UR-1505, a New Salicylate, Blocks T Cell Activation through Nuclear Factor of Activated T Cells

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ABSTRACT

2-Hydroxy-4(-2,2,3,3,3-pentafluoropropoxy)-benzoic acid (UR-1505), a new molecule chemically related to salicylic acid, has immunomodulator properties and is currently under clinical development for treatment of atopic dermatitis. The present work describes the immunomodulatory profile of UR-1505. UR-1505 targets T cells, inhibiting their proliferation and cytokine production by blocking nuclear factor of activated T cells (NF-AT) DNA-binding activity. The effects of UR-1505 (100–300 μM) on T cell proliferation seems to be dependent on the stimulus, because UR-1505 inhibited CD3/CD28-induced T-cell proliferation, increased $p27^{KIP}$ levels, and induced G_1/S cell arrest but, interestingly, did not inhibit the Janus tyrosine kinase/signal transducer and activator of transcription–induced T-cell proliferation. These data suggest that UR-1505 acts by means of a specific mechanism inhibiting T cell activation depending on T cell receptor signaling pathway. Furthermore, the antiproliferative effects of UR-1505 are not a consequence of decreased cell viability. In addition to the inhibition of T-cell proliferation, UR-1505 decreased, in a dose-dependent manner, the production of interleukin (IL)-5 and interferon (IFN)-γ in activated T cells, and this effect was produced at the transcriptional level. Because T-cell proliferation and cytokine production were regulated through NF-AT, we examined the effect of UR-1505 on this transcription factor. According to its effect on IL-5 and IFN-γ mRNA expression, UR-1505 specifically inhibited NF-AT DNA binding without effect on nuclear factor-κB and activator protein-1 activities. The effect of UR-1505 on NF-AT is not attributable to a blockade of nuclear import. In conclusion, UR-1505 is a new immunomodulator agent that specifically inhibits NF-AT activation. Because NF-AT regulates the transcription of most genes involved in lymphocyte activation, its selective inactivation results in both decreased T-cell proliferation and cytokine production.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used. It has only recently been accepted that the therapeutic effects of NSAIDs, salicylates included, were a consequence of the inhibition of cyclooxygenase (COX) activity, preventing the production of prostaglandins. However, evidence has begun to show that additional mechanisms, independent of COX inhibition, are involved in the anti-inflammatory properties of salicylates (Tegeder et al., 2001).

These additional and COX-independent, effects of salicylates mainly lead to an attenuation of pro-inflammatory gene expression by inhibiting different signal pathways (Tegeder et al., 2001). In that sense, the most well known is the inhibition of NF-κB activation (Kopp and Ghosh, 1994). Another effect of salicylates on transcription factor activity is the inhibition of CCAAT/enhancer-binding protein β and STAT-6 transcription factors (Perez-G et al., 2002). In addition to their effects on gene expression, acetylsalicylic acid is reported to block T-cell activation (Paccani et al., 2002), which plays a key and central role in autoimmune diseases. However, this effect was observed at supraphysiological concentrations. Knowing the key role of T-cell activation in autoimmune diseases, the blockade of T cell activation and

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; NF-κB, nuclear factor-κB; STAT, signal transducer and activator of transcription; CsA, cyclosporin A; UR-1505, 2-hydroxy-4–2,2,3,3,3-pentafluoropropoxybenzoic acid; NF-AT, nuclear factor of activated T cells; AP-1, activator protein 1; IL, interleukin; IFN, interferon; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; FACS, fluorescence-activated cell sorting; JAK, Janus tyrosine kinase; ELISA, enzyme-linked immunosorbent assay; Cdk, cyclin-dependent kinase; PCR, polymerase chain reaction; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; TCR, T cell receptor; rh, recombinant human.

subsequent cytokine production may be a useful treatment for autoimmune diseases. In fact, several immunosuppressive agents, such as methotrexate, cyclosporin A (CsA), or tacrolimus, are used for the treatment of autoimmune diseases (Saxne and Wollheim, 2003; Gremese and Ferraccioli, 2004; Pacor et al., 2004).

UR-1505 (Fig. 1) is a novel pentafluoropropoxy derivative of salicylic acid that has been selected from a series of salicylate derivatives, according to their activity as inhibitors of lymphocyte activation. These effects may be mediated through the nuclear factor of activated T cells (NF-AT) because this transcription factor is known to mediate the expression of the majority of genes expressed in lymphocytes as well as lymphocyte proliferation. The anti-inflammatory properties of UR-1505 in the trinitrobenzenesulfonic acid-induced colitis model will be reported elsewhere (E. Bailón, D. Camuesco, A. Nieto, Á. Concha, A. Fernandez de Arriba, J. Roman, I. Ramis, M. Merlos, A. Zarzuelo, J. Galvez, et al., manuscript in preparation), and it is suggested that the anti-inflammatory effect was mediated by blocking of Th1-cell activation.

The NF-AT family of transcription factors comprises five proteins evolutionary related to Rel/NF-κB family. Four proteins NF-ATc2 (NF-AT1), NF-ATc1 (NF-AT2), NF-ATc3 (NF-AT4), and NF-ATc4 (NF-AT3) are dependent on Ca^{2+}/calmodulin-dependent serine phosphatase calcineurin (Rao et al., 1997; Hogan et al., 2006), whereas the fifth (NF-AT5) is not regulated by calcineurin. Among these, NF-AT1 and NF-AT2 are the most expressed NF-AT proteins in mature T cells, regulating the expression of most, if not all, cytokines expressed by T cells. Activation of these members is regulated by their subcellular localization. In resting T cells, without Ca^{2+} signaling, NF-AT proteins are highly phosphorylated, remaining in the cytoplasm. Once activated, cytoplasmic Ca^{2+} levels increase, and calcineurin becomes activated and dephosphorylates NF-AT proteins, which exposes the nuclear localization signal and masks the nuclear export signal, with the consequent nuclear import of NF-AT. In the nucleus, NF-AT proteins bind to the DNA and regulate the gene transcription in cooperation with other transcription factors, such as AP-1 (Rao et al., 1997; Serfling et al., 2000; Macian, 2005). Once in the nucleus, NF-AT activity is down-regulated by means of serine/threonine kinases that phosphorylate NF-AT proteins, promoting their export to the cytoplasm.

NF-AT proteins are expressed not only in T cells but also in other immune cell types, such as B cells (Choi et al., 1994), mast cells (Prieschl et al., 1995), and natural killer cells (Aramburu et al., 1995). NF-AT is essential for T-cell activation and regulates cell-cycle control (Macian, 2005) and the production and expression of cytokines involved in the immune response (IL-2, IL-5, IL-4, IFN-γ, and others) (Serfling et al., 2000).

The aim of this study was to determine the effects of

UR-1505 on T-cell activation in both T cell proliferation and cytokine production.

Materials and Methods

Reagents. RPMI 1640 and other cellular culture products were obtained from Invitrogen (Paisley, Scotland, UK). All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. UR-1505 was synthesized at J. Uriach y Compañía, S.A (Barcelona, Spain).

ELISA kits for IL-5, CycleTEST Plus DNA reagent kit, Transfactor kits for p65, and NF-ATc1 were obtained from BD Biosciences (San Jose, CA). The WST-1 kit was obtained from Roche Diagnostics (Barcelona, Spain). The NF-AT activation kit was obtained from Cellomics (Pittsburgh, PA). Cell Proliferation Biotrak ELISA System was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Bradford reagent was from Bio-Rad Laboratories (Munich, Germany). TRIzol reagent was from Invitrogen. Anti CD28 and anti CD3 were from R&D Systems (Minneapolis, MN).

Cell Culture and Treatment. Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum in a humidified 5% CO_2 atmosphere at 37°C. Experiments with Jurkat cells were performed in RPMI 1640 medium and 2% FBS. Jurkat cells were stimulated with PMA (50 nM) and ionomycin (3 μM) in the presence or absence of UR-1505 (100–300 μM) or CsA (1 μM). Human T cells were isolated from peripheral blood by means of a Ficoll gradient (GE Healthcare), followed by a negative selection using MACS immunogenetic beads [Pan T Cell Isolation kit human; Miltenyi Biotec Inc. (Auburn, CA)]. A highly pure population of T cells was obtained [>98%, as determined by fluorescence-activated cell sorting (FACS) analysis]. Human isolated T cells were either frozen for further studies or cultured in RPMI 1640 medium supplemented with 10% FBS. T cells were stimulated with plate-coated anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml) plus rhIL-2 (25 ng/ml) (R&D Systems) in the presence or absence of UR-1505 or CsA. HeLa cells were cultured in minimal essential medium supplemented with 20% fetal bovine serum.

T Cell Proliferation Assays. We have studied the effect of UR-1505 on two different models of T cell proliferation, depending on the proliferative stimulus. First, human isolated T cells were cultured in 96-well plates (100,000 cells/well) in 100 μl of RPMI/10% FBS and were stimulated with coated anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml) plus rhIL-2 (25 ng/ml) for up to 96 h; this proliferation was dependent on TCR engagement and the cosignal provided by CD28.

Second, JAK/STAT-dependent proliferation was studied in human isolated T cells that had been growing in medium containing IL-2 (10 ng/ml) for 7 days to overexpress IL2 receptors. At day 7, human isolated T cells were plated on 96-well microplates and stimulated with IL-2 (1 ng/ml) for another 96 h, so as to detect the proliferation exclusively caused by 10 ng/ml IL-2 through its overexpressed receptor and JAK/STAT.

BrdU was added in both cases approximately 15 h before T cells reached 24, 48, 72, and 96 h of incubation, at a final concentration of 10 μM. During this labeling period, BrdU instead of thymidine was incorporated into the DNA of growing cells. Quantification of BrdU incorporation to DNA in growing cells is used as a measure of cell proliferation. BrdU was determined by ELISA according to manufacturer instructions.

Jurkat Cell Cycle Assay. Jurkat cells in log-phase growth were treated with UR-1505 (10–500 μM). After 24 h, cells were pulsed with BrdU (10 μM, 1 h), harvested by centrifugation, resuspended (1 × 10^6 cells/ml) in PBS, fixed in ice-cold 70% ethanol, and further processed and stained with 20 μl of anti-BrdU-fluorescein isothiocyanate according to the manufacturer's instructions (BD Biosciences) and with 5 μg/ml propidium iodide. Within 3 h after staining, the fractions of cells in G_0/G1, S, and G_2/M phases of the cell
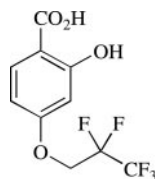

Fig. 1. Chemical structure of UR-1505.

cycle were quantified by FACS analysis performed with a FACSCalibur flow cytometer (BD Biosciences).

p27^{KIP1} Levels in Jurkat Cells. Accumulation of the cyclin-dependent kinase (Cdk) inhibitor p27 was analyzed in Jurkat cells. Cells were incubated for 24 h with 0.1% FBS (quiescent cells) or with 10% FBS (proliferating cells) plus vehicle or UR-1505 (3–30 μM). p27 levels were determined by immunoblot or flow cytometry after cell staining with phycoerythrin conjugate p27 monoclonal antibody according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA).

Viability Assays. Human isolated T cell viability was determined using the Reagent WST-1 (Roche Diagnostics) according to the manufacturer's instructions.

Cytokine Production. Human isolated T cells (1 × 10^6) were stimulated with anti-CD3, anti-CD28, and rIL-2 for up to 96 h in the presence or absence of UR-1505 (30–300 μM). Every 24 h, supernatants were obtained and immediately frozen until cytokine determination. Cytokine concentration in the supernatants was determined by flow cytometry (cytometric bead array; BD Biosciences).

Real-Time RT-PCR. Total RNA was obtained from human isolated T cells (4 × 10^6 cells) and Jurkat cells (5 × 10^6 cells) using TRIzol reagent according to the manufacturer's instructions. cDNA was generated using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) from 1 μg of total RNA. The reaction mix contained 1× RT buffer, 5.5 mM MgCl$_2$, 500 μM deoxyNTP mixtures, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl Multiscribe Reverse Transcriptase. PCR was performed in a 20-μl reaction mixture that contained 40 ng of reverse-transcribed cDNA, PCR master mix, and the needed amounts of primers and probe included in IL-5 and IFN-γ TaqMan Gene Expression Assays (Applied Biosystems). The PCR and subsequent analysis were done with the ABI PRISM 7700 apparatus (Applied Biosystems).

Cycle threshold (C$_T$) values were calculated for IL-5, IFN-γ and 18S rRNA. The relative IL-5 and IFN-γ transcripts in treated (T) and control samples (C) were expressed as $2^{-\Delta\Delta C_T}$, in that $\Delta\Delta C_T = \Delta C_T(T) - \Delta C_T(C)$ and $\Delta C_T = C_T$ (IL5 or IFN-γ) − C$_T$ (18S rRNA).

Nuclear Extract Preparation. Nuclear extracts from Jurkat cells (4 × 10^6) and human isolated T cells (4–6 × 10^6) were prepared by lysing cells with Nonidet P-40 followed by differential centrifugation. In brief, at the end of incubations, cells were collected by centrifugation at 1200 rpm for 10 min at room temperature and washed with ice-cold PBS. Then, cells were resuspended in buffer consisting of 10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin and kept on ice for 15 min. Cells were then lysed with Nonidet P-40 (10%), and the nuclear pellet was recovered after centrifugation at 13,000g at 4°C for 30 s. The nuclear pellet was resuspended in ice-cold buffer containing 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin and stored at −80°C. Protein concentration was determined by Bradford assay.

AP-1 DNA-Binding Activity. Activation of transcription factor AP-1 was determined by electrophoretic mobility shift assay (EMSA) using a consensus oligonucleotide of AP-1 (5′-CGCTTGATGAGT-CAGCCGGAA-3′) (Promega). Probes were labeled with T4 polynucleotide kinase (Promega, Madison, WI) with [γ-^{32}P]ATP (3000 Ci/mmol, 250 μCi) (GE Healthcare). In brief, 3 μl of H$_2$O, 2 μl of consensus sequence of oligonucleotide (~20 ng), 1 μl of 10× buffer enzyme, 1 μl of T4 enzyme (5 U/μl) and 3 μl of [γ-^{32}P]ATP were mixed in an Eppendorf tube and incubated at 37°C for 1 h. Oligonucleotides labeled were purified using NICK columns (GE Healthcare) and stored at −20°C.

Binding reactions included 10 μg of nuclear extracts in incubation buffer (10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 1 mM EDTA, and 4%
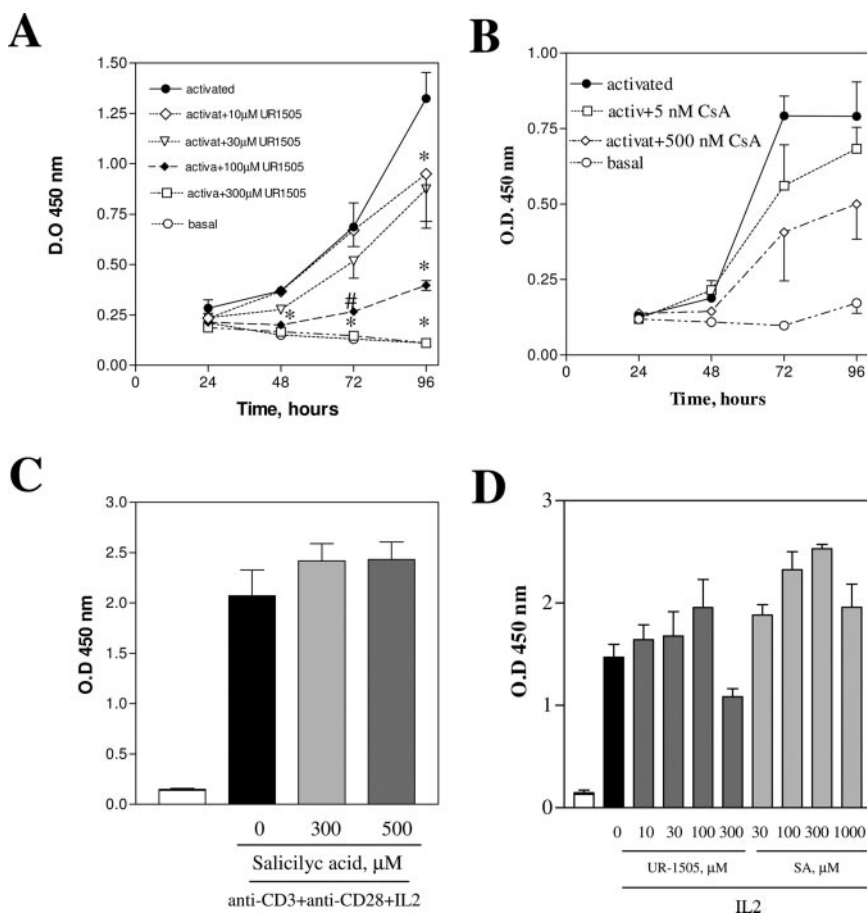
Fig. 2. UR-1505 specifically inhibited TCR-induced T-cell proliferation, having a minor effect on JAK/STAT-induced T-cell proliferation. A, human isolated T cells were stimulated with coated anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml) plus rIL-2 (25 ng/ml) for up to 96 h, and the effect of increasing concentrations of UR-1505 (10–300 μM) on T-cell proliferation was assayed. B and C, effect of CsA for up to 96 h (5 and 500 nM) and salicylic acid after 96 h (300–500 μM) on TCR induced T-cell proliferation. Finally, the effect of UR-1505 (10–300 μM) and salicylic acid (30–1000 μM) after 96 h on JAK/STAT-dependent T-cell proliferation was assayed in human isolated T cells, as detailed in *Materials and Methods*. D, data shown are the mean ± S.E.M. of three different experiments done in triplicate. ∗, $p <$ 0.001 versus activated T cells; #, $p <$ 0.01 versus activated T cells

glycerol), 1 μg of poly(dI-dC) (GE Healthcare), and labeled oligonucleotide (~30,000 cpm). The mixture was electrophoresed in a 6% nondenaturing polyacrylamide gel for 2 to 3 h at 175 V, and the gel was autoradiographed using Hyperfilm MP (GE Healthcare) at −20°C overnight.

NF-AT and NF-κB Activities. NF-AT and NF-κB activities were analyzed with the commercially available ELISA kits TransFactor NF-ATc1 colorimetric kit and TransFactor NFκB p65 colorimetric kit (BD Biosciences). In brief, nuclear extracts (10 μg of protein) from Jurkat cells or human isolated T cells were introduced into the wells of 96-well microtitre plates precoated with oligonucleotide containing the NF-AT and NF-κB consensus sequence (5′-GAGGAAAATTTG-3′, 5′-GGGG-TATTTCC-3′) in a volume of 50 μl for 1 h at room temperature. After washing three times, 100 μl of monoclonal antibody against NF-ATc1 or

p65 was added to the appropriate wells and incubated for a further 1 h at room temperature. Anti-IgG horseradish peroxidase-conjugate in a volume of 100 μl was then added and incubated for another 1 h at room temperature. Absorbance at 450 nm was measured after the addition of 1 M sulfuric acid. Specificity was verified by competition assays and by mutated oligonucleotides.

Immunocytochemistry. HeLa cells (1×10^4) were plated on eight-well culture coverslips (BD Biosciences) and cultured overnight. Then, cells were preincubated with UR-1505 (300 μM) or CsA (1 μM) for 30 min at 37°C and stimulated or not with ionomycin (1 μM) for another 40 min. Cells were immediately fixed with 3% paraformaldehyde (prewarmed at 37°C) for 20 min at room temperature. Immunocytochemistry was performed according to the manufacturer's instructions.
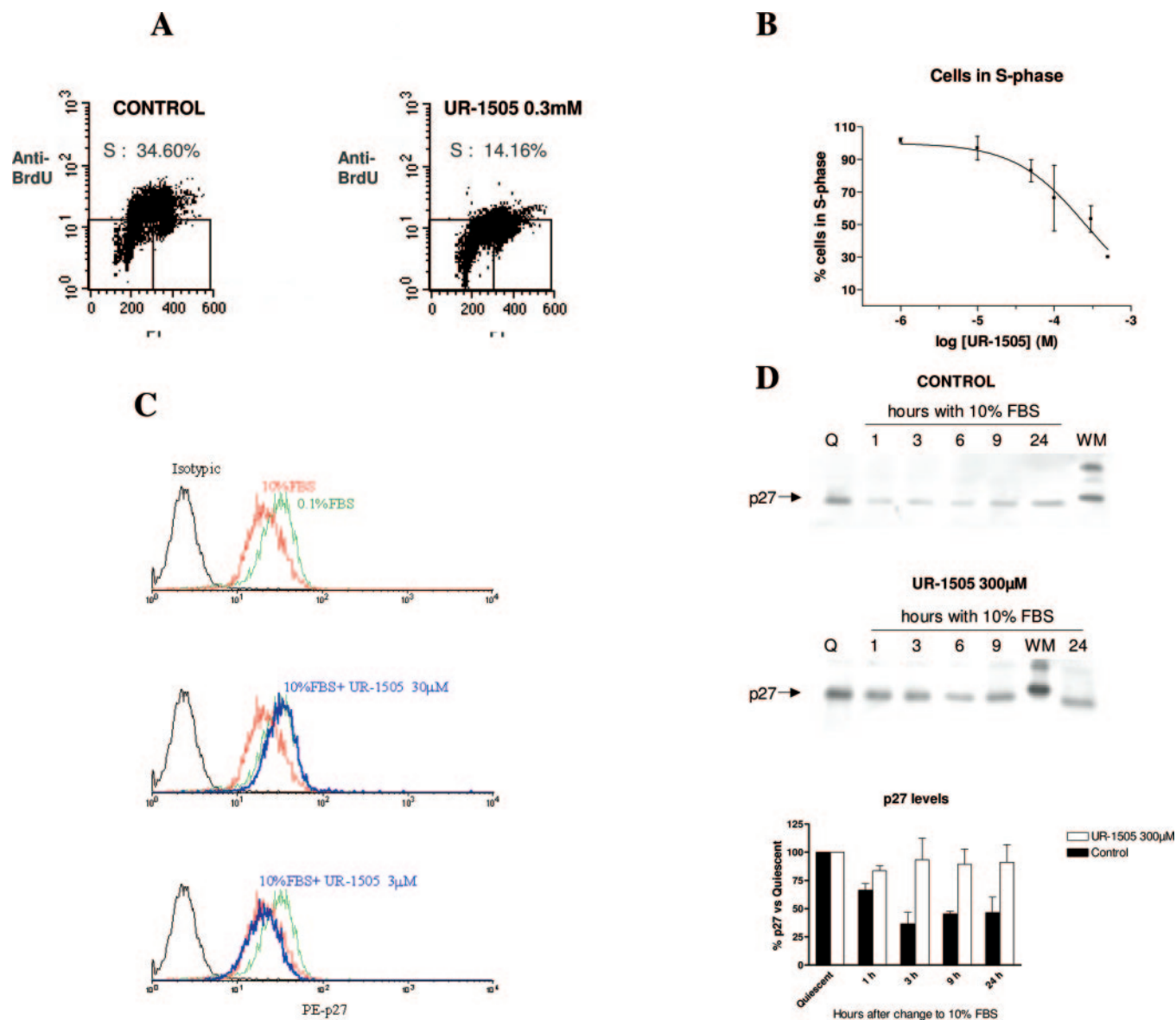

Fig. 3. Effect of UR-1505 on cell cycle and p27^{KIP1} levels. A, percentage of cells in the S phase of cell cycle in asynchronously growing Jurkat cells treated with UR-1505 (0.3 mM) or vehicle for 24 h. After incubation, cells were pulsed with BrdU (1 h) and then stained with anti-BrdU-fluorescein isothiocyanate and propidium iodide followed by FACS analysis. B, effect of UR-1505 in the percentage of Jurkat cells in the S phase of cell cycle. Experimental details as above. C, representative histograms showing the effects of UR-1505 on p27 levels in Jurkat cells. Cells were incubated for 24 h with 0.1% FBS (quiescent cells), 10% FBS (proliferating cells), or 10% FBS plus UR-1505. After 24 h, cells were stained with phycoerythrin-conjugated p27 monoclonal antibody or with an isotypic control followed by FACS analysis. D, immunoblots showing the inhibition of p27 degradation by UR-1505 in HeLa cells. Cells were changed from a quiescent environment (0.1% FBS, 48 h) to a proliferating one (10% FBS) in presence of UR-1505 (0.3 mM) or vehicle. At different times, cells were harvested and immunoblots were performed with antibodies against p27. Column graph shows the quantification of two independent experiments (mean ± S.D.).

Results

UR-1505 Inhibited TCR-Induced T-Cell Proliferation. To investigate the effect of UR-1505 on T-cell activation, we first evaluated the effect of UR-1505 on TCR-induced human isolated T cell proliferation. TCR engagement by antigen and CD28 cosignal allow T cells to proliferate through TCR signaling pathway. Therefore, to induce cellular proliferation, human isolated T cells were stimulated through T cell receptor with coated anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) + IL-2 (25 ng/ml) for up to 96 h in the presence or absence of UR-1505. Under these conditions, human isolated T cells clearly proliferated, as shown in Fig. 2, A–C. UR-1505 (10–300 μM) inhibited the CD3/CD28 induced human isolated T cell proliferation in a dose-dependent manner, whereas salicylic acid treatment for 96 h (300–500 μM) did not (Fig. 2, A and C). The inhibition achieved by 300 μM UR-1505 was 100%. In addition, we used CsA as a positive control of T-cell proliferation inhibition because it is known that the inhibitory effect of CsA on T-cell proliferation is due to calcineurin inhibition. Therefore, CsA (5–500 nM) blocked TCR signaling cascade inhibiting T-cell proliferation (Fig. 2B).

UR-1505 Did Not Inhibit JAK/STAT-Induced T-Cell Proliferation. To assess whether the inhibition of T-cell proliferation was due to an interference of UR-1505 with TCR and CD28 receptor engagement, we examined the effect of UR-1505 on JAK/STAT-dependent T cell proliferation. To induce cell proliferation, human isolated T cells were exclusively incubated with IL-2, as detailed under *Materials and Methods*. Under these conditions, T cell proliferation was not dependent on TCR signaling pathway but was controlled through the JAK/STAT signaling pathway. JAK/STAT-dependent T cell proliferation was not affected by UR-1505, because 100 μM failed to inhibit human isolated T cell proliferation, and the highest concentration of UR-1505 assayed (300 μM) had only a minor effect on cell proliferation after 96 h (Fig. 2D). It has to be mentioned that 300 μM UR-1505 absolutely abrogated the TCR-dependent T-cell proliferation after 24 h (Fig. 2A). CsA also failed to inhibit T-cell proliferation under these conditions; however, a JAK inhibitor reduced IL-2 induced lymphocyte proliferation (data not shown).

UR-1505 Induces Cell Cycle Arrest through p27^{KIP1} Accumulation. Cosignaling with TCR/CD3 and CD28 promoted progression of T cells through the cell cycle. To this end, a decrease in the levels of Cdk inhibitor p27^{KIP1} was required to permit cell passage from the G_1 to the S phase of cell cycle.

UR-1505 induced G_1 arrest of growing Jurkat cells, inhibiting in a dose-dependent manner their progression into S phase of the cell cycle (Figs. 3, A and B). This G_1 arrest seems to be mediated by an UR-1505–promoted accumulation of p27^{KIP1}. As shown in Fig. 3C, UR-1505 concentration-dependently increased p27^{KIP1} levels in log-phase proliferating Jurkat cells. Levels of p27^{KIP1} in UR-1505 (30 μM)-treated cells were similar to those of arrested cells incubated in low (0.1%) FBS.

Moreover, the effects of UR-1505 on p27^{KIP1} concentration seem to be due to inhibition of degradation of this Cdk inhibitor. As shown in Fig. 3D, incubation of HeLa cells in 0.1% FBS (48 h) induced cell quiescence and a marked increased in

p27^{KIP1} levels. Changing these cells to a 10% FBS medium resulted in a time-dependent degradation of p27^{KIP1}, which correlated with the onset of cell proliferation. However, when 10% FBS medium was supplemented with UR-1505 (300 μM), p27^{KIP1} degradation was inhibited, leaving the cells in a quiescent mode.

UR-1505 Did Not Decrease T-Cell Viability. To discard the idea that the antiproliferative effect of UR-1505 was not due to an increase in cellular toxicity in human isolated T cells, we have examined the human isolated T cell viability by means of WST-1 assay. Starting from 48 h stimulation with anti-CD3 and anti-CD28 + hrIL-2, we have observed a higher mitochondrial activity of human isolated T cells as a result of an increase in cell number caused by increased cell proliferation. It is noteworthy that UR-1505 did not reduce the human isolated T cell viability at concentrations up to 300 μM (Fig. 4), because the reduction of mitochondrial activity was always higher than basal values (nonstimulated lymphocytes) but not below that.

Effect of UR-1505 on Cytokine Production in Activated T Cells. Isolated human peripheral blood T cells were activated in the same way as for proliferation and viability studies [that is, by cross-linking the TCR with anti-CD3 (1 μg/ml) plus a costimulation provided by anti-CD28 (1 μg/ml) antibodies for up to 96 h in the presence or absence of UR-1505 (30–300 μM)]. Activation of human isolated T cells resulted in an increased production of IFN-γ, IL-5, IL-4, and tumor necrosis factor-α, whereas nonactivated T cells did not produce detectable levels of any cytokine (Fig. 5A). The increased secretion of cytokines was observed from 24 h (Fig. 5B), although it was more evident from 48 h (Fig. 5A).

Treatment with UR-1505 (30–300 μM) reduced the cytokine production by activated T cells (Fig. 5, A and B). The most important inhibitory effect was observed for IFN-γ and IL-5. It is noteworthy that the inhibition of IFN-γ and IL-5 production showed the same pattern than the inhibition of cell proliferation in human isolated T cells. In Fig. 5B, we
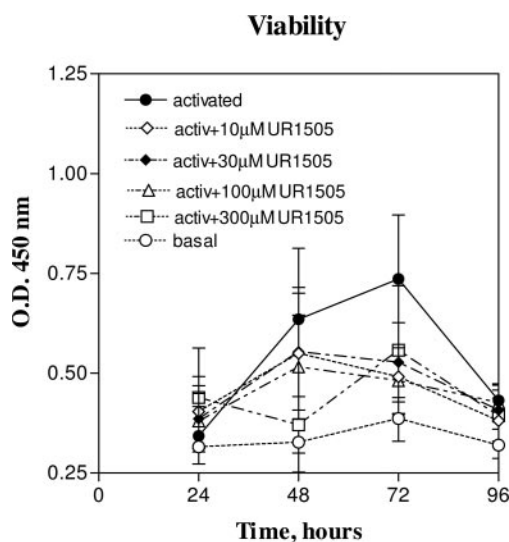

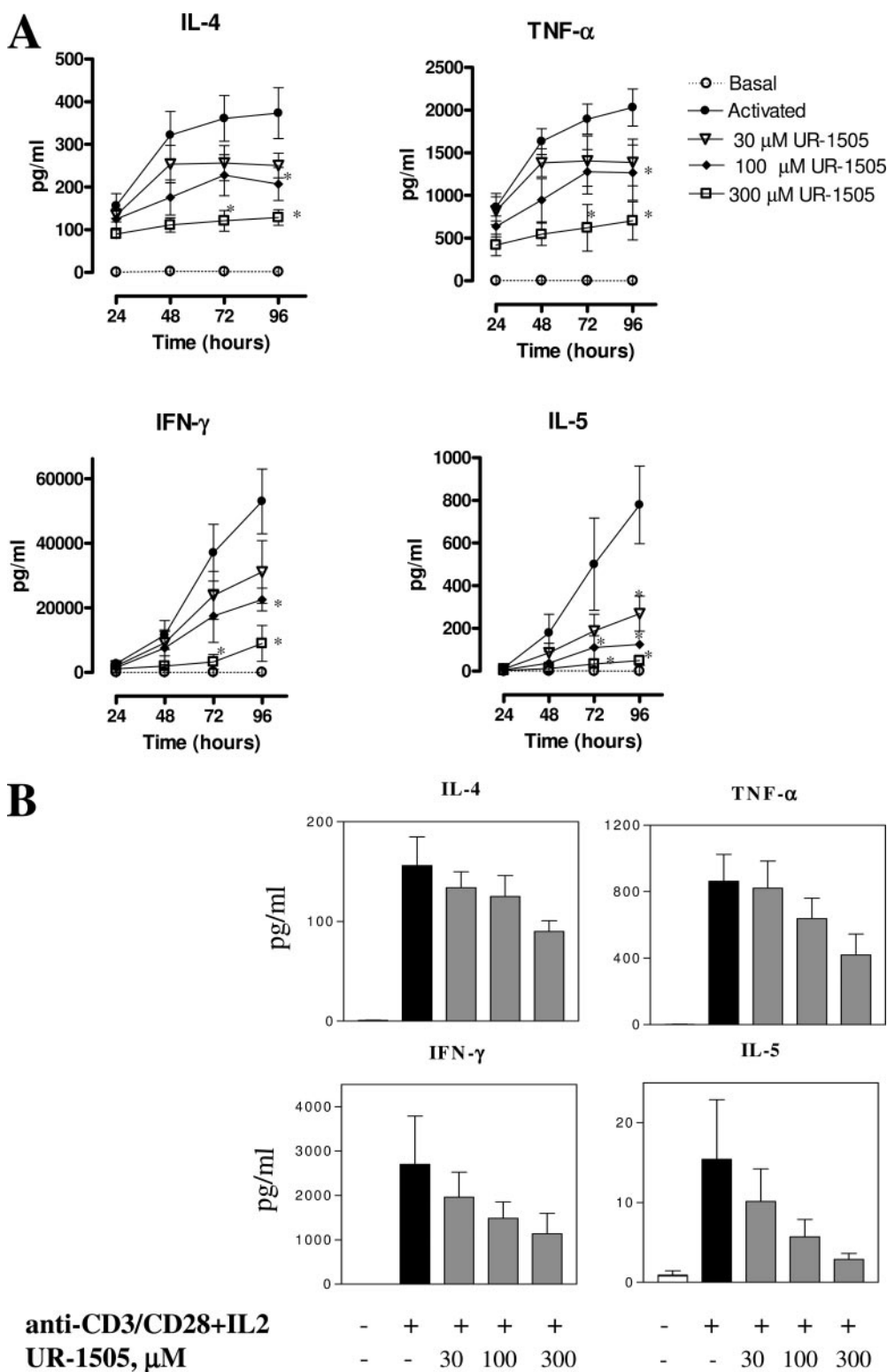**Fig. 4.** UR-1505 did not decrease T cell viability. Human isolated T cells were stimulated with anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml) plus IL-2 (25 ng/ml) for up to 96 h in the presence or not of UR-1505 (10–300 μM). Every 24 h, mitochondrial activity was measured by WST-1 Reagent (Roche). Results shown are a representative experiment performed in triplicate.

focused on the inhibitory effect of UR-1505 on cytokine secretion at 24 h. At this early time point, there was already inhibition of IL-5 and IFN-γ by UR-1505 (100–300 μM).

UR-1505 Decreased IL-5 and IFN-γ Gene Expression.
Because IL-5 and IFN-γ were the cytokines most inhibited by UR-1505 and to determine whether the inhibition of IL-5 and IFN-γ production was consequence of a decreased gene expression, we analyzed the effect of UR-1505 on IL-5 and

IFN-γ mRNA levels in T cells. For this, T cells, human isolated T cells, and Jurkat cells were preincubated with UR-1505 (300 μM), CsA (1 μM), or tacrolimus (1 μM) for 30 min and then both types of T cells were stimulated. The stimulus was different for each type of T cell; human isolated T cells were stimulated by cross-linking the TCR with anti-CD3 plus a costimulation provided by anti-CD28 antibodies, whereas Jurkat cells were stimulated pharmacologically for

Fig. 5. UR-1505 reduced cytokine production in T cells. A, human isolated T-cells were stimulated with anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml) plus IL-2 (25 ng/ml) for up to 96 h in the presence or not of UR-1505 (30–300 μM) and cultured supernatants collected every 24 h for IL-4, tumor necrosis factor-α, IL-5, and IFN-γ determination. B, effect of UR-1505 (30–300 μM) on IFN-γ and IL-5 production by anti-CD3/anti-CD28–stimulated human isolated T cells after 24 h. Results shown are a representative experiment performed in triplicate. *, $p < 0.05$ versus activated T cells.

24 h with PMA (50 nM) plus ionomycin (3 μM), which mimics the engagement of TCR with anti-CD3 and anti-CD28. The stimuli are equivalent because ionomycin increases intracellular Ca^{2+} levels and PMA activates mitogen-activated protein kinase, the same signaling pathways that are activated after TCR engagement with anti-CD3 and the cosignal allowed by anti-CD28. At the end of experiments, total RNA was obtained and reverse-transcribed to cDNA, and IFN-γ and IL-5 gene expression was quantified by real-time PCR. As shown in Fig. 6, A and B, UR-1505 (300 μM) reduced the gene expression of both genes in activated T cells. The inhibition on IL-5 and IFN-γ was observed both in Jurkat (Fig. 6A) and human isolated T cells (Fig. 6B). As expected, CsA (1 μM) and tacrolimus (1 μM) inhibited the expression of the genes in Jurkat cells and in human isolated T cells, respectively.

UR-1505 Specifically Inhibited NF-AT DNA-Binding Activity in Both Human Isolated T Cells and Jurkat Cells. Most of the genes involved in the immune response, including IL-5 and IFN-γ, are transcriptionally regulated by NF-AT, AP-1, and NF-κB binding sites located in their promoter regions. Furthermore, these transcription factors, especially NF-AT, play a key role during T-cell activation. Thus, we have studied the effect of UR-1505 on NF-AT, AP-1, and NF-κB DNA-binding in T cells. For this, we used two cellular models: human isolated T cells activated with anti-CD3/anti-CD28 (both at 1 μg/ml) for 6 h and Jurkat cells

pharmacologically stimulated for 4 h with PMA (50 nM) plus ionomycin (3 μM). T cells have been preincubated or not with UR-1505 (100–300 μM) or CsA (1 μM) 30 min before stimulation. Activated human isolated T cells and pharmacologically stimulated Jurkat cells showed increased DNA binding for all three transcription factors (NF-AT, AP-1, and NF-κB) (Fig. 7, A and B).

UR-1505 (100–300 μM) reduced NF-AT DNA binding, whereas NF-κB and AP-1 DNA-binding activities remained unaffected in both Jurkat cells and human isolated T cells (Fig. 7, A and B). CsA is a well known immunosuppressive drug that targets NF-AT activity through calcineurin inhibition. In our model, CsA (1 μM) strongly inhibited NF-AT DNA binding and, in addition, reduced partially NF-κB DNA-binding.

UR-1505 Had No Effect on Ionomycin Induced-NF-AT Translocation to the Nucleus. The finding of a diminished NF-AT DNA-binding does not allow us to distinguish between a block of NF-AT import (as CsA does) or an enhanced export. To ascertain whether UR-1505 modulated NF-AT import, we performed immunocytochemistry experiments with HeLa cells. This human cell line has been widely used for NF-AT translocation experiments. As shown in Fig. 8, NF-AT localization was cytosolic in nonstimulated HeLa cells, whereas an important NF-AT translocation to the nucleus was observed after stimulation with ionomycin (1 μM). As expected, the calcineurin inhibitor CsA (1 μM) prevented
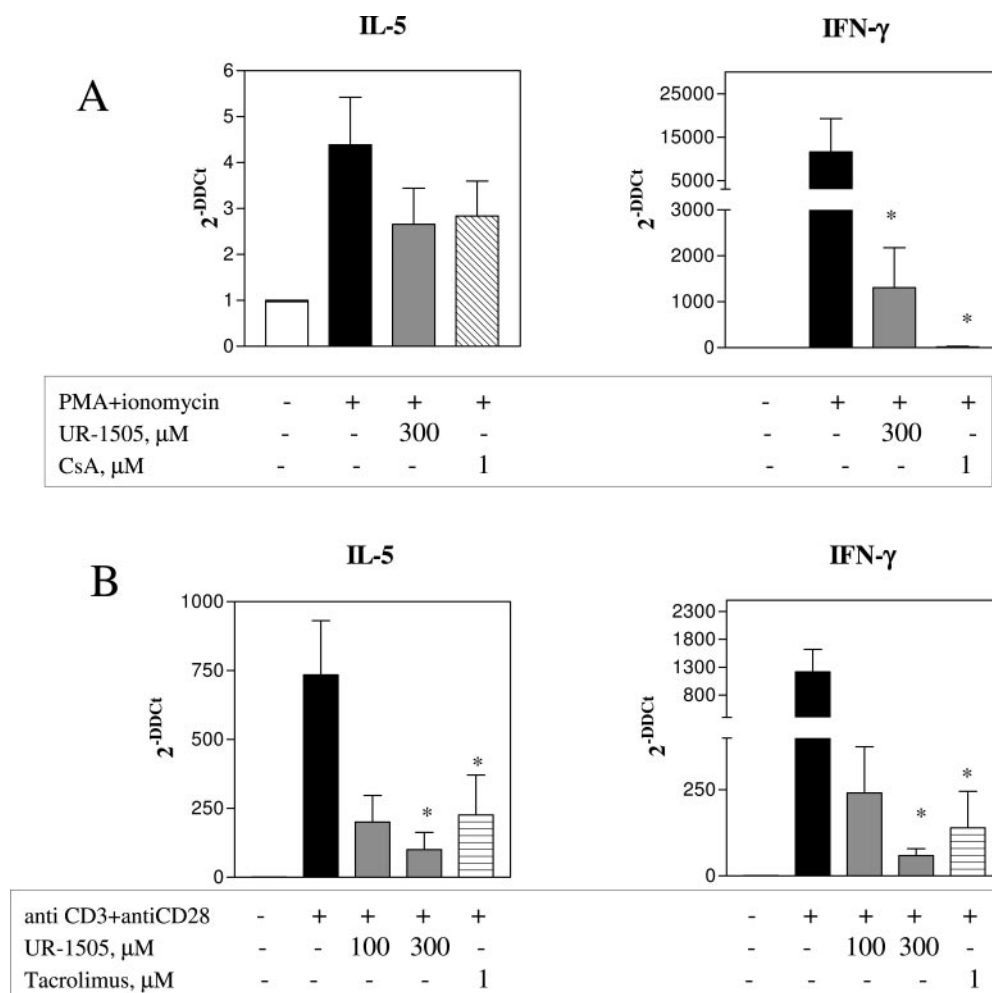

IL-5				
PMA+ionomycin	-	+	+	+
UR-1505, μM	-	-	300	-
CsA, μM	-	-	-	1

IFN-γ				
PMA+ionomycin	-	+	+	+
UR-1505, μM	-	-	300	-
CsA, μM	-	-	-	1

IL-5					
anti CD3+antiCD28	-	+	+	+	+
UR-1505, μM	-	-	100	300	-
Tacrolimus, μM	-	-	-	-	1

IFN-γ					
anti CD3+antiCD28	-	+	+	+	+
UR-1505, μM	-	-	100	300	-
Tacrolimus, μM	-	-	-	-	1

Fig. 6. Inhibition of IL-5 and IFN-γ gene expression by UR-1505. IL-5 and IFN-γ gene expression were determined by real-time RT-PCR analysis in Jurkat cells stimulated pharmacologically with PMA (50 nM) + ionomycin (3 μM) for 24 h (A) and in human isolated T cells stimulated with anti-CD3 and anti-CD28 both at 1 μg/ml for 24 h (B). In both cases, T cells were pretreated with UR-1505 (300 μM) or CsA (1 μM) in the case of Jurkat or tacrolimus (1 μM) in the case of human isolated T cells 30 min before stimulation. Relative amounts of IL-5 and IFN-γ mRNA were calculated and normalized with the endogenous control 18S rRNA. Data shown ($2^{-\Delta\Delta C_T}$) are the mean ± S.E.M. of three different experiments done in duplicate. *, $p < 0.05$ versus activated T cells.

the ionomycin-induced NF-AT translocation into the nucleus. However, UR-1505 (300 μM) failed to inhibit NF-AT nuclear translocation, because the fluorescence had been detected principally in the nucleus (Fig. 8). Indeed, salicylates are known to not inhibit NF-AT translocation to the nucleus (Aceves et al., 2004).

Nuclear Export Blockade Had No Effect on UR-1505–Induced NF-AT Inhibition. To determine whether the inhibitory effect of UR-1505 on NF-AT DNA-binding was the consequence of an increased export, we used leptomycin B as a general inhibitor of exportin (CRM1)-dependent export. As shown in Fig. 9, treatment with leptomycin B did not abolish the inhibitory effect of UR-1505 on NF-AT DNA binding,

suggesting that UR-1505 did not increase NF-AT nuclear export.

Effect of UR-1505 on p38, GSK, and CK1 Activities. The effect of UR-1505 on the kinases involved in nuclear export of NF-AT was evaluated (Upstate Discovery, Dundee, UK). As shown in Table 1, UR-1505 (300 μM) did not affect p38, GSK, or CK1 activities.

Discussion

In this study, we have shown that UR-1505, a new immunomodulator agent chemically related to salicylic acid, blocked T-cell activation, which might be a useful strategy for
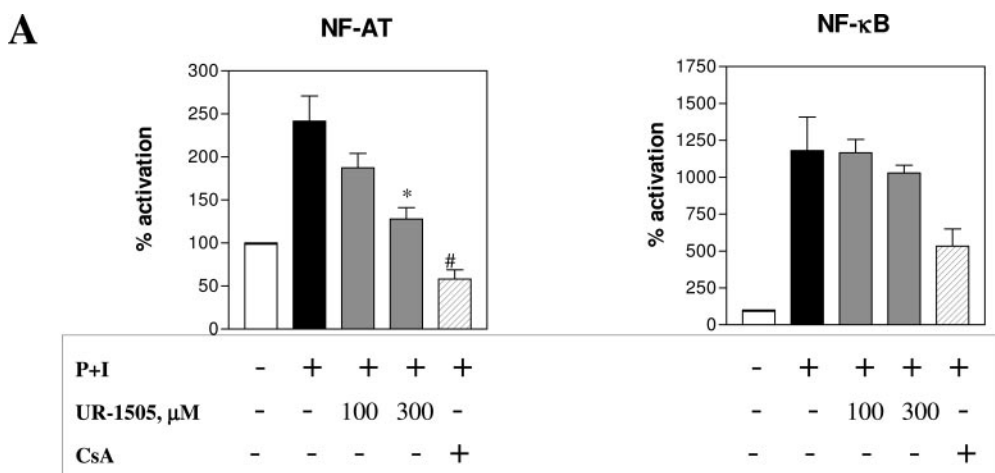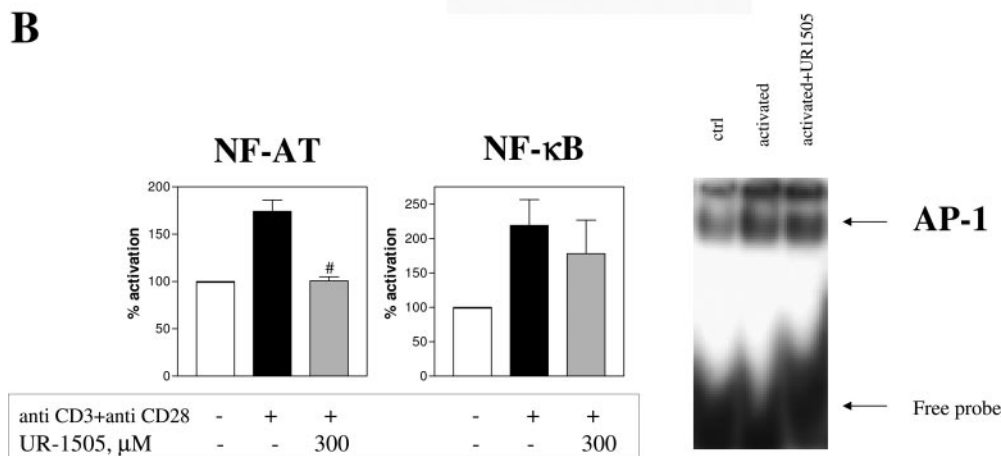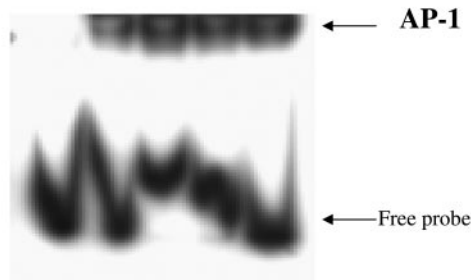
Fig. 7. UR-1505 decreased NF-AT DNA binding without effect on NF-κB and AP-1 in T cells. A, Jurkat cells were incubated with the indicated concentrations of UR-1505 and CsA (1 μM) for 30 min and then stimulated pharmacologically with PMA (50 nM) plus ionomycin (3 μM) for 4 h. B, human isolated T cells were stimulated with anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml) plus IL2 (25 ng/ml) for 6 h and incubated in the presence or absence of 300 μM UR-1505. The nuclear NF-AT and NF-κB DNA binding were detected by ELISA assays, and AP-1 was examined by EMSA. Data shown are the mean $\pm$ S.E.M. of three to five different experiments. For AP-1 experiments, a representative EMSA is shown. $*, p < 0.01$; $\#, p < 0.001$ versus activated T cells.

the treatment of autoimmune diseases, given the key role of T-cell activation in the onset of these diseases (Chow et al., 2005; Goudy and Tisch, 2005; Howard et al., 2005; Walter and Santamaría, 2005). It is important that the prevention of T-cell activation produced by UR-1505 was done in a lower range of concentrations (0.1 –0.3 mM) than those reported previously for acetylsalicylic acid (2– 4 mM) (Paccani et al.,
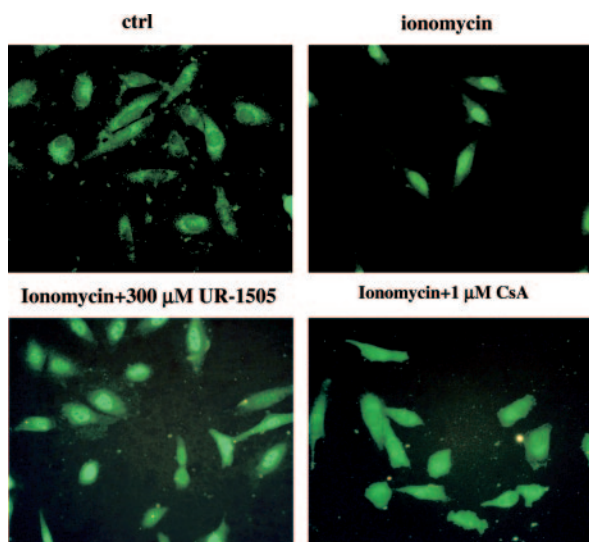

Fig. 8. UR-1505 has no effect on ionomycin-induced NF-AT nuclear import in HeLa cells. HeLa cells were attached overnight and were then preincubated with 300 μM UR-1505 or 1 μM CsA for 30 min, then stimulated with 1 μM ionomycin for 40 min. NF-AT localization was studied by immunocytochemistry. The images shown are representative of three separate experiments.
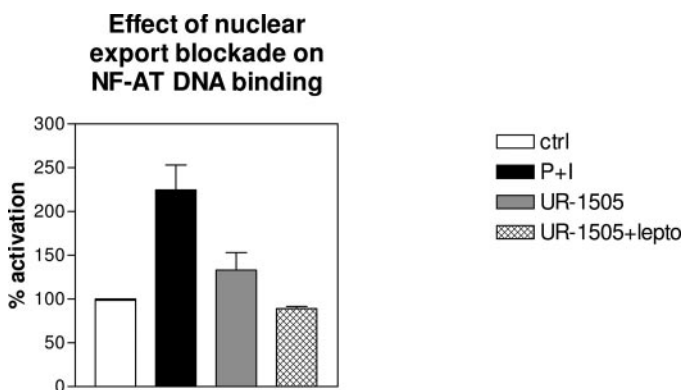

Fig. 9. Nuclear export blockade had no effect on UR-1505 inhibition of NF-AT DNA-binding. Jurkat cells were treated in the presence or absence of leptomycin B (20 ng/ml), with UR-1505 (300 μM) for 30 min and then stimulated with PMA + ionomycin for 4 h. At the end, nuclear extracts were obtained and NF-AT DNA binding determined. Results are the mean ± S.E.M. of three separate experiments.

TABLE 1
Effect of UR-1505 on the activity of p38, GSK, and CK1 kinases
The effect of UR-1505 at 300 μM, expressed as a percentage of activity compared with that of control incubations (100%) was measured. ATP was used at 100 μM in all assays.

Kinase	% Control
GSK	81
p38α	90
p38β	120
p38γ	100
p38δ	95
CK1	92

2002). Our results indicate that blockade of T-cell activation by UR-1505 results in the inhibition of both T-cell proliferation and cytokine production, and both effects seem to be mediated through the TCR/NFAT signaling pathway.

UR-1505 inhibits T-cell proliferation acting on some point(s) of the TCR signaling cascade because UR-1505 inhibits in a dose-dependent manner the T cell proliferation induced by TCR activation with anti-CD3 and anti-CD28 but does not inhibit the proliferation induced by JAK-STAT (IL-2-dependent cell proliferation). More important is the fact that UR-1505 does not inhibit cell proliferation in macrophages (Fernández de Arriba et al., 2005). On the other hand, salicylic acid was unable to inhibit T cell proliferation independently of the stimulus at any concentration assayed.

It has been reported that tacrolimus, another well known inhibitor of the TCR signaling cascade, blocks proliferation of endothelial cells through up-regulation of p27 (Matter et al., 2006). In that sense, our data shown that UR-1505 induces cell cycle arrest at G_1/S, increasing p27^{KIP1} levels in proliferating Jurkat cells, and the accumulation of p27 produces arrest in the G_1 phase (Mohapatra et al., 2001). It should be emphasized that the antiproliferative effect of UR-1505 is not a consequence of decreased T cell viability.

Activated T cells, in addition to proliferating, produce a great array of cytokines. The enhanced production of cytokines is reduced by pretreatment with UR-1505, which is the most important inhibitory effect observed on IL-5 and IFN-γ production, each with a key role in the immune response. This inhibition is produced at transcriptional level because the mRNAs of both genes are reduced after pretreatment with UR-1505.

To understand the mechanisms involved in the inhibition of cytokine gene expression, we have studied the effect of UR-1505 on NF-AT, NF-κB, and AP-1 DNA-binding activities in T cells. Our results revealed that UR-1505 specifically inhibits NF-AT DNA-binding in T cells without effect on other transcription factors such as NF-κB and AP-1. The selective NF-AT inhibition explains the decreased expression of IL-5 and IFN-γ, because their expression is modulated through NF-AT sites in their promoter regions (Lee et al., 1995; Hogan et al., 2006). Indeed, two NF-AT inhibitors as CsA and tacrolimus completely abrogate IL-5 and IFN-γ gene expression.

Inhibition of NF-AT seems to be a common point in the prevention of both T-cell proliferation and cytokine gene expression by UR-1505 because NF-AT is known to modulate the expression of hundreds of genes in T cells (Peng et al., 2001; Macian, 2005) and to promote the transition from G_1 to S phase once TCR is engaged (Feske et al., 2001; Appleman et al., 2002; Caetano et al., 2002; Lipskaia and Lompre, 2004). In addition, UR-1505 inhibits T cell proliferation and cytokine expression in a range of concentrations similar to that needed to prevent NF-AT activation. Indeed, UR-1505 follows a pattern similar to that of CsA, because it inhibits only TCR-dependent cell proliferation (and does not inhibit JAK/STAT-dependent proliferation) and prevents NF-AT DNA binding, without effect on AP-1 DNA binding activity. UR-1505 does not inhibit NF-κB, although our results shown that CsA has a partial inhibitory effect on NF-κB DNA-binding, according to previous reports that CsA inhibits NF-κB by inhibition of proteosome activity (Meyer et al., 1997).

Our results are in agreement with previous data reporting that salicylates inhibit cell proliferation through inhibition of Ca^{2+}/calcineurin/NFAT pathway (Núñez et al., 2006) because this pathway is activated after TCR engagement. On the other hand, Aceves et al. (2004) have shown that salicylates prevent NF-AT-dependent gene expression. Therefore, NF-AT is a target for salicylates and inhibition of NF-AT results in decreased cell proliferation and cytokine production.

The inhibition of NF-AT produced by UR-1505 may also explain the previously reported specificity of UR-1505 inhibiting splenocyte proliferation without effect on murine bone marrow-derived macrophage proliferation (Fernández de Arriba et al., 2005), because NF-AT plays a central role in T lymphocytes, whereas NF-κB seems to be more relevant in macrophages.

The precise mechanism through which UR-1505 inhibits NF-AT activation is not completely known. NF-AT may be regulated at different levels: phosphorylation/dephosphorylation, subcellular localization, DNA binding activity, and transactivation (Rao et al., 1997; Aceves et al., 2004). Among these possibilities, our data indicate that UR-1505 acts at the level of DNA binding and/or transactivation, because UR-1505 failed to inhibit NF-AT translocation into the nucleus in response to ionomycin stimulation of HeLa cells. This precludes an inhibitory effect of UR-1505 on calcineurin activity. By contrast, CsA, a genuine calcineurin inhibitor, blocks ionomycin-induced NF-AT nuclear translocation in HeLa cells. Therefore, although the pattern of UR-1505 and CsA as inhibitors of T cell activation is similar, the mechanisms involved are different. Furthermore, these results are in agreement with those of Aceves et al. (2004), who reported that salicylates inhibit NF-AT–dependent transcription without effect on ionomycin-induced NF-AT nuclear localization. Besides nuclear import, another important point of regulation of NF-AT is the reversible phosphorylation of NF-AT proteins by specific kinases affecting its nuclear export rates. In that sense, phosphorylation of NF-AT by p38, GSK, and CK1 in some residues is known to lead to NF-AT export to cytoplasm and decreased NF-AT DNA binding (Beals et al., 1997; Chow et al., 1997; Kiani et al., 2000). Therefore, inhibition of NF-AT DNA binding by UR-1505 may be the consequence of increased kinase activity that enhanced NF-AT nuclear export.

In that sense, we have determined that UR-1505 has no effect on in vitro kinase activity of p38, GSK-3 and CK1. Indeed, blockade of nuclear export by leptomycin B has no consequence on decreased NF-AT DNA binding, suggesting that the mechanism of UR-1505 is not mediated by an increase of NF-AT export.

Although in most cases NF-AT rephosphorylation induces its inactivation through nuclear export, there are increasing data showing that phosphorylation in specific residues belonging to the NF-AT regulatory domain increases its transcriptional activity, improving their DNA binding by means of comformational changes and/or by recruiting cofactors as p300. In that sense, Okamura et al. (2000) have reported an inducible phosphorylation in the transactivation domain of NF-ATc2 after stimulation of T cells with PMA plus ionomycin, which enhances its transcriptional activity (Okamura et al., 2000). Furthermore, Pim kinase I (Rainio et al., 2002), JNK (Ortega-Pérez et al., 2005), and Cot kinase (de Gregorio

et al., 2001) phosphorylate NF-ATc1 and NF-ATc2, increasing their transcriptional activities. Therefore, a reasonable possibility is that UR-1505 may inhibit NF-AT DNA binding, reducing the activity of the inducible kinase that increases NF-AT transactivation (Okamura et al., 2000). Studies are in progress to further identify the kinases targeted by UR-1505.

In conclusion, our results showed that UR-1505 inhibits T-cell activation, a phenomenon characterized by inhibition of both T-cell proliferation and cytokine production, and both effects seem to be the consequence of NF-AT inhibition. Furthermore, our data indicate that the mechanism of action of UR-1505 is different from typical calcineurin inhibitors. Because the adverse effects of CsA and tacrolimus (nephrotoxicity, neurotoxicity, diabetogenicity, and gastrointestinal toxicity) are due to the calcineurin inhibition in cells outside the immune system (Fung et al., 1991; Dumont et al., 1992; Ho et al., 1996), UR-1505 may be safer than calcineurin inhibitors. UR-1505 clinical development is in progress for atopic dermatitis. Clinical results in this indication and others will show whether UR-1505 could be a safer and effective alternative to calcineurin inhibitors.

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