

Endothelial Cells Stimulate T Cell NFAT Nuclear Translocation in the Presence of Cyclosporin A: Involvement of the wnt/Glycogen Synthase Kinase-3 β Pathway¹

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T cells resistant to the immunosuppressive drug cyclosporin A (CsA) may be important mediators of chronic graft rejection. We previously reported that T cells activated in the presence of endothelial cells (EC) develop resistance to CsA, and initiate IL-2 secretion within 8–12 h of triggering. CsA normally blocks the phosphatase, calcineurin, thus preventing nuclear translocation of the transcription factor, NFAT. We find that in the presence but not the absence of EC, NFAT1 can be detected in the nuclei of CsA-treated T cells within 8 h of triggering, reaching a maximal level of 60% of control by 24 h. Glycogen synthase kinase-3 β (GSK-3 β), which rephosphorylates NFAT and promotes nuclear export, is inhibited by EC costimulation. GSK-3 β is a component of the wnt signaling pathway, and EC express wnt-5a and T cells express frizzled-5, a wnt-5a receptor. Wnt-5a promotes T cell NFAT nuclear accumulation in the presence of CsA, an effect mimicked by Li⁺, a potent inhibitor of GSK-3 β . The protein kinase C agonist PMA dramatically synergizes with both EC and wnt-5a in stimulating T cell IL-2 synthesis, and inhibition of either protein kinase C by Ro-31-8425 or G-proteins by pertussis toxin effectively blocks the actions of wnt-5a on T cells. Finally, a secreted, dominant-negative form of frizzled-5 blocks EC-mediated CsA resistance. Thus, EC promote CsA-resistant nuclear localization of NFAT and subsequent IL-2 synthesis through a noncanonical wnt-dependent pathway. *The Journal of Immunology*, 2002, 169: 3717–3725.

T cells that become resistant to immunosuppressive drugs such as cyclosporin A (CsA)³ and FK506 may have a major role in driving chronic graft rejection. We are interested in the role of endothelial cells (EC) in the development of T cell resistance to these drugs (1, 2).

T cell activation requires an Ag-specific signal mediated via the TCR/CD3 complex, and non-Ag-specific signals provided by costimulatory molecules such as CD2 and CD28. Upon receipt of these signals, a series of intracellular events is triggered, including Ca²⁺ mobilization and protein kinase C (PKC) activation, leading to cytokine production and cell proliferation. Critical to this process is the synthesis or translocation of several transcription factors, including those of the AP-1, NF- κ B, and NFAT families. Members of the NFAT family preexist in the cytoplasm and, upon activation, translocate to the nucleus where they act in synergy with AP-1 and possibly other proteins (3–7) to transactivate several cytokine and cell surface receptor genes, including IL-2 (8), IL-4 (9, 10), TNF- α (11, 12), and CD40 ligand (13).

Members of the NFAT family are widely expressed by cells of the immune system; however, in normal human T cells, only NFAT1 (NFATp), NFAT2 (NFATc), and NFAT4 have been ob-

served (14). NFAT1 is the predominant species in normal T cells and is constitutively expressed. NFAT4 is also present constitutively, but its expression is extremely low and is not enhanced upon stimulation. NFAT2 is induced following T cell stimulation to the same level as NFAT1; however, in nuclear extracts from activated T cells, nearly all the NFAT that bound to a probe corresponding to the distal NFAT site of the human IL-2 promoter was attributable to NFAT1 (14). In contrast to normal T cells, NFAT2 seems to play the major role in driving IL-2 transcription in Jurkat T cells (15).

The activity of NFAT proteins is regulated by their phosphorylation state, which is controlled by the opposing action of the Ca²⁺-dependent Ser/Thr phosphatase, calcineurin (CaN), and the Ser/Thr kinase, glycogen synthase kinase-3 β (GSK-3 β). In resting T cells, NFAT is phosphorylated and localized to the cytoplasm; upon cell stimulation, it is dephosphorylated by CaN, resulting in nuclear translocation and enhanced DNA-binding affinity (16–18). The activity of CaN is highly sensitive to the immunosuppressive drugs CsA and FK506 (4, 18–20), which act to prevent CaN-dependent nuclear translocation of NFAT. Nuclear NFAT is rapidly rephosphorylated by a priming kinase and GSK-3 β , facilitating its rapid nuclear export (21). The activity of GSK-3 β is decreased following T cell activation with PMA (22), or by activation of the phosphatidylinositol 3-kinase or wnt signaling pathways (23–25). Furthermore, wnt signaling has been shown to activate NFAT in *Xenopus* (26), and overexpression of a constitutively active form of GSK-3 β in mouse T cells results in reduced IL-2 synthesis and proliferation (27). The duration of nuclear residence of NFAT has been correlated with the pattern of cytokine expression in T cells, suggesting that fine tuning of the pathways regulating this transcription factor is critical for cell differentiation (28).

Graft arteriosclerosis, the chronic inflammation of the vessels in transplanted organs (29, 30), has been attributed to presentation of allogeneic MHC molecules by graft EC to the host immune system

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³ Abbreviations used in this paper: CsA, cyclosporin A; EC, endothelial cell; CaN, calcineurin; GSK-3 β , glycogen synthase kinase-3 β ; PKC, protein kinase C; OA, okadaic acid; CHO, Chinese hamster ovary; Fz, frizzled; sFz-5, secreted sFz-5; HA, hemagglutinin; CRD, cysteine-rich domain.

(1). Interestingly, human EC can activate resting CD4⁺ T cells in culture and provide signals resulting in T cell resistance to CsA and FK506, suggesting a mechanism for bypassing immunosuppression in vivo (2, 31, 32). T cell resistance to CsA requires contact with EC and is dependent on EC expression of CD2 ligands (2). Although a combination of PMA and CD28 mAb has also been shown to induce CsA resistance in T cells (33, 34), CD28 ligands are not expressed by EC, and Abs or fusion proteins that block CD28, B7.1, and B7.2 do not block EC-mediated T cell CsA resistance (2). Interestingly, in T cells made resistant to CsA by PMA + CD28 mAb treatment, nuclear NFAT is still observed (14, 35).

In the present study, we asked the question whether T cell NFAT becomes localized in the nucleus during EC-mediated CsA resistance, and if so, by what mechanism. We find that in the presence of EC, neither CsA nor a competitive CaN-blocking peptide are able to block the accumulation of NFAT in the nucleus of activated T cells. Furthermore, the ability of EC to induce T cell CsA resistance involves activation of the wnt/GSK3- β pathway.

Materials and Methods

Reagents

PHA, pertussis toxin, and CsA were from Sigma-Aldrich (St. Louis, MO). PMA, ionomycin, and Ro-31-8425 were from Calbiochem (La Jolla, CA). Abs to HLA-DR (HB145) and CD11b (HB204) were purified from culture supernatants of cells obtained from American Type Culture Collection (Rockville, MD). Polyclonal rabbit anti-NFATp (67.1) was a generous gift from Dr. A. Rao (Harvard Medical School, Boston, MA), and goat anti-rabbit IgG-FITC was from Sigma-Aldrich.

The NFAT "sprite" (SHSPRIEITPSH) and control (SGSGSPAI AIAPSH) peptides were synthesized by Chiron Technologies (Melbourne, Australia). Both were modified by an N-terminal acetylation and a C-terminal amidation to prevent proteolytic degradation and extend their intracellular half-lives. The hemagglutinin (HA)-tagged GSK-3 β expression plasmid was a kind gift from Dr. G. Crabtree (Stanford University, Stanford, CA).

Cell isolation and culture

Human EC were isolated from umbilical veins and cultured as previously described on gelatin-coated tissue culture plastic in Medium 199 supplemented with 20% FBS, penicillin/streptomycin (all from Life Technologies, Grand Island, NY), endothelial cell growth supplement (Collaborative Biomedical, Bedford, MA), and heparin (Sigma-Aldrich; Ref. 36). EC were used between passages 3 and 5.

PBMC were obtained from heparinized venous blood by centrifugation over Lymphocyte Separation Medium (Organon Technika, Durham, NC). Purified T cells were isolated from PBMC by negative selection over magnetic beads (BioSource International, Camarillo, CA), using Abs to CD11b and HLA-DR. The purity of the T cell population was assessed by two-color FACS analysis using directly conjugated Abs to CD3, CD4, CD8, CD14, CD19, CD56, and HLA-DR (BD Biosciences, Mountain View, CA). Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences). We consistently obtained <2% B cells and monocytes, and >93% CD3⁺ T cells.

T cell coculture with EC or Chinese hamster ovary (CHO) cells

EC were plated and grown to confluence in gelatin-coated 96-, 24-, or 6-well plates, onto FBS-coated glass coverslips, or onto fibronectin-coated glass chamber slides (Fisher, Tustin, CA) and grown to confluence. CHO cells were plated in 24-well plates or on glass chamber slides and grown to confluence. Medium was removed and 1–4 \times 10⁵ or 5–10 \times 10⁶ resting T cells added in RPMI 1640 containing 10% FBS, penicillin/streptomycin (all from Life Technologies), and 50 μ M 2-ME (Sigma-Aldrich). PHA, PMA, ionomycin, LiCl, and CsA were added as indicated. In some experiments, cells were transfected or pretreated with drug before coculture (see below).

At the times indicated, the cells were stained for NFAT or lysed for kinase assay, and/or the supernatants were assayed for IL-2 by bioassay using IL-2-dependent HT-2 cells as previously described (36). The ID₅₀ values, which represent the CsA concentration that reduces IL-2 secretion to 50% of the level secreted in the absence of CsA, were determined as previously described (2).

Immunofluorescent staining

For NFAT staining, coverslips were gently washed to remove nonadherent cells. Adherent cells were fixed with 4% paraformaldehyde for 10 min, permeabilized for 10 min with 10 \times permeabilizing solution (BD Biosciences), and then incubated 1 h with 67.1 (α NFAT1) or normal rabbit serum diluted 1/1000 in PBS/1% BSA/1% goat serum. Ab binding was visualized by a 30-min incubation with goat anti-rabbit IgG-FITC (1/200 in PBS/1% BSA/1% goat serum). Coverslips were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and viewed by epifluorescence on a Zeiss Axiophot (Zeiss, Oberkochen, Germany). Quantitative data were obtained by scoring cells in at least five randomly chosen fields as nuclear or cytoplasmic for NFAT. Photographs were shot on Kodak Ektachrome 400ASA (Kodak, Rochester, NY) and the slides digitally scanned. Areas of interest were cropped and the contrast adjusted using Adobe Photoshop (Adobe Systems, Mountain View, CA). The images were not altered in any other way.

PCR and cloning of wnt-5a and frizzled (Fz)-5

RNA from resting or activated T cells (1 \times 10⁷ cells) and EC (1 \times 10⁶ cells) was isolated using the TRIzol method (Life Technologies). cDNA was prepared from 2 μ g of random-primed RNA using Superscript II RT (Life Technologies). Fz-5 was amplified using the following primers: fz-5 upper, GGGCCCGTTCGTGTGCAAGTGTGCG, and fz-5 lower, GCAGG GCCCGTGGTCTCGTAGTGGA. Full-length wnt-5a was amplified by PCR using the primers: upper, 5'-CCCCAAGCTTAAGCCCAGGAGT TGC-3' and lower, 5'-CCGGGATTCACCCACTACTTGCACA-3', cloned into the *Hind*III and *Eco*RI sites of pcDNA-3.1(+) (Invitrogen, Carlsbad, CA), and confirmed by sequencing. For cloning of the Fz-5 extracellular domain (secreted Fz-5, sFz-5), pcDNA-3.1(+) was first modified to carry a FLAG tag by insertion of the FLAG sequence between the restriction enzyme Asp 718 and *Eco*RI sites. The extracellular domain was amplified by PCR using the primers: upper, 5'-TAGCTGTAGCCGATTCTCTGCTGTGT-3' and lower, 5'-TAGCTAAGCTTCCAGAAGGTGGCGAA-3', cloned into the *Nhe*I and *Hind*III sites of the FLAG/pcDNA-3.1(+) vector and confirmed by sequencing. Expression of FLAG/sFz-5 was confirmed by Western blot. The PCR were conducted for 35–40 cycles at an annealing temperature of 68°C for Fz-5, 59°C for wnt-5a, and 55°C for sFz-5. GAPDH was used as a positive control, and no-RT controls were run to confirm lack of genomic contamination.

Peptide delivery to resting T cells

Resting PBMC were loaded with 0.5 mM control or SPRIEIT peptide by electroporation at 250 mV, 960 μ F, and allowed to rest 1 h at 37°C before coculture. Electroporation did not significantly affect cell viability as IL-2 production was reduced by <5% in cells electroporated with control peptide compared with nonelectroporated cells (data not shown). Using a FITC-labeled protein (Ig) as a marker, we estimate that ~50% of the cells are electroporated under these conditions (data not shown).

Transfections

Transient transfections of EC or CHO cells were achieved using Lipofectamine (Life Technologies) or electroporation (EC). For transfection by Lipofectamine, EC were plated at 50% confluence and maintained without a change of medium for 4 days to synchronize the cells in G₁; CHO cells were maintained in log phase growth. Cells were then trypsinized and replated at 40% confluence on 6-well plates with fresh growth medium. Twenty-six to 30 h later, cells were washed twice with serum-free medium and incubated with 1 μ g of DNA in Optimem medium (Life Technologies). After 90 min at 37°C, cells were washed once and incubated overnight with EC or CHO growth medium. For electroporation of EC, cells were maintained in log phase growth. A total of 1 \times 10⁶ cells were suspended in 500 μ l M199/5% FBS with 5–10 μ g of DNA and pulsed once at 250 mV, 960 μ F. Cotransfection with a green fluorescent protein plasmid showed typical transfection efficiencies of 10–30% for Lipofectamine-transfected EC, and 50–70% for CHO cells and electroporated EC.

Resting T cells were transfected using the Nucleofector system (Amaxa Biosystems, Germany), a modified form of electroporation that delivers the DNA directly into the nucleus. The procedure provided by the manufacturer was followed. Briefly, 5 \times 10⁶ T cells were suspended in 100 μ l Human T Cell Nucleofector solution containing 5 μ g DNA and pulsed once under program U-14. Typical transfection efficiencies were 35–50%, as determined by cotransfection with a green fluorescent protein plasmid.

Kinase assay

For GSK-3 β kinase assay, resting T cells were transfected with an HA-tagged GSK-3 β expression plasmid. Following treatment, cells were collected and resuspended in lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 μ M NaF, 0.1 mM Na₂VO₄, and protease inhibitor mixture (Roche, Indianapolis, IN)). Lysates were pre-cleared with 75 μ l protein A agarose (Sigma-Aldrich), then added to 75 μ l protein A agarose preincubated with anti-HA Ab. Following incubation for 1 h at 4°C, the beads were pelleted and washed once with lysis buffer and once with kinase assay buffer (8 mM MOPS (pH 7.4), 10 mM Mg acetate, and 2 mM EDTA). After washing, the beads were resuspended in 40 μ l kinase assay buffer containing 10 μ Ci [γ -³²P]ATP, 125 μ M unlabeled ATP, and 200 μ M GSK-3 β substrate or control peptide (Upstate Biotechnology, Lake Placid, NY). Reactions were incubated for 30 min at 30°C and stopped by pelleting and adding the supernatant to 20 μ l of 40% TCA. Reactions were spotted onto P-81 filters and washed three times with 0.75% H₃PO₄ and once with acetone before quantitation by scintillation counting.

Results

CsA resistance requires 8–12 h to develop and correlates with nuclear localization of NFAT

CsA is a potent inhibitor of IL-2 synthesis, with a half maximal effect (ID₅₀) of 1–3 ng/ml in most assays. However, T cells polyclonally activated in the presence of EC are largely resistant to CsA, the ID₅₀ increasing 30- to 100-fold to 100–300 ng/ml (2). Resistance is not due to either sequestration of CsA by EC or to degradation of CsA, as CsA preincubated with EC for 24 h is equally as effective as freshly prepared CsA (Ref. 2 and data not shown).

The major immunosuppressive action of CsA is to block cytokine production by inhibiting nuclear translocation of the transcription factor NFAT. Therefore, CsA-resistant IL-2 synthesis could be due to mechanisms that circumvent the block imposed by CsA, thus allowing NFAT translocation, or be due to the presence of redundantly acting transcription factors compensating for the absence of nuclear NFAT. To answer this question, we costimulated T cells with EC in the presence or absence of CsA, and examined over time the location of NFAT and the production of IL-2. Through the first 8 h, the T cells were completely sensitive to CsA and no IL-2 was produced, regardless of the presence or absence of EC (Fig. 1*a*). However, between 8 and 12 h, T cells in the presence (but not the absence) of EC began to develop resistance to the drug and started secreting IL-2. By 24 h in the presence or absence of CsA, IL-2 levels had reached a plateau which was maintained to at least 30 h (Fig. 1*a* and data not shown), suggesting that the lower levels seen in the presence of CsA are not due simply to a delay in the onset of synthesis.

NFAT localization was determined in parallel by immunofluorescence using the NFAT1-specific Ab, mAb 67.1. Fig. 1*b* shows a representative field from an EC-T cell coculture in the presence of CsA at 24 h. Interestingly, many PHA-activated T cells adherent to EC show nuclear staining for NFAT. In the absence of CsA, NFAT was detectable in the nucleus of 42% of the EC-adherent T cells by 4 h, and this increased to almost 60% by 24 h (Fig. 1*c*). In stark contrast, CsA completely blocked the translocation of NFAT at early times; only 2% of EC-adherent T cells demonstrated nuclear NFAT at 4 h. However, within the next 4 h, this population increased substantially, so that by 8 h, 16% of the T cells had NFAT in the nucleus compared with 50% of cells stimulated in the absence of CsA. By 24 h, 44% of T cells had nuclear NFAT in the presence of CsA compared with 60% in the absence of CsA. In the absence of EC, IL-2 synthesis was completely blocked by CsA and very little (<1%) nuclear translocation of NFAT was observed. In these studies, we have mostly concentrated on NFAT1;

however, NFAT2 also translocated to the nucleus in response to EC-derived signals in the presence of CsA (data not shown).

These data indicate that in the presence of CsA, NFAT can still enter the nucleus of T cells costimulated with EC, and that the time course of its accumulation in the nucleus correlates well with the induction of IL-2 secretion between 8 and 12 h.

EC also provide resistance to a CaN-blocking peptide

When introduced into T cells, the 7-aa peptide ¹¹⁰SPRIEIT (sprite), contained in the regulatory domain of NFAT1, blocks recognition and dephosphorylation of NFAT1 by CaN and subsequent nuclear localization (37). This sequence is conserved in NFAT2, and a similar sequence is present in NFAT3 and NFAT4. T cells were transfected with SPRIEIT or control peptide and examined for NFAT localization and IL-2 secretion following 12 h of stimulation in the presence or absence of EC. Peptides were protected from degradation by N- and C-terminal blocking (see *Materials and Methods*). In the absence of EC, the SPRIEIT peptide reduced IL-2 secretion by 45% relative to control peptide (Fig. 2), and NFAT translocation by 41% (data not shown). Because our transfection efficiency was ~50% (data not shown), this corresponds to inhibition of IL-2 secretion by transfected cells of >90%. In the presence of CsA, no IL-2 was detectable. In contrast, in the presence of EC, T cells were completely resistant to the effects of SPRIEIT peptide, and were mostly resistant to CsA inhibition; IL-2 secretion was only blocked by 27%. In summary, the presence of EC permits IL-2 secretion when CaN-mediated NFAT translocation is blocked by three different methods: CsA, the SPRIEIT peptide, and FK506 (2).

We have not assayed T cell CaN activity directly in our system, as it is impossible to separate activated T cells from EC with sufficient purity to overcome contamination from the much larger EC. However, based on previous reports using CsA or SPRIEIT peptide (37), we are confident that CaN activity is blocked in our system. Although we have seen an increase in CaN mRNA in activated T cells, this is not likely to be sufficient to overcome the high concentrations of CsA or FK506 that EC signals can overcome. Indeed, T cells transfected with a CaN expression plasmid and expressing high levels of the enzyme had an ID₅₀ only 2-fold higher than control cells (38). This is in comparison to the 100-fold (3 ng/ml to >300 ng/ml) increase we see in the presence of EC.

There is now good evidence that phosphatases other than CaN are also critical for induction of IL-2 synthesis, as okadaic acid (OA), which blocks Ser/Thr phosphatases such as PP1, PP2A, and PP5, blocks activation of IL-2 transcription (39). This pathway appears to be independent of CaN, which is not blocked by OA, but presumably also targets NFAT, as activity of an NFAT reporter was completely inhibited by OA (39). We reasoned that if NFAT export from the nucleus were blocked, then slow nuclear accumulation of NFAT under the influence of these phosphatases could account for CsA-resistant cytokine synthesis. This could be achieved by inhibition of GSK-3 β activity, possibly by EC activation of the wnt pathway in T cells.

As a first test of this hypothesis, we activated T cells in the presence of CsA and tested the ability of Li⁺, a potent GSK-3 β inhibitor (40), to abrogate inhibition of IL-2 synthesis by CsA. Treatment of T cells with CsA blocked IL-2 secretion by >95%. This effect was reversed by Li⁺, which reduced the ability of CsA to inhibit IL-2 synthesis from 95% to ~50% (Fig. 3*a*). Although EC act as costimulatory cells, greatly enhancing IL-2 production, their ability to block the effect of CsA was similar to that of Li⁺. In the presence of EC, CsA inhibited IL-2 secretion by 31%, compared with 50% in the presence of Li⁺, and >95% when neither

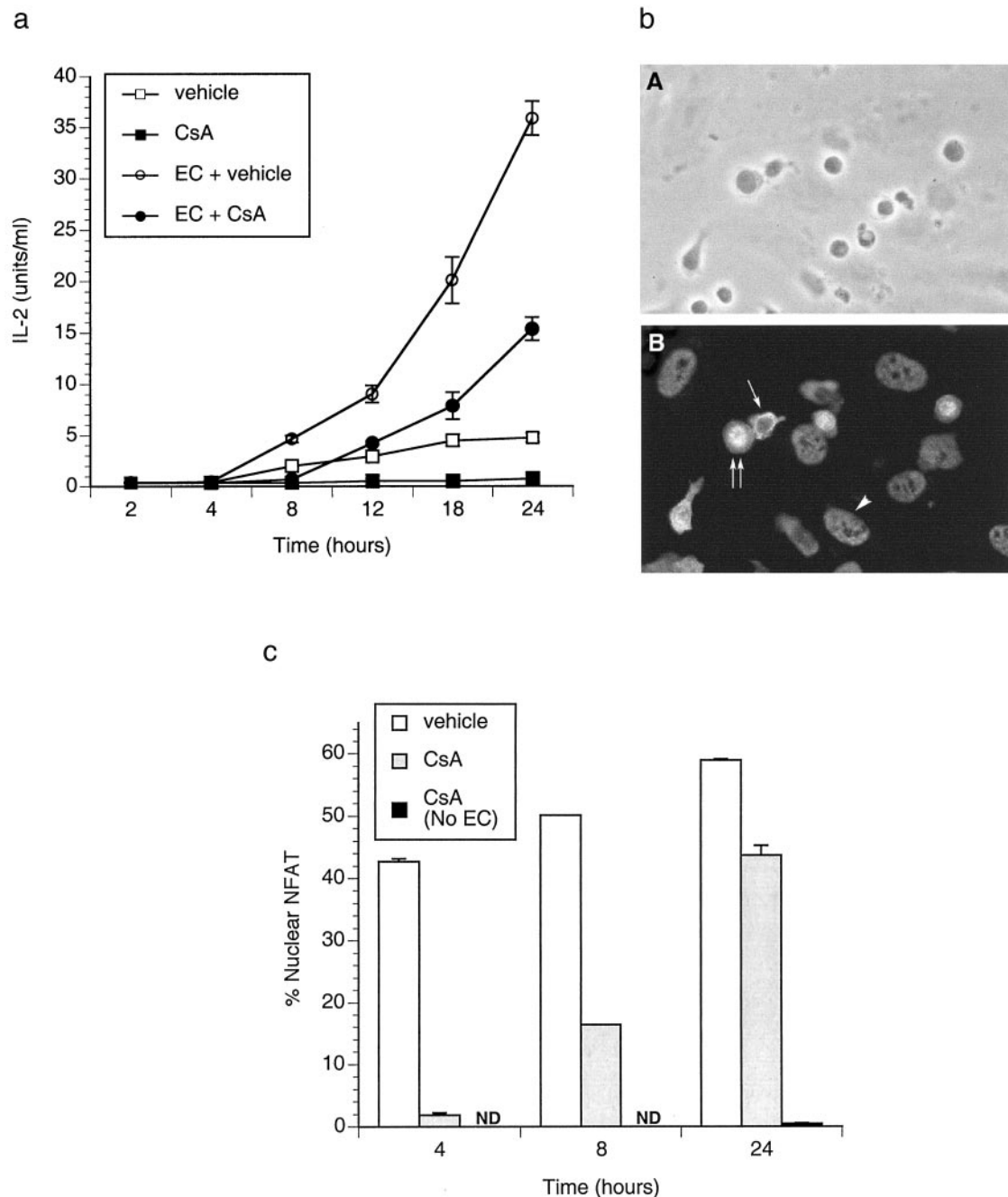


FIGURE 1. EC induce CsA-resistant IL-2 synthesis and nuclear localization of NFAT in T cells. *a*, Resting T cells were activated with 5 $\mu\text{g/ml}$ PHA in the presence or absence of EC, and 100 ng/ml CsA or vehicle control. At the times indicated, supernatants were collected for IL-2 bioassay. In both the presence and absence of CsA, IL-2 levels reach a plateau at 24 h and remain constant out to 30 h (data not shown). One of four similar experiments. *b*, Resting T cells were stimulated with 5 $\mu\text{g/ml}$ PHA in the presence of EC and 100 ng/ml CsA. After 24 h, adherent cells were fixed, permeabilized, and stained for NFAT1 (NFATp). Single arrow indicates a T cell with NFAT in the cytoplasm only. The double arrow indicates a T cell with nuclear NFAT. Arrowhead indicates an NFAT-positive EC nucleus. Note the much larger size compared with T cell nuclei. *c*, Resting T cells were added to EC plated on glass coverslips or chamber slides and activated with 5 $\mu\text{g/ml}$ PHA in the presence or absence of 100 ng/ml CsA. In some cases, T cells alone were collected by cytopsin. Cells were fixed, permeabilized, and stained for NFAT1 at the times indicated. For each point, 150–300 adherent T cells were evaluated for NFAT localization. The percentage of T cells with NFAT in the nucleus is plotted, normalized to the maximum value in the absence of CsA. Average of three experiments.

EC nor Li^+ were present. Interestingly, there was no additional effect of EC and Li^+ (data not shown). We found a similar effect of Li^+ on NFAT localization. T cells activated in the presence of CsA exhibited a 30% reduction in nuclear localization of NFAT when Li^+ was also present, compared with a 90% reduction in the absence of Li^+ (Fig. 3*b*). Thus, Li^+ maintains NFAT in the nu-

cleus and induces resistance to CsA. Augmentation of IL-2 synthesis by Li^+ was recently reported (27).

Given that the effect of treatment with Li^+ was similar to that observed in the presence of EC, we wished to determine the effect of EC costimulation on GSK-3 β activity in T cells. It was not possible to measure endogenous GSK-3 β in T cells due to the

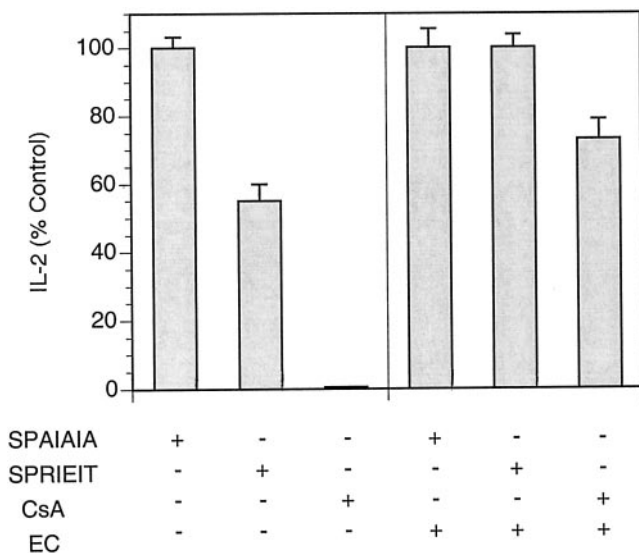


FIGURE 2. EC costimulation provides resistance to a CaN-blocking peptide. Resting PBMC, transfected with 0.5 mM SPRIEIT or control peptide, both acetylated and amidated to extend intracellular half-life, were activated for 12 h with 5 μ g/ml PHA in the presence or absence of EC and 100 ng/ml CsA or vehicle control. Following stimulation, culture supernatants were collected and assayed for IL-2 by HT-2 bioassay. In the absence of EC, control peptide-treated T cells generated 7 U/ml IL-2; in the presence of EC, control peptide-treated cells produced 38 U/ml. Nontransfected cells produced equivalent levels of IL-2 as control-transfected cells. Transfection efficiency was assessed with FITC-Ig and was ~50% (data not shown). SEs for the nonnormalized data were <10% of the mean. One of four similar experiments.

difficulty in separating EC and activated T cells. EC contain 10- to 20-fold more GSK-3 β activity than T cells (data not shown) resulting in uninterpretable data with as little as 5% EC contamination of the T cell preparation. Therefore, T cells were transfected with an HA-tagged GSK-3 β expression plasmid and incubated 4 h in the presence or absence of EC. Tagged GSK-3 β was then immunoprecipitated with anti-HA, and its activity assessed by kinase assay. As shown in Table I, GSK-3 β activity was inhibited by 30–70% in the presence of EC, demonstrating that T cell GSK-3 β is regulated by EC costimulatory signals.

In many cells, GSK-3 β is a downstream target of the wnt signaling pathway. It has been reported that EC express several wnts, including wnt-5a (41). Using RT-PCR, we have confirmed expression of wnt-5a by both resting and cytokine-activated EC, and demonstrated expression of Fz-5, a receptor for wnt-5a (42), in T cells activated with PMA + ionomycin or EC + PHA (Fig. 4*a* and data not shown). Interestingly, PHA alone did not induce Fz-5 expression, suggesting a requirement for costimulation. To test whether wnt-5a mimicked the effect of Li⁺ and targeted NFAT in T cells, we transfected CHO cells with an expression construct for wnt-5a, and examined their ability to induce CsA-resistant nuclear localization of NFAT in ionomycin-treated T cells. In the absence of wnt-5a signaling, CsA reduced nuclear localization of NFAT by >90% (Fig. 4*b*). However, in the presence of wnt-5a, we only observed a 50% reduction, indicating that wnt-5a can provide a signal to T cells resulting in nuclear localization of NFAT in the presence of CsA.

Costimulation by EC activates a PKC-dependent pathway

Wnt-5a has been reported to act primarily through PKC and Ca²⁺, rather than by stabilization of β -catenin (43–45), and GSK-3 β is a direct downstream target of PKC (23, 46). Moreover, PKC stim-

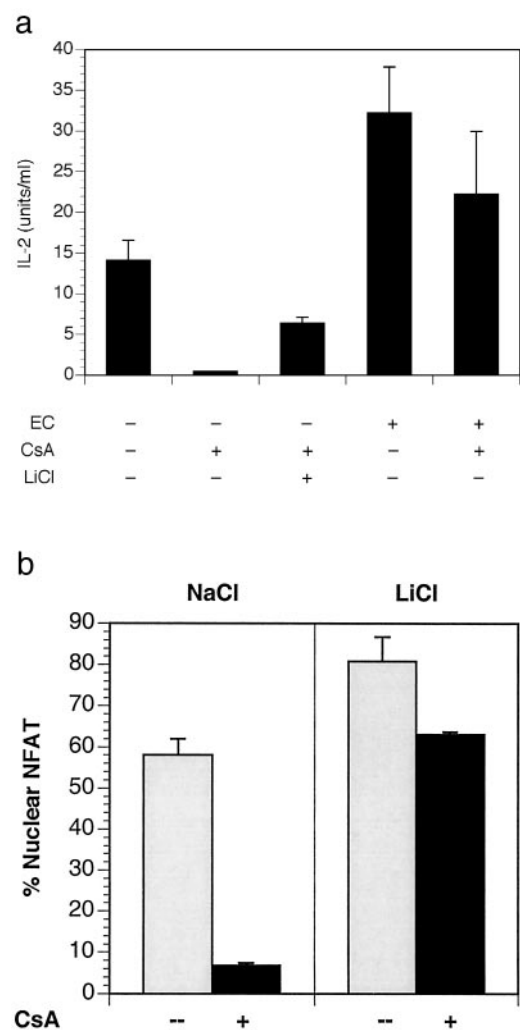


FIGURE 3. LiCl promotes CsA-resistant IL-2 synthesis and NFAT nuclear accumulation. *a*, T cells were stimulated with PHA (5 μ g/ml), either alone or with EC. Where indicated, CsA (100 ng/ml) or LiCl (10 mM) were added at the start of culture. Supernatants were harvested at 24 h and assayed for IL-2 by HT-2 bioassay. Shown are means and SDs of triplicate samples. One of three similar experiments. *b*, Resting T cells were stimulated for 24 h with 1 μ g/ml ionomycin in the presence of 10 mM LiCl or NaCl and the presence or absence of 100 ng/ml CsA. Following stimulation, cells were collected by cytospin, then fixed, permeabilized, and stained for NFAT. For each condition, 100–150 cells were evaluated for NFAT localization. Data were normalized to the maximum value in the absence of CsA. Average of three experiments.

ulation of the wnt/adenomatous polyposis coli/ β -catenin proliferative signaling pathway has been demonstrated *in vivo* (47). Previously, we had noted that chronic pretreatment of T cells with 25–50 ng/ml of the PKC activator PMA made the T cells largely refractory to subsequent EC costimulatory signals, presumably due to down-regulation of the enzyme (Refs. 48 and 49, and our unpublished observations). Addition of the specific PKC inhibitor Ro-31-8425 to cocultures resulted in the complete inhibition of T cell IL-2 synthesis, making it impossible to assess the contribution of EC costimulation through this pathway (data not shown). Therefore, we looked for synergy between EC and PMA. We examined the ability of EC to enhance IL-2 secretion by T cells stimulated for 24 h over a range of PMA concentrations, with ionomycin held constant at 1 μ g/ml. EC modestly enhanced IL-2 secretion at all PMA concentrations above 0.05 ng/ml, but with a particularly

Table I. EC costimulatory signals inhibit GSK-3 β activity in T cells

Expt.	GSK-3 β Activity (cpm) ^a		% Inhibition
	T cells	T cells + EC	
1	13,167	3,860	71
2	8,450	4,413	48
3	61,494	45,164	28
4	45,570	22,912	50

^a Resting T cells transfected with an HA-tagged GSK-3 β expression plasmid were incubated 4 h in the presence or absence of EC. Cell lysates were prepared, HA-tagged GSK-3 β was immunoprecipitated, and activity was determined by kinase assay. Thus, only the activity of GSK-3 β in the T cells was assayed.

striking effect at 0.2 ng/ml (Fig. 5). This result has been entirely reproducible between 0.2 and 0.4 ng/ml over a series of six experiments, and suggests that T cell PKC is indeed a target of EC costimulation. We believe that the absence of a similarly high degree of synergy at increased PMA concentrations, those typically used to activate T cells, reflects the occurrence of PMA-induced PKC down-regulation (48, 49).

We next wished to determine whether wnt-5a could synergize with PKC in T cells. As a control, we examined the role of the known EC costimulatory molecule, CD2, (36) in this pathway. We

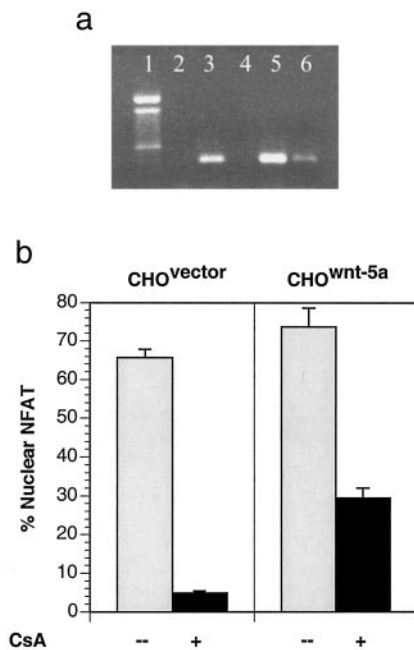


FIGURE 4. Activated T cells express transcripts for Fz-5 and the Fz-5 ligand wnt-5a, which induces nuclear accumulation of NFAT in the presence of CsA. *a*, Resting T cells or EC were cultured together or alone as indicated for 24 h and then harvested for RNA preparation. RT-PCR was performed using primers specific for Fz-5. Lane 1, marker; lane 2, resting T cells; lane 3, T cells activated with 25 ng/ml PMA and 1 μ g/ml ionomycin; lane 4, T cells activated with 5 μ g/ml PHA; lane 5, T cells activated with 5 μ g/ml PHA in the presence of EC; lane 6, resting EC. Control reactions were run without RT to confirm lack of genomic contamination. Amplification with GAPDH primers confirmed the presence of cDNA in all reactions (data not shown). *b*, Resting T cells were stimulated with 1 μ g/ml ionomycin in the presence of wnt-5a- or vector-transfected CHO cells, and the presence or absence of 100 ng/ml CsA. After 24 h of stimulation, adherent cells were fixed, permeabilized, and stained for NFAT. A total of 100–150 cells were evaluated for NFAT localization under each condition. Data were normalized to the maximum value in the absence of CsA. Average of three experiments.

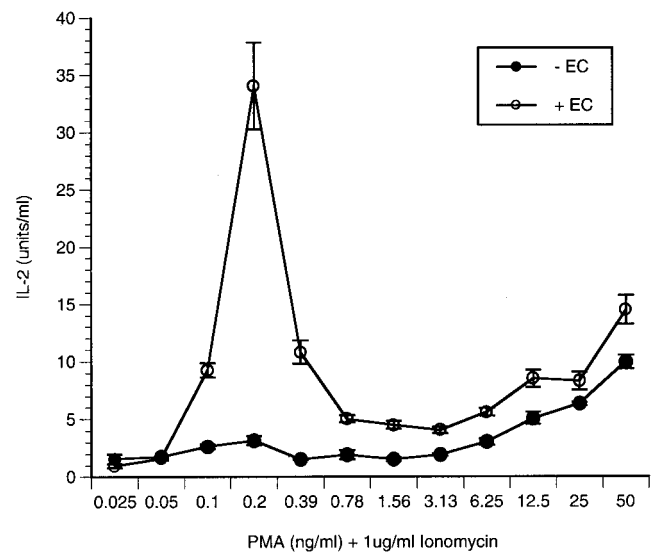


FIGURE 5. EC costimulation synergizes with the PKC agonist PMA. Resting T cells were incubated with EC, ionomycin (1 μ g/ml), and the indicated concentrations of PMA for 24 h. IL-2 was assayed by HT-2. One of six similar experiments.

found that EC enhancement of IL-2 secretion at 0.2 ng/ml PMA was not inhibited by a CD2 blocking Ab. Similarly, the addition of a stimulatory pair of CD2 Abs did not mimic the effect of EC (data not shown). In contrast, wnt-5a-transfected CHO cells effectively enhanced IL-2 secretion at low doses of PMA, although the peak augmentation occurred at a slightly higher PMA concentration than with EC (Fig. 6). These data suggest that at least part of the EC costimulatory signal operates through a non-CD2 pathway that targets PKC, and implicates wnts (possibly wnt-5a) as this signal.

Inhibition of PKC or G-proteins blocks wnt-mediated CsA resistance

The noncanonical wnts, those that do not act through β -catenin, have been shown to target PKC and to require G-protein activity

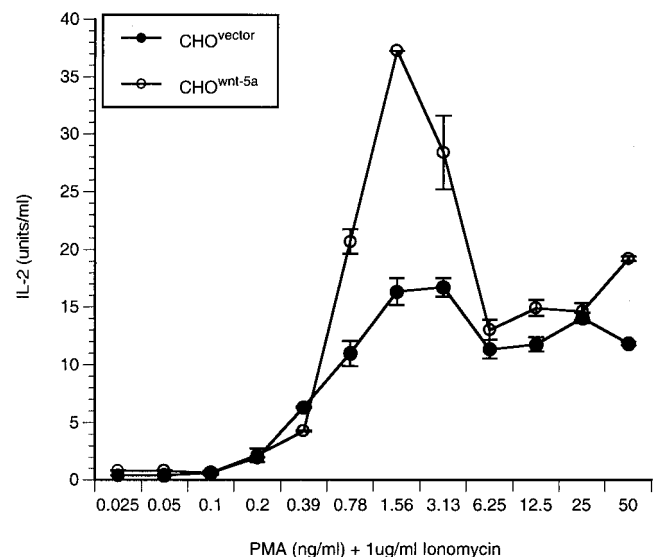


FIGURE 6. wnt-5a synergizes with the PKC agonist PMA. Resting T cells were stimulated with 1 μ g/ml ionomycin and the indicated concentration of PMA in the presence of wnt-5a- or vector-transfected CHO cells. After 24 h, culture supernatants were collected and assayed for IL-2 by HT-2. One of four similar experiments.

(43–45, 50). We further investigated the involvement of a noncanonical wnt pathway by examining the effect of PKC or G-protein inhibition on the ability of wnt-5a to potentiate nuclear accumulation of NFAT in the presence of CsA. Again, ionomycin was used to drive NFAT into the nucleus. In the presence of wnt-5a signaling, 45% of T cells stimulated with ionomycin exhibit CsA-resistant nuclear localization of NFAT, compared with only 1.5% in the absence of wnt-5a (Fig. 7). However, when PKC activity was inhibited by the addition of Ro-31-8425, or by prolonged pretreatment of the T cells with PMA, this effect of wnt-5a was almost completely abrogated. The addition of pertussis toxin, which blocks both $G\alpha_i$ - and $G\beta\gamma$ -mediated signaling by targeting $G\alpha_o$ and $G\alpha_i$ subunits, also dramatically inhibited CsA-resistant nuclear localization of NFAT in the presence of wnt-5a. These data are entirely consistent with previous reports of noncanonical wnts, such as wnt-5a, acting through PKC and G-proteins, and moreover, support the hypothesis that EC-derived wnts may activate this pathway in T cells.

Having demonstrated that wnt-5a is sufficient to confer CsA resistance on T cells, we wished to test whether the wnt pathway is necessary for EC-mediated CsA resistance, by blocking endogenous EC-derived wnt signaling. We transfected EC with an expression plasmid for the cysteine-rich domain (CRD)/extracellular domain of Fz-5 (sFz-5), a known receptor for wnt-5a, or with a control plasmid (Fig. 8). Expression of the Fz-5 CRD was confirmed by Western blot (data not shown). The CRD of Fz-5 has previously been shown to act as a dominant-negative capable of blocking wnt-5a signaling (42). In the presence of control-transfected EC, T cells were resistant to CsA, and synthesized IL-2 with an ID_{50} for CsA of >150 ng/ml. However, in the presence of EC expressing the Fz-5 extracellular domain, the dose-response curve was shifted to the left; the ID_{50} dropping >4-fold to ~30–40 ng/ml. A blocking Ab to Fz5 failed to reduce the ID_{50} , suggesting that wnt-5a does not mediate CsA resistance through Fz-5; however, its ability to bind Fz-5 allows the soluble receptor to effectively prevent wnt-5a from signaling through the appropriate re-

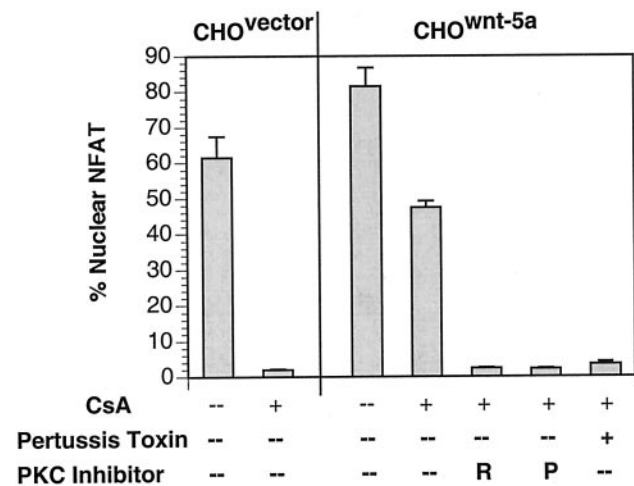


FIGURE 7. Inhibitors of PKC or G-proteins block wnt-5a-mediated CsA-resistant nuclear accumulation of NFAT. Resting T cells in the presence of wnt-5a- or vector-transfected CHO cells were stimulated with 1 μ g/ml ionomycin in the presence or absence of the indicated inhibitor. CsA was used at 100 ng/ml, Ro-31-8425 at 300 ng/ml, and pertussis toxin at 10 ng/ml. PKC down-regulation was achieved by treating T cells overnight with 50 ng/ml PMA. R, Ro-31-8425; P, PMA. After culture for 6 or 18 h, adherent cells were fixed, permeabilized, and stained for NFAT. A total of 100–150 cells were evaluated for NFAT localization under each condition. Average of three experiments.

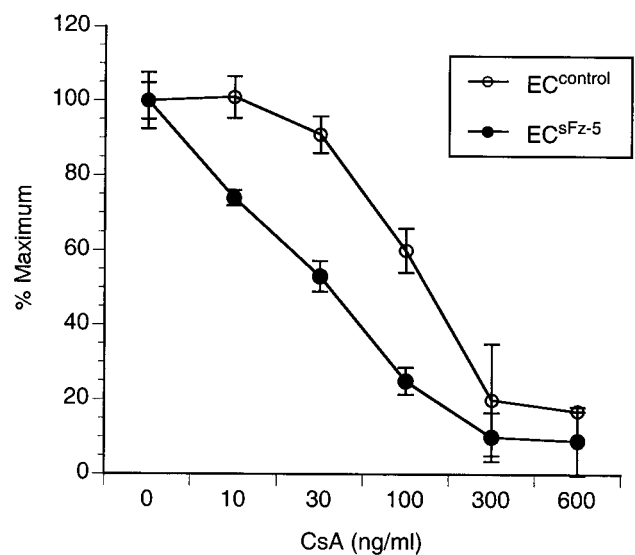


FIGURE 8. sFz-5 reduces EC-mediated CsA-resistant IL-2 synthesis. Resting T cells were incubated with control or sFz-5-transfected EC, 5 μ g/ml PHA, and the indicated concentrations of CsA for 24 h. Supernatants were then harvested for analysis of IL-2 by HT-2 bioassay. sFz-5 expression was confirmed by Western blot (data not shown). One of three similar experiments.

ceptor (data not shown). Therefore, these data confirm that wnt signaling is both necessary and sufficient for EC-mediated T cell resistance to CsA, although contributions from additional pathways have not been ruled out.

Discussion

Previous studies have demonstrated that EC costimulation can induce CsA-resistant IL-2 synthesis by T cells (2). As the major immunosuppressive action of CsA is to block nuclear translocation of the transcription factor NFAT, we tested the hypothesis that EC costimulation bypasses this block and allows NFAT translocation in the presence of CsA. In this study, we confirm this hypothesis by showing EC-induced accumulation of NFAT in the nucleus of CsA-treated T cells, and provide evidence of a role for wnt signaling.

There is now compelling evidence that GSK-3 β regulates NFAT nuclear localization. Direct phosphorylation of NFAT by GSK-3 β has been shown (21), and it was recently demonstrated that Li⁺, a potent inhibitor of GSK-3 β activity, increases the level of nuclear NFAT and prolongs IL-2 synthesis (27). We find that both EC and Li⁺ inhibit GSK-3 β activity and allow nuclear accumulation of NFAT, and IL-2 synthesis, in the presence of CsA. EC reduce GSK-3 β activity by 30–70% (Table I), similar to the 60–70% reduction we see with Li⁺ (data not shown). Li⁺ has previously been shown in NT2N neurons to reduce GSK-3 β activity by up to 80% (51). Therefore, these data implicate GSK-3 β in CsA resistance. Numerous previous studies have demonstrated GSK-3 β inactivation in response to wnts (23, 25, 45). We used several approaches to test the importance of the wnt pathway in CsA resistance, and find that wnt-5a is both expressed by EC and can stimulate NFAT nuclear accumulation in the presence of CsA. Moreover, we provide data suggesting that, in our system, it is activation of the noncanonical wnt pathway in T cells that is responsible for the regulation of NFAT localization.

Our data support a model (Fig. 9) in which NFAT slowly accumulates in the nucleus under the influence of phosphatases not sensitive to CsA. The presumably weaker activity of these phosphatases toward NFAT compared with CaN may explain the delay

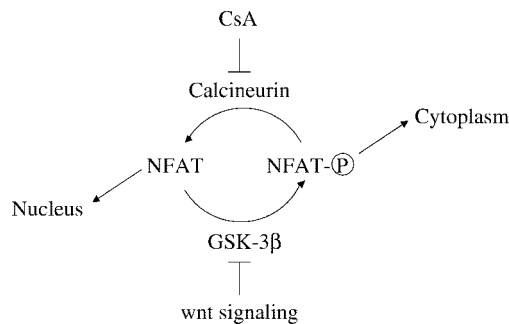


FIGURE 9. Proposed role for wnt signaling in CsA resistance. In activated T cells, NFAT is driven into the nucleus by the action of the phosphatase, CaN. GSK-3 β then rephosphorylates NFAT, promoting its export. The balance between these two enzymes thus helps to set the level of nuclear NFAT. In the presence of CsA-mediated inhibition of CaN, NFAT that reaches the nucleus, presumably by the action of other, less efficient phosphatases, is maintained there by wnt-mediated inhibition of GSK-3 β .

in onset of resistance to CsA. We have confirmed the importance of OA-sensitive phosphatases in T cell activation (data not shown), although their specific targets are still to be determined. In this model, NFAT is subsequently maintained in the nucleus due to an inhibition of GSK-3 β activity as a result of EC-derived wnt signaling. The reduction in CsA-resistant IL-2 synthesis in the presence of a dominant-negative Fz receptor is strong evidence suggesting the important role of endogenous wnt signaling in the generation of resistance in this system.

We have presented evidence that wnt-5a is operating through a PKC-dependent pathway in T cells. The best characterized wnt-dependent signaling cascade is the canonical, or β -catenin, pathway (reviewed in Ref. 45). This regulates many developmental processes, including body patterning, and involves a multiprotein complex containing axin and dishevelled that targets the LEF/TCF transcription factor family. The planar cell polarity pathway was first revealed in *Drosophila*, and likewise involves dishevelled; however, the major downstream target appears to be the c-Jun N-terminal kinase pathway (52). Recently, a third pathway has emerged, mostly studied in *Xenopus*, that is triggered by noncanonical wnts and involves activation of G-proteins and PKC (43–45, 53). There is also good in vitro and in vivo evidence that PKC can directly target GSK-3 β , leading to inactivation (23, 46, 47). Our data strongly suggest a role for this third pathway in the generation of T cell resistance to CsA.

Interestingly, NFAT was recently shown to be a wnt target in *Xenopus*, where noncanonical wnt signaling leads to elevated Ca²⁺ levels and CaN activation (26). Therefore, it is possible that NFAT may be regulated by wnts in T cells through two distinct mechanisms—up-regulation of CaN activity and down-regulation of GSK-3 β activity. However, this Ca²⁺-dependent pathway is unlikely to account for CsA resistance as it involves activation of CaN—the CsA target.

Currently, we cannot say which wnt and Fz pair is most important for mediating resistance. Fz receptors are promiscuous, binding more than one wnt; and the wnts, likewise, can bind to more than one receptor. Wnt-5a can mediate CsA-resistant NFAT nuclear accumulation, and sFz-5 can inhibit CsA-resistant IL-2 synthesis, but a blocking Ab to Fz-5 cannot. This suggests that an alternative Fz that also binds wnt-5a may be involved (54). Only the development of new reagents and the definition of physiologic receptor-ligand pairings will answer this question definitively.

Very little is known about the role of wnt signaling in the immune system. A recent report described a role for wnts in regu-

lating mouse B lymphocyte proliferation, although the Fz receptor mediating the effect was not identified (55). Wnts have also been implicated in human hematopoiesis, where wnts-5a, -2b, and -10b were shown to enhance the proliferation of progenitor cells (56) and in thymocyte development, where wnt activation of β -catenin was observed (57). Again, the Fz receptors were not identified. Interestingly, although much work has been done on LEF/TCF in T cells and the role of β -catenin in modulating the activities of these transcription factors, the receptors and ligands upstream of β -catenin have not been extensively studied.

Ghosh et al. (35) demonstrated that CsA-resistant IL-2 synthesis in response to PMA + CD28 mAb stimulation also involved NFAT translocation. Human EC do not express ligands for CD28, but do appear to target a PKC-dependent pathway (Fig. 5) and presumably provide other, necessary signals that allow for full T cell activation and IL-2 synthesis. It is possible that the combination of PMA and CD28 also targets GSK-3 β for down-regulation. We have previously demonstrated that EC do not merely degrade CsA and that EC-T cell contact is required for CsA-resistant IL-2 synthesis (32).

The chronic immune inflammation that is a hallmark of graft arteriosclerosis occurs in the presence of continued administration of CsA to patients. We have suggested that EC may be important initiators of graft arteriosclerosis in transplanted organs by presenting graft alloantigens to host T cells and inducing resistance to CsA (1, 58). In support of this, we have found that in SCID mice grafted with vascularized human skin, destruction of the human vasculature by cotransplanted allogeneic human T cells is not blocked by CsA (58); only a combination of CsA and rapamycin is protective. In this study, we present evidence that EC-mediated CsA resistance can be induced by wnt signaling; however, we have no evidence that this pathway is active during Ag presentation by classical APC. Indeed, activation of T cells by peripheral blood adherent cells, which are mostly monocyte/macrophages, does not induce resistance to CsA (2). Rather, we suggest that in the unique setting of a vascularized transplant, where the patient is receiving CsA or FK506 immunosuppression, the ability of EC-derived wnt signaling to affect NFAT localization may have profound consequences on local T cell activation and graft survival.

Acknowledgments

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References

- Salomon, R. N., C. C. Hughes, F. J. Schoen, D. D. Payne, J. S. Pober, and P. Libby. 1991. Human coronary transplantation-associated arteriosclerosis: evidence for a chronic immune reaction to activated graft endothelial cells. *Am. J. Pathol.* 138:791.
- Karmann, K., J. S. Pober, and C. C. Hughes. 1994. Endothelial cell-induced resistance to cyclosporin A in human peripheral blood T cells requires contact-dependent interactions involving CD2 but not CD28. *J. Immunol.* 153:3929.
- Jain, J., P. G. McCaffrey, V. E. Valge-Archer, and A. Rao. 1992. Nuclear factor of activated T cells contains Fos and Jun. *Nature* 356:801.
- Jain, J., P. G. McCaffrey, Z. Miner, T. K. Kerppola, J. N. Lambert, G. L. Verdine, T. Curran, and A. Rao. 1993. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with fos and jun. *Nature* 365:352.
- Hodge, M. R., A. M. Ranger, F. Charles de la Brousse, T. Hoey, M. J. Grusby, and L. H. Glimcher. 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity* 4:397.
- Garcia-Rodriguez, C., and A. Rao. 1998. Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). *J. Exp. Med.* 187:2031.
- Decker, E. L., C. Skerka, and P. F. Zipfel. 1998. The early growth response protein (EGR-1) regulates interleukin-2 transcription by synergistic interaction with the nuclear factor of activated T cells. *J. Biol. Chem.* 273:26923.

8. Jain, J., C. Loh, and A. Rao. 1995. Transcriptional regulation of the IL-2 gene. *Curr. Opin. Immunol.* 7:333.
9. Chuvpilo, S., C. Schomburg, R. Gerwig, A. Heinfing, R. Reeves, F. Grummt, and E. Serfling. 1993. Multiple closely-linked NFAT/octamer and HMG I(Y) binding sites are part of the interleukin-4 promoter. *Nucleic Acids Res.* 21:5694.
10. Szabo, S. J., J. S. Gold, T. L. Murphy, and K. M. Murphy. 1993. Identification of cis-acting regulatory elements controlling interleukin-4 gene expression in T cells: roles for NF-Y and NF-ATc. *Mol. Cell Biol.* 13:4793.
11. Goldfeld, A. E., P. G. McCaffrey, J. L. Strominger, and A. Rao. 1993. Identification of a novel cyclosporin-sensitive element in the human tumor necrosis factor α gene promoter. *J. Exp. Med.* 178:1365.
12. McCaffrey, P. G., A. E. Goldfeld, and A. Rao. 1994. The role of NFATp in cyclosporin A-sensitive tumor necrosis factor- α gene transcription. *J. Biol. Chem.* 269:30445.
13. Tsytsykova, A. V., E. N. Tsitsikov, and R. S. Geha. 1996. The CD40L promoter contains nuclear factor of activated T cells-binding motifs which require AP-1 binding for activation of transcription. *J. Biol. Chem.* 271:3763.
14. Lyakh, L., P. Ghosh, and N. R. Rice. 1997. Expression of NFAT-family proteins in normal human T cells. *Mol. Cell Biol.* 17:2475.
15. Northrop, J. P., S. N. Ho, L. Chen, D. J. Thomas, L. A. Timmerman, G. P. Nolan, A. Admon, and G. R. Crabtree. 1994. NF-AT components define a family of transcription factors targeted in T cell activation. *Nature* 369:497.
16. Loh, C., J. A. Carew, J. Kim, P. G. Hogan, and A. Rao. 1996. T-cell receptor stimulation elicits an early phase of activation and a later phase of deactivation of the transcription factor NFAT1. *Mol. Cell Biol.* 16:3945.
17. Park, J., N. R. Yaseen, P. G. Hogan, A. Rao, and S. Sharma. 1995. Phosphorylation of the transcription factor NFATp inhibits its DNA binding activity in cyclosporin A-treated human B and T cells. *J. Biol. Chem.* 270:20653.
18. Shaw, K. T.-Y., A. M. Ho, A. Raghavan, J. Kim, J. Jain, J. Park, S. Sharma, A. Rao, and P. G. Hogan. 1995. Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells. *Proc. Natl. Acad. Sci. USA* 92:11205.
19. Luo, C., K. T.-Y. Shaw, A. Raghavan, J. Aramburu, F. Garcia-Cozar, B. A. Perrino, P. G. Hogan, and A. Rao. 1996. Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import. *Proc. Natl. Acad. Sci. USA* 93:8907.
20. Wesselborg, S., D. A. Fruman, J. K. Sagoo, B. E. Bierer, and S. J. Burakoff. 1996. Identification of a physical interaction between calcineurin and nuclear factor of activated T cells (NFATp). *J. Biol. Chem.* 271:1274.
21. Beals, C. R., C. M. Sheridan, C. W. Turck, P. Gardner, and G. R. Crabtree. 1997. Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* 275:1930.
22. Welsh, G. L., S. Miyamoto, N. T. Price, B. Safer, and C. G. Proud. 1996. T-cell activation leads to rapid stimulation of translation initiation factor eIF2B and inactivation of glycogen synthase kinase-3. *J. Biol. Chem.* 271:11410.
23. Cook, D., M. J. Fry, K. Hughes, R. Sumathipala, J. R. Woodgett, and T. C. Dale. 1996. Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. *EMBO J.* 15:4526.
24. Itoh, K., V. E. Krupnik, and S. Y. Sokol. 1998. Axis determination in *Xenopus* involves biochemical interactions of axin, glycogen synthase kinase 3 and β -catenin. *Curr. Biol.* 8:591.
25. Ruel, L., V. Stambolic, A. Ali, A. S. Manoukian, and J. R. Woodgett. 1999. Regulation of the protein kinase activity of Shaggy(Zeste-white3) by components of the wingless pathway in *Drosophila* cells and embryos. *J. Biol. Chem.* 274:21790.
26. Saneyoshi, T., S. Kume, Y. Amasaki, and K. Mikoshiba. 2002. The wnt/calcium pathway activates NF-AT and promotes ventral cell fate in *Xenopus* embryos. *Nature* 417:295.
27. Ohteki, T., M. Parsons, A. Zakarian, R. G. Jones, L. T. Nguyen, J. R. Woodgett, and P. S. Ohashi. 2000. Negative regulation of T cell proliferation and interleukin 2 production by the serine threonine kinase GSK-3. *J. Exp. Med.* 192:99.
28. Feske, S., R. Draeger, H. H. Peter, K. Eichmann, and A. Rao. 2000. The duration of nuclear residence of NFAT determines the pattern of cytokine expression in human SCID T cells. *J. Immunol.* 165:297.
29. Billingham, M. E. 1987. Cardiac transplant atherosclerosis. *Transplantation Proc.* 19:19.
30. Uretsky, B. F., S. Murali, P. S. Reddy, B. Rabin, A. Lee, B. P. Griffith, R. L. Hardesty, A. Trento, and H. T. Bahnson. 1987. Development of coronary artery disease in cardiac transplant patients receiving immunosuppressive therapy with cyclosporine and prednisone. *Circulation* 76:827.
31. Murphy, L. L., M. M. Mazanet, A. C. Taylor, J. Mestas, and C. C. Hughes. 1999. Single-cell analysis of costimulation by B cells, endothelial cells, and fibroblasts demonstrates heterogeneity in responses of CD4⁺ memory T cells. *Cell. Immunol.* 194:150.
32. Savage, C. O. S., C. C. W. Hughes, R. B. Pepinsky, B. P. Wallner, A. S. Freedman, and J. S. Pober. 1991. Endothelial cell lymphocyte function-associated Ag-3 and an unidentified ligand act in concert to provide costimulation to human peripheral blood CD4⁺ T cells. *Cell. Immunol.* 137:150.
33. Thompson, C. B., T. Lindsten, J. A. Ledbetter, S. L. Kunkel, H. A. Young, S. G. Emerson, J. M. Leiden, and C. H. June. 1989. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc. Natl. Acad. Sci. USA* 86:1333.
34. June, C. H., J. A. Ledbetter, M. M. Gillespie, T. Lindsten, and C. B. Thompson. 1987. T cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell Biol.* 7:4472.
35. Ghosh, P., A. Sica, M. Cippitelli, J. Subleski, R. Lahesmaa, H. A. Young, and N. R. Rice. 1996. Activation of nuclear factor of activated T cells in a cyclosporin A-resistant pathway. *J. Biol. Chem.* 271:7700.
36. Hughes, C. C. W., C. O. S. Savage, and J. S. Pober. 1990. Endothelial cells augment T cell interleukin 2 production by a contact-dependent mechanism involving CD2/LFA-3 interaction. *J. Exp. Med.* 171:1453.
37. Aramburu, J., F. Garcia-Cozar, A. Raghavan, H. Okamura, A. Rao, and P. G. Hogan. 1998. Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. *Mol. Cell* 1:627.
38. Clipstone, N. A., and G. R. Crabtree. 1992. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* 357:695.
39. Nebl, G., S. C. Meuer, and Y. Samstag. 1998. Cyclosporin A-resistant transactivation of the IL-2 promoter requires activity of okadaic acid-sensitive serine/threonine phosphatases. *J. Immunol.* 161:1803.
40. Klein, P. S., and D. A. Melton. 1996. A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. USA* 93:8455.
41. Wright, M., M. Aikawa, W. Szeto, and J. Papkoff. 1999. Identification of a wnt-responsive signal transduction pathway in primary endothelial cells. *Biochim. Biophys. Acta* 263:384.
42. He, X., J. P. Saint-Jeannet, Y. Wang, J. Nathans, I. Dawid, and H. Varmus. 1997. A member of the Frizzled protein family mediates axis induction by wnt-5A. *Science* 275:1652.
43. Chen, R. H., W. V. Ding, and F. McCormick. 2000. wnt signaling to β -catenin involves two interactive components: glycogen synthase kinase-3 β inhibition and activation of protein kinase C. *J. Biol. Chem.* 275:17894.
44. Kuhl, M., L. C. Sheldahl, M. Park, J. R. Miller, and R. T. Moon. 2000. The wnt/Ca²⁺ pathway: a new vertebrate wnt signaling pathway takes shape. *Trends Genet.* 16:279.
45. Miller, J. R., A. M. Hocking, J. D. Brown, and R. T. Moon. 1999. Mechanism and function of signal transduction by the wnt/ β -catenin and wnt/Ca²⁺ pathways. *Oncogene* 18:7860.
46. Goode, N., K. Hughes, J. R. Woodgett, and P. J. Parker. 1992. Differential regulation of glycogen synthase kinase-3 β by protein kinase C isotypes. *J. Biol. Chem.* 267:16878.
47. Murray, N. R., L. A. Davidson, R. S. Chapkin, W. C. Gustafson, D. G. Schattenberg, and A. P. Fields. 1999. Overexpression of protein kinase C β II induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis. *J. Cell Biol.* 145:699.
48. Adams, J. C., and W. J. Gullick. 1989. Differences in phorbol-ester-induced down-regulation of protein kinase C between cell lines. *Biochem. J.* 257:905.
49. Olivier, A. R., and P. J. Parker. 1992. Identification of multiple PKC isoforms in Swiss 3T3 cells: differential down-regulation by phorbol ester. *J. Cell. Physiol.* 152:240.
50. Malbon, C. C., H. Wang, and R. T. Moon. 2001. wnt signaling and heterotrimeric G-proteins: strange bedfellows or a classic romance? *Biochim. Biophys. Acta* 287:589.
51. Hong, M., D. C. R. Chen, P. S. Klein, and V. M.-Y. Lee. 1997. Lithium reduces τ phosphorylation by inhibition of glycogen synthase kinase-3. *J. Biol. Chem.* 272:25326.
52. Boutros, M., N. Paricio, D. I. Strutt, and M. Mlodzik. 1998. Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell* 94:109.
53. Winklbauer, R., A. Medina, R. K. Swain, and H. Steinbeisser. 2001. Frizzled-7 signalling controls tissue separation during *Xenopus* gastrulation. *Nature* 413:856.
54. Sheldahl, L. C., M. Park, C. C. Malbon, and R. T. Moon. 1999. Protein kinase C is differentially stimulated by wnt and frizzled homologs in a G-protein-dependent manner. *Curr. Biol.* 9:695.
55. Reya, T., M. O'Riordan, R. Okamura, E. Devaney, K. Willert, R. Nusse, and R. Grosschedl. 2000. wnt signaling regulates B lymphocyte proliferation through a Lef-1 dependent mechanism. *Immunity* 13:15.
56. Van Den Berg, D. J., A. K. Sharma, E. Bruno, and R. Hoffman. 1998. Role of members of the wnt gene family in human hematopoiesis. *Blood* 92:3189.
57. Staal, F. J., J. Meeldijk, P. Moerer, P. Jay, B. C. van de Weerd, S. Vainio, G. P. Nolan, and H. Clevers. 2001. wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. *Eur. J. Immunol.* 31:285.
58. Murray, A. G., J. S. Schechner, D. E. Epperson, P. Sultan, J. M. McNiff, C. C. Hughes, M. I. Lorber, P. W. Askenase, and J. S. Pober. 1998. Dermal microvascular injury in the human peripheral blood lymphocyte reconstituted-severe combined immunodeficient (HuPBL-SCID) mouse/skin allograft model is T cell mediated and inhibited by a combination of cyclosporine and rapamycin. *Am. J. Pathol.* 153:627.