jurkat T cells (clone E6-1, ATCC, Rockville, MD, USA) and MOLT-4 T cells (ATCC, Rockville, MD, USA) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 U/ml of penicillin, 10 μg/ml streptomycin (Invitrogen Corporation, Grand Island, NY, USA), in a 37°C

and 5% CO_2 environment. Jurkat cells were resuspended at 1×10^6 cells/ml during treatment.

Reagents and antibodies

Akt-specific inhibitor (Akt inhibitor III – (AI–III)) was purchased from Calbiochem (La Jolla, CA, USA), PI3/Akt inhibitor LY-294002 was purchased from Biomol (Plymouth Meeting, PA, USA). Anti-human FasL NOK-2 antibody and the isotype IgG_{2a} were purchased from BD Biosciences Pharmigen (San Diego, CA, USA). PHA was purchased from Sigma (St Louis, MO, USA). Caspase-8 inhibitor II was purchased from Calbiochem (La Jolla, CA, USA). Fas-L (C-178), FAS (B-10), caspase-3 and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-FLIPγ/δ (191–209) antibody was purchased from Calbiochem (La Jolla, CA, USA). FADD and Bid antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Caspase-8 (Ab-3) antibody was purchased from oncogene (La Jolla, CA, USA). JC-1 mitochondrial potential sensor was purchased from Molecular Probes (Eugene, OR, USA).

Akt activity assay

Akt activity was measured by one of two methods: (a) enzymatic assay or (b) ELISA assay. For the enzymatic assay, treated Jurkat cells were collected after 6 h and lysed in lysis buffer (PBS pH 7.4, 1% NP-40, 200 μM sodium orthovanadate, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 4 μg/ml benzamidine, 50 mM potassium fluoride, 1 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 μM okadaic acid) and centrifuged at $15\,000 \times g$ for 10 min at 4°C. Akt protein was immunoprecipitated from total cell extracts. Purified Akt substrate was added and the kinase reaction was started by adding 10 μCi [^{32}P]ATP diluted with 0.5 mM cold ATP and samples were incubated for 15 min at 37°C. After centrifugation, equal aliquots of the supernatants were transferred to Pierce Phosphocellulose Unit, washed three times with binding buffer. The radioactivity bound to the membrane was then counted as a measure of Akt activity. Additionally, we examined Akt phosphorylation to evaluate Akt activity. Total cell lysates were prepared from treated Jurkat cells (after 6 h treatment) and Akt phosphorylation was measured using an ELISA Kit (Biosource International, Camarillo, CA, USA) according to the manufacturer's procedures.

Trypan blue dye exclusion

To measure viability, cells were stained with trypan blue dye and then counted by a method described elsewhere.[44]

DNA fragmentation ELISA assay

Treated Jurkat cells or MOLT-4 cells were lysed after 6 h to measure apoptosis. DNA fragmentation was quantitated using the Cell Death ELISA kit (Roche, Indianapolis, IN, USA). The assay is based on quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This allows for the determination of mono- and oligonucleosomes present in the cytoplasmic fraction of cell lysates. The sample was placed into a streptavidin-coated microtiter plate. A mixture of antihistone–biotin-labeled antibody and anti-DNA peroxidase-conjugated antibody was added and incubated for 2 h. After removal of the antibodies by a washing step, the amount of nucleosome-bound fragmented DNA was quantified by the peroxidase retained in the immunocomplex using ABTS (9, 2, 2′-Azino-di [3-ethylbenzthiazolin-sulfonate]) as a substrate.

Immunoprecipitation and Western blot analysis

For immunoprecipitation (IP) of the CD95 DISC, 25×10^6 Jurkat T cells were untreated or treated with AI–III 10 or 25 μM for 3 h. Following treatment, cells were lysed with IP lysis buffer (25 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 10 mM NaF, 1% Triton X-100, 0.5 mM phenylmethylsulfonylfluoride and 1 μg/ml of leupeptin, pepstatin and leucine thiol), and the lysate (300μg of protein/per sample) was incubated with 0.4 μg of anti-FAS (B-10) antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) overnight rotation at 4°C. The following day, 20 μl of protein A/G Plus agarose from Santa Cruz Biotechnology (Santa Cruz, CA, USA), was added for 2 h rotation at 4°C, and then immunoprecipitates were washed four times with IP lysis buffer, then separated on a 10% SDS-PAGE and subjected to standard immunoblotting procedures. The polyvinylidene difluoride membrane was immunoblotted with an anti-FADD antibody and with an anti-caspase-8 antibody.

For Western blot analysis, following treatment, cells were lysed and total cellular proteins (60 μg of protein) were resolved on 12% SDS-PAGE and subjected to standard immunoblotting procedures. The primary antibodies used were Fas-L (C-178), anti-FLIPγ/δ, anti-caspase-3, anti-Bid and β-actin. The appropriate secondary antibodies were used at 1 : 5000 for all the antibodies. Protein signals were visualized using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA), as directed by the manufacturer. The molecular sizes of the developed proteins were estimated by comparison with prestained protein markers (Invitrogen, Carlsbad, CA, USA).

RNA isolation, RT-PCR and real-time PCR analysis

RT-PCR assays were used to assess FasL mRNA levels in Jurkat cells. Total RNA was isolated from treated cells after 3 and 6 h, using TRIZOL (Invitrogen, Carlsbad, CA, USA). For real-time PCR, the first-strand cDNA was synthesized using TaqMan Reverse transcription reagents (Applied Biosystems). The reverse transcription was carried out using 1 × Taqman RT buffer, 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM random hexamer, 8 U of RNase inhibitor and 25 U of Multiscribe Reverse Transcriptase with 200 ng of total RNA. The RT conditions were 10 min at 25°C, 30 min at 48°C and 5 min at 95°C. Reactions in which the enzyme or RNA were omitted were used as negative controls. Real-time PCR was performed with an ABI prism 7000 sequence detection system and SYBR green I dye reagents. The specific primers were designed for human GAPDH and Fas ligand using Primer3 software program. The following primers were used in real-time PCR:

hGAPDH-RT-FP: 5′ TGGGCTACACTGAGCACCAG 3′
hGAPDH-RT-RP: 5′ GGGTGTCGCTGTTGAAGTCA 3′
hFasL-RT-FP: 5′ GGCCTGTGTCTCCTTGTGAT 3′
hFasL-RT- RP: 5′ TGCCAGCTCCTTCTGTAGGT 3′

The parameter Ct (threshold cycle) was defined as the fraction cycle number at which the fluorescence passed the threshold. The relative gene expression of FasL was analyzed using $2^{-\Delta\Delta Ct}$ method[45] by normalizing with GAPDH gene expression in all the experiments.

For regular PCR, RNA concentration was determined spectrophotometrically and 1 μg of total RNA was used for RT. Reverse

transcription was performed using a commercially available cDNA Cycle kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, 5 μl of cDNA products were amplified in 50 μl of 50 mM KCl, 10 mM Tris–HCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U AmpliTaq® DNA polymerase (Perkin-Elmer, Foster City, CA, USA) and 0.45 μM of human FasL primers pair or β-actin (R&D Systems, Minneapolis, MN, USA). The parameters for PCR were as follows: 96°C for 4 min, 33 cycles of 1 min of denaturation at 94°C, 2 : 30 min of annealing at 59°C and 40 s of extension at 72°C. The 239 bp amplified FasL and the 528 bp amplified β-actin RT-PCR products were analyzed by electrophoresis in 10% polyacrylamide gels and semi-quantitated by densitometric analysis using the Quantity One 4.2 (BIORAD) software.

Assessment of mitochondrial transmembrane potential ($\Delta\Psi_m$)

To monitor changes in mitochondrial membrane potential ($\Delta\Psi_m$), 1×10^6 Jurkat T cells were treated with increasing concentrations of AI–III (10–15 and 25 μM) for 6 h, in one group of cells, caspase-8 inhibitor (100 μM) was added 1 h prior to the addition of AI–III; after treatment cells were incubated with JC-1 dye (5 μg/ml) at 37°C for 20 min. Then cells were washed twice with cold 1 × PBS. Depending on the state of the $\Delta\Psi_m$, this cyanine dye accumulates in the mitochondrial matrix and forms aggregates that have characteristic absorption and emission spectra. Changes in mitochondrial $\Delta\Psi_m$ were analyzed by FACSCalibur flow cytometry (BD Biosciences). Fluorescence was induced with an argon laser (excitation wavelength, 488 nm) and the green fluorescence was collected through 585/42 nm (FL-2) and 530/30 nm (FL-1) bandpass filters. At least, 2×10^4 events were acquired and analyzed using the CellQuest analysis program (BD Biosciences).

Caspase activity assay

To measure caspase-8 activity, cytoplasmic extracts were prepared from Jurkat cells treated for 8 h, and analyzed using the CASPASE-8 Colorimetric Activity Assay kit (Chemicon International, Temecula, CA, USA) as directed by the manufacturer.

FasL neutralization assay

FasL-dependent cell death was evaluated by a neutralization assay. Briefly, Jurkat cells were treated with LY or AI–III, and 1 h after treatment the NOK-2 antibody or the isotype control was added to the cells and then harvested after 6 h for DNA fragmentation measurement using the Cell Death ELISA kit (Roche, Indianapolis, IN, USA).

Statistical analysis

All data are expressed as mean ± S.E.M. The method of analysis used was unpaired analysis of variance with the Tukey–Kramer multiple-comparison test. Differences were considered statistically significant for $P < 0.05$.

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