High Resolution Mapping of Prostaglandin E_2 Signaling Pathways in T cell Subsets

A Phosphoflow Cytometry Approach

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Thesis submitted for the degree of
Master of Science in Molecular Bioscience
60 credits

Department of Biosciences
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

May / 17

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2017

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http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo

Acknowledgements

The work presented in this master Thesis was performed at the Norwegian Centre for Molecular Medicine (NCMM), University of Oslo, with the supervision of Professor Kjetil Taskén and Anna Mari Lone. Foremost, I wish to thank Kjetil Taskén for giving me the opportunity to be a part of his research group. Thank you for your guidance and navigation during my lab work, for our discussions and your valuable input during the writing process. I would also like to give special thanks to my supervisor Anna Mari Lone for our countless conversations and discussions throughout the past year, for all the help regarding practical lab work and for great feedback during the writing process. I would also like to thank both of you for the trust you have showed me, and for allowing me to be a part of your research project, it has truly been an exciting year!

Furthermore, I would like to send a big thanks to the people I have shared office with this past year, Eirin, Ellen, Johanne and Paul, thank you for making our office a great work environment and for all the lunches, laughs and conversations throughout the year. I would also like to acknowledge the other members of the Taskén group, for helping me through the challenges I have encountered along the way, both big and small, and for creating a great social atmosphere. A special thanks to Marianne Enger for having an answer to all my flow cytometer - related questions, and for the technical assistance of Gladys Tjørhom and Martine Schrøder.

To my friends and Hallgrim, thank you for your continuous encouragement and for making me late night dinners. Finally, to my family, mamma, pappa and my sister, thank you for your unconditional support and for always encouraging me to do my best.

Oslo, May 2017
Marthe Jøntvedt Jørgensen

Abstract

Prostaglandin E_2 (PGE_2) is one of the most abundant metabolites of arachidonic acid and is well known for its multifaceted role in inflammation as well as for regulating normal physiological immune homeostasis. In addition to its role in inflammation, it is known to play a detrimental role in certain cancer types, in particular colorectal cancer, where PGE_2 promotes cancer progression through several mechanisms. Elucidating PGE_2 signaling patterns in specific cells types will increase our knowledge of the molecular basis of a wide range of diseases, from autoimmunity to cancer.

To improve our understanding of PGE_2's actions, a high - throughput technique combining fluorescent cell barcoding with phosphoflow cytometry was employed. This provided an extensive overview of the signaling networks PGE_2 initiates in primary T cells. These studies have been performed in four different subsets of T cells ($CD4^+CD45RA^+$, $CD4^+CD45RO^+$, $CD8^+CD45RA^+$ and $CD8^+CD45RO^+$). PGE_2 signaling patterns were characterized individually in different cell types and potential functional outcomes of PGE_2 signaling in these cell types were investigated. PGE_2 is known to elicit its functions through four different receptors called EP1 - 4. By high - throughput methods such as flow cytometry together with over 30 phosphoepitope specific antibodies, the aim was to assess and investigate the individual signaling pathways downstream of PGE_2's four receptors. To gain insight into the individual signaling pathways initiated by each receptor, T cells were exposed to highly specific agonists and antagonists of these receptors. This approach revealed crosstalk and highlighted shared and distinct signaling molecules between the four receptors. In total, we present a detailed overview of signaling molecules and pathways initiated by PGE_2 in four receptors as well as signaling differences between the four different T cell subsets. The study contributes to the understanding of the prostaglandin's impact on T cell function in health and disease and provides tools for further investigation of PGE_2 - induced signaling.

Abbreviations

3D - Three - dimentional
AKAP - A - kinase anchoring protein
APC - Antigen presenting cell
Akt/PKB - Protein kinase B
ATP - Adenosine triphosphate
CAMKII - Calcium - dependent kinase II
CD - Cluster of differentiation
cAMP - Cyclic adenosine 3',5' monophosphate
$CD3^+$ - CD3 positive
$CD4^+$ - CD4 positive
$CD45RA^+$ - CD45RA positive
$CD45RO^+$ - CD45RO positive
$CD8^+$ - CD8 positive
cAMP - Cyclic adenosine 3',5' monophosphate
cGMP - Cyclic guanosine 3','5 monophosphate
COX - Cyclooxygenase
CRC - Colorectal cancer
CREB - cAMP responsive element binding protein
Csk - C - terminal Src kinase
DAG - Diacylglycerol
DC - Dendritic cell
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
EC50 - Halfmax effective concentration
EGFR - Epidermal growth factor receptor
EP receptor - E - type prostaglandin receptor
Epac - Exchange protein directly activated by
Erk - Extracellular signal - regulated kinase
FCB - Fluorescent cell barcoding
FCS - Fetal calf serum
FOXP3 - Forheadbox P3
FSC - Forward scatter
GDP - Guanosine diphosphate
GPCR - G protein - coupled receptor
GSK3α - Glycogen synthase kinase 3α
GTP - Guanosine triphosphate
h - Hour
IC50 - Halfmax inhibitory concentration
IFN - Interferon
Ig - Immunoglobulin
IL - Interleukin
IP_3 - Inositol triphosphate
kDa - Kilodalton
Lck - Lymphocyte - specific protein tyrosine kinase
MAPK - Mitogen associated protein kinase
MEK - MAPK kinase
MHC - Major histocompatibility complex
MS - Mass spectrometry
mTor - Mammalian target of rapamycin
NDRG1 - N-myc downstream regulated gene 1
NK - Natural killer
NSAID - Non - steroid anti - inflammatory drug
PAGE - Polyacrylamide gel electrophoresis
PBMC - Peripheral blood mononuclear cell
PBS - Phosphate - buffered saline
PGE_2 - Prostaglandin E_2
PGES - PGE synthase
PI3K - Phosphoinositide 3 - kinase
PKA - Protein kinase A
PKC - Protein kinase C
PLC - Phospholipase C
PLCγ1 - Phospholipase Cγ1
PTM - Post translational modification
Rap - Receptor associated protein
RII - Type II regulatory subunit of PKA
RNA - Ribonucleic acid
S - Serine
SDS - Sodiumdodecyl sulfate

SEM - Standard error of the mean

Ser - Serine

SiRNA - Small interference ribonucleic acid

Src - Proto - oncogene tyrosine protein kinase/sarcoma

SSC - Side scatter

T - Threonine

T cells - T lymphocytes

TCR - T cell receptor

TFH - Follicular helper T cell

T_H1 - T helper type 1

T_H17 - T helper type 17

T_H2 - T helper type 2

Thr - Threonine

Treg - Regulatory T cell

Tyr - Tyrosine

Wnt - Wingless intergration 1

Y - Tyrosine

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1. Introduction

A key feature of the human body is the ability to respond to the environment and situations surrounding us. Information must be collected, processed and interpreted and decisions based on the interpretations are made. These processes are all performed by cells, the smallest functional unit in the body. All multicellular organisms depend on a constant balance between growth, proliferation and death by their cells for proper function. Furthermore, cellular functions such as metabolism, gene regulation, protein turnover and cell differentiation and specialization must be properly regulated. To achieve this, the different components in the body must communicate constantly. Communication inside the human body happens within a cell, between cells and between tissues.

1.1. Cell signaling

To communicate, signals travel in the circulation (hormones), between tissues and convey information that can go between and inside cells. These signals are an important source of information and by investigating them one can get a better understanding of how a cell works and what tasks it performs. Signals between and within cells are mediated by a variety of molecules and ions, which can either be bound to the cell surface or secreted by the cells. These molecules may for instance be growth factors or mitogens that induce growth, proliferation, survival factors that inhibit the apoptosis process (programmed cell death) or death - inducing signals that promote apoptosis. The delicate balance of whether a cell should divide or die is controlled by the various signaling molecules the cell receives (Figure 1).

Under specific conditions, the balance between growth and death of cells may be disturbed, leading to proliferation disorders such as cancer. Thus, understanding the way cells communicate with each other and unraveling the specific signaling pathways is key to our understanding of different types of disorders and diseases as well as treatment.

Cell - cell communication proceeds in different ways. Signaling can happen through direct or indirect contact between two cells or between a cell and the extracellular matrix; and these connections play an important role in regulating the behavior of cells in tissue. Although direct interactions between cells are critical, a large portion of the signaling happens through molecules secreted by cells. Different types of such indirect interactions can be distinguished by the distance over which the signals are transmitted. In endocrine

signaling hormones are produced by specialized endocrine cells and secreted into bloodstream, acting on target cells at distant body sites. In contrast, some signaling molecules act only locally on nearby cells. In paracrine signaling, a signaling molecule is released by one cell and acts on its neighboring cell. This type of signaling is critical between neurons where neurotransmitters are secreted at the synapse relaying the signal to the post synaptic cell. Lastly, some signaling molecules act on the same cell where it was produced, a process called autocrine signaling. This type of signaling is important for immune cells, where certain T lymphocytes respond to the pathogenic environment by secreting a growth factor which induces their own proliferation, thereby increasing the amount of responding T lymphocytes. This amplifies the immune response to clear out the pathogen.

Signals received by a cell will generate various responses depending on the received signal. This is a complicated process where the cells must interpret the multitude of signals they receive from other cells; the critical point in relaying the signals is converting the message from one form to another. Upon binding of molecules to the cell surface, signaling cascades within the cells are activated. These cascades can involve changes in protein intracellular location, generation of second messengers or various modifications on molecules without changing their location. This dynamic process is called signal transduction.

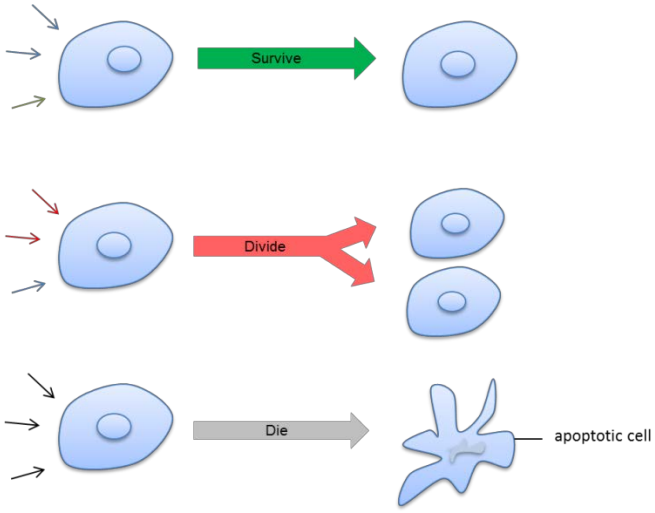

Figure 1 Multicellular organisms depend on multiple extracellular signals.
Extracellular signals can alter a cells behavior and determine whether it should divide, differentiate, die or just survive. Examples of extracellular signals include a variety of molecules such as hormones, lipids, ions and small molecules. Modified from Essential Cell Biology, second edition, p538 [1].

1.1.1. Signal transduction

How cells sense and respond to outside stimuli has been a key question in biology for centuries. Receptors that span the cell membrane enable the cell to both sense and transduce signals, in addition to directly connecting the outside of the cell membrane to the cells interior. They are extremely important as they allow cells to communicate and react to the outside environment, they receive external signals and generate new intracellular signals in response. In order to do that, they must be able to bind signal molecules known as ligands. A ligand will have affinity for a specific receptor and upon binding it will trigger changes inside the cell.

In this Thesis, a particular subset of the membrane receptor family, called G protein - coupled receptors (GPCRs), is studied. GPCRs are the largest family of cell surface receptors with more than 700 members in the human body. This family represents interesting targets in modern pharmacology as it constitutes the most important class of pharmacological targets in all clinical areas [2]. GPCRs control a large number of physiological processes and differ in signal transduction, ligand diversity and have a unique tissue - dependent expression pattern [2, 3]. Especially important are the GPCR - mediated functions within the brain and the peripheral nervous system where GPCRs have been linked with emotions, learning and memory processes. In addition, GPCRs play an important role in thermoregulation, sexual behavior and immune function [4-7] .
GPCRs can bind a variety of extracellular signaling moleules such as hormones, paracrine mediators and neurotransmitters. Despite the diversity in ligands, the GPCRs have a similar structure, where each receptor contains one single polypeptide chain that spans the cell surface membrane seven times. Upon binding of its cognate ligand, GPCRs undergo conformational changes that activate a G protein located on the cytosolic side of the plasma membrane. Heterotrimeric G proteins contain three subunits - α, β and γ. In the unstimulated state, the latter two are tethered to the plasma membrane whereas the α - subunit has bound GDP. Upon stimulation of the receptor, the G_α - bound GDP is exchanged with GTP, which activates the α - subunit. The βγ - complex is also able to transduce signals received by the GPCRs by the release from the plasma membrane. Upon activation, these subunits will interact with various target molecules and a chain of activated chemical messengers (second messengers) will relay the signals generated from the GPCR, a process called intracellular signal transduction [1].

Although signal effector molecules are essential for signal transduction, other molecules play important roles as well. These are called second messengers, and are small molecules that relay the signal initiated by a receptor. In addition to transferring the signal, second messengers serve to amplify the strength of the signal. Three major classes of second messengers exist, and all three are relevant for this Thesis. The first group is cyclic nucleotides such as cAMP and cGMP generated by specific cyclases, the second group is lipids and lipid - derived second messengers such as inositol - 3 phosphate (IP_3) and diacylglycerol (DAG), which are generated by phospholipase C (PLC), while the third group is ions such as sodium, potassium, calcium and magnesium among others [8]. Calcium and magnesium play a direct role as dynamic intracellular messengers that regulate specific proteins during signal transduction.

Signaling molecules that contribute to convey the signal relayed from the second messengers, may be activated or inactivated by the addition of a chemical group - for instance acetate, phosphate or sugars - to one or several of the amino acids on the polypeptide chain. These modifications are called post - translational modifications (PTMs). One of the most common ways of altering protein activity in a non - permanent manner is the addition of phosphate groups to serines, threonines or tyrosines on one or several sites in the protein, a process called phosphorylation. This process is catalyzed by a large group of enzymes called kinases, which phosphorylate distinct sites (defined by their amino acid sequence) on target proteins, while the reverse process, dephosphorylation, is catalyzed by phosphatases [9]. Phosphorylation may act as a molecular switch to turn on or off protein activity; and also convey information; however the effect varies among proteins and even among different sites on the same protein. Phosphorylation may lead to increased or decreased catalysis, or increased or decreased binding capacity to target proteins [9].

Most signaling pathways are comprised of an extensive number of proteins and other molecules interacting in various ways. In addition, pathways may interact with each other, thus forming complex signaling networks. Even a single type of extracellular signal binding to only one type of G - protein coupled receptor usually activates multiple parallel signaling pathways, and can alter many aspects of cellular behavior such as shape, movement, metabolism and gene expression [1].

1.2. The immune system

Intercellular communication and signal transduction are highly important for control of immune function. The immune system provides protection against pathogens and other dangerous particles that can cause infection. Children born without a functional immune system are subject to recurring infections and may die within the first year of age [10]. Cells of the immune system circulate in the blood and lymphoid tissue and constantly work to recognize antigens derived from infectious agents (such as bacteria, virus, fungi or internal parasites) or cancerous cells. Upon infection, the innate immune system is activated. It is the second line of defense when the outer barriers (such as skin, mucus membranes etc.) fail to eliminate the intruder. It mediates fast, unspecific responses with no long - term memory.

Sometimes sophisticated pathogens can overrule the innate immune system and it fails to clear out the pathogen. Luckily the body is equipped with a third line of defense, namely the adaptive immune system. This type of immune defense works more slowly, but has the ability to generate highly specific responses towards a specific pathogen. In addition to provide long - term memory, allowing more rapid secondary responses. The functions of the adaptive immune system are carried out by specific types of lymphocytes, called B and T cells. B cells initiate an antibody response, secreting antibodies that will bind to epitopes on the pathogen that initiated their production, opsonizing pathogens for engulfment by phagocytes and preventing further damage. T cells are central regulators in adaptive immunity and carry out both humoral and cell - mediated immune responses through production of cytokines as well as cytolytic activity of effector T cells [10]. Different aspects of signaling in T cells have been studied in this Thesis and T cells will therefore be the focus in this introduction.

1.2.1. T cells

T cell responses can be either humoral response by providing T cell help to the B cells or cell - mediated immune responses. The T cells perform their immunosurveillance by migrating between blood, the lymphatic system and peripheral lymphoid organs, constantly looking for antigens [10]. This migration is facilitated by chemokines secreted by surrounding cells. There are two major types of T cells discussed in this Thesis, cytotoxic T cells that typically express CD8 on their surfaces and helper T cells that express CD4. T cells can recognize infections within normal cells and cancerous cells

with mutations as well as activate other parts of the immune system to fight the pathogen by recognizing host cells presenting pathogenic or foreign (non - self) molecules on their surface. T helper cells carry out multiple functions, including activation of the innate immune system, B lymphocytes, cytotoxic T cells as well as nonimmune cells , while CD8 cytotoxic T cells are important for host defense against viruses and other cytosolic pathogens as they kill infected cells [10, 11].

To activate T cells, the T cell receptor (TCR) must be engaged along with several co - receptors. The T cell receptor complex is composed of two chains, usually TCR-α and TCR-β that bind antigens and form the core T cell receptor. They associate with invariant polypeptides called CD3ε, CD3γ, CD3δ and the ζ chain [10]. These are necessary for the transduction of signals to the cell's interior after the TCR has bound antigen. CD4 and CD8 proteins serve as co - receptors aiding the interaction of the TCR with antigens presented on major histocompatility complex (MHC) - II and (MHC) - I, respectively. All healthy, nucleated cells express MCH - I, and if the cell is infected, foreign antigens presented on the MHC - I - expressing cell will activate cytotoxic T cells. MHC - II is expressed on designated antigen presenting cells (APCs) such as macrophages and dendritic cells, and is necessary for activating T helper cells. This generates a highly specific immune response [10]. Naïve $CD4^+$ T cells respond to presented antigens by developing into distinct effector cell lineages such as T helper type I (T_H1), (T_H2) , T_H17, follicular helper T cell (T_{FH}) and regulatory T cells [11]. The fate of the $CD4^+$ T cells is determined by the antigen, the strength of the T cell receptor signaling and the cytokine environment.

The T cells fate and function is determined downstream of the TCR, in an extensive signaling pathway involving several phosphorylation steps and different mediators. An appropriate T cell response must be properly switched on and then off when the pathogen is cleared. T cells undergo effector expansion during an infection, and then when the pathogen is cleared, the activated T cell population contracts to a stable number of memory T cells. These cells are long - lived and provide lifelong protection against pathogens. The activation and differential status of human T cells have classically been distinguished by different CD45 isoforms. Naïve T cells express CD45RA whereas memory T cells express CD45RO [12]. Although this is a simplification of the two classes, it is sufficient information for the work in this Thesis.

Proper regulation of the different immune responses is key to survival. Regulation of the immune responses often happens through regulatory T cells (Tregs). Tregs have a suppressive effect in the immune system and play an important role in maintaining self - tolerance. They make cytokines that are immunosuppressive, such as IL - 4, IL - 10 and TGF - β [10]. Tregs have been associated with tumor formation. They expand and migrate to the tumor site, or they can expand within the tumor microenvironment (peripherally induced Tregs) and exert their immunosuppressive functions on anti - tumor immune functions mediated by NK cells, CD4 and CD8 positive T cells [13]. In addition to the various mediators of the immune system mentioned above, several other immune modulators or regulators can alter different aspects of immune functions.

1.3. Inflammation, prostaglandins and cancer

Inflammation is one of the immune system's most powerful weapons against infections and is a carefully coordinated process designed to eliminate pathogens that enter the body. The inflammation response is usually initiated as a response to tissue injury and is set into motion by a set of paracrine signals. These signals induce the activation and migration of leukocytes (neutrophils, monocytes and eosinophils) from blood vessels into the infection site, activation of platelets that regulate vascular permeability and chemotactic factors derived from the activated platelets that initiate granulation tissue formation. In addition, inflammatory signals activate fibroblasts and proteolytic enzymes which are necessary for remodeling the extracellular matrix. From the outside this inflammatory response is perceived as redness, pain, swelling and heat from the damaged tissue, which are classical signs of inflammation [10].

Arachidonic - acid - derived eicosanoids are important mediators of the inflammation response. One such group of lipid mediators is the prostanoids, including the prostaglandins (PGs) and thromboxanes (TXs). During inflammation responses described above, prostanoid levels increase immediately prior to recruitment of leukocytes. Furthermore, cells of the innate immune system, including macrophages, sentinel dendritic cells (DCs) and neutrophils are major producers of prostanoids which act locally on target cells. During an inflammatory response, the level and profile of prostanoid production can change dramatically. Prostanoids were originally characterized as pro - inflammatory mediators as they can induce inflammatory changes when injected into tissue [14]. The pro - inflammatory functions of prostaglandins lead to increased blood flow enhancing edema formation, in addition to increasing vascular permeability

promoting leukocyte infiltration of damaged tissue [14]. As our understanding of prostanoid biology evolves, it has become clear that these mediators can both promote and inhibit inflammation [14]. Thus, they can be thought of as regulators of the complex signaling network that makes up an inflammation process. Their overall impact in an inflammatory response will depend on multiple factors, including the level of immune cell activation, the presence of other mediators and the physiological state of the organism. Cytokines can function as either pro - or anti - inflammatory that fine - tune the inflammation process [15]. Throughout an inflammation response there is a continuous activation of chemotactic cytokines called chemokines that coordinate and dictate the natural process of an inflammation and migration of leukocytes to inflamed tissue. Usually inflammation associated with wound healing is self - limiting and tissue homeostasis is achieved once the pathogen is cleared. However, dysregulation of any of the governing inflammation factors can lead to abnormalities and ultimately, pathogenesis [16]. In addition, it is now well - established that long - standing inflammation promotes tumor progression and tumor formation [17]. Tumor cells have the ability to produce inflammatory mediators (cytokines, chemokines and prostaglandins) to aid their own growth and proliferation. In addition, these inflammatory mediators recruit other leukocytes such as neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes to the tumor tissue. These cells are capable of producing an assorted array of cytokines, angiogenic and lymphangiogenic growth factors and proteases, all of which contributes to neoplastic progression [18, 19].

An important regulator of inflammation also associated with cancer progression is cyclooxygenase – 2 (COX – 2), which is also responsible for prostanoid production. The COX - pathway produces five major prostanoids: prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin I_2 (PGI_2) and thromboxane A_2 (TXA_2), which are important for early phase as well as the resolution of inflammation as discussed above. COX - 2 has been shown to play a key role in tumor progression and has been targeted in many cancers; including colorectal cancer, breast cancer, colon cancer, prostate cancer and esophageal cancer [20, 21]. Perhaps some of the best evidence of the significance of inflammation and prostanoids during tumor progression comes from studies of long term use of non - steroidal anti - inflammatory drugs (NSAIDs) on colorectal cancer patients. Much data demonstrates that the use of these drugs reduces colon cancer risk with 40-50 % and may be preventative for lung, esophagus and stomach

cancer [22]. The function of COX enzymes, NSAIDs and tumor immune evasion will be discussed in more detail in the section COX enzymes, PGE_2 and colorectal cancer.

1.4. The role of prostaglandin E_2 in immune functions

Prostaglandins are potent lipid signaling molecules that play crucial pro - and anti - inflammatory roles in numerous processes in the body. They have important roles in diverse physiological processes and are involved in several organs and physiological processes such as gastric acid secretion and motility in the gastrointestinal tract, blood flow in kidneys, inflammation, T cell - mediated immunity and the induction of labor and other reproductive processes [23, 24]. Prostaglandins are produced from arachidonic acid derived from phospholipids in the plasma membrane (Figure 2). Arachidonic acid is released from the plasma membrane by phospholipase A_2 and converted to PGH_2 by the cyclooxygenase enzymes COX - 1 and COX - 2. Cell - specific prostaglandin synthases (PG synthases) convert PGH_2 into different prostaglandins, including PGI_2, $PGF_{2\alpha}$, PGD_2, and PGE_2 [25]. They are all mediators produced in response to various extracellular stimuli and exert a variety of physiological actions [26]. Two isoforms of COX, COX - 1 and COX - 2 exist. While COX - 1 is constitutively expressed and viewed as a housekeeping protein, COX - 2 is not normally expressed in most tissues. Instead it is induced by inflammatory stimuli, hormones and growth factors and is considered the rate - limiting step in prostaglandin synthesis during pathophysiological responses like inflammation, chronic infections and cancer [27]. Investigation of prostaglandins may enhance our understanding of immune regulation networks and thus, contribute to our understanding of different immune processes as well as other physiological processes.

One major regulator of the different immune responses is the arachidonic acid metabolite prostaglandin E_2 (PGE_2). It exerts effects both in innate and adaptive immune responses. PGE_2 like other prostanoids is produced from COX - 1/COX - 2 (Figure 2). PGE_2 is the most abundant prostaglandin found in the human body and it can be produced by many cells types in the body, with epithelia, fibroblasts and infiltrating inflammatory cells representing the major sources of PGE_2 in the course of an immune response [28] .

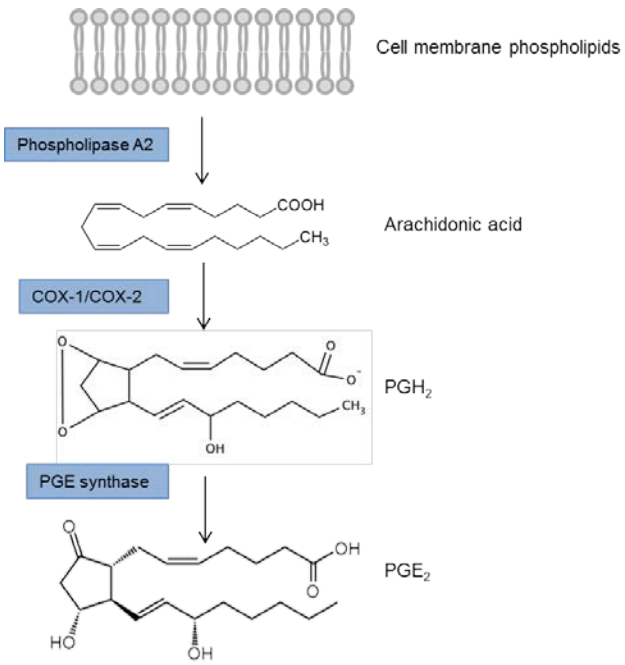

Figure 2 PGE_2 synthesis. *Phospholipase A_2 converts phospholipids found in the plasma membrane to arachidonic acid. The acid is the substrate of COX-1/COX-2 that converts it into PGH_2. PGH_2 is the intermediate in the biosynthesis of all prostaglandins. PGH_2 is converted to biologically active PGE_2 by PGE synthase. Adapted from Kalinski, 2012 [28].*

PGE_2 exerts its effect through four different GPCRs called E - type prostaglandin (EP) receptors 1- 4. They are distinguished by their G protein composition and the signaling pathways they initiate upon ligand binding. Studies have suggested that EP2 and EP4 are the main receptors to mediate actions of PGE_2 in human naïve $CD4^+$ T cells and they are upregulated in response to T cell activation [29]. Studies of the actions of PGE_2 as an immune modulator have escalated in the past decades and some of its rather controversial roles have been revealed.

1.4.1. Paradoxes of PGE_2 immune function

By binding to one of the four EP receptors, PGE_2 can alter the functions of many cell types, including macrophages, dendritic cells and T and B lymphocytes leading to both pro and anti - inflammatory effects. Prostaglandins play a key role in the generation of the inflammatory response, and their biosynthesis is significantly increased in inflamed tissue. While the pro - inflammatory role of PGE_2 during the acute inflammatory response is well - established, its role in resolution of inflammation is more controversial [24, 30].

Generally, PGE_2 has been recognized as a pro - inflammatory mediator of active inflammation. However, the biolipid has the ability to precisely suppress immune responses associated with chronic inflammation and cancer [31]. This demonstrates the paradoxes of PGE_2 functions as it both aids and suppresses immune functions.

In its pro - inflammatory role, PGE_2 induces hallmarks of inflammation such as redness and swelling by increasing bloodflow into the inflamed tissue, by augmentation of arterial dilation and increased microvascular permeability. PGE_2 also contributes to cytokine production in dendritic cells and induces their migration and their ability to augment T cell activation [32]. Furthermore, PGE_2 has been reported to drive the differentiation of T helper cells into T_H1 and T_H2 T cells [33, 34]. Notably, PGE_2 has also been shown to downregulate expression of IFNγ in T cells, and actually inhibit differentiation to T_H1 cell type [35]. Recent studies have shown that the strength of the TCR stimulation can influence whether or not T_H1 cell fate is promoted by PGE_2 [33]. Although it has been subject to debate, PGE_2 is thought to contribute to the T_H17 fate determination in addition to both T_H1 and T_H2 differentiation [29, 36]. Utilizing the ability of PGE_2 to modulate T_H responses and development could be very beneficial for treating disorders in which there is a dysregulated T_H response.

On the other hand, some reports suggest an anti - inflammatory role for PGE_2, where PGE_2 has been shown to suppress the effector functions of dendritic cells and B and T cells [37-41]. In addition, PGE_2 has also been reported to exert anti - inflammatory effects on innate immune cells like neutrophils, macrophages and natural killer (NK) cells [42, 43].

Several reports on the mechanism whereby PGE_2 suppresses T cell function, activation, expansion and differentiation have been published. Furthermore, PGE_2 has a direct inhibitory effect on T cell IL - 2 production [44]. IL - 2 is a crucial component of the cytokine cocktail that is required for proper T cell development and differentiation into effector cells; without IL - 2, effector T cell proliferation and activation is inhibited. Moreover, it has been reported that PGE_2 induces a TCR inhibitory pathway in T cells aided by the cAMP - PKA complex which also results in reduced IL - 2 production [45]. By binding to either the EP2 or EP4 receptor, PGE_2 induces local pools of cAMP. cAMP activates PKA which in turn activates an inhibitory pathway in T cells which suppresses T cell functions including IL - 2 production. Activated PKA phosphorylates C - terminal Src kinase (Csk kinase), which leads to phosphorylation of Lck to inhibit its activity. Lck usually acts to promote TCR signaling, thus Lck inhibition attenuates TCR signaling, proliferation and functions in effector T cells. Additional reports have shown that elevated levels of activated PKA lead to hypophosphorylation of CD3ζ chain, indicating low TCR activity. TCR activity was shown to be restored with the use of a PKA type I

antagonist, indicating that high PKA activity levels reduce TCR activity [46]. Furthermore, it has been shown that PGE_2 inhibits cytotoxic T cell proliferation and its ability to produce IFN-γ through a cAMP - dependent pathway [47].

This dual role of PGE_2 and its receptors in modulating inflammatory responses has been described in several disorders. In addition to having important, although controversial roles in immune functions, PGE_2 has also been shown to play a crucial role in the neoplastic process [48, 49]. The evidence indicates that this prostaglandin possesses potent tumor - promoting activity. Revealing PGE_2's many and complex signaling pathways may improve our understanding of anti - tumor immunity and tumor immune evasion. This can in the future lead to the development of strategies for prevention, discovery and treatment of cancer.

1.4.2. COX enzymes, PGE_2 and colorectal cancer

The COX enzymes convert arachidonic acid to the common precursor of all prostaglandins, PGH_2 (see Figure 2). As mentioned in the previous section, two isoforms of COX enzymes exist. While COX-1 is considered a housekeeping protein, COX - 2 expression studies confirms that COX - 2 is an inducible enzyme that produces prostaglandins during inflammation and conditions that promotes tumor formation and is associated with poorer survival prognosis among colorectal cancer patients [50, 51]. Because of this, there have been several studies on COX - 2 and its role in colorectal cancer (CRC), where its actions have a major impact.

Cancer of the colon and rectum is a heterogeneous disease and is the third most commonly diagnosed cancer throughout the world [52]. The pathogenic mechanism underlying CRC development is multifactorial and the majority of cases are sporadic in origin. Factors such as genetic changes, epigenetic changes, chronic inflammation and diet contribute to the development of CRC [52].

A large body of evidence demonstrates overexpression of COX - 2 in CRC tumor tissue, compared to normal colonic mucosa [53, 54]. In addition, COX - 2 overexpression has been associated with other cancer types such as breast, prostate and lung cancer [55, 56]. Because of these findings several studies have been performed to investigate the effects of inhibition of COX enzymes. Studies have shown that inhibition by non - steroidal anti - inflammatory drugs (NSAIDs) or aspirin, which inhibit both COX - 1 and COX - 2

reduces the risk of CRC and may be beneficial to large populations in groups at risk [57]. Since the discovery of NSAIDs and their effects in cancer, there has been a great deal of interest in identifying components of the prostaglandin synthesis pathway as well as their function.

Even though NSAIDs show promising functions, recent studies have shown that prolonged effects of COX -2 inhibitors may have unfavorable effects on the overall physiology. Although COX - 2 inhibitors suppress PGE_2 production, they also inhibit production of other prostaglandins which are important for overall homeostasis of the body and inhibiting the production of these may have several severe effects. Some studies have shown that treatment with COX - 2 inhibitors may affect platelet function leading to increased risk for coronary thrombosis and stroke. Both NSAIDs and to some extent COX-2 inhibitors have been shown to induce gastrointestinal bleeding [58, 59]. Specific PG synthase inhibitors avoid unspecific inhibition of prostaglandin synthesis and studies of these are starting to emerge [60].

Reports suggest that the pro - tumorigenic effect that COX enzymes display is largely connected to elevated levels of PGE_2 [61]. Increased levels of PGE_2 increase the size of adenomas in an inheritable type of CRC called familial adenomatous polyposis in a concentration dependent manner [62], suggesting a correlation between tumor growth and PGE_2 levels.

Cancer cells must evade antitumor immune responses to advance in the neoplastic process and to become malignant. Within the tumor, the microenvironment consists of surrounding blood vessels, fibroblasts, signaling molecules and immune cells among other cells. Within the tumor microenvironment, the immune cells are continuously exposed to antigens generated by the tumor cells, due to the tumor cells altered characteristics. This provokes an immunosuppressive environment where effector T cell functions are inhibited. In fact, many of these immunosuppressive pathways converge on elevated cAMP levels in effector T cells and several of these pathways have been shown to be initiated by PGE_2. Previous studies in this lab showed that in addition to tumor cells, peripherally induced or adaptive Tregs express COX - 2 and thus, produce PGE_2 [63]. Furthermore, PGE_2 has been shown to work in an autocrine manner and stimulate FOXP3 expression in Tregs, promoting Tregs suppressive functions [63]. FOXP3 is a transcription factor expressed mostly in Tregs and plays an important role in their

suppressive function. Thus, Tregs exposed to chronic antigen stimulation contribute to chronic inflammation and immunosuppression, both of which involve synthesis and secretion of PGE_2 [63]. Furthermore, the lab showed that regulatory T cells inhibit antitumor immunity in a COX - 2 - PGE_2 - dependent manner in patients with both primary and metastatic colorectal cancer [48, 49]. In addition to tumor cells and Tregs, other cell types also secrete PGE_2. In fact, myeloid derived suppressor cells (MDSCs) have been shown to express high levels of COX - 2 and are a major source of PGE_2 in cancer [64]. PGE_2 aids MDSCs in their suppressive functions such as production of suppressive mediators and inhibition of $CD8^+$ T cell function, within the tumor microenvironment [64].

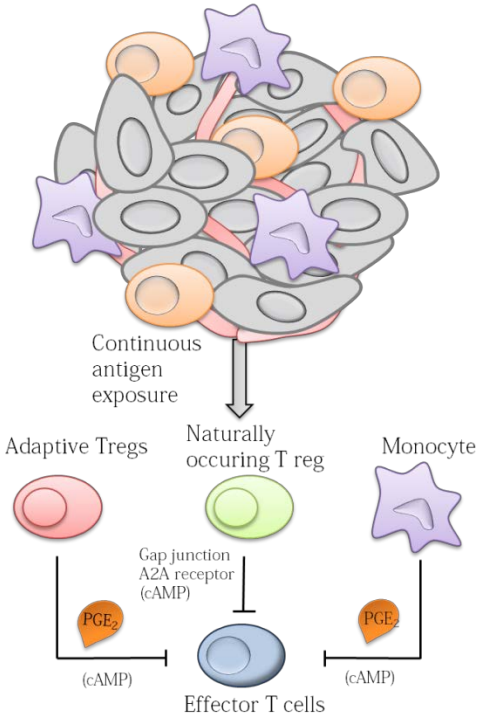

Figure 3 Adaptive Tregs and monocytes produce and secrete PGE_2 which contributes to the immunosupressive environment generated by cancer cells. *Within the tumor microenvironment adaptive Tregs and monocytes have been shown to express COX - 2 and produce PGE_2. PGE_2 inhibits functions of T effector cells by engaging EP2 and EP4 receptors on their cell surface. Figure adapted from J.D. Scott et, al. 2013 [65].*

Interactions between PGE_2 and specific immune cells contribute to the generation of the immunosuppressive environment that appears during tumor formation. In total, PGE_2 actions seem to be pro-carcinogenic as well as interrupting important anti - tumor immune responses.

1.4.3. Prostaglandin E_2 (EP) receptors

In this Thesis, specific signaling pathways initiated by PGE_2 are investigated. PGE_2 can bind to four GPCRs called E - type prostaglandin receptors 1-4 (EP1-4). The four receptors activate distinct signaling pathways and exert different functions, and they have restricted expression patterns that are confined to specific tissues [66].

The G protein - coupled receptors contain a 7 transmembrane - spanning domain. The N - terminal of the receptor faces the extracellular environment and the C - terminal tail faces inwards into the cytoplasm. The different receptors couple to different heterotrimeric G proteins, which results in the activation of different signaling cascades. Upon activation of the receptor, intracellular signaling cascades are initiated, these cascades may have a high degree of crosstalk and include involvement of other pathways which makes them complicated to characterize.

The EP1 receptor is thought to be coupled to $G_{\alpha q}$ which utilizes Ca^{2+} as a second messenger, as well as inositol 1,4,5 - triphosphate generation (IP_3). The generation of IP_3 and DAG (diacylglycerol) is initiated upon activation of the EP1 receptor which leads to the activation of phospholipase C (PLC) and Ca^{2+} release from intracellular stores (Figure 4) [67]. In addition, two splicing variants of EP1 have been reported as well as cases of transactivation of EP1 [68, 69]. One study in hepatocarcinoma cells showed that PGE_2 activates the MEK/ERK and PI3K/Akt pathways by EP1 - mediated transactivation of epidermal growth factor (EGF-R), thus promoting cell proliferation and invasion and contributing to the complexity of the EP1 - mediated signal [68].

EP2 and EP4 activate $G_{\alpha s}$ which in turn is known to upregulate cAMP (Figure 4) [70]. The typical signaling pathway initiated by cAMP involves PKA [67]. In resting cells, PKA exists as a tetramer, including two regulatory subunits (α and β type I, and α and β type II) and two catalytic subunits (α and β). When these are bound together, the catalytic subunits are inactive. Upon activation by binding of cAMP, the two regulatory subunits dissociate from the catalytic subunits, allowing their activation. The catalytic subunits then go on to phosphorylate other target proteins. PKA may be located in the cytoplasm or associated with cellular structures and organelles depending on the regulatory type of PKA [71].

In addition to the PKA pathway, EP2 and EP4 receptors may activate other pathways, such as the GSK3/β - catenin pathway [72], or other proteins such as the membrane - associated exchange protein activated by cAMP, Epac - 1 and Epac - 2. Epac proteins may activate Rap (a Ras homolog) by substituting GDP for GTP. The cAMP - Epac - Rap pathway is involved in several cellular processes such as cell motility and gene expression [73]. Although there are clear similarities between EP2 and EP4 signaling, there are differences in ligand sensitivity. EP2 is reported to have a lower affinity for its natural ligand compared to EP4 receptor. In addition, the duration of the signal differs between the two receptors. In particular, a study in Chinese hamster ovary cells showed that EP4 is rapidly desensitized following its PGE_2 interaction, whereas EP2 is resistant to ligand - induced desensitization, and is able to mediate PGE_2 functions over prolonged periods [74]. Moreover, the EP4 receptor is reported to have the ability to activate phosphatidylinositol - 3 kinase (PI3K) signaling pathways through the βγ - complex, leading to phosphorylation of the extracellular signal - regulated kinases (ERKs) and induction of early growth response factor - 1 (EGR - 1) in a HEK-293 cell line transfected with the human EP4 receptor [75]. The EP4 receptor is also thought to have the ability to bind $G_{\alpha i}$ proteins in addition to its regular binding partner $G_{\alpha s}$ [76]. Interestingly, recent studies in colon cancer cells have shown that PGE_2 signaling through EP2 receptors can also activate both PI3K and Akt via the free βγ subunit of the G protein [77]. Regarding the EP2 receptor, a novel signaling pathway which can transactivate the EGF receptor has also been reported in normal epithelia and colon cancer cell lines, and has been shown to lead to increased migration and invasion of colon cancer cells [69].

The EP3 receptor also influences cAMP production. EP3 is thought to be $G_{\alpha i}$ - linked and inhibits the actions of adenylyl cyclase, thus inhibiting cAMP production (Figure 4) [67]. The inhibition of cAMP production is a relevant source of regulation because of cAMPs many targets [78]. In addition, EP3 is also thought to have multiple splicing variants that can activate contrasting second messenger signaling. Most of the splicing variants are thought to be $G_{\alpha i}$ coupled, but some may in fact be $G_{\alpha s}$ coupled receptors [79].

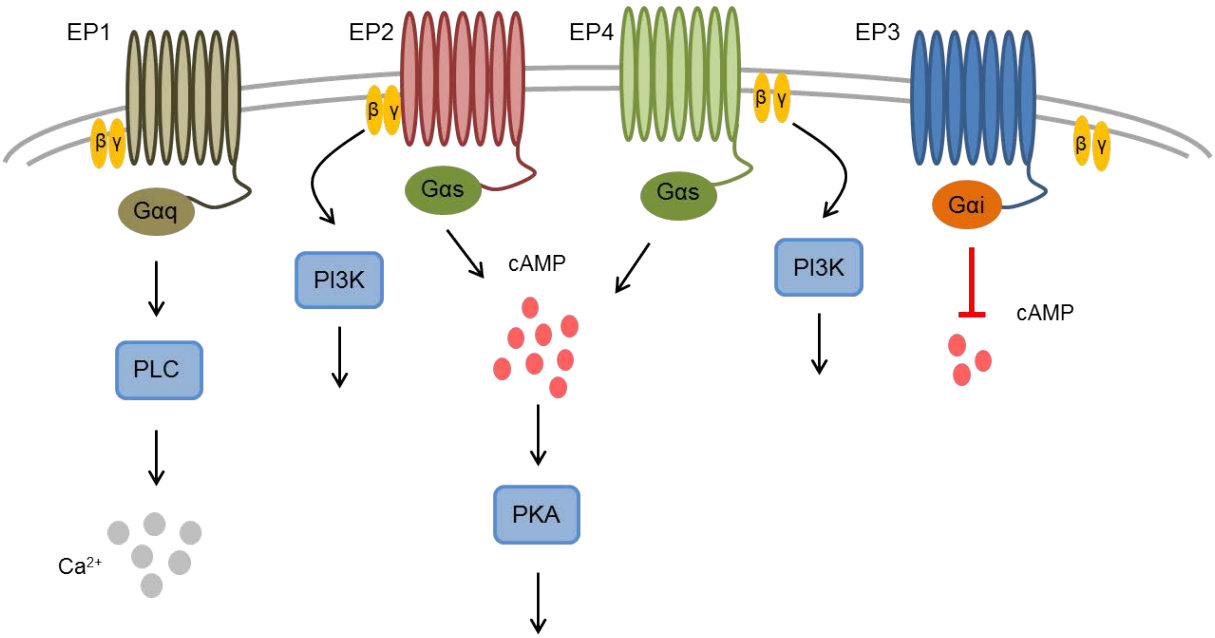

Figure 4 Overview of the classical signaling events generated from four EP receptors. *PGE_2 can bind to four distinct GPCRs, activating several different pathways. This figure displays a simplified scheme of signaling events downstream of the four receptors. Engagement of EP2 and EP4 leads to increased intracellular cAMP levels, whereas EP3 signaling inhibits cAMP generation. The EP1 receptor is known to signal through PLC which leads to increased intracellular Ca^{2+}.*

Detailed analysis of the four receptors can be made by using specific agonists or antagonists of the receptors. Agonists are compounds that can bind to a specific receptor and elicit the same biological effect as the natural ligand. Conversely, antagonists will upon binding to the receptor inhibit its biological activity. There are several prostanoid based agonists and antagonists on the market as therapeutical agents and for research use [80].

Agonists bind to the same binding site as the natural ligand of the receptor, leading to either a full or partial response. An agonist generating a submaximal response is called a partial agonist and generates a much weaker signal compared to a full agonist or the natural ligand and thus, partly antagonizes the signals of a stronger agonist. Furthermore, there is an additional type of agonist called inverse agonist. This term is applied when the receptor has a basal activity in the absence of a bound ligand and binding from either the natural ligand or an agonist will increase the level of activity. However, if an inverse agonist binds, the activity is decreased below the basal level. It is different from an antagonist in the way that the inverse agonist does not block the receptor but activates it in the opposite way. Furthermore, an inverse agonist will have an effect without another agonist present, while antagonists only carry out its effects in the presence of an agonist.

As the name implies, an antagonist inhibits the effect of the natural ligand and blocks receptor activity. Several types of antagonists exist, including competitive antagonists, which bind to the same site as the natural ligand and inhibit its effect. On the other hand, non-competitive antagonists bind to a different site on the receptor, inducing a conformational change that inhibits binding of the natural ligand [81].

Structural, pharmacological and functional differences as well as unique expression patterns that exist between the four subtypes of EP receptors determine the biological effects of PGE_2. Although the receptors are all GPCRs, the affinities for their natural ligand PGE_2 varies greatly between receptor subtypes and the affinity may depend on the state of the coupling of G protein subunits [82]. Apart from these differences there are also the phenomena of receptor desensitization and internalization that may regulate preferential signaling through certain EP receptors.
Clearly, many diverse factors govern the outcome of signaling through EP receptors and all these factors play a critical role in determining the differential biological effects of PGE_2.

1.5. Phosphoproteomic study

To generate a comprehensive overview of the signaling pathways initiated by PGE_2 in T cells, a phosphoproteomic study was performed [46]. This study described the PGE_2 - induced phosphoproteome in $CD3^+$ T cells and identified approximately 250 regulated phosphosites [46]. In a second phosphoproteomics study, signaling pathways downstream of specific EP receptors were investigated by utilizing receptor specific agonists in $CD4^+$, $CD8^+$ and T regulatory cells (Lone *et al.* manuscript in preparation). The large scale profiling performed in these studies, makes it possible to investigate dynamic changes in protein phosphorylation, revealing thousands of phosphorylation sites (phosphosites) on hundreds of proteins.

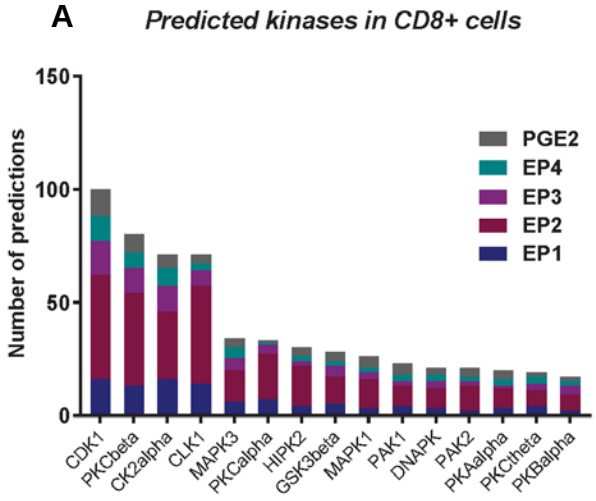

Figure 5 Overview of predicted kinases in CD8$^+$ T cells generated from the phospho - proteomic study. *The figure displays the 15 highest predicted kinases generated from PGE_2 or EP agonist stimulation. Kinases regulating the changing phosphosites were predicted using NetworKIN (Figure from Lone et al., manuscript in preparation).*

The original phosphoproteomic study [46] revealed an overrepresentation of PKA as a possible activated kinase, with 41 regulated phosphosites being predicted as possible PKA substrates. Other highly predicted kinases were CAMKII, PKB/Akt and glycogen synthase kinase 3 (GSK-3). Figure 5 displays predicted kinases investigated in the second phosphoproteomic study (Lone et. al, manuscript in preparation). In this second study, a high degree of crosstalk and overlapping phospho-sites were displayed for the four activated receptors (EP1-4), making the investigation of individual pathways more challenging. Mass spectrometry - based phosphoproteomic analyses are highly valuable in their ability to generate an unbiased view of the phosphorylation status of a large number of phosphorylated sites. Being an untargeted technique, it also allows identification and quantitative measurements of the phosphorylation status of many previously unidentified phosphopeptides. The approach is, however, limited in the number of individual samples which can be processed. Thus, a phosphoflow cytometry approach where a selection of phospho – epitope specific antibodies is used to measure phosphorylation status of various proteins is a valuable complementary technique that enables higher throughput and the ability to investigate multiple time points, stimulation conditions and cell types in the same experiments.

1.6. Phosphoflow cytometry

Phosphoflow cytometry is a phospho - specific flow cytometry approach which enables measurement of phosphorylation events on a single cell - level, by relying on phosphoepitope specific antibodies. Thus, the flow cytometer has the ability to measure phosphorylation events simultaneously in individual cells, enabling complex biochemical signaling networks to be investigated in heterogeneous cell populations, such as peripheral blood. This approach enables simultaneous monitoring of various cell surface markers and investigation of complex signaling pathways triggered by a specific stimulus. In this way, cell types of interest (for instance T cells, B cells, monocytes and subtypes of these) can be identified and analyzed. A flow cytometer equipped with up to 5 lasers can record as many as 20 different channels. These channels can be used to detect either surface markers or intracellular markers in the cell. Furthermore, by combining flow cytometry with fluorescent cell barcoding (FCB), different treatment conditions, like stimulation courses, temporal and concentration - dependent responses can be analyzed [83]. By utilizing FCB with 3 fluorophores at 4 different concentrations, up to 64 different samples can be pooled and analyzed simultaneously with the flow cytometer. This technique lowers costs by reducing antibody consumption and shortening run - time; in addition to increasing the quality of the data and comparability by enabling the FCB populations to be analyzed as one sample (see Figure 6). Phosphoepitope specific antibodies can be applied individually to the barcoded cell samples, enabling thorough investigation of intracellular proteins involved in a signaling pathway. Data generated from the flow cytometer is converted into FCS files which can be analyzed by the cloud - based software Cytobank [84]. In Cytobank, cell populations are gated to define each population in the sample and to enable investigation of phosphorylation generated from a single stimulation condition (see Figure 6). The median fluorescence intensity is calculated for each of the phospho - specific antibody channels to assess phosphorylation levels in a specific stimulation condition.

Taking these parameters into consideration, several thousand data points can be collected from only one experiment, making this high throughput method a very valuable technique for analyzing protein phosphorylation and intracellular signaling.

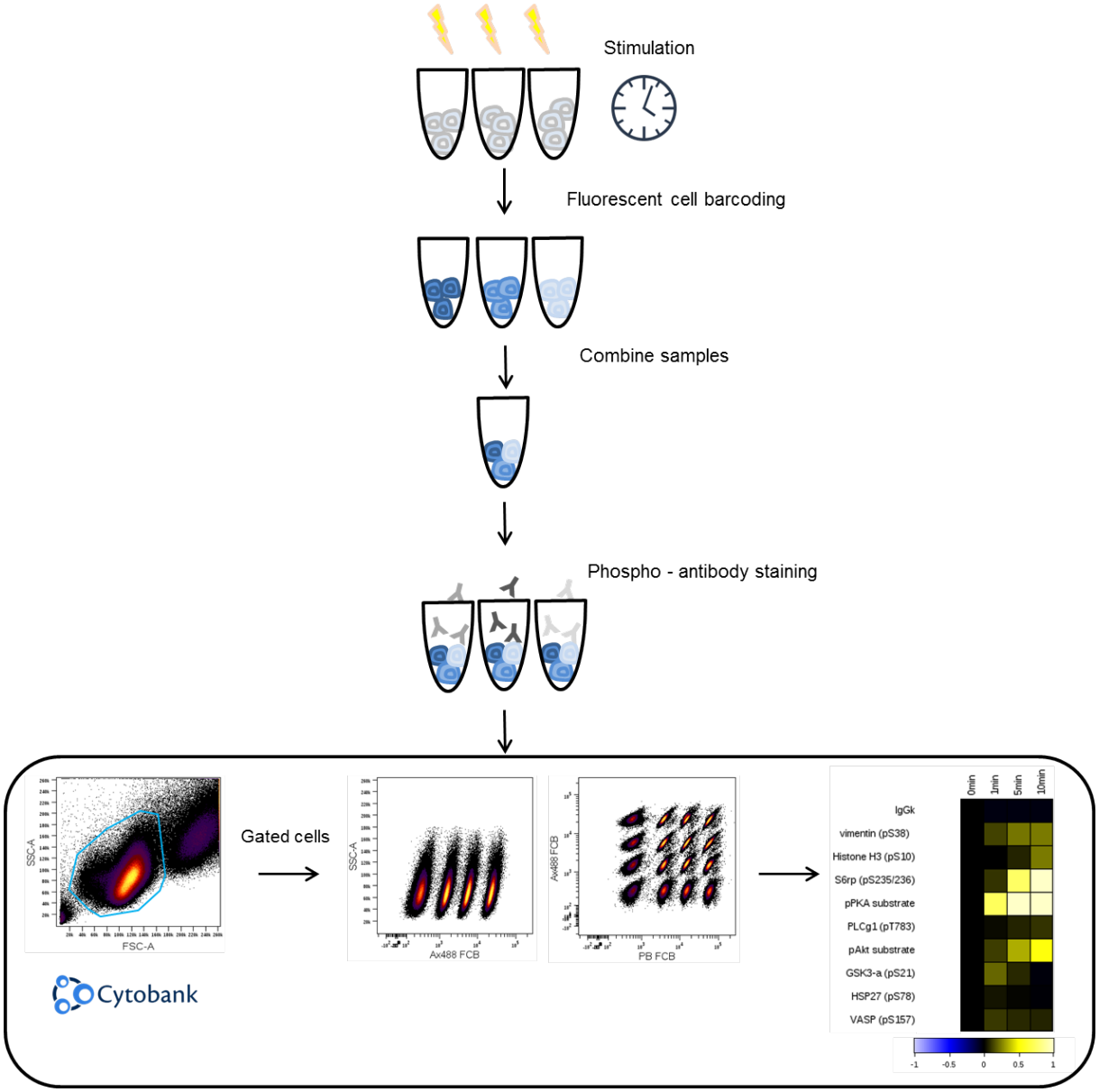

Figure 6 Work flow for phosphoflow cytometry. *T cells from healthy blood donors are purified and separated into tubes that are stimulated in different ways. Cells that have been subjected to different stimulation conditions are then barcoded with different fluorescent dyes, giving each condition a unique fluorescent signal. Cells are then permeabilized and phospho - specific antibodies are used to stain intracellular proteins. In addition, various cell surface markers are also stained to identify cell populations. Samples are analyzed on the flow cytometer where the fluorescence signals are detected. Data generated from the flow cytometer are analyzed in Cytobank. Each FCB channel can be visualized in Cytobank and gated separately to allow deconvolution of the original stimulation conditions.*

2. Aims of the study

This Thesis is based on previous work performed in the Taskèn group, where two phosphoproteomic studies have provided insight into the regulation of phosphosites, kinases and signaling pathways by PGE_2 in various T cell subsets (Lone *et al*., manuscript in preparation) [46]. However, mass spectrometry is limited in the number of conditions and individual samples which can be processed, thus a complementary, high throughput phosphoflow cytometry - based approach was employed to further investigate predicted kinases, phosphosites and signaling pathways activated downstream of PGE_2's four receptors. The aims of the Thesis were:

1. Explore and characterize specific phosphoepitopes downstream in PGE_2 - induced signaling in various T cell populations using phosphoflow cytometry.
   1.1. Establish a panel of phosphoepitope specific antibodies suitable for studying intracellular signaling induced by PGE_2 in T cells.
   1.2. Explore variations of PGE_2 signaling in four different human T cell subsets ($CD4^+$ $CD45RA^+$, $CD4^+$ $CD45RO^+$, $CD8^+$ $CD45RA^+$ and $CD8^+$ $CD45RO^+$).
   1.3. Study phosphorylation kinetics of specific phosphoepitopes and investigate the involvement of multiple kinases predicted by the phosphoproteomic study in PGE_2 - induced signaling, using kinase inhibitors.

2. Use specific agonists and antagonists of the four EP receptors to investigate individual pathways and phosphorylation sites activated by each of the receptors in four T cell subsets.
   2.1. Study the pharmacological characteristics of four EP agonists and antagonists in human T cell subsets.
   2.2. Investigate differences in signaling pathways triggered by the four EP receptors and duration of signaling elicited by the different receptor agonists.

3. Materials and Methods

3.1. Western blotting

Western blotting was used as a molecular biology method that provides information about identity of protein and their relative levels in cell lysate samples. Western blotting was used to assess the presence and levels of the four PGE_2 receptors (EP1-4) in T cells. For the detection of EP1, EP2 and EP4 receptors, receptor EP_1 Receptor Polyclonal Antibody, EP_2 Receptor Polyclonal Antibody and EP_4 Receptor (C-Term) Polyclonal Antibody were used - all from Cayman Chemicals. For the EP3 receptor, PTGER3 Antibody (Novus Biologicals) or EP3 Antibody (H-200) (Santa Cruz Biotechnology) was used. Actin Antibody (C-11) was used as a control (Santa Cruz Biotechnology). CD4 (CD4 Antibody, H-370, Santa Cruz Biotechnology) and CD8 (CD8-α Antibody, H-160, Santa Cruz Biotechnology) proteins were also stained, as controls for sample purity.

To perform western blots, $CD4^+$, $CD8^+$ and $CD3^+$ T cells were purified from two buffy coats as described in the T cell purification section. Cells were then lysed using RIPA Buffer (1x PBS, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS) and protein concentrations were measured by PierceTM BSA Protein Assay Kit (#23225, Thermo Fisher Scientific) with EnVision Multilabel Reader (Perkin Elmer). Proteins were then separated by size by Sodium Dodecyl Sulphate - polyacrylamide gel electrophoresis (SDS - PAGE). Loading buffer (SDS, β - mercaptoethanol, bromophenol blue and glycerol) was added to the protein sample to denature and add negative charge to the protein in addition to break the disulfide bonds. 1X loading dye were added to proteins with a concentration of 2 μg/ul, and 40 μl of the sample was loaded on a 10 % CriterionTM TGXTM Precast Gels (#5671033, BioRad) and an electrical field of 120V was applied for 1h 20 min, forcing proteins to travel through the gel. Protein mobility is dependent on molecular weight, as smaller molecules will be able to travel faster through the polymerized matrix than larger ones.

After SDS - PAGE gel electrophoresis, proteins were transferred to protein - binding PVDF membranes (#IPVH00010, Milipore). Mostly wet transfer was performed, although semi - dry transfer was performed once on a Trans - Blot Turbo blotting system

(BioRad) according to the manufacturer's instructions. For wet transfer, the PVDF membrane was prewetted in methanol prior to the transfer. The gel and the membrane were placed adjacent to each other in the transfer stack and an electrical conductive buffer (Towbin's buffer) was applied. A voltage of 100V was applied for 1h during which time the tank was kept in a cold room to prevent overheating and melting of the gel.

After transfer, membranes were blocked with 5 % dry milk in TBS-T with 0,2 % Na - Azid to prevent unspecific binding of antibodies to the membrane. Visualization of target protein was performed using primary and secondary antibodies. Primary antibodies used for probing were diluted appropriately in 5 % dry milk in TBS-T and staining proceeded for 1h in room temperature. The membrane was then washed twice with TBS-T (2x5 min) followed by secondary antibody staining with either Peroxidase AffiniPure Goat Anti - Rabbit IgG (#118923, Jackson ImmunoResearch) or anti - Goat IgG, H&L (Rabbit) (#401515, Calbiochem) for 1h at room temperature and washed twice with TBS-T(2x5 min). Membranes were developed using horse radish peroxidase (HRP) substrates such as SuperSignal™ West Dura Extended Duration Substrate (#34080) or SuperSignal™ West Pico Chemiluminescent Substrate (#34080), both from Thermo Fischer Scientific. The results were either visualized by AGFA Curix 60 film processor or ChemiDoc™ Touch Imaging System from BioRad.

3.2. T cell purification

Primary T cells were purified from buffy coats from healthy blood donors obtained from Oslo University Hospital Blood Centre, Oslo, Norway. The buffy coat is a concentrated leukocyte suspension; it contains most of the white blood cells from a whole blood sample. To purify T cells from buffy coats, various RosetteSep Enrichment Kits (StemCell Technologies) were used. RosetteSep allows cell isolation directly from whole blood or buffy coats. Advantages of this method are that the isolated cells are functional, flow cytometry - compatible, and unlabeled with antibodies or magnetic beads, thus avoiding the risk of activating or damaging the cells. RosetteSep crosslinks unwanted cells to red blood cells, forming immunorosettes (erythrocytes are arranged around a central cell to form a cluster that resembles a flower). Upon gradient centrifugation with Lymphoprep ™ (#12KAS03, Axis Shield), these immunorosettes form a pellet due to polysaccharides in Lymphoprep™, which enhance erythrocyte aggregation. Mono nuclear cells (MNCs) with lower densities, such as T cells, are left untouched and highly

purified at the interface between the plasma and the density gradient medium, where they can be harvested. If not otherwise indicated RosetteSep™ Human T Cell Enrichment Cocktail (#15061) was used, although RosetteSep™ Human $CD4^+$ T Cell Enrichment Cocktail (#14062) and RosetteSep™ Human $CD8^+$ T Cell Enrichment Cocktail (#15063) were used for western blot.

Cells were washed in PBS (with 2 % FCS) and resuspended in RPMI 1640 GlutaMAX medium (#61870-010, Life technologies) for counting. Cells were counted and viability was checked by Trypan Blue (#1753468, Life Technologies) staining on a Countess™ automated cell counter (Invitrogen™).

3.3. Stimulation and fixation

Cells were either stimulated on the same day as purification or the next day, after overnight incubation in a 37 ^{0}C, with 5 % CO_2 humidified atmosphere. The number of cells obtained from Lymphoprep™ varied from donor to donor, but was usually in the range 100 - 500 $\cdot 10^6$ cells.

Cells were washed with appropriate medium for stimulation (RPMI 1640 GlutaMAX medium, Life Technologies). Exact stimulation conditions varied from experiment to experiment to allow investigation of proteins involved in specific signaling pathways.

Cells were divided into 1.5 mL tubes so that each condition had approximately the same cell number (2 - 5*10^6). They were pre - equilibrated in a 37 ^{0}C water bath for 10 min prior to stimulation. Where indicated, antagonists were added 5 min prior to stimulation and inhibitors 30 min prior to stimulation. Stimulating agents and molecules were added to cells with a concentration of 0.3 % DMSO or lower. After stimulation, cells were monitored for a time course of 0 - 60 min. The positive control was usually stimulation with a high concentration (10 μM) of PGE_2 (#P5640, Sigma Aldrich). Unstimulated cells with the same amount of DMSO as stimulated samples were used as a negative control. Some unstimulated cells were set aside, fixed immediately and left in the water bath for 10 min to use for controls later in the experiment. Directly after stimulation cells were fixed using pre - warmed BD Phosphoflow Fix Buffer I (#557870, BD Biosciences) for at least 10 min at 37 ^{0}C. Immediately upon fixation the signaling ceases and the cells die. After fixation the cells were washed twice with PBS and barcoded.

3.4. Fluorescent cell barcoding (FCB)

Fluorescent cell barcoding (FCB) enables high throughput, high content flow cytometry by multiplexing samples prior to staining and acquisition on the cytometer. Individual cell samples are barcoded, or labeled, with unique signatures (different colors and concentrations) of fluorescent dyes. The dyes are NAS - coupled which allows unspecific binding of primary amines on cell surfaces. Thus, each cell sample will obtain a single fluorescent signature and a unique dye intensity distribution and samples can be combined and analyzed simultaneously. By mixing samples prior to staining, antibody consumption is typically reduced 10 - to 100 - fold. In addition, data robustness is increased through the combination of control and treated samples, which minimizes pipetting error, staining variation, and the need for normalization. Finally, speed of acquisition is enhanced, enabling large experiments to be run with standard cytometer hardware.

Three dimentional (3D) barcoding was performed by using three small molecule fluorescent dyes: Pacific Blue (#P10163, Thermo Fisher Scientific), Alexa Fluor® 488 (#A3005) and Pacific Orange (P30253) both from Molecular Probes/Life Technologies. The fixed samples were stained for 30min at room temperature with different combinations of these dyes and washed twice with flow buffer (PBS + 2 % FCS) to remove excess dyes, combined into one tube and permeabilized (with ice cold BD Phosphoflow Perm Buffer III; #558050, BD Biosciences) and put for storage at - 80^{0}C freezer.

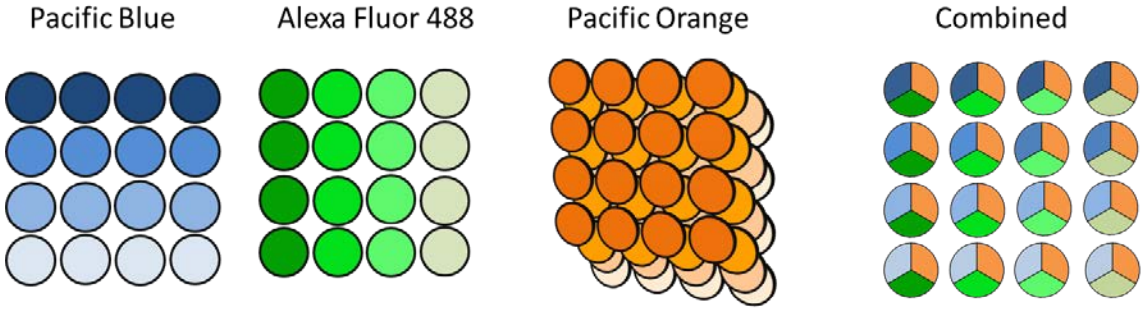

Figure 7 3D barcoding pattern. *Together these dyes generated a single fluorescent signature for each sample. This made it possible to determine each stimulation condition in the experiment by the flow cytometer and the analyzing program Cytobank. For each quadrant (16 samples) a different concentration of Pacific Orange was applied to generate a total of 64 unique fluorescent dyes.*

3.5. Staining of samples

One of the most common ways of studying cell characteristics is to use fluorophore - labeled antibodies. The labeled antibody can bind to specific molecules on the cell surface or in the cell's interior. Antibodies are highly specific to their designated epitopes and are a convenient way to visualize proteins of interest. Thus, to investigate intracellular proteins involved in signaling, antibodies conjugated to a fluorophore were used. Fluorophores emit light upon excitation and the signal may be detected by a flow cytometer.

Barcoded and permeabilized cells were washed once with flow buffer (PBS + 2 % FCS) and split into separate wells of a 96 well v - bottom plate and stained with the appropriate antibodies. All cells were stained with a mastermix containing three antibodies against surface markers: CD3 PerCP Antibody Clone SK7 (#345766), CD4 PE.Cy7 antibody Clone (#348809) SK3 and CD45RA APC-H7 Mouse Anti - Human antibody (#560043) all from BD Biosciences. CD3 is a protein that makes up most of the T cell receptor and all T cells should express this protein. Staining with CD4 was used to distinguish between T helper cells which express CD4 protein and T - cytotoxic cells which display CD8 protein. CD45RA protein distinguished between memory T - cells and naïve T cells. In this way, cell populations in the sample were easily distinguished with the flow cytometer. Phospho protein - specific antibodies which were Alexa Fluor ® 647 conjugated, were used to stain cells at room temperature, in the dark for 30 min. After 30 min incubation, cells were washed twice with PBS + 2 % FCS, suspended in 200 uL PBS + 2 % FCS. Unconjugated antibodies were used to stain cells for 30 min at room temperature, followed by 2x wash with flow buffer and an additional incubation with Alexa Fluor® 647 - conjugated secondary antibody (#A21240 or #A21245, Invitrogen) was performed for another 30 min in the dark. Cells were then washed twice with PBS + 2 % FCS, and resuspended in 200 uL PBS + 2 % FCS and subjected to the flow cytometry.
Tables of the antibody panel used in the experiments can be found in the Appendix (Supplementary Table 2).

3.6. Analysis by phosphoflow cytometry

An LSR Fortessa flow cytometer (BD Biosciences) was used for analysis and was equipped with 4 lasers. Flow cytometers are used to analyze multiple parameters in individual cells in a heterogeneous population. Cells are passed through a laser beam one by one by hydrodynamic focusing and light from each cell is captured in detectors as it passes through. This provides information about individual cells, such as size, complexity, phenotype and health. Light scattered in forward direction (forward scatter) by cells passing through the laser beam, provides information about cell size. Light scattered at larger angles, for example to the side is called side scatter, this light is collected at several detectors and represents the cell's granularity or complexity. The scattered light intensity is converted into a voltage pulse and information is represented in a two dimensional scatter plot which displays cell size and granularity.

Different parameters were determined in the instrument prior to analysis. Barcoding dye in addition to fluorophore conjugate upon laser excitation emitted wave lengths that were separated in channels. The excitation signals from the fluorophores were detected by photomultiplier tubes (PMTs), and voltage to amplify the excitation signal of each fluorophore was applied. Voltage (to these PMTs) must be applied because the original signal generated from the excitation is too weak, thus by enhancing the electrical current the signal is detected by the instrument.

The scatterplot has a logarithmic scale. This compresses the scale for positive events and broadens the scale for negative events which makes it easier to distinguish between positive and negative events. Unstained and stained controls are included in the analysis, they are important for determining the positive and negative populations of the fluorophores (Figure 9A).

Before proceeding with the analysis, compensation for the different channels in use was set.Compensation is a mathematical process that subtracts the false fluorescence generated from the positive florescence derived from the fluorochrome. It takes into account the spectral overlap that may occur between two or more fluorochromes (Figure 8). If emission spectra overlap, the detector will detect light from several fluorochromes in one channel and it will give a false positive signal in both channels. This may disturb the analysis, and may give false results. Compensation was performed for each antibody coupled fluorochrome using compensation beads from BD Biosciences. As for the

barcoding fluorochromes, a set of separately stained cells was used. For compensation both positive and negative signals are determined in a population with one given fluorochrome. Signal detected in one channel from one specific fluorochrome will therefore be true fluorescence, and not spillover signals from other channels.

For each sample 150 000 - 1 000 000 events where recorded. The data was analyzed using Cytobank.

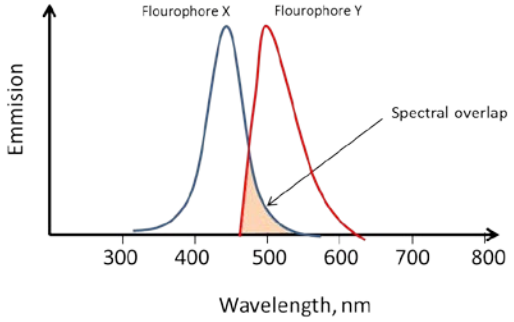

Figure 8 Spectral overlap.

Some fluorochromes have emission spectra that overlap to some extent. This "spillover" may be removed by compensation.

3.7. Gating strategy

Data were analyzed using Cytobank (https://cellmass.cytobank.org/). Cytobank is a cloud - based platform that enables analysis and visualization of multiple single - cell data sets simultaneously.

Different populations of interest were defined through "gating". A gate is a shape that is drawn around a population or a cell region on a one - or two parameter plot. Live cells were selected by plotting side scatter area against forward scatter area. Following isolation, it is important to gate out the dying or abnormal cells. Singlet cells were then selected on a plot of forward scatter area against forward scatter width. Cells may cluster together and generate doublets; these will give an incorrect signal during the interpretation of the data and should be gated out. These doublets can be detected by disproportions between height (H), width (W), and area (A) generated by the voltage pulse. Thus plotting forward scatter area against forward scatter width (in addition to SSC - A and SSC - W) it's evident which cells have a larger area and width compared to the main population and can be gated out (Figure 9B).

Barcoded populations were most readily gated from a two - dimensional plot with forward or side scatter as one parameter and the barcoding channel as the other parameter, this makes each barcoding population visible in the plot (Figure 9). Each concentration of the three barcoding dyes can be distinguished as well as the barcoding pattern for each condition in the experiment. In Cytobank, the FCS files were then split by population to make the final analysis, and four new populations were gated for: $CD4^+$ and $CD8^+$ cells as well as $CD45RA^+$ and $CD45RO^+$ cells.

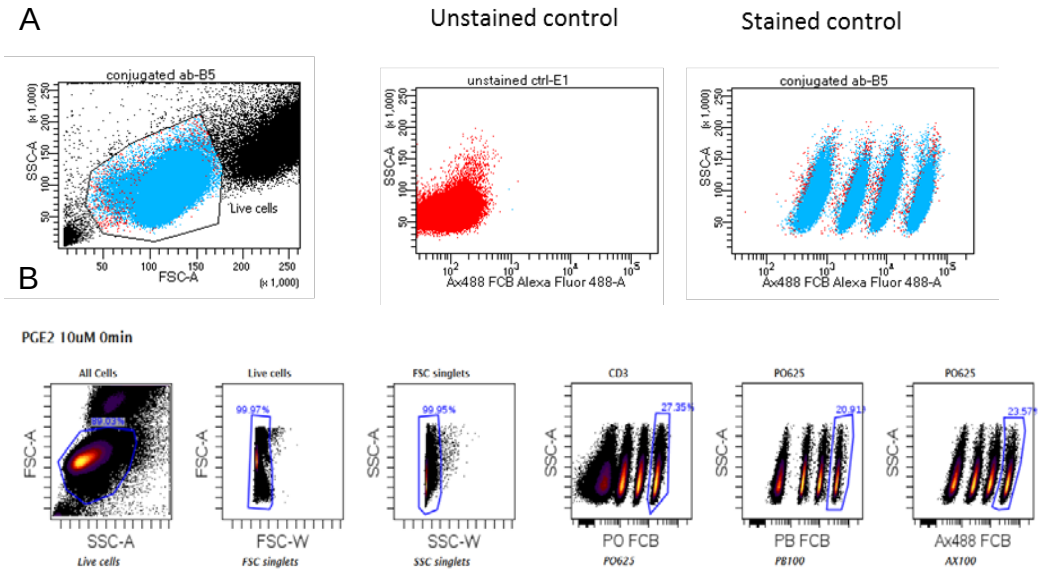

Figure 9 Gating hierarchy. *A) Live lymphocytes are gated as the middle population between FSC-A and SSC-A, and unstained and stained controls are defined in the cytometer before the analysis. B) In Cytobank, further gating and analysis were performed, singlets are selected by plotting FSC-A against FSC-W, the same is done for SSC. In this example, a specific stimulation condition (PGE_2 stimulation after 0 min) can be isolated based on its fluorescent signature, these cells have the highest concentration of Pacific Blue, Alexa Fluor 488 and Pacific Orange.*

3.8. Statistical analysis and concentration response curves

All statistical and concentration response analysis was performed in GraphPad Prism 7.02. Equations used for concentration responses curves were nonlinear regression (curve fit) log (agonist/antagonist) vs. response (three parameters). Hill coefficient value was set to 1. Equation for calculating concentration response curves with agonist:

Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)))

Equation for calculating concentration response curves with antagonist:

Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50)))

The intention of using these equations is to determine the EC50/IC50 of the agonist/antagonist. EC50/IC50 is determined as the concentration that provokes a response half way between the maximal (top) response and the lowest (bottom) response.

3.9. Agonist and antagonist experiments

Agonists and antagonists were used to investigate specific signaling nodes downstream of each EP receptor. Each agonist and antagonist used in the experiments were dissolved to the desired stock concentration in DMSO, aliquoted and stored in a -80^{0}C freezer. For experiments, they were diluted to appropriate concentrations in cell media (GlutaMax 1640, Life Technologies) to give a final DMSO concentration of 0.1 - 0.2 %. All agonists and two of the antagonists were provided under an MTA with ONO Pharmaceuticals. The agonists were ONO-DI-004, ONO-AE1-259-01, ONO-AE-248, ONO-AE1-329 for EP1, EP2, EP3 and EP4 respectively. Regarding the agonist experiments, an optimized concentration of each agonist was previously determined in the lab. These concentrations were 1 μM, 40 nM, 50 nM and 52 nM for EP1, EP2, EP3 and EP4 receptors, respectively. The set of antagonists were ONO-8713 for the EP1 receptor ONO-AE3-240 for the EP3 (MTA with ONO Pharmaceuticals), TG4-155 (#17639) and EP4 ONO-AE3-208 (#14522) for EP2 and EP4 receptors respectively (both from Cayman Chemicals).

A series of experiments was performed to optimize conditions for use of the antagonists as well as to investigate other properties. In particular antagonists were tested at different concentrations always with a final concentration of 0.1 % DMSO. The antagonists were added to cell samples 5 min prior to stimulation with PGE_2 and stimulation proceeded at 37^{0}C in water bath. Various concentrations of PGE_2 in combination with antagonists were tested as well as incubation time. At a later stage, titration of each agonist and antagonist was performed to identify the optimal concentration for activation/inhibition of various pathways. In these experiments, eight different concentrations of each agonist/antagonist were included to determine EC_{50}/IC_{50}. The phosphorylation status of various proteins downstream of each receptor was used as the readout.

3.10. Kinase inhibitor assay

To investigate which established downstream signaling nodes are regulated by which kinases and which phosphosites belong to which pathway, several different kinase inhibitors were employed.

In total, 15 different kinase inhibitors were investigated (see Supplementary Table 1 in the Appendix). The kinases investigated were known to work downstream of some or all of the receptors of interest, or were predicted to be involved in EP signaling pathways based on previous MS - study. $CD3^+$ T cells were purified from buffy coat as described in the section T Cell Purification. The cells were counted and viability was measured. The cells were divided into tubes according to the number of inhibitors and controls included in the experiment. Cells were equilibrated for 10 min in the water bath at 37 ^{0}C and then preincubated with respective inhibitor for 30 min prior to stimulation. A 10 μM concentration of PGE_2 was used to stimulate the receptors of interest and to achieve elevated signals from the proteins involved. Cells were fixed at time points 0, 3, 7, 10, 30 and 60 min. Barcoding, antibody staining and analysis on the flow cytometer was performed as described in sections Barcoding, Staining with Antibodies and Analysis on Flow Cytometer.

3.11. Antibody titration

Antibodies were purchased from several different manufacturers and companies. To find the optimal concentrations for the phosphoflow experiments described here, the antibodies were titrated. Titrating antibodies is important since the conjugates may vary from manufacturer to manufacturer and also from lot to lot from the same manufacturer. Titrating antibodies permits accurate measurements of fluorescence, and the signal to be maximized while the noise is reduced. The same set of conditions is used for the titration as the performed experiments, e.g the same number of cells, the same volumes per stain and the same length of staining.

In this antibody titration setup, primary $CD3^+$ T cells were purified as described in the section T Cell Purification. For the stimulation, four different conditions were used. Two different phosphatase inhibitors were used, Calyculin A and Pervanadate. Calyculin A (#9902S, Cell Signalling Technology) inhibits two major serine/threonine phosphatases - PP1 and PP2A. Pervanadate (Na_3VO_4, #S6508, Sigma Aldrich and 1 % hydrogen peroxide, #H1009, Sigma Aldrich) inhibits protein - tyrosine phosphatases (PTPs). The

last condition was phorbol 12 - myristate 13 - acetate (PMA, #P8139, Sigma Aldrich) together with Ionomycin (#I0634, Sigma Aldrich). PMA activates PKC signaling and Ionomycin triggers calcium release. The different stimulation conditions increases the overall phosphorylation status of the cell in different ways, thus allowing the phospho – epitope specific antibodies to bind to the phosphoepitope of interest in at least one of the stimulation conditions.

All cells were stimulated in a 37 ^{0}C water bath. For each condition, 1 mL of cells were fixed with 1 mL of BD Phosphoflow Fix Buffer I (#557870, BD Biosciences). The first tube which contained unstimulated cells, was fixed after 10 minutes at 37 ^{0}C. The second tube was stimulated with Calyculin A (1 µM) for 10 minutes at 37 ^{0}C then fixed. The third tube was stimulated with Pervanadate (10 µL of a stock of 0.1 M sodium orthovanadate Na_3VO_4 was mixed with 90 µL of 1 % hydrogen peroxide to obtain a 100x solution; 10 uL of 100x solution was then added to cells) for 5 min, followed by fixation. The fourth tube was stimulated by adding 10 µL PMA (a 10 mM stock solution was prepared from phorbol-12-myristate-13-acetate (PMA) and diluted 1:1200 in media) and 10 µL Ionomycin (1mg/ml stock solution from ionomycin calcium salt in DMSO was diluted 1:10 in media) to cells and incubating for 10 min at 37 ^{0}C, followed by fixation

Fixed cells were washed in PBS + 2 % FCS and the different stimulation conditions were barcoded as described in the section Flourescent Cell Barcoding. For each antibody, five different concentrations of the were tested, where the highest concentration was recommended by the vendor.

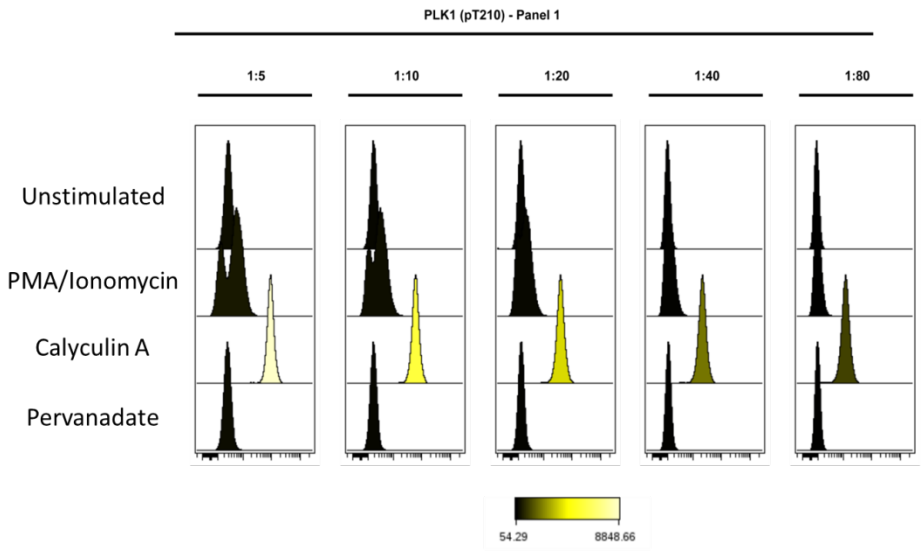

Figure 10: Titration with five different concentrations of anti - phospho PLK1 (pT210) antibody.
PLK1 (pT210) binds to phosphorylated threonin at position number 210 in the PLK1 amino acid sequence. As Calyculin A phosphorylates serines and threonines, a signal from the antibody is observed under this stimulation condition.

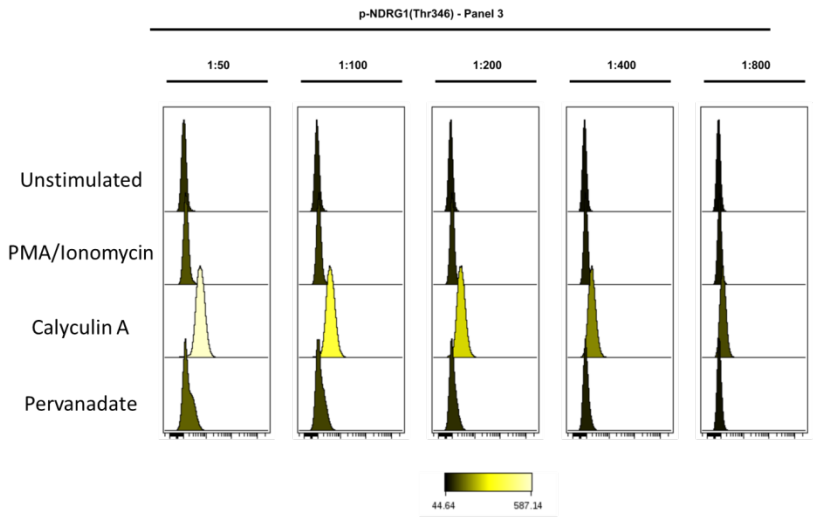

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Figure 11 Titration of anti - phospho NDRG1 (Thr346) with five different concentrations of antibody.
Phospho - NDRG1 (Thr346) was phosphorylated in the presence of Calyculin A as this reagent phosphorylates threonins, signal decreases with reduced concentration of the antibody.

As observed and previously stated the signal from the different antibodies varies. The figures above represent a few examples of the titrated antibodies. For PLK1 the recommended dilution was 1:5, but as Figure 1 displays 1:20 may also be an effective concentration. Choosing concentrations is a compromise between good separation between stimulated and unstimulated samples and an appropriate signal intensity. As observed for PKD1 and several of the antibodies, the intensity of the signal decreases for each dilution. As shown in Figure 11, Thr346 for NDRG1 was phospohorylated at in the presence of Calyculin A, and has the appropriate signal with 1:100 dilution of antibody.

4. Results

4.1. Four EP receptors are expressed in T cells

Western blotting was used to visualize and determine the expression of the four PGE_2 receptors in primary T cells. EP1 - EP4 follow restricted expression patterns that are confined to each specific tissue because of distinct signaling pathways and function elicited by the four receptors [66]. With respect to the tissue distribution and cellular localization of the four receptors, great variations have been demonstrated between tissues. EP2, EP3 and EP4 have been shown to be widely distributed in almost all tissues, whereas EP1 expression is thought to be restricted to specific organs such as lung, kidney and stomach [80]. Due to this variation, it was essential to determine whether the four receptors were expressed in primary T cells before proceeding with studies on EP1 - EP4 signaling in these cells. $CD4^+$ and $CD8^+$ T cells were purified from a healthy blood donor using the RosetteSep Enrichment Kit as described in the section "T cell purification" in Materials and Methods.

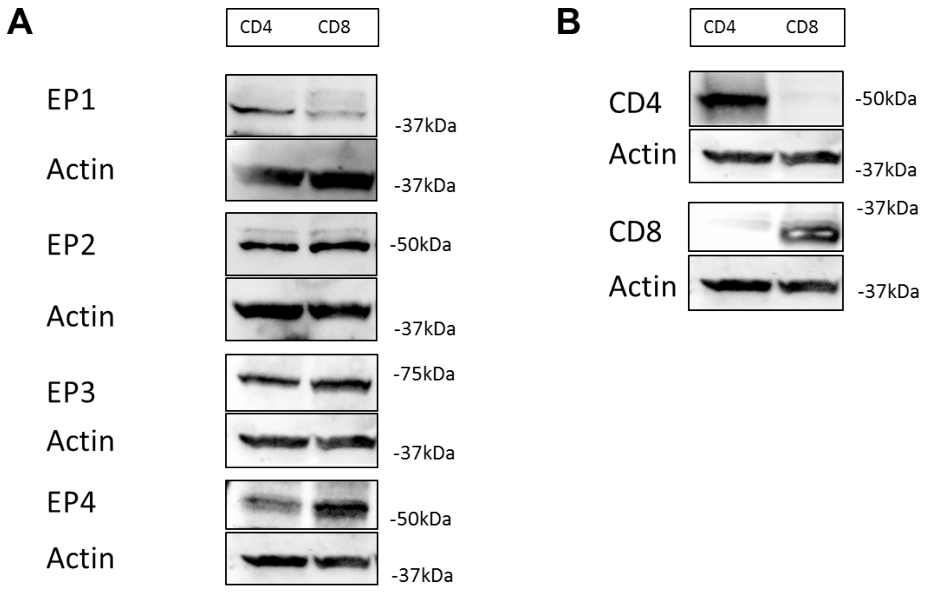


Figure 12 Western blot of EP1-EP4 in primary T cells. *A) The presence of the four receptors of interest was examined by western blot. $CD4^+$ and $CD8^+$ cells were purified from the buffy coat of a healthy blood donor. As a control, the housekeeping protein actin was detected. B) To check for sample purity, the samples were also stained with anti - CD4 and - CD8 antibodies. CD4 protein is only expressed in $CD4^+$ T cells and CD8 protein is present only in $CD8^+$ T cells.*

The expression of the four EP receptors in primary T cells was examined by western blot (Figure 12A). SDS - PAGE mobility of the different receptors was as expected from the literature, with EP1 migrating at 42kDa, and EP2, EP3 and EP4 migrating at 52, 62 and 53kDa, respectively. However, there are some conflicts between different reports about the expected molecular weight of EP3 and EP4 by SDS – PAGE. This may be due to expression of different splice variants or various modifications on the receptors, as described in the Introduction. The purpose of this experiment was to determine the presence of the four EP receptors in T cells, which we confirmed. However the expression levels of the different receptors cannot be compared from this experiment (n = 1). More observations from different donors should be made and one must recognize that the signal in western blot by different antibodies cannot be directly compared. However, the results may indicate different expression patterns in different T cell subsets, as it may appear that EP2 and EP4 - receptors are expressed at higher levels in $CD8^+$ T cells than in $CD4^+$ T cells. The co - expression of more than one PGE_2 receptor or isoform adds to the diversity of effects already inherent to prostaglandin receptor simulation. Thus, these results indicate the potential for multiple roles of PGE_2 in T lymphocytes, as all four receptors are expressed.

As a purity control, CD4 and CD8 proteins were detected (Figure 12B). The majority of CD4 proteins are expressed in $CD4^+$ T cells, while CD8 proteins are only expressed in $CD8^+$ T cells. The results display correct distribution of the two proteins representing the two different T cell populations, and confirm the purity of the samples.

4.2. PGE_2 signaling through four the EP receptors

Phosphoflow cytometry was used to investigate the PGE_2 - induced phosphorylation signaling events under a variety of different experimental stimulation conditions. These conditions included PGE_2 concentrations, agonist and antagonist concentrations as well as different stimulation time points. Phosphoflow in combination with FCB allowed analysis of a large number of samples and had the capacity to generate single cell information on the phosphorylation status of more than 30 phosphoepitopes using specific antibodies. In this way, signaling profiles in different cell types, at various time points, were investigated.

The phosphoepitope specific antibodies used to assess the phosphorylation status of a number of phosphorylation sites in pathways downstream of EP1 - EP4 were selected based on a previous mass spectrometry study performed in the lab (Lone *et al.*, manuscript in preparation). This study examined 8000 phosphopeptides in $CD4^+$ and $CD8^+$ T cells and revealed a number of EP1 - EP4 agonist - regulated phosphorylation sites downstream of each receptor. A selection of the identified phosphorylation sites from each receptor was chosen to investigate the downstream signaling pathways of the individual receptors by phosphoflow cytometry.

A phosphorylation site (phosphosite) describes the short amino acid sequence on a molecule that is recognized by kinases and is subjected to phosphorylation (addition of a phosphate group). In the present study, a phosphoepitope is defined here as the specific amino acid that is phosphorylated at a phosphosite. A challenge in studying novel phosphosites that are part of a signaling pathway is the limited access to such phospho – epitope specific antibodies suitable for phosphoflow. Antibodies to better known and previously studied phosphoepitopes are commercially available, and produced by several vendors. Thus, in some cases where antibodies against novel phosphosites identified in the mass spectrometry study could not be obtained, other antibodies against more well - known phosphoepitopes on the same or a related protein were also used. In addition, some phospho - specific antibodies were added to the panel based on the literature on PGE_2 signaling in T cells as well as other cell types. An overview of the established antibody panel used to study EP1 – EP4 signaling in the presented work is shown in Supplementary Table 2 in the Appendix. Table 1 lists the phosphoepitopes specifically discussed in this Thesis, and includes an overview of their regulation and functional role.

Table 1 Overview of phosphoepitopes investigated in this Thesis. *Several phosphoepitopes were investigated during this Thesis work, however not all responded to PGE_2 stimulation and were excluded from this table. This table displays an overview of the phosphoepitopes that are shown and discussed in the following sections, their biological function and whether phosphorylation of the phosphoepitopes inhibit or activate the function of the protein.*

Molecule (phosphoepitope)	Regulation by phosphorylation	Regulated by	Biological function	References
Protein Kinase A (PKA)	PKA substrate consensus phosphorylation sequence RRXpS/T.	cAMP	Activated by cAMP. Phosphorylates a large number of substrates in cytoplasm and nucleus.	[85]
PKA RIIα (Ser99)	Autophosphorylated, and releases the bound catalytic subunits.	cAMP	Type II regulatory subunit of PKA, binds catalytic subunit when inactivated. Found in intracellular compartments such as mitochondria, nucleus and centrosome.	[71, 86]
PKA RIIβ (Ser114)	Autophosphorylated, and releases the bound catalytic subunits.	cAMP	Type II regulatory subunit of PKA, binds catalytic subunit when inactivated. Found in intracellular compartments such as mitochondria, nucleus and centrosome.	[71, 86]
PKA - C (Thr197)	Induced enzymatic activity.	cAMP	Catalytic subunit of PKA. Phosphorylates several target proteins.	[87]
S6 ribosomal protein (Ser235/236)	Activating site, induce assembly of preinitiation complex.	RSK, S6K1, S6K1	Control cell growth through translation of selective classes of mRNAs.	[88]
S6 ribosomal proten (Ser240)	Activating site, induce assembly of preinitiation complex.	S6K1, S6K2	Control cell growth through translation of selective classes of mRNAs.	[88]
GSK3α (Ser21)	Enzymatic activity inhibited.	PKA, Akt	Regulate glucose homeostasis. Regulate level and transcriptional activity of β-catenin.	[89]
VASP (Ser157)	Induced enzymatic activity, intracellular localization.	PKA, PKC, PKG	Actin associated protein involved in cytoskeleton remodeling.	[90]
Vimentin (Ser38)	Induced activity.	PKA, CAMK2, PKC, Akt,PAK	Intermediate filament protein, regulate cytoskeletal reorganization.	[91-93]
NDRG1 (Thr 346)	Induced activity.	SGK1, PKA	Promotes proliferation and correlates with invasion. Involved in DNA damage response and regulation of apoptosis.	[94]
CREB (Ser133)/ATF-1 (Ser63)	Induced activity, transcriptional activation.	PKA, Akt, PKC, GSK3b, p90RSK	Transcription factor, binds to cAMP - responsive element (CRE), inducing transcription of various target genes.	[95, 96]
PLCγ-1 (Tyr783)	Induced activity.	EGFR, Lck, SLP76, DYN2, Syk	Produce DAG and IP3, in response to growth factor receptor and immune system receptors.	[97, 98]
Akt	Akt substrate consensus phosphorylation sequence RXRXXpS/pT.	Akt1, Akt2, Ak3	Regulate cell survival and metabolism in many different signaling pathways. Over 160 protein substrates are known.	[99, 100]
Histone H3 (Ser10)	Transcriptional activation.	MSK, Ras-MAPK, PKA, Akt1	Core component of nucleosomes that compact DNA into chromatin, regulate transcription.	[101-103]
Histone H3 (Ser28)	Transcriptional activation.	MSK,PKA	Core component of nucleosomes that compact DNA into chromatin, regulate transcription.	[101, 103]
HSP27 (Ser78)	Induced activity.	MAPKAPK2, p38, PKA, Akt, PKC, PKD	Heat shock protein that modulates actin polymerization and reorganization.	[104, 105]

Stimulation with PGE_2 resulted in phosphorylation of different phosphoepitopes on specific proteins in the signaling pathways at various time points. Phosphorylation signals were also observed to have varying signal intensities over time. Thus, increased or reduced phosphorylation each phosphoepitope peaked in a time - dependent manner. Each phosphoepitope investigated was monitored at several time points up to 60 min. Time courses are a helpful tool to assess the order of the phosphorylation of the different phosphoepitopes. For example, if a phosphorylation signal peaks at 5 minutes of stimulation, while another phosphoepitope shows a response after 30 minutes, one can hypothesize that one of the phosphorylation events occurs upstream of the other. Moreover, phosphorylation signals obtained after 30 - 60 minutes can be an indication of proteins having undergone processes such as nuclear translocation and other localization processes.

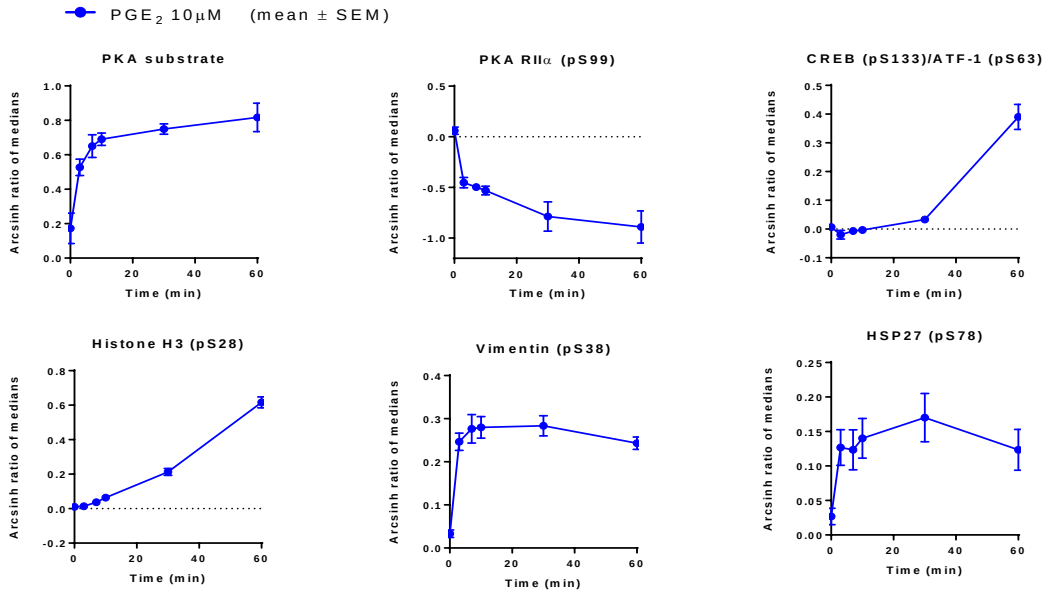


Figure 13 Phosphoflow analysis of PGE_2 - induced phosphorylation in T cells over a 60- minute time course. *$CD3^+$ T cells were purified from buffy coats from healthy blood donors and stimulated with 10 µM PGE_2 and fixed at various time points (0, 3, 7, 10, 30 and 60 min). The cells were then barcoded and permeabilized before they were stained with antibodies directed towards $CD3^+$, $CD4^+$ and $CD45RA^+$ surface markers and one of the different phospho - antibodies or IgGκ as an isotype control (not shown). The fluorescence signal was then detected by the flow cytometer and analyzed in Cytobank. Signal intensities were calculated relative to the unstimulated sample. Phosphorylation kinetics of six different phosphoepitopes regulated by PGE_2 (n = 3, mean ± SEM) in $CD8^+$ $CD45RO^+$ T cells are shown.*

The signal intensities obtained from each phosphoepitope were measured as arcsinh ratio of medians. By using the arcsinh equation, negative numbers generated from the flow cytometry analysis can be transformed while displaying the data in a log - like fashion. Negative numbers from flow cytometry is interesting to look at as they represent a possible reduction in the phosphorylation signal. In addition, the arcsinh scale is used to de - emphasize the noise near zero. Spread in peaks near zero can occur due to fluorophore spectral properties, cytometer settings and compensation.

The phosphorylation status of the various phosphoepitopes is highly time - dependent (Figure 13). The various phosphoepitopes demonstrate different phosphorylation kinetics, and the phosphorylation signal varies over time. Proteins such as vimentin and heat shock protein 27 (HSP27) are phosphorylated at a much earlier point compared to phosphoepitopes on cyclic AMP - responsive element - binding protein 1 (CREB) and histone H3, which indicates that both CREB and histone H3 are further downstream in the activated pathway(s). HSP27 and vimentin phosphorylation at Ser78 and Ser38 respectively, were observed to be maximal at 3 - 10 minutes after stimulation, followed by a decrease in signal. On the other hand, both CREB and histone H3 had low responses before a maximal peak at 60 minutes. Phosphorylation of serine 133 on CREB induces binding of this protein to the cAMP response element (CRE) where CREB functions as a transcription factor for a set of specific genes. Histone phosphorylation is regarded as the dynamic components of the machinery responsible for regulating gene transcription, as it has a major influence on chromatin structure. Thus, these proteins establish a link between transcriptional regulation and PGE_2 [101] .

The anti - phospho PKA substrate antibody recognizes phosphorylated PKA substrates (Figure 13), which contain a consensus sequence, RRXpS/pT. PKA consists of four subunits, two regulatory and two catalytic subunits. Upon phosphorylation by cAMP, the regulatory subunits dissociate from the catalytic subunits, which are then free to phosphorylate specific target molecules. By tracking PKA substrate phosphorylation, a maximum phosphorylation after 10 minutes of stimulation was observed, and the phosphorylation remained after 60 min of stimulation. In line with the information presented in the Introduction, these results confirm the involvement of PKA during PGE_2 - induced signaling. An additional phosphoepitope specific for PKA was investigated, called PKA regulatory subunit IIα Ser99, (while the others will be further discussed later

in this section). Phosphorylation of this amino acid was reduced in the presence of PGE_2 (Figure 13). T cells express two isoforms of PKA, type I and type II, each with two types of subunits - α or β. Each type may have different compositions of the subunits and thus be functionally different. In T cells, type I PKA has been found to be associated with the plasma membrane, whereas type II can be found in subcellular compartments such as nucleus, centrosome, Golgi bodies and mitochondria [86]. Moreover, PKA type I - mediated signaling elicited by cAMP in effector/memory T cells has been shown to correlate with reduced proximal T cell receptor (TCR) signaling as well as reduced proliferation and cytokine production [46, 106]. PGE_2 - induced phosphorylation of PKA substrates as shown here, can possibly contribute to the inhibition of normal T cell function. The phosphorylation of PKA substrates as well as the reduced phosphorylation of regulatory subunit PKA IIα displayed above indicates several functional differences between the two PKA types, in which both are regulated by PGE_2 (this will be further discussed in the next sections).

4.2.1. Distinct T cell populations differ in their responses to PGE_2 stimulation.

The findings described in the previous section were observed in a specific T cell population, named $CD8^+$ $CD45RO^+$ (cytotoxic memory T cells). Signaling profiles of $CD4^+$ and $CD8^+$ naïve T cells ($CD45RA^+$) and $CD4^+$ and $CD8^+$ memory T cells ($CD45RO^+$) were investigated and different phosphorylation patterns and intensities were observed in the four T cell subsets investigated during PGE_2 stimulation. To allow several channels available for FCB as well as other conditions in the flow cytometer, $CD8^+$ T cells were identified as $CD4^-$ T cells using a CD4 surface marker and CD45RA/RO positive cells were identified by the presence or absence of a CD45RA marker. By investigating several populations within the large classes of T cells such as $CD4^+$ and $CD8^+$ functional differences of PGE_2 may be revealed, because opposing signal responses in the large populations may be prevented. The signal intensity and phosphorylation of the proteins displayed in Figure 14 were observed to be cell type - dependent. $CD8^+$ $CD45RO^+$ ($CD4^-$ $CD45RA^-$) cells represent activated memory cytotoxic T cells. This cell type showed a higher sensitivity and more prolonged response towards PGE_2 stimulation for almost all phosphoepitopes investigated.

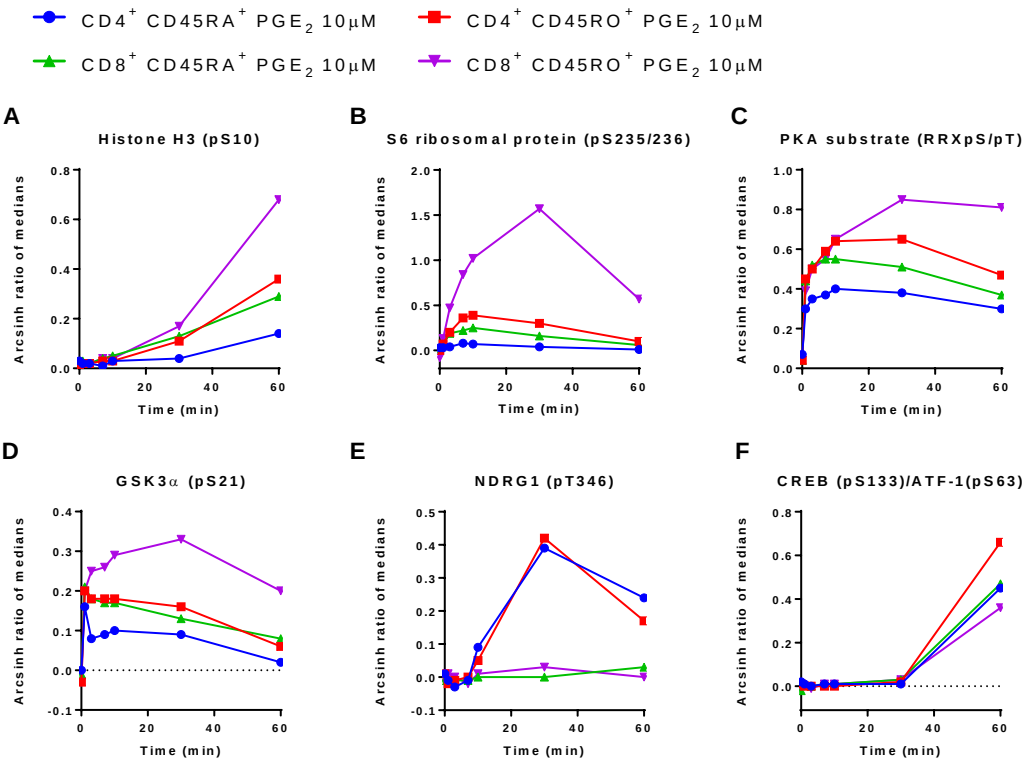


Figure 14 Distinct T cell subtypes show different responses to PGE_2 stimulation. *The different T cell subsets display different sensitivities to PGE_2. Cells were stimulated with 10 µM PGE_2 and the experiment was performed as described in Figure 13. Figures A - F display six different phosphoepitopes regulated under these conditions. A-D shows phosphoepitopes with the highest response in $CD8^+$ $CD45RO^+$ T cells, while E-F have the highest phosphorylation signal in $CD4^+$ $CD45RO^+$ T cells. Data presented is representative for three independent experiments with different donors.*

Intriguingly, N - myc downstream - regulated gene 1 (NDRG1 (pT346)) and CREB (pS133) had the highest signal intensity in $CD4^+$ T cells (Figure 14E-F) indicating functional differences of PGE_2 in the four T cell subsets. In addition, the phosphoepitopes with the highest signal in $CD8^+$ $CD45RO^+$ also indicate possible functional differences in the four T cell subsets. Phosphorylation of threonine 346 on NDRG1 (pT346) is known to be induced under a variety of stress and cell - growth regulation conditions and it has been reported to be involved in T cell clonal anergy [107]. S6 ribosomal protein and glycogen synthase kinase - 3 α (GSK3α (pS21)) are reported substrates of PKA [46] and their phosphorylation seems to be most prominent in $CD8^+$ $CD45RO^+$ T cells (Figure 14B and D). Phosphorylation of the S6 ribosomal protein, a component of the 40S ribosomal subunit at Ser235/236 shows a maximal peak at 30 min before decreasing slightly at 60 minutes. Phosphorylation of serine 235/236 leads to induced translation and the protein

plays an important role in controlling cell growth and proliferation. These findings indicate that PGE_2 regulates translation of specific proteins in T cells [108]. In contrast, phosphorylation of serine 21 on GSK3α, which also seems to be mediated by PKA, inhibits its activity [46]. These results demonstrate the regulation of the level of phosphorylation of different phosphoepitopes in the different T cell subtypes in the presence of 10 µM PGE_2. This high concentration was chosen in order to provoke and visualize even weakly PGE_2 - regulated phosphoepitopes. However, this concentration is not physiologically relevant. Thus, to further characterize kinetics of PGE_2 responses, increasing concentrations (0.01, 0.1, 1.0 and 10 µM PGE_2) were investigated in combination with temporal studies (time points 0, 1, 3, 7, 10, 30, and 60 minutes).

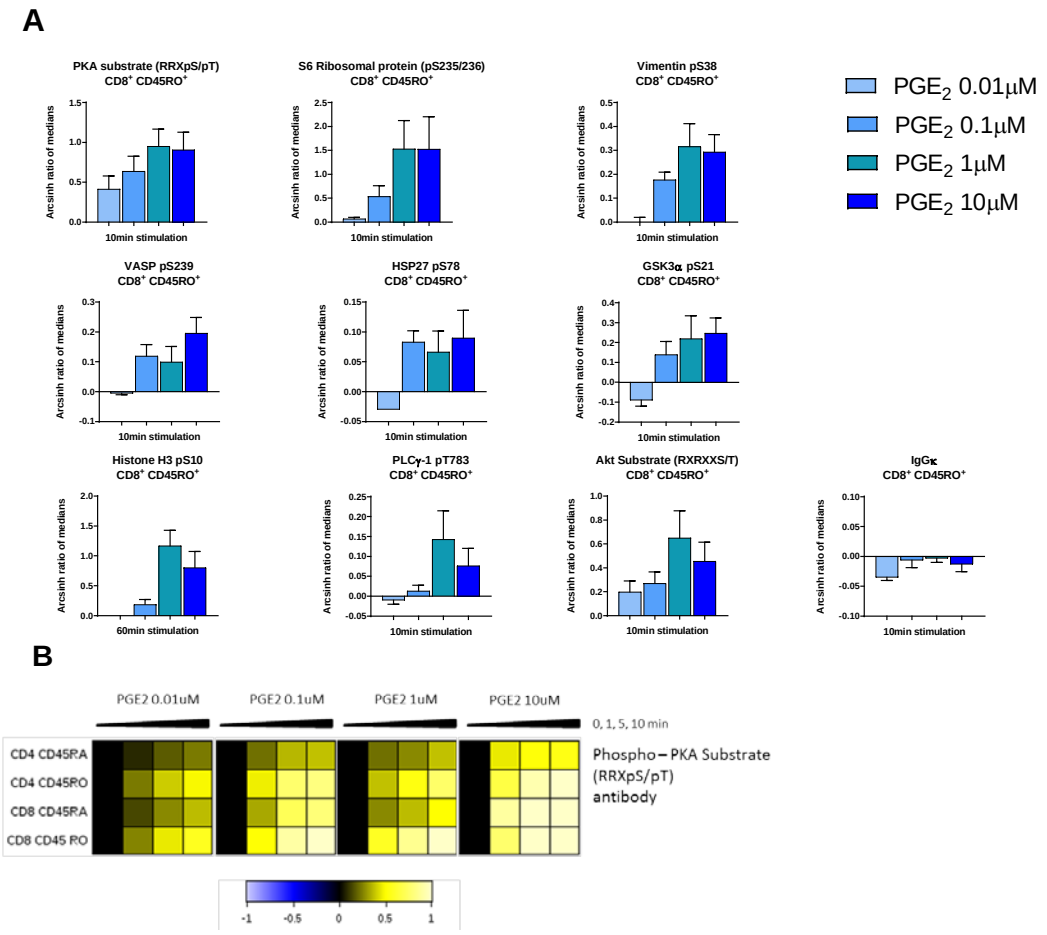


Figure 15 Phosphorylation patterns of various phosphoepitopes with increasing concentrations of PGE_2. *$CD3^+$ T cells were purified from buffy coats and stimulated with increasing concentrations of PGE_2 (0.01 - 10 µM), and performed as described in 13. Signal intensities (arcsinh ratio) were calculated with T = 0 as a reference. A) Phosphorylation responses generated with increasing PGE_2 concentrations and selected time points (n = 3, mean ± SEM). B) Heatmaps are suitable for visualizing phosphoflow data. Phospho - PKA substrate (RRXpS/pT) responses to stimulation with increasing PGE_2 concentrations are shown and signal intensity is displayed as arcsinh ratio.*

Concentrations ranging from 0.01 - 10 μM PGE_2 had various effects on the phosphorylation status of the investigated phosphoepitopes in T cells. Figure 15 shows results $CD8^+$ memory T cells ($CD8^+$ $CD45RO^+$), as this cell type displayed the highest sensitivity to PGE_2 for most of the proteins investigated, as shown in Figure 14. To achieve robust signaling readouts, 1 μM PGE_2 was required, based on observations using the ten phosphoepitope specific antibodies in the panel at the time. As observed in Figure 15, 1 μM of PGE_2 induced acceptable phosphorylation of all the phosphoepitopes displayed above, but did not saturate the signal like 10 μM PGE_2.

As PGE_2 has the possibility to bind to four receptors, the signaling pathways initiated, and the contribution to phosphorylation generated from each receptor remains unclear.

In theory, these results may involve signals generated by all four receptors, some of the receptors or from only one receptor depending on their expression, affinity and binding kinetics. As stated in the introduction, EP2 and EP4 are $G_{\alpha s}$ - coupled and are known to activate PKA. In contrast, EP3 is coupled to $G_{\alpha i}$, which is known to downregulate PKA signaling. In this way, stimulation with PGE_2 may cause opposing signals that can cancel each other out. Thus, to get a clearer view of the differences between each receptor and the pathways they initiate, specific agonists and antagonists provided by ONO Pharmaceuticals were employed.

4.3. E - Type Prostaglandin (EP) receptor agonist titrations

By using agonists and antagonists, individual pathways from each of the EP receptors can be investigated. There is an important distinction between agonists and antagonists. An agonist can bind and activate the receptor, whereas a competitive antagonist can bind at the same site without causing activation and thus block the activation of the receptor by its cognate ligand. The effect of an agonist or an antagonist is usually measured by two parameters, affinity and efficacy. An agonist has the ability to occupy a specific receptor resulting in activation of the receptor. The tendency of a drug to bind to its respective receptor is governed by its affinity, whereas the tendency to activate the receptor upon binding is called efficacy. Agonists possess high efficacy, whereas antagonists will have close to zero efficacy [81].

In this Thesis, the four receptors of interest were stimulated with various concentrations of agonists as well as antagonists to study downstream signaling of each individual receptor. The specificity of the agonists and antagonists were initially considered to be sufficient for the work presented here, and they were considered to be the most relevant for the present study. The compounds specificity in addition to other characteristics will be further discussed in the Discussion. In Figure 16, an overview of the phosphoepitopes was observed to be regulated by PGE_2 or one of the four agonists is displayed as a heatmap. Concentrations of the agonists to be used were determined based on the EC_{50} values of second messenger production provided by ONO Pharmaceuticals. Heatmaps of flow cytometry data are a convenient way to visualize many of the parameters that are included in such experiments including time, concentrations, cell types and phospho - specific antibodies.

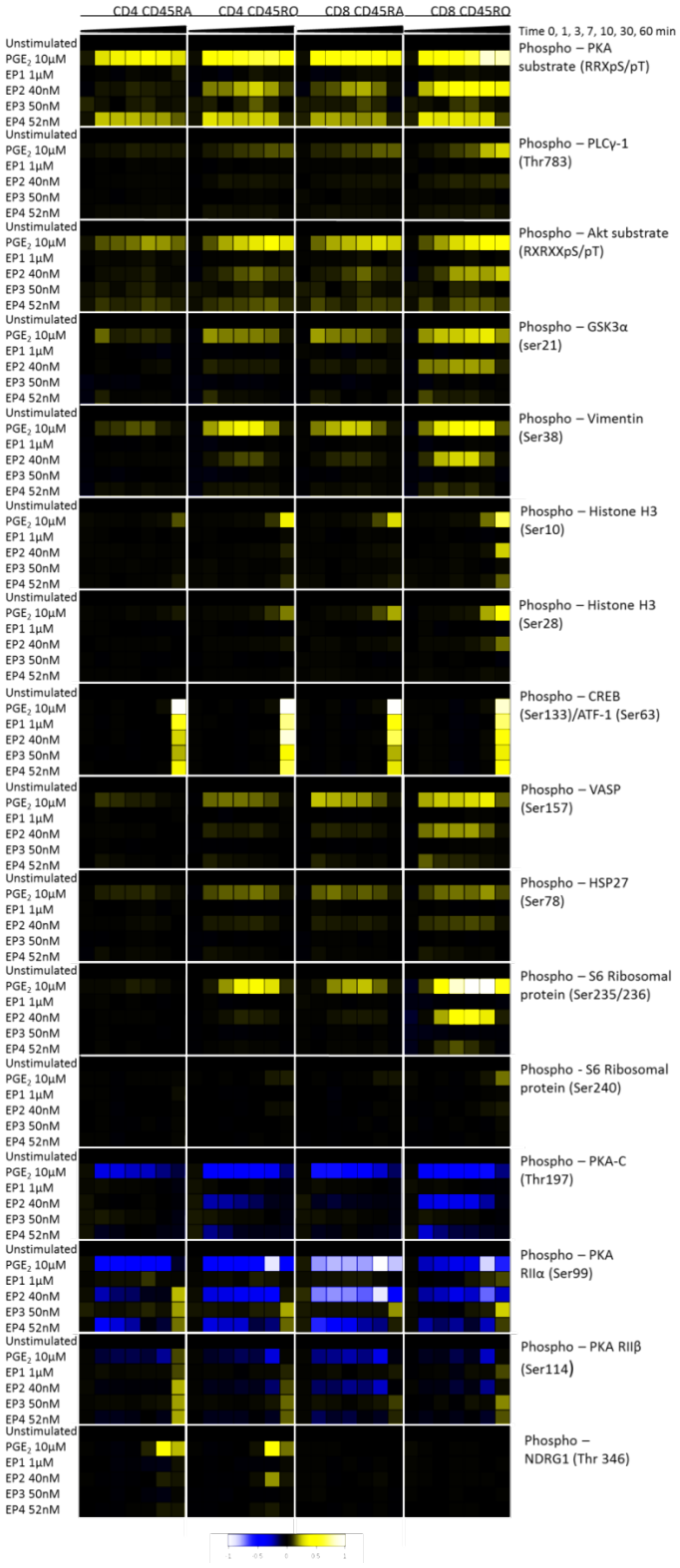


Figure 16 Phosphoflow cytometry provides detailed insight into PGE_2 signaling. *$CD3^+$ T cells were purified from buffy coats and stimulated with either PGE_2 10 μM, EP1 agonist (ONO-DI-004) 1 μM, EP2 agonist (ONO-AE1-259-01) 40 nM, EP3 agonist (ONO-AE-248) 50 nM or EP4 agonist (ONO-AE1-329) 52 nM and the experiment was performed as described in Figure 13. Phosphorylation under these conditions was investigated in four T cell subsets ($CD4^+$ $CD45RA^+$, $CD4^+$ $CD45RA^-$, $CD4^-$ $CD45RA^+$and $CD4^-$ $CD45RA^-$). Warmer colors (yellow) indicate an increase in phospho - specific epitope antibody signal and cooler colors (blue) indicate a decrease in phospho - specific epitope antibody signal. The unstimulated sample was used to calculate the arcsinh ratio of medians. IgGκ was used as an isotype control (not shown).*

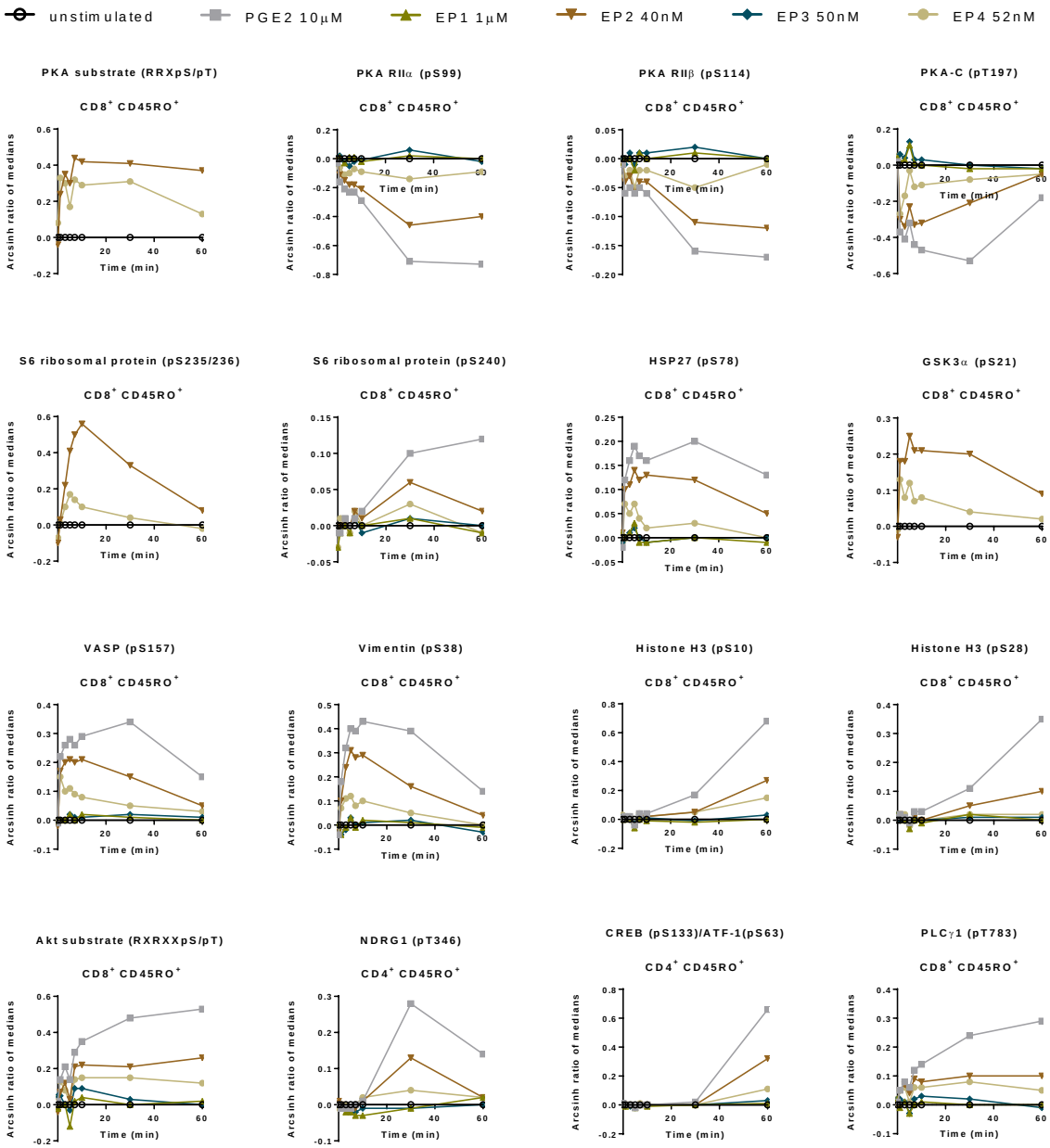


Figure 17 Analysis of stimulation with PGE_2 or one of the four EP agonists, showing phosphorylation kinetics. *$CD3^+$ T cells were stimulated under five individual conditions (Unstimulated (DMSO), PGE_2 10 µM, EP1 agonist 1 µM, EP2 agonist 40 nM, EP3 agonist 50 nM and EP4 agonist 50 nM). The experiment was performed as described in Figure 13 and stained with 28 phosphoepitope specific antibodies, whereof 16 exhibited a response to one or several of the mentioned conditions. The unstimulated sample was used as reference to calculate the fluorescence intensity signal, displayed as arcsinh ratio of medians. Agonists were ONO-DI-004 (EP1), ONO-AE1-259-01 (EP2), ONO-AE-248 (EP3) and ONO-AE1-329 (EP4).*

As observed in Figures 16 and 17, both PGE_2 and EP2 agonist provoke phosphorylation or dephosphorylation of several of the phosphoepitopes investigated. Although EP4 agonist elicits less phosphorylation of the phosphoepitopes compared to PGE_2 and EP2 agonist, it shows similar phosphorylation patterns. Such similarities are explained by the

coupling to the same $G_{\alpha s}$ protein and thus, they initiate the same signaling pathways. Both EP1 and EP3 agonists elicit phosphorylation of much fewer phosphoepitopes compared to EP2 and EP4 agonists (Figure 16). For a complete overview of the final panel of phospho - specific antibodies used in the experiments, see Supplementary Table 2 in the Appendix. Different phosphorylation kinetics such as intensity and signal duration over time were observed from the experiment displayed in Figure 17. The EP2 and EP4 receptors seem to mediate the majority of the PGE_2 - induced signaling in both $CD4^+$ and $CD8^+$ memory T cells (Figure 17) (a complete overview of the signaling generated in the four T cell subtypes is provided in Supplementary Figure 1 in the Appendix). Notably, phosphorylation and dephosphorylation regulated by the EP2 receptor seem to persist over a more prolonged time period, compared with responses elicited by the EP4 receptor, which appear to be more acute.

To investigate the individual receptor - activated responses in more detail, concentration responses over a wider concentration range of agonists were next assessed. $CD3^+$ T cells were purified from buffy coats and stimulated with eight different concentrations of a given agonist. The positive control was cells stimulated with 10 µM of PGE_2. The negative control was an unstimulated sample, containing the same amount of DMSO as the stimulated samples (0.1 %), and fixed at the same time points as the above samples (0, 3, 7, 10, 30 and 60 minutes). Samples were barcoded and permeabilized and stained with phosphoepitope specific antibodies before analysis on the flow cytometer.

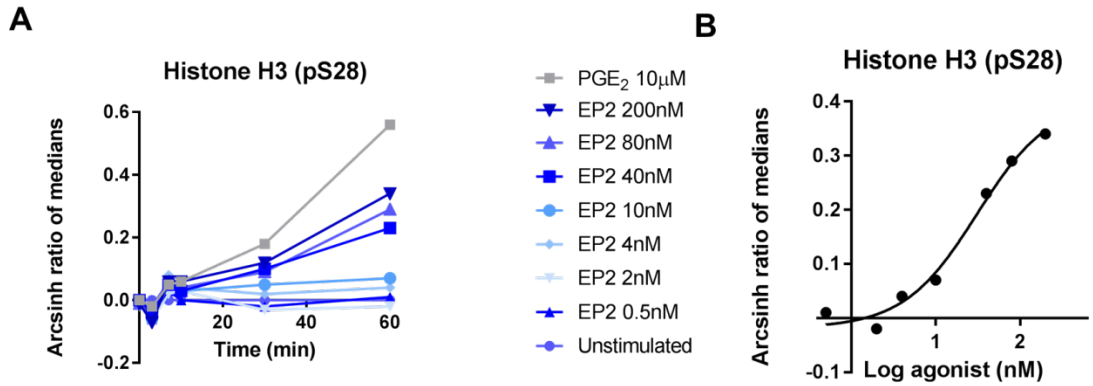


Figure 18 EP2 agonist (ONO-AE1-259-01) titration of anti - phospho Histone H3 (Ser28) responses. *(A) $CD3^+$ T cells were purified from a buffy coat and stimulated with eight different concentrations of EP2 agonist (ONO-AE1-259-01) for 0, 3, 7, 10, 30 and 60 minutes in $CD8^+$ $CD45RO^+$ T cells. One of the initial eight concentrations (1 nM) was defined as an outlier and excluded from the analysis. Unstimulated cells and cells stimulated with 10 µM PGE_2 were used as controls. Arcsin ratio of the median of stimulated samples were calculated relative to unstimulated sample.ONO-AE1-259-01 is reported to increase cellular cAMP with an EC_{50} of 4 nM, while in this experiment phosphorylation of histone H3 had an EC_{50} value of 30.87 nM (B) Concentration responses were calculated with GraphPad Prism using nonlinear regression and y vs. x (response (three parameters) vs. log(agonist)) after 60 min stimulation with ONO-AE1-259-01 (n = 1).*

Concentration response curves can be generated by titrating increasing concentrations of the agonist (18A) and concentration dependent response curves (Figure 18B) were made in Graphpad Prism by converting the concentration values into log values and using nonlinear regression (response (three parameters) vs. log(agonist)). Concentration response curves allowed determination of half maximal effective concentration (EC_{50}) for a specific compound, a specific phospho – epitope and time points. EC_{50} is determined as the concentration that provokes a response halfway between the basal (bottom) and maximal (top) signal and is commonly used as a measurement of an agonists potency (Figure 19) [109]. This model assumes that concentration response curves have a standard slope style (Hill coefficient 1.0). Furthermore, the concentration - dependent responses of a specific phosphoepitope generated by an agonist should be reversed when cells are treated with the corresponding antagonist, blocking the receptor activity. This can be used to confirm the regulation of the phosphoepitope by the specific receptor. Notably, an antagonist will have a concentration response curve that determines half maximal inhibitory concentration (IC_{50}).

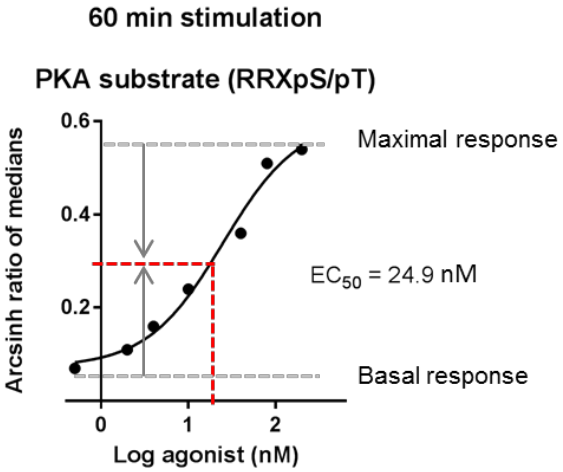


Figure 19 EC_{50} calculations for EP2 agonist (ONO-AE1-259-01) after 60 min of stimulation, for PKA substrates. *A concentration response curve for PKA substrate phosphorylation after 60 minutes of EP2 agonist stimulation in $CD8^+$ $CD45RO^+$ T cells. The EC_{50} can be calculated by determining where the response is halfway between basal and maximal response on the Y - axis, and read the corresponding agonist concentration as its log value on the X - axis. The value from the X- axis must then be converted to concentration value. The EC_{50} value was calculated in GraphPad Prism using nonlinear regression (Response (three parameters) vs. log (agonist)) and had the value of 24.9 nM at 60 min stimulation for this phosphoepitope.*

The EP2 receptor was activated by increasing concentrations of ONO-AE1-259-01, which resulted in a simultaneous increase in PKA substrate phosphorylation. The red line in Figure 19 is determined as the response halfway between basal and maximal responses. EC_{50} may be calculated by hand, using the value on the X - axis that represents the halfway response of the curve. Because this is a logarithmic scale, the value from the X - axis must be converted to an appropriate concentration value.

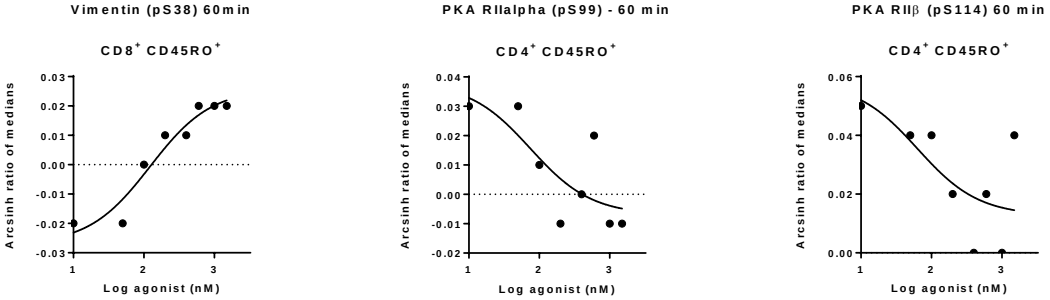
As previously discussed in this section, the phosphorylation status of the various sites was time dependent. Thus, the curve fit and EC_{50} values vary between different time points, even when looking at the same phosphoepitope, as phosphorylation is a dynamic process that may change rapidly (see Supplementary Figure 2 in the Appendix). R^2 is used as an indicator of how well the curve fits the data points involved. The R^2 value for Figure 19 is 0.9849. When $R^2 = 1$, all data points lie exactly on the curve with no scatter. Adequate concentration responses indicate that a given agonist regulates the phosphorylation of an investigated phosphoepitope.

4.3.1. EP1 agonist (ONO-DI-004) and EP3 agonist (ONO-AE-248)

The EP1 agonist provided by ONO Pharmaceuticals had a molecular weight of 422.55 kDa and was reported to cause an increase in intracellular Ca^{2+} level with an EC_{50} value of 400 nM. The EP3 agonist had a molecular weight of 380.52 kDa and was known to signal through $G_{\alpha i}$ and inhibit cAMP production. The EP3 agonist was reported to cause an increase in intracellular Ca^{2+} with an EC_{50} value of 5 nM. Experiments were performed with EP1 and EP3 agonists to determine EC_{50} values for the various phospho - specific epitopes in the panel.

Eight different concentrations were chosen, ranging from 10 to 1500 nM for EP1, which included four concentrations below the previously reported EC_{50} and three concentrations above. In the EP3 agonist experiment, concentrations ranged from 0.1 to 500 nM, also including concentrations below and above the previously reported EC_{50}. As discussed above, responses from the phospho - specific antibodies are time dependent, and different time points were chosen to capture the maximal response.

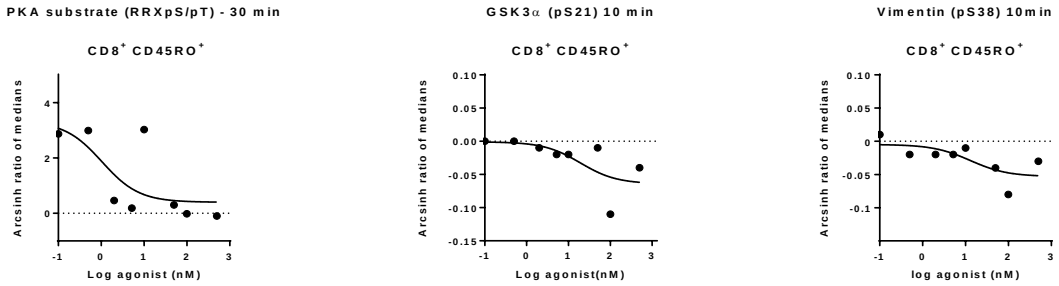
A



B

ONO-DI-004 dose responses		
Phospho – epitope	**EC50**	**R - square**
Vimentin (Ser38) 60 min	122.5	**0.93**
PKA RIIβ (Ser114) 60 min	63.43	0.47
PKA RIIα (Ser99) 60 min	74.84	0.58

C



D

ONO-AE-248 dose responses		
Phospho – epitope	**EC50**	**R - square**
PKA substrate (RRXpS/pT) 30 min	1.04	0.47
GSK3α (Ser21) 10 min	19.15	0.45
Vimentin (Ser38) 10 min	12.91	0.52

Figure 20 Phosphoepitopes regulated by the EP1 and EP3 receptors in T cell subsets. *A) $CD3^+$ T cells were purified from buffy coat using RosetteSep Enrichment Kits, and stimulated with increasing concentrations of EP1 agonist (ONO-DI-004) (10, 50, 100, 200, 400, 600, 1000 and 1500 nM) for varying periods of time (0, 3, 7, 10, 30 and 60 min). Cells were then barcoded, permeabilized and stained with phosphoepitope specific antibodies as well as antibodies against cell surface markers. The fluorescence signals were detected by the flow cytometer and the results were analyzed in Cytobank. B) EC_{50} and R^2 values calculated from the respective graphs in A), $R^2 > 0.80$ are marked in red. C) The experiment was performed as in A) but here the cells were stimulated with increasing concentrations of EP3 agonist (ONO-AE-248) (0.1, 0.5, 2, 5, 10, 50, 100 and 500 nM). In D) EC_{50} and R^2 values for the graphs in C) are displayed. Concentration response curves were made in GraphPad Prism using nonlinear regression (response (three parameters) vs. log (agonist)).*

The calculated EC_{50} values for the EP1 agonist for the three phosphoepitopes displayed above ranged from 63.43 to 122.5 nM (Figure 20B). Although some of the phosphoepitopes in the antibody panel were predicted to be involved in EP1 signaling, either from previous mass spectrometry studies or the literature, examination of their phosphorylation responses generated only a few convincing concentration response curves. However, interesting results were observed for the phosphorylation of vimentin (pS38), an intermediate filament protein. Phosphorylation on Ser38 responded to increasing concentrations of EP1 agonist, indicating that the phosphorylation of this phosphoepitope is regulated by EP1 and possibly regulated by the activation of the PI3K pathway, which is thought to be the main signaling pathway activated through EP1 [67]. Interestingly, studies in macrophages have shown that the PI3K pathway can contribute to cell migration by phosphorylation of vimentin [91]. This finding can also indicate crosstalk between the receptors, as phosphorylation of Ser38 also happens through EP2 and EP4 receptors. A trend in PKA type II (IIα (S99) and IIβ (S114)) phosphorylation was also observed with EP1 agonist titration (Figure 20A). These phosphoepitopes display the same pattern when stimulated with PGE_2, or EP2 and EP4 agonists, possibly indicating that EP1 also signals through other G proteins or that the βγ complex is activated by both EP1, EP2 and EP4 and triggers PI3K activation.

The EP3 agonist did not generate concentration - dependent responses with an adequate R^2 or curve fit. Nonetheless, some phosphorylation trends were observed with the EP3 agonist titration. As stated in the Introduction, the EP3 receptor is $G_{\alpha i}$ – coupled, resulting in adenylyl cyclase inhibition and a decrease in cAMP levels. PKA is activated by cAMP, and reduced levels of cAMP will result in reduced phosphorylation by PKA, including its autophosphorylation. Thus, when the EP3 receptor is activated a reduction in PKA phosphorylation was expected. As predicted from the literature, and observed from Figure 20C, PKA as well as some of its expected substrates displays a small decrease in phosphorylation when exposed to increased concentrations of EP3 agonist. However, the R^2 - values are low and EC_{50} cannot be accurately determined. The weak concentration responses may be explained by expression levels of EP1 and EP3 receptors in T cells, which are thought to be lower than those of the other two receptors. Furthermore, studies suggest that EP2 and EP4 are the main receptor subtypes that mediate the actions of PGE_2 in human $CD4^+$ T cells [29].

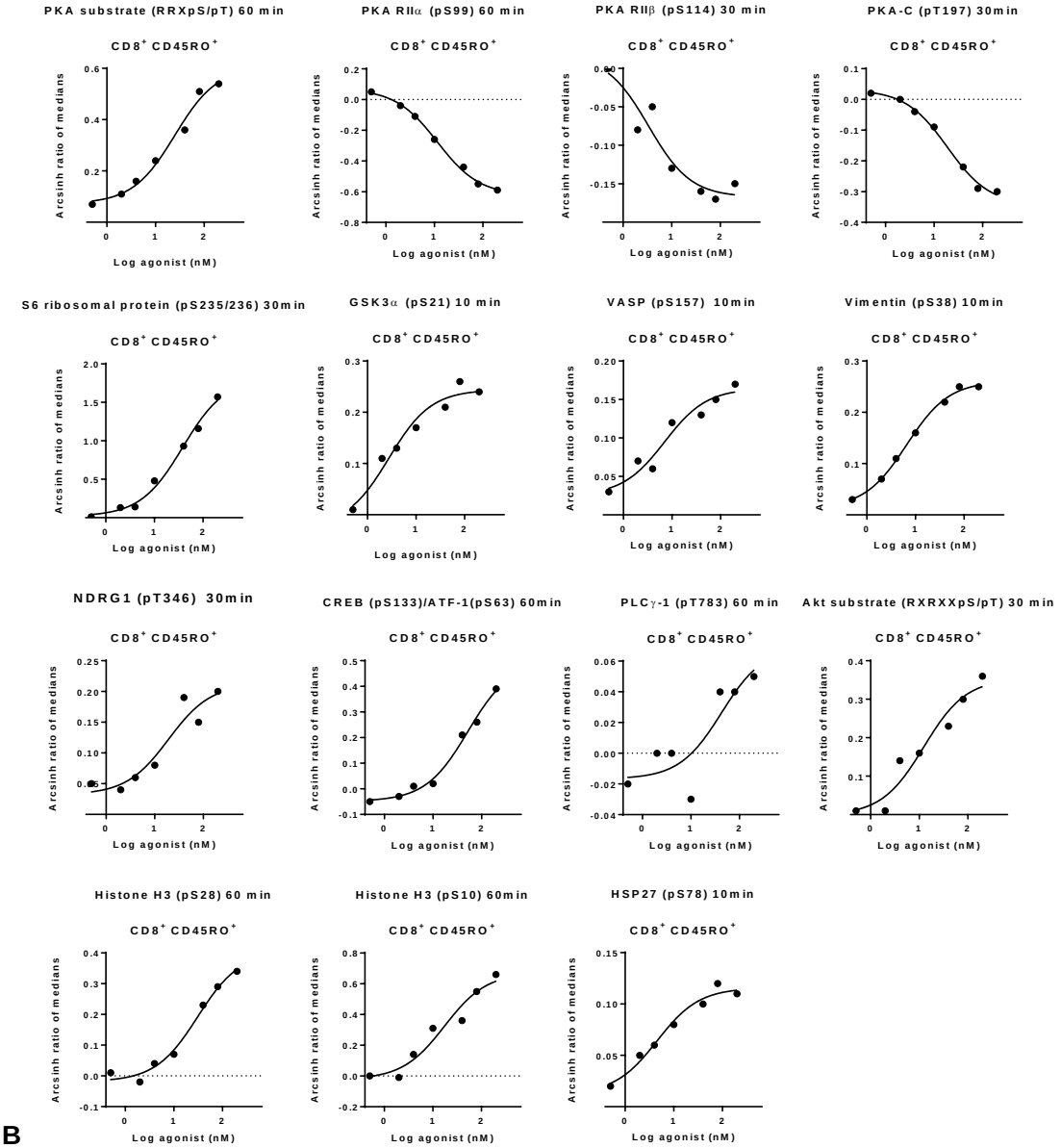
4.3.2. EP2 agonist (ONO-AE1-259-01)

The EP2 agonist was obtained through an MTA with ONO Pharmaceuticals and had a molecular weight of 432.96 kDa. As stated in the Introduction, the EP2 receptor is $G_{\alpha s}$ - coupled and activation is expected to increase levels of cAMP and activate PKA. The agonist is reported to cause an increase in intracellular cAMP with an EC_{50} value of 4 nM. T cells were purified as described in Materials and Methods and stimulated with eight increasing concentrations of EP2 agonist, ranging from 0.5 nM to 200 nM.

Many of the phospho - specific antibodies in the panel recognize and bind PKA - regulated phosphoepitopes. As expected, EP2 agonist stimulation resulted in the regulation of many of these monitored sites and several concentration response curves are thus reported from this experiment (Figure 21A).

Compared with both EP1 and EP3 agonists (Figure 20A), the EP2 agonist regulated many more sites. EC_{50} values ranged between 2.76 to 52.06 nM. These low EC_{50} values indicate high potency of the agonist (Figure 21B). A low EC_{50} value implies that less agonist is needed to provoke the half - maximal response of the receptor.

A



B

ONO-AE1-259-01 dose responses		
Phospho - epitope	**EC50**	**R - square**
PKA substrate (RRXpS/pT) 60 min	5.09	**0.96**
PKA RIIα (Ser99) 60 min	11.56	**0.99**
PKA RIIβ (Ser114) 30 min	3.34	**0.90**
PKA-C (Thr197) 30 min	18.92	**0.99**
S6 Ribosomal protein (Ser235/236) 30 min	38.28	**0.99**
GSK3α (Ser21) 10 min	2.76	**0.96**
Vasp (Ser157) 10 min	7.04	**0.94**
Vimentin (Ser38) 10 min	6.63	**0.99**
NDRG1 (Thr346) 30 min	19.24	**0.90**
PLCγ-1 (Thr783) 60 min	42.70	0.77
Akt substrate (RXRXXpS/pT) 30 min	12.43	**0.94**
CREB (Ser133)/ATF-1 (Ser63) 60 min	52.06	**0.99**
Histone H3 (Ser28) 60 min	30.87	**0.98**
Histone H3 (Ser10) 60 min	17.41	**0.94**
HSP27 (Ser78) 10 min	4.55	**0.97**

Figure 21 Concentration response curves for 15 phosphoepitopes stimulated with increasing concentrations of EP2 agonist (ONO-AE1-259-01). *A) 15 phosphoepitopes (from a panel of 28 examined antibodies, see Supplementary Table 2) displayed increased or decreased phosphorylation with increasing ONO-AE1-259-01 concentrations (0.5, 2, 4, 10, 40, 80 and 200 nM). The results indicate the involvement of these phosphoepitopes in the downstream signaling of the EP2 receptor. B) Displays EC_{50} and R^2 values for the phosphoepitopes shown in A and R^2- values above 0.80 are marked in red.*

As expected from the literature, the EP2 agonist induced PKA substrate phosphorylation (Figure 21A). Although PKA type I is predicted to be the predominant isoform in T cells [110], phosphorylation of PKA regulatory subunit Iiα (pS99) and β (pS114) subunits was observed to be reduced by EP2 agonist stimulation. This is counterintuitive because the phosphoepitopes Ser99 and Ser114 lie within the inhibitory cavity of the regulatory subunit and phosphorylation of these has been reported to induce activation of the PKA II holoenzyme [111, 112]. The observed decrease in phosphorylation of these might be due to a post - activation phenomenon. In particular, after 30 min of stimulation, the PKA subunits may already have been phosphorylated, and are now becoming dephosphorylated. PKA - autophosphorylation is a rapid response to cAMP and after 30 min of stimulation the signal may start to terminate, which is observed as a reduction in the phosphorylation of the subunits. The activation of PKA is confirmed by phosphorylation of PKA substrates, which showed a concentration - dependent response curve and shows sustained phosphorylation after 60 min of stimulation by the EP2 agonist. These findings indicate involvement of several types of PKA during PGE_2 signaling, in addition to involve of both α and β subunits.

Phosphorylation by PKA of numerous targets intersects with mitogenic signaling pathways [113] and increased cAMP levels in T cells often correlates with inhibition of immune responses as discussed in the Introduction. Several of the phosphoepitopes observed to be regulated by EP2 receptor have various reported functional roles in T cells. It is interesting to note that GSK3α is reported to be a possible substrate of PKA and its phosphorylation site Ser21 inhibits the activity of GSK3α [114]. GSK3 is known as a regulator of the hormonal control of glucose homeostasis, and part of the Wnt - pathway [115]. In addition, it has also been associated with regulation of cytotoxic T cell function [116]. Furthermore, phosphorylation of the actin regulatory protein vasodilator - stimulated phosphoprotein (VASP) has been shown to have a regulatory role in physiologically important processes such as T cell polarization and transendothelial migration [93]. VASP is also thought to be a PKA substrate and phosphorylation of Ser157 activates the protein. Several other phosphoepitopes investigated are also known substrates of PKA, such as HSP27 (pS78), CREB (pS133) and histone H3 (pS10 and pS28). Phosphorylation of these sites induces the activation of the proteins. PLCγ-1 is a membrane - bound enzyme that was observed to be phosphorylated by EP2 agonist stimulation on position Tyr783 (Figure 21A). Upon activation, PLCγ-1 cleaves

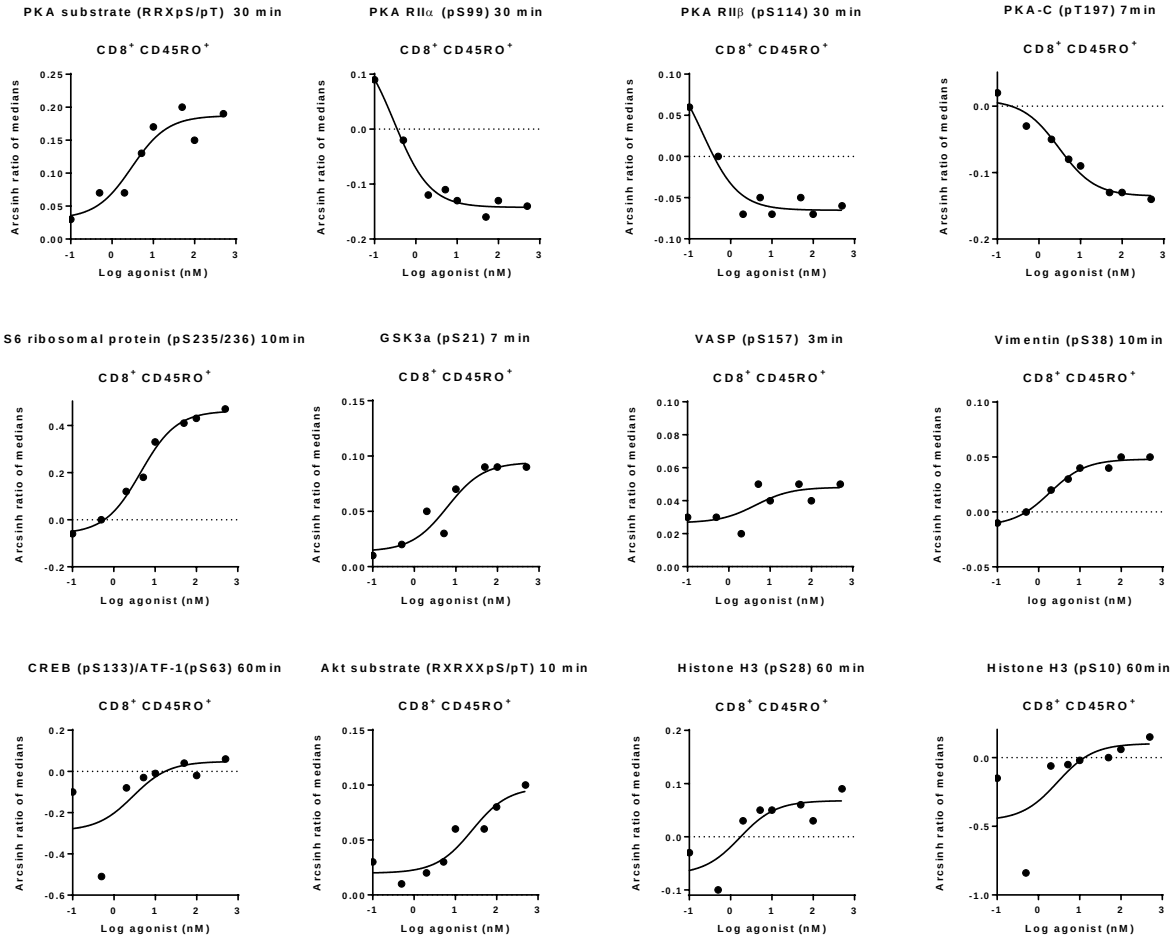
phosphatidylinositol 4,5 - biphosphate (PIP_2) to IP3 and DAG, which both act as small intracellular mediators [117]. Moreover, DAG can be further cleaved to release arachidonic acid, which can either act as a signal on its own or be used in the synthesis of other eicosanoids, including prostaglandins. Phosphorylation of Tyr783 on PLCγ-1 is essential for induction of phospholipase activity [117].

Akt substrates containing the consensus sequence RXRXXpS/pT showed increased phosphorylation in response to increased concentrations of the EP2 agonist. Although Akt is not predicted to be activated as a result of increased cAMP levels, a recent study indicated that the PI3K pathway and the activation of Akt can be initiated by the βγ - complex of the heterotrimeric $G_{\alpha s}$ protein - coupled receptor, leading to phosphorylation and inactivation of GSK3 [77]. These events may lead to stabilization and nuclear translocation of β - catenin and increased expression of growth promoting genes in epithelial cells. EP2 agonist treatment also leads to the phosphorylation of S6 ribosomal protein on Ser235 and Ser236 on the proteins C - terminus. These sites are thought to be mitogenic phosphorylation sites which induce cell proliferation and increased protein synthesis as mentioned above.

4.3.3. EP4 agonist (ONO-AE1-329)

As discussed in the Introduction, the EP4 receptor, like EP2 is $G_{\alpha s}$ coupled and known to increase levels of cAMP and activate the PKA pathway. It is therefore reasonable to expect similarities in the signaling pathways downstream of EP2 and EP4. The EP4 receptor agonist was also obtained from ONO Pharmaceuticals and has a molecular weight of 454.60 kDa. It is reported to cause an increase in intracellular cAMP with an EC_{50} value of 5.2 nM. $CD3^+$ T cells were stimulated with eight increasing concentrations of EP4 agonist ranging from 0.1 - 500 nM. Concentrations were chosen based on the information given by ONO Pharmaceuticals and the reported EC_{50} value. Cells were then fixed, barcoded, permeabilized and stained with phosphoepitope specific antibodies and antibodies against cell surface markers as in previous experiments. The fluorescence signals were detected by the flow cytometer and the data was analyzed in Cytobank.

A



B

ONO-AE1-329 dose responses		
Phosphosite	**EC50**	**R - square**
PKA substrate (RRXpS/pT) 30 min	3.00	**0.90**
PKA RIIα (Ser99) 10 min	0.54	**0.92**
PKA RIIβ (Ser114) 30 min	0.21	**0.93**
PKA-C (Thr197) 7 min	3.04	**0.96**
S6 Ribosomal protein (Ser235/236) 10 min	4.48	**0.99**
GSK3α (Ser21) 7 min	6.27	**0.89**
Vasp (Ser157) 3 min	4.48	0.55
Vimentin (Ser38) 10 min	1.86	**0.98**
CREB (Ser133)/ATF-1 (Ser63) 60 min	2.89	0.50
Akt substrate (RXRXXpS/pT) 10 min	25.54	**0.88**
Histone H3 (Ser28) 60 min	1.68	0.70
Histone H3 (Ser10) 60 min	2.91	0.48

Figure 22 Concentration response curves for phosphorylation of phosphoepitopes in CD8$^+$ CD45RO$^+$ T cells stimulated with increasing concentrations of EP4 agonist (ONO-AE1-329). *A) 12 phosphoepitopes (from a panel of 28 antibodies (Supplementary Table 2)) that display concentration - dependent responses to treatment with EP4 agonist (ONO-AE1-329) at concentrations of 0.1, 0.5, 2, 5.2, 10, 50, 100 and 500 nM. B) EC_{50} and R^2 values for the phosphoepitopes shown in A. B) Values above 0.80 are marked in red.*

The EP4 agonist was observed to induce phosphorylation of PKA substrates containing the sequence RRXpS/pT, as well as other known substrates of PKA (Figure 22), which confirms that some of the signaling initiated by EP4 receptor goes through the PKA signaling node. In addition, many of the same phosphoepitopes observed regulated by the EP2 receptor was observed for EP4 agonist as expected. Although there were similarities between these two receptors in terms of what phosphoepitopes they regulated, the results from these experiments also indicated that the two receptors differ in sensitivity and duration of the generated signal. Notably, time - dependent maxima were observed earlier for EP4 than for EP2 agonist stimulation, which may indicate that EP4 agonist stimulation induces a more acute response than EP2 agonist stimulation. In line with this, the concentration response curves for given phosphoepitopes are taken from earlier timepoints for EP4 stimulation (Figure 22) than for EP2 agonist stimulation (Figure 21).

With regards to the differing sensitivities of EP2 versus EP4, Figure 16 and 17 showed that the phosphorylation signals during EP4 agonist stimulation are weaker compared to signals generated by EP2 agonist stimulation. Indeed, there have been reports where comparison of the two $G_{\alpha s}$ proteins - coupled receptors and their relative ability to increase cAMP levels demonstrate a much weaker $G_{\alpha s}$ - coupling by EP4 compared to the EP2 receptor [72]. In addition, agonist/ligand induced internalization of the receptor could also explain the desensitization that occurs during the activation of the EP4 receptor and thus the shorter duration of this signal [74]. Interestingly, phosphorylation of NDRG1 (pT346), PLCγ-1 (pT783) and HSP27 (pS78) did not generate a suitable concentration response with increasing concentrations of EP4 agonist indicating that these phosphoepitopes may be EP2 - specific. Thus despite EP2 and EP4 regulating many of the same phosphoepitopes, the EP2 agonist might specifically activate additional pathways that differ from the EP4 - activated signaling. These findings indicate functional differences for the EP2 and EP4 receptors, as they differ in signal duration and some phosphoepitopes are regulated specifically by one of the receptors. The EP4 receptor seems to have quite low EC_{50} values (ranging from 0.21 - 6.27 nM when excluding a possible outlier with EC_{50} value of 25.54 nM) which indicates high potency of the agonist. Thus, weak potency of the agonist cannot explain why there are weaker signals from the EP4 receptor, and it must be due to other receptor characteristics such as internalization, early termination of the signal or weaker coupling to $G_{\alpha s}$.

Results from all the agonist experiments, taken together, indicate that the majority of PGE_2 - induced signaling in primary T cells occurs through the EP2 and EP4 receptors, although all receptors are present at the plasma membrane (Figure 12). This can be due to a variety of reasons; the EP1 and EP3 receptors might only be activated under specific conditions, in addition to activation by the presence of the ligand, PGE_2 or the respective agonists. The binding affinity of PGE_2 to each receptor varies, and EP1 is reported to have the lowest binding affinity [82]. However, variations in PGE_2 binding affinity do not provide an explanation for the observed low signals from the EP1 and EP3 receptors when they are activated with agonists. The agonists were reported to have high affinity for the given receptor and to have relatively low EC_{50} values and should therefore be able to activate the downstream signaling of the receptor. Signaling through EP1 and EP3 receptors in T cells and the possible functional significance of such signaling are not as thoroughly documented as for EP2 and EP4 and, thus, information about regulated phosphoepitopes is limited.

4.4. E - Type Prostaglandin (EP) receptor antagonist titrations

To further examine the involvement and their regulation of the investigated phosphoepitopes in PGE_2 - signaling, specific antagonists of the four receptors were used. An antagonist works in the opposite way of an agonist and blocks the activation of the specific receptor in the presence of activating stimuli such as PGE_2 or the agonists. By definition, full antagonists do not display any efficacy in activating the receptor. Another type of antagonists, known as partial agonists can both partially activate receptors and act as antagonists. The antagonists used in the present study were observed to be less potent than the agonists; consequently higher concentrations of antagonists were needed. All four antagonists used in these experiments were competitive antagonists, meaning they bind to the same site as the natural ligand (PGE_2) and block activation of the receptor. By using agonist - antagonist pairs, specific regulation of various phosphoepitopes can be further examined by first looking at the induction of signals, and then observing the reverse process when treating with antagonist in the presence of PGE_2 or agonist. $CD3^+$ T cells were purified from buffy coats as previously described and the cells were treated with eight increasing concentrations of antagonists in the presence of PGE_2 at 37 ^{0}C. Antagonists were added 5 minutes prior to PGE_2 stimulation to ensure proper antagonist contact with the receptor before stimulation. The potency of an antagonist can be

investigated by measuring the half maximal inhibitory concentration (IC_{50}). The IC_{50} for antagonists are dependent on the concentrations of PGE_2, as the response is dependent on the PGE_2 concentration. The PGE_2 concentration was set to 1 μM as this amount of PGE_2 resulted in suitable phosphorylation responses but did not saturate the signal (Figure 15).

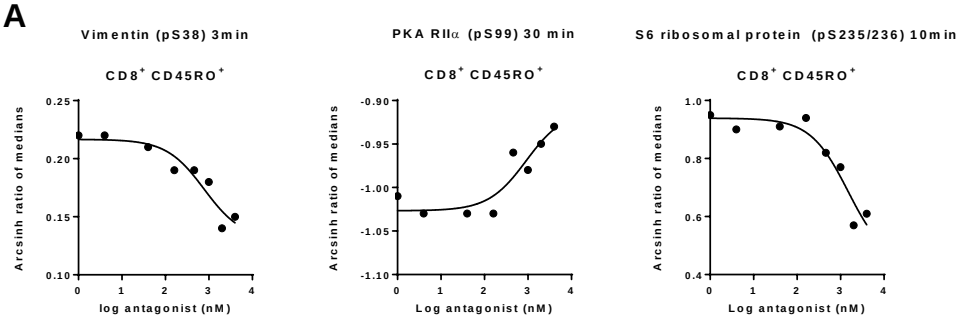
Although sensitivity and affinity to PGE_2 varies among the four receptors, only one concentration of PGE_2 was used to ensure identical conditions for all receptors. The same time points were monitored as for the agonists - 0, 3, 7, 10, 30 and 60 minutes. After stimulation, cells were barcoded, permeabilized, stained with phosphoepitope specific antibodies and analyzed on the flow cytometer. Concentration response curves were made in Graphpad Prism. Below is a selection of results from the various antagonist titrations; each phosphoepitope is displayed at only one specific time point (maximal response), since phosphorylation is a dynamic process that differs for the various phosphoepitopes monitored.

The antagonists investigated were all full antagonists and did not activate the receptors in the absence of any other stimulus. Thus, they are not partial agonists. Treatment with the antagonists alone did not show any significant response from the selected phosphoepitopes. See Supplementary Figure 3 in Appendix for an overview of the examined responses from the four antagonists.

4.4.1. EP1 antagonist (ONO-AE-340) and EP3 antagonist (ONO-AE3-240)

The EP1 antagonist was provided by ONO Pharmaceuticals and had a molecular weight of 523.52 kDa. The antagonist was reported to inhibit the increase in intracellular Ca^{2+} elicited by stimulation with 100 nM PGE_2 with an IC_{50} value of 460 nM. The EP3 antagonist had a molecular weight of 531.64 kDa. The EP3 antagonist was reported to inhibit an increase in intracellular Ca^{2+} in the presence of 10 nM PGE_2 with an IC_{50} value of 1.5 nM. This compound was reported to have some affinity for the EP4 receptor with a K_i value of 58 nM. Thus, very high concentrations of this antagonist may cause some cross - reaction with the signaling through the EP4 receptor.

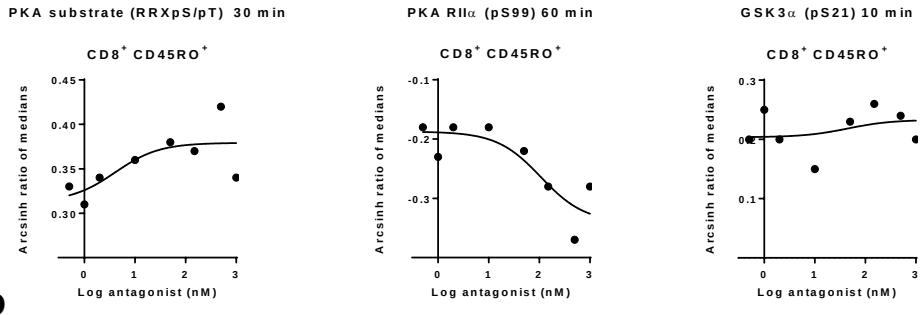
A



B

ONO-AE-340 dose responses		
Phospho - epitope	**IC50**	**R - square**
Vimentin (Ser38) 3 min	780.8	**0.90**
PKA RIIα (Ser99) 30 min	912.1	**0.85**
S6 Ribosomal protein (Ser235/236) 30 min	731.5	**0.87**

C



D

ONO-AE3-240 dose responses		
Phospho - epitope	**IC50**	**R - square**
PKA substrate (RRXpS/pT) 30 min	4.07	0.55
PKA RIIα (Ser99) 60 min	107.3	0.75
GSK3α (Ser21) 10 min	44.71	0.11

Figure 23 EP1 antagonist (ONO-AE-340) and EP3 antagonist (ONO-AE3-240) concentration response curves. *$CD3^+$ T cells were purified from buffy coats and treated with either A) eight concentrations of EP1 antagonist (ONO-AE-340) 1, 4, 10, 40, 160, 460, 1000, 2000 and 4000 nM or C) eight concentrations of EP3 antagonist (ONO-AE3-240) 0.5, 1, 2, 10, 50, 150, 500 and 1000 nM. B) IC_{50} and R^2 values of the phosphoepitopes displayed in A). D) IC_{50} and R^2 values of the phosphoepitopes displayed in C) and R^2 values over 0.80 are shown in red.*

Displayed in Figure 23 are the concentration - dependent response curves resulting from EP1 antagonist (Figure 23A) and EP3 antagonist titrations (Figure 23C). Overall, the observed IC_{50} for both of the antagonists in Figure 23 are higher compared to observed EC_{50} (Figure 20), which suggests that in this case, the antagonists are less potent than the agonists and require higher concentrations to achieve suitable effects. The observed IC_{50}

values were also higher compared to the theoretical values provided by ONO Pharmaceuticals. However, the stimulation conditions used here had much higher PGE_2 concentrations than those employed by ONO Pharmaceuticals for IC_{50} value determinations. This is known to affect the observed IC_{50} values. Also, the difference in IC_{50} values could also be due to differences in the effects that were measured as downstream phosphorylation levels in this study versus second messenger responses in ONO Pharmaceuticals IC_{50} value determinations.

The results show that the EP1 receptor antagonist downregulates phosphorylation of serine 38 on vimentin with increasing concentrations. These results corroborate with the results from the EP1 agonist experiment, where Ser38 is phosphorylated by the EP1 agonist, providing additional evidence that this protein is activated upon EP1 receptor activation. In addition, a few other phosphoepitopes generated suitable concentration responses with EP1 antagonist titration. Examples include PKA regulatory subunit IIα protein (pS99) and S6 ribosomal protein (pS235/236). However, for the two latter phosphoepitopes it is difficult to assess their involvement in EP1 activated pathways as they did not respond significantly to EP1 receptor agonist titrations. The finding might be due to a phenomenon called biased agonists [118]. G protein - coupled receptors usually activates multiple signaling pathways and agonist activation will probably never produce a complete and equal activation of the receptor, compared to the natural ligand [118]. Instead, agonists may favor one or more of the possible downstream pathways, hence the term “biased agonists”. A way to prove that the EP1 agonist employed here is biased would be to treat cells with PGE_2 and add antagonists for EP2, EP3 and EP4 and investigate the downstream signaling going through EP1 upon PGE_2 activation and compare the responses to EP1 agonist activation. The contradictory results using EP1 agonist and antagonist could also be due to possible lack of specificity of the antagonist, which will be further discussed in the Discussion.

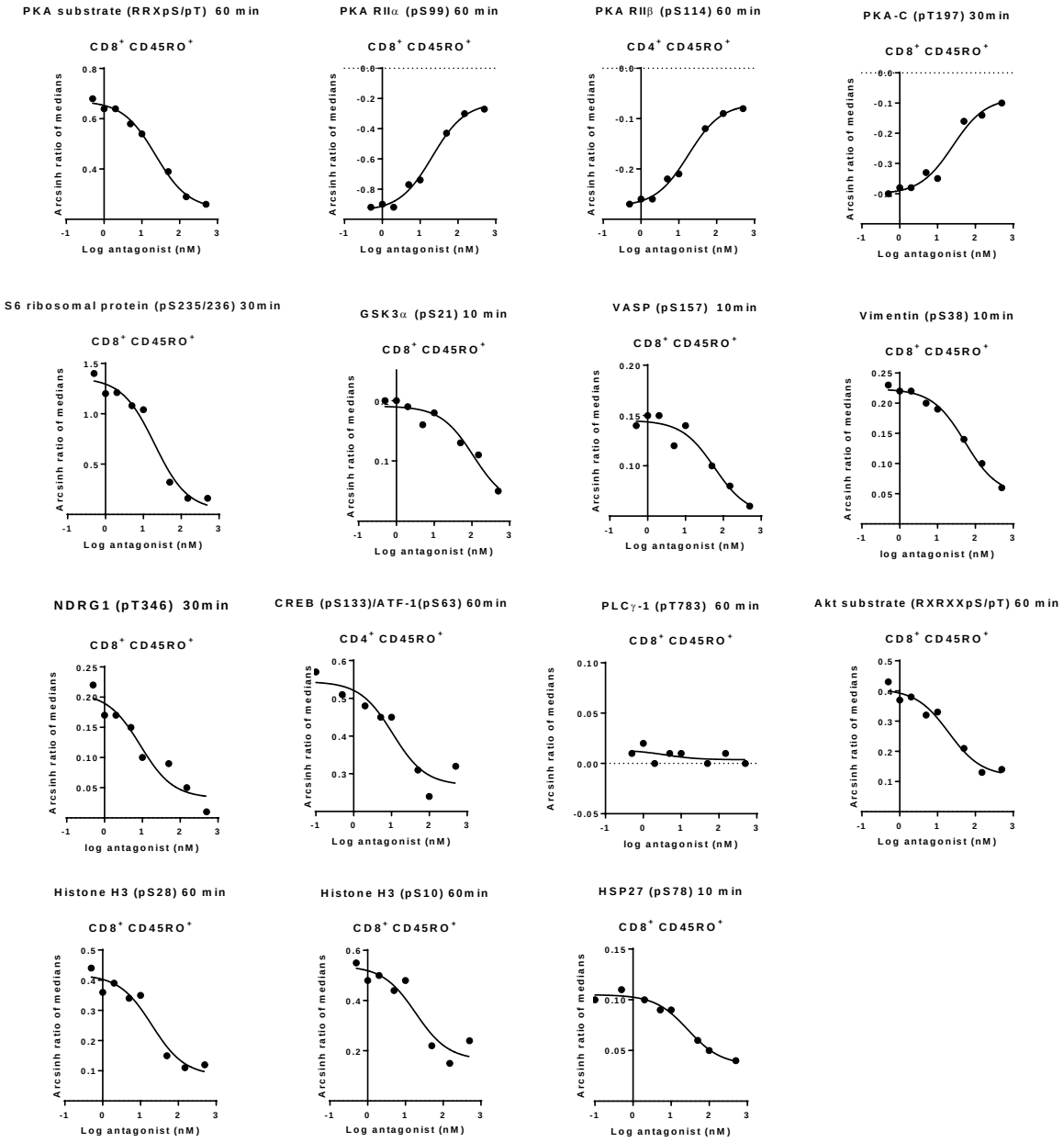
The EP3 antagonist did not generate concentration response curves with suitable fit (R^2 was lower than 0.80 for all curve fits). However, some phosphorylation trends are observed in these results that supports the findings from agonist stimulation experiments. Although R^2 values are low, both PKA substrate in general (as assessed with PKA consensus sequence - specific antibody) and the known PKA substrate GSK3α display a trend towards increased phosphorylation with antagonist treatment as expected. The EP3

receptor is $G_{\alpha i}$ - coupled and inhibits adenylyl cyclase, leading to a reduction in cAMP levels. By exposing the receptor to an antagonist, the decrease in cAMP levels should be blocked. Thus, an increase in PKA substrate phosphorylation upon antagonist treatment, as displayed in Figure 23C is expected.

4.4.2. EP2 antagonist (TG4-155)

The EP2 antagonist was obtained from Cayman Chemicals (#17639) and had a molecular weight of 394.5 kDa. Because of limited information about the IC_{50} value of this antagonist, several optimization experiments were performed to determine a concentration range for the antagonist response curves. The titration values ranged from 0.1 - 500 nM. The results below show significantly more concentration - dependent responses of the various phosphoepitopes compared to the EP1 and EP3 receptor antagonists. This was also expected, considering that the EP2 agonist generated more concentration - dependent responses of phosphorylation of the investigated phosphoepitopes.

A



B

TG4-155 dose responses		
Phospho - epitope	**IC50**	**R - square**
PKA substrate (RRXpS/pT) 60 min	22.94	**0.99**
PKA RIIα (Ser99) 60 min	20.83	**0.99**
PKA RIIβ (Ser114) 30 min	140.3	**0.92**
PKA-C (Thr197) 30 min	25.25	**0.97**
S6 Ribosomal protein (Ser235/236) 30 min	19.84	**0.98**
GSK3α (Ser21) 10 min	110.0	**0.94**
Vasp (Ser157) 10 min	58.36	**0.94**
Vimentin (Ser38) 10 min	53.29	**0.99**
NDRG1 (Thr346) 30 min	8.86	**0.91**
CREB (Ser133)/ATF-1 (Ser63) 60 min	10.72	**0.91**
PLCγ-1 (Thr783) 60 min	3.82	0.23
Akt substrate (RXRXXpS/pT) 60 min	19.75	**0.96**
Histone H3 (Ser28) 60 min	20.00	**0.95**
Histone H3 (Ser10) 60 min	18.63	**0.90**
HSP27 (Ser78) 10 min	27.65	**0.98**

Figure 24 EP2 antagonist (TG4-155) concentration - dependent responses. *A) Phosphorylation of 15 selected phosphoepitopes (out of 28 antibodies shown in Supplementary Table 2) in response to PGE_2 stimulus were examined in the presence of 8 increasing concentrations of the EP2 antagonist (TG4-155), 0.5, 1, 2, 5, 10, 50, 150 and 500 nM. These phosphoepitopes are the same sites that were shown to be regulated by the EP2 agonist. B) IC_{50} and R^2 values of the phospho - sites shown in A) and R^2 greater than 0.80 are marked in red.*

Of the 15 agonist concentration responses shown in Figure 21, 14 of the same phosphoepitopes displayed the expected reverse phosphorylation patterns in the presence of EP2 antagonist (Figure 24A). PKA substrates containing the sequence RRXpS/pT show reduced phosphorylation in response to increasing concentrations of the antagonist in the presence of PGE_2. Other substrates of PKA such as GSK3α (pS21), CREB (pS133), HSP27 (pS78), VASP (pS157) and S6 ribosomal protein (pS235/236) also display reversed phosphorylation patterns compared to those in Figure 21, as expected. PLCγ-1(pS783) did not show a concentration - dependent response curve in the presence of EP2 antagonist (Figure 24), but this is probably due to the relatively weak signal from this phosphoepitope upon stimulation with 1 μM PGE_2, which did not provoke a detectable phosphorylation response. Furthermore, NDRG1 (pT346), Akt (RXRXXpS/pT) and two different histone phosphorylation sites (pS10 and pS28) also show reduced phosphorylation as a response to increased concentrations of the EP2 antagonist.

Both of the type II PKA regulatory subunits IIα (pS99) and IIβ (pS114) and a phosphoepitope on the catalytic subunit (PKA-C (pT197)) generated reversed phosphorylation patterns upon treatment with EP2 antagonist in the presence of PGE_2 compared to concentration response curves generated by the EP2 agonist (Figure 21). They show increased phosphorylation in response to increased concentrations of EP2 antagonist, which confirms that they are targets of PGE_2 and downstream of the EP2 receptor. Phosphorylation of Thr197 on the catalytic subunit of PKA is necessary for optimal enzymatic activity and activates the enzyme. The findings here may indicate that the response is a post - activating response of the enzyme as previously discussed. Ser38 on vimentin also displays a reduction in phosphorylation with increasing concentrations of EP2 antagonist, the same effect observed upon EP1 antagonist treatment (Figure 23), which may indicate that the EP1 and EP2 receptor initiate a similar pathway, or different pathways that both converge on vimentin.

As mentioned in the Introduction, the EP2 receptor has been reported to initiate a PI3K/Akt signaling pathway through the βγ - complex [77]. This is in line with the present study and can explain the reduced phosphorylation of Akt substrates, upon EP2 antagonist stimulation. In addition, CREB (pS133) has been reported to be a substrate of Akt, thus reduced phosphorylation of Ser133 on CREB in the presence of EP2 antagonist can also indicate the activation of the Akt - pathway by the EP2 receptor [95].

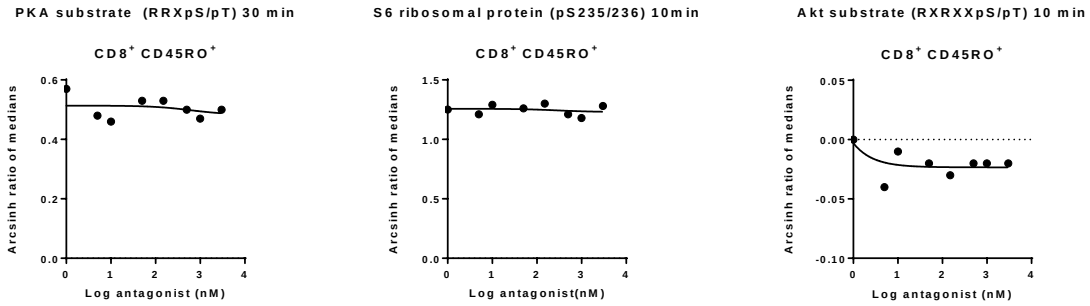
Phosphorylation of CREB at Ser133 induces its transcriptional activity and in addition to Akt, several serine/threonine kinases have been shown to promote this phosphorylation, including PKA and PKC [96]. In addition, CREB is thought to have various roles in the immune system and its activation promotes proliferation and survival and differentially regulates the various $CD4^+$ T cell subset responses [96].

In summary, these results support the results observed with the EP2 agonist by providing opposite phosphorylation patterns of the various phosphoepitopes.

4.4.3. EP4 antagonist (ONO-AE3-208)

The EP4 antagonist had a molecular weight of 404.4 kDa. This antagonist was purchased from Cayman Chemicals (#14522) and according to their product information, had a K_i value of 1.3 nM. Eight different concentrations of the EP4 antagonist were used to establish concentration response curves for phosphorylation of the various phosphoepitopes.

A



B

ONO-AE2-208 dose responses		
Phospho-epitope	**IC50**	**R - square**
PKA substrate (RRXpS/pT) 30 min	602.9	0.072
S6 Ribosomal protein (Ser235/236)10 min	Ambiguous	0.47
Akt substrate (RXRXXpS/pT) 10 min	Interrupted	-

Figure 25 No concentration responses were generated from treatment with EP4 antagonist in the presence of PGE_2. *A) Three selected concentration response curves generated from the experiment are displayed. Because there were no or few concentration response curves showing actual responses, only three of the most promising ones are shown. Cells were stimulated with eight different concentrations of EP4 antagonist, 1, 5, 10, 50, 100, 500, 1000 and 3000 nM in the presence of 1 μM PGE_2. B) IC_{50} and R^2 values of the three phosphoepitopes.*

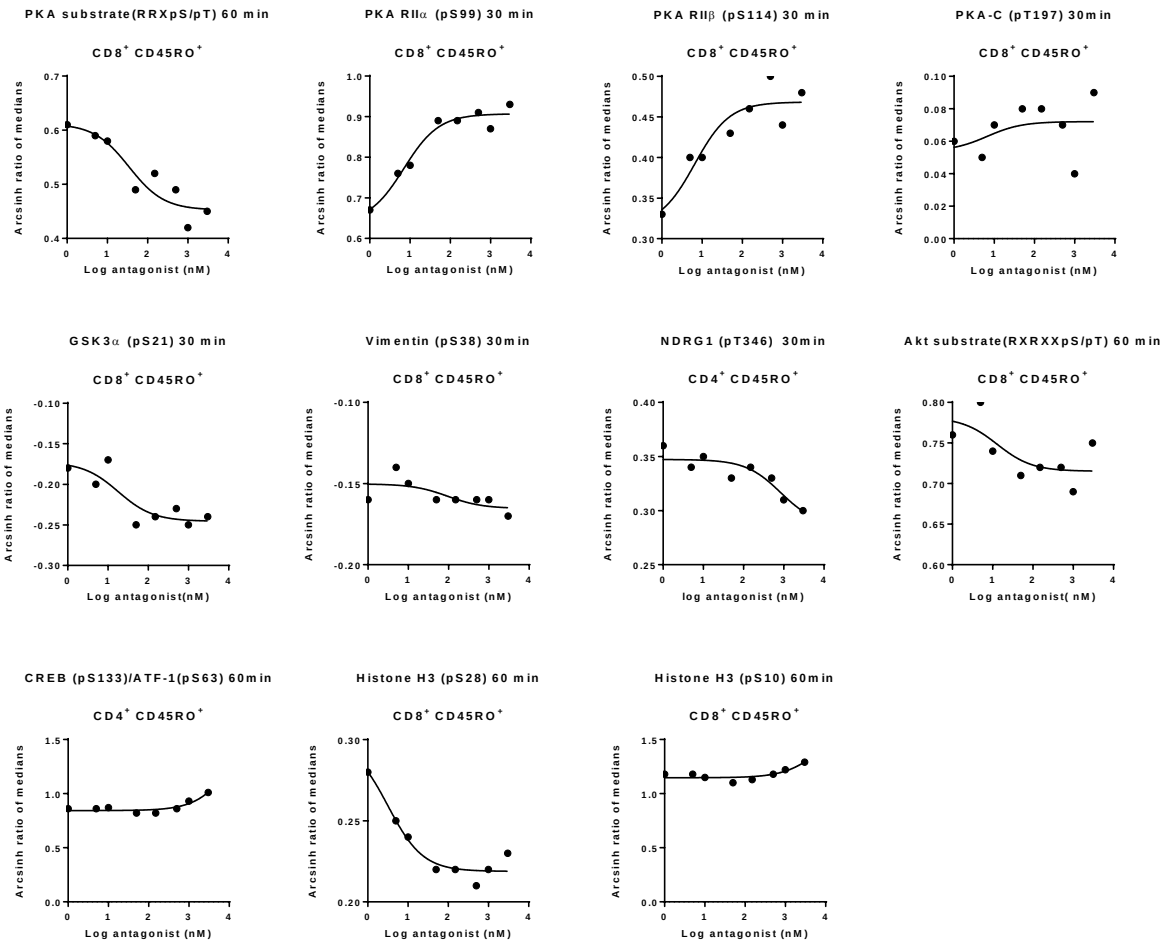
The experiment was performed as previously described in this section and the cells were stimulated with eight increasing concentrations of the EP4 antagonist in the presence of 1 μM PGE_2. Concentration response curves from the EP4 antagonist experiment could be expected, considering that the various phosphoepitopes stimulated with EP4 agonist displayed in Figure 22 generated several concentration - dependent responses. Intriguingly, they were not. In fact, exposing $CD3^+$ T cells to eight different concentrations of the EP4 antagonist ranging from 1 - 3000 nM did not generate any significant concentration - dependent responses as one would expect based on the EP4 agonist experiment (see Supplementary Figure 4 in the Appendix for a complete overview of the results from this experiment).

To obtain sufficient signals from the phosphoepitopes, the EP4 receptor was stimulated with 1 μM PGE_2. This concentration was chosen as it was thought to be around $EC_{80\text{-}100}$ for the various phosphoepitopes. However, this response was an aggregated effect from the four receptors, and information about EC_{50} for each of the four receptors was not possible to extract from that particular experiment (Figure 15). One hypothesis to explain the findings above (Figure 25) is that PGE_2 has a higher affinity for the EP4 receptor than the EP2 receptor, i.e. PGE_2 binds more strongly to the EP4 receptor compared to the EP2 receptor. Indeed, reports suggest that the EP4 receptor has a higher affinity for PGE_2 than the EP2 receptor does. PGE_2 has a dissociation constant (K_d) of 0.59-1.12 nM for the EP4 receptor while the value for EP2 ranges from 12.9 - 13 nM [82]. A high dissociation constant indicates that a higher concentration of ligand is needed for binding, thus a lower K_d value indicates higher binding strength, because less ligand is needed for binding. Because of the strong binding of PGE_2 to the EP4 receptor, even high concentrations of EP4 antagonist may not manage to displace PGE_2 and block the receptor activity, leading to the unexpected lack of concentration - dependent responses for the EP4 antagonist in the presence of PGE_2.

An EP4 agonist could possibly be easier to displace than PGE_2, the natural ligand of the EP4 receptor. Thus, an additional experiment was performed, where T cells were stimulated with EP4 agonist instead of PGE_2 and increasing concentrations of the EP4 antagonist were added in. The agonist concentration used for the stimulation was the EC_{80} concentration of the EP4 agonist (determined from an average of the EC_{80} values from the

different phosphoepitopes in the results in Figure 23). In this experiment, the antagonist successfully blocked the actions of the EP4 - specific agonist.

A



B

ONO-AE3-208 + ONO – AE1-329 dose responses		
Phospho – epitope	**IC50**	**R - square**
PKA substrate (RRXpS/pT) 60 min	11.76	**0.95**
PKA RIIα (Ser99) 30 min	6.80	**0.93**
PKA RIIβ (Ser114) 30 min	28.81	**0.87**
PKA-C (Thr197) 7 min	4.48	0.65
S6 Ribosomal protein (Ser235/236) 10 min	6.67	**0.86**
GSK3α (Ser21) 10 min	21.55	**0.83**
VASP (pS157) 10min	7.15	0.64
Vimentin (Ser38) 3 min	190.8	0.24
NDRG1 (Thr346) 30 min	14.05	**0.95**
Vimentin (Ser38) 10 min	14.22	0.45
Akt substrate (RXRXXpS/pT) 60 min	7.61	0.52
Histone H3 (Ser28) 60 min	5.56	0.58
Histone H3 (Ser10) 60 min	14893	**0.88**

Figure 26 Concentration - dependent effects of EP4 antagonist in the presence of EP4 agonist. *A) Concentration response curves from 12 investigated phosphoepitopes. $CD3^+$ T cells were stimulated with the same concentrations of EP4 antagonist as in Figure 25(ONO-AE3-208) in the presence of 50 nM of the EP4 agonist (ONO-AE1-329), fixed, barcoded and permeabilized. Samples were then stained with antibodies and analyzed on the flow cytometer. B) EC_{50} and R^2 values of the phosphoepitopes displayed in A) and R^2 values greater than 0.80 are shown in red.*

When comparing the concentration response curves in Figure 26 with those in Figure 25 (and Supplementary Figure 4) there is a clear difference in the EP4 antagonist response between cells stimulated with EP4 agonist and those stimulated with PGE_2. The results from the experiment where cells were stimulated with EP4 agonist and antagonist (Figure 26) show a number of concentration response curves. In particular, PKA substrates display a reduction in phosphorylation upon treatment with EP4 antagonist, as expected. Type II PKA regulatory subunits α (pS99) and β (pS114) show a concentration - dependent phosphorylation as a response to increased antagonist concentrations. Furthermore, known PKA substrates such as S6 ribosomal protein (pS235/236), VASP (pS157) and GSK3α (pS21) display reduced phosphorylation in the presence of increasing concentrations of EP4 antagonist, indicating that these proteins are also activated during EP4 receptor signaling. Interestingly, both CREB (pS133) and a phosphorylation site on histone H3 (pS10) did not generate concentration dependent responses as one would have expected from the agonist response curves in Figure 23. However, this observation might be attributable to a biased agonist as previously discussed in this section.

In summary, interesting findings were made by using EP1 - 4 specific agonists and antagonists to stimulate each receptor individually. Many phosphoepitopes were found to be part of the downstream signaling pathways triggered by the EP2 and EP4 receptors. Although there were some exceptions, most of the signaling seemed to go through the PKA signaling node. Some crosstalk between the EP1, EP2 and EP4 receptors was observed, and appeared to involve the phosphorylation of vimentin, which may indicate overlapping pathway(s) between these three receptors. A summary of the concentration - response curves for all agonist and antagonists investigated in sections 4.3 and 4.4 and the EC_{50}/IC_{50} of various phosphoepitopes investigated with two time points each are provided in Supplementary Table 3 - 7 in the Appendix.

4.5. Multiple kinases regulate PGE_2 - induced signaling pathways

To assess which kinases phosphorylated the various phosphoepitopes discussed in this Thesis, experiments with kinase inhibitors were performed. This Thesis demonstrates the phosphorylation of many proteins in the PKA signaling pathway in response to stimulation of the four PGE_2 receptors. Are these phosphoepitopes regulated by other kinases? Is one phosphoepitope regulated by multiple kinases and is such regulation similar in the different T cell subtypes? These questions were investigated using 15 different kinase inhibitors, and assessing the effect of these inhibitors on downstream signaling generated by PGE_2 stimulation.

The kinase inhibitors were chosen based on the results shown in this Thesis, from the mass spectrometry study (Lone *et al.*, manuscript in preparation), as well as by looking into the literature on the various signaling pathways activated by PGE_2. The assembled set of kinase inhibitors included inhibitors towards PKA, PI3 kinase, GSK3, EGF - receptor, CDKs and more. A full overview of the different kinase inhibitors and their effects is available in Supplementary Table 1 in the Appendix.

$CD3^+$ T cells were incubated with inhibitors in a water bath at 37 ^{0}C for 30 min prior to stimulation with 10 μM PGE_2. A rather high concentration of PGE_2 (10 μM) was chosen to achieve high phosphorylation levels of the various phosphoepitopes, which would make it easier to observe a reduction in the signal. Concentrations of the various inhibitors used were determined based on information from the vendor in addition to literature.

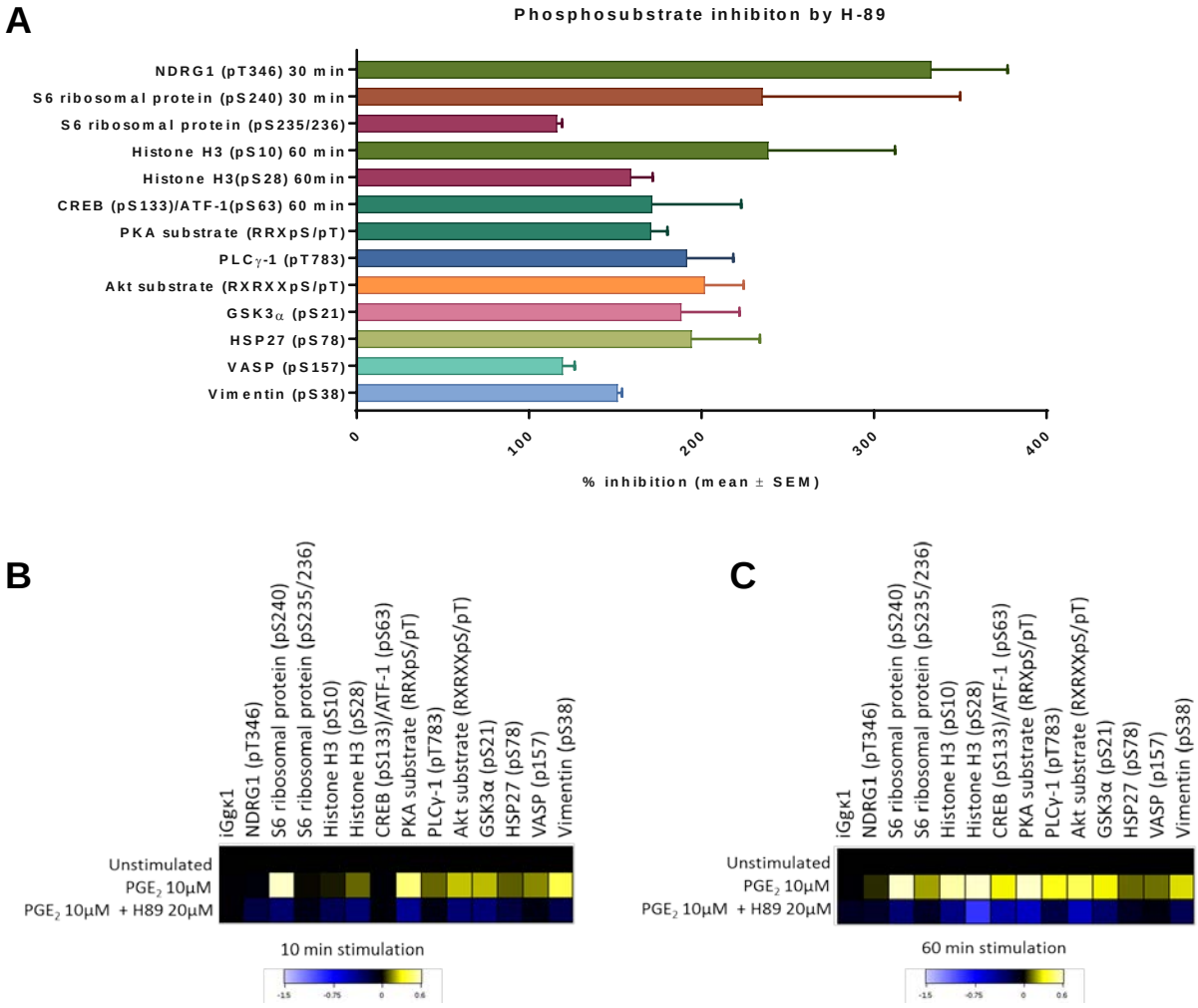


Figure 27 The PKA inhibitor, H-89 inhibits phosphorylation many of the PGE_2 - induced phosphorylation sites. *A) Phosphorylation of 13 phosphoepitopes downstream of PGE_2 was inhibited by 100 % or more by the PKA inhibitor H-89. The inhibitor was added at 20 µM to cells 30 minutes, prior to stimulation with 10 µM PGE_2 at 37 °C. Percent inhibition was calculated as the signal relative to PGE_2 stimulation for each phosphoepitope. Phosphorylation response was measured after 0, 3, 7, 10, 30 and 60 min, responses after 10 min are shown if not otherwise specified in $CD8^+$ $CD45RO^+$ T cells, with the exception of NDRG1 ($CD4^+$ $CD45RO^+$) (mean + SEM, n = 3). B) Heatmap illustration of the 13 regulated phosphoepitopes after 10 min stimulation with and without H-89 in $CD8^+$ $CD45RO^+$ T cells. C) Heatmap of the 13 phosphorylated sites after 60 minutes of stimulation, blue colors indicates a decrease in the signal whereas yellow colors indicate an increase in phosphorylation in $CD8^+$ $CD45RO^+$ T cells, signals were calculated relative to the unstimulated control (0.3 % DMSO).*

H-89 is a potent inhibitor of PKA and is known to inhibit PKA competitively at the ATP binding site thus interfering with the phosphorylation process of PKA [119]. H-89 also inhibits a number of other kinases [120], but is relatively specific in combination with cAMP stimulus. Phosphorylation of various phosphoepitopes induced by PGE_2 was abrogated in the presence of the PKA inhibitor (Figure 27A). The inhibition was measured to over 100 % for many of the phosphoepitopes. These observations indicate that there is a high basal activity of PKA (or other kinases) in these cells and that by

treating them with H-89, both the induced and basal phosphorylation of PKA as well as its substrates is decreased (see also Supplementary Figure 5). PKA has been shown to be constitutively active in a previous PGE_2 - related study [46]. These results indicate a significant involvement of the PKA signaling node downstream of PGE_2's four receptors. The observations from these inhibitor studies also correlate with the results from the agonist/antagonist experiments where concentration response curves are observed for the various phosphoepitopes downstream of PKA.

As mentioned, treatment with high concentrations of H-89 is reported to inhibit several other kinases in addition to PKA [119, 120]. Several other kinase inhibitors in addition to H-89 were assessed to obtain a greater overview of the kinases involved in the intracellular signaling initiated by PGE_2. These results give a strong indication of the involvement of multiple kinases downstream of the four receptors. These kinases were seen to regulate the phosphorylation of different phosphoepitopes. In addition, the results from the inhibitor studies revealed different T cell subtype - dependent activities of specific kinases.

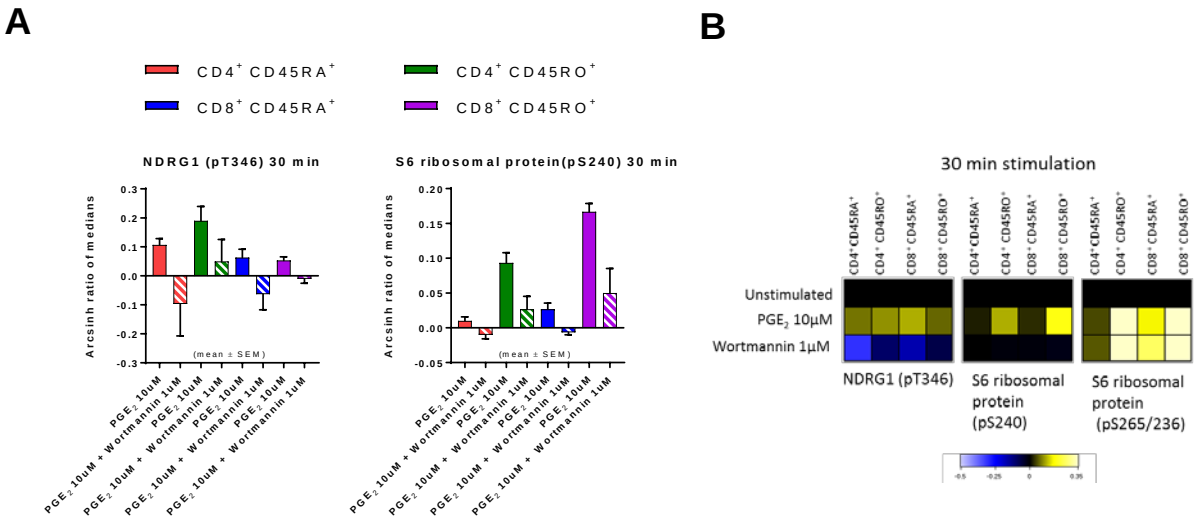


Figure 28 Phosphorylation of several phosphoepitopes is regulated by multiple kinases activated during PGE_2 signaling. *A) Phosphorylation of NDRG1 (T346) and S6 ribosomal protein (S240) is reduced in the presence of the PI3K inhibitor (stroked bars) wortmannin (1 μM), indicating that these phosphoepitopes are regulated by PI3K in addition to PKA. The response was measured in four different T cell types which is indicated by the four different colors (mean ± SEM, n = 3). B) Interestingly, the phosphoepitopes S235/236 on S6 ribosomal protein is not affected by the presence of wortmannin, indicating that multiple kinases are necessary for the regulation of S6 ribosomal protein.*

In addition to the regulation by PKA, several phosphoepitopes were observed to be regulated by PI3 kinase (Figure 28 and 29). As mentioned in the Introduction, both EP2 and EP4 receptor are able to activate the PI3 kinase pathway through the βγ - complex. These observations suggest that PGE_2 is able to activate multiple pathways downstream of its receptors and regulation of specific phosphoepitopes were regulated by different kinases in different T cell types. Notably, regulation of phosphorylation of CREB (pS133) by various kinases seemed to be T cell type - dependent.

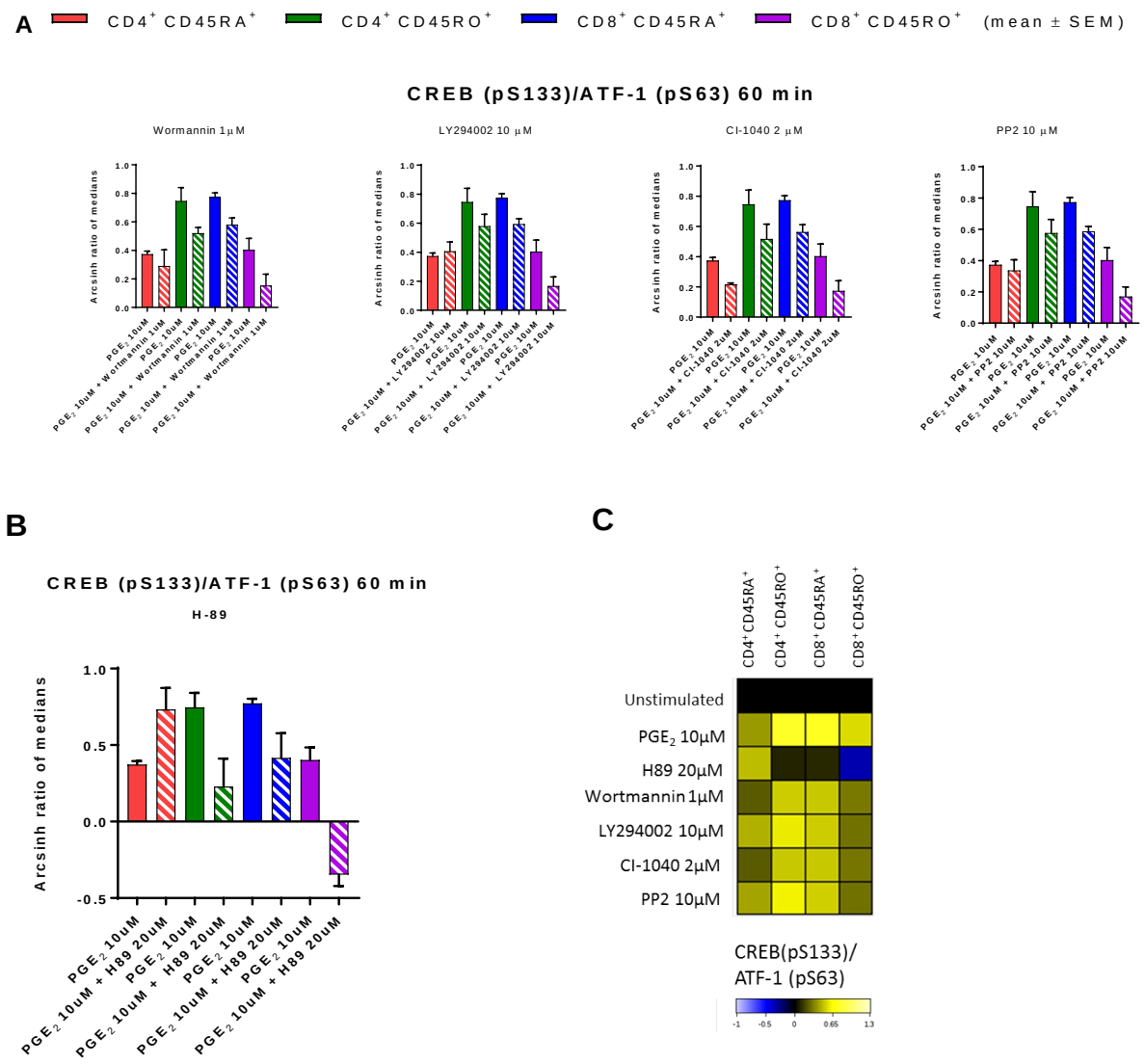


Figure 29 Phosphorylation of CREB (pS133) is T cell subtype dependent. *A) Phosphorylation of CREB is reduced in the presence of wortmannin (1 µM), LY294002 (10 µM), CI-1040 (2 µM) and PP2 (10 µM). The regulation was observed to be T cell subtype - dependent, where phosphorylation in $CD8^+$ T cells was the most sensitive to the presence of the various inhibitors (mean ± SEM, n = 3). B) T cell subtype phosphorylation responses to H-89 stimulation showed that $CD8^+$ $CD45RO^+$ T cells were most sensitive to the inhibitor (mean ± SEM, n = 3). The different T cell subsets are represented in the four different colors displayed in the figure. C) Heatmap displaying CREB (pS133) phosphorylation in the different T cell types during stimulation with five different kinase inhibitors. All samples were normalized to DMSO control (unstimulated sample).*

Phosphorylation of CREB (pS133) was observed to be regulated by at least four kinases, including MEK1/2, PI3K, Src and PKA (Figure 29A and B). LY294002 is reported to be a more specific PI3K inhibitor compared to wortmannin, it inhibits class I α/δ/β isoform of the PI3 kinase, while wortmannin inhibits both class I and II of the PI3 kinase family[121]. However, both of these inhibitors are reported to inhibit other kinases as well, and more specific kinase inhibitors should be employed to determine which isoform of the kinase takes part in the regulation. CI-1040 is a MEK1/MEK2 inhibitor, which is reported to block the phosphorylation of ERK [122]. A broad range of extracellular stimuli such as cytokines, mitogens and growth factors activates this pathway. In addition, there is reported cross - talk between the cAMP and the MAPK activated pathways, indicating that these pathways intersect at some point downstream in the cell [123].
Src - kinase has been shown to regulate Erk activity and its downstream targets such as CREB (pS133) [124, 125]. Notably, PKA seems to be the major kinase that regulates CREB phosphorylation, especially in $CD8^+$ $CD45RO^+$ T cells. Inhibition of other kinases has smaller but still substantial effects on the phosphorylation status of this phosphoepitope.

The regulation of CREB (pS133) phosphorylation was observed to be T cell type dependent, where $CD8^+$ T cells displayed the most significant reduction in phosphorylation of CREB (pS133) in the presence of each of the four kinase inhibitors (Figure 29A). The cell type $CD8^+$ $CD45RO^+$ was observed to be more sensitive to H-89 compared to the other three cell types. Interestingly, the cell type $CD4^+$ $CD45RA^+$ seemed unaffected by the presence of the various inhibitors, which can indicate other regulation mechanisms of the phosphorylation of CREB (pS133) in this cell type (Figure 29A - C). Thus, which kinases that is more important for the regulation of phosphorylation of CREB (pS133) depends on T cell type. The extensive regulation of CREB (pS133) activation might be due to the many signaling pathways that converge on this key effector molecule, which regulate many target genes [96]. These findings provide evidence that PGE_2 activates distinct pathways in different T cell types and that multiple kinases are involved in these pathways. Further studies are needed to investigate whether completely distinct pathways are activated or whether there are overlapping pathways initiated by PGE_2 in the various T cell types.

5. Discussion

In the present study, PGE_2 signaling in various T cell subsets from healthy blood donors was investigated. The phosphoflow approach employed in this Thesis revealed several PGE_2 - regulated phosphoepitopes downstream of the four receptors (EP1 - 4). The method generated an extensive overview of the signaling network initiated by PGE_2 and allowed measurements under multiple experimental conditions such as concentrations, time kinetics and T cell subtypes. Many of the phosphoepitopes discussed in this Thesis were selected based on the results from a previous proteomic study performed in this lab (Lone *et al.*, manuscript in preparation). The MS study revealed many phosphosites regulated by each of the four receptors. Interestingly, few of the specific phosphoepitope antibodies in the panel in the present study generated signals upon EP1 and EP3 receptor agonist stimulation. This observation could have several explanations. EP1 and EP3 may function as regulatory receptors that buffer the PGE_2 response by just binding ligand, where activation only happens under specific conditions. It could also be due to the EP receptors cell type - specific distribution. Although, expression of EP1 and EP3 receptor in primary T cells was confirmed by western blot (Figure 12), quantification of the expression levels were not performed, and this is known to vary greatly between different cells and tissues [80]. It may therefore be that low levels of receptor prohibited us from observing signaling as the sensitivity of the assay was not sufficient. Lastly, it may be that EP1 and EP3 signaling proceeds through other phosphoepitopes than the ones in our panel.

5.1. PGE_2 - induced signaling goes through EP2 and EP4 receptor

The documentation of the functions of EP1 and EP3 receptors in primary T cells is limited. Whilst mRNA of all EP - receptors has been found in murine T cells, the expression of EP1 and EP3 in human T cells has not been fully documented [41]. Recent studies have shown that immune functions regulated by PGE_2 in murine T cells are mainly mediated by the EP2 and EP4 receptor [29]. This appears also to be the case in humans based on the studies performed in this Thesis, where a large group of phosphoepitopes was observed to be regulated during EP2 and EP4 receptor stimulation. Very few phosphoepitopes was observed to be regulated during EP1 and EP3 receptor stimulation. What could then be the functions for low levels of EP1 and EP3 receptor in T

cells? One study claims that the EP1 receptor mediates T_H1 differentiation of naïve $CD4^+$ murine T cells [126]. However, further studies are needed to understand EP1's role in T cells. Regarding the EP3 receptor, because of EP3's inhibitory effect on cAMP signaling, the receptor is thought to counteract EP2/EP4 - mediated signaling and possess regulatory functions. The EP3 receptor is challenging to characterize because of the existence of multiple EP3 receptor isoforms generated from alternative splicing. They are coupled to different proteins, including $G_{\alpha s,}$ $G_{\alpha i}$ and $G_{\alpha q}$ [79]. Interestingly, EP3 receptor stimulation with either agonist or antagonist did not reveal any significant phosphorylation of phosphoepitopes, and its activation could thus not be observed by studying the kinetics of substrate phosphorylation. Notably, it is possible that EP3 signaling may proceed by non - phosphorylating mechanisms [127]. In conclusion, many of the PGE_2 - mediated functions in primary human T cells seem to be mediated predominantly by EP2 and EP4 receptors.

5.2. Functional differences between EP2 and EP4 receptors

EP2 and EP4 agonist and antagonist experiments confirmed the involvement of several substrates of PKA as well as other unknown regulated phosphoepitopes. Although there are biochemical similarities between EP2 and EP4 receptors in addition to regulating the same signaling molecules, they were observed to differ in signal duration and ligand affinity. EP2 receptor was observed to signal over prolonged periods and mediate PGE_2 effects even after 60 minutes of stimulation. Thus, the EP2 receptor can convey PGE_2 functions at later time points. In contrast, EP4 signals for a shorter period of time and mediates a more acute response. The brief response of EP4 - induced signaling observed in this Thesis could be due to ligand - induced internalization and desensitization of the receptor. The EP4 receptor possesses a long C - terminal tail that contains more serines and threonines compared to the EP2 receptor. This C - terminal tail can be subjected to phosphorylation which causes the receptor to lose its ability to activate the G protein. Indeed, the long C - terminal of the EP4 receptor contributes to its susceptibility to rapid agonist - induced internalization, while the EP2 receptor undergoes neither rapid agonist - induced internalization nor desensitization owing to a shorter C - terminal sequence [74].
Although EP2 and EP4 receptor functions appear similar, there are reports suggesting functional differences between the EP2 and EP4 receptors. The major difference is that EP4 can associate with both $G_{\alpha s}$ and $G_{\alpha i}$, whereas EP2 associates with $G_{\alpha s}$ only [76]. The

same study also reported that the interaction between EP4 and PI3K could be mediated by the pertussis toxin - sensitive G protein ($G_{\alpha i}$). However, further studies are necessary to clarify whether this mechanism is the one involved in EP4 signaling. Furthermore, the EP4 receptor is thought to be regulated by β - arrestin, which mediates signal transduction and desensitization - related functions [128]. The fact that there are differences in sensitivity and susceptibility to desensitization between the two receptors indicates functional differences in PGE_2 - mediated signaling and allows for adaptable patterns of responses in different T cell types at various stages as well as in different types of immune responses. Such differences contribute to increased flexibility of the PGE_2 receptor system and indicate that in addition to the regulation of PGE_2 levels in the tissue, regulation at the level of the receptors gene expression can also regulate PGE_2 responsiveness. This is beneficial for exploiting approaches for targeted therapies in various human diseases and antagonists or agonists affecting any of the four receptors allow for differential suppression or stimulation of different aspects of PGE_2 activity.

5.3. Agonist and antagonist characteristics

By establishing concentration - dependent response curves from agonist and antagonist titrations several phosphoepitopes involved downstream of the respective receptors could be confirmed. A total of over 40 phosphoepitope specific antibodies were initially used to investigate phosphorylation initiated by PGE_2. 28 of them were chosen for further studies with agonist and antagonist titrations (see Supplementary Table 2 in the Appendix). Of the 28 antibodies, 15 showed concentration - dependent responses during agonist and/or antagonist titration. All 15 antibodies showed concentration - dependent responses during EP2 agonist stimulation and antagonist treatment in the presence of PGE_2, and EC_{50}/IC_{50} values could be determined. The same 15 phosphoepitope - specific antibodies also showed regulation through the EP4 receptor during agonist stimulation, whereas only 10 of the same antibodies showed an opposite response during EP4 antagonist titration in the presence of EP4 agonist.

5.3.1. EP4 receptor characteristics

The EP4 receptor was observed to have several unique characteristics compared to the other three receptors. Interestingly, in the presence of the EP4 agonist but not in the presence of PGE_2, the EP4 receptor antagonist generated concentration responses to 10 of the phospho - specific antibodies. As mentioned in the Results, this effect could be due to

the EP4 receptor binding affinity, as the EP4 receptor is known to bind PGE_2 with a low K_d - value, thus with high affinity [82]. Because of the high binding affinity, it is difficult for an antagonist to displace a rather high concentration of the natural ligand, and no concentration response of the EP4 antagonist could be observed. Furthermore, the agonist - antagonist experiment showed that it is easier to displace the agonist (at an EC_{80} concentration) than the natural ligand. Thus, the hypothesis suggested in the Results, may provide an explanation for the finding.
Another hypothesis to explain the findings in Figure 25 could be based on the similarities between the EP2 and EP4 receptor. By stimulating the cells with PGE_2 instead of a specific agonist, all four of the receptors could in theory be engaged. Because EP2 and EP4 both activate the $G_{\alpha s}$ protein and generate rather similar signaling pathways, blocking only EP4 receptor using a specific antagonist would not be sufficient to inhibit the phosphorylation signal, as it would also come from the activated EP2 receptor.

Several experiments could be performed to provide useful information to explain the EP4 receptor characteristics. For example, an experiment where cells were treated with the same EP4 antagonist concentrations as used in the present study in the presence of a lower PGE_2 concentration could be performed to investigate if a lower concentration of PGE_2 could be easier displaced by the EP4 antagonist. In addition, to test the hypothesis where the PGE_2 - induced signaling goes through the EP2 receptor, cells could be treated with PGE_2 and a high concentration of the EP2 antagonist in combination with the same increasing concentrations of the EP4 antagonist. In this way, signaling through EP2 receptor is blocked and one can observe if this has an effect on the concentration - dependent response curves. This experiment could also provide information about whether the EP2 receptor is the major receptor that mediates PGE_2 - induced signaling, either due to higher levels of expression of the EP2 receptor or because of other characteristics of the receptor.

5.3.2. EC_{50} and IC_{50}

Several characteristics of the specific EP agonist - antagonist pairs were explored. By generating concentration response curves, EC_{50} and IC_{50} values can be determined. A low EC_{50} value indicates high potency of the agonist as low concentrations of the agonist are needed to reach the half - maximal response. Low IC_{50} values indicate high potency of the

antagonists, as low concentrations are needed to reach the half - maximal inhibitory concentration. Limited readouts for both the EP1 and EP3 agonists and antagonists made it challenging to accurately determine EC_{50}/IC_{50} as well as other properties of the agonists and antagonists for these receptors. As the specificity of the compounds were regarded as sufficient for the present study, comparison of EC_{50} values for EP1, EP2 and EP4 on phosphorylation on vimentin (S38) could be made, because of the observed downstream cross - talk between these three receptors (see the Specificity section for further discussion). The observed EC_{50} values for phosphorylation of vimentin by the three receptor agonists were 122.2 nM, 6.63 nM and 1.86 nM for EP1, EP2 and EP4 agonist, respectively. This comparison allows for determination of the potency of the agonists. An agonist with higher EC_{50} values has lower potency, because higher concentrations are needed for activation. Thus, potency of the three receptor agonists can be arranged accordingly: EP4>EP2>EP1. However, this finding does not precisely determine which receptor the signal from the agonist came from. It could for example be that EP1 agonist signaled through the EP2 receptor (see discussion of specificity). Overall, the observed EC_{50} - values for the EP4 agonist were low for the investigated phosphoepitopes and thus, EP4 agonist displays the highest potency among the four agonists in this study, in addition to the receptor being a high affinity receptor as discussed above. Overall, the antagonists had higher IC_{50} - values compared to the observed EC_{50} values, indicating that the antagonists have lower potency. The observed EC_{50} values for EP2 agonist ranged from 3.34 -52.06 nM, while IC_{50} - values for the EP2 antagonist ranged from 3.82 - 140.3 nM. However, these values are calculated relative to PGE_2 concentration (1 μM), and observed IC_{50} would be lower with lower PGE_2 concentration.

5.3.3. Therapeutic targets

Because of limited documentation on the agonist - antagonist pairs used in the present study, comparison to other similar studies is difficult. Characterization and usage of agonists and antagonists of the EP receptors has been shown to have several therapeutic effects and could be important for drug development. In fact, the same EP2 agonist (ONO-AE-259) used in the present study was reported to increase retinal arteriolar diameter and fundus (eye) blood flow in a rat model of retina degeneration [129], thus playing a protective role in disease models. Mori *et al.* also reports a protective role of the EP2 agonist ONO-AE-259, where it was found to protect against excitotoxicity and

glutamate - induced neurotoxicity in rat retina [130]. Furthermore, PGE_2 is also thought to contribute to neurodegenerative diseases, such as epilepsy and ALS by inducing pro - inflammatory functions through EP2 receptor [131]. A study performed by Jiang *et al*. showed that the EP2 antagonist (TG4-155) substantially blocked neuronal damage on hippocampus during seizures [132].

In a study where the prostanoids role in inflamed bowel disease (IBD) was investigated, the researchers found that the PGE_2 - EP4 receptor signaling played a critical role. It was reported to maintain mucosal integrity and the study showed that inhibition of the EP4 signaling by using a EP4 antagonist severely affected the recovery from colitis and the disease became prolonged [133]. Furthermore, mice with reduced PGE_2 synthesis develop systemic inflammation, a severe illness that can lead to sepsis and septic shock, which was prevented by treatment with an EP4 agonist [134]. Studies investigating therapeutic targets in breast cancer suggest a role for the EP4 receptor as a possible target. Treatment with an EP4 antagonist reduced the tumor - initiating capacity and inhibited mammosphere growth [135]. Interestingly, the COX-1/COX-2 inhibitor indomethacin did not affect the sphere size, suggesting that the sphere formation is not directly coupled to PGE_2 synthesis but rather to the PGE_2 - mediated cell signaling [135].

Thus, PGE_2's many physiological functions in health and disease make it an appropriate therapeutic target where both agonism and antagonism strategies may be explored for therapeutic developments. Nonetheless, this needs to be tailored according to cell type because of the heterogeneous functions of this prostaglandin throughout the body and further studies are needed both *in vitro* and *in vivo* to advance the EP receptors as targets for drug discovery.

5.3.4. Specificity

The specificity of the agonists and antagonists is an important aspect to consider in the work presented here. Except for the EP3 antagonist, no potential cross - reactions were reported by ONO Pharmaceuticals. As mentioned in the Results section, the specificity of the compounds was assumed to be sufficient for the work presented in this Thesis and estimated to be the best ones available at the time. Nonetheless, all of the agonists and antagonists must to some extent be thought to have some cross - reactivity with the other receptors. The observed cross - talk between the EP1, EP2 and EP4 agonist - induced

phosphorylation of vimentin could indicate that the EP1 agonist is promiscuous and partly activates the EP2 or EP4 receptor which resulted in the observed phosphorylation response. The phosphorylation response of vimentin induced by the EP1 agonist was observed to have very low arcsinh ratio values, ranging from -0.02 to 0.02. Notably, the EP1 antagonist was observed to reduce phosphorylation of many known PKA and EP2 receptor targets in the presence of PGE_2, including effects on S6 ribosomal protein and PKA RIIα subunit, which may also indicate cross reactions as these phosphoepitopes are not known targets of EP1 signaling.

A way to test the specificity of an agonist, for example the EP2 agonist, could be to treat the cells with EP2 agonist in combination with the EP2 antagonist, and observe phosphorylation responses; if the agonist is specific, no phosphorylation responses of known EP2 receptor - targets should be observed. A way to test for antagonist specificity could be to treat the cells with for example EP2 agonist in combination with EP1, EP3 and EP4 antagonist, in this experiment phosphorylation responses only induced by the EP2 receptor should be observed. If the EP2 agonist - induced responses are lower in this condition, compared to a control sample where cells are treated only with the EP2 agonist, one can assume that one of the antagonists had an inhibiting effect on the EP2 receptor.

5.4. Cross - talk between EP receptors may contribute to additional regulation of the receptor system

As already mentioned, some cross - talk between EP1, EP2 and EP4 was observed in the present study. Although the specificity of the agonists should be further evaluated, the fact that the three receptors may intersect at some point downstream in the T cells opens for interesting thoughts and theories. Why do these pathways overlap? When and under which conditions? And what are the functional benefits of such cross - talk? Cross - talk and the engagement of multiple pathways in T cells are essential for T cell receptor activation, proliferation and effector functions and also ensures redundancy [136]. Although signaling overlap between these three EP receptors remains to be studied in more detail, both EP1 and EP3 receptors are reported to mediate cross - talk to differentially regulate thromboxane receptor (TP) - mediated signaling in human erythroleukaemic cells [137]. Thus, cross - talk can contribute to specifically regulate the activity of any of the three EP - initiated pathways. In the present study, the three

receptors EP1, EP2, and EP4 all induced phosphorylation of serine 38 on vimentin. Vimentin is an important intermediate filament and plays a role in lymphocyte adhesion and transmigration [138]. The protein is a known substrate of PKA, thus phosphorylation through the EP2 and EP4 receptor was expected. However, EP1 is not known to activate PKA, which may indicate that a novel pathway through the EP1 receptor converges on vimentin. Alternatively, the result could indicate low specificity of the EP1 agonist as already discussed in section 5.3.4. As already stated, EP1 signaling in T cells is poorly documented and it is therefore challenging to predict which pathways could be involved. However, one study claims that vimentin is phosphorylated by calcium/calmodulin dependent protein kinase II (CAMKII) in differentiated smooth muscle cells [139]. Additionally, this kinase was predicted to be involved in PGE_2 signaling in T cells, specifically downstream of the EP1 receptor. [46]. Thus, CAMKII could be responsible for the phosphorylation of vimentin downstream of EP1 receptor. However, additional studies are necessary to further characterize pathways downstream of EP1 receptor and the mechanisms behind the observed cross - talk.

5.5. Different T cell subtypes mediated different responses to PGE_2 stimulation

PGE_2 was observed to initiate different phosphorylation patterns in different T cell subsets; the observation was made both by PGE_2 - induced stimulation and with the kinase inhibitor assay. The T cell type $CD8^+$ $CD45RO^+$ (cytotoxic memory T cells) was observed to be the most sensitive cell type to PGE_2 or agonist stimulation, indicating that this cell type mediates many of the PGE_2 - induced immune functions. These results indicate functional differences of PGE_2 in various T cell subsets. The differences in downstream signaling can also contribute to explain the many paradoxes of PGE_2 in immune regulation, where it has both pro - and anti-inflammatory functions [78]. The differences were observed to be either between $CD4^+$ and $CD8^+$ T cells or between naïve and memory T cells ($CD45RA^+$/$CD45RO^+$). PGE_2 exerts different functions in $CD4^+$ and $CD8^+$ T cells. In particular, PGE_2 has been shown to indirectly suppress antitumor cytotoxic T cells by altering the cytokine expression of dendritic cells (DCs) and in addition, being able to suppress the ability of fully developed $CD8^+$ T cells to interact with their targets and eliminate tumor cells [140, 141]. PGE_2 has also been shown to play a role in both T_H1 and T_H2 cell fate determination, which naturally would need unique

and specific signaling pathways. In $CD4^+$ T cells, PGE_2 suppresses T_H1 functions, but drives the T_H2 T cell differentiation, by inducing production of T_H2 - derived cytokines IL-4, IL-5, IL-10 and IL-13 [142], while suppressing T_H production of IFNγ and the responsiveness to IL-12 [34, 143].

Memory T cells acquire distinct phenotypic and functional properties that makes them able to mediate enhanced secondary immune responses by more rapidly recalling responses upon antigen encounter [10]. Naturally, they also differ from non - memory T cells in intracellular signaling and sensitivity to various molecules. In the present study, the intracellular - phosphorylation kinetics of various T cell subtypes were observed. $CD45RO^+$ T cells were observed to display a greater response to PGE_2 and EP agonist stimulation compared to $CD45RA^+$ T cells. A study reported that pleural $CD45RO^+$ T cells highly express EP1-4 receptors and that PGE_2 is shown to maintain the survival of $CD45RO^+$ T cells in the inflamed human pleural space [144]. Thus, a reason why a higher sensitivity to PGE_2 was observed in $CD45RO^+$ T cells could be due to higher expression levels of the four receptors in this cell type. Although $CD8^+$ $CD45RO^+$ cells seemed to have the most prominent signaling responses during PGE_2 stimulation, there were some exceptions. Both NDRG1 (pT346) and CREB (pS133) phosphorylation had a stronger response in $CD4^+$ $CD45RO^+$ T cells, indicating that PGE_2 induces different pathways depending on T cell type, and that some of the differences observed are probably not only due to varying expression levels in the different cell types. However, further studies are needed to establish the complete pathways in these T cell types and PGE_2's functional role in these.

Several studies report that stimulating cells with lower concentrations of PGE_2 can actually drive T_H1 and T_H17 development [33, 145], contradicting the assertions above. A recent study by Sreeramkumar *et al.* claims that low levels of PGE_2 are required for optimal $CD4^+$ T cell activation, and that the biolipid provides additive co - stimulatory signaling through Akt activation [145]. In addition, blockade of EP receptors *in vivo* impairs T cell activation and development of T cell mediated inflammatory responses [145]. The same study also reports that T cells are capable of secreting picomolar concentrations of PGE_2 upon T cell activation, contradicting the 'classical' view of PGE_2 as an immunosuppressive molecule. These findings indicate that the concentration of PGE_2 in the surrounding environment could be essential for whether it works as a pro - or

anti -inflammatory mediator. In line with the present study, the authors showed that Akt signaling in T cells was reduced upon stimulation with EP2 and EP4 specific antagonists [145]. Sreeramkumar *et al.* also showed that EP2 and EP4 receptors are upregulated following T cell activation in murine T cells. Thus, increased sensitivity to PGE_2 in $CD45RO^+$ memory T cells as observed in the present study could be due to upregulated expression of the receptors as already mentioned. They also predict that an Akt - dependent pathway could contribute to promoting T cells to attain an optimal activation threshold. In the present study, CREB (pS133), NDRG1(pS346) and S6 ribosomal protein (pS240) phosphorylation were observed to be downstream of the PI3K - Akt signaling pathway and could thus possibly be part of this optimal T cell - activation pathway reported by Sreeramkumar *et al.* [145].

5.6. PGE_2 initiates multiple pathways, including PKA and PI3K

The present study revealed multiple phosphoepitopes regulated by the activation of EP2 and EP4 receptor. PGE_2 seems to regulate protein synthesis on a translational and transcriptional level, by inducing phosphorylation of histone H3, CREB and several activating sites on S6 ribosomal protein. In $CD8^+$ T cells, TCR - induced phosphorylation of S6 ribosomal protein is thought to be regulated by the mTor, PI3K and MAPK pathways [146]. In addition, S6 ribosomal protein has been reported to be a substrate for PKA [46]. In line with this, several kinases were observed to be involved in phosphorylation of S6 ribosomal protein in the present study as well, indicating that multiple pathways are necessary to fully activate the protein. As observed in the present study, phosphorylation of Ser240 on S6 ribosomal protein was inhibited by a PI3K and PKA inhibitor, while phosphorylation of Ser235/235 was inhibited by the PKA inhibitor and not the PI3K inhibitor (Figure 30).

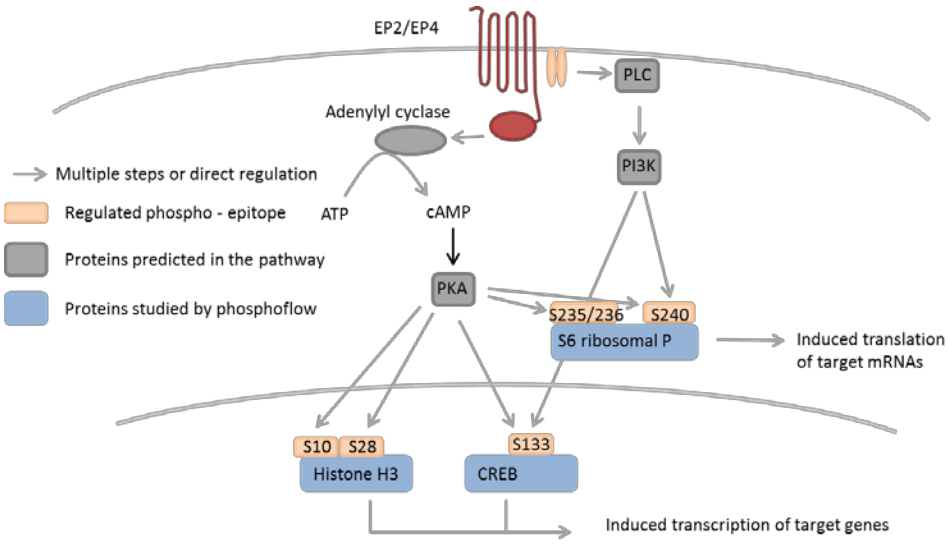


Figure 30 Regulation of transcription and translation by PGE_2 in T cells. *A cartoon of how PGE_2 regulates both transcription and translation in T cells, by phosphorylation of activating sites on S6 ribosomal protein, histone H3 and CREB.*

Histone H3 (pS10 and pS28) and CREB (pS133) were observed to be phosphorylated after 60 min of PGE_2 stimulation. CREB regulates many T cell specific genes and is important for T cell function. From studies including a transgenic mouse strain with a CREB dominant - negative mutation, mice showed impaired T_H function, especially $CD4^+$ T cells were defective in their ability to produce both T_H1 (IFN-γ) and T_H2 (IL-4) effector cytokines [147]. Agreeing with the present study, higher signals of CREB (pS133) phosphorylation in $CD4^+$ T cells during PGE_2 stimulation were observed, which can indicate an important role for CREB (pS133) phosphorylation in $CD4^+$ T cell function and development. Histone phosphorylation of Ser10 is reported to be essential in condensation of chromosomes during mitosis and meiosis and regulation of cell division [148]. Both phosphoepitopes (S10 and S28) are known to induce transcription of various target genes. These findings connect PGE_2 to regulation of translation and transcription in primary T cells.

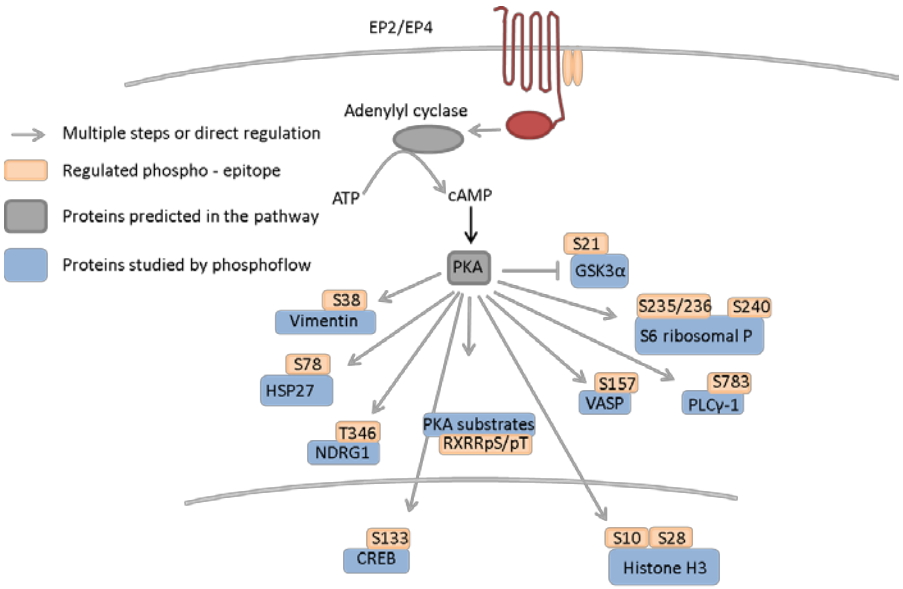


Figure 31 Overview of the various phosphoepitopes regulated by PKA. *Activated PKA type I downstream of the EP2 or EP4 receptor regulates many different proteins.*

A large portion of the signaling through EP2 and EP4 was observed to go through the PKA signaling node (Figure 31). Phosphorylation of these phosphoepitopes was also inhibited in the presence of a PKA inhibitor, confirming their activity downstream of the PKA signaling node. Phosphorylation of VASP (pS157) and vimentin (pS38) is thought to induce rearrangement of the cytoskeleton (see Table 1) These sites are phosphorylated and thus the proteins are activated in the presence of PGE_2. PKA substrates containing the motif RRXpS/pT displayed phosphorylation after 3 minutes of PGE_2 stimulation, and this phosphorylation lasted at least 1 h, indicating that PGE_2 can mediate prolonged functions in T cells through activation of PKA substrates.

Although both PKA type I and PKA type II were regulated by PGE_2, they displayed opposing signals. While phosphorylation of PKA substrate was strongly phosphorylated as expected, both IIα (pS99) and IIβ (pS114) subunits of PKA type II regulatory subunit showed a reduction in phosphorylation in the presence of PGE_2, indicating reduced activity. This may indicate various roles of PGE_2. PKA type I is reported to mediate the inhibitory effect of cAMP which was discussed in the Introduction, while PKA type II is known to have a different subcellular localization in compartments such as nucleus, Golgi, mitochondria and ER [86]. Compartmentalization of PKA is mediated through specific A - kinase - anchoring proteins (AKAPs). Thus, PGE_2 may regulate several types of PKA, which has different properties, including different subcellular localizations and functions within the cell. Another hypothesis could be that the observed decrease in phosphorylation of PKA type II regulatory subunit could be a measurement of post -

activating phosphorylation and several earlier time points could be applied to measure the phosphorylation status in more detail. The phosphorylation and activation of PKA by cAMP is rapidly induced and the observed decrease below baseline can be the termination of the PKA signal after its activation. The activation is also confirmed as the observed PKA substrate showed a stable phosphorylation even after 60 min.

5.6.1. PGE_2 - induced phosphorylation is regulated by multiple kinases

Perturbation experiments with kinase inhibitors revealed the involvement of multiple kinases during PGE_2 stimulation, indicating a quite extensive signaling network involving many kinases and even more targets. As many as 13 phosphoepitopes were observed to be regulated by PKA, indicating that the majority of PGE_2 - induced signaling investigated in this study goes via the PKA signaling node. Interestingly, phosphorylation of S6 ribosomal protein, NDRG1 and CREB was inhibited by several kinase inhibitors, in addition to the PKA inhibitor H-89, such as the PI3K inhibitors (wortmannin and LY294002), Src inhibitor (PP2) and MEK1/MEK2 inhibitor (CI-1040). Wortmannin and LY294002 are regarded as “unspecific” PI3K inhibitors that are known to inhibit several isoforms of PI3K [121].

These experiments have three biological replicates, though further examination and optimization of concentration of the various inhibitors are needed to further study the involvement of multiple kinases downstream of PGE_2 - induced signaling. For instance, to investigate which PI3K isoform that contributes to the regulation, additional experiments are needed using more specific inhibitors, for instance idelalisib (cal101) which inhibits the δ - isoform of PI3K [149]. Interestingly, the sensitivity towards the various inhibitors was T cell subtype - dependent. Phosphorylation of serine 133 on CREB was observed to be strongly inhibited by the aforementioned mentioned inhibitors in the two subtypes of $CD8^+$ T cells compared to $CD4^+$ T cell subtypes. These observations indicate that PGE_2 activates different pathways in the T cell subsets to differentially regulate various target molecules such as CREB. However, the exact regulation and the functional outcome of these pathways remains unclear and additional functional studies are needed to investigate this further.

By revealing PGE_2 - induced signaling and functions in T cells, therapeutic targets of PGE_2 may be exposed. In the present study, 15 phosphorylation sites were explored and characterized. There are presumably more than 15 phosphoepitopes involved in PGE_2 - induced signaling and further characterization of the signaling downstream of PGE_2 is needed to reveal possible therapeutical targets. However, the present study contributes to the much needed characterization of the intracellular signaling induced by PGE_2 and has revealed several novel phosphoepitopes as well as examined some of their regulation by multiple kinases in primary human T cells.

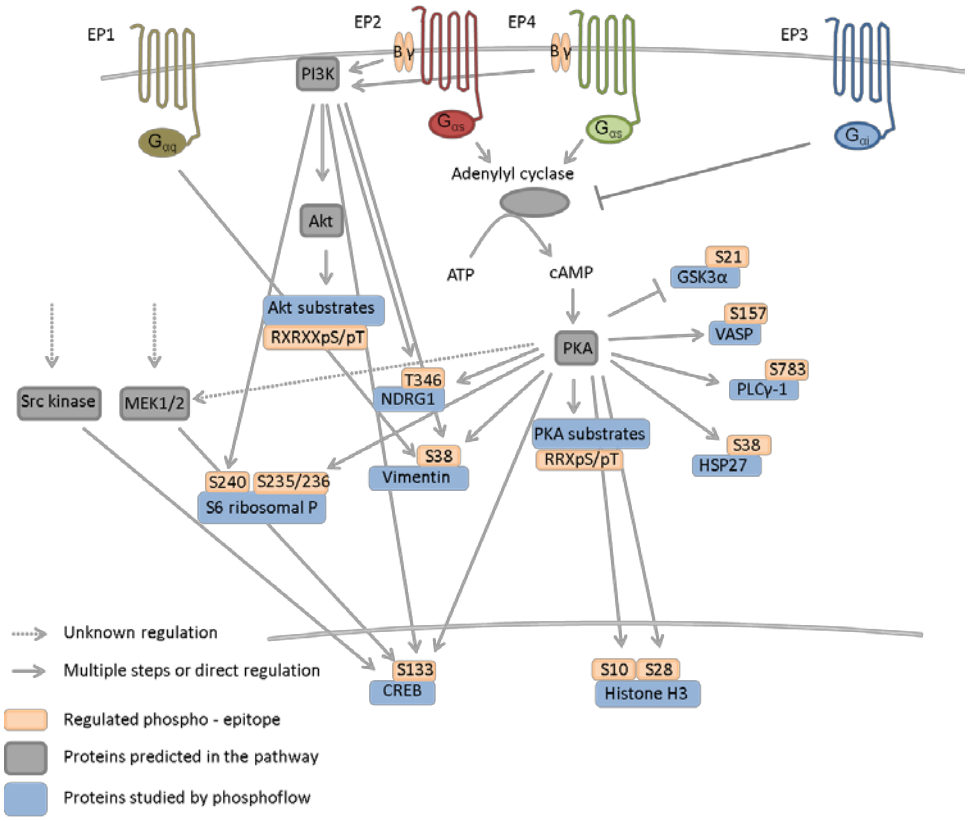


Figure 32 Overview of phosphoepitopes and their pathways initiated by PGE_2 stimulation. *Overview of EP2/EP4 activated phosphoepitopes investigated by phosphoflow cytometry and their possible down - or upstream targets based on the information obtained from experiments in the present study.*

The phosphoflow cytometry approach provides a suitable tool to investigate phosphorylation - based intracellular signaling. By using this method one can not only track the phosphorylation sites at high kinetic resolution, but also observe the activation status of the signaling hubs downstream of the investigated receptor. Thus, phosphoflow is a suitable high - throughput strategy when studying complex signaling networks. However, one of the drawbacks with the method is the limited availability of phosphoepitope specific antibodies. Thus, exploring uncharacterized areas of phosphorylation based signaling networks is challenging and makes it a more biased

approach compared to mass spectrometry studies. However, these two approaches complement each other nicely, and enable exploration of uncharacterized phosphoepitopes as well as generating high resolution data on the phosphorylation status. Overall, phosphoflow cytometry represents a reliable approach to study phosphorylation - based signaling on a single cell level in a complex cell sample.

5.7. Conclusion and future perspectives

As previously mentioned, a phosphoflow cytometry approach can be thought of as a biased approach to study intracellular signaling as you are limited to studying proteins which have suitable antibodies available. Thus, revealing novel phosphoepitopes is challenging with this method, and additional, more unbiased approaches are necessary to fully map the signaling pathways activated by PGE_2. In addition, the experiments mentioned here in the Discussion regarding specificity and EP4 receptor characteristics should be performed to provide additional information about the agonists and antagonists. More functional studies are necessary to expose the functional roles of the regulated phosphoepitopes characterized in this Thesis. In addition, focusing on more specific subsets of $CD4^+$ T cells such as T_H1 and T_H2 cells and their PGE_2 - induced signaling would be beneficial for understanding the multifaceted role PGE_2 seems to have in these cells. Furthermore, detailed expression and functional studies on EP1 and EP3 receptors in T cells must be implemented to reveal their role in PGE_2 - regulated immune functions. Expression levels of EP2 and EP4 and their relative proportions in different human T cells should also be investigated further, to understand why different pathways and sensitivity towards PGE_2 varies between T cell subsets.

In summary, it is clear that PGE_2 initiates extensive and multiple signaling pathways through its four receptors. In addition, there was observed possible crosstalk between these pathways, contributing to the complexity of the signaling network. Although further studies are needed to map PGE_2 - induced signaling in even more detail, this Thesis presents an overview with high resolution of many of the proteins involved in PGE_2 - regulated T cell functions and provides an antibody panel suitable for studying phosphorylation - based PGE_2 – induced signaling. Whether PGE_2 acts as a pro - or anti - inflammatory mediator will continue to be debated, and further functional studies are needed to reveal the role of the four EP receptors in T cell functions. However, the

present study indicates that the majority of PGE_2 - induced signaling in T cells goes through the EP2 and EP4 receptor. Furthermore, the work performed in this Thesis identified novel phosphoepitopes downstream of the four EP receptors, demonstrated that the majority of signaling goes through the PKA signaling node, established the involvement of multiple kinases in regulation of phosphoepitopes, and demonstrated different phosphorylation kinetics and PGE_2 - sensitivities in four different T cell subsets. In addition, characterization of four agonists and four antagonists of the four PGE_2 receptors has been carried out, which can be further used in the investigation of PGE_2 - induced signaling. Finally, the present study contributes to our knowledge of the overall signaling network induced by PGE_2 in human T cells, and provides tools for further investigation of PGE_2 - induced signaling in health and disease.

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Appendix

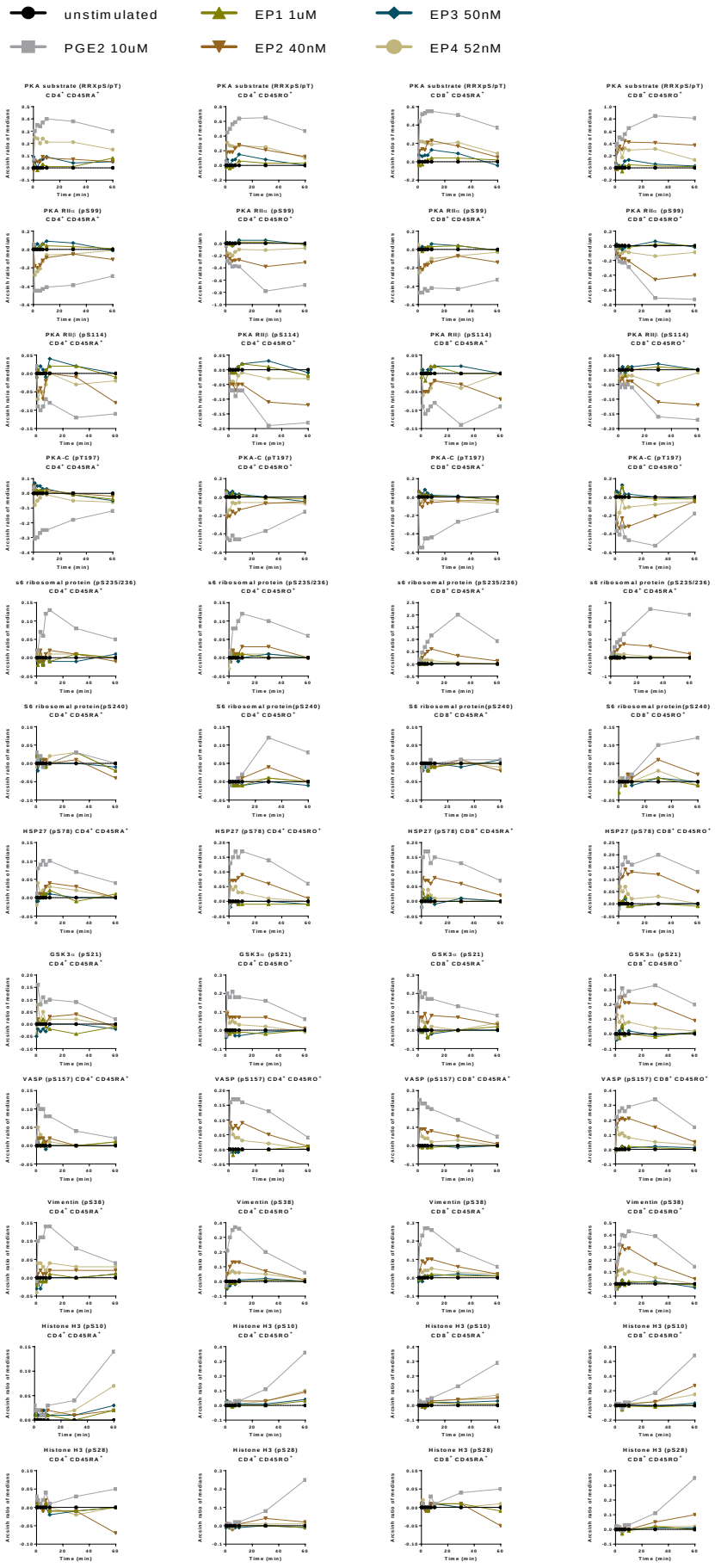
Supplementary Table 1 Kinase inhibitors. *Kinase inhibitors used in the experiments in section 4.5 in the Results.*

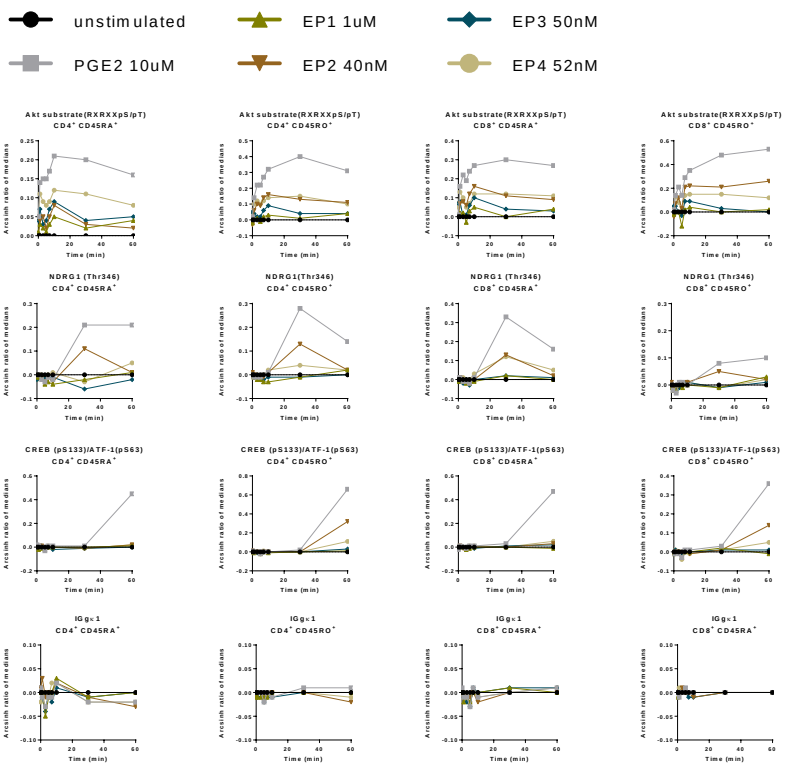
Kinase	Name	Manufacturer	Catalog #	Concentration
PKA	H-89	Cayman Chemicals	10010556	20uM
Akt	Akt Inhibitor VIII	Sigma Aldrich	A6730	5uM
PI3K	Wortmannin	Sigma Aldrich	W1628	1uM
PI3K	LY294002	Cell Signal Technologies		10uM
GSK3	CT99021	Axon Medchem	Axon 1386	2uM
Src	PP2	Calbiochem/Merck Milipore	529573	10uM
MEK1/2	CI-4010	Santa Cruz Biotechnology	Sc-202759	2uM
MEK1/2	PD325901	Axon Medchem	Axon1408	100nM
CDK1/cyclin B	RO-3306	Sigma Aldrich	SML0569	10uM
EGF - Receptor	AG1478	Selleck Chemicals	S2728	10uM
CLK1	TG003	Selleck Chemicals	S7320	10uM
PKC	Gö 6983	Tocris	2285	5uM
CK2	TBB	Tocris	2275	10uM
Erk 1/2	SCH772984	MedChem Express	HY-50846	1uM
PAK 1/2/3	FRAX597	MedChem Express	HY-15542A	5uM

Supplementary Table 2 Final antibody panel. *Panel of phospho – epitope specific antibodies used in agonist and antagonist titration experiments.*

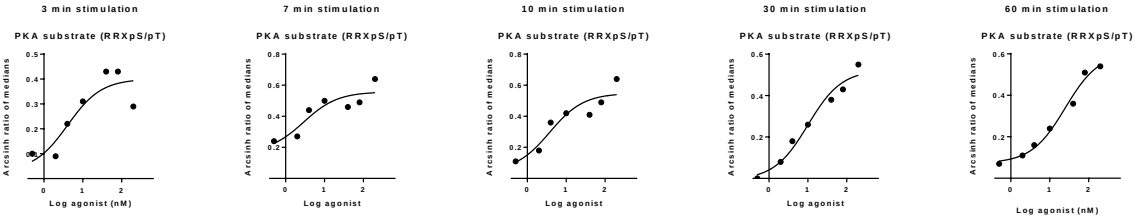
Antigen	Fluorophore	Producer	Catalog #	Isotype
IgG_1 Kappa (isotype control)	Ax647	BD Biosciences	557783	Mouse (BALB/c) IgG1 K
S6-Ribos. Prot. (pS235/236)	Ax647	Cell Signaling Technology	4851	Rabbit
Histone H3 (pS10)	Ax647	Cell Signaling Technology	9716	Rabbit
p -p90RSK(Ser380)	Ax647	Cell Signaling Technology	13575	Rabbit
p - Histone H2A.X(Ser139)	Ax647	Cell Signaling Technology	9720	Rabbit
CREB (pS133)/ATF-1(pS63)	Ax647	BD Biosciences	558434	Mouse
mTOR (pS2448)	Ax647	BD Biosciences	564242	Mouse
IRF - 7 (pS477/pS479)	Ax647	BD Biosciences	558630	Mouse
PDPK1 (pS241)	Ax647	BD Biosciences	560091	Mouse
Histone H3(pS28)	Ax647	BD Biosciences	558217	Rat
p - NDRG1(Thr346)	Ax647	Cell Signaling Technology	7497	Rabbit
S6 (pS240)	Ax647	BD Biosciences	560432	Mouse
PLK1 (pT210)	Ax647	BD Biosciences	558447	Mouse
PLC-γ2 (pY759)	Ax647	BD Biosciences	558498	Mouse
Smad2 (pS465/467)/smad3(pS423/425)	Ax647	BD Biosciences	562696	Mouse
EGF-Receptor (pY845)	Ax647	BD Biosciences	558523	Mouse
EGF-Receptor (pY1173)	Ax647	BD Biosciences	558524	Mouse
PKA RIIα (pS99)	Ax647	BD Biosciences	560164	Mouse
PKA[RIIβ] (pS114)	Ax647	BD Biosciences	560205	Mouse
Secondary antibodies				
Antigen	**Fluorophore**	**Producer**	**Cat#**	**Isotype**
pPKA Substrate (RRXS/T)	Ax647	Cell Signaling Technology	9624	Rabbit
p-PLCγ-1 (pT783)	Ax647	Cell Signaling Technology	2821	Rabbit
p-Akt subs. (RXRXXS/T)	Ax647	Cell Signaling Technology	9614	Rabbit
p-GSK3α (pS21)	Ax647	Cell Signaling Technology	9316	Rabbit
p-HSP27 (pS78)	Ax647	Cell Signaling Technology	2405	Rabbit
p-VASP (pS157)	Ax647	Cell Signaling Technology	3111	Rabbit
p-Vimentin (pS38)	Ax647	abcam	Ab52942	Rabbit
p-PKA-C (pT197)	Ax647	Cell Signaling Technology.	4781	Rabbit
p-Erk 1/2 (HTGFLpTEpYVAT)	Ax647	Sigma	M9692	Mouse
Surface markers				
Antigen	**Fluorophore**	**Producer**	**Cat#**	**Isotype**
CD3 (SK7)	PerCP	BD Biosciences	345766	Mouse
CD4 (SK3)	PE cy7	BD Biosciences	348809	Mouse
CD45RA (HI100)	APC - H7	BD Biosciences	560674	Mouse

Appendix


unstimulated
EP1 1uM
EP3 50nM
PGE2 10uM
EP2 40nM
EP4 52nM
PKA substrate (RRXpS/pT) CD4+ CD45RA+
PKA substrate (RRXpS/pT) CD4+ CD45RO+
PKA substrate (RRXpS/pT) CD8+ CD45RA+
PKA substrate (RRXpS/pT) CD8+ CD45RO+
PKA RIIα (pS99) CD4+ CD45RA+
PKA RIIα (pS99) CD4+ CD45RO+
PKA RIIα (pS99) CD8+ CD45RA+
PKA RIIα (pS99) CD8+ CD45RO+
PKA RIIβ (pS114) CD4+ CD45RA+
PKA RIIβ (pS114) CD4+ CD45RO+
PKA RIIβ (pS114) CD8+ CD45RA+
PKA RIIβ (pS114) CD8+ CD45RO+
PKA-C (pT197) CD4+ CD45RA+
PKA-C (pT197) CD4+ CD45RO+
PKA-C (pT197) CD8+ CD45RA+
PKA-C (pT197) CD8+ CD45RO+
s6 ribosomal protein (pS235/236) CD4+ CD45RA+
s6 ribosomal protein (pS235/236) CD4+ CD45RO+
s6 ribosomal protein (pS235/236) CD8+ CD45RA+
s6 ribosomal protein (pS235/236) CD4+ CD45RA+
S6 ribosomal protein(pS240) CD4+ CD45RA+
S6 ribosomal protein(pS240) CD4+ CD45RO+
S6 ribosomal protein(pS240) CD8+ CD45RA+
S6 ribosomal protein(pS240) CD8+ CD45RO+
HSP27 (pS78) CD4+ CD45RA+
HSP27 (pS78) CD4+ CD45RO+
HSP27 (pS78) CD8+ CD45RA+
HSP27 (pS78) CD8+ CD45RO+
GSK3α (pS21) CD4+ CD45RA+
GSK3α (pS21) CD4+ CD45RO+
GSK3α (pS21) CD8+ CD45RA+
GSK3α (pS21) CD8+ CD45RO+
VASP (pS157) CD4+ CD45RA+
VASP (pS157) CD4+ CD45RO+
VASP (pS157) CD8+ CD45RA+
VASP (pS157) CD8+ CD45RO+
Vimentin (pS38) CD4+ CD45RA+
Vimentin (pS38) CD4+ CD45RO+
Vimentin (pS38) CD8+ CD45RA+
Vimentin (pS38) CD8+ CD45RO+
Histone H3 (pS10) CD4+ CD45RA+
Histone H3 (pS10) CD4+ CD45RO+
Histone H3 (pS10) CD8+ CD45RA+
Histone H3 (pS10) CD8+ CD45RO+
Histone H3 (pS28) CD4+ CD45RA+
Histone H3 (pS28) CD4+ CD45RO+
Histone H3 (pS28) CD8+ CD45RA+
Histone H3 (pS28) CD8+ CD45RO+
Arcsinh ratio of medians
Time (min)



Supplementary Figure 1 Overview of phosphorylation in different cell types. *16 phosphosites were regulated during PGE_2 or agonist stimulation. Displayed above is the signal intensities measured from the 16 phosphosites in four different T cell subsets ($CD4^+$ $CD45RA^+$, $CD4^+$ $CD45RO^+$, $CD8^+$ $CD45RA^+$, $CD8^+$ $CD45RO^+$). The experiment was performed as described in Figure 17. IgGκ was used as isotype control.*



Supplementary Figure 2 Concentration response curves of PKA phosphorylation on various time points of EP2 agonist stimulation. *The experiment was performed as described in figure 18 and signal intensities are calculated relative to the unstimulated sample.*

Supplementary Table 3 EP1 agonist (ONO-DI-004) and antagonist (ONO-8713) response curves in $CD8^+$ $CD45RO^+$ T cells (best fit value). *EC_{50}/IC_{50} and R^2 - values of 28 investigated phospho - sites each with two selected time points*

ONO-DI-004 concentration responses			**ONO-8713 concentration responses**	
Phospho – epitope	**EC50**	**R - square**	**IC50**	**R - square**
Histone H2A.X (Ser139) 30 min	Not converged	-	14833	0.13
Histone H2A.X(Ser139) 60 min	Interrupted	-	Ambiguous	0.65
CREB (Ser133)/ATF-1 (Ser63) 30 min	Not converged	-	Interrupted	-
CREB (Ser133)/ATF-1 (Ser63) 60 min	179.1	0.21	Ambiguous	**0.84**
Erk1/2 (activation loop) 3 min	47.95	0.41	840.4	**0.89**
Erk1/2 (activation loop) 7 min	Not converged	-	Ambiguous	0.59
Histone H3 (Ser10) 30 min	198.2	0.16	Interrupted	-
Histone H3 (Ser10) 60 min	204.4	0.16	Ambiguous	0.44
Vasp (Ser157) 10 min	118.1	0.26	Ambiguous	0.41
Vasp (Ser157) 30 min	64.16	0.35	480.6	0.07
PKA substrate (RRXpS/pT) 10 min	Not converged	-	Not converged	-
PKA substrate (RRXpS/pT) 30 min	128.3	0.22	$3.18E^{018}$	-0.13
PKA RIIα (Ser99) 10 min	Not converged	-	3218	0.51
PKA RIIα (Ser99) 30 min	211.4	0.13	912.1	**0.85**
PLCγ-1 (Thr783) 10 min	70.94	0.56	9.16	0.04
PLCγ-1 (Thr783) 60 min	66.24	0.11	Not converged	-
Akt substrate (RXRXXpS/pT) 30 min	167.3	0.34	61860	0.41
Akt substrate (RXRXXpS/pT) 60 min	224.1	0.25	Interrupted	-
Histone H3 (Ser28) 30 min	Interrupted	-	Ambiguous	0.71
Histone H3 (Ser28) 60 min	71.28	0.05	Ambiguous	0.61
GSK3α (Ser21) 10 min	65.85	0.24	4182	0.12
GSK3α (Ser21) 30 min	96.25	0.24	Ambiguous	0.48
PKA RIIβ (Ser114) 7 min	Not converged	-	1131	0.13
PKA RIIβ (Ser114) 30 min	480-1	0.03	Ambiguous	**0.81**
CD4 CD45RO NDRG1 (Thr346) 30 min	159.4	0.006	2683	0.64
CD4 CD45RA NDRG1 (Thr346) 60 min	196.9	0.08	567.6	0.61
Vimentin (Ser38) 10 min	Interrupted	-	11136	0.69
Vimentin (Ser38) 60 min	122.5	**0.93**	3903	0.197
PKA-C (Thr197) 10 min	Interrupted	-	5332	0.16
PKA-C (Thr197) 30 min	Not converged	-	858.6	0.49
S6 Ribosomal protein (Ser235/236) 10 min	620.5	0.64	1488	**0.91**
S6 Ribosomal protein (Ser235/236) 30 min	133.7	0.09	731.5	**0.87**
Smad2 (pS465/467)/Smad3 (pS423/425) 30 min	192.1	0.27	239.1	0.08
Smad2 (pS465/467)/Smad3 (pS423/425) 60 min	584.9	0.51	Ambiguous	0.44
90RSK (Ser380) 10 min	Interrupted	-	Interrupted	-
90RSK (Ser380) 60 min	199.9	0.40	10702	0.88
EGF – Receptor (Tyr845) 30 min	Not converged	-	54459	0.08
EGF – Receptor (Tyr845) 60 min	Not converged	-	24.5	0.87
EGF – Receptor (Tyr1173) 30 min	Not converged	-	Not converged	-
EGF – Receptor (Tyr1173) 60 min	Interrupted	-	377.8	0.63

Supplementary Table 4 EP2 agonist (ONO-AE1-259-01) and antagonist (TG-155) response curves in CD8$^+$ CD45RO$^+$ T cells (Best fit values). *EC_{50}/IC_{50} and R^2 - values of 28 investigated phospho - sites each with two selected time points.*

ONO-AE1-259-01 concentration responses			**TG4-155 concentration responses**	
Phospho – epitope	**EC50**	**R - square**	**IC50**	**R - square**
Histone H2A.X (Ser139) 30 min	Interrupted	-	7.99	0.14
Histone H2A.X(Ser139) 60 min	0.88	0.08	6.14	0.48
CREB (Ser133)/ATF-1 (Ser63) 30 min	80.86	**0.88**	30.32	0.003
CREB (Ser133)/ATF-1 (Ser63) 60 min	25.36	**0.92**	88.95	**0.84**
Erk1/2 (activation loop) 3 min	12.46	0.04	1.14	0.24
Erk1/2 (activation loop) 7 min	73,60	0.07	3.95	0.03
Histone H3 (Ser10) 30 min	26.62	**0.85**	23.82	0.27
Histone H3 (Ser10) 60 min	17.41	**0.94**	18.63	**0.90**
Vasp (Ser157) 10 min	7.04	**0.94**	58.36	**0.94**
Vasp (Ser157) 30 min	12.19	**0.92**	23.83	**0.94**
PKA substrate (RRXpS/pT) 10 min	3.77	0.87	30.70	0.72
PKA substrate (RRXpS/pT) 30 min	10.04	**0.97**	32.29	**0.81**
PKA RIIα (Ser99) 10 min	2.10	**0.98**	74.62	**0.98**
PKA RIIα (Ser99) 30 min	5.36	**0.98**	20.83	**0.99**
PLCγ-1 (Thr783) 10 min	0.33	**0.96**	Not converged	-
PLCγ-1 (Thr783) 60 min	42.70	0.77	3.82	0.23
Akt substrate (RXRXXpS/pT) 30 min	12.43	**0.94**	27.23	0.67
Akt substrate (RXRXXpS/pT) 60 min	20.02	**0.99**	19.75	**0.96**
Histone H3 (Ser28) 30 min	8.56	**0.95**	34.95	**0.83**
Histone H3 (Ser28) 60 min	30.87	**0.98**	20.00	**0.95**
GSK3α (Ser21) 10 min	2.76	**0.96**	110	**0.94**
GSK3α (Ser21) 30 min	13.61	**0.95**	44.13	**0.95**
PKA RIIβ (Ser114) 7 min	2.47	**0.89**	11.54	0.58
PKA RIIβ (Ser114) 30 min	3.34	**0.90**	140.3	**0.92**
CD4 CD45RO NDRG1 (Thr346) 30 min	19.24	**0.90**	8.86	**0.91**
CD4 CD45RA NDRG1 (Thr346) 60 min	22.96	**0.87**	3.01	0.41
Vimentin (Ser38) 10 min	6.63	**0.99**	53.29	**0.99**
Vimentin (Ser38) 30 min	30.19	**0.96**	19.58	**0.97**
PKA-C (Thr197) 10 min	4.50	**0.99**	44.81	**0.97**
PKA-C (Thr197) 30 min	18.92	**0.99**	25.25	**0.97**
S6 Ribosomal protein (Ser235/236) 10 min	7.31	**0.97**	31.28	**0.94**
S6 Ribosomal protein (Ser235/236) 30 min	38.28	**0.99**	19.84	**0.98**
Smad2 (pS465/467)/Smad3 (pS423/425) 30 min	Ambiguous	0.24	4.22	0.06
Smad2 (pS465/467)/Smad3 (pS423/425) 60 min	0.74	0.55	2.85	**0.81**
90RSK (Ser380) 10 min	2.80	0.53	Interrupted	-
90RSK (Ser380) 60 min	Ambiguous	0.65	1368	**0.84**
HSP27 (pS78) 10 min	4.55	0.97	27.65	**0.98**
HSP27 (pS78) 30 min	4.51	0.96	14.92	**0.85**

Supplementary Table 5 EP3 agonist (ONO-AE-248) and antagonist (ONO-AE3-240) response curves in CD8$^+$ CD45RO$^+$ T cells (Best – fit values). *EC_{50}/IC_{50} and R^2 - values of 28 investigated phospho - sites each with two selected time points.*

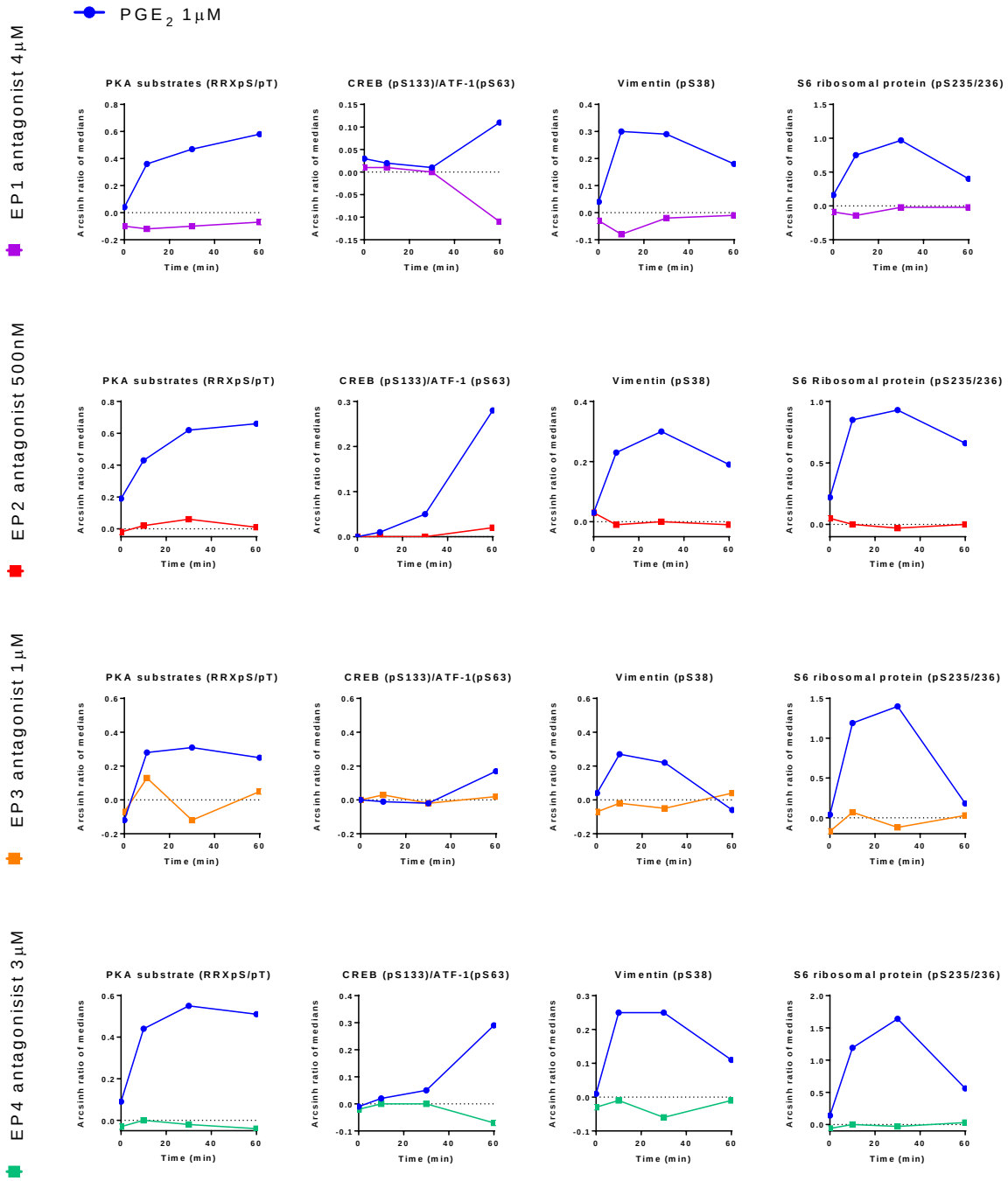
ONO-AE-248 concentration responses			**ONO-AE3-240 concentration responses**	
Phospho – epitope	**EC50**	**R - square**	**IC50**	**R - square**
Histone H2A.X (Ser139) 30 min	Not converged	-	96.46	0.46
Histone H2A.X(Ser139) 60 min	Interrupted	-	13.91	0.68
CREB (Ser133)/ATF-1 (Ser63) 30 min	Not converged	-	Interrupted	-
CREB (Ser133)/ATF-1 (Ser63) 60 min	Ambiguous	**0.90**	Ambiguous	0.58
Erk1/2 (activation loop) 3 min	1.81	0.69	4.31	0.54
Erk1/2 (activation loop) 7 min	Ambiguous	0.79	1.7	0.17
Histone H3 (Ser10) 30 min	Interrupted	-	Interrupted	0.47
Histone H3 (Ser10) 60 min	Ambiguous	0.61	26.42	0.37
Vasp (Ser157) 10 min	3.86	0.42	Ambiguous	0.61
Vasp (Ser157) 30 min	Interrupted	-	Interrupted	0.07
PKA substrate (RRXpS/pT) 10 min	0.30	0.24	0.37	0.47
PKA substrate (RRXpS/pT) 30 min	1.04	0.47	4.07	0.55
PKA RIIα (Ser99) 10 min	11.4	0.21	2.27	0.69
PKA RIIα (Ser99) 30 min	Not converged	-	1816	**0.87**
PLCγ-1 (Thr783) 10 min	Ambiguous	0.72	Ambiguous	0.52
PLCγ-1 (Thr783) 60 min	0.50	0.19	Not converged	-
Akt substrate (RXRXXpS/pT) 30 min	Ambiguous	0.10	Interrupted	-
Akt substrate (RXRXXpS/pT) 60 min	0.29	0.05	Not converged	-
Histone H3 (Ser28) 30 min	12.44	0.20	12.08	0.03
Histone H3 (Ser28) 60 min	4.07	0.03	Not converged	-
GSK3α (Ser21) 10 min	19.15	0.45	44.71	0.11
GSK3α (Ser21) 30 min	Ambiguous	0.46	869.4	0.04
PKA RIIβ (Ser114) 7 min	Ambiguos	**0.86**	19.07	0.56
PKA RIIβ (Ser114) 30 min	Not converged	-	626.4	**0.87**
CD4 CD45RO NDRG1 (Thr346) 30 min	100.2	0.03	1310	0.54
CD4 CD45RA NDRG1 (Thr346) 60 min	183.2	0.06	Ambiguous	0.58
Vimentin (Ser38) 10 min	12.91	0.52	1.65	0.50
Vimentin (Ser38) 60 min	0.78	0.24	21.73	0.74
PKA-C (Thr197) 10 min	6.16	0.02	74.21	0.59
PKA-C (Thr197) 30 min	52.15	0.23	114.9	0.47
S6 Ribosomal protein (Ser235/236) 10 min	Interrupted	-	Ambiguous	0.93
S6 Ribosomal protein (Ser235/236) 30 min	492.4	0.08	Interrupted	-
Smad2 (pS465/467)/Smad3 (pS423/425) 30 min	Interrupted	-	200.6	0.08
Smad2 (pS465/467)/Smad3 (pS423/425) 60 min	Ambiguous	0.75	Not converged	-
90RSK (Ser380) 10 min	63.04	0.07	Not converged	-
90RSK (Ser380) 60 min	1.28	0.08	12.01	**0.83**
EGF – Receptor (Tyr845) 30 min	2.103	0.21	107.0	0.47
EGF – Receptor (Tyr845) 60 min	1.11	0.62	Not converged	-
EGF – Receptor (Tyr1173) 30 min	6.37	0.30	70.70	0.65
EGF – Receptor (Tyr1173) 60 min	201.7	0.36	32.45	**0.88**

Supplementary Table 6 EP4 agonist (ONO-AE1-329) and antagonist (ONO-AE3-208) response curves in CD8$^+$ CD45RO$^+$ T cells (Best – fit value). *EC_{50}/IC_{50} and R^2 - values of 28 investigated phospho - sites each with two selected time points.*

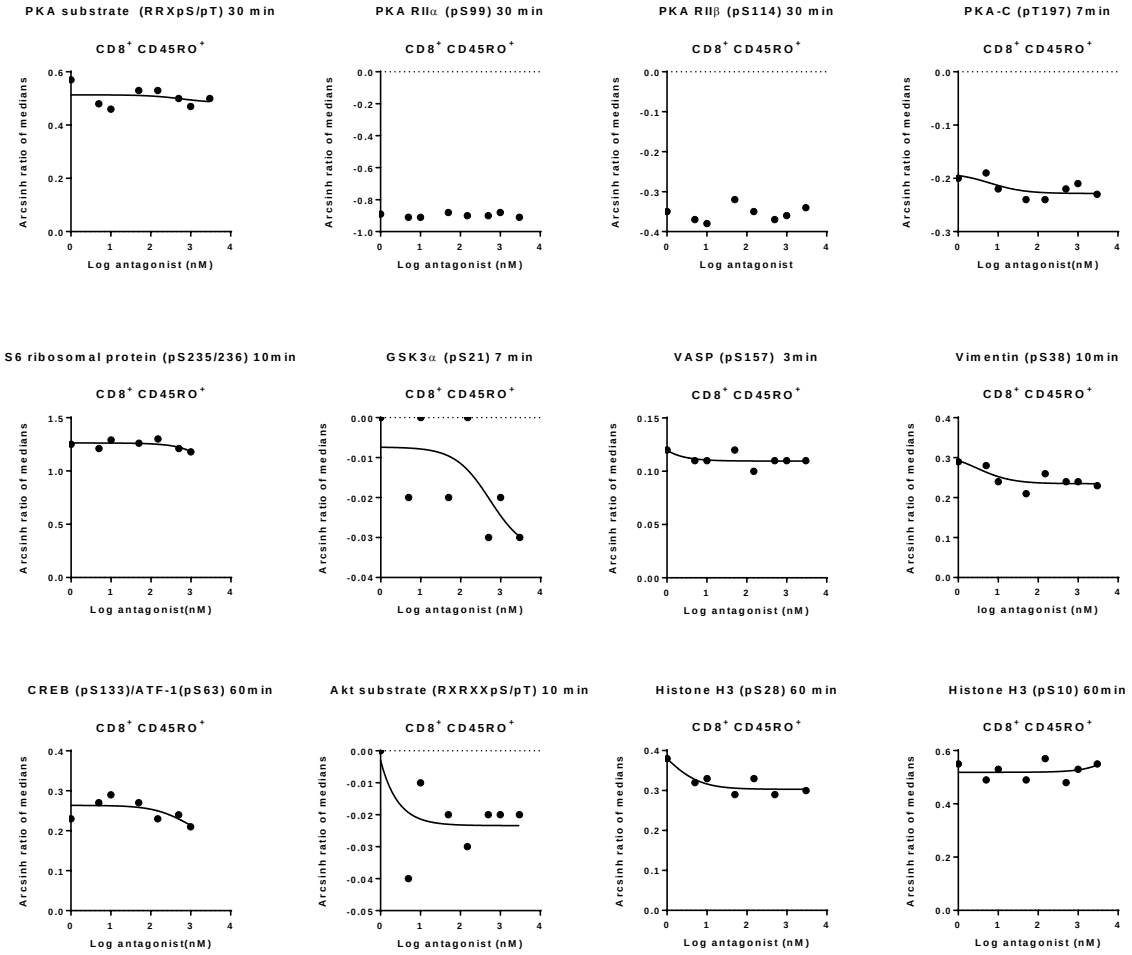
ONO-AE1-329 concentration responses			**ONO-AE3-208 concentration responses**	
Phospho – epitope	**EC50**	**R - square**	**IC50**	**R - square**
Histone H2A.X (Ser139) 30 min	38.3	0.14	0.26	0.31
Histone H2A.X(Ser139) 60 min	2.38	0.33	Interrupted	-
CREB (Ser133)/ATF-1 (Ser63) 30 min	Interrupted	-	Interrupted	-
CREB (Ser133)/ATF-1 (Ser63) 60 min	2.89	0.50	850.2	0.47
Erk1/2 (activation loop) 3 min	0.11	0.28	2.39	0.69
Erk1/2 (activation loop) 7 min	2.21	0.73	6.89	0.25
Histone H3 (Ser10) 30 min	7.27	0.72	2.20	0.20
Histone H3 (Ser10) 60 min	2.91	0.49	Interrupted	-
Vasp (Ser157) 10 min	10.28	0.20	14.65	0.78
Vasp (Ser157) 30 min	Interrupted		31.61	0.02
PKA substrate (RRXpS/pT) 10 min	5.46	**0.88**	Not converged	-
PKA substrate (RRXpS/pT) 30 min	3.00	**0.90**	602.9	0.07
PKA RIIα (Ser99) 10 min	0.54	**0.92**	4.51	0.04
PKA RIIα (Ser99) 30 min	0.30	**0.97**	Not converged	-
PLCγ-1 (Thr783) 10 min	12.72	**0.82**	0.49	0.32
PLCγ-1 (Thr783) 60 min	2.44	0.18	Interrupted	-
Akt substrate (RXRXXpS/pT) 10 min	25.54	**0.88**	Interrupted	-
Akt substrate (RXRXXpS/pT) 30 min	2.78	0.68	Not converged	-
Histone H3 (Ser28) 30 min	4.45	**0.86**	594.6	0.12
Histone H3 (Ser28) 60 min	1.68	0.70	0.87	0.77
GSK3α (Ser21) 10 min	2.74	0.79	0.43	0.32
GSK3α (Ser21) 30 min	0.33	**0.82**	17.39	0.24
PKA RIIβ (Ser114) 7 min	0.26	**0.87**	Interrupted	-
PKA RIIβ (Ser114) 30 min	0.21	**0.93**	Not converged	-
CD4 CD45RO NDRG1 (Thr346) 30 min	Not converged	-	379.4	0.0005
CD4 CD45RA NDRG1 (Thr346) 60 min	6.38	0.55	Interrupted	-
Vimentin (Ser38) 7 min	4.96	**0.80**	51.46	0.56
Vimentin (Ser38) 10 min	1.86	**0.98**	2.98	0.61
PKA-C (Thr197) 10 min	2.92	**0.91**	Not converged	-
PKA-C (Thr197) 30 min	1.74	**0.89**	362.9	0.17
S6 Ribosomal protein (Ser235/236) 10 min	4.48	**0.99**	Ambiguous	0.47
S6 Ribosomal protein (Ser235/236) 30 min	2.76	**0.81**	6.67	0.71
Smad2 (pS465/467)/Smad3 (pS423/425) 30 min	5.69	0.77	Not converged	-
Smad2 (pS465/467)/Smad3 (pS423/425) 60 min	2.53	0.32	0.19	0.62
90RSK (Ser380) 10 min	Interrupted	-	27.11	0.14
90RSK (Ser380) 60 min	5.37	0.44	7120	0.57
EGF – Receptor (Tyr845) 30 min	43.76	0.63	Not converged	-
EGF – Receptor (Tyr845) 60 min	2.55	0.37	Ambiguous	0.51
EGF – Receptor (Tyr1173) 30 min	159.2	0.07	Ambiguous	0.42
EGF – Receptor (Tyr1173) 60 min	3.91	0.42	280.2	0.42

Supplementary Table 7 EP4 agonist (ONO-AE1-329) response curves and EP4 antagonist (ONO-AE3-208 + ONO – AE1-329) response curves (Best – fit values) in the presence of EP4 agonist (ONO-AE1-329 in CD8$^+$ CD45RO$^+$ T cells.). *EC_{50}/IC_{50} and R^2 - values of 28 investigated phospho - sites each with two selected time points.*

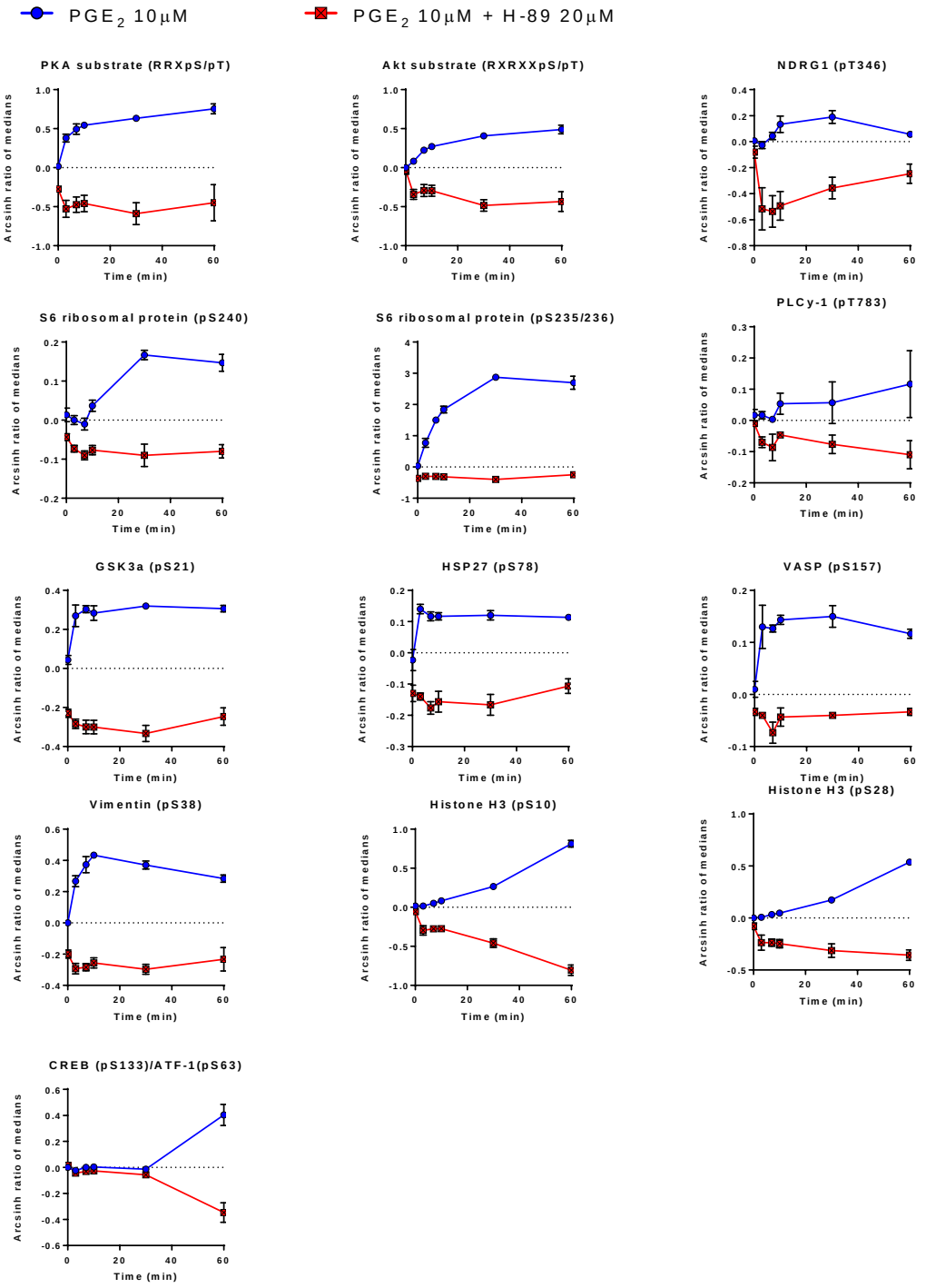
ONO-AE1-329 concentration responses			**ONO-AE3-208 + ONO – AE1-329 concentration responses**	
Phospho – epitope	**EC50**	**R - square**	**IC50**	**R - square**
Histone H2A.X (Ser139) 30 min	38.3	0.14	Interrupted	-
Histone H2A.X(Ser139) 60 min	2.38	0.33	Ambiguous	**0.85**
CREB (Ser133)/ATF-1 (Ser63) 30 min	Interrupted	-	1954	0.25
CREB (Ser133)/ATF-1 (Ser63) 60 min	2.89	0.50	26553	**0.93**
Erk1/2 (activation loop) 3 min	0.11	0.28	165.0	0.12
Erk1/2 (activation loop) 7 min	2.21	0.73	Not converged	-
Histone H3 (Ser10) 30 min	7.27	0.72	Ambiguous	0.41
Histone H3 (Ser10) 60 min	2.91	0.49	14893	**0.88**
Vasp (Ser157) 10 min	10.28	0.20	7.17	0.64
Vasp (Ser157) 30 min	Interrupted		174.6	0.04
PKA substrate (RRXpS/pT) 10 min	5.46	**0.88**	Ambiguous	0.63
PKA substrate (RRXpS/pT) 30 min	3.00	**0.90**	11.76	**0.95 (60min)**
PKA RIIα (Ser99) 10 min	0.54	**0.92**	7.82	**0.87**
PKA RIIα (Ser99) 30 min	0.30	**0.97**	6.80	**0.93**
PLCγ-1 (Thr783) 10 min	12.72	**0.82**	7.01	0.11
PLCγ-1 (Thr783) 60 min	2.44	0.18	18.57	0.29
Akt substrate (RXRXXpS/pT) 10 min	25.54	**0.88**	Interrupted	-
Akt substrate (RXRXXpS/pT) 30 min	2.78	0.68	18.06	0.04
Histone H3 (Ser28) 30 min	4.45	**0.86**	Interrupted	-
Histone H3 (Ser28) 60 min	1.68	0.70	5.56	0.58
GSK3α (Ser21) 10 min	2.74	0.79	21.55	**0.83**
GSK3α (Ser21) 30 min	0.33	**0.82**	25.66	0.46
PKA RIIβ (Ser114) 7 min	0.26	**0.87**	12,55	**0.85**
PKA RIIβ (Ser114) 30 min	0.21	**0.93**	28.81	**0.87**
CD4 CD45RO NDRG1 (Thr346) 30 min	Not converged	-	86.92	0.39
CD4 CD45RA NDRG1 (Thr346) 60 min	6.38	0.55	52.21	0.20
Vimentin (Ser38) 7 min	4.96	**0.80**	1.00	0.76
Vimentin (Ser38) 10 min	1.86	**0.98**	14.22	0.45
PKA-C (Thr197) 10 min	2.92	**0.91**	Ambiguous	0.75
PKA-C (Thr197) 30 min	1.74	**0.89**	Ambiguous	0.59
S6 Ribosomal protein (Ser235/236) 10 min	4.48	**0.99**	6.67	**0.86**
S6 Ribosomal protein (Ser235/236) 30 min	2.76	**0.81**	10.99	**0.85**
Smad2 (pS465/467)/Smad3 (pS423/425) 30 min	5.69	0.77	92.8	0.52
Smad2 (pS465/467)/Smad3 (pS423/425) 60 min	2.53	0.32	450.6	0.54
90RSK (Ser380) 10 min	Interrupted	-	1.39	0.06
90RSK (Ser380) 60 min	5.37	0.44	3449	**0.93**
EGF – Receptor (Tyr845) 30 min	43.76	0.63	581.4	0.51
EGF – Receptor (Tyr845) 60 min	2.55	0.37	Not converged	-
EGF – Receptor (Tyr1173) 30 min	159.2	0.07	Interrupted	-
EGF – Receptor (Tyr1173) 60 min	3.91	0.42	3688	**0.94**



Supplementary Figure 3 Investigation of antagonist characteristics. *A) Cells treated with the highest concentration of any of the four antagonists, without any other stimulation factor, showing no significant response. PGE_2 were added as a separate condition to compare responses (n = 1).*



Supplementary Figure 4 EP4 antagonist titration in the presence of 1 μM PGE_2. *The experiment did not generate the concentration – dependent response curves as expected. The results show no effect in phosphorylation status of the investigated phosphoepitopes in the presence of increasing concentrations of EP4 antagonist (1, 5, 10, 50, 100, 500, 1000 and 3000 nM).*



Supplementary Figure 5 H-89 inhibits phosphorylation of 14 phosphoepitopes. *Phosphorylation of the investigated phosphoepitopes was reduced to below basal signaling in the presence of the PKA inhibitor H89. The experiment was performed as described in Figure 27A.*