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Resistance of ex vivo expanded $CD3^+CD56^+$ T cells to Fas-mediated apoptosis

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Abstract A variety of malignancies express Fas ligand (FasL), which can induce apoptosis in effector lymphocytes and may limit the success of cellular immunotherapy. Our laboratory has been investigating a population of ex vivo activated T cells, termed cytokine-induced killer (CIK) cells. These cells share functional and phenotypic properties with natural killer cells and a subset of cytolytic cells have the phenotype $CD3^+CD56^+$. CIK cells expand in culture, have significant antitumor activity and are presently being tested in phase I/II clinical trials. In this study, we investigated the sensitivity of CIK cells to Fas-mediated apoptosis. Fas engagement leads to apoptosis in small numbers of CIK cells and does not significantly influence antitumor cytotoxicity. CIK cells will undergo apoptosis following Fas engagement when protein synthesis is inhibited, suggesting the expression of antiapoptotic genes. Evaluation of antiapoptotic gene transcripts shows an up-regulation in the expression of cFLIP, Bcl-2, Bcl-xL, DAD1 and survivin. Resistance to Fas-mediated apoptosis may come about through an in vitro selection for Fas resistance, since CIK cells synthesize FasL and supernatant from CIK cultures contains biologically active soluble FasL, which can be inhibited with Fas:Fc. These results indicate that CIK cells are a suitable form of immunotherapy against FasL-positive tumors.

Key words Immunotherapy · CD95 · Lymphocyte activation · Apoptosis · Gene expression

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Introduction

Escape from immune detection is thought to be critical in the development of malignancies. Consistent with this, tumor cells synthesize a variety of both secreted and cell-surface proteins that may undermine the endogenous immune response. For instance, malignant cells have been shown to secrete vascular endothelial growth factor, transforming growth factor β, and interleukin-10, which may inhibit T cell growth and cytokine production, and diminish the cytotoxic potential of effector cells [13]. In addition, neoplasms may reduce their immunogenicity by the down-regulation of surface molecules involved in cellular adhesion and antigen presentation [13]. Recently it has been recognized that some tumor cells express Fas ligand (FasL), which can induce apoptosis in tumor-infiltrating lymphocytes (TIL) [4, 43, 64], acting as yet another mechanism of immune escape.

The Fas/FasL system was first appreciated in 1989 when Yonehara generated a monoclonal antibody that could rapidly induce apoptosis in certain cell lines [68]. This antibody was directed against the Fas receptor (Fas, CD95) and further work established that cross-linking of Fas by its natural ligand (FasL) also induces apoptosis [59]. FasL belongs to the tumor necrosis factor superfamily [60] and is a type II membrane protein that exists as either a membrane-bound molecule or in a soluble form after proteolytic processing by metalloproteases [26, 62]. In the peripheral immune compartment, the Fas/FasL system has at least two distinct physiological roles. First, along with granzyme/perforin, FasL is one of the major effector mechanisms used by activated T cells and natural killer (NK) cells to kill tumor targets [25]. Second, the expression of Fas and FasL is essential for the regulation of immune responses, playing a pivotal role in the dampening of an immune response [34]. Upon stimulation of the CD3/TCR complex, resting peripheral blood T cells become activated and up-regulate both the Fas receptor and FasL

[30]. Despite this, they do not undergo Fas-mediated apoptosis in the early stages of an immune response [34]. After a period of either chronic stimulation or reactivation through the T cell receptor (TCR), T cells become susceptible to Fas crosslinking and undergo apoptosis in either an autocrine or a paracrine fashion [2, 5, 12, 24, 45]. Such a system allows for rapid T cell expansion in the first phase of an immune response and the subsequent reduction once the offending agent is eliminated [34]. While it was originally thought that the expression of FasL was limited to lymphocytes [59] it is now clear that other cells express this molecule, including malignant cells.

FasL is variably expressed on multiple malignancies including melanoma, ovarian carcinoma, lung carcinoma, colon adenocarcinoma, astrocytoma, glioma, leukemia and lymphoma [4, 14, 39, 41, 42, 46, 49, 57, 58, 63, 67]. O'Connell and coworkers have determined that FasL present on the colon adenocarcinoma cell SW620 is functional, inducing apoptosis in the Jurkat T cell line [42]. Given these findings, they proposed the concept of a "Fas counterattack" where malignant cells use FasL to eliminate tumor-reactive lymphocytes. This same group has recently evaluated a series of human esophageal malignancies and found detectable FasL in each sample tested. These tumors had variable expression of FasL in different histological sections of the tumor. In the areas where FasL expression was high there was a reduction in the number of tumor-infiltrating lymphocytes and an increase in lymphocyte apoptosis, supporting the concept that tumor cell FasL can impede an incoming immune response [4]. Few studies have correlated FasL expression by tumor cells with clinical outcome, but there is some suggestion that clinically aggressive tumors, such as those derived from the brain and pancreas, frequently express FasL [19, 63]. Furthermore, FasL may play a role in the metastatic progression of tumors since, in a small series by Shiraki et al., only two of seven primary colon adenocarcinoma lesions had detectable FasL while four of four metastatic samples were positive for FasL [58]. Taken together, these results suggest that tumor cell expression of FasL may aid in the evasion of an effective immune response, code for a more aggressive disease and facilitate the development of metastases.

Our laboratory has characterized a population of ex vivo activated T cells, that share phenotypic and functional properties with NK cells and have been termed cytokine-induced killer (CIK) cells. These activated T cells are generated by the timed addition of interferon γ (IFNγ), OKT-3 and interleukin-2 (IL-2) [53–55]. They attain the maximal antitumor response after 21–28 days in culture and the cells with the most antitumor activity co-express the NK cell marker CD56 (i.e. $CD3^+CD56^+$) [33, 54]. Without prior exposure to tumor cells, CIK cells display TCR-independent cytotoxicity against a variety of tumor cell lines [33, 38, 54, 56], using mainly a perforin-based mechanism (unpublished findings). Using a severe combined immunodeficiency (SCID) mouse/human lymphoma model, we have previously determined that CIK cells were superior to lymphokine-activated killer (LAK) cells in the eradication of human lymphoma [33]. Unlike LAK cells, which have poor in vitro expansion, CIK cells proliferate to yield large numbers of effector cells in culture [33]. Furthermore, CIK cells can be generated from patients with chronic myelogenous leukemia and are also able to eradicate autologous leukemia or autologous Epstein-Barr-virus-transformed B cells engrafted in SCID mice [21]. The potent antitumor activity and expandability of CIK cells make them an attractive candidate cell population for immunotherapy. Knowing that tumor cells may express FasL and that activated lymphocytes are commonly sensitive to Fas-mediated apoptosis, we investigated the susceptibility of CIK cells to Fas-mediated apoptosis. In this report we show that T cells activated under these culture conditions are resistant to Fas-mediated apoptosis.

Materials and methods

Generation of CIK cells and cell culture

CIK cells were generated as previously described [33]; briefly, mononuclear cells were isolated from healthy donors by Ficoll-Hypaque density centrifugation and washed three times with phosphate-buffered saline (PBS). The final product was resuspended at 2×10^6 cells/ml in complete RPMI medium (cRPMI) consisting of 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml 2-mercaptoethanol at 37 °C, 5% CO_2. On day 0, cells were activated with IFNγ (1000 U/ml, Genentech, South San Francisco, Calif.) and the following day cells were stimulated with OKT-3 (25 ng/ml; OrthoBioTech, Raritan, N.J.) and recombinant (r) IL-2 (300 U/ml, Chiron, Emmeryville, Calif.). Thereafter, cells were stimulated with rIL-2 (300 U/ml) every 3–5 days and fresh medium was added to maintain a cell density of $(1.5–2) \times 10^6$/ml for a total of 28 days.

The T cell leukemia and B cell lymphoma cell lines, Jurkat and SU-DHL4 respectively, were maintained in cRPMI. The colon adenocarcinoma cell lines HT-29 and SW-620, were grown in complete Dulbecco's modified Eagle's medium consisting of 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml 2-mercaptoethanol.

Flow cytometry

CIK cells were stained with mAb against CD3, CD16 coupled with fluorescein isothiocyanate (FITC) and mAb against CD56, CD95 coupled with phycoerythin (PE) (Becton Dickinson, San Jose, Calif.). Samples containing 1×10^6 cells were stained with the above antibodies or isotype controls for 15 min at 4 °C. Excess antibody was removed and cells were evaluated by fluorescence-activated cell sorting (FACS) analysis at the Stanford Shared FACS Facility (Stanford University Medical School), in a FASCscan instrument (Becton Dickinson, San Jose, Calif.). Data was analyzed by FlowJo software (v 2.5.1, Tree Star Inc., San Carlos, Calif.).

Apoptosis assays

CIK cells or Jurkat T cells were subjected to Ficoll-Hypaque density centrifugation to remove nonviable cells. Cells were washed with PBS, resuspended at 1×10^6 cells/ml and cultured for 24 h before further manipulation. The Fas receptor antibody, CH11 (1 μg/ml; Immunotech, France), was added to cultures and incubated for 18 h at 37 °C, 5% CO_2 before evaluation for apoptosis. In protein synthesis inhibition experiments, CIK cells were treated as above and

incubated in either the presence or absence of cyclohexamide (1–1.5 μg/ml for 18 h), with or without CH11 (1 μg/ml).

For evaluation of apoptosis, cells were stained with FITC-labeled annexin (Apoptosis Detection Kit, R&D Systems, Minneapolis, Minn.) and propidium iodide according to the manufacturer's recommendations prior to FACS analysis. Alternatively, cells were stained with Apo2.7 (Immunotech, France). Briefly, 1×10^6 cells were permeabilized with digitonin (100 μg/ml) in PBS on ice for 20 min, washed once, resuspended in PBS and stained with the PE-conjugated Apo2.7 antibody for 15 min. Excess antibody was removed and cells were resuspended in PBS and analyzed by FACS. Results of apoptosis assays were expressed as the percentage specific apoptosis by the following formula:

Specific apoptosis (%) = [(experimental apoptosis) − (spontaneous apopotosis)]/(100 − spontaneous apoptosis).

Co-culture with FasL-transduced myoblasts

Either CIK cells or Jurkat cells were co-cultured with murine myoblasts transfected with murine FasL or empty vector as previously described (a generous gift from Helen Blau) [20]. Briefly, 2×10^5 myoblasts, expressing or not expressing FasL, were plated in 35-mm culture dishes. Approximately 3 h later and when the myoblasts were adherent, the medium was removed and either CIK cells or Jurkat T cells (1×10^5) were combined with the myoblasts for 4 h in cRPMI at 37 °C, 5% CO_2. The non-adherent cells (Jurkat or CIK cells) were retrieved and assayed for apoptosis using annexin V and propidium iodide as described above.

^{51}Cr-release assay

Tumor targets were labeled with ^{51}Cr (Dupont-NEN, Boston, Mass.) by incubating 1×10^6 cells in 300 μCi ^{51}Cr for 1–2 h at 37 °C, 5% CO_2. The labeled cells were washed three times with PBS and plated in 96-well plates at a concentration of 1×10^4 cells/ml in triplicate. When adherent adenocarcinoma cells were used, targets were added to 96-well flat-bottom plates and allowed to adhere for 30–60 min prior to the addition of effector cells. Effector cells were added at specified ratios (either 10:1 or 40:1) and incubated for 4 h at 37 °C, 5% CO_2. In some experiments the Fas-stimulating antibody CH11 (final concentration, 1 μg/ml) was added immediately after effector cells had been combined with tumor target cells. At the completion of the assay, plates were centrifuged and the supernatant was counted using a gamma counter. The percentage specific ^{51}Cr lysis was calculated from the following equation:

Specific lysis (%) = 100 × [(test release) − (spontaneous release)]/[(maximal release) − (spontaneous release)].

CIK supernatant experiments

Supernatant from CIK cell cultures was harvested at day 28 of culture. To remove cell fragments or debris, supernatant was centrifuged at 100 *g* and stored at 4 °C. Jurkat cells were subjected to Ficoll centrifugation (as above) to remove nonviable cells, resuspended at 0.3×10^6 cells/ml in cRPMI and cultured for 24 h before further evaluation. These cells were washed once, resuspended in either cRPMI or CIK supernant in the presence or absence of Fas:Fc (5 μg/ml; R&D Systems, Minneapolis, Minn.) or mouse IgG2bκ (5 μg/ml; Sigma) for 48 h, and analyzed for apoptosis by FACS staining with annexin V and propidium iodide as described above.

RNase protection assay

On various days of culture, 2×10^7 cells were collected for RNA isolation using the RNeasy Kit (Qiagen, Chatsworth, Calif.). An RNase protection assay was performed by using 2.5 μg total RNA and the RiboQuant multiprobe ribonuclease protection assay (Pharmingen, San Diego, Calif.). The RNase protection assay was performed according to the manufacturer's specifications and the template sets Apo2, -3 and -4 were used.

Reverse transcription/polymerase chain reaction (RT-PCR)

For each assay time, 1 μg total RNA was isolated by using the RNeasy Mini Kit (Qiagen, Santa Clarita, Calif.) and transcribed with Superscript II RNase H$^-$ reverse transcriptase (Gibco BRL, Gaithersburg, Md.) using random hexamers. A 0.5-μl cDNA sample was amplified with recombinant Taq polymerase (Gibco BRL) for 35 cycles. The following primers and internal probes were used: (1) GAPDH-For, 5′-GTGAAGGTCGGAGTCAACG-3′ and GADPH-Rev, 5′-GGTGAAGACGCCAGTGGACTC-3′ (2) survivin-For, 5′-AGGACCACCGCATCTCTAC-3′, Survivin-Rev, 5′-ACTTTCTTCGCAGTTTCCTC-3′ and 5′-CACTGCCCCAC-TGAGAAC-3′ (internal probe) (3) Fas-receptor-For, 5′-ATGCT-GGGCATCTGGACCCT-3′, Fas-receptor-Rev, 5′-TCTAGACC-AAGCTTTGGATTTC-3′ and 5′-GGATTTAAGGTTGGATT-CA-3′ (internal probe) and (4) cFLIP-For, 5′-GGGAGAAGT-AAAGAACAAAG,-3′ cFLIP-Rev, 5′-CTGAGGCACAATCA-CAGCAT-3′ and 5′-GAGACACCTTCACTTCCCTTG-3′ (internal probe). PCR products were separated on 2% agarose, blotted to Hybond N+ membrane (Amersham, Arlington Height, Ill.), probed with the ^{32}P-end-labeled internal oligonucleotide and exposed to Biomax MR (Kodak, Rochester, N.Y.) overnight.

Results

Cytokine-induced killer cells express increasing levels of Fas receptor while in culture

The culture conditions used to generate CIK cells involve the sequential addition of INFγ, OKT-3 and IL-2. Since each of these reagents is known to stimulate T cells and may sensitize lymphocytes to undergo apoptosis [30, 32, 47], we evaluated the mRNA expression of the Fas receptor over time in CIK cultures. There was a steady increase in the mRNA expression of the Fas receptor over the first 21 days of culture (Fig. 1A). This increase correlated with the cell-surface expression of the Fas receptor as measured by FACS analysis (Fig. 1B, C).

CIK cells are resistant to Fas-mediated apoptosis

Given the increased expression of the Fas receptor over time, we evaluated the sensitivity of CIK cells to undergo Fas-mediated apoptosis. At the initiation of cultures, CIK cells have little or no cytotoxicity, but by days 21–28 they recognize a variety of tumor targets without prior exposure [33, 38, 54, 55]. Since CIK cells would be most clinically useful on days 21–28, they were tested for sensitivity to Fas crosslinking at this time. Either CIK cells or Jurkat cells were treated with the anti-Fas antibody, CH11 (1 μg/ml) and apoptosis was measured by annexin V and propidium iodide. Treatment of the Jurkat cells with CH11 yielded a significant increase in the percentage specific apoptosis. This was in contrast to low numbers of CIK cells (typically less than

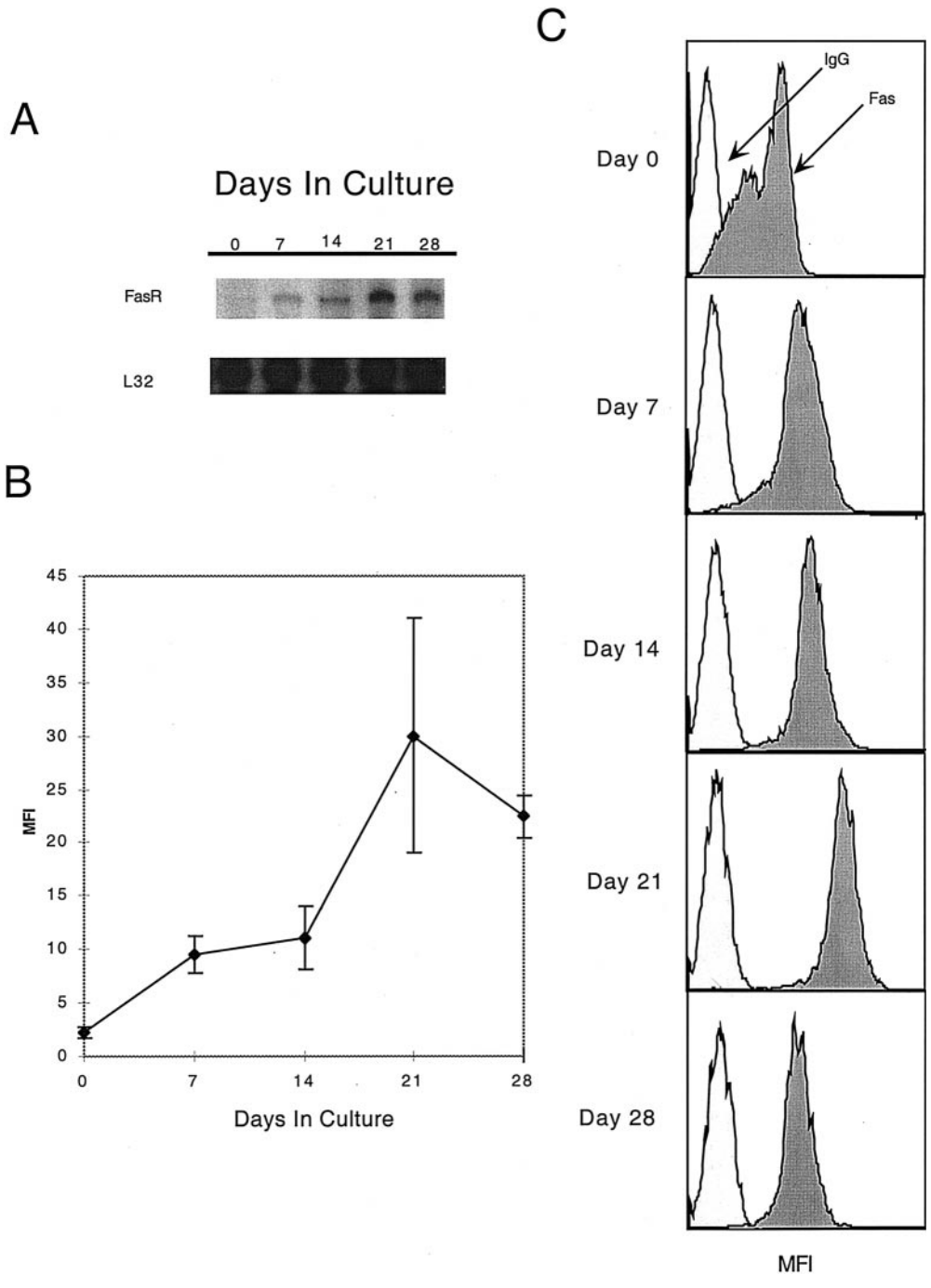

Fig. 1A–C Cytokine-induced killer (CIK) cells up-regulate the Fas receptor as they become activated. **A** Peripheral blood lymphocytes were grown under CIK conditions and total RNA was harvested on days 0, 7, 14, 21 and 28 for RNase protection assay, probing for the Fas receptor. Data are representative of three individual experiments using separate donors. **B** Over the same period of time, cell-surface expression of the Fas receptor was determined by fluorescence-activated cell sorting (FACS) analysis. Data represent average mean fluorescent intensity ± the standard deviation and are derived from nine separate CIK cultures. **C** FACS histogram demonstrating the uniform up-regulation of the Fas receptor on all cells in CIK cultures. Data are representative of three separate experiments using different lymphocyte donors. *MFI* mean fluorescence index

15%) that underwent CH11-induced apoptosis (Fig. 2A). Apoptosis was confirmed using the Apo2.7 antibody, which recognizes a mitochondrial membrane protein exposed during apoptosis [72] (data not shown).

Since membrane-bound FasL may be more physiologically relevant, coculture experiments were performed with murine myoblasts engineered to express high levels of murine FasL, as previously described [20]. Murine cell lines were chosen for this assay since human CIK cells do not lyse murine targets (unpublished observations). Conversely, murine FasL does interact with the human Fas receptor and induces apoptosis in human cell lines [20]. Therefore this assay allows us to examine the effect of membrane-bound FasL on target cells without any confounding apoptosis induced by CIK cells. Figure 2B shows, that while slightly higher levels of apoptosis were obtained in both the CIK cells and the Jurkat cells, the majority of CIK cells remained viable at the end of the culture period, suggesting that the CH11 antibody accurately reflects membrane-bound FasL in this system.

Previous work from our laboratory has shown that CIK cells are a heterogeneous population of activated T cells and that the cells with the most antitumor activity coexpress CD3 and the NK cell-surface marker CD56

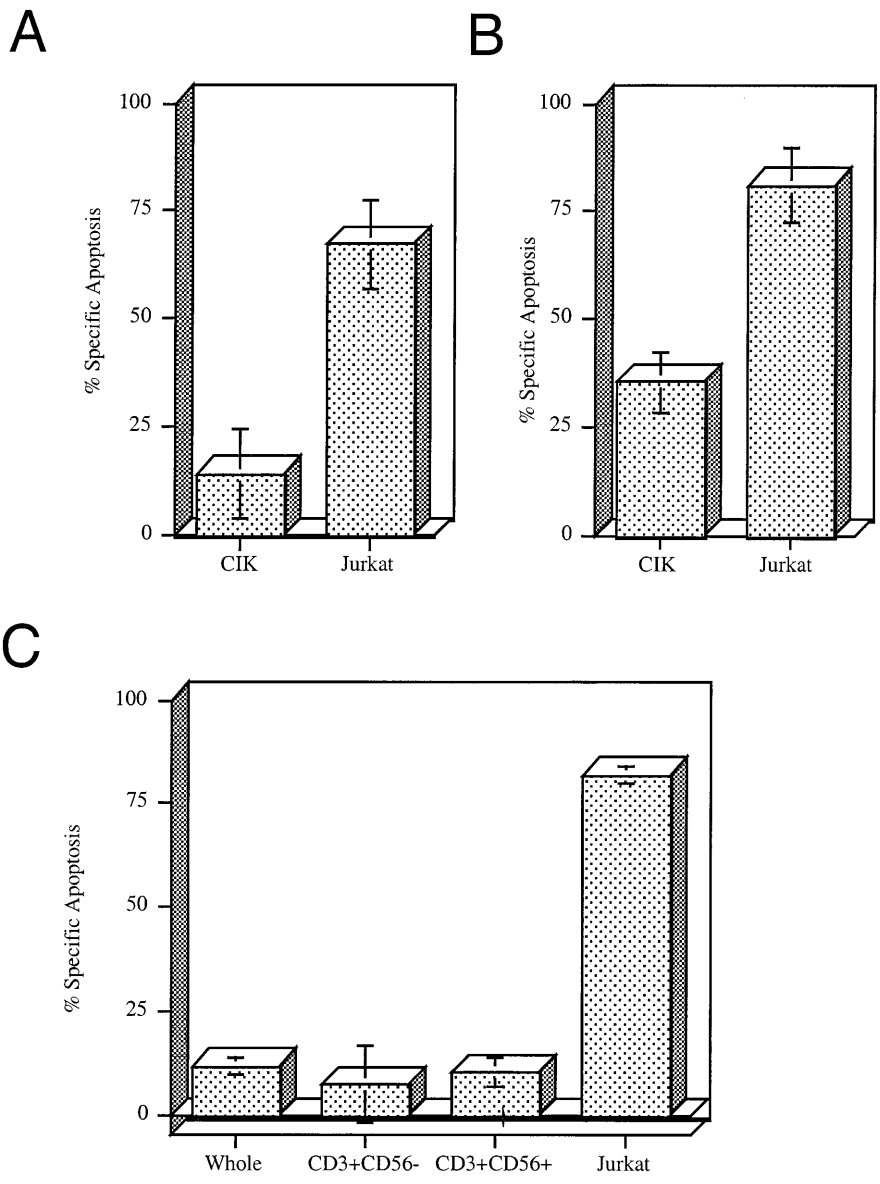

Fig. 2A–C Fas engagement induces apoptosis in small numbers of CIK cells. **A** CIK cells or Jurkat T cells were treated with the CH11 antibody (1 μg/ml) for 18 h and analyzed by annexin V and propidium iodide staining. Results are presented as the average specific apoptosis ± standard deviation and represent more than ten individual donors of CIK cells. **B** 1×10^5 CIK cells or Jurkat T cells were incubated with 2×10^5 murine myoblasts transduced with either murine FasL or empty vector (control) for 4 h and analyzed by FACS analysis for apoptosis using annexin V and propidium iodide. Specific apoptosis was calculated by using the empty vector value as baseline. Results are average specific apoptosis ± standard deviation of five separate CIK donors. **C** To investigate the sensitivity of $CD3^+CD56^+$ cells to Fas crosslinking, CIK cells were FACS sorted on day 28 of using fluorescein-isothiocyanate-labeled CD5 and phycoerythrin-labeled CD56 to achieve populations of cells ($CD3^+CD56^-$ and $CD3^+CD56^+$) that were more than 95% pure. These purified populations, unsorted CIK cell cultures and Jurkat cells were incubated with CH11 (1 μg/ml) and evaluated for apoptosis as above. The results shown are from two separate experiments and are presented as mean specific apoptosis ± standard deviation

[33, 54, 55]. $CD3^+CD56^+$ cells account for 1%–5% of the resting peripheral blood lymphocytes [44, 52]. When T cells are grown under CIK conditions, $CD3^+CD56^+$ cells expand up to 1000-fold and account for 10%–40% of the cells in culture [33]. Given that small numbers of CIK cells undergo Fas-mediated apoptosis (Fig. 2A, B) and that the $CD3^+CD56^+$ cells represent the minority of cells in culture, we investigated whether this population ($CD3^+CD56^+$) might be more or less likely to undergo Fas-mediated apoptosis. CIK cultures were FACS sorted to achieve pure populations (above 95%) of $CD3^+CD56^+$ and $CD3^+CD56^-$ cells, which were then treated with the CH11 antibody to induce apoptosis. Figure 2C shows that both the sorted populations ($CD3^+CD56^-$ or $CD3^+CD56^+$) and the unsorted CIK cells have a similar level of specific apoptosis after treatment with CH11. Therefore, $CD3^+CD56^+$ cells are overall resistant to Fas-mediated apoptosis but, like unsorted CIK cultures or $CD3^+CD56^-$ cells, small amounts of cells are sensitive to Fas receptor cross-linking.

Fas engagement does not inhibit antitumor cytotoxicity

To investigate the impact of Fas engagement on antitumor cytotoxicity, CIK cells were used in a standard ^{51}Cr-release assay in the presence or absence of exogenously added Fas-stimulating antibodies (CH11). To ensure that the CH11 antibody did not induce apoptosis in the tumor targets, we chose the SU-DHL4 cell line, which expresses the Fas receptor but is resistant to Fas-mediated apoptosis (unpublished observations). Figure 3A, shows that there was no change in the cytotoxicity of CIK cells when the Fas-stimulating

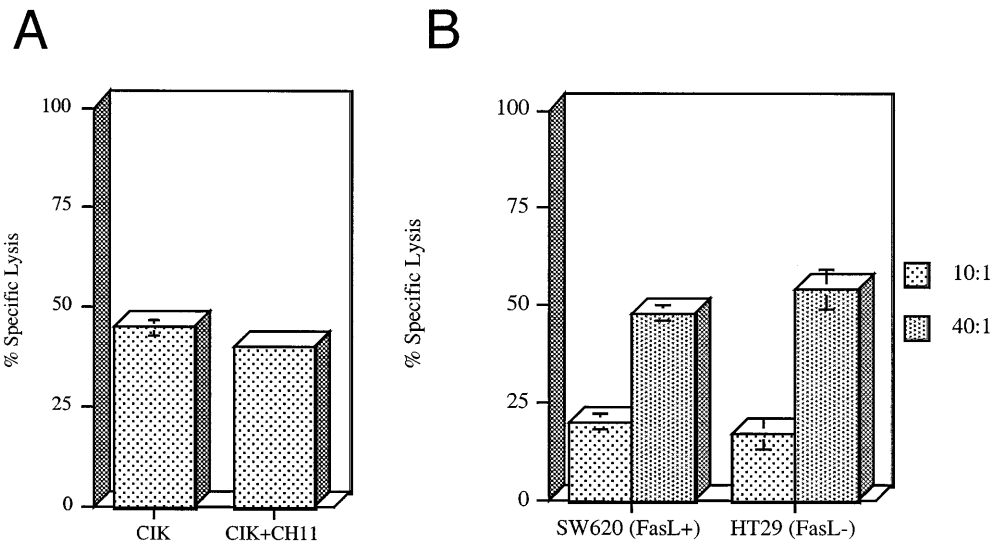

Fig. 3A, B Fas engagement does not effect antitumor cytotoxicity. **A** CIK cells were incubated with the SU-DHL4 tumor cell line in a standard ^{51}Cr-release assay (E:T ratio = 10:1), in the presence or absence of CH11 (1 μg/ml) or control antibody (1 μg/ml, not shown). **B** CIK cells were incubated with the colon adenocarcinoma cell lines SW-620 and HT29 (FasL$^+$ and FasL$^-$ respectively) with a standard ^{51}Cr-release assay (E:T ratio = 10:1 and 40:1). Results are mean specific lysis ± standard deviation of triplicates and are representative of three separate experiments

antibody (CH11) was added to the ^{51}Cr-release assay. To confirm further that FasL expression of tumor cells had no impact on tumor cytolysis by CIK cells, we used the colon adenocarcinoma cell lines SW620 and HT-29, which have previously been shown to express and not express FasL respectively [42, 58]. While there was some variability among donors, we found no significant difference in the CIK cell killing of either FasL$^+$ or FasL$^-$ tumor targets (Fig. 3B). Since CIK cells mainly exert their antitumor activity through a granzyme/perforin pathway (Hope and Negrin, manuscript in preparation), these data suggest that Fas engagement does not effect granule exocytosis.

CIK cells express FasL and CIK supernant contains soluble FasL

Activation of T cells through the CD3/TCR complex leads to an up-regulation of FasL [30]. Since OKT-3 is used in the generation of CIK cells, we hypothesized that the expression of FasL may lead to the deletion of Fas-sensitive cells and allow for the selection of a population of lymphocytes resistant to Fas crosslinking. To characterize FasL expression over time in CIK cultures, RNA was collected at various times and analyzed by an RNase protection assay. At day 0, there was no detectable FasL expression, but over the course of 28 days a significant increase was detected (Fig. 4A).

CIK cells were also evaluated for the cell-surface expression of FasL. Small numbers (less than 5%) of FasL$^+$ cells were detected by FACS analysis (data not shown). Given the likely possibility that FasL was cleaved from the cell surface by metalloproteases [26, 49], we tested the ability of the CIK cell supernatant to induce apoptosis in the Jurkat cell line. Figure 4B shows that the majority of Jurkat cells undergo apoptosis when incubated with supernant derived from CIK cultures. This apoptosis-inducing activity was partially inhibited by a soluble, decoy Fas receptor (Fas:Fc fusion protein), confirming that soluble FasL is present and active in CIK supernatant and may drive the selection for Fas-resistant CIK cells.

Expression of mRNA species involved in signaling through the Fas receptor

Resistance to Fas-mediated apoptosis could be secondary to differential splicing of the Fas receptor. Such variants may code for soluble receptor [8] or a truncated intracellular signaling domain, which is ineffective in transmitting an internal signal [7]. Given this, we evaluated CIK cells for alternate splicing of the Fas receptor using RT-PCR and no new splice variants were detected (data not shown). A reduced expression of genes involved in downstream signaling of the Fas receptor could also explain the resistance to Fas-mediated apoptosis. To investigate this, total RNA was collected at various times during culture and analyzed by RNase protection assay. The expression of caspase-8 was readily detected at baseline and was present throughout the culture period, in contrast, Fas associated protein with death domain (FADD) was not detected at baseline or over the 28-day culture period. Occasionally FADD was detected late in culture or upon overexposure of the autoradiograph (not shown). Given these results, it is unlikely that changes in the expression of the Fas receptor or the proximal adapter molecules (caspase-8 and FADD) can account for the resistance to Fas-mediated apoptosis in these cells.

Active protein synthesis is required for the Fas-resistant phenotype in CIK cells

To explore further the resistance of CIK cells to Fas-meditated apoptosis we investigated whether active protein synthesis was necessary for the Fas-resistant

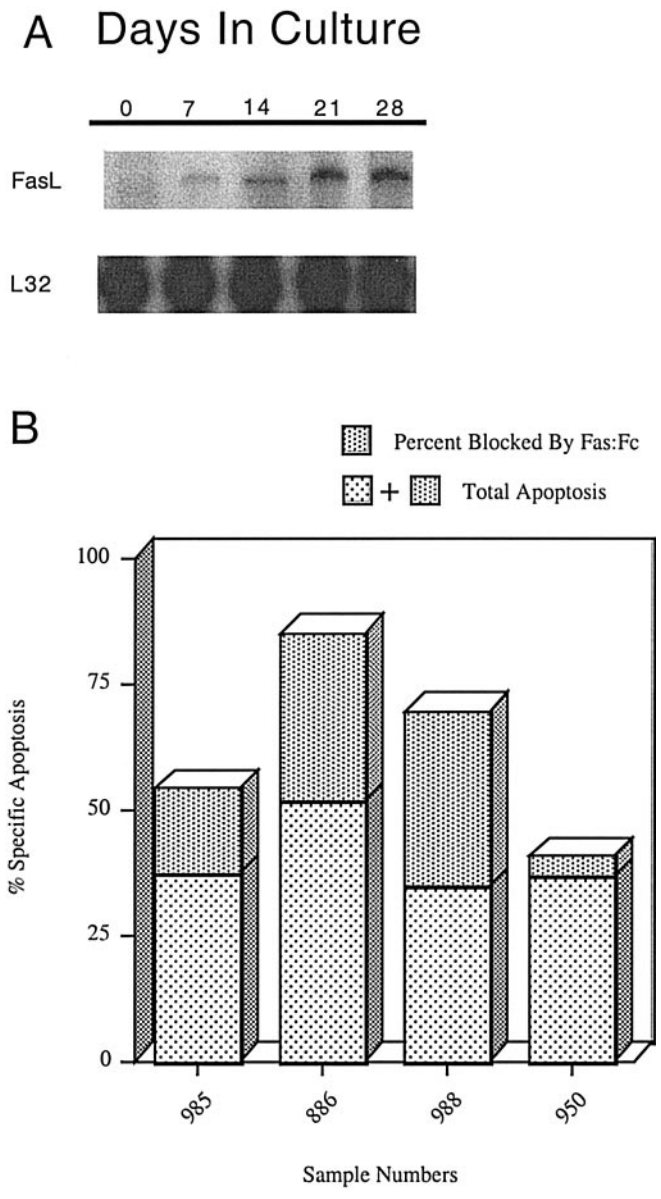

Fig. 4A, B CIK cells express FasL and CIK cell supernatant induces Fas-mediated apoptosis in Jurkat cells. **A** Total RNA was harvested from CIK cells at initiation of cultures and at weekly intervals for evaluation of FasL by RNase protection assay. **B** Jurkat cells were first treated with Ficoll to remove nonviable cells and incubated at 0.3×10^6 for 24 h. They were then washed with phosphate-buffered saline and incubated in complete RPMI medium or CIK supernant for 48 h in the presence or absence of Fas:Fc (5 μg/ml) or control antibody (5 μg/ml) (not shown)

phenotype. Figure 5 shows that, when CIK cells are incubated with either the protein synthesis inhibitor cyclohexamide or with the Fas-stimulating antibody CH11, there was a slight increase in the specific apoptosis (approximately 5% and 20% respectively). In contrast, the combination of CH11 and cyclohexamide resulted in a significant increase in the number of cells undergoing apoptosis (75%), suggesting that active protein synthesis is necessary for the Fas-resistant phenotype.

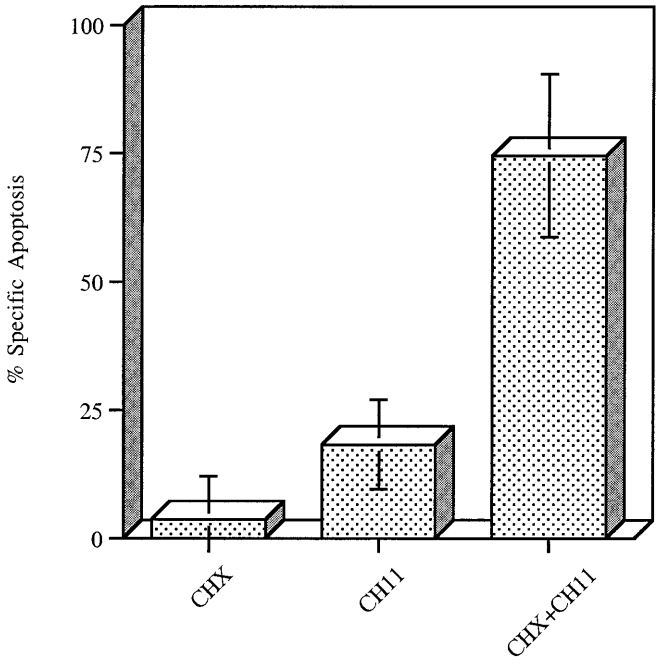

Fig. 5 Protein synthesis is required for resistance to Fas signaling in CIK cells. CIK cells were incubated for 18 h with cyclohexamide (*CHX*) alone (1–1.5 μg/ml), CH11 (1 μg/ml) or CHX + CH11 (1–1.5 μg/ml and 1 μg/ml respectively) and assayed for apoptosis with annexin V and propidium iodide. The results are mean specific apoptosis ± standard deviation from three separate experiments

The expression of antiapoptotic genes in CIK cells

Since CIK cells were sensitive to apoptosis in the presence of a protein synthesis inhibitor (cyclohexamide), we investigated the expression of genes coding for an antiapoptotic phenotype. Given the resistance to Fas crosslinking, we evaluated genes known to inhibit Fas signaling specifically. We first examined the recently cloned tyrosine phospatase, FAP. Overexpression of FAP protects cells from Fas-mediated apoptosis [51]. FAP expression was not detectable at baseline and was generally not found later in culture, suggesting that FAP is not responsible for the resistance to Fas-mediated apopotosis in CIK cells (Fig. 6A). In addition to FAP, we examined the expression of cFLIP, a protein known to inhibit Fas signal transduction by interfering with FADD/caspase-8 interactions [22]. A semi-quantitative RT-PCR showed a detectable cFLIP message at baseline, which increased between days 7 and 28 of culture (Fig. 6B).

In addition to inhibitors of the Fas signaling molecules, the expression of genes that code for a global resistance to apoptosis was examined. The well-characterized antiapoptotic genes encoding Bcl-2 and Bcl-X_L were both up-regulated over time in culture (Fig. 7A). We also tested for the expression of DAD-1, a novel protein that was cloned from an apoptosis-resistant temperature-sensitive mutant [27, 69]. While the mRNA was barely detectable at baseline, there was a clear up-regulation over time in CIK cultures (Fig. 7B). Lastly,

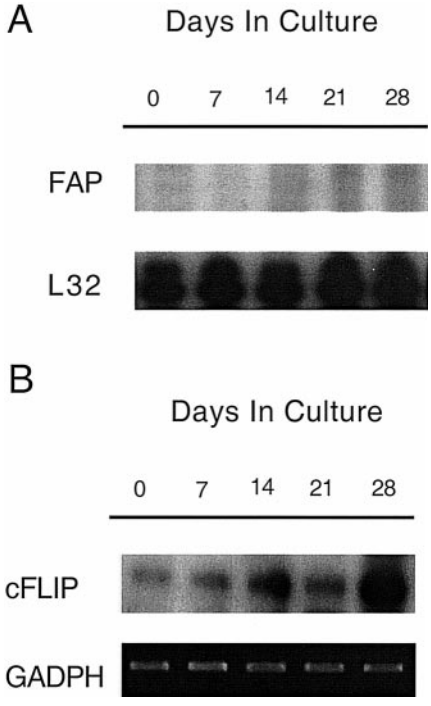

Fig. 6A, B CIK cells do not express FAP, but do express cFLIP. T cells were grown under CIK conditions and RNA was harvested at various times and analyzed for FAP and cFLIP by RNase protection assay and RT-PCR, respectively. RT-PCR was performed using cDNA that had been equalized by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression. The data are representative of two separate experiments each with different CIK cell donors

we assayed for the expression of survivin, a newly characterized protein in the baculovirus IAP apoptosis inhibitor family. While survivin was not detected at baseline, it was readily detectable throughout the 28 days of CIK culture (Fig. 7C).

Discussion

The expression of FasL by tumors raises the possibility that malignant cells could disable an immune response through Fas signaling. Supporting this, recent reports have documented that tumor-infiltrating lymphocytes may undergo Fas-mediated apoptosis when interacting with autologous tumors that express FasL [4, 43, 64] and tumor-specific cytotoxic T cells undergo apoptosis upon contact with FasL-expressing targets [71]. Moreover, many investigators have shown that a number of chemotheraputic agents may function, at least in part, through the up-regulation of FasL on the tumor cells [15, 17, 18]. Given that future immunotheraputic protocols will most likely call for cytoreduction with chemotherapy prior to immunotherapy, the expression of FasL by tumors and the resistance of immune effectors to FasL may be of critical importance.

Previous studies have shown that peripheral blood T cells are initially resistant to Fas-mediated apoptosis but

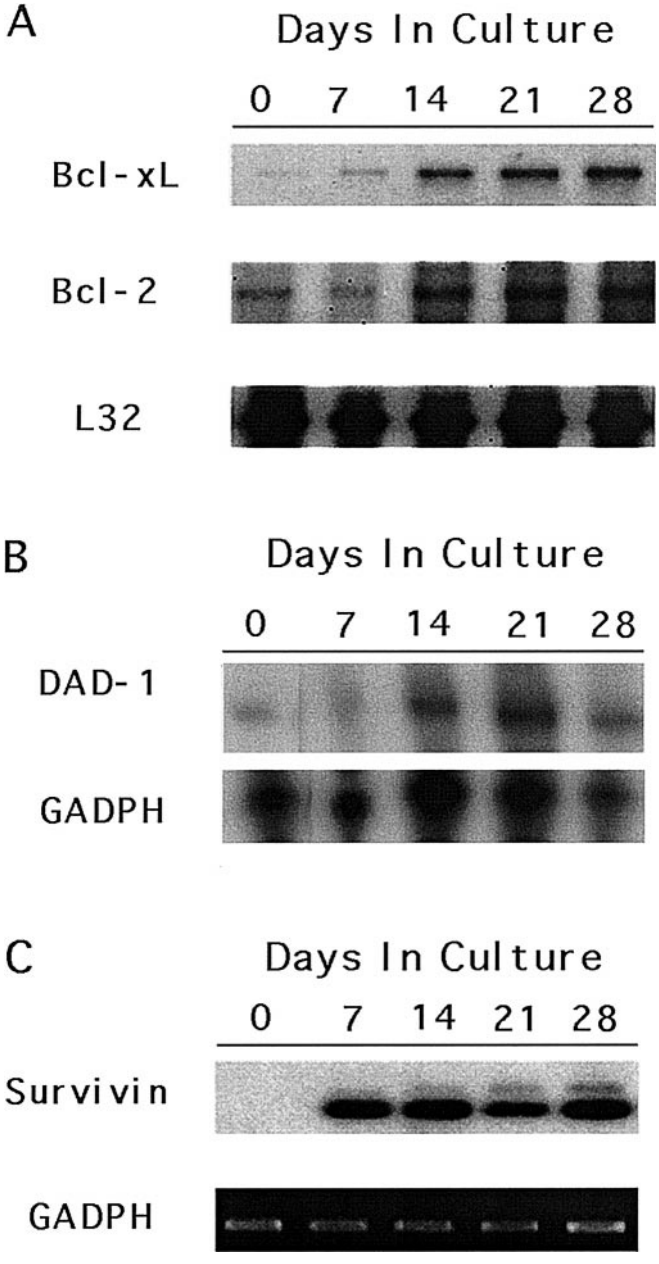

Fig. 7A–C CIK cell expression of antiapoptotic genes. **A, B** Samples were obtained at various times during CIK culture and probed for Bcl-xL, Bcl-2 and DAD1 expression using an RNase protection assay. **C** RT-PCR for survivin was performed using cDNA that had been equalized by GAPDH expression. Results are representative of two or more experiments

then become susceptible to Fas crosslinking after activation [28]. Given this, we set out to determine the sensitivity of peripheral blood lymphocytes (PBL) to Fas signaling after 21–28 days of activation under CIK culture conditions. Our results show that, over time in culture, CIK cells up-regulate both the Fas receptor and FasL. Despite this, the majority of cells are resistant to Fas receptor crosslinking at the time of maximal antitumor cytotoxicity (days 21–28). To generate CIK cells, PBL are stimulated with IFNγ, followed by OKT-3 and

IL-2. This combination of cytokines may select for a population of Fas-resistant cells. In support of this, IFNγ has been shown to increase the sensitivity of tumor cells to Fas-mediated apoptosis, converting Fas-resistant cells into Fas-sensitive ones [6, 48, 50]. In addition, multiple previous studies have addressed the topic of activation-induced cell death (AICD), where TCR stimulation of previously activated T cells results in the sensitization to Fas-mediated apoptosis [2, 35, 66, 74]. We hypothesize that, in CIK cultures, both Fas-sensitive and previously activated T cells found in peripheral blood are eliminated in the early stages of culture through either IFNγ, AICD or both. Moreover, the expression of either membrane-bound or proteolytically solublized FasL may exert continued selective pressure for Fas-resistant cells. Supernatant from CIK cultures is capable of inducing apoptosis in Jurkat cells, and this activity is partially attenuated by a decoy Fas receptor (Fas:Fc) (Fig. 4B). These results confirm that soluble FasL in CIK supernatant is biologically active, but also suggest that CIK cells secrete other molecules capable of inducing apoptosis. We have recently evaluated CIK cells for the expression of TNF-related apoptosis-inducing ligand (TRAIL) and found it to be up-regulated over time in CIK cultures (data not shown), and TRAIL has recently been shown to induce apoptosis in Jurkat cells [36]. Therefore, the expression of TRAIL or other members in the TNF superfamily may account for the activity of CIK supernant that is not inhibited by soluble Fas receptor.

While the selective pressure exerted by the culture conditions may explain the resistance of CIK cells to Fas-mediated apoptosis, it does not define the cellular mechanisms responsible for this phenotype. In this work we have demonstrated multiple points along the Fas signaling cascade that may be acting either independently or together to block Fas signal transduction and apoptosis. Fas signaling occurs after trimerization of the Fas receptor with either FasL or agonist antibodies. Trimerization of the Fas receptor allows for the recruitment of FADD and then caspase-8, thereby initiating apopotosis [9, 40]. Using an RNase protection assay, we found that CIK cells express abundant amounts of caspase-8, but that FADD was generally only detectable upon overexposure of the autoradiograph. The congenital absence of FADD ($FADD^{-/-}$ mouse) or truncated forms of FADD (lacking caspase-8-binding activity) have previously been shown to inhibit Fas-mediated apoptosis [10, 73]. While low levels of FADD may partially explain the resistance of CIK cells to Fas-mediated apoptosis, our results with cyclohexamide strongly suggest that active protein synthesis is necessary for the Fas-resistant phenotype in CIK cells (Fig. 5). Given this, we evaluated the gene transcription of proteins known to inhibit Fas signal transduction. Molecules that interfere with FADD/caspase-8 interactions, such as FAP and cFLIP, have been identified [22, 51]. While CIK cells do not express FAP, they do express cFLIP, identifying another point in the Fas signaling pathway where the transmission of an apoptotic signal may be blocked. We also found the expression of Bcl-2 and Bcl-xL mRNA also to be up-regulated over the culture period. These proteins are thought to inhibit apoptosis by the stabilization of the mitochondrial permeability transition pore complex [37, 70]. Further, it has recently been shown that Bcl-2 and Bcl-xL may protect from Fas-mediated apoptosis by directly interacting with proteins downstream of the Fas receptor (caspase-8) [11], but that, at least in some instances, the overexpression of Bcl-2 did not correlate with resistance to Fas stimulation [23].

Lastly, we examined the expression of two unrelated antiapoptotic gene products, DAD1 and survivin. DAD1 has been localized to the endoplasmic reticulum and has oligosaccharyltransferase activity, but its exact mechanism of apoptosis inhibition has not been defined [16, 27, 69]. In humans, DAD1 is located on human chromosome 14 between the genes encoding the TCR and the granzyme B complex, leading to speculation that this antiapoptosis gene may play a role in T cell selection [65]. Prior to this report DAD1 expression in either resting or activated T cells has not been addressed. Our results show that, while there is low expression in peripheral blood lymphocytes, DAD1 is significantly up-regulated in CIK cultures. The other antiapoptosis protein, survivin, belongs to the family of baculovirus IAP apoptosis inhibitors [3]. Like other members of the IAP apoptosis inhibitors, survivin functions by binding and inhibiting the activity of the centrally acting caspase-3 and caspase-7, thereby allowing resistance not only to Fas signaling but also to other agents such as Bax and etoposide [61]. Survivin expression is linked to the cell cycle and the antiapoptotic effect is dependent upon its association with microtubules [31]. In humans, survivin mRNA expression has been found almost exclusively in either fetal or malignant tissues, with low-level expression detected in the placenta and thymus [1, 3]. A recent report has also demonstrated that the murine homologue of survivin, TIAP is expressed in T cells upon activation with CD3 crosslinking antibodies and IL-2 [29]. Our results support and extend these findings since we have found survivin expression in primary human activated T cells stimulated in a similar manner.

Much of the recent work has focused on the resistance of malignant cells to Fas-mediated apoptosis and little is known about the effectors that may serve as suitable forms of immunotherapy for FasL-expressing tumors. CIK cells are easily expanded and have significant cytotoxicity against autologous tumor targets and in vivo activity in several animal models of cancer. This form of immunotherapy is currently being evaluated in phase I/II clinical trials. Through these studies we have shown that T cells activated over a period of 21–28 days with IFNγ, OKT-3 and IL-2 are resistant to Fas receptor crosslinking and display cytotoxicity against tumors in the presence of Fas-crosslinking antibodies or against tumors known to express FasL. Resistance to Fas ligation in CIK cells requires protein synthesis and

may come about through an in vitro selection for Fas-resistant cells. The up-regulation of multiple antiapoptotic genes, including those encoding cFLIP, Bcl-2, Bcl-xL, DAD1 and survivin, may account for such resistance.

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