

Prostaglandin E₂ Impairs CD4⁺ T Cell Activation by Inhibition of Ick: Implications in Hodgkin's Lymphoma

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Abstract

Many tumors, including Hodgkin's lymphoma, are associated with decreased cellular immunity and elevated levels of prostaglandin E₂ (PGE₂), a known inhibitor of CD4⁺ T cell activation, suggested to be involved in immune deviation in cancer. To address the molecular mechanisms tumor-derived PGE₂ might have on primary human CD4⁺ T cells, we used a whole genome-based transcriptional approach and show that PGE₂ severely limited changes of gene expression induced by signaling through the T cell receptor and CD28. This data suggests an interference of PGE₂ at an early step of T cell receptor signaling; indeed, PGE₂ stimulation of T cells leads to inactivation of Ick and reduced phosphorylation of ZAP70. Antiapoptotic genes escaped PGE₂-induced inhibition resulting in partial protection from apoptosis in response to irradiation or Fas-mediated signaling. As a functional consequence, PGE₂-treated CD4⁺ T cells are arrested in the cell cycle associated with up-regulation of the cyclin/cyclin-dependent kinase inhibitor p27^{kip1}. Most importantly, CD4⁺ T cells in Hodgkin's lymphoma show similar regulation of genes that were altered *in vitro* by PGE₂ in T cells from healthy individuals. These data strongly suggest that PGE₂ is an important factor leading to CD4⁺ T cell impairment observed in Hodgkin's lymphoma. (Cancer Res 2006; 66(2): 1114-22)

Introduction

Prostaglandins are lipid molecules generated from arachidonic acid by cyclooxygenases and cell-specific prostaglandin synthases regulating numerous processes including modulation of immune function (1–3). Prostaglandin E₂ (PGE₂) is produced by many different cell types, including malignant cells, thereby playing an essential role in the carcinogenesis of different tumors associated with chronic inflammatory responses (4–6). Deletion of the respective prostaglandin receptors leads to reduced carcinogenesis and enhanced antitumor immunity (7). Elevated levels of PGE₂ have also been found in patients with Hodgkin's lymphoma, which is suggested to be partially responsible for decreased cellular immune function in these patients (8, 9).

It is well known that PGE₂ has diverse effects on CD4⁺ T cells leading to inhibition of T cell activation (10). Less is known about

the mechanism of PGE₂-induced T cell inhibition. Four different PGE₂ receptors termed EP₁, EP₂, EP₃, and EP₄ are differentially expressed on different cell types and even differently regulated by inflammatory agents or PGE₂ itself (11, 12). These receptors couple through their intracellular sequences to specific G proteins with diverse second messenger signaling pathways. EP₂ and EP₄ receptors couple to a G_s-type G protein leading to stimulation of cyclic AMP (cAMP; refs. 13, 14), whereas the EP₁ receptor leads to increases of intracellular calcium (15, 16). To date, there are seven EP₃ splice variants identified in humans. Originally, the EP₃ receptor was described to couple to a G_i-type G protein leading to inhibition of intracellular cAMP, but subsequently, it was shown that individual splice variants also lead to stimulation of cAMP and inositol 1,4,5-trisphosphate (IP₃) generation (17, 18). There is still a limited understanding of how these potentially conflicting signals are integrated and coordinated within the cell and little is known which of the receptors is mediating the PGE₂-induced inhibition of CD4⁺ T cell activation. Using specific agonists and antagonists for cAMP and its target protein kinase A (PKA) it was indirectly shown that the inhibitory effects of PGE₂ could be mediated by an increase of intracellular cAMP (1), suggesting G_s-coupled EP receptors like EP₂ and EP₄ to be responsible for PGE₂-mediated inhibition of T cell activation. cAMP itself has been recognized as an important second messenger regulating immune responses through inhibition of various T cell functions like cytokine production and proliferation. The best described substrate of cAMP is PKA (19), and it has been shown that antigen-specific T cell proliferation and cytokine production are also inhibited by PKA type I (20). Recently, it was shown that PKA-I in response to cAMP activates the COOH-terminal src kinase (Csk; ref. 21). Activated Csk subsequently phosphorylates the COOH-terminal inhibitory tyrosine residue in Ick and thereby acts as a negative regulator of T cell receptor (TCR) signaling (22–24). It has also been shown that activated PKA regulates cell proliferation via alteration of the cyclin/cyclin-dependent kinase (CDK) complex (25, 26). This kinase complex is involved in the phosphorylation and inactivation of the retinoblastoma protein thereby allowing progression to enter the S phase of the cell cycle. Activated PKA reduces cyclin D₃ expression and induces expression of the Cdk inhibitor p27^{kip1}. However, a direct effect of PGE₂ leading to inhibition of Ick or elevation of p27^{kip1} has thus far not been shown in primary human CD4⁺ T cells.

In this report, we used exclusively primary human CD4⁺ T cells to study the effects of PGE₂ on T cell activation. Whole genome-based transcriptional analysis revealed an inhibition of a large number of TCR/CD28-regulated genes by PGE₂, suggesting that PGE₂ interferes with TCR/CD28 during an early step of signal transduction. We show that PGE₂ leads to an inhibition of the src-kinase Ick resulting in reduced phosphorylation of ZAP70. Additionally,

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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we show that the inhibitory effects of PGE₂ are in fact dependent on TCR signaling because CD4⁺ T cells activated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, thereby circumventing the TCR, are not affected by PGE₂.

By assessing genome-wide transcription profiles in CD4⁺ T cells derived from Hodgkin's lymphoma specimens, we found a similar transcriptional regulation of genes also altered in CD4⁺ T cells from healthy donors in response to PGE₂ treatment *in vitro*. Thus, PGE₂ might be an important mediator of impaired cellular immunity in patients with Hodgkin's lymphoma.

Materials and Methods

Isolation of CD4⁺ T cells and stimulation. Blood samples were collected from healthy blood donors at the Center for Transfusion Medicine (University of Cologne, Cologne, Germany) after informed written consent was obtained. CD4⁺ T cells were isolated by negative selection using RosetteSep CD4⁺ enrichment kit (StemCell Technologies, Meylan, France). The purity of the isolated CD4⁺ T cells was determined by flow cytometry and was routinely >80% CD3⁺ and CD4⁺, but was negative for CD8. Cells were cultured in RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotics (Invitrogen Life Technologies, Karlsruhe, Germany) and stimulated at 37°C by mixing with artificial antigen-presenting cells (aAPC) at a ratio of 1:3 (cells/beads) comprised of magnetic beads (DynaL Biotech, Oslo, Norway) coated with the following antibodies: anti-CD3 (OKT3), anti-CD28 (9.3), and anti-MHC class I (W6/32). For most experiments, these aAPCs were coated with suboptimal anti-CD3 (5%), suboptimal anti-CD28 (14%), and anti-MHC-I constituting the remaining 81% of protein added to the bead, as previously described (27). In some experiments, CD4⁺ T cells were stimulated with 20 ng/mL PMA and 1 μmol/L ionomycin (Sigma-Aldrich, Munich, Germany).

CD4⁺ T cells from lymph node specimens were isolated by homogenizing the lymph node in RPMI. CD4⁺ T cells were purified using Miltenyi magnetic cell sorting columns (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. All samples were taken after informed consent following Institutional Review Board approval.

RNA preparation, microarray hybridization, and microarray data processing. CD4⁺ T cells or subpopulations thereof (CD4⁺CD25⁻ and CD4⁺CD25⁺) were either left unstimulated or stimulated with magnetic beads coated with CD3/MHC-I or CD3/CD28/MHC-I. PGE₂-treated cells were also stimulated with CD3/CD28/MHC-I and PGE₂ was added in a concentration of 1 μmol/L. After 8 hours, magnetic beads were removed and cells were lysed in TRIzol reagent (Invitrogen Life Technologies). RNA isolation and quantification was done as described previously (28). Biotin-labeled cRNA preparation was done using the Ambion Illumina RNA amplification kit (Ambion Europe, Huntington, Cambridgeshire, United Kingdom). cRNA (1.5 μg) was hybridized to Sentrix whole genome bead chips 6 × 2 (Illumina, San Diego, CA) and scanned on Illumina BeadStation 500×. For data collection and statistical analysis, we used Illumina BeadStudio software and dCHIP 1.3. The following filtering criteria were used for selection of differentially expressed genes: fold change ≥ 2, absolute difference in signal intensity between group means ≥ 50, *P* ≤ 0.05 (paired *t* test) and present call ≥ 25%. For gene ontology assessment and pathway visualization, GenMAPP, GeneSpring, and BioRag (<http://www.biorag.org/>) software was used.

Microarrays of CD4⁺ T cells isolated from lymph node specimens were done on the Affymetrix platform (Affymetrix, Santa Clara, CA). RNA isolation, quantification and target preparation was done according to standard protocols for small samples and cRNA was hybridized to HG-U133A arrays. Cross-validation of data from the two different array platforms used revealed a valid comparability of both methods.³

Cell lysis and Western blotting. Primary human CD4⁺ T cells (1 × 10⁷ per sample) were cultured in RPMI/FCS and stimulated with aAPC with and without the addition of PGE₂. Cells were lysed for 30 minutes on ice with 50 μL lysis buffer [5 mL Triton 1% (Promega, Mannheim, Germany), 750 μL 150 mmol/L NaCl (Sigma-Aldrich), 250 μL 50 mmol/L Tris-HCl (Invitrogen), 50 μL phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich), protease inhibitor (Roche, Basel, Switzerland), 10 μL PMSF 1 mol/L (Sigma-Aldrich)] and pelleted for 10 minutes at 14,000 rpm at 4°C. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with the following antibodies: anti-Bcl-x_L (sc-8392), anti-p27 (sc-1641; both from Santa Cruz Biotechnologies, Santa Cruz, CA), anti-Ick (pY505), anti-p-ZAP70 (both from BD PharMingen, San Diego, CA) and anti-actin (Chemicon, Temecula, CA). As secondary antibodies, horseradish peroxidase-conjugated antimouse IgG and horseradish peroxidase-conjugated antirabbit IgG (both from DakoCytomation, Glostrup, Denmark) were used.

Cytometric bead array for cytokines. The concentration of tumor necrosis factor-α (TNF-α) and IFN-γ in cell culture supernatants was measured using the human Th₁/Th₂ cytokine kit II (BD PharMingen). In brief, capture beads were mixed with culture supernatants and phycoerythrin detection reagent and incubated for 3 hours at room temperature. The beads were then washed with wash buffer and analyzed according to the manufacturer's recommendation.

Results

PGE₂ leads to inhibition of CD4⁺ T cell proliferation and cytokine secretion. Although much effort has been undertaken to analyze the mechanism of PGE₂-mediated inhibition of T cell activation, most of the previous work has been done using tumor cell lines deficient in key signaling enzymes or deficient in the complete expression of EP receptors that are expressed on the surface of primary human CD4⁺ T cells (29). In fact, when analyzing EP receptor expression on the human T cell line Jurkat in comparison to primary human T cells, we observed expression of all four EP receptors on primary human CD4⁺ T cells, whereas Jurkat cells lacked expression of EP₁ (data not shown). Therefore, we chose to use only primary human CD4⁺ T cells for further analysis.

To analyze the effects of PGE₂ on primary human CD4⁺ T cells, we established a system in which freshly isolated T cells were stimulated with aAPCs in the form of magnetic beads coated with antibodies against CD3 and CD28 to deliver TCR stimulation (signal 1) and costimulation via CD80/CD86 (signal 2). This system allows one to vary the amounts of anti-CD3 and anti-CD28 coated on the bead surface, thereby mimicking differential strength of TCR and costimulatory signaling. We first used optimal (albeit unphysiologically high) TCR/CD28 stimulation by preparing aAPC coated with only anti-CD3 and anti-CD28 antibodies at a ratio of 1:1 on the bead surface. To analyze T cell proliferation, CD4⁺ T cells were labeled with 5,6-carboxyfluorescein-diacetate-succinimidyl-ester (CFSE) and subsequently stimulated with these aAPC. After 4 days of culture, CFSE dilution was determined by flow cytometry. Under these conditions, we did not observe a significant inhibition of CD4⁺ T cell proliferation in the presence of up to 1 μmol/L PGE₂ (Fig. 1A, *top*). To address the inhibitory effects of PGE₂ on CD4⁺ T cells under more physiological conditions, the amount of CD3 and CD28 coated on the bead surface was subsequently reduced to 5% anti-CD3 and 14% anti-CD28 of the total protein added to the bead. Anti-MHC-I was used as a binding, but not signaling, antibody to ensure a consistent and suboptimal loading of anti-CD3 and anti-CD28 antibodies to the bead surface. Furthermore, beads coated with anti-MHC-I antibody have been shown not to alter the gene expression profile of resting human T cells (30).

³ Unpublished data.

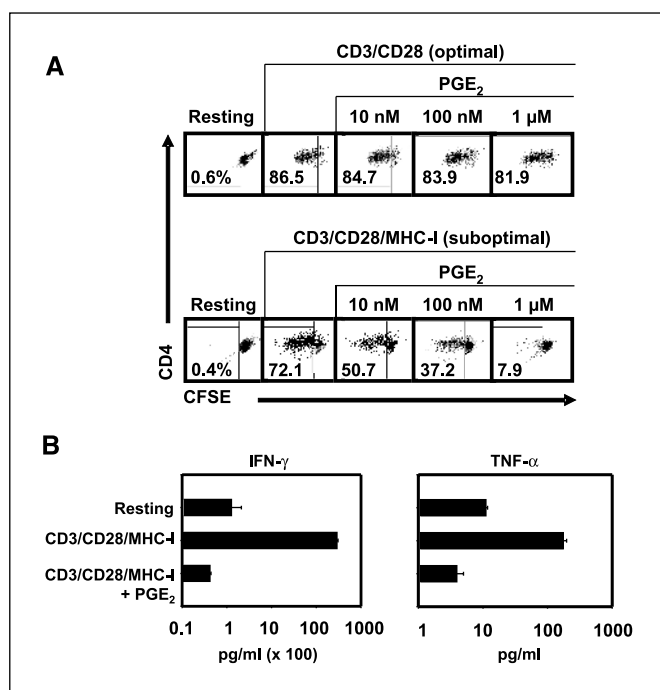


Figure 1. Inhibition of T cell proliferation and cytokine secretion by PGE₂. **A**, freshly isolated primary human CD4⁺ T cells were labeled with CFSE and left unstimulated or stimulated with the indicated magnetic beads in the absence or presence of the indicated concentrations of PGE₂. After 4 days, the CFSE dilution was analyzed by flow cytometry. The overall percentage of dividing cells is displayed in the dot plot. **B**, CD4⁺ T cells were stimulated as indicated with or without 1 μmol/L PGE₂. After 4 days of incubation, the concentration of IFN- γ and TNF- α was determined using flow cytometric bead assays. The presented data is representative of at least three independent experiments; bars, triplicates of one representative experiment.

As shown in Fig. 1, stimulation of primary CD4⁺ T cells with CD3/CD28/MHC-I resulted in robust T cell expansion and cytokine secretion as previously described (31). The addition of PGE₂ to the cultures inhibited T cell proliferation in a concentration-dependent manner (Fig. 1A, *bottom*); 1 μmol/L PGE₂ resulted in complete inhibition of T cell proliferation and this concentration was used in subsequent experiments. To analyze the effect of PGE₂ on cytokine secretion, CD4⁺ T cells were stimulated as above and cytokines were measured in the collected supernatants. As shown in Fig. 1B, 1 μmol/L PGE₂ resulted in strong inhibition of secretion of the cytokines IFN- γ and TNF- α .

To exclude the effect of regulatory CD4⁺ T cells on PGE₂-mediated inhibition of T cell proliferation, conventional CD4⁺CD25⁻ T cells were stimulated with CD3/CD28/MHC-I in the presence or absence of PGE₂ and proliferation was assessed by CFSE dilution. Similar to the total CD4⁺ T cell population, CD4⁺CD25⁻ T cell proliferation was inhibited by PGE₂ (data not shown).

PGE₂ induces overall leveling of transcriptional changes during T cell stimulation. To determine PGE₂-mediated signaling events, we first did a whole genome-based transcriptional analysis. CD4⁺ T cells were either left unstimulated or stimulated with beads coated with CD3/MHC-I as a control or CD3/CD28/MHC-I with or without the addition of 1 μmol/L PGE₂. Using stringent filter criteria, we detected no significant changes in the transcriptional profile after stimulation with CD3/MHC-I compared with resting cells (data not shown). However, 1,037 genes were significantly up-regulated compared with resting cells after stimulation with CD3/CD28/MHC-I. After the addition of PGE₂, only 191 genes were

detected to be up-regulated. Using Venn diagrams to visualize intersections, 177 of the 191 genes up-regulated after PGE₂ treatment were also regulated after CD3/CD28/MHC-I stimulation, and only 14 genes were up-regulated in a CD3/CD28/MHC-I-independent fashion. Interestingly, PGE₂ signaling targeted the genes that were down-regulated by CD3/CD28 less than those up-regulated. Five hundred and seventy-one genes were significantly down-regulated after stimulation with CD3/CD28/MHC-I beads, whereas 238 were down-regulated after additional PGE₂ treatment. Two hundred and nineteen of the genes down-regulated after the addition of PGE₂ were also found to be down-regulated after CD3/CD28/MHC-I stimulation, and 19 genes were down-regulated independently from CD3/CD28 (Fig. 2A). Using GeneMapp and BioRag software as tools to allocate regulated genes to different cellular pathways, we found a distinct up-regulation of genes belonging to metabolic and cellular signaling pathways after stimulating the cells with CD3/CD28/MHC-I beads, as expected. Additional treatment with PGE₂ significantly reduced the number of genes associated with all pathways connected to CD3/CD28/MHC-I stimulation (Fig. 2B). This general leveling of CD3/CD28-regulated genes suggests an interference of PGE₂-mediated signals during an early step of CD3/CD28-mediated signal transduction.

Genes belonging to antiapoptotic pathways escape PGE₂-mediated inhibition of TCR signaling. After stimulation of T cells with CD3/CD28/MHC-I, different genes belonging to apoptotic pathways were transcriptionally regulated in comparison with unstimulated cells. These include genes encoding for TNF- α , TNF- β , Fas-L, and CD40-L with rather proapoptotic features but also for Bcl₂, Bcl-x_L, and Bcl₂A1 with antiapoptotic attributes (Fig. 3, *top*). By analyzing unstimulated versus CD3/CD28/MHC-I-stimulated cells in the presence of PGE₂ we found that PGE₂ interfered with the majority of genes transcriptionally regulated after CD3/CD28/MHC-I stimulation, however, the antiapoptotic genes Bcl₂, Bcl-x_L, and Bcl₂A1 escaped this PGE₂-mediated inhibition. Bcl₂ and Bcl₂A1 showed the same or even stronger up-regulation compared with stimulation with CD3/CD28/MHC-I alone, Bcl-x_L was found being strongly (14.91-fold) up-regulated after CD3/CD28/MHC-I and still 10.6-fold after additional treatment with PGE₂. However, the up-regulation of TNF- α , TNF- β , and Fas-L was inhibited by PGE₂ (Fig. 3, *bottom*). This data supports previous reports that PGE₂ treatment leads to a blockade of Fas-L induction in T cells (32). The escape of antiapoptotic genes from PGE₂-mediated inhibition may suggest that these genes are differently regulated by CD3/CD28 or might be less affected by PGE₂-mediated signals.

Genes regulating cell cycle progression are significantly regulated by PGE₂. Regulation of cell cycle and cell proliferation constitute other pathways of genes significantly regulated after additional PGE₂ treatment. In a direct comparison of regulated genes after stimulation with CD3/CD28/MHC-I in the absence or presence of PGE₂, we found genes acting as negative regulators of cell proliferation (e.g., FTH1; fold change, 2.67) or cell cycle (e.g., RGS2; fold change, 2.78) up-regulated after PGE₂ treatment. Recently it was shown that FTH1 secreted from melanoma cells may suppress immune responses by preferential activation of regulatory CD4⁺ CD25⁺ T cells producing IL-10 and leading to changes in antigen-presenting cells (33, 34). RGS2 affects cell cycle progression by interaction with Ga_q and stimulates its GTPase activity (35). The cell cycle inhibitor GADD45 α (36, 37) was also up-regulated (2.06-fold) after PGE₂, but this did not reach statistical significance between the different samples.

Taken together, the genome-wide analysis of transcriptional changes induced by PGE₂ in human CD4⁺ T cells revealed at least three major aspects: (a) PGE₂ interfered with the majority of genes regulated after TCR/CD28 stimulation rather than having a unique transcriptional profile suggesting an impairment of TCR/CD28 signaling after PGE₂, (b) the escape of antiapoptotic genes from PGE₂-mediated inhibition suggests that PGE₂ might protect T cells from apoptosis, and (c) genes belonging to pathways negatively regulating cell cycle progression and cell proliferation were significantly regulated by PGE₂, suggesting that the cells are alive but not capable of proliferating. Next, we were interested to investigate the functional consequences of the transcriptional changes induced by PGE₂.

PGE₂ induces partial protection from apoptosis in human CD4⁺ T cells. First, we aimed to evaluate the transcriptional up-regulation of Bcl-x_L observed in the microarray experiments on protein level using Western blot. As shown in Fig. 4A, stimulation with CD3/CD28/MHC-I induced a strong up-regulation of Bcl-x_L, which was sustained after additional treatment with PGE₂.

To study the functional aspects of sustained expression of antiapoptotic proteins, CD4⁺ T cells were left unstimulated or stimulated with CD3/CD28/MHC-I with or without PGE₂. After 3 days of incubation, samples were irradiated with 26 Gy and incubated for another 24 hours before staining the cells with Annexin V and 7-AAD to determine the percentage of apoptotic and necrotic cells. As shown in Fig. 4B (left), the addition of PGE₂ during stimulation with CD3/CD28/MHC-I reduced the percentage of apoptotic cells after irradiation nearly to levels observed in resting cells. In a different set of experiments, cells were stimulated as above and incubated with Fas-L or control IgM for an additional 24 hours. As shown in Fig. 4B (right), PGE₂ treatment reduced the sensitivity of the cells for Fas-L-induced apoptosis. Taken together,

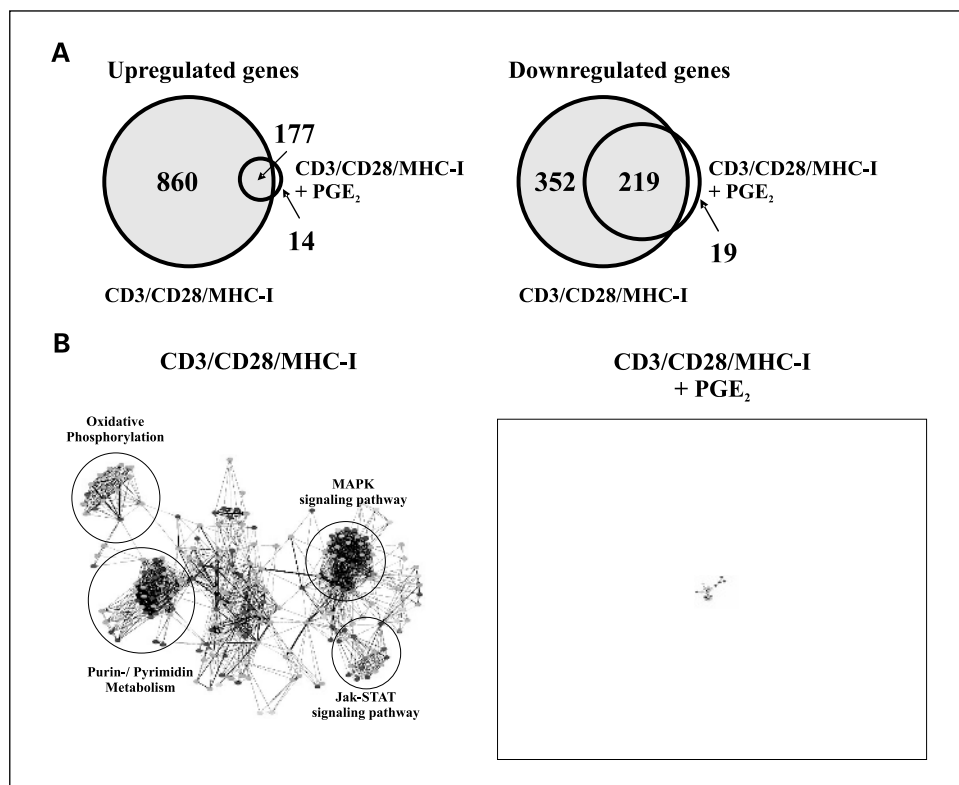
these functional results support the data obtained in microarray experiments.

PGE₂ inhibits cell cycle entry of CD4⁺ T cells associated with elevated levels of p27^{kip1}. Two hallmarks of T cell unresponsiveness are partial protection from apoptosis and significantly reduced transcriptional changes following encounters with antigens (38) similar to what we observed in T cells stimulated by TCR/CD28 in the presence of PGE₂. CD4⁺ T cell unresponsiveness has been described to be associated with elevated levels of the cell cycle inhibitor p27^{kip1}. Intracellular levels of p27^{kip1} are regulated by transcription, posttranslational modification, and ubiquitin-dependent degradation. We detected a slight but not significant transcriptional up-regulation of p27^{kip1} (1.67-fold) in transcriptome experiments when comparing T cell stimulation in the absence or presence of PGE₂. This observation was confirmed on the protein level. Figure 4C shows that p27^{kip1} is down-regulated in CD4⁺ T cells after stimulation with CD3/CD28/MHC-I. The addition of PGE₂ restored the amount of p27^{kip1} to levels observed in resting cells.

We next assessed whether these changes in expression of p27^{kip1} are associated with differential cell cycle regulation (Fig. 4D). After 3 days of stimulation, cells were fixed with paraformaldehyde and stained with propidium iodide to analyze the DNA content of individual cells. After stimulation with CD3/CD28/MHC-I, 42.4% of the cells were in G₁, 42.6% in S phase, and 14.5% were in G₂ phase of the cell cycle. However, after additional treatment with PGE₂, 80.8% of the total cell population remained in G₁, whereas only 14.7% entered S phase, respectively, 5.5% were in the G₂ phase of the cell cycle. Thus, after PGE₂ treatment, the majority of cells remain in G₁ phase and do not proceed through the cell cycle.

PGE₂ interferes with early TCR signaling events by phosphorylation of Ick⁵⁰⁵. We next wanted to evaluate how PGE₂ leads to the general leveling of gene transcription induced by

Figure 2. Transcriptional profile of primary human CD4⁺ T cells after PGE₂ treatment. CD4⁺ T cells were stimulated as indicated. After 8 hours, beads were removed and cells were lysed. RNA was hybridized overnight to Sentrix whole genome bead chips and scanned with Illumina BeadStation. Data was analyzed using dCHIP 1.3, GeneSpring and BioRag software. A, number of genes up-regulated or down-regulated after stimulation with CD3/CD28/MHC-I in the absence or presence of PGE₂ compared with resting cells. B, significantly regulated genes allocated to cellular metabolic and regulatory pathways (pathway source KEGG; BioRag) after stimulation with CD3/CD28/MHC-I with and without additional PGE₂ compared with resting cells. Data represents the mean of four independent donors.



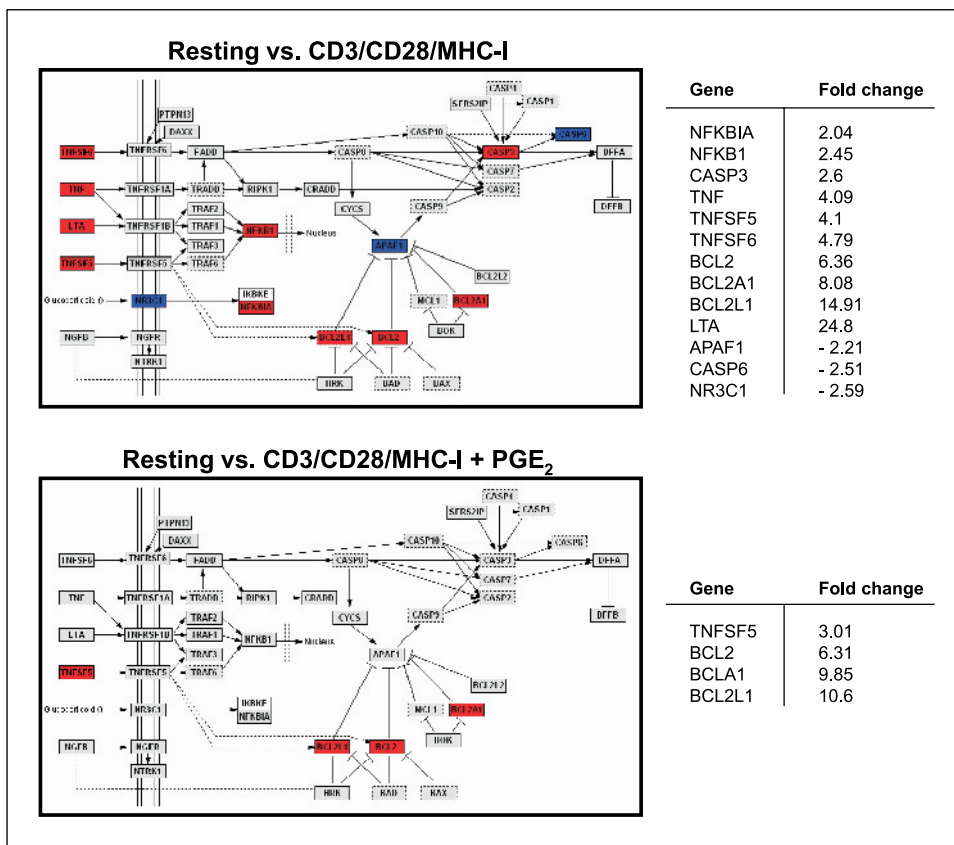


Figure 3. Regulated genes in apoptotic pathways after PGE₂ treatment. GeneMapp software was used to allocate significantly regulated genes to distinct pathways. The displayed maps were modified from GeneMapp Hs_Apoptosis_KEGG (gene database Hs-Std_20041021.gdb). *Top*, highlighted are the significantly up-regulated (red) and the significantly down-regulated (blue) genes after stimulation with CD3/CD28/MHC-I compared with unstimulated cells. *Bottom*, significantly regulated genes after stimulation with CD3/CD28/MHC-I + PGE₂ in comparison with resting cells. The tables show fold changes of regulated genes.

TCR/CD28 stimulation (see Fig. 2). Increasing evidence relates the inhibitory effects of PGE₂ to an elevation of intracytoplasmatic cAMP and further to activation of the cAMP-dependent target PKA. This hypothesis is due to (indirect) experiments with specific agonists and antagonists of the adenylate cyclase or PKA (20, 29). Indeed, we were able to corroborate these findings when using aAPC and primary human CD4⁺ T cells as well as the respective cAMP and PKA agonists (data not shown). However, a direct approach to confirm the hypothesis that PGE₂ is acting on CD4⁺ T cells via cAMP and PKA would be to analyze directly the effects of PGE₂ on possible downstream targets of PKA. As mentioned above, PKA activation ultimately leads to phosphorylation and thereby inactivation of Ick. p-Ick⁵⁰⁵ could therefore be used to monitor PKA-dependent signaling.

As shown in Fig. 5A (left), Ick is phosphorylated at position 505 in resting CD4⁺ T cells. Activation of the cells for 5 minutes with CD3/CD28/MHC-I reduced the amounts of p-Ick⁵⁰⁵, however, additional treatment with PGE₂ restored the levels of p-Ick⁵⁰⁵ to levels observed in resting cells. To confirm that elevated levels of p-Ick⁵⁰⁵ in fact lead to impaired TCR signaling, we studied downstream TCR signals. As shown in Fig. 5A (right), stimulation with CD3/CD28/MHC-I for 5 minutes resulted in increased phosphorylation of ZAP70, which was reduced after additional treatment with PGE₂.

If interference with early events in TCR signaling via phosphorylation of Ick⁵⁰⁵ is the mechanism by which PGE₂ acts to inhibit CD4⁺ T cell activation, bypassing the TCR by using the mitogens, PMA and ionomycin, should not result in the inhibition of T cell activation by PGE₂. We therefore stimulated freshly isolated CD4⁺ T cells with CD3/CD28/MHC-I as a control or PMA/ionomycin with and without increasing concentrations of PGE₂. As shown

in Fig. 5B, T cell proliferation was inhibited by PGE₂ in a concentration-dependent manner after stimulation with CD3/CD28/MHC-I. However, stimulation with PMA/ionomycin rendered the cells unresponsive for PGE₂-mediated inhibition. Thus, PGE₂-mediated proliferation arrest is dependent on stimulation of T cells via the TCR. This data further suggests that the mechanism of PGE₂-mediated T cell inhibition acts via impairment of TCR signaling.

CD4⁺ T cells in Hodgkin's lymphoma show transcriptional changes associated with PGE₂ signaling. As the potentially best tumor model studying chronic inflammatory responses in close proximity to tumor cells, we assessed T cells derived from lymph node biopsies of patients with Hodgkin's lymphoma. Elevated levels of PGE₂ have been found in patients with Hodgkin's lymphoma (9). Monocytes, which have been described as being the source of higher PGE₂ concentrations, and impaired cellular immune functions described in Hodgkin's lymphoma have been attributed in part to PGE₂ (8). Therefore, we were interested in comparing the transcriptional profile of PGE₂-treated CD4⁺ T cells from healthy donors with the transcriptional profile of CD4⁺ T cells from patients with Hodgkin's lymphoma. By analyzing CD4⁺ T cells from healthy donors and comparing unstimulated cells versus cells stimulated with CD3/CD28/MHC-I in the absence or presence of PGE₂, we found a number of genes independently regulated after treatment with PGE₂ (Fig. 2A; Supplemental Table S1). We used these genes as a filter prior to comparing the transcriptional profiles of CD4⁺ T cells isolated from lymph nodes of either five patients with Hodgkin's lymphoma or five patients with reactive lymph nodes. On the basis of this list of genes, CD4⁺ T cells from patients with Hodgkin's lymphoma present with a distinct transcriptional profile and cluster separately from CD4⁺ T

cells isolated from reactive lymph nodes, as shown in Fig. 6A. The transcriptional changes between the two groups mirror significantly transcriptional changes after PGE₂ treatment of healthy donor cells because many genes independently up-regulated after PGE₂ treatment *in vitro* were also found to be up-regulated in patients with Hodgkin's lymphoma and vice versa (Supplemental Table S2).

An alternative possibility to analyze the transcriptional changes after treatment with PGE₂ is to directly compare CD3/CD28/

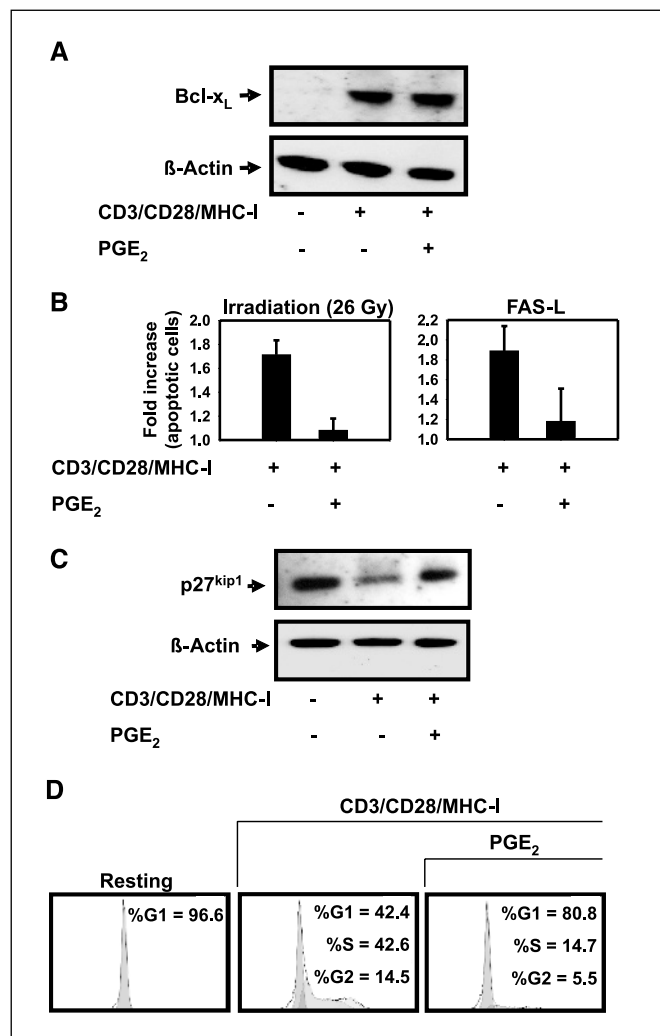


Figure 4. PGE₂ leads to partial apoptosis resistance and blocks cell cycle progression. **A**, CD4⁺ cells were stimulated as indicated. After 24 hours, cells were lysed, proteins were separated by SDS-PAGE and transferred to nitrocellulose. Bcl-x_L was detected by Western blotting with a Bcl-x_L-specific antibody (top). The membrane was then stripped and reprobbed with anti-actin to show equal loading of protein (bottom). **B**, CD4⁺ T cells were stimulated with the indicated beads. After 3 days, samples were irradiated (26 Gy) and incubated for an additional 24 hours. Cells were subsequently stained with Annexin-V and 7-AAD to detect the percentage of apoptotic and necrotic cells; shown is the fold increase of apoptotic and necrotic cells over resting cells (left). Prestimulated cells were incubated with Fas-L or control IgM for an additional 24 hours (right). Fold increase of apoptotic and necrotic cells over baseline (incubation with control IgM). **C**, CD4⁺ T cells were stimulated as indicated. After 24 hours, cells were lysed and proteins were separated with SDS-PAGE. p27^{kip1} expression was detected by Western blotting with a p27^{kip1}-specific antibody (top). Subsequently, the membrane was stripped and blotted with anti-actin to show equal loading of protein (bottom). **D**, CD4⁺ T cells were left unstimulated or stimulated with CD3/CD28/MHC-I with or without additional PGE₂. After 3 days, cells were stained with propidium iodide and cell cycle progression was analyzed by flow cytometry. Representative of three different experiments.

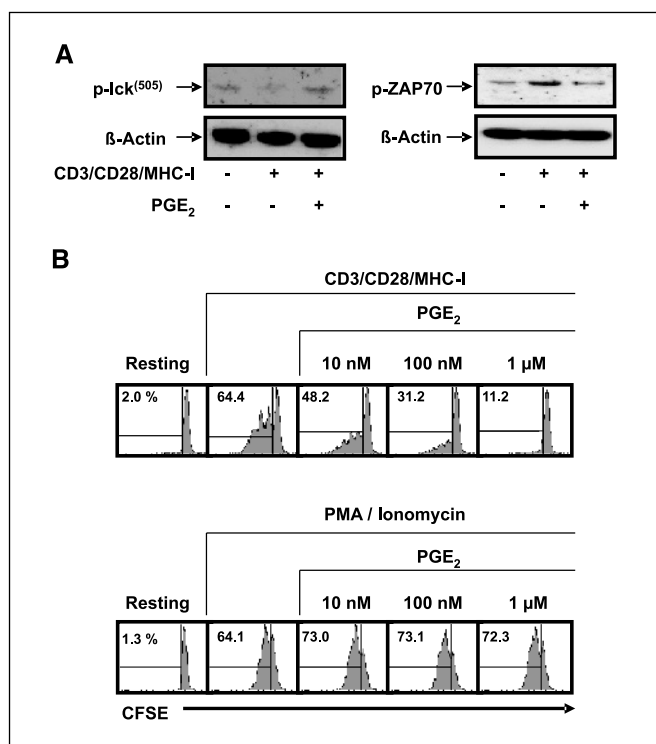


Figure 5. PGE₂ treatment of CD4⁺ T cells inhibits TCR-mediated signaling by phosphorylation of Ick⁵⁰⁵. **A**, CD4⁺ T cells were stimulated with CD3/CD28/MHC-I beads with and without additional PGE₂. After 5 minutes, beads were removed and cells were lysed. Proteins were separated with SDS-PAGE and transferred to nitrocellulose. p-Ick⁵⁰⁵ (left) and p-ZAP70 (right) were identified by Western blotting with p-Ick⁵⁰⁵- and p-ZAP70-specific antibodies. To show equal loading of protein, membranes were stripped and reprobbed with anti-actin (bottom). **B**, CD4⁺ T cells were labeled with CFSE and stimulated with CD3/CD28/MHC-I or PMA/ionomycin in the absence or presence of the indicated concentrations of PGE₂. After 6 days of incubation, T cell proliferation was analyzed by flow cytometry. Representative of three different experiments.

MHC-I-stimulated CD4⁺ T cells in the presence or absence of PGE₂. This analysis results in a distinct list of genes regulated by PGE₂. When using these genes as a filter, transcriptional profiles of CD4⁺ T cells from patients with Hodgkin's lymphoma also cluster separately from CD4⁺ T cells derived from reactive lymph nodes (Fig. 6B). By the use of other filter criteria (e.g., a list of cluster of CD markers or nuclear factor κB target genes) samples from patients with Hodgkin's lymphoma were not separated from patients with reactive lymph nodes (data not shown). To exclude the possibility that different ratios of T cell subpopulations from the different samples were responsible for the observed differences, we isolated CD4⁺CD25⁺ regulatory and CD4⁺CD25⁻ conventional T cells from healthy donors and obtained transcriptional profiles. We used regulatory T cells as an example because they are particularly enriched in Hodgkin's lymphoma (39). We then extracted the data for genes specifically regulated by PGE₂ (Supplemental Table S1) and used these genes to cluster regulatory and conventional T cells. Using several different clustering approaches, these genes never did segregate the two different T cell subpopulations (data not shown). Taken together, this data strongly suggests that CD4⁺ T cells in Hodgkin's lymphoma lymph nodes are under the influence of PGE₂ leading to the transcriptional changes observed in human CD4⁺ T cells in the presence of PGE₂ *in vitro*.

Discussion

PGE₂ has been implicated as a potential inhibitor of T cell function in the context of malignant disease (40, 41). Albeit the outcome of PGE₂ signaling is well established, the molecular mechanisms involved are still not completely understood. The present study was designed to determine the potential mechanisms of PGE₂ leading to inhibition of CD4⁺ T cell activation. We exclusively used primary human CD4⁺ T cells to achieve more physiologic conditions compared with cell line models defective in key enzymes of T cell signaling (42, 43). Analysis of the transcriptional profile after PGE₂ treatment revealed that PGE₂ leads to a general interference with the great majority of genes regulated by signals 1 and 2 rather than to a generation of a unique PGE₂-associated transcriptional profile. This interference affected both genes up-regulated and down-regulated after CD3/CD28 stimulation suggesting an inhibition of early TCR-mediated signaling events. We showed that PGE₂ leads to phosphorylation of Ick⁵⁰⁵, thereby inactivating the most proximal event of TCR signaling. Moreover, we were able to show that PGE₂-mediated inhibition of T cell activation is in fact dependent on activation of the cells via the T cell receptor because activation of T cells bypassing the TCR renders the cells unresponsive to PGE₂-mediated inhibition. Alternatively, when providing unphysiologically high TCR/CD28 signals, the effect of PGE₂ could also be overcome.

Integrating previous data, we would suggest the following model of PGE₂-mediated T cell inhibition. Increasing evidence correlates PGE₂-mediated events to elevated levels of intracytoplasmic cAMP and subsequent activation of PKA (20, 29). This data results from experiments using specific agonists and antagonists of cAMP and

the cAMP-dependent substrates, PKA and EPAC. The fact that specific agonists of cAMP and PKA, but not EPAC, mirrored PGE₂-mediated inhibition of T cell activation as well as that this inhibition was abrogated under the influence of specific antagonists of the adenylate cyclase or PKA provided indirect evidence of the cAMP/PKA pathway linked to the PGE₂ receptors EP₂ and EP₄ to be responsible for PGE₂-mediated signaling. Increase of cAMP and stimulation of PKA by respective chemical agonists can regulate the expression of p27^{kip1} and cyclin D₃ to suppress proliferation of leukemic T cell lines and human peripheral blood lymphocytes (25, 26). Additionally, PKA type I, in response to cAMP, activates Csk in Jurkat cells and peripheral T cells (44). Activated Csk was shown to phosphorylate the COOH-terminal inhibitory tyrosine residue in Ick (22–24), thereby acting as a negative regulator of TCR signaling. Altogether, these data suggested that PGE₂ might be able to inhibit T cell activation via up-regulation of cAMP, activation of PKA, and subsequent activation of Csk inducing phosphorylation of Ick⁵⁰⁵, thereby inhibiting TCR-mediated T cell activation. Here, we directly show that PGE₂-mediated T cell inhibition is indeed mediated by inactivation of Ick. This is associated with reduced ZAP70 phosphorylation and followed by a general leveling of the TCR-mediated transcriptional response.

Interestingly, transcripts for the antiapoptotic proteins Bcl₂, Bcl-x₁, and Bcl2A1 were not affected or were significantly less affected by PGE₂ treatment, and PGE₂-treated cells were in part protected from apoptosis induced by irradiation or Fas-L. Our observations are in line with a previous report by Seddon et al. (45). In this report, the peripheral T cell pool of p56^{lck} (Ick)-deficient mice was reconstituted by the expression of an inducible Ick transgene. After switching off the transgene, a continued survival

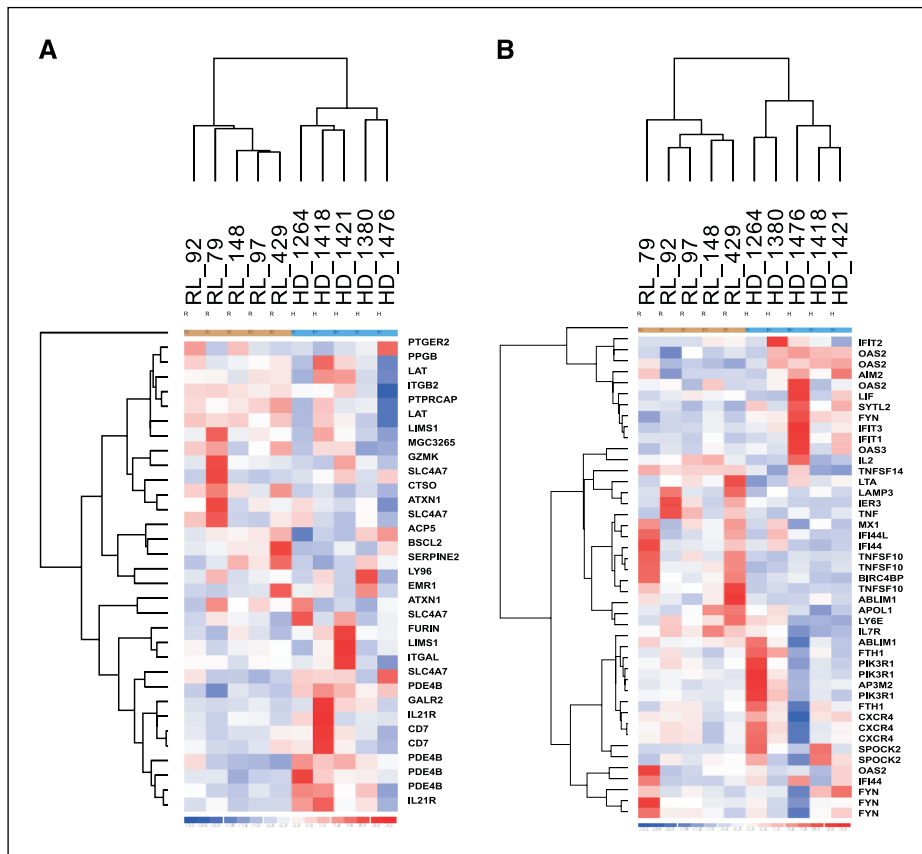


Figure 6. CD4⁺ transcriptional profiles from patients with Hodgkin's lymphoma segregate from reactive lymph nodes on the basis of PGE₂-regulated genes. CD4⁺ T cells were isolated from the lymph nodes of five different patients with Hodgkin's lymphoma or reactive lymph nodes and cRNA was hybridized to HG-133A Affymetrix arrays. A, when comparing the transcription profiles of unstimulated versus stimulated cells in the absence or presence of PGE₂, a number of genes were found to be regulated independently from CD3/CD28/MHC-I. These genes were allocated to Affymetrix probesets. The corresponding probesets were used to cluster the different transcription profiles of CD4⁺ T cells from Hodgkin's lymphoma and reactive lymph nodes. B, genes differentially regulated in a direct comparison of CD3/CD28/MHC-I stimulated T cells in the absence or presence of PGE₂ were used as a filter prior to clustering the different samples.

of peripheral naïve T cells was observed, confirming that T cell survival was independent of lck. However, the essential role of lck for homeostatic T cell proliferation became obvious because lck-deficient T cells did not proliferate in a T cell-deficient host environment in the absence of lck. These data further suggest that survival and TCR-driven homeostatic expansion possibly depend on different signals.

Cyclooxygenase-2 (COX-2) overexpression and PGE₂ secretion is described to be associated with reduced T cell and dendritic cell functions in different solid tumors, especially lung (40), breast (41), and colon carcinoma (46). Selective COX-2 inhibitors are tested in clinical trials for prevention and tumor therapy (47–49) and preclinical data suggests that COX-2 inhibitors might act additively or synergistically with the specific chemotherapeutic agents used in the treatment of these tumors.

It is known that Hodgkin's lymphoma is associated with decreased cellular immunity (50, 51) and that lymphocyte function influences the prognosis (52). Furthermore, it is known that monocytes produce increased amounts of PGE₂ in these patients (53, 54). We show that the transcriptional profile of CD4⁺ T cells in patients with Hodgkin's lymphoma has significant similarities to CD4⁺ T cells of healthy donors treated with PGE₂. Moreover, on the basis of genes independently regulated after PGE₂ treatment of CD4⁺ T cells from healthy donors, T cells originating from the lymph nodes of patients with Hodgkin's lymphoma could be separated from reactive lymph nodes by cluster analysis. This data further suggests the influence of PGE₂ on CD4⁺ T cells in Hodgkin's

lymphoma and supports the hypothesis that PGE₂ might contribute to impaired T cell function in these patients.

In conclusion, our data provide direct evidence that PGE₂ affects TCR/CD28-driven signals by inactivation of lck resulting in impaired activation of downstream signals as shown by decreased phosphorylation of ZAP70. The functional consequence is a state of CD4⁺ T cell unresponsiveness despite sufficient CD28 costimulation. Based on our observations, we postulate PGE₂ as an important factor responsible for impaired CD4⁺ T cell function in Hodgkin's lymphoma, and potentially also in other tumors associated with increased levels of PGE₂. Current and future work will elucidate whether T cells in other PGE₂-associated tumors also show hallmarks of impaired T cell function. Moreover, the identification of these central mechanisms leading to T cell inhibition might open new avenues towards active cancer immunotherapy by reversing PGE₂-mediated signaling events.

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