Systems approach reveals distinct and shared signaling networks of the four PGE₂ receptors in T cells*

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Abstract

Prostaglandin E₂ (PGE₂) promotes an immunosuppressive microenvironment in cancer, partly by signaling through four receptors (EP₁, EP₂, EP₃, and EP₄) on T cells. Here, we comprehensively characterized PGE₂ signaling networks in helper, cytotoxic, and regulatory T cells using a phosphoproteomics and phosphoflow cytometry approach. We identified ~1500 PGE₂-regulated phosphosites and several important EP₁₋₄ signaling nodes, including PKC, CK2, PKA, PI3K, and Src. T cell subtypes exhibited distinct signaling pathways, with

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the strongest signaling in EP₂-stimulated CD8⁺ cells. EP₂ and EP₄, both of which signal through G_{αs}, induced similar signaling outputs, but with distinct kinetics and intensity. Functional predictions from the observed phosphosite changes revealed PGE₂ regulation of key cellular and immunological processes. Finally, network modeling suggested signal integration between the receptors, and a substantial contribution from G protein-independent signaling. This study offers a comprehensive view of the different PGE₂-regulated phosphoproteomes in T cell subsets, providing a valuable resource for further research on this physiologically and pathophysiologically important signaling system.

Introduction

Prostaglandin E₂ (PGE₂) is the most abundant prostanoid in the human body and plays a crucial role in maintaining immune homeostasis as well as in pathophysiological settings, including cancer and chronic inflammatory conditions [1, 2]. PGE₂ is increased in colorectal, lung, breast, and pancreatic cancers [3], where it is produced by tumor cells or induced regulatory T cells (Tregs) [4] and promotes tumor growth by stimulating angiogenesis, cell invasion, and metastasis while inhibiting tumor cell apoptosis [5]. PGE₂ also contributes to the formation of an immunosuppressive tumor microenvironment through effects on multiple immune cell types [6-13].

Because of its significance in cancer, methods for interfering with PGE₂ signaling are being explored as cancer prevention and treatment. In particular, inhibition of cyclooxygenase 1 (COX1) and COX2, which control the rate-limiting step in the biosynthesis of PGE₂, reduces the incidence of colorectal cancer [14-16] and improves survival if treatment is initiated after diagnosis [17, 18]. Individual PGE₂ receptors – in particular EP₁, EP₂, and EP₄ – have also been targeted with antagonists for increased specificity [19, 20]. There also appears to be synergy between PGE₂ targeting and cancer immunotherapy. For instance, COX inhibitors enhance the effect of immune checkpoint blockade [21], and a peptide that blocks an inhibitory PGE₂ signaling pathway augments the antitumor efficacy of chimeric antigen receptor (CAR) T cells [22]. There is also interest in combining EP

antagonists with immunotherapy, and a selective EP₄ antagonist is currently in Phase I Clinical Trials (ClinicalTrials.gov, NCT03155061) [23] in combination with Nivolumab in patients with solid tumors. There is thus ample basis for pursuing PGE₂-targeted cancer therapy alone or in combination with other immunotherapies. However, given the plethora of tissue-, cell-, and receptor-specific effects of PGE₂ in health and disease, it is crucial to have a comprehensive understanding of the signaling events and biological functions regulated by PGE₂ to avoid unintended side effects of blocking specific PGE₂-regulated pathways.

Prostaglandin E₂ (PGE₂) signals through four distinct G protein–coupled receptors: EP₁, EP₂, EP₃ and EP₄ [19, 24], all of which appear to be present on T cells, with EP₂ and EP₄ being the most highly abundant [25-27]. EP₁ is G_{αq}-linked and signals mainly through phospholipase C (PLC), leading in turn to protein kinase C (PKC) activation [2, 20, 24]. EP₂ and EP₄ both couple to the stimulatory G protein, G_{αs}, which activates adenylyl cyclase, thereby increasing intracellular cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA). In addition, EP₄ couples to G_{αi}, which inhibits PKA and is also associated with the release of the βγ subunits of the G protein complex, thus triggering phosphoinositide 3-kinase (PI3K) signaling [28-30]. EP₃ couples mainly with G_{αi}, but it exists in multiple isoforms, some of which couple to alternative G_α proteins and pathways [20, 24]. These receptors also signal through G protein–independent pathways such as β-arrestin signaling [31-36]. Together, these myriad intracellular signaling options resulting from one extracellular stimulus presents an interesting problem in signal integration and functional output.

We have been particularly interested in PGE₂ signaling in T cells because this plays an important role in many pathophysiological settings, including cancer and chronic inflammatory conditions [1, 2, 37]. PGE₂ suppresses immune function by promoting Treg generation, recruitment, and proliferation [9, 38]; by inhibiting CD8⁺ T cell cytotoxicity by stimulating the production of CD94-NKG2A heterodimers (CD94/NKG2A) [39]; by inhibiting T cell receptor (TCR)-dependent interferon γ (IFNγ) release from CD8⁺ T cells [40]; and by promoting a shift from an anti-tumor T helper type 1 (Th1) response to an

immunosuppressive Th2 response [9, 41]. In addition, our lab has characterized a PGE₂-regulated inhibitory cAMP-PKA pathway in effector T cells with importance in disease [1, 4, 42-47]. PGE₂ can also have a proinflammatory, cancer-promoting function in T cells [2, 48]. For instance, PGE₂ promotes interleukin 23 (IL-23)-induced Th17 differentiation and proliferation [26, 49-51], induces Th1 differentiation through a PI3K-Akt signaling pathway when strong TCR signaling is also present [50], and promotes T cell proliferation through the induction of costimulatory molecules on dendritic cells (DCs) [52].

We have previously studied PGE₂ signaling pathways in primary (CD3⁺) T cells using phosphoproteomics [53], and a few other mass spectrometry (MS)-based studies on PGE₂ stimulation have been performed in Jurkat T cells [54, 55] and fibroblasts [56]. In these studies, all four EPs were stimulated concurrently by PGE₂, so that the individual contributions of each receptor could not be assessed. In a normal physiological context, PGE₂ would of course be the natural stimulus for this receptor system, and signaling would proceed through each of the receptors that is present on an individual cell. However, given the interest in targeting specific receptors in cancer therapy and other conditions, as well as an academic interest from a signaling network perspective in understanding how this four-receptor system integrates signals from individual receptors, it would also be valuable to have a better understanding of the specific signaling occurring through each receptor. Here, we therefore chart, in a system-wide manner, the signaling elicited by each of the four PGE₂ receptors individually to obtain a detailed map of the EP signalosomes and comprehensive PGE₂-regulated phosphoproteomes in primary T cell subtypes, including helper (CD4⁺) T cells, cytotoxic (CD8⁺) T cells, and Tregs. To this end, we stimulated cells with receptor-specific agonists and studied the signaling that was elicited using MS-based phosphoproteomics and multiplexed phosphoflow cytometry. Here, we present a global and detailed view of the signaling nodes, pathways, and networks regulated in CD4⁺ T cells, CD8⁺ T cells, and Tregs upon triggering EP₁, EP₂, EP₃, or EP₄. This system-wide view of the contributions from and cross-talk between the different receptors sheds light on an important immunoregulatory network and provides a context in which the systems pharmacology of

targeting PGE₂ or its receptors can be assessed. Further, the current study provides a valuable resource for targeted studies of PGE₂ signaling mechanisms and biological functions in T cells.

RESULTS

PGE₂ receptors EP₁₋₄ regulate unique and overlapping phosphosites in T cell subsets

To map the PGE₂-regulated phosphoproteome in T cells, we stimulated CD4⁺ T cells, CD8⁺ T cells, and Tregs from healthy donors with agonists highly specific for each receptor (table S1) or PGE₂ and then performed phosphoproteomics using a label-free strategy with Ti⁴⁺-immobilized metal ion affinity chromatography (IMAC) enrichment (fig. S1, A to E) [55]. In total, we identified more than 21,000 phosphopeptides (data file S1) and quantified 5000-8000 unique phosphosites with very high reproducibility across each studied cell type. The Pearson correlation coefficients between biological replicates were between 0.7-0.96, and for a given condition, about 50-60% of the phosphosites could be quantified in each of the 5 biological replicates (fig. S2). The quantitative analysis revealed a stronger and distinct response in CD8⁺ cells compared to CD4⁺ cells and Tregs (Fig. 1A-C). In particular, we observed regulation (an increase or decrease in phosphorylation) of more than 1000 phosphosites upon stimulation of CD8⁺ T cells with the EP₂ agonist (Fig. 1D). In this cell type, more regulated sites were observed when stimulating with EP₂ agonist than with PGE₂, likely due to the inhibitory effect on G_{as} signaling of simultaneous PGE₂ signaling through the G_{ai}-coupled EP₃ receptor. In terms of signaling differences between cell types, the lower number of regulated sites in Tregs may be due to the high basal amounts of cAMP in this cell type [57], which could dampen the cAMP-dependent arm of the PGE₂ response. The phosphoproteomes regulated by the different receptors in a given cell type showed substantial overlap, indicating crosstalk and possible signal integration between receptors. However, many regulated phosphosites were unique to each receptor (Fig. 1E, fig S3A, B). For instance, in CD8⁺ cells, the EP₂ receptor uniquely regulated 706 phosphosites, and in addition regulated 398 sites that were also regulated by one or more of the other receptors.

Thus, both unique and shared signaling pathways are present downstream of the individual receptors.

Identifying kinases that control the PGE₂-regulated phosphoproteomes

To further understand how the EP-regulated phosphoproteomes are controlled, we used NetPhorest [58] to predict which kinases phosphorylate the regulated phosphosites (Fig. 1F, fig S4, table S2). In both CD8⁺ and CD4⁺ cells, the PKC, cyclin-dependent kinase (CDK), CDK-like kinase (CLK), casein kinase 2 (CK2), and mitogen-activated protein kinase (MAPK) groups were the most highly predicted. PKA, which is known to be a key regulator of PGE₂ signaling, was also highly predicted in each cell type. Although the absolute number of predictions for a given kinase varied between cell types, the patterns in the predicted kinases between different stimulation conditions (receptor-specific agonist or PGE₂) remained similar. The sequence motifs of the regulated phosphosites were then examined using IceLogos and corresponded well with the predicted kinases. The predominant IceLogo for the regulated phosphosites was RRXSP for all stimulation conditions (Fig. 1G, top row; fig. S5), with some variations between stimulation conditions and cell types. Surmising that this logo is likely a composite of multiple motifs, we isolated all regulated sites with proline in the +1 position (Fig. 1G, bottom row left; fig. S6) and found that these sites often also contained a proline in the -2 position. This would agree well with phosphorylation sites for extracellular signal-regulated kinase 1 (ERK1, also known as MAPK3), ERK2 (also known as MAPK1), or CDK2, -4, or -5 [59], all of which are kinases predicted by NetPhorest.

For the regulated phosphosites without proline in position +1 (Fig. 1G, bottom row right; fig. S7), a strong RRXpS motif emerged in CD8⁺ cells, likely accounting for the PKA prediction. In contrast, in non-proline phosphosites that increased in abundance upon stimulation in CD4⁺ cells, we mainly observed a different motif, pSDXE (fig. S7), which is consistent with CK2 phosphorylation [60]. This corroborates the prediction from NetPhorest that there is a higher relative proportion of CK2 phosphorylation events in CD4⁺ cells than in CD8⁺ cells. In general, phosphorylation motifs and kinase predictions were similar between

stimulation conditions and cell types, implying that the signaling pathways originating from each receptor proceed through many of the same kinase nodes, allowing for receptor cross-talk and signal integration.

EP₁₋₄ regulate different biological functions

Next, we used the “Predict Functional Phosphosites” (PFP) [61] tool to identify regulated phosphosites with a known biological function according to the PhosphoSitePlus database (Table 1) and sites predicted to be biologically relevant by at least one of the four algorithms in the program (table S3). Of the sites with known biological function, many are involved in signaling, and several are related to the kinases predicted above. For instance, we saw regulation of sites on Ca²⁺/calmodulin-dependent protein kinase 2 (CAMK2), CDK9, p21-activated kinase 1 (PAK1), MAP3K5, protein kinase C θ (PRKCQ), protein kinase D2 (PRKD2), ribosomal protein S6 kinase A3 (RPS6KA3), S6 ribosomal protein (S6RP), rapidly accelerated fibrosarcoma 1 (RAF1), and regulator of G protein signaling 3 (RGS3). Further, we observed many PKA-related regulated sites, for instance PKA regulatory subunit RI α (PRKAR1A) Ser⁷⁷ and Ser⁸³, protein tyrosine phosphatase non-receptor type 7 (PTPN7) Ser⁴⁴, vasodilator-stimulated phosphoprotein (VASP) Ser²³⁹, and vimentin (VIM) Ser²⁶ and Ser⁷³. Many of the regulated sites are also particularly interesting in a T cell context, for instance Ser⁶⁹⁷ and Ser⁷⁰⁶ of CD44, Tyr⁴²⁰ of the kinase FYN, Tyr³⁹⁴ and Tyr⁵⁰⁵ of the kinase LCK, and Tyr³¹⁷ of phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG1).

Cellular functions regulated by the EPs

To further understand the range of biological processes regulated by the four PGE₂ receptors in T cells, the ClueGO software was used to analyze the regulated proteins for involvement in cellular functions (Fig. 2A, fig. S8, table S4). In CD4⁺ T cells and Tregs, no significant GO term was enriched, whereas for CD8⁺ T cells, all stimulation conditions showed enrichment for regulation of cytoskeleton organization, mRNA processing, cell-cell

adhesion, cell polarity, and small GTPase-mediated signal transduction. Proteins involved in these various processes were also present in the list of biologically active phosphosites (Table 1 and table S3). For instance, several proteins related to small GTPase-mediated signal transduction, such as Rho guanine nucleotide exchange factors (ARHGEFs) 2, 6, and 7, Arrestin β 1 (ARRB1), nuclear receptor coactivator 3 (NCOA3), Rab GTPase-binding effector protein 1 (RABEP1), RAF1, RGS3, and tripartite motif-containing 28 (TRIM28), were observed among the regulated biologically active sites (Table 1 and table S3).

As expected, several immune processes were also highly predicted by ClueGO (Fig. 2B, fig. S9, table S5). For EP₂, the most highly predicted immune process was TCR signaling, which corroborated previous findings that EP₂ inhibits TCR signaling [1]. This process was also highly enriched in PGE₂-stimulated CD8⁺ cells. Other highly enriched immune processes included T cell activation (for EP₁, EP₃, and PGE₂), establishment of T cell polarity (EP₃, EP₄, and PGE₂), thymic T cell selection and T cell differentiation in thymus (EP₁, EP₃, and EP₄), lymphocyte migration (EP₄) and lymphocyte proliferation (EP₃).

Receptor- and cell type-specific regulation of phosphosites

The specific signaling elicited through each receptor was examined in more detail using an approach that combines fluorescent cell barcoding with phospho-flow cytometry [62]. This technique allows for high-throughput monitoring of phosphorylation events resulting from a given stimulus or combination of stimuli, yielding information on signaling kinetics, magnitudes, and differences across T cell subtypes. A panel of 16 phospho-specific antibodies was established based on known PGE₂ signaling pathways in T cells as well as the current phosphoproteomics study. The signaling elicited by specific agonists of each of the four EPs was monitored over time in CD4⁺ and CD8⁺ naïve (CD45RA⁺) and effector/memory T cells (CD45RO⁺) T cells (Fig. 3A, B, figs. S10, S11, S12A-C and S13A-C).

Many readouts in the phosphoflow cytometry panel, including glycogen synthase kinase 3 α (GSK3A) pSer²¹ [63], VASP pSer¹⁵⁷ [64], VIM pSer³⁸ [65], Histone H3 pSer¹⁰ [66], cAMP response element-binding protein 1 (CREB1) pSer¹³³ [67], and heat shock protein B1

(HSPB1) pSer⁷⁸ [68], can be directly phosphorylated by PKA, which is activated immediately downstream of EP₂ and EP₄, following G_{as} activation and cAMP production by adenylyl cyclase. As expected, the abundance of these phosphorylated PKA substrates was strongly increased by EP₂ or EP₄ agonist stimulation. A further three phosphoflow readouts were phosphosites on different subunits of PKA [PRKAR2A (pSer⁹⁹), PRKAR2B RIIb (pSer¹¹⁴), and PRKACA (pThr¹⁹⁷)], of which the first two are thought to be autophosphorylated [69, 70] and the latter is an activating site that appears to be phosphorylated by PDK1 in vivo [71, 72]. Counterintuitively, these three phosphosites demonstrated decreased abundance upon treatment with EP₂ or EP₄ agonists, though this observed reduction is likely due to post-activation desensitization.

Of the remaining monitored sites, several can be downstream of PI3K-Akt pathways, which in turn can be activated by G_{βγ} signaling [73-75] and β-arrestin signaling [76, 77]. In particular, the EP₄ receptor, when coupled to G_{ai}, is known to trigger a PI3K pathway through G_{βγ} signaling [28]. Potential PI3K-Akt-regulated phosphoflow readouts included N-Myc downstream regulated 1 (NDRG1) pThr³⁴⁶, which can be phosphorylated by serum and glucocorticoid-regulated kinase 1 (SGK1) downstream of PI3K [78] or by Akt downstream of CD28 [79], as well as S6RP (pSer²⁴⁰), which can be phosphorylated by ribosomal protein S6 kinase (p70S6K) downstream of PI3K and mechanistic target of rapamycin (mTOR) [80]. The other monitored phosphosite on S6RP, pSer^{235/236}, is thought to be phosphorylated by a different kinase, namely p90S6K, operating downstream of ERK [80]. In agreement with previous studies [80], distinct kinetics were observed for the two phosphorylation events on S6RP, with the Ser²⁴⁰ phosphorylation site displaying a slower response than Ser^{235/236}, suggesting that two different kinases with different kinetics are operating in T cells as well.

The two final phosphoflow readouts, phospholipase C γ1 (PLCG1) pTyr⁷⁸³ and Histone H3 pSer²⁸, have several potential regulatory kinases, but it is thought that PLCG1 phosphorylation at Tyr⁷⁸³ is downstream of TCR activation [81, 82] and that Histone H3 pSer²⁸ is phosphorylated by Aurora B kinase, mitogen and stress activated kinase 1 and 2

(MSK1/2) [66], or MAPKs [83]. Many of the readouts discussed above as PKA or Akt substrates may also be phosphorylated by other kinases, depending on context.

Overall, the monitored phosphoflow readouts responded most strongly to EP₂ and EP₄ agonist stimulation, with smaller or sometimes absent responses to EP₁ and EP₃. Control stimulation with PGE₂, as expected, led to a robust response for all readouts. The addition of receptor antagonists reversed the signaling elicited by the agonists (fig. S14). The strong responses to EP₄ and, especially, EP₂ agonist stimulation, may be due to the higher abundances of EP₂ and EP₄ than of EP₁ and EP₃ in peripheral blood T cells [26], though all appear to be present [25] (fig. S1).

The kinetics of phosphorylation events varied considerably between readouts in this study, with some showing early (VIM pSer³⁸, VASP pSer¹⁵⁷), medium (S6RP pSer²⁴⁰, NDRG1 pThr³⁴⁶) or late (CREB1 pSer¹³³, Histone H3 pSer¹⁰ and pSer²⁸) phosphorylation responses (Fig. 3A, B). One explanation for this observation could be that it reflects different cellular locations. For instance, CREB1 and Histone H3, which exhibited slow temporal regulation, are both located in the nucleus, whereas the early and medium readouts listed above are cytoplasmic. Different signaling pathways may also have different dynamics independent of location, for instance in the case of S6RP phosphorylation by two different kinases as described above. The difference in timing may in some cases also stem from differences between G protein–dependent (G_α and G_{βγ}) signaling, which tends to occur relatively rapidly [84, 85] and G protein–independent (β-arrestin) [35, 36] signaling, although the latter may in some cases also proceed quickly [86].

The phosphoflow cytometry approach further revealed considerable differences in PGE₂-induced signaling between T cell subtypes (Fig. 3C). In particular, for the vast majority of the readouts, the signals were the strongest and most persistent in CD8⁺CD45RO⁺ cells. S6RP pSer^{235/236}, GSK3A pSer²¹, PKA substrates, and Histone H3 pSer¹⁰ were examples of this (Fig. 3C). In a few cases, however, the highest amounts of signaling were observed in CD4⁺ cells, for instance, for NDRG1 (pThr³⁴⁶) and CREB1 (pSer¹³³). These cell type–specific

differences in PGE₂ signaling in T cells may reflect differences in the abundances of individual EPs or distinct downstream signaling pathways in different cell types.

EP₂ and EP₄ regulate phosphosites with different kinetics

EP₂ and EP₄ both couple to G_{αs} and might thus be expected to signal through many of the same downstream pathways. Indeed, agonists of these receptors triggered the same readouts in our phosphoflow panel (Fig. 3A, B). However, it was striking that the signals triggered by EP₂ were substantially stronger than those from EP₄, and also persisted longer (Fig. 4A, fig. S15. A and B). To confirm that this was not an effect of different doses, potencies, or other properties of the receptor agonists, we performed an experiment to isolate PGE₂ signaling through a specific receptor by stimulating CD3⁺ cells with PGE₂ while simultaneously blocking three of the four EP receptors with specific antagonists (Fig. 4B, 4C). Using this approach, we confirmed that the same concentration of PGE₂ gave a stronger signaling response through EP₂ than through EP₄ (Fig. 4B). The signaling intensity of EP₂-isolated PGE₂ signaling, EP₄-isolated PGE₂ signaling, and PGE₂ signaling through all four receptors was then compared over time (Fig. 4C). The experiment confirmed the observation from the agonist-based studies, that EP₂ signaling had longer duration and higher intensity than EP₄ signaling, independently of any specific agonist properties. In general, PGE₂ signaling through EP₂ produced a signal equivalent to PGE₂ signaling through all four receptors, in some cases exceeding it, whereas EP₄ signaling was less intense. The observation that EP₂-isolated PGE₂ signaling sometimes produced slightly higher phosphoflow responses than PGE₂ signaling through all receptors could possibly be due to PGE₂-induced G_{αi} activation through, for instance, EP₃ and EP₄, which could temper G_{αs} signaling through EP₂ when all receptors are stimulated simultaneously.

Phosphoflow cytometry inhibitor studies

To further investigate which kinases are involved in the PGE₂ signaling pathways in T cells, phosphoflow cytometry was applied in the presence of PGE₂ stimulation and various

kinase inhibitors. First, we observed that PGE₂ regulation of all monitored readouts was inhibited by at least 100% by the PKA inhibitor H89 (Fig. 4D). Here, we defined 100% inhibition as a reduction in the phosphorylation of a given readout to the amount observed in the unstimulated sample. Any inhibition beyond 100% thus constitutes a reduction in the basal phosphorylation of these phosphosites. Several readouts were inhibited by 200% or more (Fig. 4D), indicating considerable basal PKA signaling in the absence of PGE₂ stimulation. A majority of the sites regulated by PGE₂ in T cells thus appear to be downstream of PKA, in line with the observation that the phosphoflow readouts were mainly affected by signaling through the EP₂ and EP₄ receptors, which are known to couple to G_{αs} and PKA.

In addition to H89, inhibitors of other important kinases in T cells, including PP2 (Src inhibitor), Wortmannin (PI3K inhibitor), LY294002 (PI3K inhibitor), CI-1040 (MEK inhibitor), AG1478 (EGFR inhibitor), TBB (CK2 inhibitor) and CT99021 (GSK3A and GSK3B inhibitor) were also tested (Fig. 4E). Although H89 generally gave the strongest inhibitory response, some readouts were also inhibited by other kinase inhibitors, indicating that these phosphosites are downstream of signaling pathway(s) involving several different kinases. For instance, NDRG1 pThr³⁴⁶ was inhibited by Wortmannin, LY294002 and PP2 in addition to H89, indicating regulation by PI3K and Src. S6RP pSer²⁴⁰ was also inhibited by these inhibitors as well as by CT99021 and TBB, suggesting that this phosphosite is downstream of several different pathways, also involving GSK3A/B and CK2. CREB1 pSer¹³³ was a further readout influenced by several inhibitors – most strongly by H89, Wortmannin, LY294002, CI-1040, and PP2. This is in line with the literature, in which CREB1 pSer¹³³ has been reported to be downstream of several pathways and kinases including PKA, PI3K-Akt, and PKC [67, 87]

Considerable cell type differences were observed in these inhibitor studies (Fig. 4F, fig. S16). For CREB1 pSer¹³³, there was a striking difference between the cell types in that Wortmannin, H89, and PP2 inhibited this readout substantially except in CD4⁺CD45RA⁺ cells. Another readout that exhibited a cell type-specific inhibition pattern was NDRG1

pThr³⁴⁶. This phosphosite was inhibited to below baseline by Wortmannin, H89, and PP2 in CD4⁺CD45RA⁺ cells, but only by H89 in CD4⁺CD45RO⁺ cells, indicating that there may be basal signaling through all three kinases in CD4⁺CD45RA⁺ cells, but only through PKA in CD4⁺CD45RO⁺ cells. S6RP pSer²⁴⁰ phosphorylation was also affected differently by inhibitors in different cell types. In particular, both Wortmannin and PP2 inhibited this phosphorylation to baseline or below in naïve (CD45RA⁺) CD4⁺ and CD8⁺ cells, but only by about 40-70% in effector/memory (CD45RO⁺) CD4⁺ and CD8⁺ cells. Together, these results suggest that the contributions of different signaling pathways may differ considerably between cell types.

Overview of PGE₂ signaling in T cells by network modeling

Network modeling was used to obtain an estimate of PGE₂ signaling pathways activated in CD4⁺ and CD8⁺ T cells under separate and combined stimulation conditions. To this end, an Integer Linear Programming (ILP) formulation of PHOSphorylation NEtworks for Mass Spectrometry (PHONEMeS) [88] was applied by combining the large-scale phosphoproteomic data set with a network of directed protein and kinase and phosphatase-to-substrate (K/P-S) interactions representing our prior knowledge. PHONEMeS identifies subnetworks that best explain the signal propagation resulting in the measured phosphoproteomic data for each of the experimental conditions. This allows us to extract possible paths connecting the stimulated receptors to the downstream regulated phosphosites. This approach yielded network models for each of the five different stimulation conditions and one network model when considering all the combined experimental conditions in CD4⁺ (Fig. 5A, B, figs. S17A, S18A, S19A, S20A, S21A, S22A) and CD8⁺ (Fig. 5C, figs. S17B, S18B, S19B, S20B, S21B, S22B) T cells. Modeling was not performed in Tregs, due to the low number of regulated phosphosites in this cell type, which did not permit robust analysis.

The modeled networks provided an overview of possible signaling networks through all receptors in both cell types in PGE₂-stimulated conditions and when signaling through

specific receptors was isolated by stimulating cells with PGE₂ in combination with 3 receptor-specific antagonists (Fig. 5A, figs. S21, S22). From the modeled networks it appeared that the same main pathways were active in CD4⁺ and CD8⁺ cells for a given stimulation condition. For instance, for EP₂, the main signaling appeared to proceed through PKA, Src, GSK3, CK2, and MAPK-based pathways in both cell types (Fig. 5, B and C, fig. S18). This was in line with results from the phosphoflow cytometry study, which also suggested that the difference in signaling between cell types may be more one of strength and duration of signaling rather than different pathways, with a few notable exceptions (Fig. 4F). In the phosphoproteomics results, strength of signaling appeared to translate into a larger number of observed readouts, resulting in a larger predicted network for CD8⁺ than for CD4⁺ cells, though essentially centered on the same key pathways, indicating a majority of preserved pathways between cell types.

Modeled networks for the different stimulation conditions successfully recapitulated the main expected pathways, including PKA-based pathways for EP₂ and EP₄, as well as PKC-based pathways for EP₁. A good correspondence was also observed between the predicted networks and NetPhorest-predicted kinases (Fig. 1F, table S2) and kinases implicated based on inhibitor studies (Fig. 4E). In particular, the key kinases identified in the inhibitor studies, such as PKA, Src, and PI3K were prominently present in the predicted networks for most stimulation conditions. The five most highly predicted kinase groups by NetPhorest, including PKC, CDKs, CLKs, CK2, and MAPK, were also present in the predicted networks. CLKs were only present in a few predicted networks, notably EP₁ and EP₂ signaling in CD8⁺ cells, but these were in fact the most highly predicted conditions for CLKs by NetPhorest as well. We noted that G protein-independent pathways were highly predicted in the networks. In particular, β -arrestin accounted for a substantial part of the signaling in all stimulation conditions, according to the predicted networks. Although it is known that PGE₂ can signal through β -arrestin-mediated pathways, the extent of the predicted contribution was greater than expected.

DISCUSSION

Phosphoproteomics of T lymphocytes

MS-based phosphoproteomics has undergone major developments in recent years, allowing the detection of ever-increasing numbers of phosphosites in only a few hours [89]. In T cells, a few phosphoproteomics studies have investigated signaling, including that induced by PGE₂ [53-55, 90, 91]. Here, our mapping of phosphoproteome changes in individual PGE₂ signaling pathways in three primary T cell subsets – helper T cells (CD4⁺), cytotoxic T cells (CD8⁺), and Tregs – through each of the four PGE₂ receptors identified more than 12,500 phosphopeptides and quantified changes in 5000-8000 phosphosites, which is comparable coverage to that in related studies [55].

Kinase nodes in PGE₂ signaling

The kinases predicted here show substantial overlap with previous studies and support the finding that PKA, CAMK2, Akt, GSK3, and CK2 are major contributors to PGE₂ signaling in T cells [53, 55]. PKA is not as highly predicted as in previous studies [53], which could be due to the overlapping substrate motifs of PKA, PKC and PKB (Akt) [92]. Inhibitor phosphoflow cytometry experiments certainly support a key role for PKA in PGE₂ signaling, though with the caveat that H89 is known to have some cross-reactivity with other kinases [93, 94]. When combined with cAMP stimulation, however, H89 is quite specific for PKA.

Kinase contributions differed across T cell subtypes. For instance, CK2 was more highly predicted in CD4⁺ than in CD8⁺ cells. Regulated phosphomotifs exhibited similar cell-type differences, with CD4⁺ cells having a greater proportion of acidophilic, potential CK2 target motifs and a lower proportion of basophilic, potential PKA, PKC, PKG, Akt, p70 S6 kinase, AMPK, and RSK target motifs than CD8⁺ cells. CK2 is thought to predominantly have a proinflammatory role in T cells [95-98], although its role in different subsets and is not well understood. It is notable that PGE₂ may be one of the factors regulating CK2 activity through a positive feedback loop. Overall, the high degree of conservation of EP-activated kinases between T cell subtypes likely indicates that PGE₂ has largely shared functions in

different T cell subtypes. However, there appear to be some interesting exceptions, for instance CK2, where PGE₂ may signal through distinct pathways and distinct kinase nodes depending on cell type, leading to distinct PGE₂ functional output between cell types as well.

Tyrosine-centered phosphorylation motifs

Only approximately 2% of identified phosphosites by IMAC-based MS studies are tyrosine-centered [90], and a low abundance of these sites was observed in the current study as well. A few tyrosine phosphosites were, however, regulated. Phosphorylation of the inhibitory Tyr⁵⁰⁵ site on LCK was found to be increased in all stimulation conditions in CD4⁺ cells, perhaps indicating that the inhibitory pathway involving phosphorylation of this site is more active in CD4⁺ than in CD8⁺ cells [42]. Further, phosphorylation of the activating site on Src or Fyn (Tyr⁴¹⁹ or Tyr⁴²⁰, respectively) was decreased upon EP₂ stimulation, in line with the previous observation that cAMP-PKA signaling reduces Src activity [99]. On the other hand, phosphoflow cytometry studies with inhibitors (Fig. 4B-F) suggested the activation of Src, as did network modeling, perhaps indicating transient activation.

Predicting functions of PGE₂ in T cells

A general problem in phosphoproteomics studies currently is that most phosphosites detected have no known biological function, making functional assignments challenging [92]. However, using function prediction analysis of PGE₂-regulated phosphosites, it was possible to identify some sites with known or predicted biological function (Table 1 and table S3), and gene ontology analysis further implicated PGE₂-regulated species in key cellular and immunological processes (Fig. 2). One of the most highly predicted processes in all stimulation conditions was cytoskeleton remodeling, which agrees with findings in other cell types [56]. and may be mediated partly by PKA through phosphorylation of proteins involved in cytoskeletal processes, such as VASP Ser¹⁵⁷, Ser²³⁹ and Thr²⁷⁸ [100], VIM Ser³⁸ [65] or HSPB1 Ser⁷⁸ (Fig 4). Processes related to RNA processing, including transcription, were also highly predicted (Fig. 2), in line with the observed regulation of several transcription-related

outputs (Histone H3 and CREB1) by phosphoflow cytometry, indicating that PGE₂ may also be important in regulating RNA-related cellular functions. PGE₂ signaling through the four EPs was also implicated in regulating several important immune-specific functions such as T cell proliferation (Fig. 2B), which agrees with a large literature [27, 101, 102]. In addition, a number of the regulated phosphoflow cytometry readouts, notably Akt, NDRG, S6RP, and CREB1, are also involved in cell proliferation, further supporting a role for PGE₂ in regulating T cell proliferation. TCR signaling was predicted by GO analysis in the EP₂ and PGE₂ conditions, in line with a literature on the inhibition of TCR signaling by EP₂, and to some extent EP₄, by PGE₂ [42, 43]. Finally, T cell activation was highly predicted in several conditions, as also seen in previous studies [53]. Together, our results support a broad role for PGE₂ signaling through the four EP receptors in regulating important cellular and immunological functions in T cells.

Cell type and receptor differences in PGE₂ signaling

Some signaling pathways become only very transiently activated upon PGE₂ stimulation [55], necessitating the use of a dynamic profiling technique with high temporal resolution, such as multiplexed phosphoflow cytometry. Our phosphoflow panel consisted of 16 antibodies, recognizing phosphorylated proteins involved in a number of biological processes, ranging from translational regulation (S6RP) to cytoskeletal remodeling (VASP, VIM, HSPB1), proliferation (NDRG1) and transcription (Histone H3, CREB1), echoing the functions predicted for PGE₂ in the GO analysis of regulated sites in the phosphoproteomics study.

The temporal profiles of EP₂ and EP₄ signaling differed markedly in spite of both receptors primarily coupling to G_{αs}. The shorter signal duration of EP₄ could be due to more rapid receptor internalization [103]. As for the differing signal intensity, EP₄ is thought to have weaker functional coupling to cAMP and PKA than EP₂ due to its ability to also couple to G_{αi} [30]. Thus, stimulation of EP₂ by PGE₂ leads to higher cAMP amounts than equivalent stimulation of EP₄, assuming equal abundances of the receptors [29]. In fact, EP₂ may be

present in higher amounts than EP₄ in T cells [26]. Thus, PKA-dependent readouts would be expected to change more strongly in response to EP₂ stimulation [30], as observed here. In addition, G_{ai} signaling is associated with concomitant G_{βγ} release and signaling, and in the case of EP₄, G_{ai} coupling triggers a PI3K signaling pathway and inhibits PKA [28, 104], possibly also contributing towards the observed lower signaling intensity for EP₄. We speculate that the differences in signaling intensity and kinetics between the EP₂ and EP₄ receptors may yield distinct cellular effects even though many of the sites regulated are shared between the two receptors.

Most phosphoflow cytometry readouts displayed the highest levels of signaling in CD8CD45RO cells, with a few readouts (notably NDRG1 and CREB1) showing the highest signaling levels in CD4 T cells. Inhibitor phosphoflow cytometry experiments echoed the findings from kinase predictions and motif analysis, namely that while some kinases, such as PKA, contribute strongly in all cell types, other signaling pathways contribute differentially across T cell subtypes, highlighting the diversity and complexity of PGE₂ functions in the immune system.

Evidence for G protein–dependent PGE₂ signaling pathways from network modeling

The G_α-triggered pathways for the EP receptors were likely responsible for much of the signaling observed by MS and phosphoflow and were all recapitulated in the modeled networks. For instance, EP₂ and EP₄ signaling through G_{as} and PKA-based pathways were present in the modeled networks both in CD4⁺ and CD8⁺ cells and many PKA substrates were phosphorylated in response to EP₂ and EP₄ stimulation in the phosphoflow experiments. For EP₃ on the other hand, signaling through the G_{ai} pathway would be predicted to reduce PKA activity. No clear evidence for this was observed in the phosphoflow cytometry results. This could be due to lower expression of EP₃ and EP₁ than EP₂ and EP₄ in T cells [26], EP₃-mediated activation of additional intracellular signaling pathways through G_{αq}, G_{as} and G_{α12} [20] that counterbalance G_{ai} signaling, or activation of certain adenylyl cyclase isoforms by G_{βγ} signaling, increasing cAMP and activating PKA [28]. The modeled

networks for the EP₁ receptor recapitulated the G_{αq} signaling pathway with activation of PLC and PKC.

The literature suggests that G_{βγ} signaling may be active downstream of EP₄ [28], EP₂ [105] and possibly also EP₃, when this receptor couples to G_{αi}, the G_α protein most frequently associated with G_{βγ} signaling [75, 106]. G_{βγ} subunits can affect a number of different pathways including PI3K, PKA, PAK, Raf-1, and more [75], all of which we saw evidence for in either kinase prediction, motifs or network models.

Evidence for G protein-independent PGE₂ signaling pathways from network modeling

There is some evidence in the literature that PGE₂ also triggers G protein-independent signaling. In particular, EP₂, EP₃ and EP₄ can couple to G protein-coupled receptor kinase (GRK) and β-arrestin [28, 107, 108], both of which can initiate their own signaling pathways [76, 77, 109]. For instance, EPs may transactivate EGFR through β-arrestin and Src activation [33, 107, 110-112], which in turn triggers additional signaling pathways, including PI3K-Akt, Ras-Raf and more [19]. It is unclear whether this transactivation also occurs in T cells, but in our inhibitor experiments, we observed that EGFR inhibition in T cells did in fact affect some phosphoflow cytometry readouts. EGFR transactivation was also predicted in the modeled network for the combined stimulation condition in CD8⁺ cells (fig. S22). By phosphoflow cytometry, EGFR and PI3K inhibitors affected many of the same pathways and readouts, for instance CREB1 pSer¹³³, HSPB1 pSer⁷⁸, NDRG1 pThr³⁴⁶, PKA substrates, and S6RP pSer²⁴⁰ (Fig. 4E), possibly due to pathway convergence.

β-arrestin can also trigger MAP kinases (in particular Raf and MAP3K5), PI3K, RhoA and Src signaling pathways [77]. Many of these were predicted in the modeled networks (Fig. 5A-C). In support of RhoA signaling, several ARHGEFs, which are small GTPase activators, were regulated in the current dataset (Table 1, table S3).

Other parts of the EP₁₋₄ inactivation process may also contribute to signaling output, and the data suggests some involvement of RGS proteins, for instance through the

regulation of phosphorylation of RGS3, RGS11, and RGS14 observed in the phosphoproteomics data. A few known GRK substrates were also regulated in the phosphoproteomics dataset, including NHERF and HDAC [109] and GRKs were further predicted by NetPhorest to regulate some of the phosphosites seen by MS, mostly in CD8. Thus, the current study supports an important role for G protein–independent signaling alongside G protein–dependent signaling in PGE₂ signaling in T cells.

Concluding Remarks

Here, we have conducted a systems-level study of PGE₂ signaling pathways in helper, cytotoxic, and regulatory T cell subsets. We present a comprehensive and detailed view of PGE₂-regulated signaling nodes, pathways, and networks in T cell subsets, thus improving the current understanding of PGE₂'s multifaceted role in T cells and providing a valuable resource for targeted research on this physiologically and pathophysiologically important signaling system.

MATERIALS AND METHODS

Agonists and antagonists

EP₁ agonist ONO-DI-004, EP₂ agonist ONO-AE1-259-01, EP₃ agonist ONO-AE-248, EP₄ agonist ONO-AE1-329, EP₁ antagonist ONO-8713 and EP₃ antagonist ONO-AE3-240 were provided under an MTA with ONO Pharmaceuticals. EP₂ antagonist TG4-155 (Cat. No. 17639) and EP₄ antagonist ONO-AE3-208 (Cat. No. 14522) were both from Cayman Chemicals. PGE₂ (Cat. No. P5640) was from Sigma Aldrich.

Antibodies

CD3-PerCP Clone SK7 (Cat. No. 345766), CD4-PE-Cy7 Clone SK3 (Cat. No. 348809), CD45RA APC-H7 (Cat. No. 560674), CD3-PB Clone UCHT1 (Cat. No. 558117), CD4-PerCP (Cat. No. 550631), CD8-PE-Cy7 (Cat. No. 557746), FOXP3-Ax647 (Cat. No.

560045), IgG1 Kappa-Ax647 (Cat. No. 557783), CREB1 pSer¹³³/ATF1 pSer⁶³-Ax647 (Cat. No. 558434), Histone H3 pSer²⁸-Ax647 (Cat. No. 558217), S6RP pSer²⁴⁰-Ax647 (Cat. No. 560432), PKA RII α (PRKAR2A) pSer⁹⁹-Ax647 (Cat. No. 560164) and PKA RII β (PRKAR2B) pSer¹¹⁴-Ax647 (Cat. No. 560205) were from BD. S6RP pSer^{235/236}-Ax647 (Cat. No. 4851), Histone H3 pSer¹⁰-Ax647 (Cat. No. 9716), NDRG1 pThr³⁴⁶-Ax647 (Cat. No. 7497), pPKA Substrate (RRXS/T) (Cat. No. 9624), PLC γ -1 (PLCG1) pThr⁷⁸³ (Cat. No. 2821), p-Akt substrate (RXRXXS/T) (Cat. No. 9614), GSK3 α (GSK3A) pSer²¹ (Cat. No. 9316), HSP27 (HSPB1) pSer⁷⁸ (Cat. No. 2405), VASP pSer¹⁵⁷ (Cat. No. 3111), PKA-C (PRKACA) pThr¹⁹⁷ (Cat. No. 4781) were from CST. CD25-PE Clone 4E3 (Cat. No. 130-091-024) was from Miltenyi. CD127-PECy7 clone RDR5 (Cat. No. 25-1278-73) was from eBioscience. Pacific Blue Succinimidyl Ester (Cat. No. P10163), Alexa Fluor 488 Succinimidyl Ester (Cat. No. A3005), Pacific Orange Succinimidyl Ester (Cat. No. P30253), Goat anti-mouse IgG1 secondary antibody Ax647 (Cat. No. A21240), Goat anti-rabbit IgG secondary antibody Ax647 (Cat. No. A21245) were from Thermo Fisher. Vimentin (VIM) pSer³⁸ (Cat. No. Ab52942) was from Abcam.

Kinase Inhibitors

PKA inhibitor H-89 (Cat. No. 10010556, Cayman Chemicals), PI3K inhibitor wortmannin (Cat. No. W1628, Sigma Aldrich), PI3K inhibitor LY294002 (Cat. No. 9901, CST), MEK inhibitor CI-1040 (Cat. No. Sc-202759, Santa Cruz), Src inhibitor PP2 (Cat. No. 529573, Calbiochem), GSK3 inhibitor CT99021 (Cat. No. Axon 1386, Axon Medchem), CK2 inhibitor TBB (Cat. No. 2275, Tocris), EGFR inhibitor AG1478 (Cat. No. S2728, Selleck Chemicals).

Patient material and ethical considerations

Buffy coats were obtained from anonymized healthy blood donors [Oslo University Hospital Blood Centre, Oslo, Norway; studies were approved by the Regional Ethics

Committee, all donors gave their consent and the research on human blood was carried out in accordance with the Declaration of Helsinki (2013)]

Purification of CD3, CD4, CD8 and regulatory T cells

Human peripheral blood CD3, CD4 and CD8 T cells were isolated from buffy coats from healthy blood donors using RosetteSep Enrichment Kits for CD3, CD4 or CD8 cells (StemCell Technologies) followed by gradient centrifugation with LymphoPrep™ (Axis-Shield) according to the manufacturer's protocol, but using PBS instead of 2% FCS (Thermo Fisher Scientific) in PBS during washes. For Treg isolation, CD4 cells isolated as described above were processed using a CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's protocol. Cells were then suspended to 1×10^6 cells/mL in X-VIVO 15 medium (Lonza) with 10% FCS (Invitrogen), 1x Penicillin-Streptomycin (ThermoFisher Scientific), 100nM rapamycin (Calbiochem), 500U/mL rIL-2 (Invitrogen). 100,000 cells were plated per well in 96-well plates, and 400,000 CD3/CD28 MACSibeads (T cell Activation/Expansion Kit, Miltenyi Biotec, prepared according to the manufacturer's instructions) were added. Media was replaced on day 1 after plating, then every 4-5 days until cells were harvested at day 14 post-plating. Cells were moved to larger well-plates at appropriate times during expansion.

Stimulation of Cells for MS

For stimulation, cells were suspended in RPMI 1640 GlutaMax medium (Thermo Fisher Scientific) and diluted to 20×10^6 cells/mL. One-milliliter aliquots were then equilibrated in a 37°C water bath for 30 minutes prior to stimulation. CD4, CD8 or Treg cells (20 million per condition) were stimulated with 1μM EP₁ agonist (ONO-DI-004), 0.04μM EP₂ agonist (ONO-AE1-259-01), 0.05μM EP₃ agonist ONO-AE-248 (10x EC₅₀), 0.052μM EP₄ agonist (ONO-AE1-329), 10μM PGE₂ or vehicle for 5 minutes. Cells were then centrifuged (400g, 2min, 4°C), the supernatant was removed and the pellet snap frozen on LN₂. For each cell type, buffy coats from five healthy blood donors was utilized, providing 5 biological replicates.

Protein Lysis and Digestion

Cells were lysed at 4 °C with a Bioruptor Plus (Diagenode) for 15 cycles of 30 s, in buffer containing 50 mM ammonium bicarbonate (pH 8.0), 8 M urea, 1 mM sodium orthovanadate, complete EDTA-free protease inhibitor mixture, and phosSTOP phosphatase inhibitor mixture (both Roche). Cell debris were then removed by centrifugation at 20,000g for 10 min at 4 °C. The total protein concentration was measured using a Bradford assay (Bio-Rad). Proteins were reduced with dithiothreitol at a final concentration of 4 mM at 56 °C for 25 min; subsequently samples were alkylated with iodoacetamide at a final concentration of 8 mM at RT for 30 min in the dark. Proteins were then pre-digested using Lys-C (enzyme:substrate ratio 1:100) for 4 h at 37 °C. The solution was then diluted to a final urea concentration of 2 M with 50 mM ammonium bicarbonate (pH 8.0), prior trypsin digestion at 37 °C overnight (enzyme:substrate ratio 1:100). The digestion was quenched by acidification to 5% of formic acid. The digests were desalted using Sep-Pak C18 cartridges (Waters), dried *in vacuo*, and stored at -80 °C until further use.

Phosphopeptide Enrichment by Ti^{4+} -IMAC

Ti^{4+} -IMAC material was prepared and used essentially as previously described [113]. In-parallel spin tip enrichment [55] by centrifugation at 50-100g was performed as follows: columns were conditioned using 50 μ L of loading buffer (80% acetonitrile/6% trifluoroacetic acid), 200 μ g protein digests dissolved in loading buffer were loaded and then, the columns were sequentially washed with 50 μ L of 50% ACN, 0.5% TFA containing 200 mM NaCl and 50 μ L of 50% ACN/0.1% TFA. The bound phosphopeptides were eluted into a new tube (containing 30 μ L of 10% formic acid) with 20 μ L of 10% ammonia. A final elution was performed with 10 μ L of 80% ACN/2% formic acid. The collected eluate was further acidified by the addition of 5 μ L of 100% formic acid, dried *in vacuo*, and desalted using C18-StageTips [114], prior to nLC-MS/MS analysis.

Reverse Phase Chromatography and Mass Spectrometry

Peptides were subjected to reverse phase nLC-MS/MS analysis using a Proxeon EASY-nLC 1000 (Thermo Scientific) and a LTQ-Orbitrap Elite (Thermo Scientific) or using and Agilent 1290 Infinity UHPLC system (Agilent) and an Orbitrap Fusion mass spectrometer (Thermo Scientific). Peptides were first trapped (Reposil C18, Dr Maisch, 3 μm , 2 cm x 100 μm) at 5 $\mu\text{L}/\text{min}$ with 100% solvent A (0.1% formic acid in water) before being separated on the analytical column (Agilent Poroshell 120 EC-C18, Agilent, 2.7 μm , 40 cm x 50 μm). Peptides were chromatographically separated by a 90 min gradient from 7% to 30% (or 95 min gradient from 4% to 36% for the Agilent 1290) of solvent B (0.1% formic acid in 80% ACN) at a flow rate of ~ 100 nL/min. The total measurement time for each sample was 110 min. The mass spectrometer was operated in a data-dependent mode to automatically switch between MS and MS/MS. Briefly, survey full-scan MS spectra were acquired in the Orbitrap analyzer, scanning from m/z 350 to m/z 1500 at a resolution of 60,000 using an automatic gain control setting of $1\text{e}6$ ions (or $4\text{e}5$ for the Orbitrap Fusion). Charge state screening was enabled, and precursors with either unknown or 1+ charge states were excluded. After the MS survey scan, the 20 most intense precursors were selected for subsequent CID or ETD fragmentation by a decision-tree-based method [115] with ion trap readout. The normalized collision energy for CID was set at 35%, and supplemental activation for ETD, and dynamic exclusion were enabled (40 s, or 18 s for the Agilent 1290).

Data Analysis

Raw files were processed using MaxQuant (version 1.5.2.8) [116]. Proteins and peptides were identified using a target-decoy approach with a reversed database, using the Andromeda search engine integrated into the MaxQuant environment. The database search was performed against the human Swiss-Prot database (version August, 2014) and against a common contaminants database. Default settings were used, with the following minor changes: oxidation (M), Acetyl (Protein N-term), and Phospho (STY) as variable modifications Enzyme specificity was set to trypsin with a maximum of 2 missed cleavages

and a minimum peptide length of 6 amino acids. A false discovery rate (FDR) of 1% was applied at the protein, peptide and modification level. A site localization probability of at least 0.75 was used as thresholds for the localization of phosphorylated residues. The “match between runs” feature was enabled.

Bioinformatics analysis was performed with Perseus [117], and R statistical computing software [118]. The 3 data sets were processed individually, and data were filtered to make sure that identified phosphorylation sites showed quantification value in all 5 biological replicates of at least one stimulation, and missing values were then imputed on the basis of normal distribution (down shift = 1.8, width = 0.15), as implemented in the Perseus software [117]. Significance was assessed by t-test with a permutation-based FDR of 5% and a S0 parameter of 0.1.

Venn diagrams were produced using the following tool:

<http://bioinformatics.psb.ugent.be/webtools/Venn/>. Significantly regulated phosphorylation sites were subjected to IceLogo [119], using percent difference compared to the reference set Swiss-Prot means for *Homo Sapiens*, with a significance set to 0.05. Kinases responsible for regulating the observed regulated phosphosites were predicted using the tool NetPhorest [58]. The sequence database used was “Human – Uniprot 2013/01 (MaxQuant)”. Default settings were used, with minimum score=2 and max difference=4. The max number of predictions was set to 1. The tool “Predict Functional Phosphosites” (PFP) (<http://www.kiharalab.org/web/pfp.php>) [61] was used to predict which of the regulated phosphosites observed in the current study are likely to be functional. The database was downloaded on 30/3/2013, version

“pfp_database_release_1_2_update_1__20160126.csv.zip”. Regulated phosphosites were searched against the database, and lists compiled of regulated phosphosites that were actual class positive (known-function human phosphosites from the PhosphoSitePlus database, file name: Regulatory_sites, version: 060415) and regulated phosphosites that were either actual class positive or that have at least one positive prediction (in RandomForest, BayesNet, Logistic or Multilayer Perceptron models). Gene Ontology

analysis was performed using the ClueGO cytoscape plugin [120]. Regulated proteins in each condition were searched against the KEGG, GO Biological Process-EBI-QuickGO-GOA and Wikipathways databases using the following ClueGO parameters: GO term fusion selected; show only pathways with $p \leq 0.05$; GO Tree Interval=all levels; GO term minimum number of genes=3; 4% of genes per pathway; kappa score=0.42. Regulated immune processes were identified by querying the regulated proteins against the GO ImmuneSystemProcess-EBI-QuickGO-GOA database. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium through the PRIDE partner repository [121] with the dataset identifier PXD014503.

Isolation, Stimulation and Fixation of Cells for Phosphoflow Cytometry

CD3 cells were resuspended in RPMI 1640 GlutaMAX medium (Thermo Fisher Scientific) and pre-equilibrated for 10 minutes in a 37°C water bath prior to pre-incubation with kinase inhibitors or antagonists or stimulation with EP₁₋₄ agonists, PGE₂ or DMSO control (maximum total DMSO concentration 0.3%). Antagonists were added 5 min prior to stimulation and inhibitors 30 min prior to stimulation. Agonists and antagonists were used at the following concentrations, except where indicated otherwise: EP₁ agonist ONO-DI-004 (1 µM), EP₂ agonist ONO-AE1-259-01 (40 nM), EP₃ agonist ONO-AE-248 (50 nM), EP₄ agonist ONO-AE1-329 (52 nM), EP₁ antagonist ONO-8713 (1 µM), EP₂ antagonist TG4-155 (150 nM), EP₃ antagonist ONO-AE3-240 (150 nM), EP₄ antagonist ONO-AE3-208 (100 nM). Inhibitors were used at the following concentrations: PKA inhibitor H-89 (20 µM), PI3K inhibitor Wortmannin (1 µM), PI3K inhibitor LY294002 (10 µM), MEK inhibitor CI-1040 (2 µM), Src inhibitor PP2 (10 µM), GSK3 inhibitor CT99021 (2 µM), CK2 inhibitor TBB (10 µM), EGFR inhibitor AG1478 (10 µM).

At the indicated timepoints, samples were harvested by fixation for 10 minutes with pre-warmed Phosphoflow Fix Buffer I (Cat. No. 557870, BD) at 37°C followed by two washes with PBS. An unstimulated sample was collected prior to stimulation.

Fluorescent Cell Barcoding (FCB)

Fixed cells were incubated with different concentrations of NHS-coupled Alexa Fluor 488, Pacific Orange and Pacific Blue in a 96-well V-bottom plate for 20 minutes in the dark at room temperature. Cells were washed twice with flow buffer (PBS + 2% FCS), combined and permeabilized with ice-cold Phosphoflow™ Perm Buffer III (BD, Cat. No. 558050), then stored at -80°C until analysis.

Antibody staining and phosphoflow cytometry

Permeabilized cells were thawed on ice and washed once with flow buffer. Cells were then resuspended in flow buffer and plated in a 96-well V-bottom plate. Cells were stained with PerCP-conjugated anti-CD3, PE-Cy7-conjugated anti-CD4, APC-H7-conjugated anti-CD45RA and the indicated phosphoantibodies at room temperature, in the dark for 30 minutes. Cells were then washed twice with flow buffer. For unconjugated phosphoantibodies, a second staining step was performed with Ax647-conjugated secondary antibody for 30 minutes in the dark, followed by two washes with flow buffer. Cells were then analyzed on an LSR Fortessa flow cytometer (BD Biosciences). Compensation was performed using unstimulated cells stained with Alexa Fluor 488, Pacific Orange and Pacific Blue, as well as compensation beads incubated with PerCP-, PE-Cy7-, APC-H7- and Ax647-conjugated antibodies. 150 000- 1 000 000 events were recorded per sample.

Phosphoflow Cytometry Data Analysis

The data was analyzed in Cytobank (<https://cellmass.cytobank.org/cytobank/>). Lymphocytes were selected by plotting SSC-A versus FSC-A. Singlets were selected by plotting FSC-H versus FSH-W. Each barcoding channel was then plotted against SSC-A to identify the different barcoding populations. Subsequently, cells were gated for CD3, CD4 and CD45RA. CD4 and CD8 cells were identified by the presence or absence of the CD4 marker, while CD45RA and CD45RO cells were identified by the presence or absence of the CD45RA marker. Signals for the phosphoantibodies were calculated as inverse hyperbolic

sine (arcsinh) ratios of MFIs for stimulated versus unstimulated cells. Statistical analyses were performed in GraphPad Prism 7.02 (GraphPad Software). For IC50 and EC50 calculations, a three-parameter nonlinear regression was employed, with the Hill coefficient set to 1. The equations used were $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC50} - X)))}$ and $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(X - \text{LogIC50})})$.

Network Modeling with PHONEMeS

PHONEMeS requires the use of a Prior Knowledge Network (PKN). First, the PKN is formalized as a boolean model. Then, the boolean model is trained with experimental data to find which interactions are relevant in the context of the present study. For assembling our PKN, we used OmniPath [122], a comprehensive collection of 57 pathway resources (<http://omnipathdb.org/info>). As a first step, we built the PKN by including all the K/P-S interactions from OmniPath. Since there were no connecting paths between GPCR receptors and measured phosphosites in the K/P-S network alone, we mapped proteins involved in GPCR downstream signaling (obtained from MSigDb, http://software.broadinstitute.org/gsea/msigdb/cards/REACTOME_GPCR_DOWNSTREAM_SIGNALING), [123] on the signed and directed Protein-Protein Interactions (PPI) of Omnipath to create a GPCR downstream signaling causal network. This network was then combined with the K/P-S network and a list of manually curated interactions from the literature (Data File S2). Additionally, functionally related G-Protein subunits and other proteins in the PKN were grouped together (Data File S3). This yielded a list of 26367 interactions, 2414 of which are signed and directed protein interactions, while the rest is all the set of K/P-S interactions currently present in OmniPath. We use this PKN to train and contextualize the cell type specific signalling networks for CD4 and CD8. The next step consists of preparing the data inputs for PHONEMeS. Sites which have no interaction evidence in the PKN will be ignored. Significantly regulated sites for each cell type were identified through statistical testing with a permutation-based FDR (Benjamini-Hochberg method) at a threshold value of $p_{Thresh} = 0.05$ for CD4 and CD8 cell types. We assigned to

each of the measurements i at each experimental condition j (EP₁, EP₂, EP₃, EP₄ and PGE₂) a score based on their inferred adjusted p-values $S_{i,j} = \log_2(pVal_{i,j}/pThresh)$. Significantly regulated sites (with $pVal_{i,j} < 0.05$) are assigned a negative score ($S_{i,j} < 0$), while the rest of measurements (with $pVal_{i,j} \geq 0.05$) are assigned a positive score ($S_{i,j} > 0$). An Integer Linear Programming (ILP) implementation of PHONEMeS was used for training the network to the input data. The ILP formulation consists of two main parts: an objective function and a set of linear constraints whose variables are all binaries (indicating the presence/absence of a node or interaction in the optimal solution). The objective function represents the cost function of the ILP problem. In this case it is defined as a minimization of the sum of scores assigned to each node (each node representing a specific site) on each of the conditions considered for the PHONEMeS analysis. Since this is a minimization problem, the method incorporates as many regulated sites (with negative score assigned $S_{i,j} < 0$), while at the same time penalizing the inclusion of the non-regulated measurements (which were assigned a positive score $S_{i,j} > 0$). Additionally, a set of constraints in the ILP formulation determines the set of feasible paths connecting the upstream prostaglandin receptors with the downstream measurements. In this case, a feasible path is a set of interactions present in the PKN connecting the prostaglandin receptors with the measurements through intermediate nodes. A size penalty factor ($\lambda = 0.0001$) over the number of interactions is also applied in the objective function, so as to systematically apply Occam's razor to the final set of networks. This size penalty is small compared to the scores (in absolute values) assigned to each measured node and is added such that simpler models (with fewer edges in the solution) are preferred over the larger ones. The ILP problem is solved through the CPLEX-IBM optimizer. Codes for the modelling are available at: https://github.com/saezlab/Prostaglandin_Project

Phenotyping of CD4, CD8 and Tregs for MS

To verify the purity of the isolated CD4 and CD8 cells used for MS studies, cell samples were fixed and permixed using Phosflow buffers (BD), followed by staining (20 min. at room temperature) for relevant surface markers. For CD4 cells, CD3-PerCP (1 μ L/100 μ L final

volume) and CD4-PE-Cy7 (1 μ L/100 μ L final volume), were used. For CD8 cells, CD3-PB (0.5 μ L /100 μ L final volume), CD4-PerCP (2 μ L/100 μ L final volume) and CD8-PE-Cy7 (0.2 μ L /100 μ L final volume) were used. All antibodies were from BD.

To confirm the purity of the expanded Tregs, a small sample of the cells used for MS studies were fixed and permeabilized with FOXP3 buffers (BD) according to the manufacturer's instructions, then stained for 20 minutes at room temperature with the following antibodies against cell surface markers: CD3-Pacific Blue (0.5 μ L/100 μ L final volume, BD), CD4-PerCP (2 μ L/100 μ L final volume, BD), CD25-PE (5 μ L/100 μ L final volume, Miltenyi), FOXP3-Ax647 (5 μ L /100 μ L final volume, BD), CD127-PECy7 (0.25 μ L/100 μ L final volume, eBioscience).

Treg Suppression Assay

Effector T (Teff) responder cells (CD4⁺CD25⁻), were isolated from buffy coats from healthy human donors by first purifying CD4 cells as described in the paragraph "Purification of CD4, CD8 and regulatory T cells" and then depleting CD25 cells within this population. To deplete CD25 cells, CD4 cells were first suspended in MACSi buffer (PBS with 0.5%BSA and 2mM EDTA) at 10⁷ cells per 80 μ L buffer. 20 μ L of anti-CD25 microbeads (Miltenyi) were added per 10⁷ cells and incubated on ice for 15 minutes, followed by addition of 50mL MACSi buffer and centrifugation (350g, 10min). Supernatant was removed and cells suspended in 1.5mL MACSi buffer. An LD column (Miltenyi Biotec) was placed in magnetic holder, wetted with 2mL MACSi buffer, followed by loading of cells. Cells were allowed to pass through by gravity, followed by rinsing of the column twice with 1mL MACSi buffer. Eluate was washed twice with RPMI, followed by labeling with 2 μ M CFSE (Sigma) in RPMI for 10 minutes at 37°C. 10x volume of 100% cold FCS was added to quench the labeling reaction, followed by washing with 10% FCS in RPMI.

To test suppression of Teff proliferation by regulatory T cells, 200,000 CFSE-labeled Teffs were plated in 10% FCS in RPMI in round-bottom 96-well plates together with varying numbers of Tregs. Cells were stimulated with 42,000 CD2/CD3/CD28 MACSibeads (T cell Activation/Expansion Kit, Miltenyi Biotec, prepared according to the manufacturer's

instructions). Cells were allowed to proliferate for 4 days in a 37°C incubator, then fixed with FOXP3 buffer A (BD) and frozen, then run on an LSR Fortessa.

FOXP3 promoter methylation

To assess methylation levels of the FOXP3 promoter, which is an indicator of bona fide Treg status, DNA was isolated using the DNeasy kit (Qiagen) from expanded Tregs. Cells from two healthy buffy coats were used and Tregs were isolated and expanded as described in the section “Purification of CD4, CD8 and regulatory T cells”. After DNA extraction, unmethylated cytosines were converted to uracils using sodium bisulfite conversion (Epitect Bisulfite Kit from Qiagen). Converted DNA was subjected to PCR amplification with primers Fxpro-met_F1 and Fxpro-met_R2 (Miyara, M., et al., *Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor*. Immunity, 2009. **30**(6): p. 899-911.), Taq polymerase (Invitrogen) and the following thermocycler conditions: 15 minutes at 94°C, 40 cycles of 1 minute at 94°C, 45 sec at 60°C, 1 minute at 72°C, and then a final extension period of 10 minutes at 72°C. PCR products were cleaned up using a PCR purification kit (Saveen) and cloned into a sequencing vector using the pGEM®-T Easy Vector Systems (Promega) according to the manufacturer’s protocol. At least ten clones were sequenced for each donor/cell type, and the demethylation of ten CpG methylation sites within the Treg-specific demethylated region was assessed (-256, -216, -139, -127, -114, -78, -66, -59, -44, -16).

Western blots

CD4, CD8 and Treg cells were isolated/expanded as described in the section “Purification of CD4, CD8 and regulatory T cells”. Frozen pellets were lysed in 200µL RIPA with complete protease inhibitor tablets (Roche) for 10 minutes on ice. Samples were then sonicated 10x and centrifuged (16.1g, 10min, 4°C) and the protein concentration of the supernatant determined by Bradford assay. 65ug of protein was loaded per lane on precast SDS-PAGE gels (Biorad), which were run at 120V for 1hr 45min. Transfer onto an

Immobilon-P PVDF membrane (Merck) proceeded at room temperature for 1hr at 100V. Membranes were blocked in 5% milk in TBS-T in cold room overnight. Staining with primary antibodies diluted in 5% milk in TBS-T (EP₁ Receptor Polyclonal Antibody at 1:200 (Item no 101740, Cayman Chemicals), EP₂ Receptor Polyclonal Antibody at 1:1000 (Item no 101750, Cayman Chemicals), PTGER3/EP₃ Antibody at 1:2000 (Item NBP1-00810, Novus Biologicals), PTGER4/EP₄ Antibody at 1:1000 (Item NBP1-84833, Novus Biologicals) or actin C-11 at 1:2000 (Santa Cruz) proceeded for 90 minutes at room temperature, followed by washing 3 times with TBS-T. Membranes were then incubated with Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) secondary antibody (Jackson ImmunoResearch) for 90 minutes at 1:10000 dilution in 5% milk in TBS-T, followed by 3 washes with TBS-T. Membranes were then developed with SuperSignal™ West Dura Extended Duration Substrate (Pierce).

SUPPLEMENTARY MATERIALS

Figs. S1 to S22.

Tables S1 to S5.

Data Files S1 to S3.

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other data needed to evaluate the conclusions in the paper are present in the main text or Supplementary Materials.

FIGURE LEGENDS

Fig. 1. Phosphoproteomic analysis reveals regulation of phosphosites by PGE₂ receptors in T cells. (A to C) Significantly regulated phosphoproteins in CD4⁺ (A), CD8⁺ (B), and regulatory (C) T cells stimulated with 1μM EP₁ agonist (ONO-DI-004), 0.04μM EP₂ agonist (ONO-AE1-259-01), 0.05μM EP₃ agonist ONO-AE-248 (10x EC50), 0.052μM EP₄ agonist (ONO-AE1-329), 10μM PGE₂, or vehicle. Significantly regulated phosphosites (two-way t-test, S0=0.1, FDR =5%) are shown in red. For each cell type, 5 healthy blood donors were utilized, providing 5 biological replicates. (D) Quantitation of regulated phosphosites under the different stimulation conditions in different subtypes of primary T cells. (E) Venn diagrams showing unique and overlapping phosphosites regulated under different stimulation conditions in CD4⁺ and CD8⁺ cells. (F) Groups of kinases regulating the observed changing phosphosites in CD8⁺ cells were predicted using NetPhorest. (G) IceLogos showing the phosphorylation motifs that were most increased for the CD8⁺ EP₂ stimulation condition. The IceLogo for all phosphosites that increased in abundance is shown above the IceLogos for those sites with (=) or without (≠) proline (P) in the P1 position.

Fig. 2. Gene ontology analysis identifies regulated biological processes in CD8⁺ cells.

(A) Regulated biological processes are displayed as a pie chart for two selected stimulation conditions (EP₂ and PGE₂) in CD8⁺ cells. Regulated proteins in each condition were searched against the KEGG, GO Biological Process-EBI-QuickGO-GOA, and Wikipathways databases. (B) Regulated immune processes in the EP₂ and PGE₂ stimulation conditions were identified by querying the regulated proteins against the GO ImmuneSystemProcess-EBI-QuickGO-GOA database. Regulated biological processes and pathways and associated

regulated proteins are displayed in Cerebral (cell region–based rendering and layout) format, which superimposes the interaction network on the subcellular locations of the components.

Fig. 3. Phosphoflow cytometry demonstrates receptor- and cell type–specific regulation of a panel of markers. (A) Schematic of the stimulation setup and graph key. (B) Time courses of the regulation of the 16 indicated phosphopeptides monitored by phosphoflow after stimulation. Cells were stimulated with either a single receptor-specific agonist or PGE₂, which targets all four receptors. The agonists used were 1 μM ONO-DI-004 (EP₁), 0.04 μM ONO-AE1-259-01 (EP₂), 0.05 μM ONO-AE-248 (EP₃) and 0.052 μM ONO-AE1-329 (EP₄), and PGE₂ was used at 10 μM. The symbol and color-coding key and graph axis labeling apply to all graphs in the panel. All readouts shown are from CD8⁺CD45RO⁺ cells, except CREB (pSer¹³³) and NDRG (pThr³⁴⁶), which are from CD4⁺CD45RO⁺ cells. (C) Time course for the regulation of the indicated phosphopeptides in CD8⁺CD45RA⁺, CD8⁺CD45RO⁺, CD4⁺CD45RA⁺, and CD4⁺CD45RO⁺ cells stimulated with PGE₂. Cells were stimulated with 1 μM PGE₂, except for NDRG1 pThr³⁴⁶, where stimulation was carried out with 10 μM PGE₂. Data shown is for one representative experiment; two replicates are included in the Supplementary Materials (figs. S12, S13).

Fig. 4. Signaling differences between EP₂ and EP₄ receptors and effects of kinase inhibitors. (A) Comparison of intensity and duration of signaling through EP₂ (40 nM ONO-AE1-259-01) and EP₄ (52 nM ONO-AE1-329) receptors in CD8⁺CD45RO⁺ cells. Data shown is from one representative experiment; replicates are included in the Supplementary Materials (figs. S12, S13). (B) CD3⁺ cells were stimulated with different concentrations of PGE₂ in combination with antagonists to isolate PGE₂ signaling through either EP₂ (combination 1) or EP₄ (combination 2). The graphs show the degree of phosphorylation of the indicated phosphosites relative to the concentration of PGE₂ in CD8⁺CD45RO⁺ cells. Key

findings were repeated with $n=3$ (fig. S15). **(C)** Time course of the abundances of the indicated phosphosites in CD8⁺CD45RO⁺ cells stimulated with combination 1 + PGE₂, combination 2 + PGE₂, or PGE₂ alone. **(D)** Inhibition of the phosphorylation of the 13 indicated phosphosites downstream of PGE₂ by the PKA inhibitor H89. Percent inhibition was calculated relative to 10 μ M PGE₂ stimulation, and phosphorylation responses are shown at 10 min, except for NDRG1 pThr³⁴⁶ (30 min), S6RP pSer²⁴⁰ (30 min), Histone H3 pSer¹⁰ and pSer²⁸ (60 min), and CREB1 pSer¹³³/ATF-1 pSer⁶³ (60 min). Responses were measured in CD8⁺CD45RO⁺ cells, except for NDRG1, which was measured in CD4⁺CD45RO⁺ cells. Data are mean + SEM, $n = 3$. **(E)** Heatmap showing inhibition of phosphosites after 60min of stimulation in the presence of inhibitors in CD8⁺CD45RO⁺ cells. Signals were calculated relative to the unstimulated control. Data are representative of three independent experiments. **(F)** Phosphorylation of NDRG1 (30 min), S6RP (30 min), and CREB1 (60 min) in the indicated cell types in the presence of Wortmannin, H89, or PP2. Data are mean $\pm$ SEM, $n = 3$. Samples were normalized to the unstimulated sample.

Fig. 5. Signaling networks for different stimulation conditions modeled using PHONeMeS. **(A to C)** Phosphosites observed to be regulated by phosphoproteomics were used as input for the PHONeMeS algorithm, which maps this information onto a background network constructed from known kinase and phosphatase–substrate and protein-protein interactions. PHONeMeS then optimizes the network and extracts possible paths connecting the stimulated receptors with the perturbed phosphosites by using an Integer Linear Programming (ILP) formulation, and finally evaluating the network by comparison with the data. This approach yielded network models of each of the five different stimulation conditions in CD4⁺ and CD8⁺ cells. Shown here are the modeled networks for the combined results of stimulation with the four different receptor agonists in CD4⁺ cells (A) and stimulation with only the EP₂ agonist in CD4⁺ cells (B) and CD8⁺ cells (C).

Table 1: Table of regulated phosphosites predicted to be functional. The Predict Functional Phosphosites (PFP) tool (<http://www.kiharalab.org/web/pfp.php>) was used to predict which of the regulated phosphosites identified in the current study are likely to be functional. The table shows regulated phosphosites that have known functions according to the PhosphoSitePlus database (actual class positive in PFP); the majority are also predicted to be functional by one of the four algorithms employed by PFP. For a full overview of all regulated phosphosites predicted to be functional by at least one of the four algorithms in PFP or that are known to be functional according to the PhosphoSitePlus database, see Supplementary Materials (table S2).

Phosphosite	EP1	EP2	EP3	EP4	PGE2
ABI1 S183	CD8	CD8	CD8	CD8	CD8
ARHGEF2 S886		CD8			
ARHGEF6 S225		CD8			
ARHGEF6 S488	CD8	CD8	CD8	CD8	CD8
ARHGEF7 S518	CD8	CD8	CD8	CD8	CD8
ARRB1 S412	CD8	CD8	CD8	CD8	CD8
ATXN1 S775		CD8			
BAD S118	CD8	CD8			
BANF1 S4	CD8	CD8	CD8	CD8	CD8
BANF1 T2	CD8	CD8	CD8	CD8	CD8
BANF1 T3	CD8	CD8	CD8	CD8	CD8
BTG1 S159		CD8			
CAD S1406		CD8			CD8, Treg
CALM2 S82	CD8	CD8	CD8		CD8
CAMKK1 S458	CD8	CD8			
CARHSP1 S41		CD8			
CBL S619		CD8			
CD247 Y123	CD8	CD8	CD8		
CD44 S697					Treg
CD44 S706	CD8	CD8	CD8	CD8	
CDK9 S347		CD8			
CREB1 S271		CD8			
DAP S3		CD8			
DBN1 S142		CD8			
DBNL S269	CD8	CD8	CD8		
DBNL T291		CD8			
DCK S11	CD8				CD8
DNM1L S616		CD8	CD8		
DSN1 S109		CD8			CD4, Treg
EEF1D S133	CD8	CD8	CD8		
EEF2 T57		CD8			
EIF2S2 S2	CD8	CD8	CD8	CD8	CD8
ETS1 S282				CD4	
FAM129A S602		CD8			
FLNA S1459	CD8	CD8	CD8	CD8	CD8
FLNA S2152		CD8			
FOXO1 S287		CD4			
FYN Y420		CD8			
G3BP1 S149				CD8	
HDAC7 S486		CD8			
HMGNI S7		CD8			
HNRNP S284			CD8		
ITPR1 S1598		CD8			
KIF3A S687					Treg
KLC2 S582		CD8			
LASP1 S146	CD4				
LCK Y394		CD8			
LCK Y505	CD4	CD4	CD4	CD4	CD4
LCP1 S5	CD8	CD8	CD8	CD8	CD4
LIG1 S66		CD8			CD8
LMNA S390		CD8			
LSP1 S252	CD8	CD8	CD8		
MAP3K5 S1033	CD4		CD4	CD4	CD4
MCM2 S27		CD8			
MVB12A S170		CD8	CD8		
MYH9 S1943		CD8			
NCOA2 S493	CD8	CD8			
NCOA3 S857		CD8			
NDRG1 S330		CD8			
NF2 S518	CD8	CD8	CD8	CD8	CD8
NIFK T238					Treg
NOP58 S502		CD8			
NPM1 S10		CD8			
PAG1 Y317				CD8	CD8
PAK1 S174			CD8		
PDHA1 S293	CD8	CD8	CD8	CD8	
PEA15 S104		CD8			
PEA15 S116		CD8			
PFKFB3 S461		CD4		CD4	
PIKFYVE S307		CD8			
PKN2 T958			CD8	CD8	
PPP1R12A S445			CD8		CD8
PPP1R12A T696		CD8			
PPP2R5A S41		CD8			
PRKAR1A S77		CD8			
PRKAR1A S83		CD8			
PRKCQ S676	CD8	CD8			
PRKCQ S695		CD8			
PRKCQ T538			CD8		
PRKD2 S710				CD4	
PTPN7 S44	CD8			CD8	CD8
RABEP1 S407	CD8	CD8			
RAF1 S43		CD8			
RBBP8 S327					Treg
RBL2 S1080					Treg
RGS3 S943		CD8			
RPS6 S235					CD8, Treg
RPS6 S236					CD8, Treg
RPS6KA3 S227		CD8	CD8	CD8	CD8
SAMSN1 S23		CD8			
SH3KBP1 S587					CD4
SLAMF6 Y309		CD8			
SMN1 S28				CD4	
SSB S366		CD8			
STAT1 S727		CD8	CD8	CD8	CD8
STAT5A S780		CD8	CD8		CD8

<i>STIM1 S608</i>					Treg
<i>TACC3 S558</i>					CD8
<i>TNFR S764</i>		CD8			
<i>TRIM28 S473</i>	CD8	CD8	CD8		
<i>USP20 S333</i>					Treg
<i>VASP S239</i>					CD8
<i>VIM S26</i>	CD8		CD8	CD8	CD8
<i>VIM S73</i>		CD8			
<i>ZC3HC1 S395</i>	CD8	CD8		CD8