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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: 4721–4732.

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/σ) are independent of both Ca²⁺ and DAG (1).

T lymphocytes contain up to eight different species of PKC isotypes. 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-deficient mice, or mice expressing dominant-negative TGF-1βRs on T cells, soon develop spontaneous autoimmune diseases (14, 15). Furthermore, TGF-1β-deficient 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 immunosuppressive ligands such as adenosine, IL-10, and also TGF-1β itself (18, 19). Tregs inhibit multiorgan autoimmunity induced 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

Naive CD4+ Th 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 assessed using the VarioMACS cell separator (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 each and 500 µl RPMI and added to each well of a 96 flat-bottom well plate. Before cell seeding, mAb solution was removed and plates were washed with RPMI 1640 medium. In some experiments (in 50 µl RPMI), 5 mg/ml PHA (Sigma-Aldrich) for 24 h at 4˚C; and 0.62 µg/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), and 50 ng/ml calcyclin (Sigma-Aldrich, batch 022). In some experiments, CD4+ T cells were stimulated with 5 mg/ml PHA (Sigma-Aldrich) for 24 h at 4˚C; and 0.62 µg/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), and 50 ng/ml calcyclin (Sigma-Aldrich). In some experiments, CD4+ T cells were stimulated with 5 mg/ml PHA (Sigma-Aldrich) for 24 h at 4˚C; and 0.62 µg/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), and 50 ng/ml calcyclin (Sigma-Aldrich).

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 10% FBS-containing RPMI and added to each well of a 96 flat-bottom well plate. IL-2 was added to the culture medium at a concentration of 10 ng/ml 24 h after cell seeding.

Anti-CD3 (clone x55; 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-bottom 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, in the absence of IL-2 and CD3/CD28 mAbs, alone or in combination, were diluted at 4 µg/ml each and 500 µl RPMI and added to each well of a 96 flat-bottom well plate.

Cell proliferation

Cell proliferation was studied analyzing by flow cytometry the number of cell doublings. 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 by 4 of d 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-CD95-PE, 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 (i.e., 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 kit for unstimulated T cells. Overall, we show that 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.

To generate proper controls to our RNA silencing experiments, we mutated siRNA-target 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 primer 5′-GGGCAGGATCCATCTAGGT AGTGTCCA TGGC-3′ and the end primer 5′-GGCGGTCGCA CGTTGAGAGA CACG-3′. Mutations were introduced in the siRNA-target sequences by overlap extension PCR. Two PCR products, according to the mutations in overlapping complementary ends were produced from the GFP-PKCe cDNA with the following primers: siRNA4sense, 5′-GAATAAGGGCG GCGGGCG- CGG AAacctcaca gccgctctg-3′; siRNA4antisense, 5′-TTCCGGGGCC GGCACCAATT TTCCctctt ggccaagtg-3′; siRNA5sense, 5′-TTAAAGTTGCC CCACTGGTT gcgctcgcgg tcg-3′; siRNA5antisense, 5′- AAATGGGGCG CGCTTGGGA GTATCTCAA Agcagagctc ggtggg-3′; siRNA6antisense, 5′-AAATTAGACTCGGCGGGTCAC ecagcagctgc tggggc-3′; siRNA2antisense, 5′-GGACACCCGG CGTGCTAA Ttgctggcgc ggtggg-3′; siRNA2antisense, 5′-GAATACCCGCG GCTTGCTAA Ttgctggcgc ggtggg-3′; siRNA2antisense, 5′-CTCTGAATTTG GGTCACGAA Gtggggc-3′; siRNA2antisense, 5′-CTTGAGTTTT GGGTCACGAA Gtggggc-3′. The overlapping sequence, containing a mutated version of the siRNA target sequence of mouse PKCe cDNA, is reported by using uppercase letters.

As previously described in Fig. 5D, the PCR products obtained with siRNA4sense-end primers (lane 1; 132 bp) was hybridized with the probe obtained with siRNA4antisense-start primers (lane 2; 977 bp). The overlapping product was amplified by primers start and end (lane 6; 2297 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 siRNA3sense-end lane 14 (778 bp) and siRNA3antisense-start lane 13 (2297 bp). The overlapping product was amplified by primers start and end (lane 8; 2297 bp) to yield the full-length PKCe cDNA resistant to the siRNA PKCe, numbers 3 and 4 (PKCe3R4). The same procedure was repeated in lanes 7 (PCR by siRNA2sense and end primers; 2051 bp), 8 (PCR by siRNA2antisense 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, siRNA3sense-siRNA3antisense product (lane 10; 747 bp) was hybridized with the start+siRNA3antisense amplicon (lane 11; 265 bp), both obtained using PKCe234R as template. The overlapping product was amplified by the primers start and siRNA3antisense (lane 12; 778 bp). This last PCR product was hybridized with siRNA3sense-end amplified 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 siRNAs, 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 was performed with 2× SSC and 1% SDS at 65°C for 30 min. The probe was detected by autoradiography.
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).

PKCε1234R was digested with BglII and Sall and ligated to the corresponding sites of the pEGFP-C1 vector. The sequence integrity of siRNA-resistant PKCε was tested by DNA sequencing. Table 1 shows the alignment of nucleotide sequence of human, wild type mouse, and siRNA-resistant PKCε cDNAs.

**RT-PCR**

CD4+ T cell cultures were grown for 48 h, and 2 × 10^6 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′-ATCCCAACGC CACCTTCA AAGC-3′ and antisense 5′-GGCCCA CGAATGAT AAAT-3′ primers generated an expected PCR product of 347 bp (NM-000417 GenBank locus, GI:23238195). By the product of 347 bp (NM-000417 GenBank locus, GI:455766). IL-2R sense 5′-GCCCCCATCT CCCTCAAGT-3′ and antisense 5′-AGGGGAAAGGG CGAAGAGC-3′ of 529 bp (NM-000878 GenBank locus, GI:23238195). To improve specificity and efficiency, we added 3% of DMSO to the PCR mix reaction. Moreover, to improve specificity and efficiency, we added 3% of GATA-3 (205 bp) by 5′-GTGCCTTTTT ACAA TAC-3′ and 5′-ATTGGGTTCG AGACAACAGG-3′ and antisense 5′-CTGGTCCCGT GAAATACACC-3′; cRel by 5′-ATCCCAACGC CACCTTCA AAGC-3′ and 5′-ATTGGGTTCG AGACAACAGG-3′; c-Rel by 5′-CTGGTCCCGT GAAATACACC-3′; cRel by 5′-ATCCCAACGC CACCTTCA AAGC-3′ and 5′-ATTGGGTTCG AGACAACAGG-3′. The lengths of the expected amplified regions were 318, 322, 300, 518, and 488 bp (GenBank locus NM102936; GenBank locus GI:4577121; NM001145138 GI:2232468680; NM006509 GI:35493877; and NM002908 GI:56550118).

Temperature and time were modified from the original methodology (35): CDNA was amplified by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. The extension time of the last 15 cycles was incremented by 3 s/cycle. Moreover, to improve specificity and efficiency, we added 3% of DMSO to the PCR mix reaction.

To obtain a relative quantification of gene expression, 1/10, 1/100, and 1/1000 of total cDNA was amplified, PCR products were separated in 2% agarose gel, stained with ethidium bromide, and visualized under an UV transillumination by KODAK Image Station 400MM. Band intensity of each PCR was quantified by KODAK Molecular Imaging Software 4.0 and corrected for β-actin amplified cDNA intensity.

The expression levels of the markers of polarization to Th1, Th2, Th17, and Treg were analyzed by RT-PCR. T-bet cDNA (217 bp) was amplified by 5′-CCACCCAGCCA CTACAGGATG-3′ and 5′-GGAGGCCCCC TTGGTGT3T-3′ primers; GATA-3 (205 bp) by 5′-GATCGAGGTG GACCTGGTAA-3′ and 5′-ATCCCAACGC CACCTTCA AAGC-3′; p38 by 5′-CAGCAGAGAT CGATGATCA-3′ and 5′-ATTGGGTTCG AGACAACAGG-3′; and the antisense 5′-GGCCCA CGAATGAT AAAT-3′ primers, we obtained two expected amplifieds of 386 bp (NM-000206 GenBank locus sequence, GI:4557881) and 407 bp (HUMIL2RGA GenBank locus, GI:349631). By the sense 5′-TGGACGGGTC ACCACACC TGCCCTCACTA-3′ and antisense 5′-CTAGAAGAT CGTGCTGGTG GAGATGGG GG-3′, we amplified an expected 649-bp region of β-actin mRNA (E00829 GenBank locus, GI:2216690). β-Actin was amplificationally described (30, 32).

The 387-bp region of the human PKCε cDNA, NM050440 GenBank locus (GL:47157326), was amplified by the sense 5′-CAATGGCTCTTC TTTAGATCA AA-3′ and antisense 5′-CTTCAGAGAT CGATGATCA-3′, as previously described (32).

NF-κB1 was amplified by 5′-AGATGGTGAGG AGATGGTG-3′ and the antisense GCCAATGAGAT GGTCTTGGCTG-3′ primers; NF-kB2 by 5′-AAATGAATGG TTCCGATGGA-3′ and 5′-CTTTTGGATG AAAGAATGT-3′, and the antisense 5′-GGCCCA CGAATGAT AAAT-3′ primers, we obtained two expected amplicons of 386 bp (NM-000206 GenBank locus sequence, GI:4557881) and 407 bp (HUMIL2RGA GenBank locus, GI:349631). By the sense 5′-TGGACGGGTC ACCACACC TGCCCTCACTA-3′ and antisense 5′-CTAGAAGAT CGTGCTGGTG GAGATGGG GG-3′, we amplified an expected 649-bp region of β-actin mRNA (E00829 GenBank locus, GI:2216690). β-Actin was amplificationally described (30, 32).

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**Results**

**PKCε and CD4+ T cell proliferation**

To evaluate the role of PKCε in CD4+ T lymphocyte proliferation, we transfected freshly purified cells with siRNA to PKCε (siRNA_PKCε), or with control siRNA, and then seeded them in anti-CD3− and/or anti-CD28−coated wells in the presence of IL-2. T 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 PKCε expression by specific siRNA transection did not affect the number of duplicating cells in the control (untreated) cultures (≤5%), whereas it significantly impaired the proliferation of CD3/CD28−stimulated T cells. As shown in Fig. 1B, siRNA_PKCε−transfected stimulated cultures show a significant increase of cells with zero duplications. Because different donors responded differently to the mitogen 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_PKCε−treated cultures was 161 ± 19% of normalized (100%) controls (siRNAActl−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_PKCε−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_PKCε−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_PKCε−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_PKCε−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 PKCε, 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.
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 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, siRNA_{PKCe} 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 siRNA_{PKCe}-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 Kehr et al. (10) and Wolfraim 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 siRNA_{PKCe} 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 siRNA_{PKCe} to boost TGF-1β effects is more evident in anti-CD3/CD28–stimulated cultures (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. PKCε controls cell proliferation, but not cell activation, of T CD4+ lymphocytes. A, Western blot analysis of PKCε 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 PKCε was also studied using specific anti–phospho-PKCε rabbit serum (pPKCε). B, CD4+ T cells were labeled with CFSE and transfected with control siRNA (siRNA ctrl.) or with siRNA to PKCε (siRNA PKCε). 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. siRNA ctrl. and siRNA PKCε 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 PKCε downmodulation (siRNA PKCε) 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.
siRNA transfection was not due to any significant increase of cell death (Fig. 4D).

siRNA target specificity and PKCe rescue experiments

To test the specificity of our siRNA PKCe, we choose the Jurkat cell model where, at variance with primary CD4+ T cells, PKCe is constitutively expressed. siRNA PKCe selectively impaired PKCe expression, not affecting PKCδ protein levels (Fig. 5A, 5B; *p < 0.05, ANOVA by Dunnett’s test). In parallel, rescue experiments were performed transfecting CD4+ T cells with siRNA-resistant PKCe (PKCe1234R) cDNA. As shown in Fig. 5C, the transfection of PKCe1234R, but not that of the wild type gene (CMV-PKCe), prevented siRNA PKCe-mediated sensitization to TGF-1β antiproliferative effects in CD4+ T cells.

PKCe and IL-2R expression

Because we know that the percentage of activated CD4+ T cells that expresses CD25 decreases when PKCe is downregulated (Fig. 2B), to explore the molecular mechanism activated by PKCe 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 PKCe expression by siRNAPKCe 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 siRNA PKCe–transfected cells, suggesting that an interference with NF-κB expression might be at the basis of all the observed effects of PKCe inhibition in T cells (Fig. 7B).

PKCe expression in HT

Because PKCe levels modulate CD4+ T cell proliferation and sensitivity to TGF-1β, we finally hypothesized that PKCe over-
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 undetectable PKCe levels (0.22 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 siRNAPKCe (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 siRNActrl-transfected cells with more than two duplications).

**PKCe impaired TGF-1β signaling**

It has been reported that the activation of PKCδ 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 pSmad2 and 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 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 siRNActrl-transfected cells with more than two duplications).

**FIGURE 5.** siRNA PKCe effects were target selective. A. Western blot analysis of PKCe and PKCδ expression in Jurkat cell treated with siRNA PKCe. Total protein lysates of untransfected (Control) cell cultures and of siRNA PKCe and control siRNA treated cells (siRNA ctrl.), harvested 48 h after nucleofection, were blotted for PKCe, PKCδ, and β-actin. A representative experiment is reported. B. Densitometric analysis of PKCe and PKCδ expression: mean values of three independent experiments are reported. PKCe and PKCδ expression were normalized to β-actin. *p < 0.05 versus untransfected cells (lane 1); analysis was made by ANOVA followed by Dunnet’s test. C. siRNA-resistant PKCe abolished siRNA effects. CFSE-labeled CD4+ T cell cultures, nucleofected with siRNA PKCe and with GFP-PKCe1234R (PKCe1234R) or GFP-PKCe wt, were grown in anti-CD3 + anti-CD28 mAb-coated wells for 4 d with or without 10 ng/ml TGF-1β. siRNA-resistant PKCe(PKCe1234R) prevented siRNA PKCe-induced sensitization to antiproliferative effects of TGF-1β, restoring cell proliferation. Flow cytometry analysis was performed 3 d after TGF-1β addition. The number of duplications is indicated above the histograms (from 0–5). A representative experiment is reported. D. Construction of siRNA-resistant PKCe: 0.8% agarose gel, stained with ethidium bromide and visualized under UV exposure. CMV-PKCe (lane P1) was used as template of the PCRs 1 and 2, whose amplicons are shown (lanes 1, 2). The fusion product of PCRs 1 and 2 (PKCe resistant to the siRNA number 4) was obtained (lane 3). Amplicon 3 was used as template of PCRs 4 and 5 (lanes 4, 5). The fusion product of PCRs 4 and 5 (PKCe resistant to the siRNAs number 3 and 4) was obtained (lane 6). With the same procedure, amplicon of the PKCe resistant to the siRNAs numbers 2, 3, and 4 was shown (lane 9). The PKCe resistant to our 4 siRNAs (lane 14) was obtained by fusion of 13 with 12. The amplicon shown in 12 was obtained by fusing PCR 10 to PCR 11, both obtained using 9 as template. For a better description of primers used, see Materials and Methods.
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 PKCε modulates mRNA levels of IL-2R chains.

**FIGURE 6.** PKCε 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 PKCε-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.** PKCε 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 PKCε-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.
PKCε 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).

**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 PKCε plays a key role in inflammation and immunity. In fact, using both knockout mice and PKC peptide modulators, Hucho et al. (45) demonstrated that PKCε inhibition profoundly suppresses the acute and chronic inflammatory pain responses, whereas Aksoy et al. (46) suggested that PKCε 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 PKCε inhibitors (47). Nevertheless, the role of PKCε in lymphocyte activation and proliferation is not yet clear, and even contrasting opinions can be found in the current literature (4–

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**Table I. Western blot analysis of PKCε 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>PKCε, 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>
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<tr>
<td>HC</td>
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<td>23</td>
<td>0.22 ± 0.20 (0–0.58)</td>
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<tr>
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<td>0.42 ± 0.17 (0.14–0.97)</td>
</tr>
<tr>
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<td>0.28 ± 0.25 (0–0.84)</td>
</tr>
<tr>
<td>HT</td>
<td>M</td>
<td>16</td>
<td>0.38 ± 0.29 (0–0.97)</td>
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*p = 0.026 (t test).

**FIGURE 8.** PKCε expression and signaling. A, Western blot analysis of CD4+ T cells from PBLs of four HC adults (HC, lanes a–e) and from eight HT patients (HT, lanes f–l). Filters were immunoblotted with anti-PKCε and anti-β-actin Abs. Signals obtained from lysates were compared with those produced by 5, 2.5, and 1.25 ng human recombinant PKCε (hrPKCε). Protein expression signals recovered were analyzed and reported in B as PKCε/β-actin densitometric values. C, Residual proliferation of CD4+ T cell cultures from three HT patients (HT, gray bars) and three healthy controls (HC, white bars) treated with 10 ng/ml TGF-1β. Cells were either transfected with siRNA to PKCε (siRNA PKCε) to inhibit PKCε expression or with scrambled nucleotides siRNA (siRNA ctrl.). The number of cells with two or more duplications or with zero duplications is reported as percentage of control cultures (untransfected TGF-treated lymphocytes); #, p < 0.05 versus control cultures (untransfected and TGF-untreated cells). Cell cultures were stimulated with anti-CD3 mAb (4 ng/ml). Means ± SD are reported. *p < 0.05 versus control culture (siRNA ctrl-transfected and TGF-1β–untreated cultures), analysis by ANOVA, followed by Dunnett’s test. D and E, PKCε impairs TGF-1β signaling. Western blot analysis of total and activated Smad2 and Smad3 in Jurkat cells (lanes 1–6) and in CD4 lymphocytes (lanes 7–12). Cells were transfected with GFP-PKCε or with the negative control GFP-PKCε–transfected for 2 h with TGF-1β (10 ng/ml). CD4+ lymphocytes were grown for 36 h in the presence of IL-2 and anti-CD3 + anti-CD28 stimulation; then cultures were starved for 8 h before being treated for 2 h with TGF-1β (10 ng/ml). Protein expression signals recovered from three experiments were analyzed and reported in E as pSmad2/Smad2 or pSmad3/Smad3 expression (normalized levels). Values were normalized to control cultures (untransfected and TGF-untreated cells); #, p < 0.05 versus lane 4 (untransfected TGF-treated lymphocytes); $, p < 0.05 versus lane 5 (untransfected TGF-treated Jurkat); analysis by ANOVA, followed by Dunnett’s test. F, PKCε impaired TGF-mediated T cell polarization. Resting CD4+ T cells were nucleofected with siRNA PKCε or with control siRNA (siRNA ctrl.) and then grown for 4 d on anti-CD3 and anti-CD28 mAb-coated wells. At day 1 of growing, cultures were treated with or without 10 ng/ml TGF-1β. Analysis of T-bet, GATA-3, Rorγt, and Foxp3 expression by RT-PCR in untransfected cells (lanes 1, 4), control siRNA-transfected cell cultures (siRNA ctrl; lanes 2, 5), and siRNA PKCε-transfected CD4+ cells (siRNA PKCε; lanes 3, 6) grown in absence (lanes 1–3) and in the presence of TGF-1β (lanes 4–6). 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.
Our data demonstrate that PKCe sustains human CD4+ T cell duplication in vitro. In fact, suppression of PKCe expression impaired CD3-mediated activation and proliferation: cell cultures transfected with siRNAPKCe 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 PKCe expression in CD4+ cells transfected with wild type PKCe promoted cell proliferation. This effect was specific to PKCe because it could not be observed in cells transfected with a mutated, kinase-inactive PKCe isoform (PKCe\textsuperscript{m}) and was rescued by siRNA-resistant PKCe (PKCe\textsubscript{1234R}) 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 PKCe expression. Using multiple TCR-independent T cell activators (PHA, PMA, and ionomycin), we observed that PKCe expression and activation were upregulated in stimulated CD4+ cells. Ionomycin, an activator of classical PKCs, activated PKCe as well and promoted its late upregulation, in agreement with recent data reported by P.J. Parker’s group showing that PKCe can be downstream of PKCs (49). Functionally, PHA-induced CD4+ T cell duplication was found to be mediated, in part, by PKCe activation. These data suggest that PKCe 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 PKCe is activated later (90 min after TCR ligation) than PKCo and PKC\textbeta. We show that PKCe 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 PKCe on CD25 expression. This different result might be possibly explained by the different method of PKCe inhibition (intracellular mAb instead of siRNA). In line with this concept of a late role of PKCe in cell duplication, it should be noted that it has been recently reported in different models that PKCe, by interacting with 14-3-3 protein and RhoA, promotes cytokinesis (50).

NF-\textkappaB, originally identified in B cells (38, 39), is a central transcription factor in both innate and adaptive immune responses. NF-\textkappaB is activated by a plethora of proinflammatory cytokines, chemokines, adhesion molecules, and immunoregulatory mediators. Deregulation of NF-\textkappaB has been associated with a number of disorders including arthritis, asthma, and inflammatory bowel disease (38, 51). At least two NF-\textkappaB signaling pathways exist (38, 39): the classical pathway, which is dependent on I\textkappaB\alpha and is involved in inflammatory responses and innate immunity; and the alternative pathway, which is dependent on I\textkappaBo, RelB, and p52 (involved in development, homeostasis, and activation of adaptive immunity) (38, 39). Currently, five mammalian NF-\textkappaB family members have been identified and cloned; these include NF-\textkappaB1 (p50/p105), NF-\textkappaB2 (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-\textkappaB1 and NF-\textkappaB2 only. We observed that in the absence of PKCe, CD4+ T cells failed to upregulate NF-\textkappaB1 and NF-\textkappaB2 transcription after CD3 and CD28 stimulation. Because IL-2R chain transcription is under the control of NF-\textkappaBs (38–41), we believe that PKCe upregulation is a critical event for IL-2R chain expression in activated CD4+ T cells, which operates by promoting NF-\textkappaB1 and NF-\textkappaB2 transcription.

Guber et al. (48) suggest a redundant function of PKCe 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 PKCe and PKC\textdelta in mouse and human platelets, suggesting species-specific functions of individual PKC isoforms (54). Second, it is not possible to exclude that some PKCe functions, in the case of germinal deletion of the PKCE as in knockdown mice, may be compensated by other PKCs, such as PKC\textgamma, PKC\textalpha, and PKC\textdelta, 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 PKCe expression in the heart of PKCe\textsuperscript{-/-} mice (55). In some instances, indeed, PKCs modulate each other’s expression and activity: the overexpression of PKCo in breast cancer cells, for example, increases PKC\textdelta and decreases PKC\textgamma and PKC\texteta expression (56); specifically, the overexpression of PKCe induces the recruitment of PKC\textbetaII in the development of cardiac hypertrophy (57) by promoting RACK1 expression and PKC\textbetaII–RACK1 interaction; and part of the PKCe\textgamma effects can be mediated by PKCe (49). In contrast, Castrillo et al. (7) reported that lymphocytes in PKCe\textsuperscript{-/-} 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 PKCe 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-1\beta is more active on CD3-stimulated CD4+ T cell cultures, in the absence of CD28 costimulation. We have demonstrated that the TGF-1\beta antiproliferative signaling, undetectable when CD4+ T cells are stimulated with CD3 and CD28, is enhanced by the downregulation of PKCe expression levels in CD3/CD28 and in PHA-stimulated cells. To formally prove that

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Conserved base is reported by a dot.

H, siRNA target sequences of human PKCe; R, siRNA target sequences of PKCe\textsubscript{1234R}; siRNA#1 (H2, 3, and 4), sequence of the number 1 (2, 3, and 4) siRNAPKCe; translation, amino acid sequence of siRNAPKCe target sequence; WT, siRNA target sequences of wild type mouse PKCe.
PKCε interferes with TGF-β signaling, we upregulated PKCε expression in CD4+ T cells stimulated with CD3 alone, inducing resistance to TGF-β. We also studied TGF-β downstream signaling using Jurkat cells and CD4+ T lymphocytes as model systems, focusing on Smad2 and Smad3. Differently from PKCθ, the overexpression of PKCε reduced Smad2, but not Smad3, phosphorylation. In line with previous data, demonstrating that both Smad2 and Smad3 contribute to TGF-β–induced inhibition of IL-2–sustained T cell proliferation (43), it looks reasonable that pSmad2 is downstream PKCε signaling. However, because it is well-known that addition of IL-2 can override the growth arrest of T cells mediated by TGF-β (10), we cannot exclude that the ability of PKCε to regulate IL-2R expression levels might also explain the increased TGF-β sensitivity of siRNAPKCε–transfected cells.

TGF-β1 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 Rorty expression and consequent conversion to Th17 phenotype. Moreover, recently it has been demonstrated that TGF-β1–mediated induction of Foxp3 is also dependent on both Smad2 and Smad3 signaling, which, conversely, are not involved in the TGF-β–mediated induction of Rorty (58). In this article, we show that the inhibition of PKCε expression levels does not affect CD4+ T cell polarization in the absence of TGF-β1, whereas increasing Foxp3 expression in TGF-β1–treated cells. On the contrary, siRNAPKCε downregulated Rorty expression (a Th17 marker) in TGF-β1–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-β1 is decreased in HT (25), and that some Treg subsets apparently exert a defective suppressive function in these patients. Being that PKCε is implicated in the response of CD4 T cells to both CD3-mediated proliferative stimuli and TGF-β1–antiproliferative signals, we were not surprised to find significantly higher expression levels of PKCε in HT patients than in HC. CD4+ T cells from HT patients were resistant to TGF-β1 antiproliferative effects, and the downregulation of PKCε expression restored their sensitivity to TGF-β1 on anti-CD3 stimulation.

The increased expression of PKCε 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 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.

Acknowledgments

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Disclosures

The authors have no financial interests of interest.

References


