Interleukin-4 Inhibits Caspase-3 by Regulating Several Proteins in the Fas Pathway during Initial Stages of Human T Helper 2 Cell Differentiation*[S]

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Interleukin-4 (IL-4) is the main cytokine that polarizes activated naïve CD4$^+$ T cells in the T helper 2 (Th2) direction. IL-4 also regulates the subsequent stages of Th2 cell-mediated diseases, such as allergies. We conducted a proteomics study to identify IL-4-induced differences during the initial stages of T helper cell differentiation. Primary CD4$^+$ T lymphocytes were isolated from human cord blood, activated through CD3 and CD28, and cultured in the presence or absence of IL-4. Soluble proteins were separated by two-dimensional electrophoresis and visualized by staining with autoradiography, which indicated that at least 20 proteins might be regulated by IL-4. From this minimum of 20 stained proteins, altogether 35 proteins were identified using tandem mass spectrometry. Interestingly the fragmented form of GDP dissociation inhibitor expressed in lymphocytes/Rho GDP dissociation inhibitor 2 (Ly-GDI), a known target of Caspase-3, was observed to be down-regulated in IL-4-treated cells. It was shown in further studies that IL-4 decreases Caspase-3 activity and cell death in these cells. Neutralizing Fas-Fas ligand interaction led to decreased Caspase-3 activity and lowered Ly-GDI fragmentation. We further characterized the effects of IL-4 on the expression of main regulators in the Fas-mediated pathway. We demonstrated that IL-4 decreases expression of Fas receptor and increases expression of Bid, Bcl-2, and Bcl-xL. Importantly IL-4 significantly up-regulated the short form of c-FLIP, although the levels of c-FLIP long were unaltered after IL-4 induction. Taken together, our results indicate that IL-4 inhibits caspase activity during the initial stages of human Th2 cell differentiation by regulating expression of several key players in the Fas-induced pathway. ***Molecular & Cellular Proteomics 6:238–251, 2007.***

Caspase-3 is a cysteine protease that is activated through a proteolytic cleavage by upstream caspases. Caspase-3 can be activated by two distinct pathways, which are initiated either from the mitochondria or from the cell membrane. Several triggers, such as DNA damage or cytokine withdrawal, can induce depolarization of the mitochondrial membrane, leading to the release of cytochrome *c* into the cytosol (1, 2). Released cytochrome *c* acts together with Apaf-1 and Caspase-9 to activate Caspase-3 (3). The role of Bcl-2 family members Bcl-2 and Bcl-xL in the inhibition of the mitochondrial membrane depolarization is well documented as is the prevention of depolarization by other means that are unrelated to Bcl-2 or Bcl-xL (4).

The binding of ligands to so-called death receptors can activate the caspase-mediated pathway upstream of Caspase-3 (5, 6). This has been shown to be the mechanism of activation-induced cell death, which is initiated after restimulation of effector T lymphocytes (7). Triggering of death receptor Fas (also known as CD95 or TNFRSF6) by Fas ligand (FasL)[1] leads to activation of Caspase-8 and Caspase-10, which in turn activate downstream effector caspases, such as Caspase-3 (8–11). Active Caspase-8 and Caspase-10 also have potential to induce the depolarization of the mitochondrial membrane by cleaving Bid protein, which in turn is activated and initiates the depolarization step (12, 13).

Research on mature T cell apoptosis has been very active in the past 10 years. However, apoptosis during the initial activation of T cells has largely been outside the focus of general discussion partly because T cells show increased resistance to activation-induced apoptosis at that stage (14, 15). Initially it was thought that resistance is caused by up-regulation of c-FLIP long, but according to recent studies c-FLIP short seems to be responsible for resistance to apoptosis during the initial activation of T cells (14, 16–19). On the other hand, naïve T helper cells have been reported to have increased

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[1] The abbreviations used are: FasL, Fas ligand; IL, interleukin; 2-DE, two-dimensional electrophoresis; αNAC, nascent polypeptide-associated complex α subunit; c-FLIP, cellular FADD-like IL-1β-converting enzyme-inhibitory protein; c-FLIPs, c-FLIP short; D-PBS, Dulbecco's PBS; hnRNP, heterogeneous nuclear ribonucleoprotein; GRP94, 94-kDa glucose-regulated protein; Ly-GDI, GDP dissociation inhibitor expressed in lymphocytes/Rho GDP dissociation inhibitor 2; pb, plate-bound; PI, propidium iodide; Th, T helper; TCR, T cell receptor; HRP, horseradish peroxidase; FSC/SSC, forward scatter/side scatter.

susceptibility to Fas-induced apoptosis during TCR activation, whereas Fas induction had a stimulatory effect on activated memory Th cells. Activation-induced apoptosis was similarly weak in both naïve and memory Th cells (20).

Activation of certain caspases is necessary for the proper activation of T cells. Caspase-8 is activated during T cell activation leading to proliferation of activated T cells (9). c-FLIP long is involved in Caspase-8 activation, and it is also needed for normal proliferation of activated T cells (21, 22). During the initial activation of primary human T cells, Caspase-3, -6, -7, and -8 are activated in a manner that is unrelated to apoptosis of these cells (23, 24). Caspase activation in initially activated T cells is linked to selective substrate cleavage: poly(ADP-ribose) polymerase, lamin B, and Wee1 kinase are processed, whereas 45-kDa subunit of DNA fragmentation factor (DFF45) or 140-kDa subunit of replication factor C (RFC140) are not (24). The significance of Caspase-3 activation for the initial activation of lymphocytes has not been studied in detail, but Caspase-3 is able to inhibit proliferation of B cells (25). Caspase-3-deficient T cells also show increased thymidine uptake after restimulation, but it is largely caused by reduced apoptosis in Caspase-3-deficient cells (26).

The differentiation of T helper cells into distinct effector cells is a finely balanced process that is controlled by TCR activation, co-stimulatory molecules on the surface of the antigen-presenting cell, and polarizing cytokines in the vicinity of T cells. IL-4 is the main cytokine dictating Th differentiation into the Th2 direction (for a review, see Ref. 27). Caspases also regulate T helper cell differentiation because their inhibition leads to increased IL-4 production and therefore enhanced Th2 polarization of $CD4^+$ T cells (28). In addition, when naïve T cells are co-stimulated with anti-Fas antibody during initial activation, they show increased production of Th1 type cytokine interferon-γ (29). Caspase-8 activity has been reported to prevent Th2 responses, and active Caspase-8 is required for T cell-mediated immunity against *Trypanosoma cruzi* infection (30). Caspase-3 activation has been associated with the regulation of monocyte differentiation: Caspase-3 is activated when monocytes differentiate into macrophages but remains inactive when monocytes are treated with IL-4 and granulocyte macrophage colony-stimulating factor to induce dendritic cell differentiation (31). In addition, Caspase-3 activity is required for the osteogenic differentiation of bone marrow stromal cells and for skeletal muscle differentiation (32, 33). The role of Caspase-3 in T helper cell differentiation is not known.

Despite several transcripts regulated by IL-4 during Th2 differentiation having recently been identified both in human and mouse (34–37), nothing is known about the effects of IL-4 on the proteomes of freshly activated naïve human $CD4^+$ cells. Thus, to better define changes to proteomes of human $CD4^+$ cells, we conducted a proteomics study to identify IL-4-induced differences during the initial stages of T helper cell differentiation. With this approach, we detected 20 IL-4-regulated protein spots and identified 35 proteins from these spots. Interestingly the fragmented form of Ly-GDI, a known target of Caspase-3 (38–40), was down-regulated in IL-4-treated cells. We also demonstrated that Caspase-3 activity is decreased in response to IL-4 treatment. Moreover we showed that IL-4 modifies the protein expression of several proteins regulating Caspase-3 activity, such as Fas receptor, c-FLIP short, Bid, Bcl-2, and Bcl-xL. These latter observations may provide a mechanism through which IL-4 inhibits Caspase-3 activity in these cells.

EXPERIMENTAL PROCEDURES

Isolation, Activation, and Culturing of $CD4^+$ Cells—Human umbilical cord blood was obtained from Turku University Central hospital. $CD4^+$ lymphocytes were isolated from neonatal umbilical cord blood by using Ficoll-Paque isolation (Amersham Biosciences) and $CD4^+$ isolation kit (Dynabeads M-450 Human, Dynal, Oslo, Norway). The purity of $CD4^+$ cells in the gated cell population was 98.6 $\pm$ 0.7%. 90.8 $\pm$ 4.0% of the cells were $CD45RA^+$, and 20.8 $\pm$ 7.1% were $CD45RO^+$. These figures are typical for human umbilical cord blood samples (41).

Cells were activated using anti-CD3, anti-CD28, and anti-mouse $F(ab')_2$ fragment. They were suspended in Yssel's medium containing 1% of human AB serum (the Finnish Red Cross Organization) to the concentration 10×10^6 cells/ml, incubated for 15 min at +4 °C with soluble mouse anti-CD3 (10 μg/ml, Immunotech, Marseille, France) and mouse anti-CD28 (10 μg/ml, Immunotech), and then washed with Dulbecco's PBS (D-PBS). Cells were cultured for 24 h in RPMI 1640 medium (Sigma) containing cross-linking dialyzed goat anti-mouse $F(ab')_2$ (final concentration, 10 μg/ml; BIOSOURCE, Camarillo, CA) and 1% human AB serum (Finnish Red Cross). Recombinant human IL-4 (10 ng/ml, R & D Systems, Minneapolis, MN) was added to the culture media of IL-4-treated samples.

For metabolic labeling, cells were activated as above and cultured in methionine-free RPMI 1640 medium (Sigma) including [^{35}S]methionine and [^{35}S]cysteine (Redivue Pro-Mix L-[^{35}S] *in vitro* cell labeling, Amersham Biosciences), goat dialyzed anti-mouse $F(ab')_2$ (final concentration, 10 μg/ml; BIOSOURCE), and 1% human AB serum (Finnish Red Cross). For plate-bound CD3 activation, cells were activated with plate-bound anti-CD3 (pb anti-CD3) and soluble anti-CD28 so that the 24-well plate wells were coated with 100 ng of anti-CD3 (Immunotech) in 200 μl of D-PBS for 2 h at 37 °C and washed twice with 0.5 ml of D-PBS before the cells were added to the wells. Cells were cultured in RPMI 1640 medium (Sigma) containing 1% human AB serum and 500 ng/ml mouse anti-CD28 (Immunotech).

Neutralization of Fas-FasL Interaction—For the neutralization experiments, cells were activated with plate-bound anti-CD3 and soluble anti-CD28 as described above. During coating, pb anti-CD3 concentration was either 0.5 or 2.5 μg/ml in D-PBS. Cells were cultured for 24 h and collected. Mouse anti-Fas antibody (5 μg/ml, ALX-805-010-C100, Alexis Biochemicals) or mouse anti-FasL antibody (10 μg/ml, catalog number 556371, BD Pharmingen) was included in the culture medium to inhibit Fas-FasL interaction.

Flow Cytometry—$0.1–0.5 \times 10^6$ cells were used for each flow cytometry sample. Staining was performed at +4 °C, and the cells were incubated in the dark. Samples were measured with the FACSCalibur™ system (BD Biosciences) and analyzed with CellQuest Pro (BD Biosciences). At least 10,000 cell counts were measured per sample. Active Caspase-3 was measured according to the protocol provided by the manufacturer (catalog number 550914, BD Pharmingen).

For surface staining of the Fas receptor, cells were washed once with FACS buffer (2% FCS, 0.01% azide in D-PBS) and incubated for 15 min with 20 μl of mouse anti-Fas-FITC (ALX-805-010F-T100, Alexis Biochemicals) or isotype control mouse IgG2b-FITC (catalog number MG2b01, Caltag Laboratories, Burlingame, CA). After incubation, cells were washed once with FACS buffer and once with FACS buffer without FCS and finally resuspended in 1% formalin in D-PBS.

The amount of intracellular cleaved Ly-GDI was measured with flow cytometry in the following way. Cells were washed twice with staining buffer (0.5% BSA, 0.01% azide in D-PBS) and fixed with 4% paraformaldehyde in D-PBS for 15 min. Cells were washed once with staining buffer, permeabilized for 10 min with a permeabilization buffer (0.5% saponin, 0.5% BSA, 0.01% azide in D-PBS), and incubated for 20 min with 5 μl of mouse anti-D4-GDI (cleavage product-specific, Alexis Biochemicals). Cells were washed four times with permeabilization buffer and incubated with goat $F(ab')_2$ anti-mouse-FITC (M35001, Caltag Laboratories) for 20 min. Cells were washed four times with permeabilization buffer and once with staining buffer and resuspended in staining buffer.

Two-dimensional Electrophoresis (2-DE) and Protein Detection—Cells were lysed and proteins were separated with 2-DE as described previously (42). Briefly soluble proteins were absorbed into the 18-cm 3–10 non-linear IPG-strips (Amersham Biosciences) for 24 h at room temperature. Isoelectric focusing to a total of 40 kV-h was carried out at 20 °C, and the focused strips were equilibrated for 25 min at room temperature. The second dimension was vertical 12% SDS-PAGE with gel thickness of 1 mm. Proteins were detected with silver staining (43) and autoradiography (24 h) (44). Autoradiography images from six independent experiments were used for the comparison of protein expression levels. Gel comparison was done with the PDQuest program (version 7.0.1, Bio-Rad), and all the gel images were normalized before the comparison. If comparison was not possible due to unsuccessful protein separation during 2-DE or undetectable signal intensities of the proteins, the gel pair in question was not taken into consideration in the final analysis of that selected protein. The normalized signal intensities of protein spots were compared to find reproducible IL-4-induced differences and to determine paired *t* test values for the effect of IL-4.

Protein Identification—The protein spots with reproducible at least 2-fold regulation by IL-4 were selected for identification. Proteins were in-gel digested, and the resulting peptides were analyzed by nano-LC-MS/MS as described previously (45–48). Briefly the excised gel spot was cut into pieces, washed twice, and dehydrated with ACN. Proteins were reduced with 20 mM dithiothreitol (Sigma), alkylated with 55 mM iodoacetamide (Sigma), and in-gel digested with trypsin (sequencing grade modified trypsin, Promega Corp., Madison, WI) at +37 °C overnight. Peptides were extracted with 5% formic acid, 50% ACN and dried with a vacuum centrifuge. Peptides were dissolved in 2% HCOOH prior to MS analysis. Analyses were made with a QStar Pulsar™ ESI-hybrid quadrupole-TOF instrument (Applied Biosystems/MDS Sciex, Toronto, Canada) coupled on line with nano-HPLC (Famos, SwitchosII, and Ultimate, LC Packings, Amsterdam, Netherlands) as described previously (48).

Peak lists from the MS/MS spectra were created from the Analyst QS program (Version 1.1, Applied Biosystems) using the mascot.dll script with the following parameters: centroided mass; double and triple charged peptides as precursors only; peak threshold, 0.01% of maximum; precursor mass tolerance for grouping, 0.05; a minimum of one and a maximum of 10 cycles between groups. These spectra were analyzed using MASCOT software (Matrix Science) and searched against the Swiss-Prot database (version 50.1, June 26, 2006) using the following parameters: human-specific taxonomy; trypsin digestion with one missed cleavage; carbamidomethyl modification of cysteine as a fixed modification and oxidation of methionine as a variable modification; peptide tolerance maximum, ±0.3 Da; MS/MS tolerance maximum, ±0.2 Da; peptide charge, +2 or +3; monoisotopic mass. Keratin identifications were excluded from the list if at least two different keratins were identified from the same sample. Only proteins with a minimum of two peptides identified and a protein score of above 50 in the peptide summary report were included in the protein identification list. To detect post-translational protein modifications from spot numbers 9 and 10, the original peak list was reanalyzed with MASCOT software using the same parameters and including the following modifications in the variable modification list: acetyl (Lys), deamination (NQ), *N*-acetyl (protein), *N*-formyl (protein), oxidation (Met), oxidation (HW), phosphorylation (STY).

Western Blotting—Cells were lysed in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.1% (w/v) bromophenol blue. The SDS concentration of the lysis buffer was high enough to prevent Granzyme B-dependent activation of caspases during the cell lysis (49). SDS-PAGE and protein transfer to nitrocellulose membranes were carried out according to standard protocols (50) Blocking was achieved with 5% milk, TBS, 0.1% Tween unless otherwise specified. The following antibody dilutions were used for Western blots: 1:700 rat anti-94-kDa glucose-regulated protein (GRP94) (RT-102-P1ABX, NeoMarkers, Fremont, CA) in 5% BSA, TBS; 1:100 goat anti-heterogeneous nuclear ribonucleoprotein (hnRNP) K (sc-16554, Santa Cruz Biotechnology, Santa Cruz, CA); 1:200 goat anti-hnRNP E1 (sc-16504, Santa Cruz Biotechnology); 1:5000 rabbit anti-Ly-GDI (catalog number 556511, BD Pharmingen) in 5% milk, PBS, 0.1% Tween; 1:500 mouse anti-D4-GDI (anti-Ly-GDI, cleavage product-specific, ALX-804-264, Alexis Biochemicals); 1:500 mouse anti-c-FLIP (ALX-804-428, Alexis Biochemicals, blocking in 2.5% milk, 2.5% BSA, PBS, 0.1% Tween) in 2% BSA, PBS, 0.1% Tween; and 1:10,000 mouse anti-β-actin (catalog number A5441, Sigma). Members of Bcl-2 family were detected with Bcl-2 family sampler kit (catalog number 612742, BD Biosciences). Rabbit anti-nascent polypeptide-associated complex α subunit (αNAC) (blocking in 5% milk, TBS; antibody: 1:500 dilution in 2.5% milk, TBS) was a generous gift from Dr. René St-Arnaud (Genetics Unit, Shriners Hospital for Children, Montréal, Québec, Canada). Secondary antibodies used in this study were as follows: 1:10,000 HRP goat anti-mouse (sc-2005, Santa Cruz Biotechnology), 1:5000 HRP goat anti-rabbit (catalog number 554021, BD Pharmingen), 1:5000 HRP donkey anti-goat (sc-2020, Santa Cruz Biotechnology), 1:20,000 HRP goat anti-mouse IgG1 (catalog number 1070-05, Southern Biotech, Birmingham, AL), and 1:10,000 HRP goat anti-rat (catalog number 81-9520, Zymed Laboratories Inc., San Francisco, CA).

RESULTS

IL-4-induced Differences in the Proteomes of Activated Naïve Human T Helper Cells—In this study we investigated the effect of IL-4 on the proteomes of freshly activated naïve human T helper cells. Primary $CD4^+$ cells were isolated from the human cord blood; activated with mouse anti-CD3, mouse anti-CD28, and anti-mouse $F(ab')_2$ fragment; and metabolically labeled for 24 h in the presence or absence of IL-4. The cells were lysed, and soluble proteins were separated with 2-DE. The analysis of autoradiography gel images showed that at least 20 proteins were reproducibly differentially expressed after IL-4 induction (Fig. 1): seven were up-regulated (spot numbers 1–7) and 13 were down-regulated (spot numbers 8–20) in IL-4-treated cells. Altogether 35 unique proteins were identified in these protein spots using tandem mass spectrometry (Table I). The positions of L-plas-

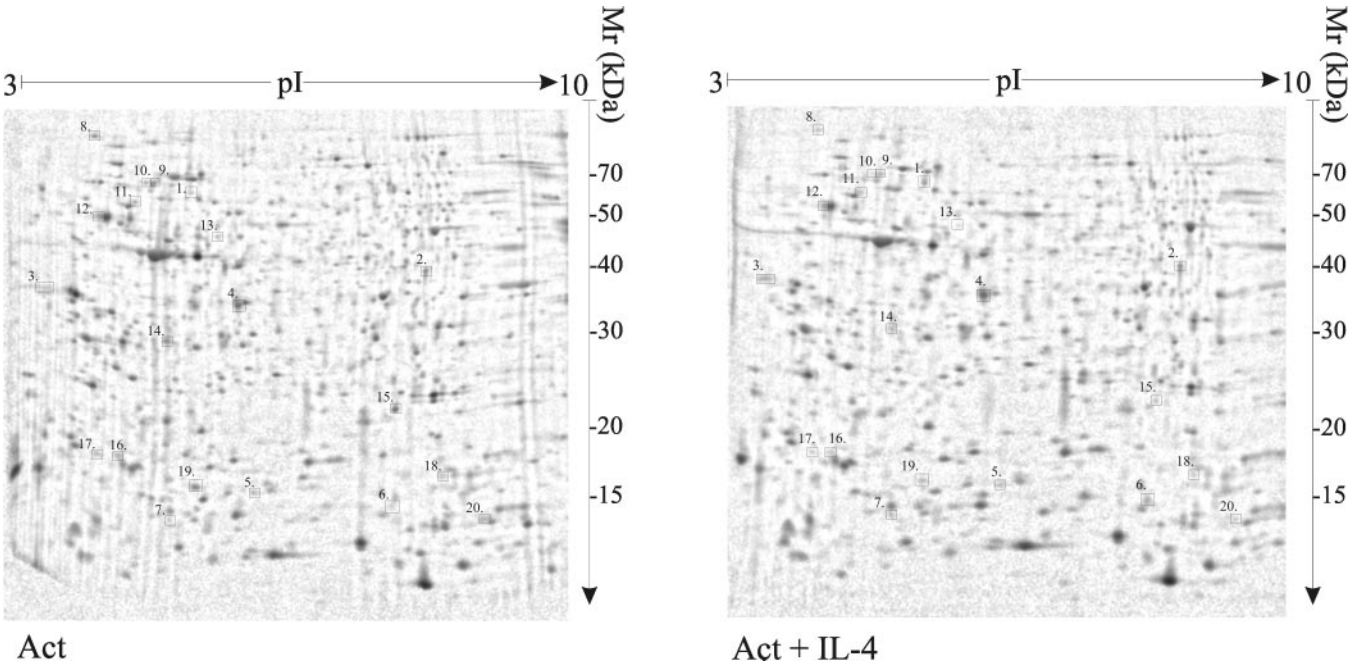

FIG. 1. **Reproducible IL-4-induced differences in the proteomes of activated primary T helper cells are indicated as 1–20 and surrounded by a □.** $CD4^+$ human T cells were isolated from cord blood; activated with soluble mouse anti-CD3, anti-CD28, and goat anti-mouse $F(ab')_2$; and metabolically labeled for 24 h in the presence (activated (*Act*) + *IL-4*) or absence of IL-4 (activated (*Act*)). Cells were lysed, and soluble proteins were separated with 2-DE. The expression of 20 proteins visualized by staining with autoradiography was reproducibly regulated by IL-4 at least by 2-fold: seven were up-regulated (*spot numbers 1–7*) and 13 were down-regulated (*spot numbers 8–20*) in IL-4-treated cells.

tin spots (numbers 9 and 10 in Fig. 1) in the 2-DE gel suggest that these spots consist of two differentially post-translationally modified forms of L-plastin. L-plastin is known to be phosphorylated on serine residues Ser-5 and Ser-7 (51–54). We could detect a possible phosphorylation of Ser-5 with tandem mass spectrometry only from protein spot number 10, which correlates with its migration to a more acidic pI in 2-DE when compared with spot number 9 (Fig. 1 and supplemental data). The same peptides from Histone H2A were identified from two different protein spots: one was down-regulated (spot number 7) and one was up-regulated (spot number 20) in IL-4-treated cells. Annexin V (theoretical mass, 36 kDa; 374 amino acids) was identified from spot numbers 16 and 17 (Fig. 1), whose molecular masses in the 2-DE gel were less than 20 kDa. The sequence coverage was amino acids 193–300 in spot number 16 and 6–116 in spot number 17, suggesting that these protein spots consist of N- and C-terminal fragments of Annexin V, respectively. The expression of both spots was down-regulated by IL-4.

The expression regulation of hnRNP K, hnRNP E1, αNAC, GRP94, and Ly-GDI by IL-4 was considered interesting (Fig. 2). These proteins were selected based on their reported functions and the availability of specific antibodies. In addition, their regulation by IL-4 in 2-DE gels was statistically significant (Table I). hnRNP K and hnRNP E1 have been shown to have a role in different stages of hematopoiesis through regulation of translation and mRNA stabilization (55), and hnRNP K is able to function as a transcription factor (56). αNAC is a subunit of a protein complex that binds to newly synthesized proteins emerging from the ribosome (57, 58). One of its isoforms acts as a transcription factor and is capable of regulating erythroid cell differentiation (59–61), and αNAC has been shown to inhibit Fas-associated death domain-mediated signaling in the absence of death receptor induction (62). Endoplasmin precursor (GRP94) functions as a co-stimulatory molecule in the immune system and is expressed on the cell surface of B cells and in particular tumor cells (63, 64). It is also localized in the endoplasmic reticulum (65). In our studies, GRP94 was not expressed on the surface of the $CD4^+$ cells as measured by flow cytometry (data not shown). When the protein levels of hnRNP K, hnRNP E1, αNAC, and GRP94 were measured with Western blotting analysis, they showed no IL-4-induced changes in their total protein expression (Fig. 2).

IL-4 Decreases the Fragmentation of Ly-GDI—Ly-GDI is a weak Rho GDP dissociation inhibitor expressed only in hematopoietic tissues and mainly in lymphocytes (66, 67). It has a role in TCR-induced, Vav1-mediated signaling (68), and its deficiency leads to decreased cell death after IL-2 withdrawal (69). Ly-GDI is a known target of Caspase-3 (38–40). The cleaved protein translocates to the nucleus, but the significance of this translocation is not known (38, 70). The molecular mass of the protein in our 2-DE gels was ~23 kDa, which is closer to the cleaved than the intact form of the protein (38,

TABLE I

IL-4-regulated proteins identified by 2-DE and tandem mass spectrometry in activated primary T helper cells

Seven protein spots were up-regulated and 13 were down-regulated in IL-4-treated cells. 35 different proteins were identified from these spots. The reproducibility ratio of at least 2-fold differences (the number of at least 2-fold differences divided by the number of total comparisons), protein accession number, and the experimental molecular mass as well as the theoretical molecular mass and pI of the protein are indicated in the columns. The confidence of the protein identification is expressed as the number of different peptides identified as well as the sequence coverage and MASCOT score of identification in the peptide summary report. Numbers in the first column correspond to the ones in Fig. 1.

Spot no.	Reproducibility of ≥2-fold differences	Identified proteins	Swiss-Prot accession number	Molecular mass		Theoretical pI	No. of matched peptides	Sequence coverage	Mascot protein score
				Experimental	Theoretical				
				kDa				%	
Up-regulated by IL-4									
1	3/5[a]	hnRNP K	P61978	64	51.2	5.39	10	27.6	233
2	3/6[b]	Poly(rC)-binding protein 1 (α-CP1) (hnRNP E1)	Q15365	40	38.0	6.66	10	30.6	245
		Fructose-bisphosphate aldolase C (EC 4.1.2.13)	P09972	40	39.7	6.41	3	9.6	99
3	4/5[a]	αNAC	Q13765	37	23.4	4.52	6	20.5	199
		Guanine nucleotide-binding protein $G_i/G_s/G_t$ β subunit 1 (transducin β chain 1)	P62873	37	38.0	5.60	3	9.4	86
4	3/5	L-Lactate dehydrogenase B chain (LDH-B)	P07195	34	36.8	5.71	22	45.9	471
		60 S acidic ribosomal protein P0 (L10E)	P05388	34	34.4	5.71	4	13.6	162
5	3/6	Not identified		14					
6	4/6	40 S ribosomal protein S12	P25398	14	14.7	6.36	54	36.6	342
		Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	P04406	14	36.1	8.58	4	14.4	199
		Histone H2B	P62807, P58876, Q99880, Q99877, Q99879, Q93079, O60814, P57053	14	13.8	10.32	3	19.2	138
		L-Lactate dehydrogenase A chain (LDH-A)	P00338	14	36.8	8.46	2	6.3	115
		Histidine triad nucleotide-binding protein 1 (adenosine 5′-monophosphoramidase)	P49773	14	13.8	6.46	2	24.8	105
		Cofilin-1 (Cofilin, non-muscle isoform) (18-kDa phosphoprotein)	P23528	14	18.6	8.46	2	18.8	98
		Cytochrome *c* oxidase polypeptide Vb, mitochondrial precursor (EC 1.9.3.1)	P10606	14	13.9		2	17.8	71
7	3/6[c]	Histone H2A	Q96QV6, P04908, Q93077, P20671, P28001, Q96KK5, Q99878, P0C0S8, Q6FI13, Q16777, Q7L7L0, P16104	14	14.1	10.90	3	17.7	73
Down-regulated by IL-4									
8	2/4[b]	Endoplasmin precursor (GRP94)	P14625	89	92.7	4.76	6	7.3	126
9	4/5	L-plastin (Plastin-2) (lymphocyte cytosolic protein 1)	P13796	67	70.7	5.20	52	72.2	1900
		Lamin-B1	P20700	67	66.5	5.11	26	52.3	1139
		Serum albumin precursor	P02768	67	71.3	5.92	3	6.2	157
10	3/5	L-plastin (Plastin-2) (lymphocyte cytosolic protein 1)	P13796	67	70.7	5.20	45	71.7	1635
		Lamin-B1	P20700	67	66.5	5.11	27	45	1010
		Serum albumin precursor	P02768	67	71.3	5.92	5	9.5	215
11	3/5	Keratin, type I cytoskeletal 9 (Cytokeratin-9) (CK-9)	P35527	54	62.3	5.19	5	8.3	163
		Actin, cytoplasmic 1/2 (β/γ-actin)	P60709, P63261	54	42.1	5.29	5	15.7	75
12	3/5	Tubulin β-2 chain	P07437	50	50.1	4.78	23	41.9	404
13	3/4[b]	Actin-like protein 3 (actin-related protein 3)	P61158	47	47.8	5.61	15	39.5	309

TABLE I—*continued*

14	4/6	Proteasome activator complex subunit 2 (proteasome activator 28-β subunit)	Q9UL46	29	27.4	5.44	5	24.4	109
15	4/6[b]	Ly-GDI (Rho GDP-dissociation inhibitor 2) (Rho GDI 2) (Rho-GDI β)	P52566	22	22.9	5.10	29	57.5	519
		Transgelin-2 (SM22-α homolog)	P37802	22	22.4	8.45	4	26.3	176
		Bullous pemphigoid antigen 1, isoforms 6/9/10 (Trabeculin-β)	O94833	22	593.8	5.49	4	0.6	54
16	3/5	Annexin A5 (Annexin V) (Lipocortin V) (Endonexin II)	P08758	18	35.8	4.94	8	18.5	200
17	2/3	Annexin A5 (Annexin V) (Lipocortin V) (Endonexin II)	P08758	18	35.8	4.94	12	25.7	374
		Small ubiquitin-related modifier 1 precursor (SUMO-1)	P63165	18	11.6	5.35	3	20.8	77
18	3/6[c]	Peptidyl-prolyl cis-trans isomerase A (PPIase A) (Rotamase A) (Cyclophilin A)	P62937	16	18.1	7.68	11	50.6	297
		Peroxiredoxin-5, mitochondrial precursor (EC 1.11.1.15) (Prx-V)	P30044	16	22.3	8.85	3	15.4	100
		Histone H3	Q16695, P68431, P84243	16	15.5	11.13	2	11.9	50
19	2/3	Ubiquitin-conjugating enzyme E2 N (EC 6.3.2.19) (ubiquitin-protein ligase N)	P61088	16	17.2	6.13	9	55.9	212
20	3/4	Hemoglobin δ subunit (hemoglobin δ chain)	P02042	14	16.0	7.97	10	67.8	275
		Actin, cytoplasmic 1/2 (β/γ-actin)	P60709, P63261	14	42.1	5.29	7	12.3	151
		Histone H2A	Q96QV6, P04908, Q93077, P20671, P28001, Q96KK5, Q99878, P0C0S8, Q6FI13, Q16777, Q7L7L0, P16104	14	14.1	10.86	3	17.7	74
		Hemoglobin α subunit (hemoglobin α chain)	P69905	14	15.2	8.73	3	28.4	60

[a] Paired *t* test value <0.01.

[b] Paired *t* test value <0.05.

[c] The difference was seen once in the opposite direction.

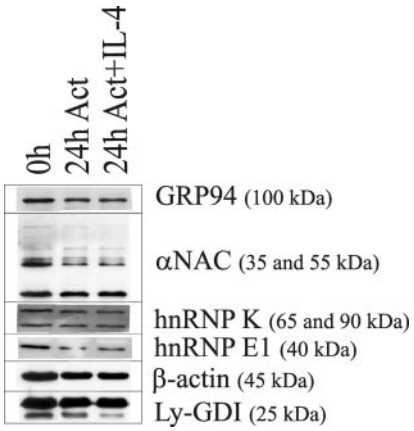

FIG. 2. **Fragmentation of Ly-GDI is decreased in IL-4-treated cells.** Human CD4$^+$ T cells were isolated and activated as described for the 2-DE separation of proteins, cultured for 24 h in the presence or absence of IL-4, and harvested. Total levels of the selected proteins were measured by Western blotting. IL-4 decreased the amount of cleaved Ly-GDI (*bottom*), but the total expression of other selected proteins was not modified by IL-4. Equal loading was confirmed by β-actin detection. Similar results were obtained from three independent experiments. *Act*, activated.

71, 72). Moreover the sequence coverage of the identification included amino acids only from the fragmented form (amino acids 22–164), suggesting that the differentially expressed protein is the cleaved form of Ly-GDI. This was confirmed by Western blotting: the amount of the fragmented form of Ly-GDI was decreased by 60% on average in activated + IL-4-treated cells compared with cells cultured without IL-4 (Fig. 2). Decreased fragmentation of Ly-GDI was also detected using an antibody specific for the cleaved form of the protein (data not shown).

IL-4 Decreases Caspase-3 Activity and Cell Death in Activated CD4$^+$ Cells—Because Ly-GDI is a known target of Caspase-3 (38–40), decreased Ly-GDI fragmentation in IL-4-treated cells implies that Caspase-3 activity is also decreased in IL-4-treated cells. To study the Caspase-3 activity in initially activated naïve T helper lymphocytes, cells that were stained with active Caspase-3-specific antibody were measured with flow cytometry. IL-4 had a dramatic effect on Caspase-3 activity, which was ~60% lower in IL-4-treated cells than in controls (Fig. 3*a*). Because Caspase-3 is linked to apoptosis, we studied the effect of IL-4 on the viability of activated CD4$^+$

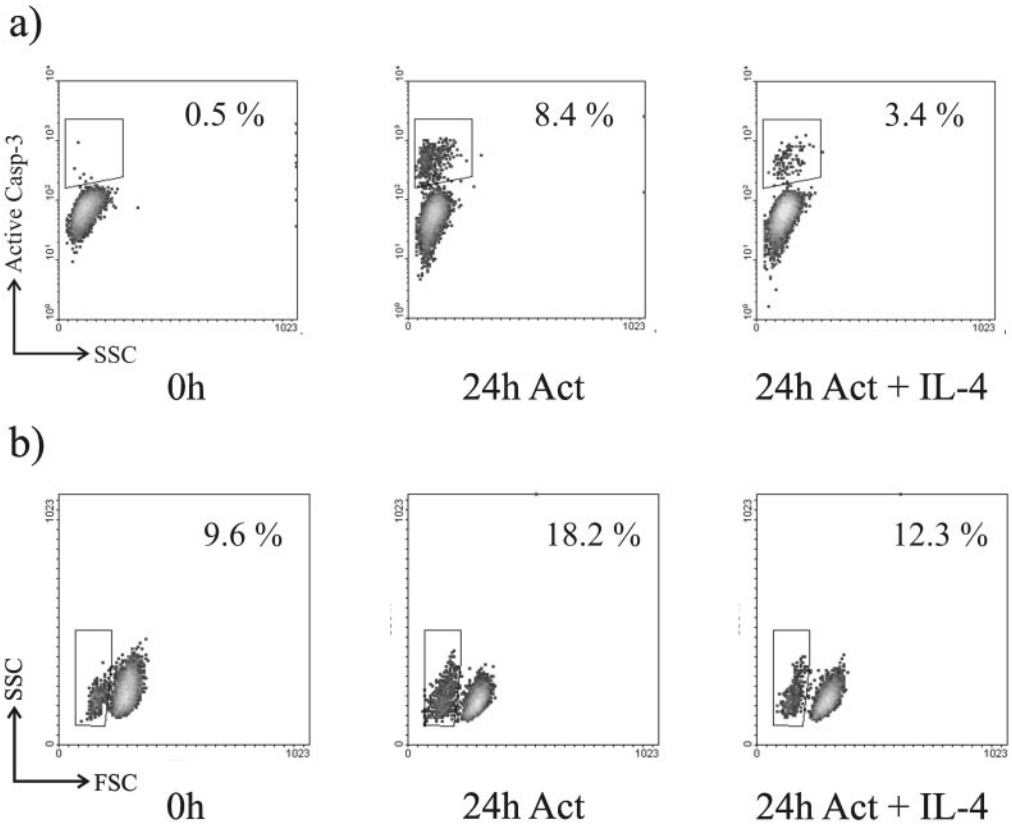

FIG. 3. **The amount of active Caspase-3 (*a*) and cell death (*b*) is decreased in IL-4-treated cells.** Human CD4$^+$ T cells were isolated and activated as described previously for the 2-DE separation of proteins, cultured for 24 h, and harvested. *a*, the percentage of active Caspase-3-positive cells was measured by flow cytometry. The amount of active Caspase-3-positive cells was 60% lower in IL-4-stimulated samples compared with controls. The results are representative of seven independent experiments. Both the effect of activation and the effect of IL-4 were statistically significant (paired *t* test value <0.01). *b*, the FSC/SSC of the fixed cells was measured by flow cytometry. IL-4 significantly reduced the proportion of dying (gated) cells. Results are representative of six independent cultures. Both the effect of activation and the effect of IL-4 were statistically significant (paired *t* test value <0.001). *Casp-3*, Caspase-3; *Act*, activated.

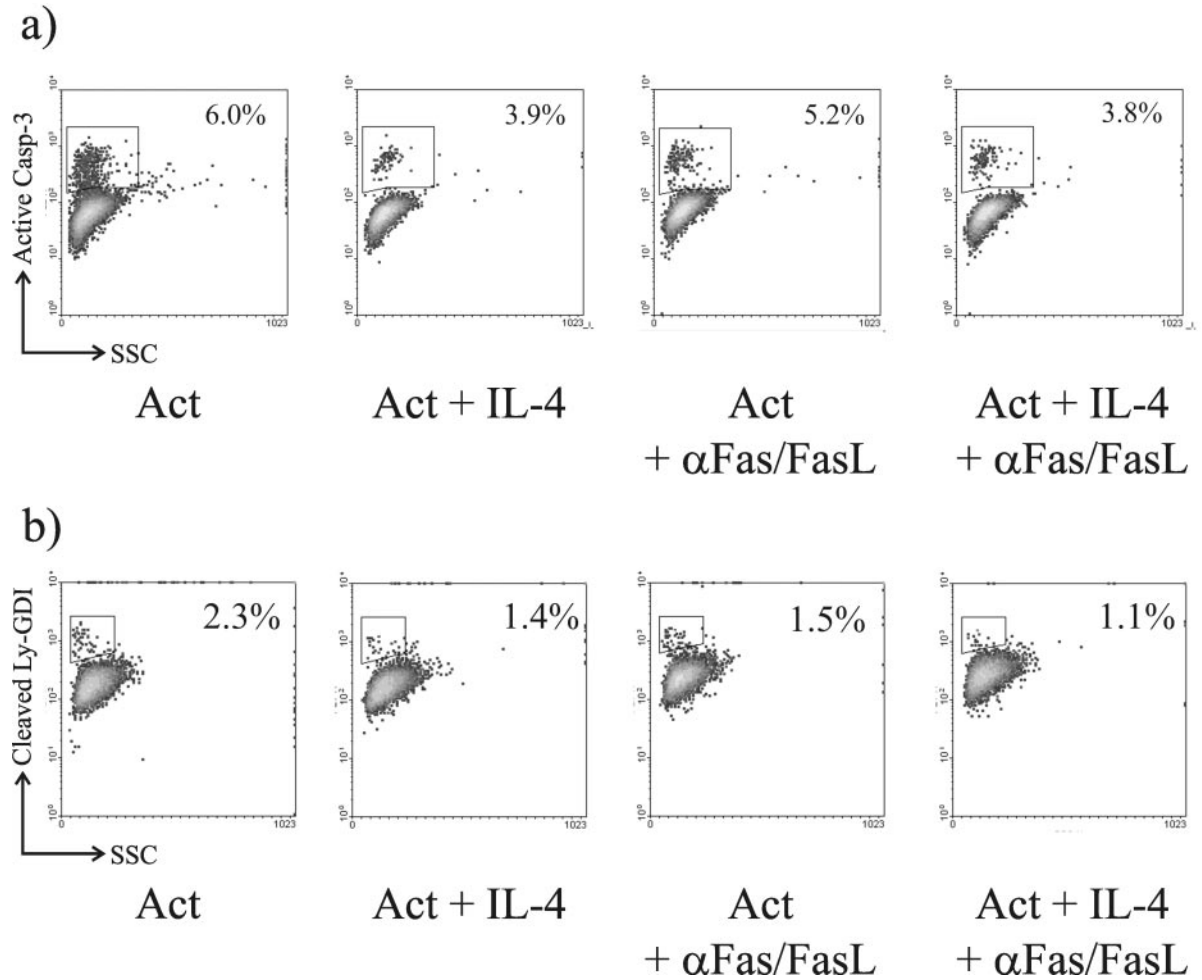

FIG. 4. **Inhibition of Fas-FasL interaction decreases both Caspase-3 activity (*a*) and Ly-GDI fragmentation (*b*) in activated CD4$^+$ T cells.** CD4$^+$ cells were activated with plate-bound anti-CD3 and soluble anti-CD28 and cultured for 24 h in the presence or absence of IL-4. Fas-FasL interaction was inhibited by adding neutralizing anti-Fas or anti-FasL antibodies (αFas/FasL) to the culture media. *a*, the amount of Caspase-3-active cells was decreased in activated samples including neutralizing antibodies. Similar results were obtained from four independent experiments. Paired *t* test values for the effect of neutralization were 0.070 for the activated samples and 0.185 for the activated + IL-4-treated samples. *b*, the amount of activated cells with high expression of cleaved Ly-GDI was decreased after neutralization of Fas-FasL interaction. We measured a cell population that was separate from background signal. Similar results were obtained from three independent experiments. Paired *t* test values for the effect of neutralization were 0.037 for the activated samples and 0.170 for the activated + IL-4-treated samples. *Casp-3*, Caspase-3; *Act*, activated.

cells. IL-4 significantly decreased the proportion of dying cells when cell death was measured by FSC/SSC of the fixed cells (73) (Fig. 3*b*). Although IL-4 had a similar effect on the double positive cells stained both by Annexin V and propidium iodide (PI), it did not decrease the amount of Annexin V$^+$ PI$^-$ cells (data not shown).

Activation of Caspase-3 Is Partly Dependent on Fas-mediated Signaling—Caspase-3 can be activated both by Fas induction and the release of proapoptotic substances from the mitochondria to the cytosol (for reviews, see *e.g.* Refs. 1, 6, and 8). To clarify the role of Fas receptor signaling in Caspase-3 induction, we performed neutralization experiments with neutralizing anti-Fas and anti-FasL antibodies. Isolated CD4$^+$ T cells were activated with pb anti-CD3 and soluble anti-CD28 and cultured for 24 h in the presence or absence of IL-4. Fas-FasL interaction was neutralized by adding anti-Fas or anti-FasL antibodies to the culture medium. The pb anti-CD3 activation method was chosen because cross-linking of neutralizing anti-Fas antibody can induce Fas signaling. Neutralization of Fas-mediated signaling decreased the amount of active Caspase-3-positive cells in activated samples cultured without IL-4 (Fig. 4*a*). Caspase-3 activity was only partially decreased in neutralized samples, so it is not entirely dependent on Fas-FasL interaction. Flow cytometry was also used to study the effect of neutralization on Ly-GDI fragmentation. Harvested cells were permeabilized and stained with an antibody specific for the fragmented form of Ly-GDI. Inhibition of Fas-FasL interaction decreased the

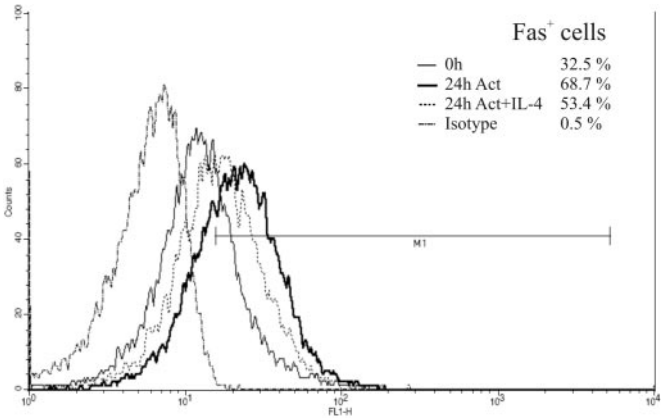

FIG. 5. **The expression level of Fas receptor is decreased in IL-4-treated cells.** Isolated $CD4^+$ cells were activated with pb anti-CD3 and anti-CD28 and cultured for 24 h in the presence or absence of IL-4. Surface expression of Fas receptor was measured with flow cytometry. Similar results were obtained from three independent experiments. *Act*, activated.

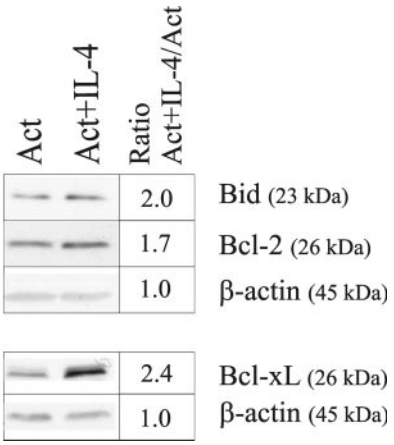

FIG. 6. **Western blotting indicates that IL-4 increases the protein expression of Bid, Bcl-2, and Bcl-xL.** Isolated $CD4^+$ cells were activated with anti-CD3, anti-CD28, and anti-mouse $F(ab')_2$ fragment (*Act*) and cultured for 24 h in the presence or absence of IL-4. The average ratios of normalized protein levels in activated + IL-4 *versus* activated cells are indicated in the *column*. Similar results were obtained from at least three independent experiments. There were no reproducible differences in the expression of Bcl-2 family members Bad, Bax, BAG-1, or Becklin (data not shown).

amount of Ly-GDI-positive cells in samples cultured without IL-4, thereby indicating lower Caspase-3 activity in neutralized samples (Fig. 4*b*). However, the differences seen in Caspase-3 activity did not correlate with the amount of cell death. When dying cells were detected using FSC/SSC or single or double stained cells in Annexin V and PI stainings, cell viability was increased only once in the samples that included neutralizing antibodies (data not shown).

IL-4 Regulates the Expression of Several Proteins That Are Known to Control Caspase-3 Activity—Triggering of Fas receptor leads to the formation of the death-inducing signaling complex and activation of initiator caspases, such as Caspase-8, which in turn can activate Caspase-3 either directly or indirectly through the activation of the mitochondrial pathway (74). We measured the protein expression of several actors in the Fas-induced signaling pathway to investigate whether IL-4 mediates its effect on Caspase-3 activity by regulating the expression of upstream effectors of Caspase-3.

To measure the expression of Fas receptor with flow cytometry, isolated $CD4^+$ lymphocytes were activated with pb anti-CD3 and soluble anti-CD28 and cultured in the presence or absence of IL-4 for 24 h. pb anti-CD3 activation was used to eliminate the background signal resulting from the binding of $F(ab')_2$ fragment to the staining antibodies. IL-4 decreased the expression of Fas receptor on activated cells (Fig. 5) in concordance with previous reports about decreased Fas mRNA expression in IL-4-stimulated $CD4^+$ cells (75).

Bcl-2 family members are known regulators of apoptosis initiated or mediated by mitochondria (4). Western blot analysis was used to study the effect of IL-4 on the expression of Bcl-2 family members Bad, Bax, BAG-1, Becklin, Bid, Bcl-2, and Bcl-xL. Isolated $CD4^+$ T cells were activated with soluble anti-CD3, anti-CD28, and anti-mouse $F(ab')_2$ fragment, cultured for 24 h in the presence or absence of IL-4, and harvested. The levels of full-length proapoptotic Bid and antiapoptotic Bcl-2 were slightly increased by IL-4 (Fig. 6). IL-4 also increased the expression of antiapoptotic Bcl-xL protein. The antibody used for Bid detection recognizes only the nonfragmented form of the protein. The IL-4-induced effect is likely to result from decreased Bid fragmentation, thereby implicating its lower activity. As Bid is a well known target of Caspase-8 and Caspase-10 (12, 13), its decreased fragmentation implicates lower Caspase-8/10 activity. No reproducible IL-4-induced effects were seen in the expression of Bcl-2 family members Bad, Bax, BAG-1, or Becklin (data not shown).

Western blot analysis was used to study the effect of IL-4 on the expression of other factors in the Fas-induced signaling pathway. As can be seen in Fig. 7, IL-4 clearly up-regulated c-FLIP short (c-FLIPs) protein, which is known to bind Caspase-8 and to inhibit its activation (14, 76, 77). IL-4 has been shown to induce c-FLIP mRNA or c-FLIP long protein expression in other cells (78–80). Importantly we showed for the first time that IL-4 up-regulates the protein expression of c-FLIPs. There is an important difference in the function of the short and long isoforms because c-FLIP long can both stimulate and inhibit Caspase-8 and -10, but c-FLIPs has only an inhibitory effect on caspase activity (22, 81). IL-4 also increased the levels of IκB-α (Fig. 7). IκB-α is degraded after Caspase-8 induction leading to the activation of NF-κB (22, 82). Increased IκB-α levels thereby implicate decreased caspase activity in IL-4-treated cells, although the effect of IL-4 on IκB-α expression might also be caused by other factors regulating the expression and turnover of this protein.

DISCUSSION

In this proteomics study of IL-4-induced differences in activated primary CD4$^+$ T cells, we observed reproducible differential expression in a minimum of 20 proteins by 2-DE after IL-4 stimulation; from these we identified 35 unique proteins. One of the IL-4-regulated proteins was Ly-GDI, which is a known target of Caspase-3 (38–40). We showed that Ly-GDI fragmentation is decreased by IL-4 in activated CD4$^+$ T cells, suggesting that IL-4 can decrease Caspase-3 activity in these cells. Caspase-3 activity was also significantly reduced by IL-4. We further demonstrated that the activation of Caspase-3 and subsequent fragmentation of Ly-GDI is partly dependent on Fas-mediated signaling. In addition, we showed that IL-4 regulates certain proteins in the Fas-mediated pathway: IL-4 stimulation decreased the expression level of Fas receptor and increased the expression level of c-FLIPs, Bcl-2, and Bcl-xL. Key factors in the Fas-induced signaling pathway and the effect of IL-4 on their expression are summarized in Fig. 8.

When measuring the expression levels of the selected proteins with one-dimensional electrophoresis Western blot analysis, only the fragmented form of Ly-GDI was found to be differentially expressed in IL-4-treated samples (Fig. 2). There are several possible explanations as to why the rest of the selected proteins showed no IL-4-induced differences in their total protein expression. For example, the differences in the 2-DE may be post-translational, or they may be masked in one-dimensional electrophoresis by other forms of the protein

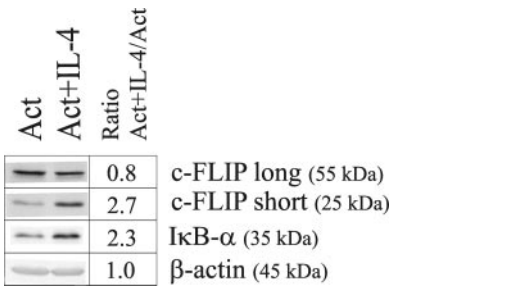

FIG. 7. **The levels of c-FLIP short and IκB-α are markedly increased by IL-4.** Isolated CD4$^+$ cells were activated with anti-CD3, anti-CD28, and anti-mouse F(ab′)$_2$ fragment (*Act*) and cultured for 24 h in the presence or absence of IL-4. The average ratios of normalized protein levels in activated + IL-4 *versus* activated cells are indicated in the *column*. Similar results were obtained from at least three independent experiments.

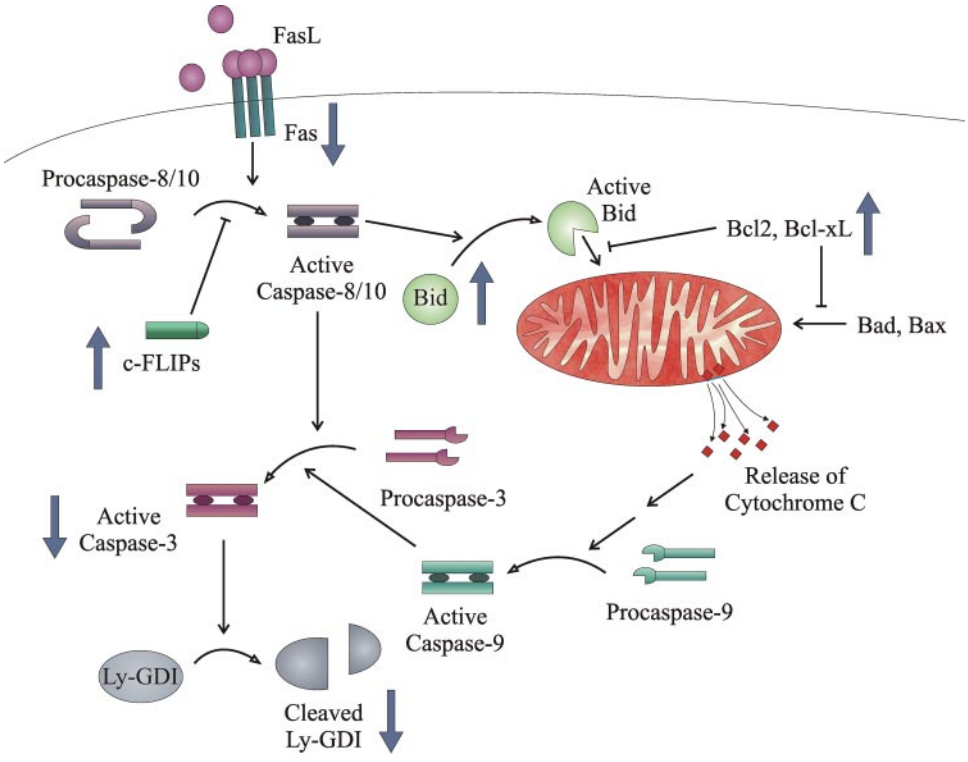

FIG. 8. **Key factors in the Fas-induced signaling pathway and the effect of IL-4 on their expression.** Binding of FasL to the Fas receptor causes the oligomerization of the receptor, which leads to the activation of initiator caspases, *i.e.* Caspase-8 or -10. These initiator caspases can directly activate Caspase-3 through a proteolytic cleavage, and they can also initiate the depolarization of mitochondrial membrane potential by cleaving Bid protein. Bid, Bad, and Bax proteins have the potential to form permeability transition pores in the outer mitochondrial membrane, leading to the release of proapoptotic substances, such as cytochrome *c*, to the cytosol. Bcl2 and Bcl-xL can inhibit the function of Bid, Bad, and Bax and thereby stabilize the mitochondrial membrane potential. Release of cytochrome *c* induces the activation of Caspase-9, which is capable of activating Caspase-3. Ly-GDI is one target protein of Caspase-3. IL-4-induced differences in protein expression are marked with *bold arrows* in the figure.

with a similar size. Other proteins identified from the same spot may be the ones that are differentially expressed after IL-4 stimulation. In addition, metabolic labeling and autoradiography used for the detection of expressed proteins in 2-DE gels do not necessarily generate similar results in comparison with methods quantifying the total protein amount. The expression of hnRNP K, αNAC, GRP94, and Ly-GDI, but not the expression of hnRNP E1, was regulated by IL-4 in one 2-DE Western experiment in a manner similar to that seen in our primary 2-DE studies (data not shown). Despite there being no differences in the total expression of hnRNP K, αNAC, or GRP94, the differences in 2-DE may still be biologically meaningful. Post-translational regulation is an important aspect of cell differentiation, and some of these proteins have already been demonstrated to have a role in differentiation in other cell systems. In addition to the fragmented form of Ly-GDI, there were two proteins identified from the 2-DE gels, namely hnRNP K and 60 S ribosomal protein P0 (spot numbers 1 and 4, respectively), that behaved in response to IL-4 in a manner opposite to that of Fas induction in Jurkat T cells in previous proteomics studies (70, 83). These findings might reflect a decreased rate of apoptosis in IL-4-stimulated cells.

IL-4 increased the expression of Bcl-2 slightly. A stronger increase could be seen in the expression of Bcl-xL (Fig. 6). Both of these antiapoptotic proteins are known to inhibit the mitochondrial depolarization step. Stat6 controls Bcl-xL gene expression both in mouse primary B cells and mast cells, and the induction of Bcl-xL correlated with the inhibition of apoptosis in these cells (84, 85) On the other hand, the induction of Bcl-2 and Bcl-xL has been reported to be unrelated to the IL-4-induced increase in cell viability of activated primary human lymphocytes because inhibition of phosphatidylinositol 3-kinase or pp70 S6 kinase inhibited the antiapoptotic function of IL-4, whereas Bcl-x and Bcl-2 induction remained normal (86). In our experiments the Bcl-2 induction by IL-4 was moderate, so its effect on Caspase-3 activity is unlikely to be very strong. The strong up-regulation of Bcl-xL by IL-4 suggests that Bcl-xL might contribute to the decreased Caspase-3 activity by inhibiting the mitochondrial depolarization step.

IL-4 has potential to regulate T cell viability, but its effects might not be so clearly antiapoptotic as have been postulated in previous studies. In murine $CD4^+$ cells, IL-4 enhances the survival of naïve T cells at least partly by maintaining Bcl-2 and Bcl-xL protein levels (84, 87, 88). IL-4 also enhances long term survival of activated wild type or CD28-deficient $CD4^+$ lymphocytes (89), induces survival of *in vivo* activated $CD4^+$ cells by inhibiting the decay of Bcl-2 and Bcl-xL (90), and rescues T cell clones from cell death induced by CD4 triggering before TCR activation (91). In all these studies, cell death was measured by PI staining, which correlates well with the results of this study (Fig. 3*b* and data not shown). On the other hand, when apoptosis is measured by staining of $AnnexinV^+$ PI^- cells, IL-4 has been shown to induce apoptosis in restimulated mouse T helper cells via an IL-2-dependent mechanism (92). When apoptosis is detected with a terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, IL-4 also increases tumor necrosis factor-mediated apoptosis of human peripheral blood lymphocytes after stimulation with *Mycobacterium tuberculosis* antigens (93). PI staining is not specific to caspase-dependent apoptosis but also detects cells that have died by other means, such as through caspase-independent apoptosis, necrosis, or autophagy (94). In this study, IL-4 decreased both Caspase-3 activity and cell death of activated T helper cells (Fig. 3), but IL-4 did not decrease the amount of early apoptotic cells that are $Annexin\ V^+\ PI^-$ (data not shown). In addition, when Fas-FasL interaction was neutralized, we observed decreased Caspase-3 activation while cell viability was increased in only one experiment (Fig. 4*a* and data not shown). This suggests that IL-4 has the potential to increase the proportion of surviving cells but presumably through mechanisms other than solely the inhibition of caspase-mediated apoptosis. Therefore, the decreased Caspase-3 activity may also have influences other than decreasing the rate of apoptosis in IL-4-treated cells. Previous studies reported that IL-4 also promotes apoptosis (92, 93), but we did not see any reproducible increase in the proportion of $AnnexinV^+$ PI^- cells in IL-4-treated samples (data not shown).

Interestingly caspase activity is related to both the activation and apoptosis of initially activated T cells. There must be a constant and tightly regulated balance between these two phenomena at this stage of T cell development. Dohrman *et al.* (95) have reported that $CD8^+$ cells show both increased caspase activity and rate of apoptosis compared with $CD4^+$ cells during the initial T cell activation. Caspase activity and apoptosis were further enhanced in c-FLIP long transgenic T cells, but the difference between $CD8^+$ and $CD4^+$ cells remained similar. Although c-FLIP long transgenic mice showed enhanced caspase activation, they were resistant to Fas-induced apoptosis after the initial activation. Other forms of T cell apoptosis, such as activation-induced cell death or spontaneous apoptosis, either remained unaltered or were increased in transgenic mice (95, 96). Although c-FLIP long-mediated activation induced proliferation in both cell types, the effect was more pronounced in $CD4^+$ T cells, whereas c-FLIP long induced apoptosis more strongly in activated $CD8^+$ T cells (95, 96). This suggests that although some level of caspase activity is well tolerated, an increase in caspase activity also correlates with a high rate of apoptosis.

c-FLIP short is known to inhibit Caspase-8 activity, which is needed for proper activation of T cells and which has an inhibitory effect on Th2 development (22, 30). The IL-4-induced up-regulation of c-FLIP short, Bid, and IκB-α (Figs. 6 and 7) suggests an overall decrease in the caspase activity, which has implications for the activation, apoptosis, and T helper cell differentiation of these activated cells.

In conclusion, we demonstrated that IL-4 regulates caspase

activity in primary naïve human $CD4^+$ T cells during the initial stage of T cell activation. We also revealed several mechanisms that IL-4 uses to inhibit the Fas-induced signaling pathway and Caspase-3 activity.

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