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Prostaglandin E₂ Affects T Cell Responses through Modulation of CD46 Expression

Karoline Kickler,* Kathryn Maltby,* Siobhán Ni Choileain,*† Jillian Stephen,* Sheila Wright,* David A. Hafler,*§,|| Henry N. Jabbour,* and Anne L. Astier*†

The ubiquitous protein CD46, a regulator of complement activity, promotes T cell activation and differentiation toward a regulatory Tr1-like phenotype. The CD46-mediated differentiation pathway is defective in several chronic inflammatory diseases, underlying the importance of CD46 in controlling T cell function and the need to understand its regulatory mechanisms. Using an RNA interference-based screening approach in primary T cells, we have identified that two members of the G protein-coupled receptor kinases were involved in regulating CD46 expression at the surface of activated cells. We have investigated the role of PGE₂, which binds to the E-prostanoid family of G protein-coupled receptors through four subtypes of receptors called EP 1–4, in the regulation of CD46 expression and function. Conflicting roles of PGE₂ in T cell functions have been reported, and the reasons for these apparent discrepancies are not well understood. We show that addition of PGE₂ strongly downregulates CD46 expression in activated T cells. Moreover, PGE₂ differentially affects T cell activation, cytokine production, and phenotype depending on the activation signals received by the T cells. This was correlated with a distinct pattern of the PGE₂ receptors expressed, with EP4 being preferentially induced by CD46 activation. Indeed, addition of an EP4 antagonist could reverse the effects observed on cytokine production after CD46 costimulation. These data demonstrate a novel role of the PGE₂–EP4 axis in CD46 functions, which might at least partly explain the diverse roles of PGE₂ in T cell functions. The Journal of Immunology, 2012, 188: 5303–5310.

CD46 is a ubiquitously expressed type I membrane protein that was first identified as a regulator of the complement cascade, preventing autolysis of cells by binding to C3b/ C4b and allowing their cleavage by protease I (1, 2). About 10 years ago, CD46 was shown to link innate immunity to acquired immunity. Indeed, costimulation of the TCR with CD46 leads to increased T cell proliferation (3) and affects T cell morphology (4) and polarity (5). Importantly, CD46 also drives Tr1 differentiation, characterized by secretion of high amounts of IL-10 (6) and granzyme B (7). IL-2 is key in CD46-mediated Tr1 differentiation, acting as a sensor to switch T cells from a Th1 to a Tr1 phenotype (8). The enzymatic processing of CD46 is a crucial feature of the CD46-mediated pathway that is involved in regulating T cell function. CD46 surface expression is strongly downregulated upon its own triggering, partly due to MMP cleavage of its ectodomain (9–11). This is followed by γ-secretase cleavage of the two cytoplasmic tails of CD46, which is important to initiate and terminate T cell responses (11, 12). This again underlines the importance of the plasticity of CD46 in controlling T cell homeostasis. Moreover, CD46-mediated Tr1 differentiation is altered in patients with multiple sclerosis (MS), characterized by an impaired IL-10 secretion upon CD3/CD46 costimulation (13–16), and the dysregulation of CD46 pathways in T cells was recently described in patients with asthma (17) and in a small group of patients with rheumatoid arthritis (RA) (8). The identification of a dysfunctional CD46 pathway in chronic inflammatory diseases highlights its importance in controlling T cell homeostasis and further underlines the need to understand its regulation and the molecular mechanisms responsible for its functions.

Using an RNA interference (RNAi)-based approach (18) to dissect the molecular pathways that regulate CD46 expression on primary human T cells, we identified two members of the serine/threonine kinase G protein-coupled receptor kinase (GRK) family involved in the regulation of CD46 expression. GRKs phosphorylate agonist-activated G protein-coupled receptors (GPCRs) (19, 20), resulting in their binding to β-arrestins and subsequent signaling impairment and internalization, a process known as desensitization (21, 22). There are seven types of GRK referred to as GRK 1–7, each with different expression profiles (21). Among them, GRK 2, 3, 5, and 6 are ubiquitously expressed, but are expressed at particularly high levels in immune cells, and have been shown to regulate inflammation (23).

In this study, we show that the knockdown of GRK2 and GRK3 strongly decreased CD46 expression and that activation of CD46 increased GRK2/3 expression levels. GRK2/3 have been shown to regulate PGE₂ receptors, among other GPCRs (24). As PGE₂ is a known modulator of T cell functions (25), we assessed the role of PGE₂ in the regulation of CD46 expression and function to demonstrate a role of GRKs in the CD46 pathway. PGE₂ notably inhibits T cell proliferation by downregulating both IL-2 and the IL-2Rα-
PGE₂ REGULATES CD46 EXPRESSION AND FUNCTION

The addition of PGE₂ to T cell cultures strongly decreased CD46 expression in activated T cells. In this study, we first demonstrate that PGE₂ differentially affected T cell responses depending on the activation status. Moreover, specific changes in the phenotype of activated T cells were observed. PGE₂ binds to the E-prostanoid family of GPCRs, through four subtypes of receptors called EP 1–4 (24). We show that CD46 and CD28 costimulation of PGE₂ have been reported, and although the local concentrations of PGE₂ are important to control T cell differentiation (28), the reasons for these apparent discrepancies are not well understood. Moreover, there are no studies on any potential effects of PGE₂ on human T cell activation.

Materials and Methods

Abs and reagents used

The Abs used for activation were anti-CD3 (OKT3; 5 μg/ml), anti-CD28 (CD28.2; 5 μg/ml), and anti-CD46 (20.6; 10 μg/ml), kindly provided by Dr. Chantal Rabourdin-Combe (INSERM, Lyon, France). PGE₂ (100 nM, 227; 300 nM) was chemically synthesized by Charnwood Molecular (Leicester, U.K.). The Abs for flow cytometry were purchased from Calbiochem (Nottingham, U.K.). The EP2 antagonist (AH6809; 10⁻²⁻⁻ g/ml) was chemically synthesized by Charnwood Molecular (Pharmacia LKB Biotechnology, Piscataway, NJ) from heparinized venous blood from healthy donors obtained after informed consent. Ethical approval was obtained from the Lothian Board Ethics Committee. CD4⁺ T cells were negatively selected using magnetic beads (purification >90%; CD4 isolation Kit II; Miltenyi Biotec, Auburn, CA). T cells (1 × 10⁵ cells/ml well) were then cultured in 24-well plates precoated with anti-CD3, anti-CD28, or anti-CD46, in RPMI 1640 containing 10% FCS. Exogenous IL-2 (10 μU/ml) was added to CD3/CD46 stimulated cells as previously described (6). In some experiments, PGE₂, selective EP2 or EP4 antagonists, and DMSO or ethanol as vehicle control (similar dilution) were added to the culture.

The RNAi Consortium RNAi library

The complete description of the RNAi Consortium lentiviral RNAi library used in this study has been reported (38) and already used to infect primary T cells (18), and it is now commercialized by Sigma-Aldrich. Human genes are targeted with ~5 short hairpin RNAs (shRNAs) expressed under the control of the U6 Pol III promoter in a lentiviral vector (pLKO.1) that also confers puromycin resistance. Plasmid DNA purification and lentiviral production were performed as described [(38) and http://www.broad.mit.edu/ori/rnc/lir].

T cell stimulation and infection

CD4⁺ T cells (2 × 10⁵ cells/well) were cultured in 96-well plates precoated with anti-CD3 (OKT3; 2.5 μg/ml) and anti-CD28 (2D10; 2.5 μg/ml) in presence of human recombinant IL-2 (Tecin, 20 U/ml; National Cancer Institute, Frederick, MD) for 24 h before infection. The infection was carried out by centrifugation for 90 min at 2300 rpm at room temperature in presence of the viral supernatant and polybrene (8 μg/ml). After removal of the virus, fresh medium was added. Infected cells were then selected by addition of puromycin (2.5 μg/ml) 48 h postinfection, and the cells were expanded in IL-2 for 10 d. Cells were then restimulated with anti-CD3/CD28 Abs in the presence of IL-2. At 24 h post-restimulation, the level of CD46 expression at the cell surface was determined by flow cytometry.

GRK2/3 detection by Western blot

Purified CD4⁺ T cells were activated in 48-well plates precoated with anti-CD3, anti-CD28, or anti-CD28 Abs and cultured for 3 d. Cells were then lysed with lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA, 1 mM PMSF, 1 mM iodoacetamide, 10 mM NaF, 0.4 mM Na₂VO₃). Cell lysates were run on an 8% SDS-PAGE and amounts of GRK2 and GRK3 detected by Western blot using anti-GRK2 Abs that cross-react with GRK3 (Cell Signaling). The membranes were then stripped and reblotted with anti-GAPDH mAb to evaluate protein quantitites. Densitometry analyses (with ImageJ) were then performed to calculate the GRK/GAPDH ratio.

Flow cytometry

The expression level of CD46, CD25, and CD69 was assessed by flow cytometry with anti-CD46–FITC, anti-CD69–PE, and anti-CD25–allophycocyanin. The presence of CTLA-4 (PE) and Foxp3 (allophycocyanin) was determined after permeabilization of the cells with 0.1% saponin. The relative expression to the staining with the isotype control was calculated by calculating the Δmean fluorescence intensity (MFI) (MFI Ab stained − MFI control Ab). Samples were run on a BD FACS Calibur. Phosphorylation of STAT3 was analyzed with an anti-p-Tyr STAT3–Pacific blue and run on a BD FACS Calibur LSR Fortessa. All flow cytometry data were analyzed using FlowJo (Tree Star).

T cell proliferation

Purified T cells (2 × 10⁵ cells/200 μl/well) were cultured in 96-well plates, precoated with anti-CD3, anti-CD28, or anti-CD46, in 10% FCS–RPMI 1640 for 72 h before addition of 1 μg/ml IL-2 (Amersham). Proliferation was determined using a Liquid Scintillation Counter (Wallac).

ELISA

Cytokine production was determined in cell culture supernatants using ELISA specific for human IL-10 (BD Pharmingen, San Jose, CA) and IFN-γ (Endogen, Cambridge, MA). Recombinant hIL-10 (BD Pharmingen) and hIFN-γ (Endogen) were used as standards.

EP 1–4 PCR

These PCRs were performed as previously described (39). Briefly, RNAs from activated T cells were extracted using the RNeasy Mini Kit (Qiagen). RNA samples were reverse-transcribed using Vilo (Invitrogen, Paisley, U.K.) according to the manufacturer’s guidelines. RT-PCR analysis of EP1, EP2, EP3, and EP4 was carried out using an ABI Prism 7500 (Applied Biosystems, Warrington, U.K.). Results are expressed relative to a standard (pooled normal human endometrial tissue cDNA) included in all reactions. Data are represented as mean ± SEM.

Statistical analyses

The groups were analyzed using the GraphPad Prism software. Data were analyzed using the Wilcoxon test. All p values are two-tailed and with a 95% confidence interval.

Results

An RNAi screen identifies GRKs as novel regulators of CD46 expression in primary human T cells

To gain new insights into the regulation of CD46 expression in primary human T cells, we carried out an RNAi screen using a subgenomic library targeting ~1000 genes, focused on kinases and phosphatases, and containing three to five shRNAs per gene, as previously described (18). Our goal was to identify novel
GRKs regulate CD46 expression in primary human T cells. The level of CD46 expression at the cell surface of T cells infected with lentivirus particles expressing a distinct and unique shRNA was determined by flow cytometry. Using a statistical Z-score (see Ref. 18) to quantify the deviation of CD46 levels from the mean of all measurements within the same plate, we selected shRNAs that significantly modulated CD46 expression compared with the cohort of control wells consisting of cells infected with control shRNAs (against nonmammalian reporter genes) (Fig. 1A). GRK2 and GRK3, two members of the GRK family, decreased CD46 expression once knocked down (Fig. 1A, circled). Two shRNA constructs out of four for GRK2 and two out of three for GRK3 led to a similar phenotype (Fig. 1A). These data were reproduced in two independent experiments performed with different donors (Fig. 1B). Representative plots showing normal CD46 expression compared with expression upon GRK2 and GRK3 knockdowns are shown in Fig. 1C. Hence, downregulation of GRKs decreased CD46 expression in activated primary human T cells.

**CD46 activation led to enhanced GRK2/3 levels**

T cell activation by PHA and anti-CD3 Abs increases GRK expression and activity (40, 41). Hence, we next assessed whether CD46 costimulation could modulate GRK2/3 levels. CD4+ T cells were activated with immobilized anti-CD3, anti-CD3/CD28, or anti-CD3/CD46 Abs for 3 d. GRK2/3 levels were then assessed by Western blots using anti-GRK2/3 Abs. T cell activation by CD28 and especially CD46 led to enhanced levels of GRKs (Fig. 1D). Hence, these data demonstrate a relationship between GRKs and the CD46 pathway.

**PGE2 decreases CD46 surface expression in activated T cells**

PGE2 is a known T cell modulator that signals through binding to the E-prostanoid family of GPCRs (namely EP 1–4). GRK2 regulates expression of the EP4 receptor (24), and we found that EP4 knockdown led to increased CD46 expression (Supplemental Fig. 1), suggesting a role of PGE2 in controlling CD46 expression. Hence, to assess whether signaling through GPCR/GRK could modulate CD46 expression, we next investigated the effect of PGE2 on CD46 expression and function. CD4+ T cells were activated with anti-CD3, anti-CD3/CD46, or anti-CD3/CD28 Abs in the presence of PGE2 or DMSO as a vehicle control, and expression of CD46 was monitored by flow cytometry. Fig. 2A represents the representative data obtained after 2 d of culture for one donor, and Fig. 2B shows the average data obtained with the different donors after 2 or 5 d of culture (n = 15). The percentage changes in CD46 expression upon addition of PGE2 (compared with expression levels in absence of PGE2 as baseline) were also calculated and are presented in Fig. 2C. As previously shown, CD46 ligation led to a strong downregulation of its expression. A slight increase in CD46 surface expression was detected when CD46 and CD28 were co-ligated, suggesting that there is a cross-talk between CD28 and CD46 that partially restores CD46 expression (Fig. 2B). The addition of PGE2 led to a slight increase in CD46 expression in unstimulated T cells after 2 d of culture (Fig. 2B, 2C). In contrast, PGE2 treatment led to decreased CD46 expression in activated T cells with the stronger reduction (~40%) observed upon CD46 costimulation. The effect of PGE2 was lost in CD3-activated T cells at day 5 (Fig. 2B). However, the decrease in CD46 expression was sustained by CD28 and CD46 costimulation, with again the stronger effect observed upon CD46 ligation. Fig. 2D shows the dose-dependent effect of PGE2 on CD46 expression. Hence, PGE2 clearly modulates CD46 expression in activated T cells.

**PGE2 differentially affects T cell activation depending on the costimulation signal**

We next assessed the role of PGE2 on T cell activation. CD4+ T cells were activated with anti-CD3, anti-CD3/CD46, or anti-CD3/CD28 Abs in the presence or absence of PGE2. First, T cell proliferation was determined by [3H]thymidine incorporation (n = 10). As expected, addition of PGE2 significantly decreased T cell proliferation in a dose-dependent manner, but the strongest effect was observed upon CD46 costimulation (Fig. 3A, 3B). Similar effect was observed when proliferation was determined by CFSE labeling (data not shown). Second, we determined whether the levels of cytokine produced by activated T cells were modified by addition of PGE2. We focused on IL-10 and IFN-γ as CD46 has the ability to switch T cells from producing IFN-γ to PGE2 decreases CD46 surface expression in activated T cells.
secreting IL-10 (8) and does not induce any Th2 cytokines (6, 42). Secretion of IL-10 and IFN-γ was quantified by ELISA, and we also calculated the IL-10/IFN-γ ratio to bypass the variations due to the changes in proliferation (n = 15). As previously described, CD46 activation promotes a Tr1-like phenotype, visualized by an increase in the IL-10/IFN-γ ratio (Fig. 3C). PGE2 drastically decreased cytokine production (Fig. 3C). However, PGE2 had different effects on the relative levels of cytokines produced depending on the activation signals received by the T cells. A trend toward an increase in IL-10/IFN-γ was observed upon CD3 activation alone, reflecting the known effect of PGE2 in the downregulation of IFN-γ. Costimulation with CD28 had no significant effect on the IL-10/IFN-γ ratio, suggesting that the lower level of cytokine production was mainly correlated with the lower proliferation of the cells. In contrast, ligation of CD46 resulted in a significant decrease in the IL-10/IFN-γ ratio in the presence of PGE2. Moreover, the effects of CD46 were dominant over CD28 as also observed upon CD3/CD28/CD46 co-stimulation. Importantly, similar trends were obtained when naive CD4+ T cells were used (data not shown). Overall, these data indicate that PGE2 exerts different effects on cytokine production that mainly depend on the activation signals received by the T cells.

**PGE2 modulates the phenotype of CD46-costimulated T cells**

We next determined whether PGE2 was affecting the phenotype of CD46-costimulated T cells. As PGE2 is known to reduce CD25 expression in CD3-activated T cells (36), we assessed the expression levels of CD25 as a control. We also determined the expression levels of CD69, another activation marker (Fig. 4A). Moreover, as PGE2 has been reported to induce regulatory T cells (Tregs) (36), we determined the expression levels of CTLA-4 (Fig. 4A), Foxp3, and CD46 (Fig. 4B). Fig. 4C represents the average change in expression upon addition of PGE2 obtained for the different donors (n = 6). A strong decrease in CD25 expression was detected for all conditions of activation, including CD46-costimulated T cells. Surprisingly, whereas CD69 expression was strongly decreased in the presence of PGE2 upon CD3 and CD28 activation, its expression was maintained when the cells were costimulated by CD46. T cell activation in the presence of PGE2 also resulted in a decrease in CTLA-4 expression, which was slightly restored upon costimulation. Lastly, PGE2 increased Foxp3 expression in both CD28 and CD46 costimulated T cells. Notably, the cells acquiring Foxp3 maintained CD46 expression although CD46 expression in CD46-costimulated T cells was downregulated by PGE2 (Fig. 4B). These data show that, depending on the activation signals, PGE2 differentially affects a variety of phenotypic markers in T cells that are involved in regulating T cell activation as well as Treg functions.

**PGE2 inhibits STAT3 phosphorylation**

PGE2 decreased CD46 expression in activated T cells, and it has been shown to modulate STAT3 signaling (43, 44). As the CD46 expression in CD3-activated T cells (36), we assessed the expression levels of CD25 as a control. We also determined the levels of CD69, another activation marker (Fig. 4A). Moreover, as PGE2 has been reported to induce regulatory T cells (Tregs) (36), we determined the expression levels of CTLA-4 (Fig. 4A), Foxp3, and CD46 (Fig. 4B). Fig. 4C represents the average change in expression upon addition of PGE2 obtained for the different donors (n = 6). A strong decrease in CD25 expression was detected for all conditions of activation, including CD46-costimulated T cells. Surprisingly, whereas CD69 expression was strongly decreased in the presence of PGE2 upon CD3 and CD28 activation, its expression was maintained when the cells were costimulated by CD46. T cell activation in the presence of PGE2 also resulted in a decrease in CTLA-4 expression, which was slightly restored upon costimulation. Lastly, PGE2 increased Foxp3 expression in both CD28 and CD46 costimulated T cells. Notably, the cells acquiring Foxp3 maintained CD46 expression although CD46 expression in CD46-costimulated T cells was downregulated by PGE2 (Fig. 4B). These data show that, depending on the activation signals, PGE2 differentially affects a variety of phenotypic markers in T cells that are involved in regulating T cell activation as well as Treg functions.

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promoter includes a STAT3 binding site, and as the direct interaction between STAT3 and CD46 has been previously demonstrated by chromatin immunoprecipitation (ChIP) assays, mutations of the STAT3 binding sites in CD46, and by the use of anti-STAT3 oligonucleotides (45, 46), we determined the levels of STAT3 phosphorylation in activated T cells. T cells were activated for 24 h in the presence or absence of PGE2 and STAT3 phosphorylation determined by flow cytometry. An increase in phosphorylation was observed in all activated T cells, including CD46-costimulated T cells. Addition of PGE2 decreased STAT3 phosphorylation, with the strongest effect observed upon CD46 ligation (Fig. 5).

Specific changes in PGE2 receptors are induced by CD46 activation
PGE2 binds to four GPCRs, namely EP1, EP2, EP3, and EP4. We determined their mRNA expression by quantitative PCR upon

FIGURE 3. PGE2 modulates T cell activation depending on the activation signals. Purified CD4+ T cells were stimulated by immobilized Abs as indicated in the presence or absence of PGE2. (A) Proliferation was determined after 4 d by [3H]thymidine incorporation (n = 10). (B) The dose effect of PGE2 on T cell proliferation of T cells activated by anti-CD3/CD28, anti-CD3/CD46, or anti-CD3/CD28/CD46 is shown (n = 3). (C) Cells were activated as in (A), and the amounts of IL-10 and IFN-γ in the supernatants were determined by ELISA (n = 15). The IL-10/IFN-γ ratio is also represented to show specific effects independent of the changes in proliferation induced by PGE2.

FIGURE 4. PGE2 modulates T cell phenotype upon activation. (A) Purified CD4+ T cells were stimulated with immobilized anti-CD3, anti-CD3/anti-CD28 (CD3.28), anti-CD3/CD46 (CD3.46), or anti-CD3/CD28/CD46 (CD3.28.46) Abs in the presence (shaded gray histograms) or absence (black line) of PGE2. After 5 d, the expression of surface CD25 and CD69 and intracellular CTLA-4 was determined by flow cytometry. (B) Expression of Foxp3 and CD46 was also determined after intracellular staining. (C) The percentage changes in CD25, CTLA-4, CD69, and Foxp3 expression upon addition of PGE2 obtained for the different donors are represented (n = 6).
T cell activation with anti-CD3, anti-CD3/CD28, and anti-CD3/CD46 Abs for 48 h (n = 3 different donors, and experiments performed at different times). As previously reported (33), only EP2 and EP4 were strongly expressed in T cells. Whereas CD3 and CD28 costimulation induced both EP2 and EP4 expression (Fig. 6A), CD46 activation only led to an increased EP4 expression, hence strongly increasing the EP4/EP2 ratio (Fig. 6B).

To understand the role of EP4 in CD46 activation, we added the selective EP4 antagonist ONO-AE2-227 to T cell culture and assessed cytokine production. Blocking EP4 led to an increase in the IL-10/IFN-γ ratio, but only when CD46 was ligated, and we did not observe any effect of the EP2 antagonist AH6809 (Fig. 6C). This further demonstrates the role of the PGE₂–EP4 axis in regulating IL-10 production by CD46-activated T cells.

**Discussion**

This study reports novel elements in the complex regulation of primary human T cell activation. Our data show that GRKs and the PGE₂–EP4 axis are involved in the CD46 pathway and that it modulates CD46 expression and functions. Knocking down GRKs led to a strong downregulation of CD46 at the surface of activated T cells. It is known that GRK levels are modulated by T cell activation (40, 41), and we report in this study that CD46 costimulation increased levels of GRK expression. Notably, decreased levels of GRK2 and GRK6 were observed in T cells from patients with RA (47) and MS (48, 49), and proinflammatory cytokines and oxygen radicals can decrease GRK2 levels in vitro (50). Hence, the levels of GRKs are highly modulated during inflammation, and this suggests the subsequent modulation of expression of CD46, a key regulator of T cell activation, in inflammatory settings. As CD46 is dysfunctional in T cells from patients with MS and RA, future investigation into the role of GRKs upon CD46 activation in these patients might highlight some of the molecular mechanisms of this defective pathway.

GRKs control GPCR signaling by phosphorylating these receptors, which provokes their internalization and degradation, a crucial feature of the plasticity of the immune system involved in the regulation of inflammation (reviewed in Ref. 23). Indeed, almost 30% of drugs on the market target GPCR (51, 52). We investigated the role of PGE₂, which signals through four subtypes of the E-prostanoid subfamily of GPCRs (EP1, EP2, EP3, and EP1–4) in T cell responses. Although the antiproliferative role of PGE₂ on T cells is well established, the role of PGE₂ in T cell differentiation is much more complex and not well understood, as it has some complex proinflammatory and immunoregulatory properties. In this study, we have compared the effects of PGE₂ in CD28 and CD46 costimulated T cells. As previously shown, PGE₂ exerted a strong antiproliferative role on all activated T cells. However, the effect was much stronger in CD46-costimulated T cells than in CD28-costimulated T cells. Despite the decrease in proliferation, activated T cells did secrete some significant levels of IL-10 and IFN-γ. In a number of studies, the addition of PGE₂ caused a marked reduction in Th1 cytokines (26, 27), whereas other studies indicate the role of PGE₂ in inducing Th1 or Th17 differentiation (28, 33). We found that the relative levels of IL-10 and IFN-γ secreted were differentially modulated depending on the activation signals received by the T cells. Whereas CD3 activation favored an increase in the IL-10/IFN-γ ratio, as previously described, we did not find any significant effects on CD28-costimulated T cells. However, there was a significant decrease in the IL-10/IFN-γ ratio upon CD46 ligation. PGE₂ therefore skews the usually regulatory cells toward a more inflammatory secretion profile. PGE₂ downregulates both IL-2 and CD25 (26). As IL-2 is required for CD46-mediated Tr1 differentiation (8), it is likely that the effect of PGE₂ on IL-2 and CD25 expression as well as the inhibition of CD46 expression contribute to inhibit IL-10 production by CD46-activated T cells. Our data also underline that the actions of PGE₂ are largely dependent on the activation of the T cells, as previously reported on the secretion of IL-3 by T cells (53). Hence, it becomes clear that PGE₂ differentially modulates T cell responses depending on the environment and signals received by the T cells. Our data highlight the role of PGE₂ in the production of IFN-γ and IL-10, which are, at least in part, determined by the regulation of expression of CD46.

PGE₂ strongly modulates the phenotype of activated T cells. We show that the addition of PGE₂ decreased CD46 expression. This was observed in all activated T cells, although the effect was much more pronounced upon CD46 ligation. Of note, we observed a slight increase in CD46 expression in unactivated T cells. Notably, PGE₂ has been found to upregulate CD55, another regulator of complement activation molecule, in colon cancer cells (54). Hence, there is a similar pattern for CD46 in resting T cells. However, the increased expression of CD55 might also partially counteract the downregulation of CD46 expression observed in

**FIGURE 5.** PGE₂ inhibits STAT3 phosphorylation. Purified CD4⁺ T cells were stimulated by immobilized Abs as indicated in the presence or absence of PGE₂. After 24 h, the level of p-Tyr STAT3 was determined by flow cytometry (representative of two independent experiments). (STAT3 staining: shaded gray areas; isotype control: black lines.)

**FIGURE 6.** T cell activation results in expression of different PGE₂ receptors depending on the activation signal. Purified CD4⁺ T cells were stimulated by immobilized Abs as indicated for 2 d. mRNA was then prepared and the expression levels of the four PGE₂ receptors (EP1–4) were determined. (A) Relative expression of EP2 and EP4 in activated T cells. The means ± SEM are shown (n = 3 donors). Samples were analyzed using the Wilcoxon test. (B) The ratio of EP4/EP2 relative expression has been plotted. CD46 activation results in an increased EP4/EP2 ratio. (C) EP4 is involved in the modulation of cytokines produced by CD46-costimulated T cells. Purified CD4⁺ T cells were stimulated by immobilized Abs as indicated in the presence of EP2 or EP4 antagonist and DMso or ethanol as a control, respectively. The levels of IL-10 and IFN-γ were quantified, and the IL-10/IFN-γ ratio is represented. Representative of three experiments.
activated cells to protect from complement attack. Importantly, as CD46 is ubiquitously expressed, it is likely that PGE2 will also modulate its expression and function in other cell types, such as dendritic cells, especially as PGE2, like CD46, has been shown to regulate IL-23 production (35, 55).

It was previously demonstrated that PGE2 decreased CD25 expression and upregulated Foxp3 levels in human T cells (36). We confirmed these findings and report that similar decreased CD25 and increased Foxp3 expression are also detected after CD46 costimulation. Moreover, PGE2 caused a strong reduction of expression of the coinhibitory molecule CTLA-4 on CD3-activated cells, but CTLA-4 expression was mostly maintained upon costimulation. CTLA-4 is expressed at high levels by Tregs and is involved in their function (56), hence the increase in Foxp3 correlates with CTLA-4 expression. PGE2 also decreased CD69 expression in activated T cells, although not in CD46-costimulated cells. CD69 is transiently induced after activation but can persist on leukocyte infiltrates in chronic inflammatory diseases. CD69-deficient mice developed exacerbated forms of arthritis, allergic asthma, and other inflammatory settings, and it was proposed that CD69 could act as a regulator of Th17 differentiation (57). The fact that PGE2 maintained CD69 in CD46-costimulated T cells is intriguing and warrants further investigation on this pathway, notably in chronic inflammatory diseases where the CD46 pathway is dysfunctional.

The different effects on cytokine production and phenotype observed in the presence of PGE2 were correlated with distinct profiles of expression of PGE2 receptors. CD3 and CD28 led to similar increase in EP2 and EP4, which is consistent with what had been shown in the mouse (58) and in human T cells (33). However, CD46 costimulation largely favored EP4 expression, and addition of an EP4 antagonist increased the IL-10/IFN-γ ratio by CD46-activated T cells, highlighting the specific role of EP4 in CD46 function. Contrasting effects of PGE2 on cytokine production have previously been shown to be mediated by specific receptors. Boniface et al. (33) show that PGE2 can act on CD3/CD28/CD2-coactivated human T cells to promote IL-17, which is mainly mediated by EP2, whereas it inhibits IFN-γ and IL-10 production mainly through EP4 signaling. Both the EP2 and the EP4 receptors couple with the Galpha(s) protein, whereas the EP1 and the EP3 receptors are known to couple to Galpha(q) and Galpha(i), respectively. However, differences in signaling between EP2 and EP4 have indeed been demonstrated, as for example EP4 but not EP2 leads to ERK phosphorylation via PI3K activation (59). It is likely that different signaling cascades are involved in the CD28 and CD46 pathways. The link between CD46 and EP4 is also intriguing as there is a special emphasis on these receptors in MS. Among PGE2 receptor-deficient mice, only the EP4-subtype knockout can significantly inhibit experimental autoimmune encephalomyelitis (29). Moreover, MS-associated single nucleotide polymorphisms in EP4 (also called PTEGR4) have been demonstrated (60). As it is now clearly established that the CD46 pathway is dysfunctional in MS (13–16), further investigations should address the question as to whether the CD46–PGE2–EP4–GRK pathway is dysfunctional in MS (13–16), further investigations should address the question as to whether the CD46–PGE2–EP4–GRK pathway is dysfunctional in MS.

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Disclosures

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References


Supp Figure S1: EP4 knockdown by RNAi led to increased CD46 and CD25 expression. Purified primary human CD4+ T cells were activated by CD3/CD28 antibodies and infected by lentiviruses coding for EP4 shRNA (tinted grey area) or control shRNA (black line), as described in the mat and method section. After expansion for 7 days in IL-2, cells were restimulated by anti-CD3/CD28 antibodies and 24hrs later, expression of CD46 and CD25 was measured by flow cytometry.