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CD4-Lck Through TCR and in the Absence of Vav Exchange Factor Induces Bax Increase and Mitochondrial Damage[1]

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In the present study, we aimed to demonstrate that CD4 may represent a critical turning point that governs the apoptotic and survival programs in T cells, without modifying the physical association with the TCR-CD3 complex. To address this issue, we have explored the possibility that the activation of CD4 may transduce apoptotic signals unless signaling effectors neutralize them. Our data show that in Jurkat T cells CD4 engagement by Leu3a mAb results in a rapid and strong increase of Lck kinase activity, subsequent alterations of mitochondrial membrane potential, and apoptosis. Critical parameters are coassociation of CD4/Lck with TCR/CD3 and up-regulation of the proapoptotic protein Bax. Indeed, Leu3a-mediated Lck activation failed to induce apoptotic features in Jurkat cells either defective for TCR/CD3 or overexpressing the antiapoptotic protein Bcl-2. Furthermore, we demonstrate that Leu3a treatment of Jurkat cells overexpressing Vav results in the inhibition of mitochondrial damage and apoptosis; this rescue effect is accompanied with a significant decrease of Bax expression observed in apoptotic cells. Our evidence that the activation of Lck activates in T cells apoptotic pathways which are counteracted by Vav, a signaling molecule that cooperates with CD28 to boost TCR signals, suggests a novel role for costimulation in protecting T cells from CD4-mediated cell death. ***The Journal of Immunology*****, 2002, 168: 6106–6112.**

T cell activation following Ag recognition occurs as the result of a multimolecular interaction between T cells and APCs, a physical site termed the "immunological synapse" (1, 2). It is easy to deduce that the biochemical events regulating T cell signaling can lead to extreme modifications such as cell growth, unresponsiveness, or cell death.

CD4 is one of the receptors on T cells that can both potentiate or suppress T cell activation. The ability of CD4 to enhance or alter signals generated by the TCR-CD3 complex has been attributed to its cytoplasmic tail that interacts with the Src family tyrosine kinase $p56^{lck}$. Indeed, following recognition of peptide-MHC complexes by TCR and CD4, Lck is activated and phosphorylates the tyrosine residue within the immunoreceptor tyrosine-based activation motif of the CD3 and ζ-chains, which is the earliest known event in T cell signaling (3). It has been suggested that CD4 binds MHC class II simultaneously or perhaps shortly after TCR-peptide-MHC engagement (4, 5), stabilizing the interactions between peptide-MHC and TCR (6, 7). The use of new technologies such as the multidimensional imaging of T cells interacting with APC has afforded new insights into T cell activation. Grakoui et al. (8) have shown the immunological synapse formation as a dynamic mechanism that allows T cells to distinguish potential antigenic ligands and the recruitment of $p56^{lck}$ to the engaged TCR by CD4 may contribute to this kinetic discrimination. More recently, Krummel et al. (9) showed unexpected CD4 engagement dynamics. The authors found that immediately after cell contact and coincident with the increase in intracellular calcium, CD4 and TCR-associated CD3ζ clustered in the interface. However, following signaling-, costimulation-, and cytoskeleton-dependent processes, CD3ζ chains were redistributed to central clusters while CD4 moved to the periphery. It is interesting to note that both papers suggest that CD4 participates in the triggering of early signals necessary in immunological synapse formation and may not be required once activation has been initiated.

In the context of the negative signals delivered by CD4, the quality of the Ag, cell cycle phase, decreased availability of Lck by TCR, defective transduction pathways, cytokine availability, and lack of costimulatory signals have been described as apoptosis inducers (10–12). However, many of these results have been obtained when the obligatory interaction between TCR and CD4 was interrupted by sequential stimulation of CD4 and TCR. Since this kind of dissociation is a rare event in physiological conditions in vivo, the role of CD4 in mediating apoptotic programs in T cells remains still unclear. The observation that CD4 should serve primarily to "boost" recognition of ligand by the TCR (9) prompted us to reconsider the role of CD4 in programmed cell death, one of the apoptotic pathways operative in T lymphocytes (13). We hypothesized that if CD4 is able to transduce both survival and apoptotic programs, both programs must be prepared at the first stage of Ag recognition and in concert with TCR. Indeed, once the engaged TCR are invested in building the stable central cluster and other costimulatory molecules are moved into the immunological synapse by cytoskeleton-mediated membrane raft movement, the apoptotic programs will be deleted and the survival programs executed.

An essential regulator of cytoskeletal rearrangements during T cell activation is Vav (14). Vav functions as a guanine nucleotide exchange factor for the Rho/Rac family of protein G (15, 16) and plays a critical role in both thymocyte development (17) and T lymphocyte activation (18). More recently, data from Wulfing et al. (19) support the role of Vav as a central regulator of T cell activation-induced actin cytoskeleton rearrangements, necessary

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for an organized accumulation of signaling molecules at the interface of APC and responding cells. Moreover, Vav overexpression has been suggested to mimic the effects of costimulatory molecules, such as CD28, in potentiating TCR signaling (20, 21). Altogether these data strongly support the hypothesis that Vav may play a role in blocking CD4-mediated apoptosis.

The present study aimed to explore the possibility that CD4 engagement, without modifying the physical association with the TCR-CD3 complex (22), induces both apoptotic and survival programs in T cells and to clarify the involved molecular mechanisms. Our results show that suicide signals, triggered in Jurkat T cells by Leu3a mAb-mediated engagement of CD4, crucially depend on CD4-TCR/CD3 coassociation. The apoptotic features observed are related to an increase of Bax expression and mitochondrial damages. Overexpression of Vav rescues T cells from CD4-mediated apoptotic programs by inhibiting up-regulation of Bax expression mediated by CD4/Lck observed in apoptotic cells. These data suggest that signaling effectors, which contribute in facilitating TCR and costimulatory molecules signaling, can neutralize CD4-triggered suicide programs.

Materials and Methods

Cell lines, Abs, and reagents

Jurkat (clone J20) and its derivative Jurkat 31.13 defective for TCR/CD3 surface expression (23) were maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin (Life Technologies, Milan, Italy). Staining of 31.13 cells with anti-CD4 Ab revealed that only 10–20% of the overall population expresses high levels of CD4. Thus, cells expressing CD4 molecules were enriched by magnetic cell sorting using beads coated with anti-CD4 Ab and following the manufacturer's recommended procedure (Miltenyi Biotec, Bologna, Italy). The Jurkat cell lines overexpressing Vav (CL9) or Bcl-2 were grown in the same culture medium supplemented with 2 mg/ml G418 (Life Technologies) or 500 ng/ml puromycin (Sigma-Aldrich, St. Louis, MO), respectively.

Mouse anti-CD4 mAb (Leu3a) was purchased from BD Biosciences (Mountain View, CA). Rabbit anti-human Bax and Bcl-2 Abs were obtained from BD PharMingen (San Diego, CA). Mouse (3A5) and rabbit anti-p56^{lck} Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-c-*myc* (9E10) Ab was obtained from Boehringer Mannheim (Monza, Italy).

PP2, a selective inhibitor of the *Src* family of tyrosine kinases, was provided by Calbiochem (La Jolla, CA). Bax antisense (24) and unrelated oligodeoxynucleotides with a natural phosphodiester backbone were synthesized by M-Medical Genenco (Florence, Italy). Sequences used were: Bax antisense oligonucleotides 5′-TCG ATC CTG GAT GAA ACC CT-3′ and 5′-TCC CCC CCC ATT CGC CCT GC-3′, and unrelated oligonucleotides 5′-GGT ATG GCT AGC ATG ACT GG-3′ and 5′-GTC GTA CTC TTC TGC ATT GA-3′.

Plasmid and cell transfection

pEF-Bos-expressing N-terminal *myc*-tagged Vav was a kind gift from Dr. A. Weiss (University of California, San Francisco, CA). pEF-pGKpuro-expressing human Bcl-2 (25) was kindly provided by Dr. A. Strasser (Walter and Eliza Hall Institute, Melbourne, Australia).

Stable transfectants overexpressing Vav wild-type (Vav CL9) was obtained by electroporating (at 260 V, 960 F) 10^7 Jurkat cells in 0.5 ml of RPMI 1640 containing 20% FCS with 30 μg of pEF-Bos-expressing N-terminal *myc*-tagged Vav. After 48 h, cells were placed in 96-well flat-bottom culture plates in RPMI 1640 medium plus G418. The Jurkat cell line, overexpressing Bcl-2, was generated by transfecting Jurkat with 30 μg of pEF Bcl-2-pGKpuro. After 24 h, cells were cultured for 3 wk with RPMI 1640 medium containing 500 ng/ml puromycin. Transfectants were analyzed for CD3, CD4, and CD28 expressions by FACS analysis (FACSCalibur; BD Biosciences) and for *myc*-Vav and Bcl-2 expression by Western blotting.

Cell stimulation, immunoprecipitation, and in vitro kinase assay

Jurkat cells were washed twice, resuspended in medium (10^8/ml), and incubated for different times at 37°C in the presence or absence of Leu3a mAb. At the end of incubation, cells were harvested and lysed for 30 min on ice in 1% Nonidet P-40 lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EGTA in the presence of inhibitors of proteases and phosphatases: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Pefabloc-sc, 50 mM NaF, 10 mM $Na_4P_2O_7$, and 1 mM $NaVO_4$. The kinase activity of immunoprecipitated p56^{lck} was assayed at 30°C for 15 min in 25 μl of kinase buffer (1 mM Tris-HCl (pH 7.5), 7.5 mM NaCl, 25 mM HEPES (pH 7.3), 10 mM $MnCl_2$, and 0.05% Nonidet P-40) in the presence of 10 μCi of [γ-^{32}P]ATP (10 Ci/mmol). The reactions were terminated with 2× Laemmli sample buffer. Samples were analyzed by SDS-PAGE gel. Gels were fixed, treated with 1 M of KOH for 1 h at 55°C to remove the alkali-labile phosphate groups from serine- and threonine-phosphorylated proteins, dried, and autoradiographed. Radioactivity in the phosphorylated proteins was quantitated by a phosphoimager.

Immunoblotting

Jurkat cells were washed twice, resuspended in medium (10^8/ml), and incubated at 37°C in the presence or absence of Leu3a mAb. At the end of incubation, cells were lysed for 30 min on ice and proteins were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with anti-Bax, or anti-Bcl-2, or anti-Vav, or anti-Lck Abs, extensively washed, and after incubation with HRP-labeled goat anti-rabbit or HRP-labeled goat anti-mouse (Amersham International, Amersham, U.K.) Abs developed with the ECL detection system (Amersham International).

Apoptosis analysis

Jurkat T cell lines were incubated for 48 h in the presence or absence of Leu3a (1:50). Apoptosis was measured by a BD Biosciences FACSCalibur flow cytometer by evaluating changes in cell dimensions measured by forward scatter height (FSC-H)[4] vs cell condensation and granularity measured by side scatter height (SSC-H), as previously described (26). The percentage of specific apoptosis was calculated as follows: percentage of specific apoptosis = 100 × (percent Leu3a-treated apoptotic cells − percent spontaneous untreated apoptotic cells)/(100 − percent spontaneous untreated apoptotic cells).

Analysis of variation in the mitochondrial transmembrane electrical potential ($\Delta\Psi_m$)

Variation in $\Delta\Psi_m$ at the single mitochondrial level was detected by using the lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Briefly, cells were incubated at 10^6 cells/ml in RPMI 1640 with 10% FCS for 15 min at 37°C with 10 μg/ml JC-1 (Molecular Probes, Eugene, OR). At the end of incubation, cells were washed twice, resuspended in PBS, and $\Delta\Psi_m$ was analyzed by a BD Biosciences FACSCalibur flow cytometer as described previously (27). Depolarization of mitochondrial membrane is accompanied by a change of JC-1 color from greenish orange (analyzed in FL-2) to green (analyzed in FL-1).

Flow cytometric analysis of Bax and Bcl-2

Cells were fixed with PBS containing 2% paraformaldehyde and subsequently permeabilized in PBS containing 0.5% BSA, 0.02% sodium azide, and 0.5% saponin. Then cells were incubated for 15 min at room temperature with rabbit anti-human Bax or Bcl-2 Abs (BD PharMingen) or isotype-matched rabbit IgG Abs (Sigma-Aldrich), washed, and stained with FITC-labeled anti-rabbit IgG (Sigma-Aldrich). Flow cytometric analysis was performed on a BD Biosciences FACSCalibur flow cytometer.

Results

CD4 engagement by Leu3a mAb activates Lck kinase and induces apoptosis by mitochondrial damage

It has been reported that in Jurkat T cells CD4 functionally interacts with the TCR-CD3 complex even in the absence of TCR engagement (28). This could be related to a high background of tyrosine phosphorylation compared with normal T cells and could mimic a low activation threshold. Thus, the engagement of CD4 in Jurkat cells could mimic the boost effect, described by Krummel et al. (9), that normally occurs when T lymphocytes interact with APC. If the early signals mediated by the interaction of CD4 with TCR/CD3 are not sustained by other costimulatory signals such as those supported by APC, the activation pathways may be switched off in favor of apoptotic pathways. To test this hypothesis, we

[4] Abbreviations used in this paper: FSC-H, forward scatter height; SSC-H, side scatter height; $\Delta\Psi_m$, mitochondrial transmembrane electrical potential; MFI, mean fluorescence intensity.

cultured Jurkat cells in the presence or absence of Leu3a, and both Lck kinase activity and apoptotic features were analyzed. To evaluate Lck activation, cells were treated with Leu3a for different times and in vitro kinase assays were performed in anti-Lck immunoprecipitates. Fig. 1*A* (*upper panel*) clearly shows that cross-linking of CD4 by Leu3a results in a rapid and strong increase of Lck kinase activity that returns to the basal level after 15 min of stimulation. No induction of Lck kinase activity was observed following immunoprecipitation of CD4 itself in unstimulated cells (data not shown). Lck protein content in each sample was also checked by Western blotting using anti-Lck Ab (Fig. 1*A*, *lower panel*). Apoptosis was analyzed on the same cells after 48 h of treatment with Leu3a by FACS analysis (Fig. *1*, *B* and *C*). Cell death was evaluated by FACS analysis measuring either changes in cell dimension vs cell granularity (Fig. 1*B*) or changes in the $\Delta\Psi_m$ (Fig. 1*C*) after staining with the lipophilic cationic probe JC-1 (27). The results obtained show that the activation of Lck by Leu3a in the absence of other signals results in a massive apoptosis of Jurkat cells, and almost all apoptotic cells showed significant alterations of the mitochondrial membrane potential. No differences in both basal apoptosis and mitochondrial membrane depolarization were observed when an isotype-matched mAb was used as control (data not shown).

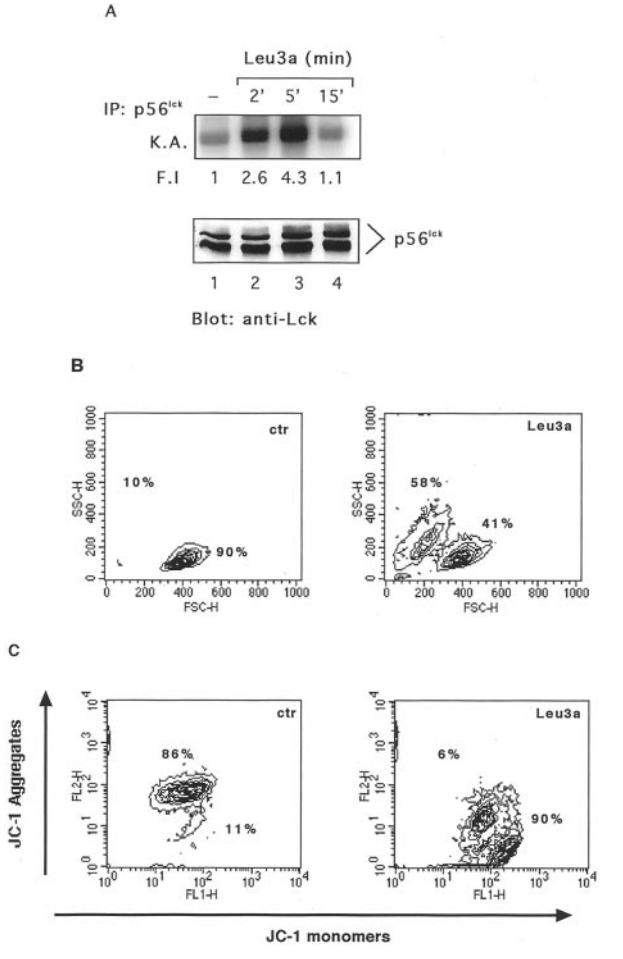

FIGURE 1. CD4 engagement by Leu3a activates p56^{lck} kinase activity and induces both mitochondria damage and apoptosis in Jurkat T cells. *A*, Jurkat cells were incubated for different times in the presence or absence of Leu3a at 37°C and the kinase activity (K.A.) of p56^{lck} was determined after immunoprecipitation using the 3A5 anti-Lck mAb (*upper panel*). Radioactivity was quantitated by a phosphoimager and expressed as fold induction (F.I.) over the basal activity. A sample of each lysate was analyzed for the expression of Lck by Western blotting using rabbit anti-Lck Abs followed by HRP-protein A (*lower panel*). The results shown represent one of three independent experiments. *B*, FACS analysis of apoptotic Jurkat cells treated for 48 h with or without (ctr, control) Leu3a. The graphs describe the distribution in the cell population of the morphological features represented by FSC-H, accounting for cell dimension, vs cell condensation and granularity, measured by SSC-H. Apoptotic cells are characterized by a lower FSC-H and higher SSC-H. Data are representative of five independent experiments. *C*, An aliquot of Jurkat cells treated as in *B* was analyzed by flow cytometry for mitochondrial membrane depolarization after staining with JC-1.

Both kinase activity of the CD4-Lck complex as well as TCR/CD3-mediated signaling are responsible for apoptosis induction

To assess whether the kinase activity of Lck was responsible of the apoptotic features observed in Leu3a-treated Jurkat cells, we used PP2, a potent selective inhibitor of the Src family tyrosine kinases. PP2 is equivalent to PP1 that has been described as a tool for examining the role of Lck and FynT tyrosine kinases vs ZAP-70 in T cell activation (29). As shown in Fig. 2*A*, the addition of PP2 to Leu3a-treated cells induced a significant reduction of specific apoptosis, supporting the need of Lck-mediated tyrosine phosphorylation in inducing apoptosis. However, the evidence that the PP2, when added to kinase assay, did not completely abolish Lck kinase

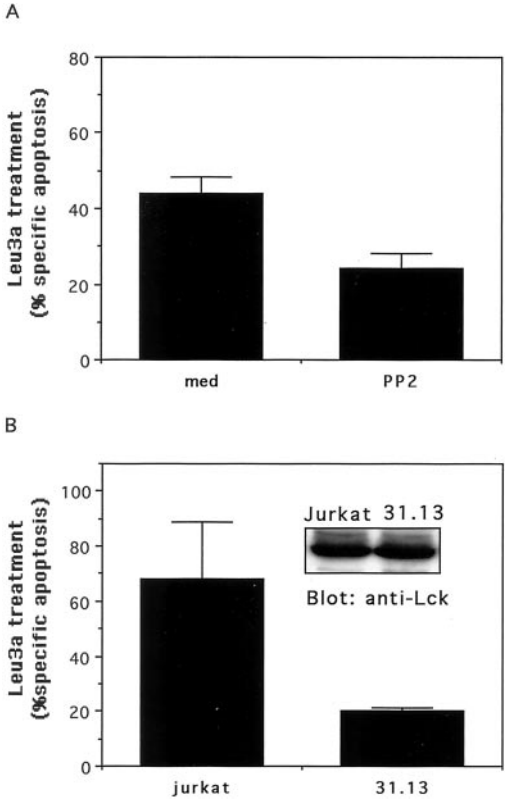

FIGURE 2. Both p56^{lck} as well as TCR/CD3-associated tyrosine kinases are necessary for Leu3a-induced apoptosis. *A*, Jurkat cells were pretreated for 30 min with medium alone (med) or 10 μM Src kinase inhibitor PP2 and then cultured for 48 h in the presence or absence of Leu3a. Apoptosis was evaluated by FACS analysis and the results, expressed as percentage of specific apoptosis, represent the mean ± SD of three independent experiments. The percentage of apoptotic cells in Leu3a-untreated cultures was ≤10%. *B*, Jurkat cells and TCR/CD3-negative Jurkat cells (31.13) were cultured for 48 h with or without Leu3a and apoptosis was analyzed as in *A*. The results, expressed as percentage of specific apoptosis, represent the mean ± SD of three independent experiments. p56^{lck} expression in Jurkat and 31.13 cells was evaluated by Western blotting (*inset*).

activity (data not shown) and did not affect totally the induction of apoptosis suggested to us that kinases different from Lck or other signals could be involved in CD4-mediated apoptosis. Therefore, we analyzed the need of the TCR-CD3 complex in favoring the execution of apoptotic pathways triggered by CD4. To do that, we performed apoptosis experiments in the Jurkat derivative subline 31.13. These cells are defective for the expression of the TCR β-chain, an event that blocks the assembly of the TCR-CD3 complex at the cell surface (23). Although both cells expressed similar levels of Lck (Fig. 2*B*, *inset*) and similar increases of Lck kinase activity following Leu3a treatment (data not shown), Leu3a-mediated Lck activation in the absence of TCR/CD3 failed to induce significant apoptosis in 31.31 cells (Fig. 2*B*). Hence, surface expression of the TCR-CD3 complex is a prerequisite not only for propagating the early tyrosine phosphorylation cascade induced by CD4 engagement (30), but also for triggering apoptotic signals.

CD4-mediated apoptosis of Jurkat cells is a Bax-dependent phenomenon

By using Ag-specific memory T cells, we have recently demonstrated that CD4 cross-linking before activation via TCR results in the up-regulation of the proapoptotic protein Bax, without modifying the levels of the anti-apoptotic protein Bcl-2 and in mitochondrial damage (11). The evidence that apoptosis observed in Leu3a-treated Jurkat cells was mitochondrion-dependent prompted us to analyze in these cells the level of Bax expression. Data reported in Fig. 3*A* clearly demonstrate that CD4/Lck activation results in up-regulation of Bax. To further confirm that a Bax increase in Leu-3a-treated cells was responsible for mitochondrion-dependent apoptosis, Jurkat cells were incubated for 48 h with medium or Leu3a in the presence of 5 μM Bax antisense oligonucleotides or unrelated oligonucleotides. The initial first hour of incubation was performed in medium without serum to increase the uptake of oligonucleotides. At the end of incubation, mitochondrial membrane depolarization was evaluated by JC-1 staining. As shown in Fig. 3*B*, Bax antisense oligonucleotides significantly inhibited Leu3a-induced mitochondrial damage. These data, along with our previous results (11), confirm that Bax can be considered an important mediator of CD4-mediated apoptosis.

Bcl-2 overexpression inhibits apoptosis induced in Jurkat cells by CD4 stimulation

The Bcl-2 family comprises death-inducing and death-inhibitory members that differ in their tissue- and activation-dependent expression patterns (31). Many of these proteins are predominantly located in the outer mitochondrial membrane regulating the mitochondrial transmembrane potential; this applies to Bcl-2, Bcl-x_L, and Bax. The ratio of death-inducing (Bax) and death-inhibitory members (Bcl-2, Bcl-x_L) determines whether a cell will respond to an apoptotic signal by mediating the disruption of the mitochondrial membrane and the release of protease activators (32, 33). Our evidence that the activation of Lck resulted in the up-regulation of Bax without modifying Bcl-2 levels (see Figs. 3*A* and 6) prompted us to verify whether the overexpression of Bcl-2 in Leu3a-treated cells could abolish mitochondrial membrane depolarization. Therefore, we stably transfected Jurkat cells to overexpress Bcl-2 (JBcl-2). Both Jurkat and JBcl-2 cells were incubated for 48 h, in the presence or absence of Leu3a, and apoptosis was evaluated by FACS analysis, measuring the variation of cell dimension (FSC-H) vs cell granularity (SSC-H). As shown in Fig. 4*A*, Bcl-2 overexpression reduced the percentage of apoptotic cells in Leu3a-treated cells. Leu3a-induced mitochondrial membrane depolarization was also reduced to 41% in Bcl-2-overexpressing cells (data not shown). These results evidence that the overexpression of Bcl-2 contrasts the apoptotic pathways activated by CD4/Lck.

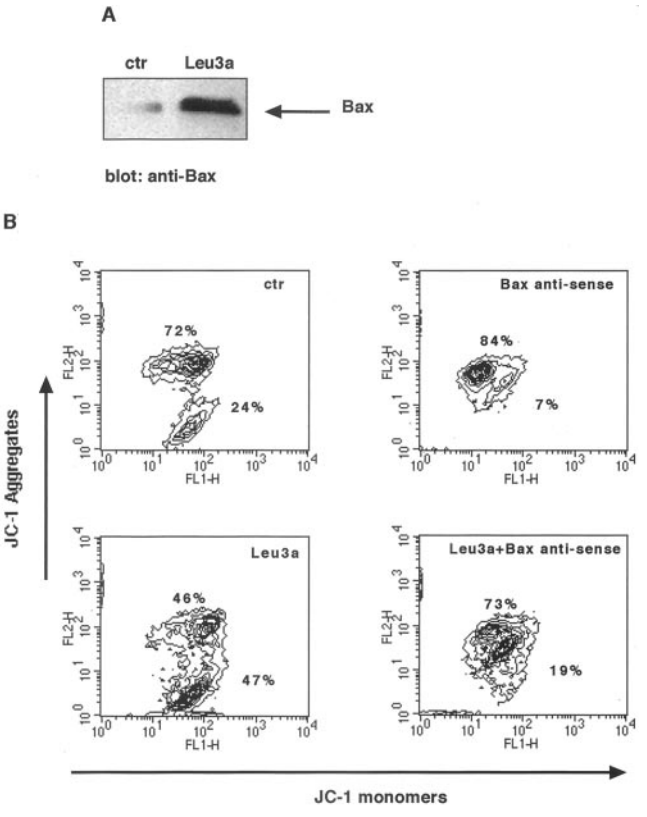

FIGURE 3. Bax antisense oligonucleotides inhibit mitochondrial damage induced by Leu3a treatment. *A*, Jurkat cells were cultured for 48 h in the presence or absence (ctr, control) of Leu3a, and Bax expression was analyzed by Western blotting using specific Abs. The results shown are representative of three independent experiments. *B*, Jurkat cells were pretreated with 5 μM Bax antisense or unrelated oligonucleotides for 1 h in RPMI 1640 without FCS before Leu3a treatment as in *A*. Control and Leu3a refer to unrelated oligonucleotide treatment. Mitochondria membrane depolarization was analyzed by flow cytometry after JC-1 staining. Results represent one of three independent experiments.

Vav overexpression rescues Jurkat cells from CD4-mediated mitochondrial damage and apoptosis by decreasing Bax expression

Vav is an intracellular signaling molecule involved in the regulation of TCR-initiated calcium signaling and gene transcription (18, 34) and in the control of actin cytoskeleton (19). We hypothesized that the apoptotic signals triggered by CD4-mediated up-regulation of Bax should be neutralized following Vav overexpression. Jurkat- and Vav-overexpressing (Vav CL9) cells were treated with Leu3a and apoptosis (Fig. 5) as well as Bax (Fig. 6*A*), and Bcl-2 (Fig. 6*B*) expressions were analyzed. As reported in Fig. 5*A*, Vav overexpression reduced the percentage of apoptotic cells induced by Leu3a treatment. The inhibition of Leu3a-induced apoptosis in Vav-overexpressing cells was also associated with reduced mitochondrial damage (data not shown). Fig. 5*B* shows the expression of Vav-*myc* in Vav CL9 cells. We next examined Bax and Bcl-2 expression by FACS analysis. As shown in Fig. 6*A*, Jurkat cells expressed a basal level of Bax (mean fluorescence intensity (MFI), 9.8) that was significantly increased following Leu3a treatment (MFI, 20.4). Vav overexpression significantly inhibited CD4/Lck-induced Bax up-regulation (MFI of untreated cells = 8.9 vs MFI of Leu3a-treated cells = 12.4). Calling δ_1 the percentage of Bax

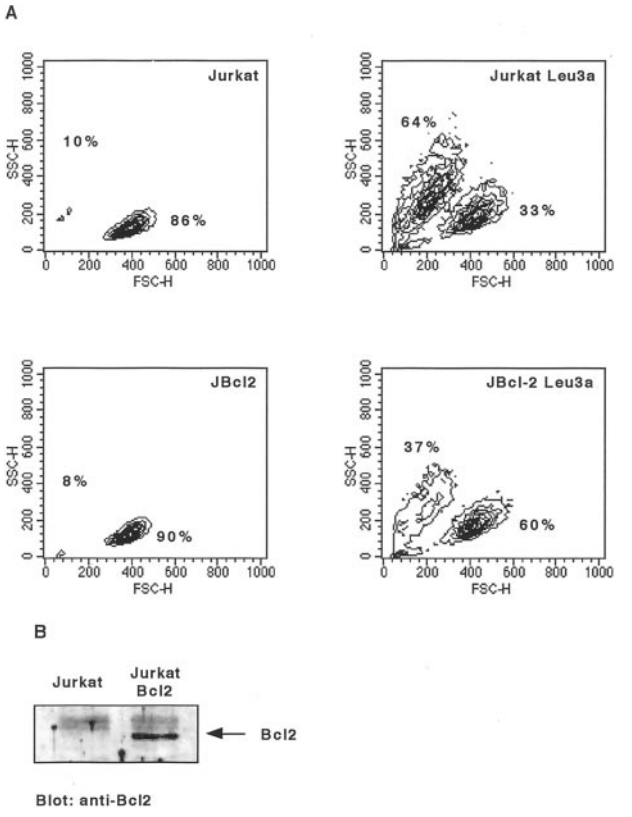

FIGURE 4. Bcl-2 overexpression protects Jurkat cells from Leu3a-induced apoptosis. *A*, FACS analysis of Jurkat cells or Bcl-2-overexpressing Jurkat cells (JBcl2) cultured for 48 h in the presence or absence of Leu3a. The percentage of apoptotic cells is indicated in the squares. Data are representative of three independent experiments. *B*, Bcl-2 expression was analyzed by Western blotting in both Jurkat cells and in Jurkat cells stably transfected with Bcl-2 vector (JBcl2).

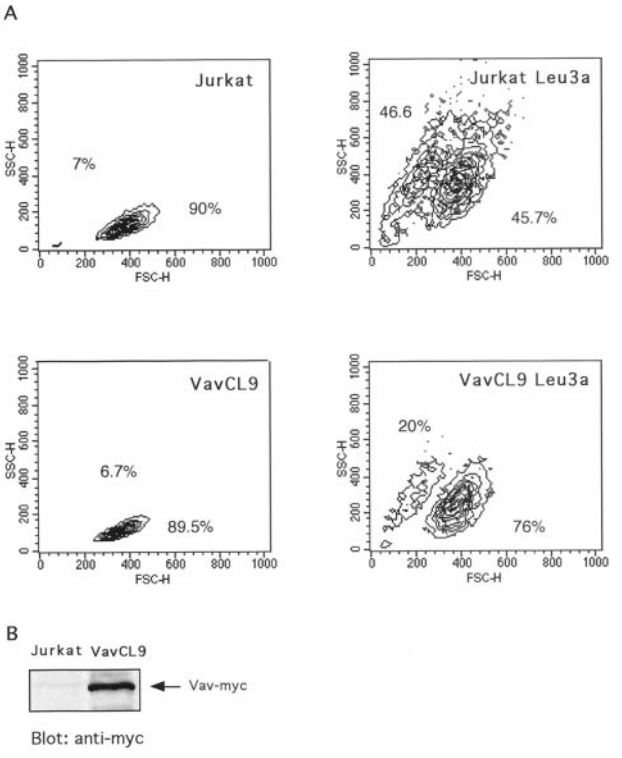

FIGURE 5. Vav exchange factor counteracts Leu3a-mediated signals inducing apoptosis and mitochondrial damage. *A*, Jurkat cells and Jurkat-stable cells transfected to overexpress Vav (Vav CL9) were incubated for 48 h with Leu3a. At the end of incubation, apoptosis was analyzed by flow cytometry. Data are representative of three independent experiments. *B*, Jurkat cells and Vav CL9 cells were lysed and *myc*-tagged Vav expression was revealed by Western blotting using anti-*myc*-specific mAb.

increase observed in Jurkat cells with respect to basal level (mean ± SD, 47.5 ± 15) and δ_2 the percentage of Bax increase observed in Vav CL9 (mean ± SD, 15.5 ± 8), δ_2 appeared to be systematically lower. Indeed, the reduction factor ($r = 1 - (\delta_2/\delta_1)$) on three independent experiments was 0.68. Similar to that observed in Jurkat cells, Leu3a did not affect Bcl-2 levels in Vav CL9 cells (Fig. 6*B*). Our evidence that Vav, a signaling molecule that cooperates with CD28 to boost TCR signals (20), counteracts CD4/Lck-mediated apoptotic signals indicates that the activation of Lck in the absence of costimulatory signals may activate in T cells apoptotic programs.

Discussion

T lymphocytes display a remarkable ability to evaluate differences in MHC-peptide complexes and altered peptide ligands. Agonist, weak agonist, or antagonist can have significant effects on T cells. It has been described that distinct early TCR-mediated signaling events can be transduced by such altered peptide ligands (35, 36), although the consequent biological effects can be significantly distinct. More recently, a correlation between the $t_{1/2}$ of the TCR-MHC-peptide interaction and the number of MHC-peptide complexes present in the immunological synapse has been found (8). The recruitment of $p56^{lck}$ to the engaged TCR by CD4 is a process envisaged to influence this kinetic discrimination (36). Thus, if CD4 plays a critical role in very early stages of immunological synapse formation and $t_{1/2}$ of the TCR-ligand interaction is the critical parameter regulating this role, CD4 could also represent a stop signal mediating the elimination of T cells incorrectly activated by altered peptides. The goal of this study was to demonstrate that CD4 represents a critical turning point that governs the life or the death of T cells.

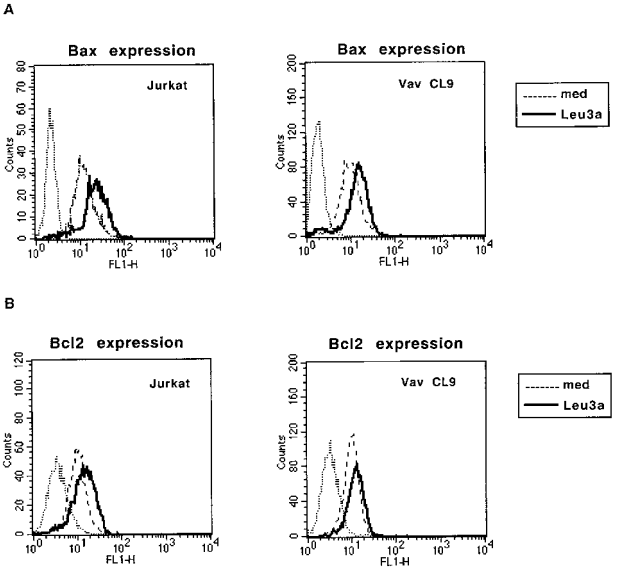

FIGURE 6. Vav overexpression blocks Leu3a-induced Bax up-regulation without affecting Bcl-2 levels. Jurkat cells and Vav CL9 cells were treated for 48 h with Leu3a and Bax expression (*A*) or Bcl-2 expression (*B*) were evaluated by FACS analysis using specific Abs. The results shown are representative of three independent experiments.

It is clear that when Th cells recognize the peptide-MHC complexes through their TCRs, CD4 binds to an Ag-independent region of the MHC (37). These interactions result in the tyrosine phosphorylation of the TCR-associated CD3 γ-, δ-, ϵ-, and ζ-chains, an event that precedes all cellular changes accompanying T cell activation. The earliest step in this process is the activation of the Src and Syk families of tyrosine kinases, such as Lck and Zap-70, respectively (3). In $CD4^+$ T cells, ~75–95% of cellular Lck associates with the cytoplasmic tail of CD4, involving ~85–95% of CD4 (38). Thus, CD4 cross-linking results in the activation of Lck kinase activity and, even in the absence of TCR engagement, supports the initiation and propagation of TCR signaling cascade (30). It is interesting to note that all of this machinery, necessary to lead to IL-2 production and to cellular growth, is also required for delivering apoptotic signaling. Indeed, our data show that Lck activity as well as TCR/CD3 expression and tyrosine phosphorylation events are necessary for inducing T cell suicide.

That CD4 signaling induces apoptosis in T cells has been known since 1990 (39). However, the large amount of evidence demonstrating that CD4 regulates apoptosis has been obtained after separate ligation of CD4 and TCR (11, 40–43). This has been a good approach to analyze the effects of CD4 antagonist ligands such as anti-CD4 Abs (44) or HIV gp120 (45, 46). Indeed, this approach has provided useful information to control autoimmune diseases and organ graft rejection and to explain $CD4^+$ T cell deletion in AIDS. However, it does not resemble the physiological role of CD4 following Ag recognition. We reasoned on the possibility that an incorrect activation signal, such as that mediated by altered peptide ligands, must be repaired through the activation of cell suicide. It has been reported that partial agonist or antagonist ligands induce a distant biochemical profile characterized by partial ζ-chain phosphorylation without activation of the associated ZAP-70 kinase (47, 48). The CD4-Lck activation in the absence of a concomitant increase in ZAP-70 phosphorylation has been considered a dominant-negative signal (49). Our evidence that Lck kinase activity is strongly induced by CD4 engagement in the absence of TCR/CD3-mediated phosphorylation events (data not shown) suggests that our system can mimic a partial activation signal.

The duration of antigenic stimulation determines the fate of naive T cells and costimulation via CD28 facilitates T cell activation by decreasing the time of commitment and by protecting T lymphocytes from cell death (50). Ag-stimulated Jurkat T cells in the absence of CD28/B7 interaction present a strong inhibition of tyrosine phosphorylation of ζ-chain and ZAP-70, confirming the need of CD28 in the earliest events of tyrosine phosphorylation in these cells (51). CD28 engagement in the same cells activates Vav to boost TCR signaling and to enhance TCR proximal signaling (20). Moreover, recent data in vivo demonstrated that Vav is an important regulator of both TCR proximal signaling events (18) and cytoskeleton rearrangements (19). In this context, Jurkat cells overexpressing Vav display cellular processes identifiable as typical lamellipodia and microspikes (20). These findings suggest that activating signals such as those mediated by the engagement of TCR/CD3 with peptide-MHC and CD4 with MHC in the absence of CD28 can represent an obstacle in the process of immunological synapse formation, and consequently the cells may be prone to apoptosize. If this obstacle is overcome by the recruitment of costimulatory signals and active cytoskeletal organization forms a scaffold for signaling components (1), the cells are rescued and the events for cell proliferation occur. Our evidence that Vav overexpression rescues T cells from the CD4-activated apoptotic pathway supports the role of cytoskeletal organization in mediating T cell commitment (1) and suggests that Vav may represent a critical element in counteracting the activation of apoptotic pathways.

It has been proposed that Bcl-2 family members regulate apoptosis like a rheostat (52). If proapoptotic factors such as Bax prevail, they form homodimers and cell death ensues. In contrast, cell survival is ensured when antiapoptotic factors such as Bcl-2 are more abundant, eventually leading to the formation of Bcl-2/Bax heterodimers (53). It has been reported that Bax, when overexpressed, translocates to the mitochondria and produces cell death (54). Activation and oligomerization of Bax results in formation of a homomultimeric pore to initiate cytochrome *c* release (55), an event that is accompanied by typical features of apoptosis. More recently, the role of Bax, as an essential gateway to mitochondria dysfunction, has been described in vivo (56). Very little is known about the molecular mechanisms that control mitochondrion-dependent apoptosis in T cells after TCR triggering. A described physiological mechanism is the absence of costimulatory signals that influence the expression of the anti-apoptotic protein $Bcl\text{-}x_L$ (57). No data are available on the possibility that other physiological mechanisms could be involved. Our evidence that in T lymphocytes CD4-Lck through TCR up-regulates Bax and induces mitochondria dysfunction, defines a new mechanism. Moreover, the observation that Vav overexpression blocks the execution of apoptotic programs by interfering with CD4-Lck-mediated signaling that leads to Bax up-regulation suggests a novel role for costimulation in protecting T cells from cell death.

In conclusion, our studies have identified the molecular mechanisms that allow CD4 to cooperate with TCR/CD3 in mediating apoptotic signals and suggest a new critical role of Vav in mediating survival signals.

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