Identification of Novel Targets of Immunosuppressive Agents by cDNA-based Microarray Analysis*

Received for publication, September 6, 2001, and in revised form, October 29, 2001
Published, JBC Papers in Press, November 1, 2001, DOI 10.1074/jbc.M108598200

Anthony D. Cristillo and Barbara E. Bierer‡

From the Laboratory of Lymphocyte Biology, NHLBI, National Institutes of Health, Bethesda, Maryland, 20892

The immunosuppressive agents cyclosporin A (CsA) and tacrolimus (FK506) bind to unrelated intracellular immunophilin receptors, cyclophilin (CyP) and FK506-binding protein (FKBP), respectively. The complexes of CsA-CyP and of FK506-FKBP both bind to and inhibit the activity of the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin. We used cDNA microarray analysis to characterize early human peripheral blood T cell transcriptional responses following antigen receptor stimulation in the absence or presence of CsA or FK506, hoping to identify novel targets dependent upon calcineurin or immunophilins or, perhaps, specific targets of either CyP or FKBP inhibitable by one drug alone. The array data failed to identify genes uniquely sensitive to only one drug, suggesting that transcriptionally regulated, immunophilin-dependent but calcineurin-independent targets fell below the limits of detection in this system. In contrast, transcript profiling identified and mRNA and protein analysis confirmed novel as well as known genes reproducibly induced or inhibited by both immunosuppressive agents. In this context, we show that transcriptional activation of STAT5a and repression of the cytokine interleukin-16 are regulated by T cell receptor engagement and dependent upon drug-immunophilin complexes and, presumably, calcineurin activity.

The activation of T lymphocytes occurs upon engagement of the T cell receptor (TcR)-CD3 complex with peptides embedded within MHC (major histocompatibility complex) proteins presented on antigen presenting cells (1–3). The complex intracellular signaling pathways stimulated by TcR-CD3 engagement have been extensively studied. Briefly, TcR cross-linking has been shown to induce the aggregation of a number of signaling molecules into a large macromolecular complex that includes members of the Src-related and Syk/ZAP-70 tyrosine kinase families, adapter proteins (LAT, Grb2, GADS), and serine/threonine and tyrosine protein kinases and phosphatases (4–8). T cell signaling results in the phospholipase C-dependent hydrolysis of inositol 3,4,5-trisphosphate that leads inter alia to calcium (Ca^{2+}) mobilization (9–11). An increase in intracellular Ca^{2+}, together with calmodulin, activates calcineurin (phosphatase 2B or PP2B (12)), a serine/threonine phosphatase that is required for the activation and nuclear translocation of a number of transcription factors (13–16) including nuclear factor of activated T cells (NFAT) (17, 18); NFAT activity regulates the transactivation of a number of cytokine and other genes, including interleukin (IL)-2, IL-3, IL-4, IL-12, inflammatory mediators (e.g. TNFα), and growth factors (e.g. granulocyte/macrophage colony-stimulating factor) (19–22). There is considerable evidence to suggest that calcineurin may have both positive and negative effects on lymphoid and nonimmune cells and that NFAT is only one of a number of transcription factors regulated by calcineurin (15, 16, 23).

Widely used in solid organ and stem cell transplantation, cyclosporin A (CsA), a fungal cyclic undecapeptide, has been shown to bind to a family of intracellular immunophilin receptors, the cyclophilins (CyP) (24, 25). Tacrolimus (FK506), an immunosuppressant structurally unrelated to CsA but with biological properties similar to those of CsA, was found to bind to members of an intracellular immunophilin family termed FK506-binding protein (FKBP) (24, 25). The CyP and FKBP immunophilin families are highly conserved, ubiquitously expressed proteins that share the ability to catalyze the cis to trans isomerization of proline residues and are thus thought to play a role in protein folding and transport (24, 26–28). Although CsA and FK506 bind to and inhibit the isomerase activity of CyPs and FKBP, respectively, it is not the inhibition of this enzymatic activity that correlates with immunosuppression (29). The complexes of CsA-CyP and, independently, of FK506-FKBP bind to and inhibit the activity of the serine/threonine phosphatase calcineurin (30, 31).

We used cDNA-based microarray analysis to obtain a more comprehensive view of CsA- and FK506-sensitive early genes in purified human peripheral blood T lymphocytes (PBL). The complexity of large-scale gene expression analysis was aided by the use of simultaneous hybridization using two different cDNA samples each labeled with a different fluorophore. We reasoned that changes in gene expression common to both CsA and FK506 treatment would likely be secondary to inactivation of calcineurin phosphatase activity. Additionally, perturbations in gene expression unique to one drug alone would potentially identify specific immunophilin (CyP or FKBP)-dependent gene targets. We failed to identify transcripts specifically regulated by only one immunosuppressive agent, suggesting that immunophilin-dependent, calcineurin-independent gene expression was below the limits of detection of our analysis. However, we did identify a number of CsA- and FK506-sensitive (and calcineurin-dependent) genes induced or inhibited following anti-CD3 mAb ligation in the presence of phorbol 12-myristate 13-acetate relative to resting cells. Although a...
number of these transcripts are well characterized (e.g. IL-2 and lymphoactin), the array data suggested a number of novel calcineurin-dependent substrates. Analysis of mRNA tran-scription and protein in both CD4+ and CD8+ T cell subpopulations, used to verify and extend these cdna microarray results, demonstrated both induction of Stat5a and inhibition of IL-16, genetic elements not previously appreciated to be affected by either CsA or FK506. Hierarchy profiles of these target genes suggested relative specificity of these drugs.

EXPERIMENTAL PROCEDURES

Cells—Human PBL were isolated from volunteer donors by sphere-cell. Cells were then subjected to reverse flow elutiation and Ficoll-Hypaque centrifugation and washed with 1x phosphate-buffered saline (PBS). PBL were resuspended in RPMI 1640 (MediTech, Herndon, VA) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 2 mM l-glutamine, 10% heat inactivated fetal calf serum (Invitrogen), 2 mM l-glutamine, 10 mM HEPES, pH 7.2, 100 units/ml penicillin, 100 µg/ml streptomycin (MediaTech), and 50 µg 2-mercaptoethanol (Bio-Rad, Hercules, CA), termed 10% RPMI, and incubated at 37 °C, 5% CO2-in-air. After overnight incubation, cells were stimulated as indicated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Calbiochem), 1 µM ionomycin (Calbiochem), plate-bound (10 µg) or soluble (100 µM) anti-CD3 mAb OKT3 (American Type Culture Collection, Manassas, VA), 1 µM anti-CD28 mAb 9.3 (the kind gift of Carl June, University of Pennsylvania, Philadelphia). CD4+ and CD8+ T lymphocytes were purified by negative selection using an indirect magnetic labeling system and the MidiMACSTM columns (Miltenyi Biotec, Auburn, CA).

RNA Preparation and cdna Microarray Analysis—Total RNA was prepared from resting or stimulated human PBL using TrizolTM (Invitrogen). Poly(A) mRNA was extracted using Oligotex mRNA midi-kit (Qiagen, Valencia, CA) and quantitated using RiboGreen™ RNA quantitation kit (Molecular Probes, Eugene, OR). cdna from resting or stimulated, CsA-, FK506-, or ethanol-treated cells were labeled with Cy3 and Cy5 fluorescent dyes for microarray hybridization. Fluorescently labeled cdna were hybridized to a human microarray (IncyteGenomics, Palo Alto, CA). Data were analyzed using GemTools™ software and are expressed as balanced differential.

RT-PCR—Total RNA was prepared from human PBL using Trizol™ (Invitrogen) and quantitated using A260 and a RiboGreen™ RNA quantitation kit (Molecular Probes). mRNA levels were assessed using the Onestep RT-PCR kit (Qiagen) using the following oligonucleotide purified primers (Biosequest Biotechnologies, Lawrence, MA; where F is forward and R is reverse): Stat5a-F, 5'-GAG TCT CAG TTC AGT GTT GGC AGC-3'; Stat5a-R, 5'-AGT CAC TAA AGC GCA ACA AGA TC-3'; IL-16-F, 5'-TGC TGG TCT TGG CAG CTT GGC-3'; IL-16-R, 5'-GTC CTC CTT AGG AGC TGT CAG CAG-3'; β-actin-F, 5'-ATC TGG TAC CAC ACC TTC TAC AAT GAG CTC CG-3'; β-actin-R, 5'-CGT ACT CCT GCT TGC TGA TCA ACC ACA GC-3'; IL2-F, 5'-CGT ACT CTT TGC TGA TCA ACC ACA GC-3'; IL2-R, 5'-CGT TGA TAT TGC TGA TTA GAT CCC TG-3'.

RT-PCR was performed using the following conditions: 50 °C for 30 min, 95 °C for 15 min, 30 cycles of (i) 94 °C for 1 min, (ii) 55 °C for 1 min, and (iii) 72 °C for 1 min, and 72 °C for 10 min. Samples were analyzed by gel electrophoresis, and bands were stained by phoshorimaging analy-sis using ImageQuant software, and mRNA levels were normalized to β-actin mRNA levels as indicated.

Western Blotting—After stimulation of human PBL or purified CD4+ or CD8+ T lymphocytes as indicated, samples were centrifuged at 466 × g for 5 min and washed once with RPMI 1640. Cells were resuspended in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 10 mM NaF, 10 mM sodium pyrophosphate, Na2VO3, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin). Samples were incubated for 20 min on ice and then centrifuged at 17,530 × g. The supernatant containing the post nuclear lysate was removed, and proteins were separated by SDS-PAGE (Pro-togel, National Diagnostics, Atlanta, GA). Electrophoresed proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), immunodecorated with goat anti-human Stat5a antobody (clone 89, Transduction Laboratories, Lexington, KY), and det-ected by enhanced chemiluminescence (ECL, Amersham Biosciences, Inc.) according to the manufacturer’s instructions.

Enzyme-linked Immunosorbent Assay—Human CD4+ and CD8+ T cells were stimulated with PMA (10 ng/ml), PMA + ionomycin (1 µM), anti-CD3 (100 ng/ml) + PMA, or anti-CD3/anti-CD28 (1 µg/ml) + PMA, cells were centrifuged for 5 min at 1500 rpm, and supernatants were collected to assay for IL-16 protein. 96-well Nunc immunoplate with Maxisorp™ surface (Nalge Nunc Int., Rochester, NY) were coated with 4 µl mouse anti-human IL-16 antibody (50 µl/well) (clone 14.1, Pharmingen, San Diego, CA), and incubated overnight at 37 °C. Wells were washed with 0.02% Tween 20 (1x PBS) and incubated with 0.5 µg/ml biotinylated goat anti-human IL-16 (100 µl/well; R&D Systems, Minneapolis, MN) for 1 h at 37 °C. Wells were washed as described above and then incubated with horseradish peroxidase-conjugated streptavidin (1:1000, 100 µl/well) for 1 h at 37 °C. Finally, 3,3',5,5'-tetramethylben-zidine liquid substrate (100 µl/well, Sigma) was added to each well (10 min) followed by the addition of 0.5x sulfuric acid (100 µl/well). The signal was detected using the Fmax colorometric plate reader (Molecular Devices).

RESULTS

Inhibition of Gene Expression in Activated Human Peripheral Blood T Cells by Immunosuppressive Agents—To identify transcriptional events regulated coordinately or differentially by the immunosuppressive agents CsA and FK506, we compared resting and activated human peripheral blood T cells cultured in the presence or absence of either vehicle or drug. Purified human T cells were stimulated using immobilized anti-CD3 (pCD3) mAb in concert with PMA, an agent used to activate classical forms of protein kinase C; each was used at concentrations and culture conditions known, and previously optimized, to induce IL-2 production and proliferation of T cells (data not shown). We isolated poly(A)1-selected RNA from appropriately cultured cells using standard methods, synthesized fluorescently Cy5- or Cy3-labeled cdna from each culture, and hybridized these to cdna microarrays using the Human-1.0 microarray (IncyteGenomics Inc.). The cdna microarray chosen represents ~10,000 human cdna clones representing unique human genes identified in the NCBI Unigene data base. The distribution of fluorescence intensity ratios between cdna prepared from resting and activated cells and between cdna from cells activated in the absence of or in the presence of either CsA or FK506 was compared and analyzed using standard methodology (Fig. 1). The majority of the genes represented were not appreciably affected either by the 4-h stimulation or by incubation with immunosuppressive agents. However, the comparison of unstimulated to anti-CD3 mAb plus PMA-stimulated cells revealed a number of genes in which expression was found to be significantly different (Fig. 1). Of genetic elements induced by anti-CD3 mAb plus PMA stimulation, ~2000 genes had differential values between 1.0 and 1.9, whereas 76 had values ≥1.7 and were thus considered induced by T cell activation. In addition, 105 genes were found to have differential values between 2.0 and 4.9, 13 genes had values between 5.0 and 9.9, and four genes had differential values greater than 10.0. An obvious advantage of this approach was that well characterized genes (e.g. IL-2, TNFα, lymphoactin) were identified that served as robust internal controls. Of genetic elements down-regulated by T cell activation, ~4,500 genes had differential values between -1.0 and -1.9, whereas 140 of them had values ≤ -1.7; 112 genes had differential values between -2.0 and -4.9, three genes had values between -5.0 and -9.9, and one gene had a differential value less than -10.0. Thus, multiple transcriptional events occur during the very early stages of T cell activation.

To identify genes in which expression levels were differentially modulated by CsA and FK506, we compared the differential expression pattern of gene elements using fluorescently
modified probes prepared from 4-h stimulated (plate-bound anti-CD3 mAb plus PMA) human PBL cultured in the presence of CsA, FK506, or vehicle alone. Twenty-seven genes were identified in which expression levels were consistently inhibited by both immunosuppressants (Table I). Among the genes identified, 22 corresponded to genes induced upon T cell activation and five showed no change with activation (compared with resting cells) but were nevertheless inhibited by drug. Again, genes previously reported to be sensitive to CsA and/or FK506 inhibition were identified, including lymphotactin, L-selectin, TNFα, and IL-2 (32–35). Importantly, we identified known (e.g. Stat5a) and novel (e.g. EST AA770150) CsA- and FK506-sensitive genetic elements that had not been reported previously.

Regulation of Stat5a mRNA and Protein in Human Peripheral Blood T Cells—To confirm the cDNA microarray data (Table I), the regulation of Stat5a mRNA was explored further. Stat5a is a member of the STAT family of transcription factors that has been shown to mediate cytokine, growth factor, and hormone responses (36, 37) and to play a critical role in cell cycle progression. Phosphorylated STAT proteins form homodimeric and heteromeric complexes that, together with other transcription factors, induce transcriptional activation of a number of target genes. Among the STAT family of proteins are two closely related Stat5 proteins, Stat5a and Stat5b, that are both activated by a number of cytokines in inducing IL-2 and IL-4 (38, 39) and that have both been shown to be immediately and transiently tyrosine-phosphorylated following T cell activation (40, 41). The importance of Stat5a and Stat5b in T cell function has been underscored by mice rendered genetically deficient in either Stat5a and Stat5b; the lymphocytes derived from these mice display defects in both T cell proliferation and function (42–44). Although the role of and factors that influence Stat5a in T cell cycle progression have been extensively studied, much less is known about the transcriptional regulation of its expression.

Stat5a mRNA from unstimulated or treated peripheral blood T cells was analyzed by reverse transcription (RT)-PCR (Fig. 2). The high basal expression of Stat5a mRNA in resting, purified human PBL (Fig. 2A, upper panel, lane 1) was enhanced by stimulation with the calcium ionophore ionomycin (lane 3) and further induced by the combination of PMA and ionomycin (lane 5). Cyclosporine pretreatment minimally reduced basal Stat5a mRNA expression (lane 2) and partially attenuated ionomycin (lane 4) and ionomycin plus PMA (lane 6)-dependent Stat5a transcriptional activation. As expected, IL-2 mRNA levels were present at very low levels in resting human PBL (Fig. 2A, lower panel, lane 1) and were induced minimally by calcium agonists (lane 3) and more significantly by PMA plus ionomycin (lane 5). As anticipated (45, 46), CsA reduced both basal and stimulated IL-2 transcription. The induction of Stat5a mRNA by PMA plus ionomycin was found to be sensitive to incubation with the inhibitor of transcription actinomycin D (ActD, Fig. 2B). This latter finding suggests that Stat5a mRNA induction was dependent, at least in part, on new mRNA transcription and was not due solely to mRNA stabilization (Fig. 2B). Furthermore, Stat5a mRNA levels in stimulated cells pretreated with both CsA and ActD was less than that found in cells pretreated with CsA alone, implying that new Stat5a mRNA synthesis contributes not only to inductive, stimulation-dependent (and CsA-inhibitable) mRNA but also to basal Stat5a expression. Similar results were noted in samples derived from cells stimulated with anti-CD3 mAb (OKT3) plus PMA (data not shown).

To determine whether the induction and suppression of Stat5a mRNA translated into changes in protein expression,
post-nuclear lysates of appropriately treated PBL were subjected to immunoblotting analysis (Fig. 2C). Compared with resting cells, Stat5a protein was induced in cells stimulated with ionomycin, PMA, PMA plus ionomycin, anti-CD3 mAb, or anti-CD3 plus anti-CD28 mAb (Fig. 2C). Although CsA pretreatment attenuated Stat5a protein expression in ionomycin-, anti-CD3 mAb plus PMA-, or anti-CD3/anti-CD28 mAb plus PMA-treated cells, CsA had no appreciable effect on PMA plus ionomycin- or PMA-only treated cells. Purified CD4+ and CD8+ subpopulations of T cells demonstrated a similar pattern of responses (Fig. 3). Note that the basal expression of Stat5a was reproducibly greater in resting purified CD8+ T cells compared with CD4+ T cells. Although some variability was noted in the extent of CsA-dependent inhibition between different healthy donors, the pattern was similar in all experiments. Taken together, these results confirm and extend the results of the cDNA microarray analysis to demonstrate CsA- and FK506-sensitive induction of Stat5a mRNA and protein following activation of both CD4+ and CD8+ T cell subpopulations.

**Gene Expression Induced by Immunosuppressive Agents in Activated Human PBL—CsA and FK506 are known to inhibit transcriptional activation of a number of cytokine and other genes. However, the ability of these immunosuppressive drugs to induce or up-regulate transcription is less well appreciated. In addition to genes inhibited by drug, the differential cDNA microarray analysis identified 21 genes that were induced similarly by both immunosuppressive agents (Table II). Of the genes up-regulated by drug, eight corresponded to genes identified as down-regulated following T cell activation. Thus, treatment with immunosuppressive agents prevented activation-induced down-modulation. Eleven genes were unchanged by T cell stimulation and two were up-regulated (and, therefore, “superinduced” by drug). Novel genes of unknown function (e.g., KIAA0985, KIAA0135, and KIAA0430) as well as known genes not previously reported to be CsA/FK506-sensitive (e.g. IL-16) were identified. Further analysis of IL-16, a cytokine not previously known to be regulated by CsA or FK506, was explored further.**

**Regulation of IL-16 mRNA and Protein Secretion in CD4+ and CD8+ T Cells—**The biologically −13-kDa active and secreted form of IL-16 is cleaved from a 67-kDa (pro-IL-16) precursor protein (47) and acts upon CD4+ T cells, monocytes, and eosinophils by binding to CD4 and potentially to other receptors. Although originally described as a product of CD8+ T lymphocytes, IL-16 has now been shown to be synthesized by both CD4+ and CD8+ T cells (48-50). Although the importance of the IL-16 protein product in regulation of cell adhesion, chemotaxis, cell cycle progression, and HIV/SIV (human immunodeficiency virus/simian immunodeficiency virus) infectivity (51-54) is well appreciated (47), the transcriptional regulation of IL-16 is poorly understood.

To verify not only the CsA and FK506 sensitivity but also the T cell activation-induced down-modulation of IL-16 mRNA (Table II), unstimulated and treated purified human CD4+ and CD8+ T cells were analyzed. Basal IL-16 mRNA transcription in both CD4+ and CD8+ T cell subpopulations was approximately comparable (Fig. 4, lanes 1 and 2). IL-16 mRNA was unchanged by treatment with PMA (lanes 2 and 12), but down-regulated by treatment with PMA plus ionomycin (lanes 3 and 13), anti-CD3 mAb plus PMA (lanes 4 and 14), and anti-CD3/anti-CD28 mAb plus PMA (lanes 5 and 15). CsA pretreatment had minimal effects on IL-16 mRNA in PMA-treated cells (lanes 7 and 17) or anti-CD3/anti-CD28 mAb plus PMA-treated cells (lanes 10 and 20) but was found to eliminate the attenuation of IL-16 mRNA in PMA plus ionomycin (lanes 8 and 18).
Stat5a mRNA and protein levels in human PBL were assessed by RT-PCR (A and B) and immunoblotting (C) assays. A, cells were treated for 6 h with either ethanol diluent (lanes 1 and 2 from left), ionomycin (Iono, lanes 3 and 4), or PMA plus ionomycin (PMA + Iono, lanes 5 and 6 from left) in the absence (lanes 1, 3, and 5 from left) or presence (lanes 2, 4, and 6) of CsA. Total RNA was prepared (See Fig. 1), and RT-PCR was carried out as described (see “Experimental Procedures”) using Stat5a- and IL-2-specific primers. Stat5a and IL-2 bands were quantitated, and values expressed in arbitrary units (a.u.) using ImageQuant software are represented graphically. B, stimulation of human PBL with PMA + ionomycin was carried out for 2, 4, 6, and 12 h in the absence or presence of CsA (1 μM, 0.5 h preincubation) and/or actinomycin D (2.5 μg/ml, 1 h preincubation) as described. C, total RNA was prepared, and RT-PCR analysis was conducted as described in A. For protein expression studies, cells were treated for 6 h with ethanol diluent (lanes 1 and 7), soluble anti-CD3 mAb, anti-CD28 mAb (designated αCD3/28) plus PMA (C, lanes 2 and 8 from left), anti-CD3 mAb plus PMA (lanes 3 and 9 from left), PMA plus ionomycin (lanes 4 and 10 from left), ionomycin alone (lanes 5 and 11 from left), and PMA alone (lanes 6 and 12 to each). Cells were stimulated in the absence (lanes 1–6) or presence (lanes 7–12) of CsA. Cells were lysed as described (see “Experimental Procedures”), and proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with a mouse anti-human Stat5a antibody (89). The Stat5a band was detected by ECL and is indicated by the arrowhead. Bands were quantitated and values expressed in arbitrary units (a.u.) using ImageQuant software and are graphically represented. The results (A, B, and C) of one experiment, representative of at least two experiments carried out using human PBL from different human donors, is shown.
stimulation. Consistent with the cDNA microarray analysis, these results demonstrate that stimulation of both CD4+ and CD8+ T cell subpopulations down-regulates IL-16 mRNA in a CsA- and FK506-sensitive fashion.

To determine whether the drug-sensitive and stimulation-dependent modulation of IL-16 mRNA translated into functional differences in IL-16 secretion, cell supernatants from stimulated CD4+ and CD8+ T cells were assayed for secreted...
IL-16 protein. Soluble IL-16 from CD4+ T cells decreased following a 6-h stimulation with PMA, PMA plus ionomycin, anti-CD3 mAb plus PMA, or anti-CD3/anti-CD28 mAb plus PMA compared with resting cells (Fig. 5, upper left panel). Although CsA addition did not affect IL-16 secretion basally or in response to anti-CD3 mAb plus PMA stimulation, CsA increased IL-16 secretion in response to PMA, PMA plus ionomycin, and anti-CD3/anti-CD28 mAb plus PMA. At 24 h following stimulation, the response differed. Although soluble IL-16 decreased in response to PMA plus ionomycin, the quantitated IL-16 remained virtually unchanged after PMA or anti-CD3/anti-CD28 mAb plus PMA treatment and was increased by anti-CD3 mAb plus PMA treatment relative to resting cells (Fig. 5, lower left panel). Surprisingly, CsA addition was found to increase secreted IL-16 levels in all stimulated conditions examined.

The pattern of responses to stimulation and to drug in CD8+ T cells differed somewhat from that of CD4+ T cells. Stimulation with PMA alone and, to a lesser extent, anti-CD3 mAb plus PMA induced IL-16 secretion at both 6 and 24 h; induction was sensitive to CsA treatment. The addition of anti-CD28 to anti-CD3 mAb plus PMA also increased measured IL-16 protein, but the response to CsA changed with time; CsA increased IL-16 at 6 h but not at 24 h. In contrast, PMA plus ionomycin decreased (6 h) or failed to change (24 h) IL-16 levels in the supernatant; CsA treatment nevertheless increased the measured amount of cytokine. IL-16 protein expression correlated with mRNA transcriptional studies under some but not all treatment conditions, consistent with the complex regulation of IL-16 protein production, processing, and/or secretion previously reported.

Gene Clusters Reveal Selectivity of CsA and FK506 Action—

The transcript profiling analysis described above identified several genes, both known and novel, that are CsA- and FK506-sensitive, allowing for the possibility that these genes share a transcriptional regulatory mechanism mediated by a common target of CsA and FK506, the calcium/calmodulin-dependent, serine/threonine phosphatase calcineurin. To compare the expression profiles of resting versus activated in the absence versus presence of drug, the data sets were analyzed using GEMTools™ software (IncyteGenomics, Inc.). This analysis software serves three purposes. First, it manages and analyzes the results of GEM™ microarray experiments by utilizing an image recognition algorithm that interprets the scanned images of a processed GEM™ array. Next, it compares the results against a data base to identify which genes are differentially expressed in the two cell samples and to what degree. Finally, it allows the data to be queried to allow clustering of target gene elements into defined enzyme, function, and pathway hierarchies. The “enzyme” hierarchy (Incyte) is based on the EC enzyme classification system, currently maintained by the IUBMB Joint Commission on Biochemical Nomenclature (76). The six groups within this hierarchy include oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. The designated “function” hierarchy (Incyte) classifies proteins based on biochemical function and/or localization. The scheme accommodates enzymes and other proteins into the following seven groups of gene elements: signal transduction and regu-
Novel T Cell Targets of Immunosuppression

![Diagram](image)

**Fig. 5. Analysis of secreted IL-16 from both CD4+ and CD8+ T cell subpopulations.** Human CD4+ and CD8+ T cells were isolated and stimulated as described in the legend for Fig. 4. The levels of IL-16 in sample supernatants were detected by ELISA as described (see “Experimental Procedures”). IL-16 levels in supernatants were interpolated by comparison with a standard curve using recombinant human IL-16 (PharMingen).

It has been approximately a decade since calcineurin was identified as the common target of CsA and FK506 action. Since that time efforts have focused on the identification of substrates of calcineurin and, in particular, of NFAT, highlighting their essential role in cytokine transcriptional activation. The production of many immediate early genes and cytokines, including IL-2, IL-3, IL-4, IL-12, granulocyte/macrophage colony-stimulating factor, and TNFα (19–22, 55, 56), has been shown to be sensitive to inhibition by the immunosuppressants CsA and FK506. The identification and analysis of downstream targets of immunosuppressants in resting T lymphocytes has been biased, however, toward genes such as cytokines, known to be regulated by the NFAT family. Here, we used cDNA microarrays to identify genes in which expression levels are regulated by T cell signaling and further regulated by a common target of CsA and FK506, which is presumably calcineurin. Although calcineurin is the only common target of CsA-CyP and FK506-FKBP known to date, it remains formally possible that any gene identified by our analysis is regulated not by calcineurin but by another, heretofore unknown, common target of CsA and FK506. Consistent with this notion, work by Matsuda and co-workers (57) demonstrates the ability of CsA and FK506 to exert their immunosuppressive effects not only by targeting calcineurin-dependent NFAT but also calcineurin-independent activation pathways for c-Jun NH2-terminal kinase (JNK) and p38; the specific target(s) of the drug-
immunophilin complexes, however, were not identified. It is also formally possible that a gene may be regulated both by a target of CsA and an unrelated target of FK506, resulting in the same transcriptional outcome (induction or inhibition by drug). We consider this latter possibility remote, as we failed to identify any genes affected by one drug and not by the other. The likelihood that, in early T cell activation, two distinct molecular targets would always converge to regulate a subset

**FIG. 6.** Enzyme, function, and pathway hierarchy profiles reveal specificity of CsA and FK506 action. GEMTools™ software (IncyteGenomics, Inc.) was used to categorize the genes in which expression levels were differentially modulated by T cell activation and by CsA and FK506 into hierarchical groups. Enzyme (A), Function (B), and Pathway (C) hierarchy plots were then constructed that showed the Fold Differential expression of target genes modulated by either T cell activation (yellow circles) or by CsA (red triangles). The treatment with FK506 (See Tables I and II) was similar to CsA in the differential expression patterns of target genes and, for purposes of clarity, is not represented in this figure.
of genes commonly and with a similar outcome (induction or inhibition) is improbable. Subsequent analysis will confirm whether the genes identified are regulated by NFAT, by another calcineurin-dependent transcription factor (e.g., NFκB, Elk-1), or by a calcineurin-independent, immunophilin-dependent target of CsA and FK506.

In addition to identifying novel gene targets common to both of the immunosuppressants CsA and FK506, we also sought to use this cDNA microarray analysis to identify genes in which transcriptional regulation was dependent upon the isomerase activity of either the cyclophilin or FKBP immunophilin families (and inhibited by drug). Although many members of these families of highly conserved proteins have been characterized (26–28), their biological roles in vivo remain largely elusive. Our study did not uncover any genes that were differentially modulated by CsA compared with FK506. Our study was limited, however, to a restricted stimulation condition using resting human peripheral blood T lymphocytes as the cell source. We cannot rule out the possibility that under different experimental conditions or using a different tissue source, immunophilin-dependent gene expression may be uncovered. It is also possible that cyclophilin and FKBP isomerase activities act only post-translationally to affect protein folding or protein transport; approaches employing proteomics will be instrumental for this analysis. The less likely alternative, that isomerase- and immunophilin-dependent gene expression is regulated indiscriminately by the class of enzymes (despite differing substrate affinities of individual members of the class), will be more appropriately addressed in yeast, where genetic variants lacking all cyclophilins, or FKBPs, or both have been generated and are viable.

Our analysis identified 27 genes in which expression levels were consistently inhibited by both immunosuppressants (Table I). Of these genes, 22 were induced upon T cell activation, whereas five showed no change with activation. In addition to these genes inhibited by drug, 21 genes were identified that were induced similarly by both immunosuppressive agents (Table II). Of these genes, eight were down-regulated following T cell activation, 11 were unchanged but induced by drug, and two were up-regulated and further induced by drug. Genes previously reported to be sensitive to CsA- and/or FK506-mediated inhibition were identified, including IL-2, lymphotactin, L-selectin, and TNFα (32–35, 58). Moreover, our findings that SLAM, IRF4, IL-16, and KIAA0135 are CsA- and FK506-sensitive are consistent with data recently reported by Feske et al. (58). Although we used resting human peripheral blood T lymphocytes stimulated in the presence or absence of drug, Feske and co-workers (58) used continuously growing T cell lines derived from human peripheral blood lymphocytes of healthy donors and severe combined immunodeficiency patients with a principal defect in T cell activation (attributed to a calcium influx defect). It is reassuring that certain identified gene elements were common to these two different studies, which used different T cell sources, different stimulation conditions, and different cDNA microarray templates; many other elements, of course, differed. The cluster analysis of our data suggested relative specificity of genetic elements regulated by the calcineurin inhibitors (Fig. 6). Our study also recovered several expressed sequence tags (ESTs) that are modulated by T cell activation and are sensitive to both CsA and FK506. These ESTs are the focus of current studies that may provide further insight into novel immunophilin- (and calcineurin)-dependent T cell signaling pathways.

One of the gene products identified as being CsA- and FK506-sensitive and selected for further investigation in our study was Stat5a. Although Stat5a protein tyrosine phosphorylation, activation, and signaling have been studied extensively, far less is known regarding the regulatory mechanisms governing Stat5a gene expression. Consistent with previous findings (59, 60), we noted high basal expression of Stat5a mRNA in human resting PBL, unaffected by drug treatment of the cells (Fig. 2, A and B). Stat5a mRNA increased in response to stimulation with the calcium ionophore ionomycin and by both PMA and ionomycin; mRNA induction was actinomycin D-sensitive (Fig. 2, A and B) and thus dependent on new message synthesis and not solely upon mRNA stabilization. That Stat5a gene expression was dependent on both calcium- and PKC-mediated pathways is consistent with a previous report demonstrating an increase in Stat5a mRNA in human peripheral blood mononuclear cells following T cell mitogen (phytohemagglutinin) stimulation (60). The data presented here revealed Stat5a to be CsA/FK506-sensitive at the level of both mRNA and protein, suggesting calcineurin-dependent regulation of Stat5a. This model would predict a calcineurin target (i.e., NFAT or other transcription factor) within the Stat5a promoter. Consistent with this model, sequence analysis of the Stat5a 5′ nucleotide sequence, upstream of the transcriptional start site, revealed one putative NFAT recognition element adjacent to a putative AP1 element. Detailed promoter analysis of the Stat5a promoter element will be required to verify whether this site mediates CsA and FK506 sensitivities.

The significance of drug regulation of Stat5a is not restricted to confirmation of the microarray results reported here. Stat5 activated by IL-2 enhances IL-2Rα gene expression and cell cycle progression (42, 61). In addition, however, the induction of Stat5a mRNA and protein upon early T cell activation (largely in advance of IL-2 production and secretion by the T cell) may influence the kinetics and requirements for activation of responding T cells, differing between resting T cells, which express basal levels of Stat5a, and activated T cells, in which Stat5a is dramatically induced. Stat5 activation has also been shown to regulate Fas ligand expression and activation-induced cell death (62). Although CsA and FK506 have been shown to regulate apoptosis by a number of mechanisms including mitochondrial permeability, NFAT-dependent Fas ligand expression, and MEK2, inhibition of Stat5a expression may be another means by which CsA and FK506 regulate cell death.

Recent work by Yamashita et al. (63) has demonstrated that Stat5 becomes physically associated with the IL-4 receptor in optimally stimulated, anti-TCR-activated Th2 cells, an event inhibitable by FK506. Moreover, inhibition of Stat5 activation resulted in diminished IL-4-induced proliferation, suggesting that IL-4-induced Stat5 activation is required for the expansion of developing Th2 cells. Our data would suggest that, in addition to calcineurin regulation of the IL-4 receptor signaling complex, events inhibitable by FK506 regulate Stat5 expression and thereby Th2 development.

Although encoded by distinct genes, Stat5a and Stat5b show 96% sequence similarity at the protein level (64) and have been shown to form heterodimers with each other and with other proteins. These heterodimers bind DNA recognition domains in the promoters of downstream genes and result in their transactivation (65) functioning as both positive and negative regulators (66). Although Stat5b mRNA was not represented within the human GEM™ microarray used, immunoblot analysis of the Stat5b protein (Fig. 2C, lane 3 from left) (67, 68) revealed, for several stimulation conditions, an expression profile similar to that of Stat5a. Stat5b protein quantitatively increased following treatment of human PBL by ionomycin as well as by PMA plus ionomycin, anti-CD3 mAb plus PMA and anti-CD3/CD28 plus PMA,
each partially inhibited by CsA in both CD4+ and CD8+ T cells. The requirements for Stat5α and Stat5β activation are similar (69, 70), and our data would suggest that regulation of Stat5α and Stat5β protein expression may also be comparable.

IL-16 was the second CsA- and FK506-sensitive gene target that we identified and investigated. IL-16 functions as a chemottractant, a modulator of T cell activation, a ligand for CD4, and thereby an inhibitor of immunodeficiency virus replication (47, 51–54, 71). We have shown down-regulation of IL-16 mRNA following T cell stimulation with PMA plus ionomycin, anti-CD3 mAb plus PMA, and anti-CD3/CD28 plus PMA, consistent with previous findings (50). Activation-dependent IL-16 down-regulation may be reversed by CsA or FK506; the transcription factors regulating IL-16 mRNA induction are not yet known. It is interesting to note that sequence analysis of the IL-16 promoter revealed several putative Elk-1 binding sites; the involvement of Elk-1, itself regulated by calcineurin, will be the subject of future analysis. Although the effects of T cell activation and CsA/FK506 pre-treatment on secreted IL-16 protein (Fig. 5) were consistent with expression patterns of IL-16 mRNA in certain stimulation conditions, several inconsistencies were also noted. The complexity of IL-16 mRNA and protein suggests that CsA and FK506 may influence IL-16 secretion by both direct (transcription-independent) and indirect (transcription-dependent) calcineurin-mediated mechanisms.

Finally, analysis of hierarchy profiles of target genes differentially regulated by T cell activation and by immunosuppressive drug treatment revealed the wide spectrum of genetic elements that was induced or inhibited as a consequence of T cell activation, consistent with previous findings (58, 72–75). In our data, we demonstrated that pre-treatment of human resting PBL with CsA or FK506 resulted in the modulation of only a limited subset of genes from particular hierarchy profiles. These findings suggest that the actions of CsA and FK506 are highly specific. Further analysis will allow definition of functional signaling pathways required to activate certain transcription factors that in turn coordinateably control gene expression. Our analysis using resting human peripheral blood T cells must now be complemented by and compared with model systems in which known proteins (e.g. calcineurin, NFAT) are genetically modified to allow clarification of signaling pathway intermediates and assignment of function.

Acknowledgments—We thank William W. Crouch for helpful discussions, and we appreciate the technical assistance of Kai Chang.

REFERENCES
1. Weiss, A., and Littman, D. R. (1994) Cell 76, 263–274
2. Chan, A. C., and Shaw, A. S. (1996) Immunity 4, 266–273
3. Espevik, T., Figari, I. S., Shalaby, M. R., Lackides, G. A., Lewis, G. D., Shepard, H. M., and Palladino, Jr., M. A. (1989) J. Exp. Med. 169, 751–756
4. Lin, J. X., Migone, T. S., Tsang, M., Friedmann, M., Weatherbee, J. A., Zhou, X. (1998) Science 289, 353–356
5. Lin, J. X., Mietz, J., Modi, W. S., John, S., and Leonard, W. J. (1996) J. Biol. Chem. 271, 1370–1377
6. Yoshimura, N., and Kahan, B. D. (1985) J. Biol. Chem. 260, 1370–1377
7. Yoshimura, N., and Kahan, B. D. (1985) Transplantation 40, 661–666
8. Kornfeld, H., Cruikshank, W. W., and Kalluri, R. (1999) J. Leukocyte Biol. 66, 425–435
9. Chupp, G. L., Wright, E. A., Wu, D., Vallen-Mashikian, M., Crouch, W. C., Center, D. M., and Cruikshank, W. W. (1997) J. Immunol. 158, 5210–5215
10. Yu, C. R., Young, H. A., and Ortaldo, J. R. (1998) J. Immunol. 161, 2114–2119
11. Matuda, S., Shibusaki, F., Takehara, K., Mori, H., Nishida, B., and Koyasu, S. (2000) EMBO Rep. 1, 428–433
12. Keskar, S. N., Bailey, D., and Crouch, W. W. (1996) Immunity 5, 1591–1599
13. Klee, C. B., Ren, H., and Wang, X. (1998) J. Immunol. 160, 13367–13370
14. Frantz, B., Nordby, E. C., Boren, G., Steffen, N., Paya, C. V., Kincade, R. L., Toce, M. J., O’Keefe, J. E., and O’Neill, E. A. (1994) EMBO J. 13, 389–393
15. Schaninger, M., Blume, R., Kruger, M., Lux, G., Oetjen, E., and Kneipel, W. (1995) J. Biol. Chem. 270, 8860–8866
65. Novak, U., Mui, A., Miyajima, A., and Paradiso, L. (1996) *J. Biol. Chem.* **271**, 18350–18354
66. Luo, G., and Yu-Lee, L. (1997) *J. Biol. Chem.* **272**, 26841–26849
67. Majka, M., Janowska-Wieczorek, A., Ratajczak, J., Kowalska, M. A., Vilaire, G., Pan, Z. K., Honczarenko, M., Marquez, L. A., Ponce, M., and Ratajczak, M. Z. (2000) *Blood* **96**, 4142–4151
68. Nelson, B. H., McIntosh, B. C., Rosencrans, L. L., and Greenberg, P. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 1878–1883
69. Kirken, R. A., Malabarba, M. G., Xu, J., DaSilva, L., Erwin, R. A., Liu, X., Hennighausen, L., Rui, H., and Farrar, W. L. (1997) *J. Biol. Chem.* **272**, 15459–15465
70. Rosenthal, L. A., Winestock, K. D., and Finlbloom, D. S. (1997) *Cell. Immunol.* **181**, 172–181
71. Mathy, N. L., Bannert, N., Norley, S. G., and Kurth, R. (2000) *J. Immunol.* **164**, 4429–4432
72. Lechner, O., Lauber, J., Franzke, A., Sarukhan, A., von Boehmer, H., and Buer, J. (2001) *Curr. Biol.* **11**, 587–595
73. Teague, T. K., Hildeman, D., Kedl, R. M., Mitchell, T., Rees, W., Schaefer, B. C., Bender, J., Kappler, J., and Marrack, P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12991–12996
74. Rogge, L., Bianchi, E., Biffi, M., Bone, E., Chang, S. Y., Alexander, H., Santini, C., Ferrari, G., Sinigaglia, L., Seiler, M., Neeb, M., Mous, J., Sinigaglia, F., and Certa, U. (2000) *Nat. Genet.* **25**, 96–101
75. Walker, J., and Rigley, K. (2000) *J. Immunol. Methods* **239**, 167–179
76. Liebecq, C. (ed) (1992) *Biochemical Nomenclature and Related Documents*, 2nd Ed., Portland Press, London, UK
Identification of Novel Targets of Immunosuppressive Agents by cDNA-based Microarray Analysis
Anthony D. Cristillo and Barbara E. Bierer

J. Biol. Chem. 2002, 277:4465-4476.
doi: 10.1074/jbc.M108598200 originally published online November 1, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108598200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 73 references, 38 of which can be accessed free at
http://www.jbc.org/content/277/6/4465.full.html#ref-list-1