Protein Kinase Cε Regulates Proliferation and Cell Sensitivity to TGF-1 β of CD4+ T Lymphocytes: Implications for Hashimoto Thyroiditis

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Protein Kinase Cε Regulates Proliferation and Cell Sensitivity to TGF-1β of CD4+ T Lymphocytes: Implications for Hashimoto Thyroiditis

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We have studied the functional role of protein kinase Cε (PKCε) in the control of human CD4+ T cell proliferation and in their response to TGF-1β. We demonstrate that PKCε sustains CD4+ T cell proliferation triggered in vitro by CD3 stimulation. Transient knockdown of PKCε expression decreases IL-2R chain transcription, and consequently cell surface expression levels of CD25. PKCε silencing in CD4 T cells potentiates the inhibitory effects of TGF-1β, whereas in contrast, the forced expression of PKCε virtually abrogates the inhibitory effects of TGF-1β. Being that PKCε is therefore implicated in the response of CD4 T cells to both CD3-mediated proliferative stimuli and TGF-1β antiproliferative signals, we studied it in Hashimoto thyroiditis (HT), a pathology characterized by abnormal lymphocyte proliferation and activation. When we analyzed CD4 T cells from HT patients, we found a significant increase of PKCε expression, accounting for their enhanced survival, proliferation, and decreased sensitivity to TGF-1β. The increased expression of PKCε in CD4+ T cells of HT patients, which is described for the first time, to our knowledge, in this article, viewed in the perspective of the physiological role of PKCε in normal Th lymphocytes, adds knowledge to the molecular pathophysiology of HT and creates potentially new pharmacological targets for the therapy of this disease. The Journal of Immunology, 2011, 187: 000–000.

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases that regulate a wide variety of cellular functions (1). The PKC family consists of at least 11 members that have been categorized into three groups based on their structure and biochemical properties, and named conventional, novel, and atypical PKCs. Conventional PKCs (α, β, βII, and γ) require Ca2+ and diacylglycerol (DAG) for their activation, novel PKCs (δ, ε, η, θ) are dependent on DAG but not Ca2+, whereas atypical PKCs (ζ, λ, η) are independent of both Ca2+ and DAG (1).

T lymphocytes contain up to eight different species of PKC isoforms. In the last few years, PKC9 has become the most interesting isoform as far as T cell activation, proliferation, and survival are concerned (for review, see Refs. 2, 3). However, previous studies have reported that PKCε, which localizes at the cell membrane of T lymphocytes after stimulation with PMA or anti-CD3 Abs (4), has a role in the expression of the transcription factors NF-AT and AP-1, which are involved in the regulation of IL-2 expression (5). The inhibition of PKCs by blocking Abs interferes with CD3- and TCR-induced IL-2 secretion, CD25 cell surface expression, and DNA synthesis of human lymphocytes (6). In particular, anti-PKCε Abs inhibit IL-2 synthesis. Of note, Castrillo et al. (7) reported that mice carrying homozygous disruption of the PKCε locus show unchanged T cell development: mature T cells have a wild type surface phenotype and are present at the expected numbers. However, T cell proliferation of PKCε−/− animals, stimulated by anti-CD3 or anti-TCR Abs, was reduced (7).

Although positively modulated by mitogenic cytokines or factors, T cell proliferation is inhibited by several classes of molecules, like ILs, nucleosides (8, 9), and cytokines (10–12), resulting in the protection from autoimmune disorders. TGF-1β is a critical cytokine that preserves immune homeostasis (13). TGF-1β−/− mice, or mice expressing dominant-negative TGF-1βRs on T cells, soon develop spontaneous autoimmune diseases (14, 15). Furthermore, TGF-1β−/−-induced T cells can educate naive T cells to develop suppressive properties, generating regulatory T cells (Tregs) (16) that are critical for maintaining peripheral tolerance (17) and secrete different antimitogenic ligands such as adenosine, IL-10, and also TGF-1β itself (18, 19). Tregs inhibit multiorgan autoimmune induction by the transfer of CD4+CD25− T cells into nude mice (20), and their depletion in vivo permits induction of autoimmunity, including thyroiditis, in otherwise resistant mouse strains (20–22).

Autoimmune thyroid diseases are the most common organ-specific autoimmune disorder, affecting 1–2% of the population, with a 5- to 10-fold increase among women. Autoimmune thyroid diseases comprise two main clinical entities: Graves’ disease and Hashimoto thyroiditis (HT) (23). HT has been recently viewed as a genetic disorder of cell-mediated immunity (23, 24). After initiation of the autoreactive immune response by CD4+ T cells, the
expansion of autoreactive T cell populations, prolonging the inflammatory response, results in a massive lymphocyte accumulation in patients’ thyroids (23, 24). It has recently been demonstrated that the serum concentration of TGF-β1 is decreased in HT (25), and that in patients with HT and Graves’ disease, some Treg subsets apparently exert a defective suppressive function (26).

Given this complex background, in this study, we have investigated the functional role of PKCe in the control of human CD4+ T cell proliferation and sensitivity to TGF-β1. PKCe expression levels were transiently modulated in primary human T cells induced in vitro by anti-CD3 and anti-CD28 mAb stimulation. Overall, we show that PKCe promotes T cell proliferation in vitro, whereas it is spontaneously expressed at significantly higher levels in CD4+ T cells from HT patients, strongly suggesting a role of this kinase in the molecular pathophysiology of Hashimoto disease.

Materials and Methods

Patients

Twenty-three HT patients (7 female and 16 male patients; mean age, 46 ± 13 y) were selected at the Internal Medicine, Vascular and Metabolic Diseases Unit of the Parma University Hospital (Table I). Selected patients were characterized by high serum levels of anti-thyroidperoxidase (>100 U/ml) and anti-thyroglobulin Abs (>300 U/ml). Patients with anti-insulin and anti-islet Abs were excluded. Twenty-seven healthy control donors (HC; 4 female and 23 male subjects; mean age, 37 ± 9 y) without previously diagnosed autoimmune diseases, viral or bacterial infection, or metabolic syndrome were used as control subjects.

Cell isolation

Naïve CD4+ T cells were isolated from human PBMC by using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec) and the VarioMACS cell Separator, as previously described (27–30). Purity of cells was immediately checked by anti-CD3-FITC and anti-CD8-PE staining and flow cytometry analysis. Only samples with a purity exceeding 97% were used.

Cell treatment

Cells were activated by seeding into anti-CD3 and/or anti-CD28 mAb-coated plates, as previously described (29, 30). In particular, 1 × 10^6/ml freshly isolated CD4+ T cells (2.5 × 10^5/well) were resuspended in 100% FBS-enriched RPMI 1640 medium containing 30 U/ml IL-2 and seeded in 96 flat-well plates. IL-2 was readded to the culture medium at the same concentration of 10 ng/ml 24 h after cell seeding.

Anti-CD3 (clone x35; Beckman Coulter) and/or anti-CD28 (clone 28.2; Beckman Coulter) mAb coating was performed for 24 h at 4°C: the two mAbs, alone or in combination, were diluted at 4 μg/ml (or 2 and 1 μg/ml in some experiments) in 50 μl RPMI and added to each well of a 96 flat-well plate. Before cell seeding, mAb solution was removed and plates gently washed with complete culture medium. Control samples were cultured exactly in the same conditions, without IL-2 and CD3/CD28 stimulation.

In some experiments, CD4+ T cells were stimulated with 5 μg/ml PHA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), and 50 μg/ml PMA (Sigma-Aldrich) for 24 and 48 h.

TGF-β1 signaling pathway was studied in the Jurkat T cell line and in CD4+ T lymphocytes. Jurkat cells were grown in serum-free medium for 18 h to generate a total inactivation of Smad2 (31), then 10 ng/ml TGF-β1 was added for 2 h to induce Smad2 phosphorylation. Because Smad3 is not detectable in Jurkat cells, to study its activation, we used CD4+ T lymphocytes grown in complete medium (RPMI 1640 + 10% FBS + IL-2) in anti-CD3 and anti-CD28–coated plates. After 36 h of culture, CD4 cells were starved for 8 h and then stimulated with TGF-β1 for 2 h. Cell were harvested and total protein lysate was analyzed by Western blot.

Cell proliferation

Cell proliferation was studied analyzing by flow cytometry the number of cell duplications. As previously described (30), cells were labeled with 10 μM CFSE, extensively washed, counted, and resuspended in complete medium. Resting (controls) and activated CFSE-labeled cells were analyzed after 4 d of culture. To exclude dead cells from analysis, we stained samples with propidium iodide (PI; 2.5 μg/ml) and CFSE fluorescence was analyzed gating on PI− cells.

Flow cytometry

Staining with anti–CD71-CY5, anti–CD19-PE, and anti–CD25-PE (Beckman Coulter) was performed according to manufacturer’s instructions. Control samples were stained with appropriate irrelevant isotype–matched control (CY5 and PE–labeled) Abs. Analysis was performed with an EPICS-XL flow cytometer, followed by the EXPO32 software analysis (Beckman Coulter), collecting at least 10,000 events/sample.

Small interfering RNA design and transfection

The respective sense and antisense RNA sequences of double-stranded small interfering RNA (siRNA), designed to target human PKCe mRNA (siRNA_PKCe), were synthesized by Silencer siRNA Construction Kit (Ambion, Austin, TX) as previously described (32, 33). Nonspecific siRNA duplexes containing the same nucleotides, but in irregular sequence (scrambled PKCe siRNA), were prepared according to the protocol and used as controls. The GFP–PKCe expression and control plasmid were kindly provided by Prof. Peter Parker (Cancer Research UK, London Research Institute). To maximize transfection efficiency, we delivered siRNAs (100 nM each) and GFP–PKCe plasmids (1 μg) using the Amaxa nucleofection technology (Amaxa, Koeln, Germany), according to the manufacturer’s protocols. In particular, resting CD4+ T cell cultures were infected by the human T cell neucleofection kit for unstimulated cells (Amaxa) with the U-014 nucleofection program. Jurkat cell cultures were transfected with GFP–PKCe and GFP–PKCε plasmids (1 μg) by CD34+ cell nucleofection kit (Amaxa) with the U-014 nucleofection program.

Design of a PKCe cDNA resistant to siRNA_PKCe

To generate proper controls to our RNA silencing experiments, we mutated siRNA–targeted sequences of mouse PKCe to obtain an siRNA-resistant PKCe cDNA (Table II). Specifically, we used the mouse PKCe full-length cDNA (722/737 conserved amino acids versus human PKCe protein) fused in-frame with the N-terminal GFP tag (34) by PCR using the start primer 5’-GGGCGATCCATATGTTGATTGC-3’ and the end primer 5’-GCGGCGTCTGA CGTCTCCACAG CAGG-3’. Mutations were introduced in the siRNA–target sequences by overlap extension PCR. Two PCR products (overlapping complementary ends) were produced from the GFP–PKCe with the following primers: siRNA4 sense, 5’-GGAAATGGCG CGGGCGGCG CGG AAaccacaa gccggtcgtc-3’; siRNA4 antisense, 5’-TTGCCGCCCC CGGGCAATTT CTCatcct ttggccttg tg-3’. siRNA3 sense, 5’-TTAAAGGT CGGCATTTT gccagaacct gcgc-3’; siRNA3 antisense, 5’- NAATGCGGATC TGAaattgtg gttgctgcg-3’; siRNA2 sense, 5’-AATTGAATCCT GCGTGTTCTG Cacaagcggac gaco-3’; siRNA2 antisense, 5’-GAACACCGGCG AGTTCAATTT gtcggtgtct gc-3’. siRNA1 sense, 5’-cccttacaa AATAGATT GTGGAACCGG gtagtctg-3’. The overlapping sequence, containing a mutated version of the siRNA target sequence of mouse PKCe cDNA, is reported by using uppercase letters.

In particular, as described in Fig. 5D, the PCR products obtained with siRNA4/sense-end primers (lane 1; 132 bp) was hybridized with the product obtained with siRNA4/antisense-end primers (lane 2; 977 bp). The overlapping product was amplified by primers start and end (lane 3; 297 bp). This PCR product is the full-length PKCe cDNA resistant to the siRNA_PKCe number 4 (PKCe4R). PKCe4R amplicon was purified on agarose gel and amplified by PCR using the primers siRNA3/sense-end (lane 4; 778 bp) and siRNA3/antisense-end (lane 5; 729 bp). The overlapping product was amplified by primers start and end (lane 6; 2297 bp) to yield the full-length PKCe cDNA resistant to the siRNA_PKCe numbers 3 and 4 (PKCe34R). The same procedure was repeated in lanes 7 (PCR by siRNA2/sense and end primers; 2051 bp), 8 (PCR by siRNA2/antisense and start primers; 265 bp), and 9 (PCR by start and end primers; 2297 bp) to obtain the full-length PKCe cDNA resistant to the siRNA_PKCe, 2, 3, and 4 (PKCe234R). Then, siRNA3 sense/siRNA3 antisense product (lane 10; 747 bp) was hybridized with the start and siRNA3 antisense amplicon (lane 11; 265 bp), both obtained using PKCe234R as template. The overlapping product was amplified by the primers start and siRNA3 antisense (lane 12; 778 bp). This last PCR product was hybridized with siRNA3 sense/end amplicon region of PKCe234R (lane 13; 1538 bp). The overlapping product was amplified by primers start and end (lane 14; 2297 bp) to yield the full-length PKCe cDNA resistant to our four siRNA_PKCe, (PKCe234R).

DNA was amplified by 10 cycles of 60 s at 94°C, 30 s at 55°C, and 1 min at 72°C. Then, additional 15 cycles were performed for 60 s at 94°C and 2 min at 72°C (extension time was incremented by 3 s/cycle). Hybridization Downloaded from http://www.jimmunol.org/ by guest on April 14, 2017
and extension of overlapping PCR products were obtained in PCR mix by 15 cycles of 60 s at 94°C, 60 s at 55°C, and 3 min at 72°C. Agarose gel purification of PCR products was performed with the GenElute agarose spin columns (Sigma-Aldrich).

PKCe1234R was digested with BglII and Sall and ligated to the corresponding sites of the pEGFP-C1 vector. The sequence integrity of siRNA-resistant PKCe was tested by DNA sequencing.

Table II shows the alignment of nucleotide sequence of human, wild type mouse, and siRNA-resistant PKCe cDNAs.

RT-PCR

CD4+ T cell cultures were grown for 48 h, and 2 × 104 cells were collected by centrifugation. Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA (1 μg) was reverse transcribed, and progressive dilutions (1/10, 1/100, 1/1000) were then subjected to PCR amplification. cDNAs of IL-2R chains were amplified using the primers previously described by Hughes-Fulford et al. (35). IL-2Ru sense 5′-ATCCACAGGG CACCATCCA AAGC-3′ and antisense 5′-TGCCC-CCCA AGAATGAT AAAT-3′ primers generated an expected PCR product of 347 bp (NM-000417 GenBank locus, GI:4557666).

IL-2Rb sense 5′-GCCCCCATCT CCTCAGAGT-3′ and antisense 5′-AGGGAGGAAG CCAAGAGC-3′ primers gave the expected PCR product of 529 bp (NM-000878 GenBank locus, GI:23238195). By the sense 5′-GGAAGCCTGG GTATTTCTGG TT-3′ and the antisense 5′-GGTG- GTGTAC ATGAAGGAAAA AA-3′ IL-2Ry cDNA primer, we obtained two expected amplions of 386 bp (NM-000206 GenBank locus sequence, GI:407345; 407 bp (HUMIL2RGA GenBank locus, GI:349631). By the sense 5′-TGGAGGGGTGC ACCACACTGG GCCACATCTA-3′ and antisense 5′-CTGAAGATC TTAGGGGTCC AGATGAGGG G-3′ primers, we amplified an expected 649-bp fragment of β-actin mRNA (E00829 GenBank locus and CD4+ T cells; 1/200 dilution) as previously described (30, 32).

The 387-bp region of the human PKCε cDNA, NM005400 GenBank locus (GI:47157326), was amplified by the sense 5′-CATGGCTGCTT TTAAGATCAA AA-3′ and antisense 5′-CCTGAGAGGT CATGA CATAC-3′ primers, as previously described (32).

NF-κB1 was amplified by 5′-AGATGTTGGAG ATGGTTTGC-3′ and the antisense GCCAATGAG TGTGTGCTGG-3′ primers; NF-κB2 by 5′-AGAGCTCTG TCTGCTATAG AT-3′ and the antisense 5′-TTACTGCA TGAAGGTTT GC-3′; the antisense 5′-CTAAG GAGGCGAGC-3′; RelA by 5′-CACTACAGG GAAGGCTTC AGATGAGGG G-3′; and the antisense 5′-GCCCCATCT CCCTCCAAGT-3′ primers with fresh pro-

The 387-bp region of the human PKCε locus (GI: 47157326), was amplified by the sense 5′-GCCCCCATCT CCCTCCAAGT-3′ and the antisense 5′-GCCACGAC CAAGAGGCGA-3′ primers, corrected for each PCR was quantified by KODAK Molecular Imaging Software 4.0 and 1/1000 of total cDNA was amplified, PCR products were separated in 2%agarose gel and extension of overlapping PCR products were obtained in PCR mix by 15 cycles of 60 s at 94°C, 60 s at 55°C, and 3 min at 72°C. Agarose gel purification of PCR products was performed with the GenElute agarose spin columns (Sigma-Aldrich).

Results

PKCe and CD4+ T cell proliferation

To evaluate the role of PKCe in CD4+ T lymphocyte proliferation, we transfected freshly purified cells with siRNA to PKCe (siRNA_PKCe), or with control siRNA, and then seeded them in anti-CD3– and/or anti-CD28–coated wells in the presence of IL-2. Cell proliferation was analyzed 4 d later, quantifying by flow cytometry the number of duplications of the viable cells. As shown in Fig. 1, control (resting) cell cultures did not proliferate at all (zero duplications), whereas 40% of stimulated cells did two or more duplications (one representative example is reported in Fig. 1A). Inhibition of PKCe expression by specific siRNA transection did not affect the number of duplicating cells in the control (unstimulated) cultures (<5%), whereas it significantly impaired the proliferation of CD3/CD28-stimulated T cells. As shown in Fig. 1B, siRNA_PKCe-transfected stimulated cultures show a significant increase of cells with zero duplications. Because different donors responded differently to the mitogenic stimulus, the number of cells with zero, one, two, four, and five duplications has been reported as percentages of siRNA-transfected control cultures (Fig. 1C). In particular, analyzing four representative donors, the relative percentage of cells with zero duplications (Fig. 1C, black bars) in siRNA_PKCe-treated cultures was 161 ± 19% of normal (100%) controls (siRNActrl-transfected cultures), whereas the number of cells with one duplication was 99 ± 34% of controls. The number of cells with two or more duplications (two, three, four, and five duplications) in siRNA_PKCe-treated cultures was 46 ± 32, 29 ± 21, 35 ± 14, and 58 ± 20% of controls, respectively.

Because nucleofection is a traumatic procedure, it significantly increased cell death of CD4+ T cells (Fig. 1D). However, viability of siRNA_PKCe-transfected cells was similar to those of control siRNA-transfected cultures (49.7 ± 18.2 versus 60.3 ± 10.7; p = 0.354, Student t test; Fig. 1E, black bar versus gray bar). There was no significant difference in cell death of the nonproliferating cell subset (zero duplications) between control and siRNA_PKCe-transfected cultures (36.6 ± 9.8 and 44.2 ± 16.7, respectively; p = 0.462, Student t test; Fig. 1F, empty bars). Similarly, no difference was observed in cell death between control and siRNA_PKCe-transfected cultures in the cell subsets doing two or more duplications (9.5 ± 8.4 and 17.4 ± 8.0, respectively; p = 0.222, Student t test; Fig. 1F, solid gray bars). This means that, in the absence of PKCe, CD4+ T cells stimulated with CD3/CD28 show a significant reduction of the number of cells able to do two or more duplications, which is not caused by increased cell death.
Flow cytometry analysis of PI-stained CD4+ T cell cultures labeled with CFSE and grown 4 d in 96-well plates coated with anti-CD3 + anti-CD28. Independent experiments are reported. Cell viability of siRNA-treated CD4 T cells: percentage of viable cells (white bar); # indicates zero, one, two, three, four, or five duplications (siRNA ctrl: CFSE fluorescence) and cell death (PI fluorescence) (siRNAPKC). Results obtained with four donors and means ± SD. *p < 0.05 versus control siRNA-treated cell cultures (normalized at 100% and omitted for clarity). **p < 0.05 versus control siRNA-treated cell cultures (normalized at 100% and omitted for clarity). Results obtained with four donors and means ± SD. x-Axis reports the number (0–5) of cell duplications. Values (y-axis) are expressed as percentages of control siRNA (siRNA ctrl)-treated cell cultures (n = 100% and omitted for clarity). #p < 0.05 versus control siRNA-treated cell cultures. A, Flow cytometry analysis of PI-stained CD4+ T cell cultures labeled with CFSE and grown 4 d in 96-well plates coated with anti-CD3 anti-CD28 mAbs. Duplication (CFSE fluorescence) and cell death (PI fluorescence) were analyzed in untransfected (Untransf.), control siRNA (siRNA ctrl.), and siRNA to PKCe (siRNAPKCe) transfected cells. E, Cell viability of siRNA-transfected CD4+ T cell cultures. Means ± SD from three independent experiments are shown. *p < 0.05 versus untransfected cell cultures (white bar); #p < 0.05 versus control siRNA-transfected cultures (gray bar). ANOVA by Dunnett’s test. F, Cell death of non-doubling cells (zero duplications) and of proliferating cells (two or more duplications). Means ± SD from three independent experiments are shown.

Because it is known that both TCR-dependent and -independent stimulation of CD4+ T cells activate PKCe (4), to better discriminate the role of PKCe in the proliferation of CD4+ T cells, we stimulated cells either with anti-CD3 alone or in a TCR-independent way. When we analyzed the expression levels of PKCe and PKCθ in CD4+ T cells stimulated in a TCR-independent way, we observed that although PHA and PMA promoted a rapid upregulation of both PKCe and PKCθ expression, ionomycin stimulation took 24 h to increase both PKCs expression (Fig. 2A). As shown in the case reported in Fig. 2B, siRNAPKCe reduced to 66% the number of cells doing two or more duplications and increased the number of cells that did not duplicate (138% of control cell cultures). Similar results were observed in PHA-treated CD4+ T cell cultures. These observations suggest that PKCe likely takes part in a proliferative signaling cascade that is common to both the TCR-dependent and -independent pathways.

Because CD4+ T cells still duplicated, although to a minor extent, in the absence of PKCe, we asked whether PKCe also promoted cell activation. Fig. 2C shows that activation markers like CD71 and CD95 are equally expressed on the cell surface in the presence or absence of PKCe, whereas CD25 is clearly downregulated. These data support the hypothesis that PKCe likely has a role in the signaling promoting CD4 T cell duplication.

In particular, the percentage of CD25+ cells in the siRNAPKCe-transfected cultures decreased from 78 to 67%, and the cell surface density of CD25, calculated as molecules of equivalent soluble fluorophore, decreased to 84.5 ± 6.2% as compared with the control siRNA-transfected cultures (Fig. 2D, 2E).

**PKCe and TGF-1β activity**

Because PKCe appears to be involved in the control of cell proliferation, we then investigated its role in the presence of anti-proliferative signals as those generated by TGF-1β. As preliminary experiments, we started evaluating the TGF-1β effects on CD4+ T lymphocytes proliferation. As shown in Fig. 3A, 10 ng/ml TGF-1β did not impair the proliferation of cell cultures stimulated with high doses of anti-CD3 and anti-CD28 mAbs. In agreement with Kehrl et al. (10) and Wolfrain et al. (11), however, TGF-1β was, on the contrary, able to inhibit proliferation in the absence of anti-CD28 costimulation. However, CD4 T lymphocyte proliferation induced by low doses of anti-CD3/CD28 was partially inhibited by TGF-1β. In particular, in the donor shown in Fig. 3A, when cells were stimulated with anti-CD3 alone at high dose, TGF-1β reduced the number of proliferating cells (i.e., those with two or more duplications) to 64% of the control; when anti-CD28 was used together with low dose (1 ng/ml) of anti-CD3, TGF-1β reduced the number of duplicating cells (i.e., those with two or more duplications) to 42% of the control (Fig. 3B).

On the basis of these observations, we then studied the effects of PKCe in this cell system. Fig. 4 shows that siRNAPKCe increased the sensitivity of CD4+ T cells to TGF-1β both when cultures were maximally stimulated with anti-CD3/CD28 or with anti-CD3 alone. The ability of siRNAPKCe to boost TGF-1β effects is more evident in anti-CD3/CD28–stimulated cells (Fig. 4A) because they are not sensitive to TGF-1β. In fact, TGF-1β treatment of control cultures (transfected with siRNA ctrl) did not interfere with cell proliferation (105 ± 14% cells with two or more duplications), whereas in the absence of PKCe, TGF-1β could reduce the number of doubling cells to 55 ± 17%. To formally prove that PKCe negatively regulates TGF-1β signaling, we transfected CD4+ T cells with the expression vectors CMV-PKCe (expressing wild type PKCe) and CMV-PKCeM (expressing a mutated PKCe lacking enzymatic activity). As expected, the best results were obtained with CD4+ T cell cultures activated by anti-CD3 alone (Fig. 4B). In fact, anti-CD3-stimulated cells were sensitive to TGF-1β inhibition (68 ± 11% of CVM-PKCeM–transfected control cells), but the forced upregulation of PKCe expression completely restored their proliferation rate (98 ± 10% of control cell cultures). Similar experiments were performed using a TCR-independent stimulation. Fig. 4C shows that TGF-1β impaired proliferation of PHA-stimulated CD4+ T cells, and the inhibition of PKCe expression increased TGF-1β antiproliferative effects, whereas on the contrary, the upregulation of PKCe expression blocked TGF-1β effects. The analysis of cell death in TGF-1β–treated cell cultures showed that their low duplication rate after
FIGURE 2. PKCe controls cell proliferation, but not cell activation, of T CD4+ lymphocytes. A, Western blot analysis of PKCe and PKCθ expression in stimulated CD4+ human T cells. A representative experiment is shown. Resting (Control), PHA (5 mg/ml), PMA (50 μg/ml), ionomycin (ionom.; 500 ng/ml), and anti-CD3 anti-CD28 (both at 4 μg/ml) mAb-stimulated CD4+ T cell cultures were grown for 24 or 48 h. Activation of PKCe was also studied using specific anti–phospho-PKCε rabbit serum (pPKCe). B, CD4+ T cells were labeled with CFSE and transfected with control siRNA (siRNActrl.) or with siRNA to PKCe (siRNA PKCe). After 4 d of stimulation with CD3 alone (4 μg/ml), with CD3 and CD28 (both at 4 μg/ml) mAbs, or with PHA (5 mg/ml), the number of cell duplications was quantified by flow cytometry. The number of duplications is indicated above the histograms (from 0–5). A representative experiment is shown. C, Flow cytometry analysis of cell surface expression of CD25, CD95, and CD71. siRNActrl. and siRNA PKCe transfected cells were activated with CD3/CD28 and grown for 4 d. The fraction of positive cells is reported in each dot plot. The appropriate irrelevant isotype-matched control (Irrl-CY5 and Irrl-PE) is shown. D, Quantification (as molecules of equivalent soluble fluorophore [MESF]) of cell surface expression of CD25 and CD95 from three independent experiments. Cells were activated with CD3 (4 μg/ml) and CD28 (4 μg/ml) for 4 d. Phenotype of resting cultures is also reported. E, Effects of PKCe downmodulation (siRNA PKCe) on CD25 and CD95 expression. Values are calculated as percentage of the control siRNA-treated cell cultures and are reported as mean ± SD of three independent experiments (the same shown in C). * p < 0.05 versus control siRNA-treated cultures, analysis by t test.
Residual proliferation of CD4+ wild type murine PKCε or with siRNA PKCε impaired PKCε (Fig. 5), lacking enzymatic activity (CMV-PKCε). Respective interference with NF-κB expression might be at the basis of all the observed effects of PKCε inhibition in T cells (Fig. 7B).

PKCε expression in HT

Because PKCε levels modulate CD4+ T cell proliferation and sensitivity to TGF-1β, we finally hypothesized that PKCε over-

FIGURE 4. PKCε reduces TGF-1β antiproliferative effects. Residual proliferation of CD4+ T cell cultures treated with 10 ng/ml TGF-1β. Cells were transfected either with siRNA to PKCε (siRNAPKCε) to inhibit PKCε expression or with the wild type murine PKCε (CMV-PKCε). Respective negative controls were represented by scrambled-nucleotides siRNA (siRNActrl.) and by a vector expressing a mutated PKCε lacking enzymatic activity (CMV-PKCent). The number of cells with two or more duplications (black bars) is reported as percentage of control untreated cultures. Means ± SD of four independent experiments are reported. *p < 0.05 versus control culture, analysis by t test. D, Flow cytometry analysis of cell death of CD4+ T cell cultures treated with 10 ng/ml TGF-1β. Cell death is reported as number of PI+ cells. Means ± SD of four independent experiments are reported.

PKCε and IL-2R expression

Because we know that the percentage of activated CD4 T cells that expresses CD25 decreases when PKCε is downregulated (Fig. 2B), to explore the molecular mechanism activated by PKCε in the promotion of cell proliferation, we focused on the transcriptional levels of IL-2R chains. Fig. 6A shows that we were indeed able to detect IL-2Rα-, β- and γ-chain mRNAs by RT-PCR from PHA-activated PBLs. Fig. 6B shows that, as expected, CD4+ resting cells do not express detectable levels of IL-2Rα or β-chains, whereas γ-chain transcripts (the 407- and 386-bp PCR products) were detectable at low levels only when a high amount of cDNA (lane 2) was tested (1/10 total cDNA). Cell activation by CD3 and CD28 stimulation promoted the expression of IL-2Rα-, β- and γ-chains. Coherently with our flow cytometry data, inhibition of PKCε expression by siRNAPKCε prevented the anti-CD3/CD28–dependent upregulation of IL-2Rα-, β-, and γ-chains, reducing the amount of amplifiable cDNA to the level of resting cells. It is well-known that NF-κB is a key transcription factor involved in IL-2R chain expression in T cells and, more in general, in T cell activation and proliferation (38-41). Using PHA-activated PBLs, we could detect the expression of NF-κB1 and NF-κB2, but not of RelA, RelB, or cRel (Fig. 7A). Interestingly, we found that NF-κB1 and NF-κB2 gene expression was downregulated in siRNAPKCε–transfected cells, suggesting that an interference with NF-κB expression might be at the basis of all the observed effects of PKCε inhibition in T cells (Fig. 7B).
expression could be involved in CD4+ T cell-mediated autoimmune diseases, like HT. Thus, we studied PKCe protein expression levels in peripheral blood-derived resting CD4+ T cells from 23 HT patients and 27 HC (Table I). PKCe expression showed an individual variability (Fig. 8) ranging from 0.00 to 0.58 ng/50 μg cell lysate. In preliminary experiments, we noted that several male subjects in the HC group showed an individual variability (Fig. 8) ranging from 0.00 to 0.1 ng/50 μg cell lysate. We therefore decided to increase the number of male subjects in both groups.

HT subjects showed significantly (p = 0.026) higher mean values of PKCe levels (0.38 ± 0.29 ng/50 μg) than HC (0.22 ± 0.20 ng/50 μg). We subsequently tried to correlate the expression levels of PKCe with TGF-1β sensitivity of CD4+ T cells of HT patients. Primary CD4+ T cell cultures were stimulated with anti-CD3 mAb. As shown in Fig. 8C, CD4+ T cells from HT patients (solid gray bars) were less sensitive to TGF-1β than those from HC. The transfection of siRNA PKCe (Table II) strongly impaired cell proliferation of HT-derived CD4+ T cells, which acquire a phenotype similar to that of HC (*p < 0.05 ANOVA-Dunnett’s test versus HT siRNA ctrl-transfected cells with more than two duplications).

**FKCe impaired TGF-1β signaling**

It has been reported that the activation of PKCe by TCR agonists is necessary and sufficient to inhibit TGF signaling on TCR activation (42). Moreover, it is known that TGF inhibits IL-2 and anti-CD3/anti-CD28-induced T cell proliferation in a Smad3-dependent manner. By contrast, Smad3 is not essential for TGF-1β-mediated inhibition of IL-2-induced T cell proliferation of lymphocytes activated by ConA (43). Looking for TGF-1β signaling intermediates potentially targeted by PKCe, we analyzed pSmad3 levels in PKCe-overexpressing Jurkat cells and in primary activated CD4+ T cell cultures treated with TGF-1β for 2 h. Of note, PKCe overexpression significantly reduced TGF-1β–induced pSmad2 levels in Jurkat cells (Fig. 8D, 8E, lane 5 versus lane 6) and in anti-CD3/anti-CD28–stimulated CD4+ cells (Fig. 8D, 8E, lane 11 versus lane 12). Although pSmad3 was not detectable in Jurkat cells, TGF-1β increased pSmad3 levels in CD4+ cells (Fig. 8E, gray bars; *p < 0.05, ANOVA-Dunnett’s test versus 1). PKCe overexpression did not affect TGF-1β–induced phosphorylation of Smad3.

TGF-1β has been shown to play an essential role in inhibiting Th1 polarization, as well as inducing both Treg and Th17
differentiation (16). In particular, the polarization of naive T cells to Th1, Th2, and Th17 T cell subpopulation is mediated by specific agonist combinations: IL-12/anti–IL-4 for Th1; IL-4/anti–IL-12 for Th2; TGF-1/IL-6 or IL-21 for Th17 (44). Although in our experiments TGF-1 was added 24 h after T cell stimulation and only IL-2 was used as a mitogenic cytokine, the knocking down of

FIGURE 6. PKCe modulates mRNA levels of IL-2R chains. A, Analysis by RT-PCR of IL-2R chain expression. RNA from PHA + IL-2–activated PBLs (3 d of cell culture) was reversed transcribed and amplified by PCR. Amplicons were separated in 2% agarose gel, stained with ethidium bromide, and visualized under UV exposure. We were able to detect mRNA of the three IL-2R chains (two transcripts were observed for IL-2Rα and IL-2Rγ). B and C, Analysis of IL-2R chain expression by RT-PCR in resting CD4+ T cells (lanes 2–4), activated and untransfected cells (Untr.; lanes 5–7), control siRNA-transfected cell cultures (siRNActrl; lanes 8–10), and siRNA PKCe-transfected CD4+ T cells (siRNAε; lanes 11–13) after 24 h of cultures. 1/10, 1/100, and 1/1000 cDNA, obtained by reverse transcription of 1 μg total RNA, was amplified and PCR products were separated on agarose gel and visualized under UV illumination after ethidium bromide staining. Length of PCR product is also reported. Lanes 2, 5, 8, and 11: PCR with 1/10 cDNA. Lanes 3, 6, 9, and 12: PCR with 1/100 cDNA. Lanes 4, 7, 10, and 13: PCR with 1/1000 cDNA. Lane 1: negative control PCR (Blank); M.W., 100-bp DNA ladder. PCR products were quantified by densitometric analysis and plotted in the graph as described in Materials and Methods. *p < 0.05 versus untransfected and activated cells, analysis by ANOVA and Dunnett’s test.

FIGURE 7. PKCe modulates mRNA levels of NF-κB. A, Analysis by RT-PCR of NF-κB family expression. RNA from PHA + IL-2–activated PBLs (after 3 d of cell culture) was reversed transcribed and amplified by PCR. Amplicons were separated in 2% agarose gel, stained with ethidium bromide, and visualized under UV exposure. We found detectable amount of cDNA of NF-κB1 and NF-κB2, but not of RelA, RelB, and cRel. B and C, Analysis of NF-κB-1 and NF-κB-2 expression by RT-PCR in resting CD4+ T cells (lanes 1–3), activated and untransfected cells (Untr.; lanes 4–6), control siRNA-transfected cell cultures (siRNActrl; lanes 7–9) and siRNA PKCe-transfected CD4+ T cells (siRNAε; lanes 10–12) after 24 h of cultures. 1/10, 1/100, and 1/1000 cDNA, obtained by reverse transcription of 1 μg total RNA, was amplified and PCR products were separated on agarose gel, visualized under UV illumination after ethidium bromide staining. Lanes 1, 4, 7, and 10: PCR with 1/10 cDNA. Lanes 2, 5, 8, and 11: PCR with 1/100 cDNA. Lanes 3, 6, 9, and 12: PCR with 1/1000 cDNA. PCR products were quantified by densitometric analysis and plotted in the graph as described in Materials and Methods. *p < 0.05 versus untransfected and activated cells, analysis by ANOVA and Dunnett’s test.
PKCe expression did not induce T-bet, GATA-3, Rorγt, or Foxp3 mRNAs, although it primed the TGF-mediated in vitro Treg polarization of human T CD4+ cells (Fig. 8F).

Table I. Western blot analysis of PKCe expression levels in CD4+ T cells from peripheral blood of adults with HT and of HC

<table>
<thead>
<tr>
<th>Description</th>
<th>Sex</th>
<th>n</th>
<th>PKCe, Mean ng/50 μg ± SD (Range)</th>
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<tr>
<td>HC</td>
<td>F</td>
<td>4</td>
<td>0.24 ± 0.24 (0–0.55)</td>
</tr>
<tr>
<td>HC</td>
<td>M</td>
<td>23</td>
<td>0.22 ± 0.20 (0–0.80)</td>
</tr>
<tr>
<td>HC</td>
<td>F+M</td>
<td>27</td>
<td>0.22 ± 0.20* (0–0.58)</td>
</tr>
<tr>
<td>HT</td>
<td>F</td>
<td>7</td>
<td>0.42 ± 0.17 (0.14–0.97)</td>
</tr>
<tr>
<td>HT</td>
<td>M</td>
<td>16</td>
<td>0.28 ± 0.25 (0–0.84)</td>
</tr>
<tr>
<td>HT</td>
<td>F+M</td>
<td>23</td>
<td>0.38 ± 0.29* (0–0.97)</td>
</tr>
</tbody>
</table>

*p = 0.026 (t test).

Discussion
PKCs, in particular, PKCθ, have a critical role in the activation and proliferation of lymphocytes (3), which, however, express several PKC isoforms that are upregulated and activated on T cell stimulation. In vivo experiments demonstrated that PKCe plays a key role in inflammation and immunity. In fact, using both knockout mice and PKC peptide modulators, Hucho et al. (45) demonstrated that PKCe inhibition profoundly suppresses the acute and chronic inflammatory pain responses, whereas Aksoy et al. (46) suggested that PKCe has a role in inflammation and immune-mediated disorders. Indeed, infiltration of macrophages and T cells into cardiac grafts, as well as parenchymal fibrosis, was decreased in animals treated with PKCe inhibitors (47). Nevertheless, the role of PKCe in lymphocyte activation and proliferation is not yet clear, and even contrasting opinions can be found in the current literature (4–
PKCε IN HUMAN CD4 LYMPHOCYTES

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Alignment</th>
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</tr>
<tr>
<td>WT</td>
<td>G ATC AAA ATC TGC GAG GCC</td>
</tr>
<tr>
<td>R</td>
<td>A T G T A T A G</td>
</tr>
<tr>
<td>H</td>
<td>G C C</td>
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<tr>
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</tr>
<tr>
<td>siRNA#2</td>
<td>G ATC GAG CTG GCT GTC TTT</td>
</tr>
<tr>
<td>WT</td>
<td>G ATC GAG CTG GCT GTC TTT</td>
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<tr>
<td>R</td>
<td>A T A C G G C</td>
</tr>
<tr>
<td>H</td>
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<td>H</td>
<td>A G C C</td>
</tr>
<tr>
<td>Translation</td>
<td>K R A</td>
</tr>
</tbody>
</table>

Conserved base is reported by a dot.

H, siRNA target sequences of human PKCε; R, siRNA target sequences of PKCε1234R; siRNA#1 (#2, #3, and #4), sequence of the number 1 (2, 3, and 4) siRNA PKCε; translation, amino acid sequence of siRNA PKCε; target sequence; WT, siRNA target sequences of wild type mouse PKCε.

7, 48). For this reason, and for its potential implications in autoimmune disease pathophysiology, we decided to study the role of PKCε in the activation and proliferation of human CD4+ T cells.

Our data demonstrate that PKCε sustains human CD4+ T cell duplication in vitro. In fact, suppression of PKCε expression impaired CD3-mediated activation and proliferation: cell cultures transfected with siRNA PKCε showed more cells that did not duplicate at all, and an overall decrease of the proliferating fraction of the cell culture, without affecting cell viability. In contrast, upregulation of PKCε expression in CD4+ cells transfected with wild type PKCε promoted cell proliferation. This effect was specific to PKCε because it could not be observed in cells transfected with a mutated, kinase-inactive PKCε isoform (PKCαm) and was rescued by siRNA-resistant PKCε (PKC1234R) expression.

Searching for a mechanistic explanation for these observations, we found that, although the cell surface expression of activation markers like CD71 and CD95 was not affected, the expected upregulation of CD25 expression was prevented in stimulated CD4+ T cells by the suppression of PKCε expression. Using multiple TCR-independent T cell activators (PHA, PMA, and ionomycin), we observed that PKCε expression and activation were upregulated in stimulated CD4+ cells. Ionomycin, an activator of classical PKCs, activated PKCε as well and promoted its late upregulation, in agreement with recent data reported by P. J. Parker’s group showing that PKCε can be downstream of PKCα (49). Functionally, PHA-induced CD4+ T cell duplication was found to be mediated, in part, by PKCε activation. These data suggest that PKCε is not involved in the early events after T cell activation, but rather acts later, promoting cell proliferation. This conclusion is in agreement with Szamel et al. (6), who compared different PKC isoforms, showing that PKCε is activated later (90 min after TCR ligation) than PKCα and PKCζ. We show that PKCε decreases IL-2R expression in stimulated CD4+ T cells both at protein (CD25 cell surface expression) and mRNA level (RT-PCR). Szamel et al. (6), on the contrary, did not observe effects of PKCε on CD25 expression. This different result might be possibly explained by the different method of PKCε inhibition (intracellular mAb instead of siRNA). In line with this concept of a late role of PKCε in cell duplication, it should be noted that it has been recently reported in different models that PKCε, by interacting with 14-3-3 protein and RhoA, promotes cytokinesis (50).

NF-κB, originally identified in B cells (38, 39), is a central transcription factor in both innate and adaptive immune responses. NF-κB is activated by a plethora of proinflammatory cytokines, chemokines, adhesion molecules, and immunoregulatory mediators. Deregulation of NF-κB has been associated with a number of disorders including arthritis, asthma, and inflammatory bowel disease (38, 51). At least two NF-κB signaling pathways exist (38, 39): the classical pathway, which is dependent on IκBβ and is involved in inflammatory responses and innate immunity; and the alternative pathway, which is dependent on IκBα, RelB, and p52 (involved in development, homeostasis, and activation of adaptive immunity) (38, 39). Currently, five mammalian NF-kB family members have been identified and cloned; these include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel (52, 53). In our hands, activated human PBLs do not express detectable amounts of RelA, RelB, or c-Rel; thus, in our model system of activated human CD4+ T cells, we analyzed the expression of NF-κB1 and NF-κB2 only. We observed that in the absence of PKCε, CD4+ T cells failed to upregulate NF-κB1 and NF-κB2 transcription after CD3 and CD28 stimulation. Because IL-2R chain transcription is under the control of NF-κB1s (38–41), we believe that PKCε upregulation is a critical event for IL-2R chain expression in activated CD4+ T cells, which operates by promoting NF-κB1 and NF-κB2 transcription.

Gruber et al. (48) suggest a redundant function of PKCε in mouse T cell proliferation, and this opinion is in apparent contrast with our findings. However, first, PKC isoforms have different roles and cellular distribution in humans and mice. Perhaps the best example of this is the reciprocal expression of PKCε and PKCδ in mouse and human platelets, suggesting species-specific functions of individual PKC isoforms (54). Second, it is not possible to exclude that some PKCε functions, in the case of germinal deletion of the PKRεε as in knockdown mice, may be compensated by other PKCs, such as PKCδ, PKCζ, and PKCβ, expressed in T cells that have relevant roles in the control of cell activation and proliferation. A good example of this is the upregulation of PKCδ expression in the heart of PKCε−/− mice (55). In some instances, indeed, PKCs modulate each other’s expression and activity: the overexpression of PKCδ in breast cancer cells, for example, increases PKCδ and decreases PKCε and PKCζ expression (56); specifically, the overexpression of PKCε induces the recruitment of PKCβII in the development of cardiac hypertrophy (57) by promoting RACK1 expression and PKCβII–RACK1 interaction; and part of the PKCα effects can be mediated by PKCε (49). In contrast, Castrillo et al. (7) reported that lymphocytes in PKCε−/− mice show a 20% reduction of activation and proliferation in response to classical mitogenic stimuli when compared with lymphocytes from wild type mice. Our data in vitro show that PKCε silencing reduces the proliferation of purified human CD4+ T cells by 50%.

T cell proliferation is finely tuned by activating and inhibiting cytokines. In agreement with previously reported data (11), the inhibiting factor TGF-β1 is more active on CD3-stimulated CD4+ T cell cultures, in the absence of CD28 costimulation. We have demonstrated that the TGF-β1 antiproliferative signaling, undetectable when CD4+ T cells are stimulated with CD3 and CD28, is enhanced by the downregulation of PKCε expression levels in CD3/CD28 and in PHA-stimulated cells. To formally prove that...
PKCe interferes with TGF-1β signaling, we upregulated PKCe expression in CD4+ T cells stimulated with CD3 alone, inducing resistance to TGF-1β. We also studied TGF-1β downstream signaling using Jurkat cells and CD4+ T lymphocytes as model systems, focusing on Smad2 and Smad3. Differently from PKCθ, the overexpression of PKCe reduced Smad2, but not Smad3, phosphorylation. In line with previous data, demonstrating that both Smad2 and Smad3 contribute to TGF-1β-induced inhibition of IL-2–sustained T cell proliferation (43), it looks reasonable that SSmad2 is downstream PKCe signaling. However, because it is well-known that addition of IL-2 can override the growth arrest of T cells mediated by TGF-1β (10), we cannot exclude that the ability of PKCe to regulate IL-2R expression levels might also explain the increased TGF-1β sensitivity of silRNA PKCe-transfected cells.

TGF-1β has been shown to play an essential role in inhibiting Th1 markers, as well as inducing both Treg and Th17 differentiation. Furthermore, Foxp3 expression can suppress Rorγt expression and consequent conversion to Th17 phenotype. Moreover, recently it has been demonstrated that TGF-1β–mediated induction of Foxp3 is also dependent on both Smad2 and Smad3 signaling, which, conversely, are not involved in the TGF-1β–mediated induction of Rorγt (58). In this article, we show that the inhibition of PKCe expression levels does not affect CD4+ T cell polarization in the absence of TGF-1β, whereas increasing Foxp3 expression in TGF-1β–treated cells. On the contrary, silRNA PKCe downregulated Rorγt expression (a Th17 marker) in TGF-1β–treated cultures, probably as a consequence of Foxp3 overexpression.

HT has recently been viewed as a genetic disorder of cell-mediated immunity (23, 24). After initiation of the autoreactive immune response by CD4+ T cells, the expansion of T cell populations results in a massive lymphocyte accumulation in patients’ thyroids (23, 24). Moreover, it has recently been demonstrated that the serum concentration of TGF-1β is decreased in HT (25), and that some Treg subsets apparently exert a defective suppressive function in these patients. Being that PKCe is implicated in the response of CD4 T cells to both CD3-mediated proliferative stimuli and TGF-1β antiproliferative signals, we were not surprised to find significantly higher expression levels of PKCe in HT patients than in HC. CD4+ T cells from HT patients were resistant to TGF-1β antiproliferative effects, and the downregulation of PKCe expression restored their sensitivity to TGF-1β on anti-CD3 stimulation. The increased expression of PKCe in CD4+ T cells of HT patients, described in this article for the first time, to our knowledge, viewed in the perspective of the physiological role of PKCe in normal TH lymphocytes, adds knowledge to the molecular pathophysiology of HT and creates potentially new pharmacological targets for the therapy of this disease.

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Disclosures

The authors have no financial conflicts of interest.

References


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