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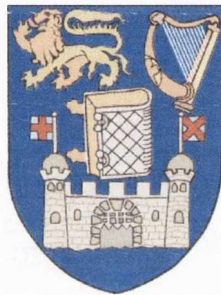
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The immunomodulatory effects of the cAMP effector Epac

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of
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Abstract

Cyclic AMP is an extremely important second messenger and is known to be a central mediator of inflammation and regulator of the immune response. For many years it was thought that the effects of cAMP on immunity were principally mediated via protein kinase A (PKA), but recently a novel target of cAMP, exchange protein directly activated by cAMP (Epac), has been identified. Relatively little is known about the effects of Epac on the immune system and therefore it is important to establish what role Epac plays in cAMP mediated immunological effects.

To determine the effects of selectively activating Epac and PKA in DCs, the specific Epac activator, 8-pCPT-2'-O-Me-cAMP (8pCPT), and PKA activator, N⁶-Bz-cAMP (N6-Bz), were used. Activation of PKA in DCs increased surface expression of MHC class II, CD86 and CD80 while CD40 expression was decreased. PKA activation decreased IL-12 production while increasing the secretion of IL-10 and IL-6. In contrast, Epac activation enhanced IL-12 secretion and increased MHC class II expression on DCs. Undifferentiated DCs, co-cultured with the Epac activator and GM-CSF, showed enhanced production of IL-12 following HK bacteria and CpG stimulation compared to the DCs differentiated in the presence of GM-CSF alone. Co-culture of these DCs with the PKA activator and GM-CSF showed suppression of IL-12 production. The effects of Epac and PKA activation on cytokine production by spleen cells were also assessed. Cells were incubated with anti-CD3 in the presence or absence of PKA and Epac activators in order to assess cytokine production. Activation of the Epac pathway resulted in increased secretion of the Th1-associated cytokine IFN- γ . In contrast, PKA activation, suppressed cytokine production by spleen cells. Glybenclamide is a sulfonylurea drug used to treat type 2 diabetes and is reported to directly activate Epac2. Treatment of DCs and spleen cells with glybenclamide induced IL-12 production by DCs and IFN- γ production from spleen cells, mimicking the effects of 8pCPT on DC and spleen cell cytokine production. Upon further investigation of the 8pCPT-induced IFN- γ production, it was found that at early timepoints CD8⁺ T cells were the source of the IFN- γ . Analysis of CCR7 and CD62L expression, as a measure of the CD8⁺ T cell subtype showed that following Epac activation with 8pCPT the CD8⁺IFN- γ ⁺ cells were CCR7⁻CD62L⁻, indicative of effector memory cells.

Several studies were carried out to assess the effects of Epac activation *in vivo*. Intraperitoneal injection of 8pCPT on five consecutive days induced IFN- γ and IL-17 production in the mediastinal lymph nodes, while N6-Bz injection induced IL-4 and IL-17 production. Further analysis of the *in vivo* effects of Epac activation showed increased IFN- γ production from CD8⁺ T cells in the peritoneum after a single injection of 8pCPT, and increased IFN- γ expression in CD8⁺ T cells, NK and NKT cells in the spleen after injecting 8pCPT for five consecutive days. Studies were carried out using either ovalbumin (OVA) or the *Mycobacterium tuberculosis*-derived Hybrid 1 (H1) antigen, in order to determine the adjuvant effects of 8pCPT. H1 is a fusion of the highly immunogenic proteins ESAT-6 and Ag85B, both derived from the causative agent of tuberculosis (TB), *Mycobacterium tuberculosis*. Epac activation did not enhance an OVA-specific IFN- γ or antibody responses, but did inhibit OVA-specific IL-4 production in spleen cells. In the case of H1, Epac activation induced a non-antigen specific IFN- γ response but enhanced H1-specific IgG2c antibody titres, suggesting that activating the Epac pathway has potential in Th1-promoting vaccines.

Abbreviations

8pCPT	8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cAMP
AC	adenylyl/adenylate cyclase
AD	Alzheimer's disease
AhR	aryl hydrocarbon receptor
AID	activation-induced cytidine deaminase
AKAP	A-kinase anchoring protein
AMP	antimicrobial peptides
APC	antigen-presenting cell
β2AR	β2 adrenergic receptor
BCR	B cell receptor
BMDC	bone marrow-derived dendritic cells
BSA	bovine serum albumin
cAMP	cyclic 3',4'-adenosine monophosphate
CD	cluster of differentiation
cDNA	complementary DNA
CIITA	MHC Class II Transcription activator
CLR	C-type lectin receptor
CNB	cyclic mononucleotide-binding
CNS	central nervous system
COX	cyclooxygenase
CpG	cytosine-phosphate-guanine
CR	catalytic region
CREB	CRE-binding protein
cRMP1	complete RPMI
CSR	class switch recombination
CT	cholera toxin
CTL	cytotoxic T-lymphocyte
CVI	common variable immunodeficiency
DB-cAMP	N ⁶ ,2'-O-Dibutyryl-adenosine-3'-5'-cyclic monophosphate·Na /dibutyryl-cAMP
DC	dendritic cell
DEP	dishevelled-Egl-10-Pleckstrin domain
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immunosorbent assay
Epac	Exchange Proteins directly activated by cAMP
FACS	fluorescence-activated cell sorting
FCS	Foetal Calf Serum
FITC	fluorescein isothiocyanate
FOXP3	forkhead transcription factor
FP	forward primer
G protein	guanine nucleotide-binding protein
GABA	γ-aminobutyric acid
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor

GLB	glybenclamide
GM-CSF	granulocyte-monocyte colony stimulating factor
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
HD	homology domain
HIV	human immunodeficiency virus
HK	heat-killed
HLX	H2.0-like homeobox
HRP	horseradish peroxidase
HSC	haematopoietic stem cell
HUVEC	human umbilical vein endothelial cell
i.p.	intraperitoneal
IBMX	3-isobutyl-1-methylxanthine
IFN	interferon
Ig	immunoglobulin
IKK	I κ -B kinase
IL	interleukin
Ion	Ionomycin
IPAF	ICE-protease activating factor
IRF	IFN regulatory factor
iTreg cell	inducible regulatory T cell
I κ B	inhibitor of NF- κ B
LAD	leukocyte adhesion deficiency disease
LB	Luria-Bertani
LFA	lymphocyte function-associated antigen
LGP2	laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LT	heat-labile enterotoxin
LT α	lymphotoxin α
Mal	MyD88 adapter-like
MDA5	melanoma differentiation-associated antigen 5
Me	methyl (-CH ₃)
MHC	major histocompatibility complex
MIIC	MHC class II compartment
MyD88	myeloid-differentiation factor 88
N ⁶ -Bz	N ⁶ -Benzoyladeniosine-3', 5'-cyclic monophosphate/N ⁶ -Benzoyl-cAMP
NAIP	neuronal apoptosis inhibitor factor
NALP	NACHT-, LRR- and pyrin-domain containing protein
NF- κ B	nuclear factor- κ B
NFAT	nuclear factor of activated T cells
NK cell	natural killer cell
NLR	NOD-like receptor
NLS	nuclear localisation sequence
NOD	nucleotide-binding oligomerisation domain-1
NSAID	non-steroidal anti-inflammatory drug
nTreg	naturally occurring regulatory T cell
OH	hydroxyl
OPD	ortho-phenylenediamine
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern

PBS	phosphate-buffered saline
pCPT	parachlorophenylthio
PDE	phosphodiesterase
PE	phycoerythrin
PGE	prostaglandin
PGE ₂	prostaglandin E ₂
PKA	cAMP-dependent protein kinase A
PKA-C	PKA catalytic subunit
PKA-R	PKA regulatory subunit
PMA	phorbol mysteric acid
PRR	pattern recognition receptor
RAG	recombination activating gene
REM	Ras-exchange motif
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I-like receptor
ROR	retinoid-related orphan receptor
ROS	reactive oxygen species
RP	reverse primer
RPMI	Roswell Park Memorial Institute
RR	regulatory region
RT-PCR	real-time polymerase chain reaction
RUNX3	runt-related transcription factor 3
SARM	sterile α and armadillo motifs
SHM	somatic hypermutation
SLE	systemic lupus erythematosus
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TGF- β	Transforming growth factor β
Th	T helper
TIR	Toll/IL-1R
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TM	transmebrane
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
TRAM	TRIF-related adaptor molecule
Treg	regulatory T cell
TRIF	Toll-receptor-associated activator of interferon
TB	Tuberculosis
UV	Ultraviolet
WGA	wheat germ agglutinin

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Chapter 1

Introduction

1.1 Vaccines and adjuvants

1.1.1 Vaccines

Ideally vaccines are formulations that induce specific, non-toxic, long lasting immune responses to prevent or treat disease [1]. Since the introduction of vaccination in the 19th century, human morbidity and mortality as a result of infections has markedly been reduced [2]. Other contributory factors include improved sanitation and provision of clean water and food to the population. The primary aim of vaccination is to induce a protective immune response against the associated pathogen.

The effort to protect against infections is not a recent development. Several cultures have been shown to have developed strategies to protect themselves against infection and death. In China in the eleventh century, a technique termed variolation was reported. This involved people snorting the ground up scabs of infected individuals to protect against smallpox. Exposure of these individuals to small doses of the pathogen, by this method, lead to protection against subsequent infection [3]. However this protection was not always guaranteed and a significant percentage of people developed smallpox as a result of the procedure.

The concept of vaccination, as currently understood, is attributed to Edward Jenner's work in 1796. He observed that milkmaids were protected against smallpox and deduced that infection with cowpox, a related virus that causes a mild illness in humans, could prevent smallpox. He tested this by inoculating a small boy, James Phipps with cowpox, followed some months later by exposure to smallpox. The boy was protected against smallpox and this led to the widespread application of vaccination to induce protection against infectious diseases [4]. Robert Koch and Louis Pasteur are considered to be the co-founders of modern bacteriology. Koch demonstrated that microbes cause disease [5] and his postulates establish the relationship between the causative agent and the disease. Koch's work inspired the next major advance in vaccination by Louis Pasteur. Pasteur demonstrated that an attenuated microbe could induce long-lived protection against infection by the nonattenuated form of that organism. He showed that pathogens could be attenuated by exposure to environmental insults such as high temperature, oxygen and chemicals

[3]. He created the first attenuated vaccine against chicken cholera in 1879, and his most famous vaccine against human rabies was developed in 1885 [6].

Vaccination of a large proportion of the population can lead to protection of the entire population, due to the “herd effect”. Herd immunity occurs when the vaccination of a portion of the population also provides protection to unvaccinated individuals (Figure 1.1) [7]. The herd immunity theory proposes that it is more difficult to maintain a chain of infection when large numbers of a population are immune. The higher the proportion of individuals who are immune, the lower the likelihood that a susceptible person will come into contact with an infected individual. Current vaccine strategies in the Western World begin at infancy and have massively reduced morbidity and mortality resulting from infectious diseases. The most successful vaccination strategy to date was co-ordinated by the World Health Organisation, and led to the eradication of smallpox, the last natural case of which was reported in Somalia in 1977. This was a major success story, culminating in the elimination of a major human pathogen. Currently efforts are being made to eradicate polio and measles, which were formerly among the foremost global infectious diseases.

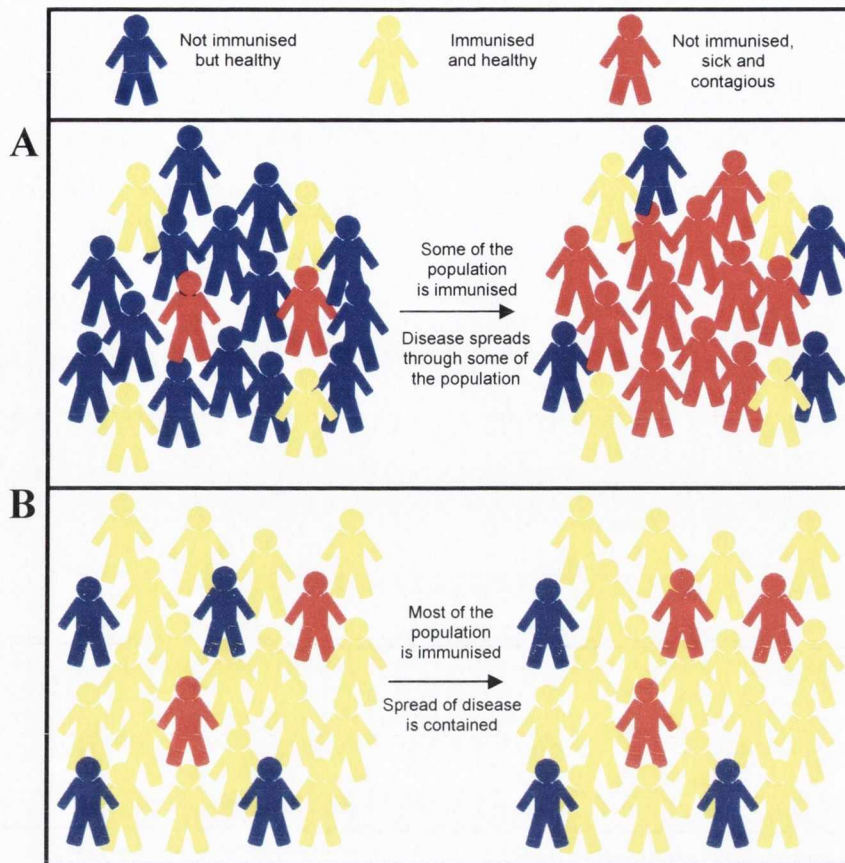


Figure 1.1: Herd immunity provides protection to unvaccinated individuals in the population. Shown in box A is a community where a portion of the population is immunised but this is not enough to confer herd immunity to the individuals and an outbreak occurs. In box B, immunisation of a certain portion of the population gives protection to most of the community. Even those who are not immunised are protected because herd immunity has been achieved and the spread of disease is contained [7].

1.1.2 Adjuvants

The first vaccines developed included attenuated whole organisms, which were very effective at inducing immunity. However, if the organism was not fully and correctly attenuated, this could lead to infection of the individual with the live pathogen, and severe side effects including death. Due to these safety concerns and advances in molecular biology, many newer vaccines contain subunits, or parts of the pathogens as opposed to the whole pathogen. These subunit vaccines based on peptides, proteins and DNA are generally poorly immunogenic and need to be administered with an adjuvant to stimulate protective immunity [8]. However, the development of efficient and safe vaccines for many diseases has been held back by the lack of appropriate adjuvants.

Adjuvants are substances used in combination with a specific antigen that produces an increased immune response in comparison to the antigen alone. Adjuvant research began with Gaston Ramon at the Pasteur Institute in the 1920s, where he showed that several substances, when co-injected, were capable of enhancing antibody responses to a bacterial toxoid [9, 10]. These substances included tapioca, inorganic salts, oil and pyogenic bacteria. Subsequently, A.T. Glennly showed that injection of diphtheria toxoid as an alum precipitate induced higher antibody responses than injection of the toxoid alone [11, 12]. This key observation about the adjuvant properties of aluminium compounds led to the use of aluminium mineral salts in adjuvant compositions [2]. Alum is the most widely used vaccine adjuvant and is very effective at promoting antibody responses and Th2 type T cell responses. This an effective approach for vaccinating against extracellular pathogens. However, a number of globally significant infections including tuberculosis (TB) and malaria require strong cell mediated immunity for protection. Adjuvants that can effectively drive such responses and not only antibody responses are required [13]. The aim of this project is to investigate the possibility of using an Exchange protein directly activated by cAMP (Epac) activator as an adjuvant to drive Th1 immune responses.

Alum has until recently been the only adjuvant approved for use in humans. After aluminium-based adjuvants, emulsions are among the most frequently used adjuvants in humans and animals. MF59 is an oil-in-water (O/W) emulsion [14], and is approved for use as an adjuvant in Europe. Alum is currently the only approved adjuvant in America, although this will probably change soon. Originally the effectiveness of adjuvants including alum, emulsions, liposomes and microparticles were attributed to a 'depot effect.' This is where the adjuvant forms a depot at the vaccination site, from which antigen can be released and the vaccine is presented continuously to the immune system. However, it is now known that an innate immune response is absolutely required to drive the adaptive immune response involving T helper cells and antibody responses [2].

1.2 The Innate Immune System

The Immune System can be subdivided into two components; the innate and the adaptive systems, with much crossover between the two. The innate immune response has traditionally been described as a rapid response while the adaptive response is slower, but provides immunological memory.

The innate immune response is the first line of defence against invading microbes. It recognises molecules associated with microbes, and this recognition triggers an inflammatory response in which certain cells of the immune system attempt to wall off the invader and halt its spread. The activity of these cells and of the chemicals they secrete leads to the redness and swelling at sites of injury and accounts for the fever, body aches and other flulike symptoms that accompany many infections.

Until recently, immunologists focused primarily on the adaptive side of the immune system. The innate system was considered a simple and basic process, while the adaptive system was considered more important as it includes a memory component. The innate system includes components such as antibacterial enzymes found in secretions such as saliva or on the skin and an interlocking set of proteins (collectively called complement) that kill bacteria. Antimicrobial peptides (AMPs) are polypeptides of fewer than 100 amino acids that have antimicrobial activity at physiological concentrations [15]. These AMPs characteristically have activity against a broad spectrum of bacteria and other microbes. Lysozyme is an AMP, which was originally described as having ‘bacteriolytic activity’ in nasal secretions by Alexander Fleming [16]. It is found in a number of secretions, such as tears, saliva, human milk and mucus. AMPs also play an important role in alerting sentinel cells of the innate immune system, e.g. dendritic cells (DCs), macrophages, monocytes and granulocytes. The production of AMPs can lead to increased chemoattraction of these cells to the site of infection; thereby creating a feed-forward loop that contributes to prevention and early clearance of infection. Release of inducible AMPs is important in the early stage of an infection as they kill invading pathogens and prevent the establishment of a focus of infection [17]. The AMPs can be divided into two subgroups; inducible AMPs and constitutive AMPs. Inducible AMPs are secreted in response to tissue injury or microbial infection by cells of the innate immune system

e.g. neutrophils, eosinophils, basophils, natural killer cells (NKs), monocytes or macrophages [17]. The constitutive AMPs are secreted at luminal linings of certain organs, and their main function is to prevent infection and to induce inflammation in cases of infection.

The complement system is also a major component of the innate system. It was first identified as a heat-sensitive factor in fresh serum that ‘complemented’ the effects of specific antibody in the lysis of bacteria and red blood cells [18]. It is now known that this system is a complex pathway of more than 30 serum proteins and cell surface receptors that interact and contribute to a range of functions from direct cell lysis to the enhancement of B and T cell responses [19, 20]. Three different pathways activate complement: classical, lectin and alternative. All three share the common step of activating the central component C3, but they differ according to the nature of recognition. The complement proteins are predominantly synthesised in the liver and enter the circulation as inactive zymogens (an enzyme precursor).

The innate immune system contains various cell types responsible for mediating different effector functions. The cell types involved in the innate and adaptive immune responses are shown in Figure 1.2. All white blood cells are known as leukocytes, which are able to move freely and interact with and capture cellular debris, foreign particles, or invading microorganisms. Most innate immune leukocytes are produced by pluripotent hematopoietic stem cells present in the bone marrow [21]. The innate leukocytes include NK cells, mast cells, eosinophils, basophils; and the phagocytic cells including macrophages, neutrophils and DCs, and function within the immune system by identifying and eliminating pathogens. Inflammation is one of the first responses of the immune system to infection or irritation. Inflammation is stimulated by chemical factors released by injured cells and serves to establish a physical barrier against the spread of infection, and to promote healing of any damaged tissue following the clearance of pathogens [22]. Chemical factors produced during inflammation (histamine, bradykinin, serotonin, leukotrienes and prostaglandins) sensitise pain receptors, cause vasodilation of the blood vessels at the scene, and attract phagocytes, especially neutrophils [22, 23]. Neutrophils release factors that summon other leukocytes e.g. cytokines. Cytokines are small, secreted soluble proteins, which mediate and regulate immune responses [24]. They are newly

produced in response to an immune stimulus and are used extensively in cellular communication.

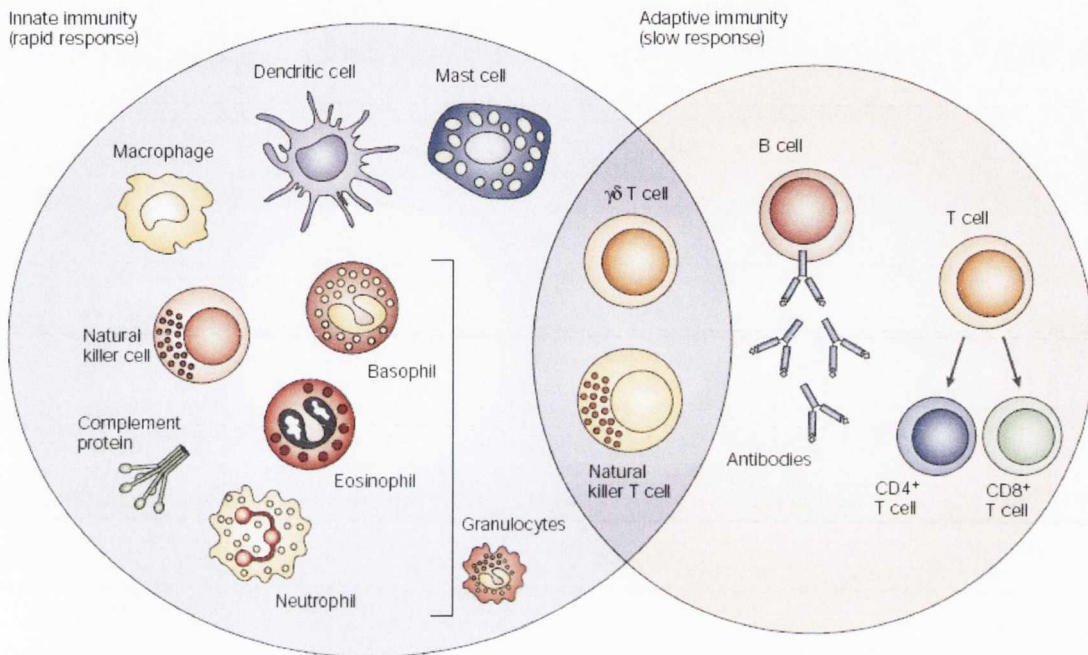


Figure 1.2: The innate and the adaptive immune responses. The innate immune response functions as the first line of defence against infection. It is composed of soluble factors and several different cell types. The soluble factors include complement proteins while the cells involved in the innate response include granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, DCs and NK cells. The adaptive response is slower to come into effect, but exhibits antigenic specificity and memory. It consists of antibodies, B cells and CD4⁺ and CD8⁺ T lymphocytes. Some cells are at the border between innate and adaptive immunity e.g. the cytotoxic lymphocytes: NK T cells and γδT cells. Figure from [25].

The principal functions of the innate immune system include:

- The recruitment of immune cells to sites of infection, through the production of chemical factors, including specialised chemical mediators, called cytokines.
- Activation of the complement cascade to identify bacteria, activate cells and to promote clearance of dead cells or antibody complexes.
- Identification and removal of foreign substances present in organs, tissues, the blood and lymph, by specialized white blood cells.
- Activation of the adaptive immune system through a process known as antigen presentation.

1.2.1 The innate response and pattern recognition receptors

A key function of the innate immune system is to recognise invading microbes and mount a quick and effective initial response. Detection of pathogens is mediated via a variety of receptors, referred to as pattern recognition receptors (PRRs) that recognise molecular patterns on pathogens called pathogen-associated molecular patterns (PAMPs). Several families of pathogen sensors have been identified; Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs) [26, 27]. In addition to the ability of TLR9 to sense DNA, there have been at least six intracellular DNA sensors reported, including: DNA-dependent activator of interferon (IFN)-regulatory factors (DAI), absent in melanoma 2 (AIM2), RNA polymerase III (Pol III, leucine-rich repeat (in Flightless I) interacting protein-1 (Lrrfip1), DExD/H box helicases (DHX9 and DHX36), and the IFN-inducible protein IFI16 [28]. All of the innate receptors, TLRs, NLRs, RLRs and CLRs, respond to microbial products. The interplay between these families ensures the efficient co-ordination of innate immune responses through either synergistic or co-operative signalling. Various levels of crosstalk between the TLR and NLR pathways have been described, most notably the description of a molecular scaffold complex, termed the inflammasome, which requires input from both pathways and leads to the activation of the proinflammatory cytokines interleukin (IL)-1 β and IL-18 [29].

1.2.2 TLRs

The TLRs are a highly conserved family of proteins that are one of the earliest surveillance mechanisms responding to infection. TLR signalling activates both the innate and adaptive systems. TLRs are transmembrane (TM) receptors that recognise PAMPs on invading microbes, which triggers a series of events that leads to the expression of many immune and inflammatory genes. The microbial structures recognised by TLRs are molecules important to the survival of the pathogen. Eliminating or chemically altering any one of these elements could cripple a pathogen, which means that the organisms cannot evade TLRs by mutating until these components are unrecognisable [30].

Upon TLR activation i.e. when the TLRs sense a pathogen, production of an array of signalling proteins (e.g. cytokines) that induce inflammation and direct the body to mount a full-fledged immune response is triggered. These protein messengers then recruit additional macrophages, DCs and other immune cells to wall off and non-specifically attack the microbes. Macrophages and DCs that have engulfed a pathogen display pathogenic peptides on their surface along with other molecules indicating that a disease-causing agent is present. This display combined with the cytokines released in response to TLR engagement, ultimately activates B and T cells that recognise those specific antigenic components, causing them to proliferate and launch a powerful and highly specific assault on the pathogen. TLRs play a central role in linking innate and adaptive immunity through actions on T cells and particularly via actions on DCs [31]. Upon signal transduction through TLRs, DCs undergo a complex differentiation programme called DC maturation, which is essential for the induction of pathogen-specific adaptive immune responses.

There are 10 TLRs (TLR1-10) in humans and 12 TLRs (TLR1-9 and TLR11-13) in mice [32, 33]. TLRs are expressed on a range of immune cells including macrophages, DCs, NK cells, B cells and certain types of T cells. TLRs are also expressed on certain non-immune cells such as epithelial cells, which lie at potential sites of entry, including the skin, respiratory, intestinal and genitourinary tracts, endothelial cells and smooth muscle cells. TLRs 3, 7, 8 and 9 are expressed intracellularly while TLRs 1, 2, 4, 5, 6 and 10 are expressed on the cell surface [29]. A diverse range of endogenous (host-derived) and exogenous (pathogen-derived)

TLR ligands has now been identified. TLRs occur as homodimers and heterodimers [34] and recognise a diverse range of PAMPs (Figure 1.3).

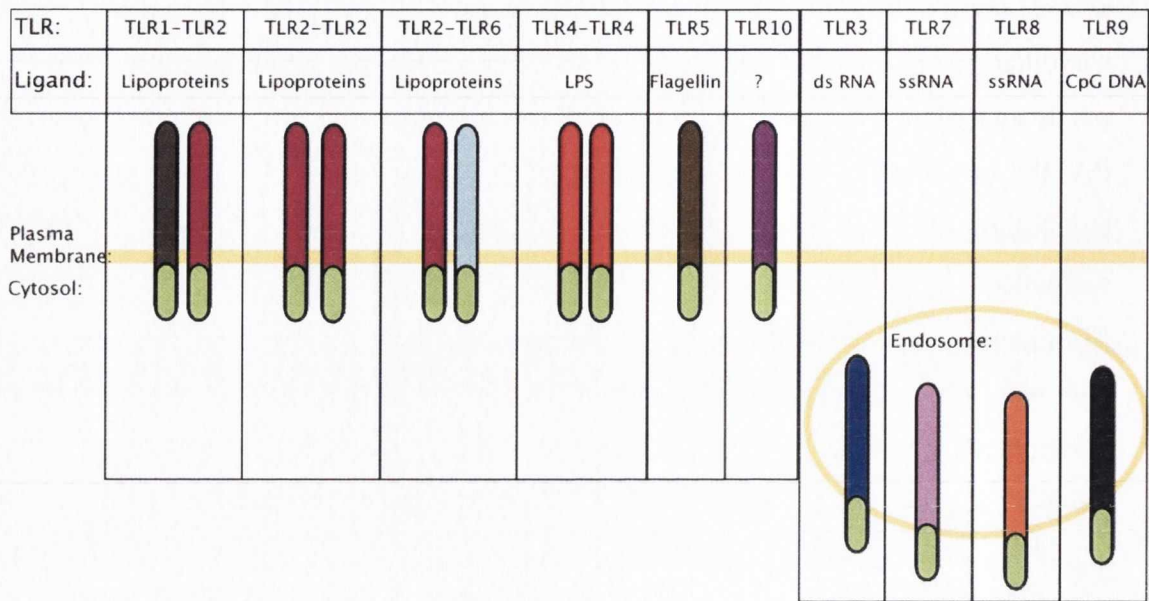


Figure 1.3: The human TLRs and their ligands. Shown here are the TLR proteins expressed in humans and their ligands. TLR2 forms a homodimer with itself and heterodimers with TLR1/TLR6 and functions to detect bacterial lipoproteins. TLR4 forms a homodimer and detects LPS; TLR5 detects Flagellin while the ligand for TLR10 is unknown. TLR3, 7, 8 and 9 are located on endosome membranes in the cytosol. TLR3 detects dsRNA, TLR7 and 8 detect ssRNA, while TLR9 detects CpG methylated DNA motifs.

Lipopolysaccharide (LPS) is a cell-wall component of gram-negative bacteria, and consists of a lipid and a polysaccharide joined by a covalent bond. It acts as an endotoxin and stimulates a powerful immune response through activation of TLR4. TLR4 acts as a homodimer, recognising a range of agonists. TLR4 is crucial for effective responses to LPS [35-37]. In humans, exposure to LPS causes fever and can lead to septic shock – a deadly vascular shutdown triggered by overwhelming destructive actions of immune cells. LPS induces this inflammatory response in part by prompting macrophages and DCs to release the cytokines TNF- α and IL-1. In sepsis a so-called “cytokine storm” is observed where there is high levels of proinflammatory cytokines and chemokines in the blood. However, there is some evidence in human septic shock that there is some immunosuppression, which indicates a difference to the mouse sepsis response [38]. Mutations of the *Lps* gene selectively hampers LPS signal transduction in C3H/HeJ mice, rendering them resistant to endotoxin i.e. these mutant mice are unable to respond to LPS as they

harbour a defective version of TLR4 [35]. Whereas normal mice die of sepsis if injected with LPS, these mutant mice survive and behave as if they have not been exposed to the molecule at all.

The recognition of PAMPs by TLRs triggers the activation of a signalling cascade, which culminates in the induction of genes involved in the inflammatory response (Figure 1.4). The five adaptor molecules are MyD88, Mal (MyD88 adapter-like)/TIRAP (TIR-domain-containing adaptor protein), TRIF (Toll-receptor-associated activator of interferon), TRAM (TRIF-related adaptor molecule) and SARM (sterile α and armadillo motifs), and they all contribute to the specificity of responses to pathogens. These adaptors couple to downstream protein kinases that ultimately lead to the activation of transcription factors such as nuclear factor- κ B (NF- κ B) and members of the interferon (IFN)-regulatory factor (IRF) family (Figure 1.4).

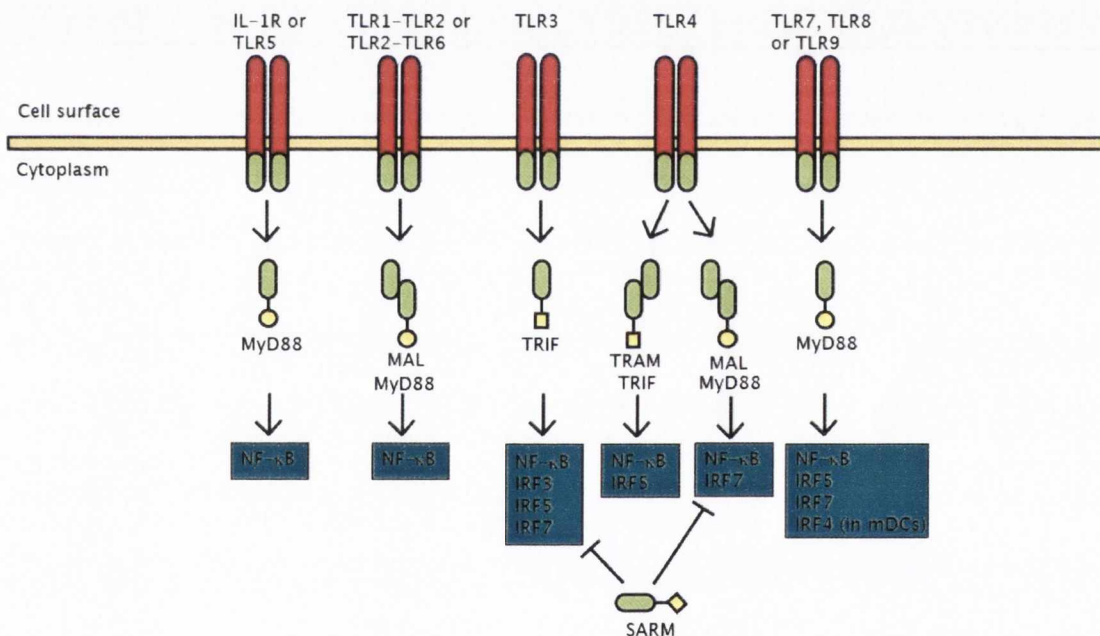


Figure 1.4: Overview of transcription-factor activation through TIR-domain-containing adaptors for the TLR/IL-1R superfamily. The receptor complexes use each adaptor differently to positively regulate transcription-factor activation. In contrast SARM inhibits TRIF-mediated transcription-factor activation. Abbreviations: (IL-1R (interleukin-1 receptor), IRF (IFN regulatory factor), mDC (myeloid DC). Figure adapted from [39].

TLRs are key inducers of the pro-forms of IL-1 β and IL-18. This is called “priming” the system for caspase-1 [40]. This priming of caspase-1 is necessary in order to allow the caspase to cleave the pro-form of the cytokine into the active cytokine. IL-1 β and

IL-18 use MyD88 to mediate their intracellular signalling effects [41]. MyD88 is therefore a central adaptor for innate immunity. MyD88 is essential for producing IL-1 β , IL-6, IL-12 and tumour necrosis factor (TNF)- α in response to LPS. MyD88 is not required for IL-18 secretion [42].

1.2.3 TLR Adaptors

TLR signalling needs to be tightly balanced and various negative regulatory mechanisms exist, ranging from extracellular decoy receptors, intracellular inhibitors, and membrane bound suppressors to degradation of TLRs and TLR-mediated apoptosis. There have been several reports on single-nucleotide polymorphisms in the adaptors that regulate their function. An example of this is the S180L variant in MAL [43-45]. Heterozygotes are protected against infectious diseases, including malaria and TB, and systemic autoimmune diseases such as systemic lupus erythematosus (SLE).

MyD88

MyD88 is the universal adaptor, used by all TLRs except TLR3, and acts to recruit the IRAK family of kinases. These ultimately trigger NF- κ B and MAPK activation. MyD88 was first shown to be involved in signalling by the type 1 IL-1 receptor (IL-1R) [46, 47] and subsequently in signalling by various TLRs [48, 49]. The crucial evidence for its involvement in TLR signalling came from MyD88 deficient mice which were found to be unresponsive to ligands for TLR2, TLR4, TLR5, TLR7 and TLR9 [50, 51]. MyD88 activation promotes NF- κ B but additional functions for MyD88 include activation of the transcription factors IRF1, IRF5 and IRF7, and also a role outside the TLRs in IFN- γ -signalling. Downstream of TLR4, there is a MyD88 dependent pathway and a MyD88-independent pathway (Figure 1.5).

MAL

MAL is recruited by TLR2 and TLR4, and its main function is to stabilise MyD88 acting as a bridge to the TLR. MAL was the second adaptor in the TIR-domain-

containing adaptor family to be discovered [52, 53]. A dominant-negative version of MAL results in the inhibition of TLR4 signalling, but not IL-1 signalling.

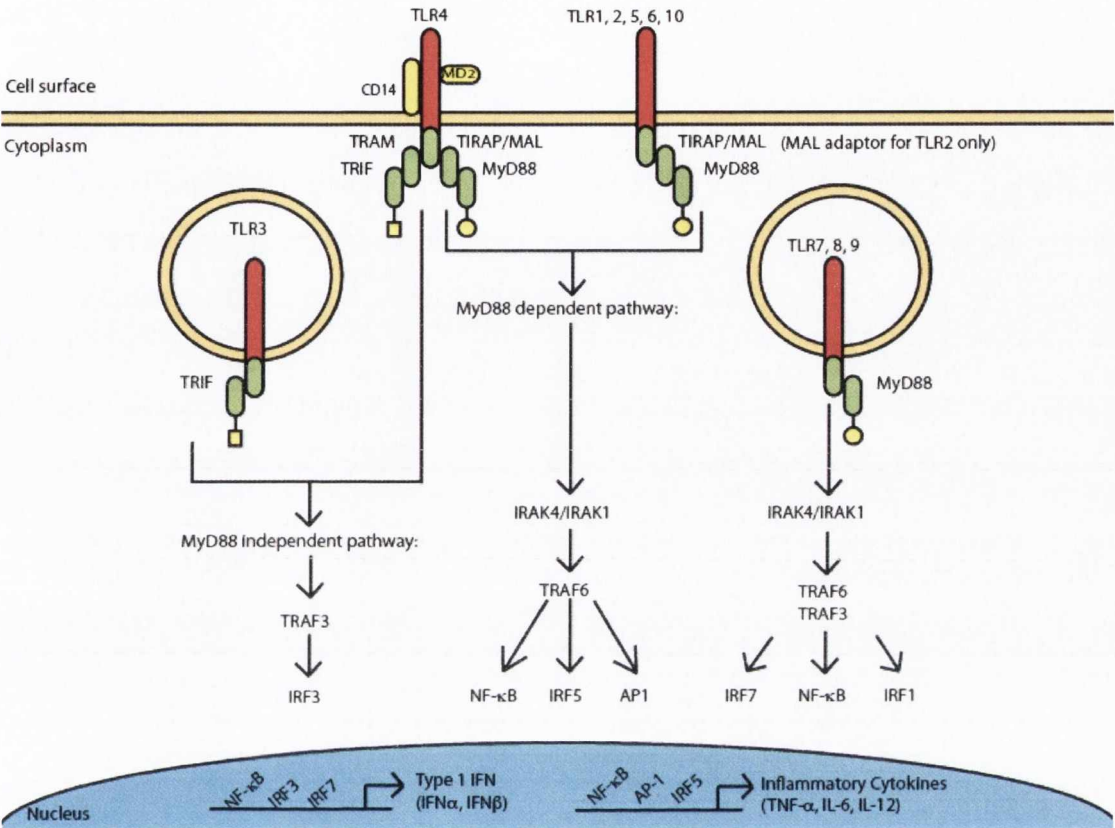


Figure 1.5: MyD88-dependent and MyD88 independent pathways downstream of TLR signalling. All the TLRs besides TLR3 signal via the MyD88 dependent pathway. This pathway is initiated by the binding of Mal to the TLR via their TIR domains after ligand binding. Mal recruits MyD88, which binds IRAK4 and IRAK1. IRAK1 is phosphorylated by itself and IRAK4 and leaves the membrane to activate TRAF6. After TRAF6 is ubiquitinated, it causes NF-κB, AP-1 and IRF-5 activation, leading to the induction of proinflammatory cytokine production. The MyD88 independent pathway (or the TRIF-dependent pathway) is used by TLR3 and TLR4 by binding TRAM at the membrane. This pathway leads to the activation of IRF3, a transcription factor that translocates to the nucleus to produce IFN-inducible genes. In plasmacytoid DCs, an alternative MyD88 pathway exists, where activation of TLR7 and TLR9 leads to the activation of TRAF6 through MyD88, IRAK1 and IRAK4. This activates IRF7, which promotes IFN-inducible genes including IFN-α.

TRIF

TRIF is used by TLR3 and also TLR4 but in the case of TLR4 another bridging adaptor called TRAM is needed. TRIF leads to IRF3 activation via recruitment of the kinase TBK-1, and this signal in the case of both TLR3 and TLR4 comes from the endosome, with TLR4 trafficking there after LPS recognition [54, 55]. MAL was initially thought to control the TLR4-mediated, MyD88-independent pathway leading to IRF3 and delayed NF- κ B activation. However, the discovery of the role of MAL as a bridging adaptor in the MyD88-dependent pathway showed that it was not involved in TLR4-induced type I IFN induction. TRIF is now known to control the TLR4-induced MyD88-independent pathway, and also is the exclusive TLR adaptor used by TLR3.

TRAM

The fourth adaptor to be identified was TRAM (also known as TICAM2) [56, 57]. TRAM functions exclusively in the TLR4 pathway and is therefore the most restricted of the adaptors in terms of TLR action. Although TRAM interacts with TRIF, its role seems to be more wide-ranging than that of TRIF for TLR4 signalling, in that the activation of signals and induction of cytokines by LPS is more impaired in TRAM-deficient cells than in TRIF-deficient cells. TRAM is essential for TLR-4 mediated TRIF-dependent pathway and appears to act as a bridging adaptor between TLR4 and TRIF [58].

SARM

The fifth adaptor SARM has been shown to inhibit TRIF-dependant TLR signalling and therefore is believed to be a negative regulator [59]. SARM is the last of the five adaptors to be assigned a role in TLR signalling and is the most evolutionarily conserved. *SARM* was initially identified in 2001 as a human gene orthologue of a *Drosophila melanogaster* protein with two sterile α -motifs (SAMs) and HEAT/armadillo repeats [60].

1.2.4 NLRs

The NLR family is a group of cytoplasmic PRRs that in humans consists of more than 23 members [61]. They recognise cytoplasmic microbial PAMPs and/or endogenous danger signals. The NLR family includes NODs (nucleotide-binding oligomerisation domain-1), CIITA (MHC Class II Transcription activator), NALPs (NACHT-, LRR- and pyrin-domain containing proteins), IPAF (ICE-protease activating factor) and NAIPs (neuronal apoptosis inhibitor factors) [61]. Ever since the discovery of NLRs in mammals, the similarity of these genes with a family of plant genes involved in immune defences has been observed [62]. The plant genes, known as R-genes (R for resistance), are crucial for the immune defence of plants against bacteria, fungi, viruses, and other pathogens. The first NLRs reported to be intracellular microbial sensors were NOD1 and NOD2 and sense iE-DAP (γ -D-Glu-mDAP) and MDP (muramyl dipeptide) respectively [63]. These are both breakdown products of peptidoglycan. Both NOD1 and NOD2, once activated, recruit and engage the kinase RIP2 through CARD-CARD interactions [64]. This then activates NF- κ B via activation of the I κ B kinase complex [65, 66]. While TLRs are associated with the plasma membrane or, in some instances with lysosomes, NLRs are localised in the cytosol.

The term inflammasome is used to describe a high molecular weight complex that activates inflammatory caspases which process the cytokine IL-1 β [67]. Caspases are proteases produced in cells as catalytically inactive zymogens and usually undergo processing during activation [68]. In mammals, the inflammatory caspases include human and murine caspase-1, human and murine caspase-12, murine caspase-11, and the two caspase-1-related human caspases, caspase-4 and caspase-5 [69, 70]. These platforms integrate cellular signals, recruit initiator caspases via their death-fold domain, and promote dimerisation of the caspases, which all lead to the formation of an active enzyme, which initiates specific signalling cascades. The inflammasomes are known to control maturation and secretion of the proinflammatory cytokines IL-1 β and IL-18 [71]. IL-1 β is involved in T cell activation, innate cell recruitment and induction of fever, while IL-18 increases NK cell cytolytic activity and IFN- γ production and influences neutrophil recruitment and activation. Currently there are

four inflammasomes defined: the NLRP1, NLRP3, IPAF and AIM2 inflammasomes [71]. There has been a lot of study into the NLRP3 inflammasome and it has been shown to be a sensor of multiple pathogens, pathogen products such as MDP, and products such as uric acid crystals and exogenous crystals such as asbestos [72-74] (Table 1.1).

NLRs, their agonists and downstream signalling pathways are shown in Table 1.1.

Receptor	Agonist	Signalling Pathway
NOD1	GM-tripeptide	NF- κ B, MAPK
	meso-lanthionine, meso-DAP, iE-DAP	
	(FK156), (FK565)	
NOD2	MDP, M-TRILys	NF- κ B, MAPK
NLRC4	Flagellin (<i>Salmonella</i> , <i>Legionella</i> , <i>Pseudomonas</i>)	Caspase-1
	Unknown (<i>Shigella</i>)	
NAIP	Unknown	Caspase-1
NLRP1b	Anthrax lethal toxin	Caspase-1
NLRP3	Bacterial RNA	Caspase-1
	Viral RNA and DNA	
	Uric acid crystals	
	LPS, MDP, ATP	
	Silica, Asbestos, Microparticles, Alum	

Table 1.1: NLR agonists and downstream signalling pathways. Table adapted from [75].

1.2.5 RLRs

Three genes encode RLRs in human and mouse genomes; retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated antigen 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [76]. Signalling through RLRs ultimately results in IRF3 and NF- κ B activation [77]. The RLRs are widely expressed and become upregulated following IFN- α/β activation. The best-known RLR is RIG-I, which senses double stranded RNA. In uninfected cells there is no dsRNA, but in virally infected cells non-self RNAs, such as dsRNA and 5'-triphosphate RNA, accumulate in the cytoplasm where they are detected by RIG-I. RIG-I binds specifically to RNA containing 5'-triphosphate [78]. In the host, RNA synthesis takes place in the nucleus and the host's RNA contains 5'-triphosphate, which is modified to be either deleted or masked through post-transcriptional RNA modification (e.g. capping of the 5'-

triphosphate end or nucleoside modification of RNA) when transported to the cytoplasm. The viral RNA binds to RIG-I via its RNA binding domain.

1.2.6 CLRs

CLRs are an important family of PRRs that are involved in the induction of specific gene expression by specific pathogens, either by modulating TLR signalling or by directly inducing gene expression [79, 80]. The term C-type lectin was introduced to distinguish between Ca^{2+} -dependent and Ca^{2+} -independent carbohydrate-binding lectins. CLRs contain at least one carbohydrate recognition domain, which is a compact structural module that determines the carbohydrate specificity of the CLR [81]. The CLR family also includes proteins that have one or more domains that are homologous to carbohydrate recognition domains but do not always bind carbohydrate structures [81]. CLRs exist both as soluble and transmembrane proteins, and are divided into two groups; group I CLRs belong to the mannose receptor family and group II CLRs belong to the asialoglycoprotein receptor family, which includes the DC-associated C-type lectin 1 (Dectin-1) and DC immunoreceptor (DCIR) subfamilies [82]. Dectin-1 senses β -glucan found in fungal cell walls [83].

1.3 Dendritic cells

The innate immune system plays a vital role in providing signals required to induce an effective adaptive immune response. The nature of the adaptive response induced mainly depends on the initial ‘danger signals’ [84] seen by the innate immune cells following infection (and vaccination). Antigen-presenting cells (APCs) are essential for initiating immune responses by presenting antigen to T cells. DCs are the key sentinel APCs for priming naïve T cells [85], and are a fundamental link between the innate and adaptive immune systems.

Several cell types are capable of presenting antigen including DCs, macrophages and B cells. DCs are regarded as the most efficient stimulators of primary immune responses and the subsequent establishment of immunologic memory. DCs have long been considered an immune cell type that is specialised for the presentation of antigen

to naïve T cells. There have been many studies carried out to determine their lineages, pathways of differentiation, and effectiveness in antigen presentation compared to that of macrophages. In the mouse the expression of the integrin CD11c on the cell surface has become a definition of DC that distinguishes them from macrophages. CD11c is known also as integrin αX , and with integrin $\beta 2$ (a.k.a. CD18), forms the complement receptor 4. While CD11c is widely employed as a marker of murine DC activated cytotoxic T-lymphocytes (CTL), NK cells and marginal zone macrophages also express low levels of CD11c [86]. A key discovery with respect to DCs was the observation that GM-CSF (alone or in combination with IL-4) can promote the expansion/differentiation of bone marrow and blood monocytes into cells with APC potential (i.e. DCs) that can be matured further with stimuli such as microbial products [87]. This finding has led to numerous studies in which GM-CSF-stimulated cells are equated with DCs and are contrasted to CSF-1-stimulated cells (macrophages) in terms of their ability to present antigen.

DCs are professional APCs that are found in the T cell areas of lymphoid tissues and as minor cellular components in most tissues. They are localised at strategic places in the body at sites of pathogen entry, and are thereby in an optimal position to capture antigens. They continuously sample the antigenic environment and are sensors of microbial invasion or tissue damage. In general, vaccination strategies try to mimic the key functions of pathogens and DCs are considered to play a central role in the mediation of adaptive immune responses by vaccination. DCs have a remarkable ability to take up antigens and stimulate major histocompatibility complex (MHC)-restricted specific immune responses.

DCs have a branched or dendritic shape [88], process and present antigens to naïve T lymphocytes, and regulate the nature of the T cell response obtained. The interaction of DCs with naïve T cells can lead to different types of effector responses, or to T cell tolerance, depending on the type of DC and its activation state. The different signals required to initiate a potent immune response are described in Figure 1.8. Initially, T cells interact transiently with DCs, scanning the surface for peptide-MHC complexes displayed on the cell surface of the APC. Upon TCR engagement by peptide-MHC complexes the T cell is activated, if there is adequate costimulation provided by

costimulatory molecules, e.g. CD80 and CD86, on the DC cell surface, which interact with CD28 expressed on T lymphocytes (Figure 1.6). CD28 synergizes with TCR signalling to induce effector functions and cytokine secretion in different T cell subsets [89].

The traditional view of DCs is that they pick up antigens in the periphery, and then migrate to the T cell areas of lymphoid organs to initiate immunity. In the steady state, most DCs in lymphoid organs arise from haematopoietic stem cells (HSCs) located in the bone marrow [90]. The earliest self-renewing HSCs in the adult bone marrow differentiate into a series of downstream precursor cells that circulate in the blood and lymph.

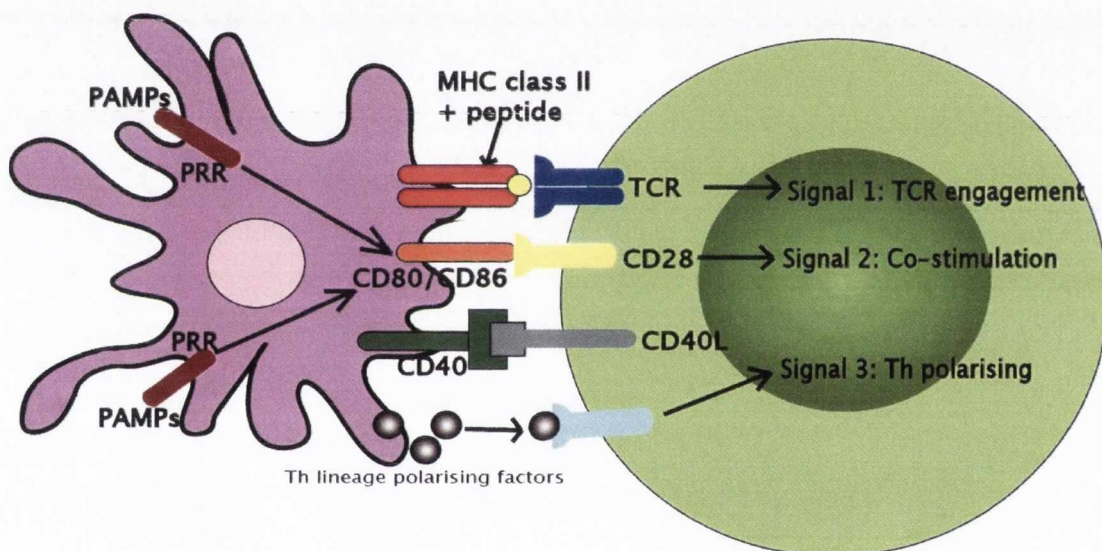


Figure 1.6: The interaction between a DC and a naïve $CD4^+$ T cell. Shown here is a simplified diagram showing DC–T cell interaction. The key interaction is the recognition of antigenic peptide–major histocompatibility complex (MHC) II dimers by T cells bearing T cell receptors (TCRs) with high affinity for the complex. In addition to the engagement of TCR with MHC–peptide complex (signal one), further signals are needed for initiation and amplification of specific T cell responses (signal two). CD80 and CD86 expressed on the DC are the ligands for CD28 on T cells. This ligation is essential to prevent tolerance. CD40L expressed on $CD4^+$ T cells enhances the activation of the DC via CD40L–CD40 interaction. Ligation of adhesion molecules stabilises the immunological synapse and plays a critical role in the signalling required to initiate a primary immunological response. The expression of co-stimulatory molecules and cytokines by APCs is tightly regulated and induced only when the APC encounters antigens associated with pathogen-associated molecular patterns. The DC secretes cytokines that polarise the T cell towards a specific Th cell lineage (signal 3).

1.3.1 DC Activation/Maturation

During their conversion from immature to mature cells, DCs undergo a number of phenotypical and functional changes. Efficient antigen presentation requires high levels of MHC complexes and co-stimulatory molecules at the cell surface. DCs upregulate co-stimulatory molecules such as CD40, CD58, CD80, CD86 upon maturation [91]. Both surface MHC Class I and Class II are upregulated. Finally, maturation is associated with a morphologic change, where the cells lose adherence and alter their shape by cytoskeleton rearrangement [92]. Immature DCs possess numerous motile, thin cytoplasmic processes or dendrites giving a large surface area. On maturation these thin processes become larger cytoplasmic veils that are continuously extended and retracted. Prolongation of the dendrites optimises the surface area for the simultaneous interaction with multiple T cells [93]. The life of the DC is ended in the lymph nodes where it is believed that the cells die shortly after antigen presentation.

The ability of DCs to regulate the immune system is dependent on DC maturation. A variety of factors can induce maturation following antigen uptake and processing within DCs, including: whole bacteria or bacterial-derived antigens (e.g. LPS), inflammatory cytokines, ligation of select cell surface receptors (e.g. CD40) and viral products (e.g. dsRNA). Exposure to pathogen components results in fully activated DCs that promote Th cell responses. This suggests that inflammation cannot replace contact with pathogen components in DC activation [94]. Maturation is paralleled by migration of DC through the afferent lymph vessels to the T cell areas of the secondary lymphoid organs. The maturation process thus continues from the initial encounter with antigen until final T cell activation in the lymph nodes.

Autoimmunity develops when the adaptive immune system reacts against self-antigens, causing destruction or altered function of the host's own healthy tissues. DCs play a crucial role in the regulation of adaptive immunity and the maintenance of immune tolerance to self-antigens. This tolerance guards against unnecessary and inappropriate activation of the immune system. For effective antigen presentation leading to a productive immune response, DCs must undergo maturation, and go from an immature phenotype to a mature one. The initiation of immune tolerance instead of

active immunity by DCs was first noted when targeting antigen to the DC surface molecule, now called DCIR2, using the mAb 33D1, resulted in tolerance if no DC activating agents or adjuvants were also given [95]. Without this co-stimulation, there was a failure to induce immune effector cells i.e. these tolerant mice had reduced ability to secrete antibodies and type I and type II cytokine responses and the mice were unresponsive to further antigen challenge, even with adjuvants. Co-stimulation is therefore necessary to overcome the immune tolerance. To produce a vaccine giving enhanced immune responses and effective immunological memory, then the factors, which induce tolerance, must be avoided or eliminated. Co-administration of factors that activate DCs (e.g. anti-CD40 or TLR ligands) permits the induction of cellular and humoral immune responses to the antigens.

Circulating DC precursors can be recruited to sites of antigen exposure by chemokines, released by tissue damage and/or microbes. Expression of chemokine receptors allows DC migration toward gradients of chemokines. Chemokine responsiveness and chemokine receptor expression are essential parts of DC recruitment to sites of inflammation and migration to lymphoid organs [96]. Immature DCs may express certain chemokine receptors so they are recruited to areas of inflammation. Following antigen acquisition and processing, DCs migrate to T cell-rich areas within lymphoid organs via blood or lymph, simultaneously undergoing maturation and modulation of chemokine and chemokine receptor expression profiles. A change in expression levels of the chemokine receptors CCR6 and CCR7 contributes to the functional shifts observed during DC maturation [97].

DCs also express adhesion molecules which allow entrance into peripheral tissues [88]. Immature DCs capture antigens through several uptake pathways. Extracellular fluid and solutes are taken up by macropinocytosis [98]. DCs can also take up antigens by receptor-mediated endocytosis. Conjugation of mannose residues to proteins and peptides has been shown to increase their uptake by DCs [99]. The third uptake mechanism used by DCs is phagocytosis. This is the method by which particulates, e.g. latex particles, microbes and also necrotic and apoptotic cells are taken up [100].

1.3.2 Antigen presentation by DCs

DCs are capable of processing both exogenous and endogenous antigens and present peptide in the context of either MHC class I or class II (Figure 1.7). Antigenic material taken up by DCs is degraded intracellularly into peptide fragments that are loaded on MHC molecules and presented to T cells in the lymph nodes. As DCs mature, they acquire the properties necessary to form and transport peptide-loaded MHC class II complexes to the cell surface [101].

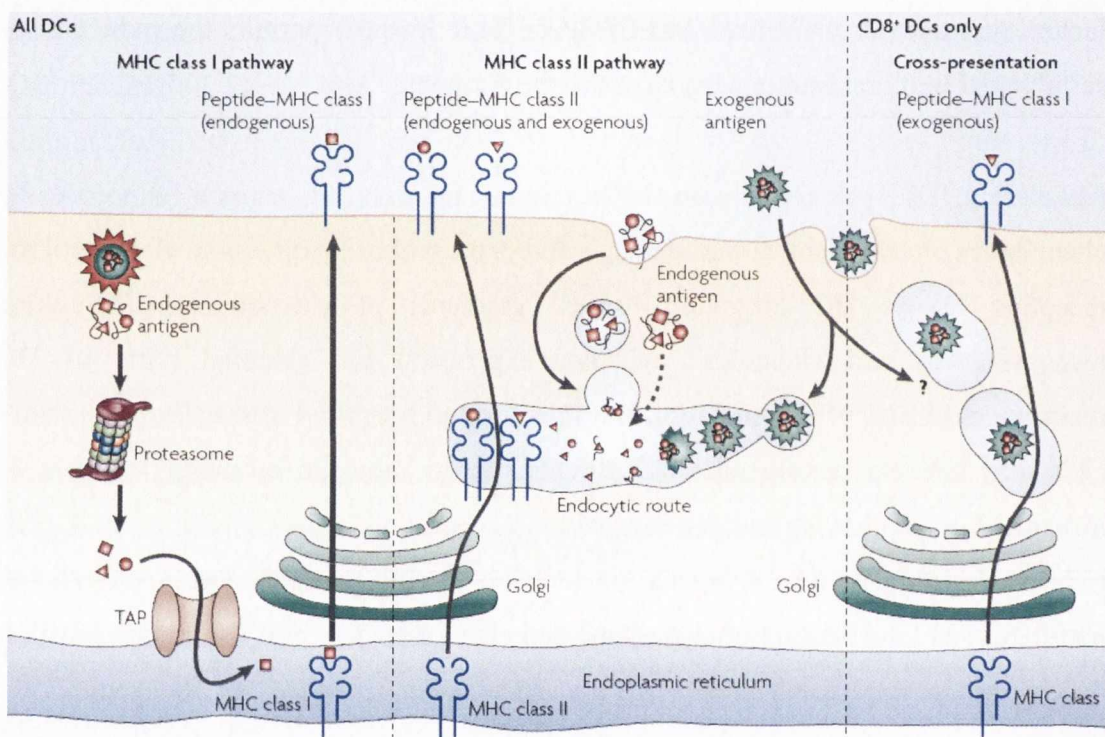


Figure 1.7: The antigen-presentation pathways in DCs. All DCs can present antigens via MHC class I and class II pathways. MHC class I molecules present peptides from cytosolic proteins, which in most DCs are usually proteins synthesised by the cell itself. MHC class II molecules obtain peptides that are generated by proteolytic degradation in endosomal compartments. These proteins are from material that was endocytosed from the extracellular environment and also some cellular components, such as plasma membrane proteins, components of the endocytic pathway and cytosolic proteins that access the endosomes by autophagy. The CD8⁺ DCs are the only known cell type capable of presenting antigens not derived from inside the cell to the MHC class I (cross-presentation) pathway. The two-pronged arrow shows that the MHC class II and the MHC class I crosspresentation pathways may 'compete' for exogenous antigens in CD8⁺ DCs, or that the way the antigen is endocytosed may determine whether it is preferentially delivered to the MHC class II pathway or the MHC class I cross-presentation pathway. From: [102]

Soluble and particulate antigens, after uptake, are directed to MHC class II compartments (MIIC) in the DC. The antigens are then degraded into peptide fragments. Antigen transport to the cell surface corresponds with increased expression of co-stimulatory molecules, such as CD80 and CD86. DCs present antigenic peptides complexed with MHC class I molecules to CD8-expressing T cells in order to generate cytotoxic cells [103]. Peptide fragments of exogenous protein acquired from outside the cell are presented by MHC class II molecules and stimulate CD4⁺ helper T cell responses. The transporter associated with protein processing (TAP-1 and -2) is a dedicated peptide transporter that helps transfer cytosolic peptides to the endoplasmic reticulum where they can then bind to MHC class I molecules. DCs consequently “cross-prime” T cells [104]. Most cells can only present MHC class I-restricted antigens using the direct presentation pathway, where proteins synthesised by the cell are degraded in the cytosol by the proteasome, bind to MHC class I molecules and are transported to the cell surface to be recognised by CD8⁺ T cells [105]. However DCs are able to cross-present antigens from other cells to CD8⁺ T cells and this is called cross-presentation. A variety of cell surface receptors expressed by immature DCs may function in antigen uptake and also present antigen via the MHC I pathway. There is some evidence for cross talk between the two MHC presentation pathways, since MHC class I also can present certain types of exogenous antigens [106].

1.3.3 T cell priming: DCs as the link between innate and adaptive immunity

T cell priming occurs in the T cell areas of the secondary lymphoid organs [88]. Cell surface receptors not only facilitate antigen uptake, but also are responsible for the clustering of DCs and T cells together in lymph nodes. DC-SIGN, a calcium-dependent, type II CLR, is a DC-specific ligand for ICAM-3 expressed on naïve T cells and can promote a transient clustering between a DC and T cell, thus allowing the DC to screen numerous T cells for an appropriately matched TCR [107]. Adhesion receptors such as lymphocyte function-associated antigen (LFA)-1, ICAM-1, LFA-3 and CD44 may also be expressed on mature DCs and promote adhesion to T cells [108].

The type of cytokines secreted by DCs varies with the different stages of DC development and maturation, thus influencing the different effector functions

characteristic of immature versus mature DCs. A wide variety of cytokines may be expressed (not necessarily at the same time) by mature DCs including IL-12, IL-1 α , IL-1 β , IL-15, IL-18, IFN- α , IFN- β , IFN- γ , IL-10, IL-6, IL-16, TNF- α , and MIF [109]. The exact cytokine repertoire expressed will depend on the nature of the stimulus, maturation stage of the DC and the existing cytokine microenvironment. The distinct cytokine patterns released by mature DCs ultimately determine their T helper cell polarising capacities [110].

1.4 The Adaptive Immune System

Adaptive immunity provides the immune system with a memory component and works in tandem with the innate response. The innate system produces cytokines or chemokines that not only induce inflammation but also recruit and activate the B and T cells that participate in the adaptive response. B cells make antibodies that specifically recognise and bind specific antigens on the surface of an invading pathogen and T cells have receptors able to recognise pathogen-derived fragments of proteins. Antigens are specific components unique to a given invader. This is called the adaptive response because over the course of an infection, it adjusts to optimally handle the particular microorganism responsible for the disease.

B and T lymphocytes are the main cellular components of the adaptive system. B cells play a major role in the humoral immune response, whereas T cells are closely involved in cell-mediated immune responses. Activated B cells secrete antibody molecules that bind to antigens and destroy the invader directly or mark it for attack [111]. T cells recognise antigens displayed on cells. Some T cells help to activate B cells and other T cells directly attack infected cells [112]. When an infection is removed these memory B and T cells persist, priming the body to ward off subsequent attacks. This ability to remember past infections allows vaccines to protect us from disease caused by pathogens. Vaccines expose the body to a disabled form of a pathogen (or harmless pieces of it) but the immune system reacts as it would to a true assault, generating protective memory cells in the process. Due to T and B cells, once the host has encountered a pathogen and survived, it becomes protected from being overtaken by the same pathogen again [113].

1.4.1 B cells

The lymphocytes in the adaptive system are known as T (*t*hymus-derived) and B (*b*ursal or *b*one marrow-derived) lymphocytes. The discovery and characterisation of B cells occurred in the mid-1960s and early 1970s. A functional division between cells in the chicken bursa of Fabricius responsible for antibody production (B cells) and cells that required an intact thymus for manifestation of delayed-type hypersensitivity (T cells) was proposed [114]. B lymphocytes (B cells) are a population of cells that express clonally diverse cell surface immunoglobulin (Ig) receptors recognizing specific antigenic epitopes. Their origin can be traced to the evolution of adaptive immunity in jawed vertebrates beginning more than 500 million years ago [115]. The development of mammalian B cells involves several stages that begin in primary lymphoid tissue (e.g. human foetal liver and foetal/adult marrow), followed by their maturation in secondary lymphoid tissue (e.g. human lymph nodes and spleen).

Antibodies are made of three structural units composed of two heavy chains and two light chains. The hypervariable region is the region responsible for the antigenic specificity of the B cell. In placental mammals there are five isotypes of antibodies: IgA, IgG, IgE, IgM and IgD which exert different biological functions. B cell development in mice [116] and humans [117] has been extensively studied, and the functional rearrangement of the Ig loci is essential. This occurs via an error-prone process involving the combinatorial rearrangement of the V, D, and J gene segments in the H chain locus and the V and J gene segments in the L chain loci [118]. In mice and humans, this occurs primarily in foetal liver and adult marrow, resulting in the development of a diverse repertoire of functional VDJH and VJL rearrangements encoding the B cell receptor (BCR). The recombination activating genes 1/2 (RAG-1/2) are responsible for the initial steps of both DNA strand breakage in both Ig and T cell receptor rearrangement [119]. A decade after the discovery of RAG1/2, it was demonstrated that class switch recombination (CSR) and somatic hypermutation (SHM) are mediated by activation-induced cytidine deaminase (AID) [120].

The clonal expansion theory is widely accepted as the way antibodies are produced from B cells [121]. This theory postulates that each lymphocyte has a single type of

receptor with a unique specificity, that the receptor must be occupied for cell activation, that the differentiated effector cells derived from an activated lymphocyte will have receptors of identical specificity to the parental cell and that the lymphocytes bearing receptors for self molecules will be deleted at an early stage [122]. In clonal selection of lymphocytes a haematopoietic stem cell undergoes differentiation and genetic rearrangement to produce immature lymphocytes with many different antigen receptors. Those that bind to self-antigens are destroyed, while the rest mature into inactive lymphocytes. Most of these will never encounter a matching foreign antigen, but those that do are activated and produce many clones of themselves.

1.4.2 T cells

The antigen-receptor complex expressed by T cells is composed of the T cell receptor (TCR) and CD3. Signalling by the TCR-CD3 complex is initiated by the recognition of peptide antigen in the context of MHC molecules on APCs. Most T lymphocytes express TCRs on the cell surface comprised of α and β chains, while a small subset express γ and δ chains (called $\gamma\delta$ T cells). These two T cell populations develop in the thymus. The $\alpha\beta$ T cells are comprised of two populations: the cytotoxic $CD8^+$ T cells responsible for killing infected cells and T helper (Th) cells expressing CD4 on the cell surface. $CD4^+$ T cells recognise their antigen in the presence of MHC class II molecules while $CD8^+$ T cells recognise their antigen in association with MHC class I.

$CD8^+$ T cells

$CD8^+$ T lymphocytes differentiate into cytotoxic T lymphocytes (CTL) capable of killing virus-infected cells [123]. $CD8^+$ T cells recognise and respond to peptides displayed on MHC class I complex. MHC class I expresses intracellular peptides. Upon activation of $CD8^+$ T cells, infected cells are eliminated by cytolysis or recruitment and activation of other immune cells. As $CD8^+$ T cells recognise intracellular peptides on MHC class I, they are important in defence against viruses and intracellular bacteria. Therefore, pathogen-specific $CD8^+$ T cell responses are important in vaccination against these types of pathogens.

CD8⁺ T cell responses can be divided into four phases. The first phase (initial activation) is where there is a stable reaction between a naïve T cell and a mature DC [124]. The mature DC must express the antigen along with appropriate costimulatory molecules. The second phase (expansion phase) is where the numbers of antigen-specific CD8⁺ T cells are increased and this lasts for 5-8 days [125]. This expansion phase is coupled with the differentiation to effector T cells that migrate around the body to protect against infection [126]. The third phase is a contraction phase, where 90-95% of effector cells are killed [125, 127]. The final phase is where the memory CD8⁺ T cell pool that survived phase three contraction, are maintained. It has been reported that the last three phases are essentially independent of antigen presentation [128, 129].

As mentioned earlier, DCs are major producers of IL-12, and this cytokine is an important mediator of Th1 polarisation in CD4⁺ T cells. It has been found that IL-12 can act with IL-18 to induce antigen-independent IFN- γ production by memory CD8⁺ T cells [130, 131]. *In vitro* and *in vivo* experiments have shown that IL-12 can act directly on CD8⁺ T cells to promote proliferation, survival, and differentiation into effector cells capable of cytotoxicity [132, 133].

CD4⁺ Th cells

CD4⁺ T cells play a central role in defence against pathogens through their actions on cells of both the innate and adaptive immune systems. Th cells play roles in promoting the production of antibodies by B cells, stimulate macrophages to develop enhanced microbe-killing activity and recruit neutrophils, eosinophils, and basophils to sites of infection and inflammation. Many of these effects are mediated by the production of cytokines and chemokines.

In 1986, two subsets of activated CD4⁺ Th cells, Th1 and Th2 cells were described [134]. These Th subsets differed from each other in their pattern of cytokine production and their functions. The original definition of Th subsets was based on stable production of cytokines by several mouse clones that were established from

immunised mice and then cloned and maintained *in vitro*. Mouse T cell clones segregated into two subsets based on the mutually exclusive production of IFN- γ for the Th1 subset or IL-4, IL-5 and IL-13 for the Th2 subset [134]. Since the original description of the Th1 and Th2 cell subsets, new subsets have been described but the Th1/Th2 designation has proven very useful for immunologists. At least four main CD4⁺ T cell subsets are determined, including Th1, Th2, Th17 and regulatory T (Treg) cells. Other subsets are proposed including follicular helper T (Tfh) cells, which are important providers of help to B cells, but the exact signals leading to their induction are not fully defined [135]. Each subset has unique cytokine production and related functions, distinct expression of cell surface receptors and characteristic transcription factors, shown in Table 1.2. T cell subsets are characterised by networks of lineage-specifying transcription factors, which bind to regulatory elements in genes that encode cytokines and other transcription factors.

Lineage	Th1	Th2	Th17	Treg
Polarising Cytokines	IL-12	IL-4	TGF- β , IL-6 IL-1, IL-23	TGF- β , IL-10
Cytokines produced	IFN- γ	IL-4, IL-5, IL-9, IL-10	IL-17, IL-21, IL-22	TGF- β , IL-10, IL-35
Transcription Factor	T-bet	GATA3	ROR γ t	FOXP3
Key signalling molecules	STAT4 STAT1	STAT6 STAT5	STAT3	STAT5
Negatively regulated by	Th2, Treg	Th1, Treg	Th1/2, Treg	
Function/Targets	IC pathogens	EC pathogens	EC pathogens	Regulation
References	[136]	[137]	[138]	[139]

Table 1.2: The CD4⁺ Th cell, lineage-defining polarising cytokines, cytokine production profiles and signalling cascade components and regulators.

Upon interaction with antigen presented by APCs such as DCs, CD4⁺ T cells can differentiate into a variety of effector subsets, as described in Table 1.2. The differentiation decision is governed predominantly by the cytokines in the microenvironment and, to some extent, by the strength of the interaction of the TCR with the antigen [140]. Th1, Th2 and Th17 cells are primarily characterised by the secretion of ‘signature’ cytokines, with Th1 cells producing IFN- γ and Th2 cells producing cytokines encoded by the ‘Th2 cytokine locus’: IL-4, IL-5 and IL-13 [141]. Th17 cells, a more recent addition to the helper T cell subsets, are characterised by the secretion of IL-17A and IL-17F, but also produce other cytokines such as IL-22 and

IL-21 [142]. The $CD4^+$ T lymphocyte subsets have different functions essential for an efficient response against different types of pathogens. Th1 cells promote cell-mediated immunity and clear intracellular pathogens, whereas Th2 responses are essential to fight extracellular organisms, including helminths [143]. Th17 cells contribute to host defence against extracellular bacteria and fungi, particularly at mucosal sites [144].

1.4.3 Differentiation of the Th cell lineages

The Th cell differentiation process is mostly driven by the combination of TCR signalling from antigen binding and by cytokines produced by the innate immune system. Naïve $CD4^+$ T cells respond to signals through the TCR and co-stimulatory molecules by activating NFAT and other transcription factors to induce production of IL-2. This leads to IL-2-induced activation of signal transducer and activator of transcription 5 (STAT5) and entry into the cell cycle. After this, the lineage that the naïve $CD4^+$ cell differentiates into is determined mostly by the cytokine environment the cell is in and the network of transcription factors that are induced downstream of cytokine signalling pathways as shown in Figure 1.8.

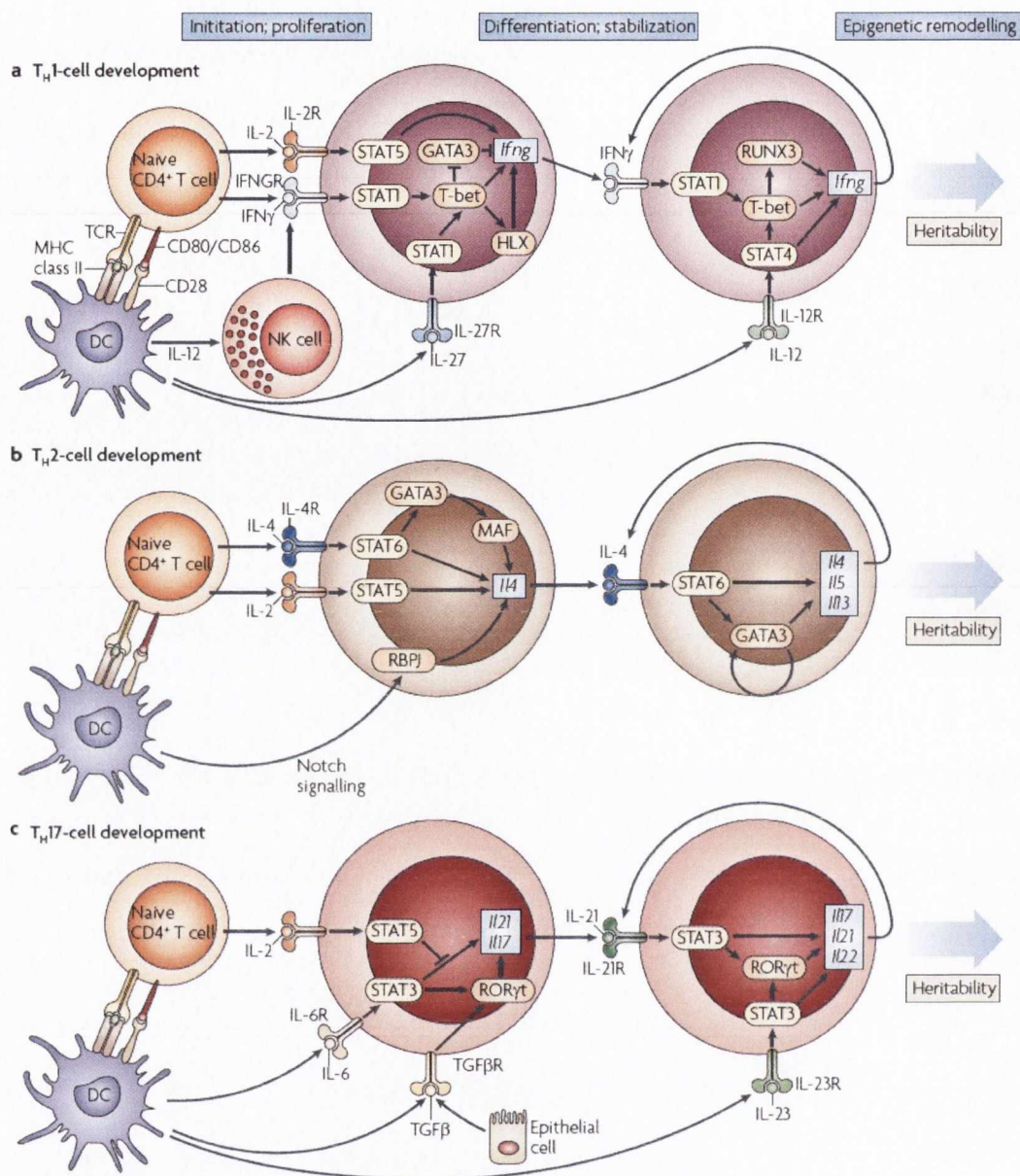


Figure 1.8: T helper cell differentiation is regulated by cytokines and transcription factors. Upon activation of CD4⁺ T cells, they can differentiate into one of several Th cell lineages. (A) IFN- γ and/or IL-27 upregulate T-bet and activate STAT1 leading to Th1 cell differentiation. T-bet induces HLX expression, and these join with other transcription factors activated upon TCR signalling, to activate transcription of *Ifn- γ* and to inhibit GATA3. This induces IL-12R expression, which binds IL-12 secreted by APCs (e.g. DCs), and thereby activates STAT4. RUNX3 is also induced by T-bet, and both RUNX3 and STAT4 drive Th1 cell differentiation. (B) Induction of GATA3 is needed for Th2 cell differentiation. STAT6 and IL-4 induce GATA3 activation, which is activated by STAT5 and STAT6 and/or RBPJ. This creates a positive-feedback loop that drives Th2 cell differentiation and the expression of IL-4, IL-5 and IL-13. (C) Activation of STAT3 induces Th17 cell differentiation, which induces IL-21 expression. This works with TGF- β signalling to induce expression of ROR- γ t, IL-17 and IL-23R, while STAT3 activation is inhibited by IL-2-induced STAT5. IL-21 and IL-23 promote the production of IL-17 and IL-22 and Th17 cell differentiation. Figure from: [143]

1.4.4 Th1 cells

Th1 cells mediate immune responses against intracellular pathogens. In humans, the cells play a particularly important role in resistance to mycobacterial infections. Th1 cells are characterized by their production of high levels of IFN- γ and low levels of IL-4 in response to antigenic stimulation. IL-12 produced by innate immune cells as well as IFN- γ produced by both NK cells and T cells promote toward Th1 cell differentiation through the action of several transcription factors (Figure 1.9).

In the initiation of Th1 responses, APCs, stimulate naïve CD4⁺ T cells. This is directed by IL-12, a heterodimeric cytokine predominantly secreted by DCs [136]. APCs that produce large amounts of IL-12 as a result of their activation promote Th1 cell differentiation by acting on both NK cells and T cells [145]. IL-12 is composed of the IL-12p40 and IL-12p35 subunits (Figure 1.10). IL-12 binds to a receptor composed of two subunits, IL-12R β 1 and IL-12 β 2 [146] and IL-12 signals are relayed to the nucleus to regulate target genes by activation of STAT4 [147]. The STAT proteins are cytoplasmic transcription factors that require phosphorylation for nuclear retention, and have an important role in the control of immune responses. IL-12R β 2 expression is induced by TCR activation and then maintained by IL-12 as well as by IFN- γ stimulation [148]. IL-12R β 1 is constitutively expressed on naïve CD4⁺ T cells and its expression is further increased in Th1 cells through an IRF1-dependent mechanism [149]. The production of IL-12 is essential for the development of Th1 responses. IL-12 activates NK cells to produce IFN- γ , which in turn activates STAT1 in the responding CD4⁺ T cells, up-regulating their T-bet expression. IL-18R α is also upregulated during Th1 differentiation. Although IL-18 is not involved in the differentiation of Th1 cells, it can synergise with IL-12 to induce IFN- γ , indicating that IL-18 plays an important role in Th1 responses [150].

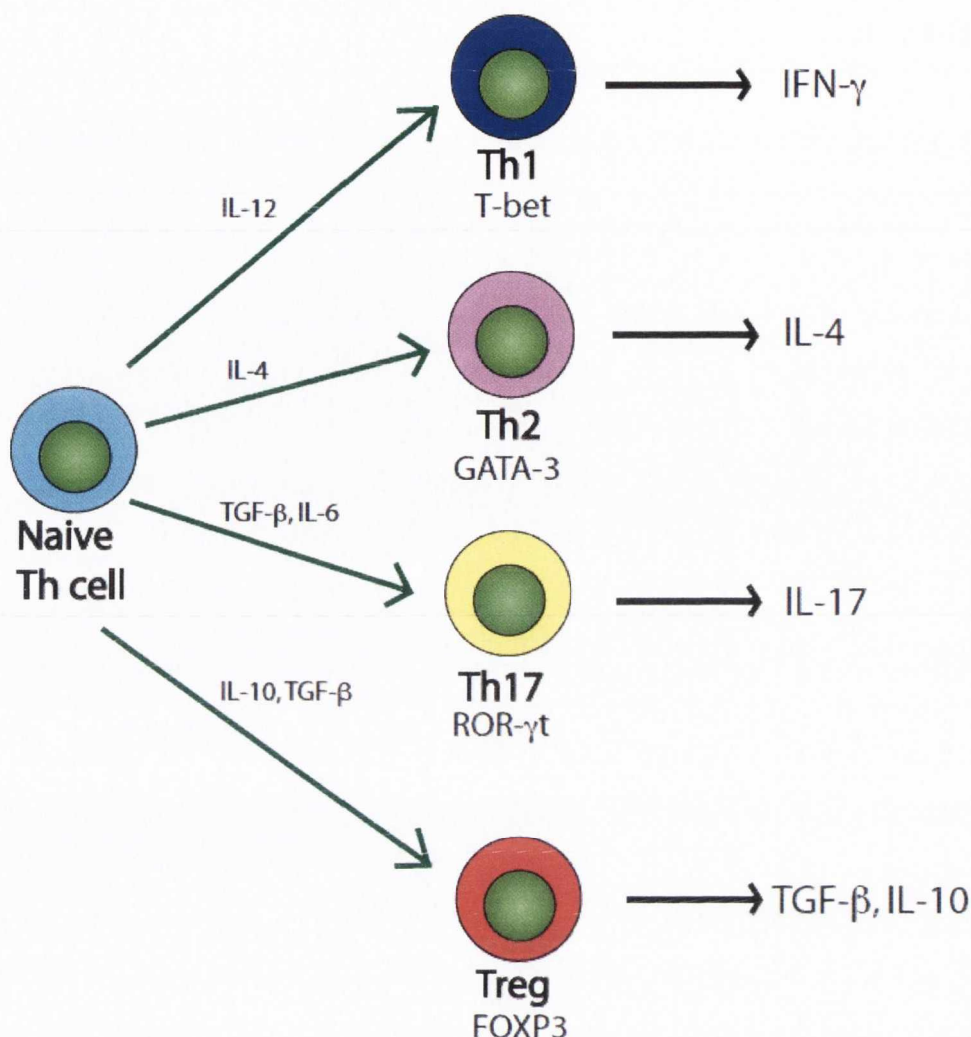


Figure 1.9: A model for CD4⁺ Th cell differentiation from naïve CD4⁺ T cells. Naïve CD4⁺ T cells, after activation by signalling through the TCR and co-stimulatory molecules, can differentiate into one of several lineages of effector Th cells, depending on the initial stimulus and the cytokine environment. These Th cell lineages produce different cytokines and have distinct immunoregulatory functions. Th1 cells differentiate in the presence of IL-12, and require activation of the transcription factor, T-bet, through STAT1. Fully committed Th1 cells produce IFN- γ and lymphotoxin through STAT4. They are involved in cell-mediated immunity against intracellular bacteria and viruses. Th2 cells differentiate in the presence of IL-4 and its characteristic transcription factor is GATA3. Th2 cells are important in humoral immunity against parasites and helminths. Th17 cells require a combination of TGF- β and proinflammatory cytokines (IL-1 β , IL-6, and/or IL-21) to differentiate from naïve CD4⁺ T cells, and ROR- γ t acts as the key transcriptional regulator. Upregulation of the IL-23 receptor makes these cells responsive to IL-23. Human Th17 cells produce IL-17A, IL-17F, IL-22, and IL-26, and are important in host protection against extracellular pathogens and in autoimmunity. In addition to effector T cells, naïve CD4⁺ T cells can also differentiate into regulatory Th cells (Treg) in the presence of IL-2 and TGF- β or IL-10. Treg cells produce the immunosuppressive cytokines, TGF- β , IL-10, and IL-35 and express the master regulator transcription factor, FOXP3.

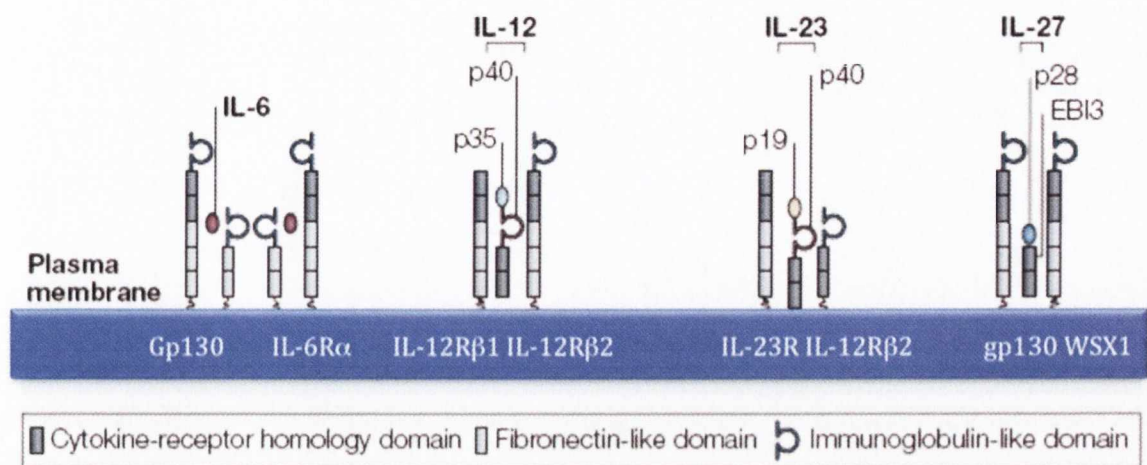


Figure 1.10: The IL-6 and IL-12 family of cytokines and their receptors. IL-6 is a monomeric cytokine that binds with gp130 (glycoprotein 130) and the IL-6 receptor α -chain (IL-6R α). IL-12 is a heterodimer composed of a light chain (IL-12p35) and a heavy chain (IL-12p40). The IL-12 receptor comprises IL-12R β 1 and IL-12R β 2, both of which are homologous to gp130. The IL-12p40 component of IL-12 can also dimerise with IL-23p19 to form IL-23. IL-12R β 2 and IL-23R come together to form the receptor for IL-23. IL-27 is also a member of this family of cytokines. IL-27 is made of EBI3 (Epstein-Barr-virus-induced molecule 3) and IL-27p28. IL-27 binds a receptor composed of gp130 and WSX1. Figure from: [151]

The major cytokine products of the Th1 subset are IFN- γ , lymphotoxin α (LT α), and IL-2. IFN- γ produced by Th1 cells is important in activating macrophages to increase their microbe killing activity. LT α has been implicated as a marker for disease progression in multiple sclerosis patients [152] and LT α -deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE) [153], the animal model of human multiple sclerosis. IL-2 production is important for CD4 $^{+}$ T cell memory. IFN- γ^{+} IL-2 $^{+}$ cells are regarded as precursors of the Th1 memory cells [154] and IL-2 stimulation of CD8 $^{+}$ cells during their priming phase is critical for CD8 $^{+}$ memory formation [155].

All of the T cell lineage differentiation pathways are controlled at the level of transcription. Th1 cell differentiation requires the expression of the transcription factor T-box expressed in T cells (T-bet, also called TBC21) [147]. T-bet $^{-/-}$ mice show strongly impaired IFN- γ production by CD4 $^{+}$ T cells and spontaneously develop asthma-like diseases [156]. T-bet induces T cell IFN- γ production and up-regulates IL-12R β 2. T-bet appears to activate the IFN- γ gene by directly binding to several of its regulatory elements. Similarly to the Th1-specific genes IL-12R β 2 and IFN- γ , T-

bet is not expressed in naïve CD4⁺ T cells, but is readily induced during Th1 cell differentiation [141].

Th1 cell development is initiated by STAT1, which is activated in response to IFN- γ and IL-27 that are produced by NK cells and APCs respectively [143]. STAT1, the major transducer of IFN- γ signalling, plays a critical role in the IFN- γ -mediated induction of T-bet [157]. Together with TCR-induced transcription factors, STAT1 induces T-bet, which in turn induces the production of IFN- γ , the activation of the transcription factors H2.0-like homeobox (HLX) and runt-related transcription factor 3 (RUNX3), and opposes the inhibitory effects of GATA-binding protein 3 (GATA3) on Th1 cell differentiation [158]. Expression of IL-12R β 2 is also induced in this process. IL-12R β 2 pairs with IL-12R β 1 to form the IL-12 receptor, thereby allowing APC-derived IL-12 to activate STAT4. STAT4, T-bet, HLX and RUNX3 then bind to and activate *Ifn γ* , which reinforces Th1 cell commitment through the activation of STAT1 in a positive-feedback loop. At the same time, T-bet and RUNX3 bind to and repress *Il4* to inhibit Th2-cell differentiation. RUNX3, a transcriptional repressor important for silencing CD4 during CD8⁺ T cell development, is also upregulated in Th1 cells. STAT4, an IL-12 signal transducer, is important for amplifying Th1 responses. In addition, STAT4 can directly induce IFN- γ -production in activated CD4⁺ T cells. This can initiate the positive feedback loop in which IFN- γ induces more IFN- γ and IL-12/STAT4.

1.4.5 Th2 cells

Th2 cells mediate host defence against extracellular parasites including helminths. They are important in the induction and persistence of asthma and other allergic diseases. Th2 cells produce IL-4, IL-5, IL-9, IL-10, IL-13, IL-25, and amphiregulin.

Th2-cell differentiation is initiated by the activation of STAT6 by IL-4 (Figure 1.8), which together with TCR-induced transcription factors binds to and activates *Gata3* [137]. GATA3 together with STAT6 activate the transcription of *Il4*, *Il5* and *Il13*. The Th2 lineage is re-enforced by the autoactivation of GATA3, the autocrine and paracrine activation of STAT6 by IL-4 and the STAT6- and GATA3-dependent

inhibition of IFN- γ expression and Th1-cell differentiation [137]. STAT6, activated by IL-4, is a major signal transducer in IL-4-mediated Th2 cell differentiation [159] [160]. *In vitro*, STAT6 activation induces high expression levels of the Th2 master regulator gene, GATA-3 [161]. Over-expression of GATA-3 in Th1 cells induces IL-4 production [162] and in the absence of GATA-3, Th2 differentiation is totally abolished *in vitro* and *in vivo* [163]. STAT6 is not the only STAT molecule required for Th2 cells, Th2 cell differentiation requires strong STAT5 signalling [164]. There are 2 STAT5 family members, STAT5a and STAT5b, both of which are important for cytokine-driven cell proliferation and cell survival and IL-2 potently stimulates STAT5 activation.

Both IL-4 and IL-2 are required for Th2 cell differentiation *in vitro* [165]. Basophils are believed to be a major source of IL-4 [166]. IL-4 can be provided exogenously, in which case IL-4-mediated STAT6 activation induces GATA-3 expression. If exogenous IL-4 is not provided, naïve CD4⁺ T cells can produce limited amounts of IL-4, as a result of TCR-mediated *Gata3* transcription and IL-2 mediated STAT5 activation. The IL-4/STAT6 pathway also induces expression of Gfi-1, a transcriptional repressor, which plays an important role in inducing GATA-3^{high} cells to grow [167]. Full differentiation of Th2 cells *in vitro* occurs when STAT5 and GATA-3 work together. Cytokines produced by Th2 cells have multiple effects on different immune cells. IL-5 plays a critical role in recruiting eosinophils [168]. IL-9 induces mucin production in epithelial cells during allergic reactions [169]. IL-10, produced by Th2 cells, suppresses Th1 cell proliferation and DC function [170]. However, the anti-inflammatory immunosuppressive cytokine IL-10 is produced by several different types of immune cells, as a regulatory mechanism. IL-13 is the effector cytokine in the removal of helminths and in the induction of airway hypersensitivity [171]. Amphiregulin is a member of the epidermal growth factor (EGF) family, which induces epithelial cell proliferation. IL-25 (also known as IL-17E) is also a Th2 cytokine, which by signalling through IL-17R β , enhances the production of IL-4, IL-5, and IL-13 by a unique c-kit⁺Fc ϵ RI⁺ nonlymphocyte population [172].

1.4.6 Th17 cells

Until recently the induction of many autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and their experimental murine models, was attributed to the action of Th1 cells. As mentioned earlier, EAE is a mouse model for multiple sclerosis, a human autoimmune disease where the immune system attacks the myelin sheath on the nerves in the central nervous system. This was thought to be a Th1-mediated disease. However, if a Th1 response was the cause of this disease then when IL-12 and IFN- γ were neutralised or knocked out, the symptoms should be alleviated. As mentioned earlier, IL-12 is composed of the IL-12p40 and IL-12p35 subunits (Figure 1.10). IL-12p40 knockout mice were resistant to EAE induction whereas the IFN- γ knockout mice were more sensitive. The discovery of IL-23, which consists of IL-12p40 paired with IL-12p19, led to a change in the understanding of how IL-12 and IL-23 affected EAE induction [173]. It was determined that IL-23 and not IL-12 has the major role in inducing EAE. Due to the linkage between IL-23 and the expression of IL-17, a new Th lineage, Th17, was soon characterised [174].

The cytokines required for Th17 differentiation and expansion have also been identified [175], as have the key transcription factors involved [176], firmly establishing Th17 cells as an independent Th cell lineage in the human and mouse. Th17 cells play important roles in clearance of extracellular bacteria and fungi, especially at mucosal surfaces [177]. However, Th17 cells are potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions. Th17 cells produce IL-17A (IL-17), IL-17F, IL-21, and IL-22. IL-17A was originally cloned as CTLA-8 and was renamed IL-17 when its receptor was cloned [178].

In 2006, it was shown that Th17 cells could be induced *in vitro* from naïve mouse CD4⁺ T cells by stimulation through their TCR in the presence of IL-6 and TGF- β [179-181]. TGF- β is critical for Th17 cell differentiation and IL-6 is produced by the cells of the innate immune system that have been activated through TLR signalling. In the presence of IL-6, TGF β induces Th17 differentiation, production of IL-21 and expression of IL-23R and ROR γ t. IL-21 can replace IL-6 in inducing ROR γ t and IL-

17 expression [182]. IL-23 was initially thought to be a differentiation factor for Th17 cells, but in actual fact its critical role is for Th17 cell survival and/or for maintaining their function. Therefore, Th17 cell differentiation consists of 3 stages: a differentiation stage, with TGF- β and IL-6; an amplification stage, mediated by IL-21; and a stabilisation stage due to IL-23.

The function of IL-18R α on Th17 cells is unclear while IL-1R1 appears critical for IL-17 production [180]. IL-1 signalling on T cells is critically required for the early programming of Th17 cell lineage and Th17 cell-mediated autoimmunity. IL-1 receptor expression in T cells, which is induced by IL-6, is necessary for the induction of EAE and for early Th17 cell differentiation *in vivo*. Moreover, IL-1 signalling in T cells was required in DC-mediated Th17 cell differentiation from naïve or regulatory precursors and IL-1 synergised with IL-6 and IL-23 to regulate Th17 cell differentiation and maintain cytokine expression in effector Th17 cells [183].

As for the Th1 and Th2 lineages, there is a ‘master’ regulator transcription factor for Th17 cells called retinoid-related orphan receptor γ t (ROR γ t) [176]. ROR γ t is induced by TGF- β in combination with the proinflammatory cytokines IL-6, IL-21, and IL-23, all of which activate STAT3 phosphorylation [184]. A related nuclear receptor, ROR α , is able to synergize with ROR γ t to promote differentiation of Th17 cells [185]. Overexpressing ROR γ t induces IL-17 production, whereas ROR γ t-deficient cells produce very little IL-17. STAT3, the major signal transducer for IL-6, IL-21 and IL-23, is indispensable for IL-17 production and deletion of STAT3 results in the loss of IL-17 producing cells [186]. STAT3 is also responsible for the induction of IL-23R. IRF4 has been reported to be critical for Th17 cell differentiation [187]. The aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor that mediates effects of environmental toxins such as dioxin has been shown to regulate Th17 cell cytokine production. It has been shown to be required for IL-22 and IL-17 production [188]. CT, as a mucosal adjuvant, induces a Th17 response, and it has been found that an antagonist of the AhR, which is required for Th17 differentiation by IL-6 and TGF- β , inhibited Th17 differentiation by CT [188].

The cytokines produced by Th17 cells exert a range of functions. IL-17 can induce the production of inflammatory cytokines including IL-6 and chemokines such as IL-8 (also known as CXCL8), and therefore has an important role in inducing inflammatory responses [178]. Both IL-17 (IL-17A) and IL-17F recruit and activate neutrophils during immune responses against extracellular bacteria and fungi. IL-21 made by Th17 cells is a stimulatory factor for Th17 cell differentiation and serves as a positive feedback amplifier for Th17 cells as does IFN- γ for Th1 and IL-4 for Th2 cells. IL-21 also acts on CD8⁺ T cells, B cells, NK cells, and DCs [189]. Not surprisingly Th17 cells express high levels of IL-23R [177], along with substantial amounts of IL-1R1 and of IL-18R α [190].

1.4.7 Treg cells

Regulatory T cells or Treg cells are crucial in maintaining self-tolerance as well as in regulating immune responses [139]. Increasing Treg numbers and/or enhancing their suppressive function may be beneficial for treating autoimmune diseases and for preventing allograft rejection. Indeed, Treg cells stimulated *in vitro* with alloantigen prevent both acute and chronic allograft rejection in mice [191]. On the other hand, depletion of Treg cells and/or inhibition of their function could enhance immunity against tumours and chronic infections. Treg cells can suppress Th1 and Th2 cell responses because mice lacking them exhibit overwhelming autoimmune disease characterised by excessive production of Th1 and Th2 cell cytokines [192].

Treg cells are characterized by the expression of the forkhead transcription factor FOXP3. FOXP3 plays a critical role in specifying and maintaining the functional programme of Treg cells [193]. In 2001, the autoimmune Scurfy mice were found to have mutations in FOXP3 [194], which results in a loss of functional Treg cells. In 2003, FOXP3 was reported as the master transcriptional regulator for Treg cells [195]. TGF- β induces FOXP3 expression [196]. Activated naïve CD4⁺ T cells stimulated by TGF- β in the absence of proinflammatory cytokines develop into iTreg cells (inducible Treg cells). Continuous expression of FOXP3 is critical for maintaining the suppressive activity of Treg cells. Reducing the amount of FOXP3 expressed may convert Treg cells to Th2-like cells, implying a close relationship between the Th2

and Treg lineages [197]. STAT5 activation by IL-2, which is important for Th2 differentiation, is also required for Treg development [198]. STAT5 may contribute to FOXP3 induction through binding to its promoter.

Treg cells exert their suppressive functions through several mechanisms, some of which require cell-cell contact [199]. This could also be due to direct killing of cells or secretion of suppressor cytokines such as IL-10 and TGF- β . The production of cytokines by the Treg cells is crucial for their function. Treg cells secrete TGF- β , IL-10, and IL-35. While Treg cells are crucial in suppressing immune cell function, CD4⁺ T cells other than Th2 and Treg cells can also produce IL-10. IL-10 production by Th1 or Th17 cells may play an important role in limiting their own effector function [200]. IL-10, IL-27, and TGF- β can induce IL-10 production [201] and IL-10 is critical for Treg-mediated prevention and therapy of inflammatory bowel disease [202].

Two major categories of FOXP3⁺ Treg cells have been identified (although there are others): the naturally occurring CD4⁺CD25⁺ Treg (nTreg) cells that arise in the thymus and the TGF- β -induced Treg (iTreg) cells produced in the periphery [203, 204]. iTreg cells are now well established as an inducible cell population that phenotypically resembles nTreg cells. The differentiation of Th17 cells may actually be linked to the differentiation of iTreg cells [205]. TGF- β is required for iTreg differentiation and is also required for nTreg development [206]. Deleting TGF- β from Treg cells results in diminished suppressive function and poor survival *in vivo* [207]. In response to TCR stimulation in the presence of TGF- β , naïve CD4⁺ T cells can differentiate into either Th17 or iTreg cells depending on the overall cytokine environment. In the absence of proinflammatory cytokines, TGF- β induces iTreg differentiation from naïve mouse CD4⁺ T cells [208]. TGF- β activates Smad3 while TCR stimulation induces NFAT activation. Smad3 and NFAT collaborate in remodelling the FOXP3 enhancer region and promote FOXP3 expression [209]. Both TGF- β and IL-2 are required for the survival and function of Treg cells even after they have differentiated.

1.4.8 Crosstalk between Th cell lineages

Extensive crosstalk between the Th cell lineages exists. There is positive feedback by cytokines involved in Th differentiation and cross inhibition of some lineages by the other lineages.

Both IFN- γ and IL-4 mutually suppress each other's signalling. TGF- β was found to suppress both Th1 and Th2 differentiation and both IL-4 and IFN- γ inhibit Th17 differentiation [210]. Additionally, IL-27, a member of the heterodimeric IL-12 cytokine family, promotes Th1 cell differentiation by inducing T-bet and IL-12R β 2 [211] and at the same time inhibits Th17 cell differentiation in a STAT1-dependent manner. The loss of T-bet strongly favours IL-17 expression both in CD4⁺ and CD8⁺ T cells [212].

As discussed earlier, the Th17 and Treg cell programmes are intrinsically linked and which lineage is induced depends on the cytokine environment. TGF- β inhibits Th1 and Th2 cell differentiation and promotes Treg cell and Th17 cell lineage commitment by inducing the expression of the transcription factors FOXP3 and ROR γ t, which are required for Treg and Th17 cell lineage commitment respectively [213]. FOXP3 inhibits ROR γ t and therefore blocks Th17 development, whereas in the presence of IL-6, STAT3 is activated, which inhibits the expression of FOXP3 and its interactions with ROR γ t. This results in an increase in the expression of ROR γ t (as well as ROR α) [185], and Th17 cell differentiation is favoured. Once the Th17 cell differentiation programme has been initiated, the T cells produce IL-21, which activates STAT3 and induces the expression of IL-23R. This allows APC-derived IL-23 to activate STAT3, which dampens IL-10 production, drives IL-22 production and stabilises Th17 cell differentiation and commitment.

Mice lacking TGF- β lack both FOXP3⁺ Treg and Th17 cells and have overwhelming autoimmunity largely caused by uncontrolled Th1 cell activity [214]. These findings suggest an intimate link between the Treg and Th17 cell programmes of differentiation, and indeed there is evidence for such a relationship from both *in vitro* and *in vivo* studies [208]. There have been experiments suggesting that FOXP3 may

antagonise ROR γ t-induced IL-17 expression. T cells that co-express ROR γ t and FOXP3 have been identified *in vivo* in both mice and humans. These FOXP3⁺ROR γ t⁺ cells may also exist as a transient population that can give rise to either Treg or Th17 cells [205]. It has also been found that ROR γ t and FOXP3 can interact with each other. After *in vitro* induction of ROR γ t and FOXP3 by TGF- β , cells do not express IL-17 but have the dual potential to differentiate into either the Th17 or Treg cell lineage depending on the cytokine environment. In the presence of proinflammatory cytokines (IL-6, IL-21, or IL-23) and low concentrations of TGF- β , ROR γ t expression is further upregulated, whereas FOXP3 expression and function are inhibited. This relieves repression of ROR γ t activity by FOXP3 in favour of Th17 cell lineage specification. In contrast, in the absence of proinflammatory cytokines, high concentrations of TGF- β are optimal for FOXP3 expression and thus tip the balance toward Treg cell differentiation [205]. This shift toward Treg cells is enhanced by IL-2 and retinoic acid. Both inhibit Th17 cell differentiation by reducing ROR γ t expression and enhancing TGF- β -induced FOXP3 expression, thus influencing Th17-Treg cell specification [215].

1.4.9 The effector functions of the Th cell subtypes

Th1 cells work primarily with macrophages, increasing their ability to kill infected cells and also the proliferation of CD8⁺ T cells [216]. Th1 cells also promote the production of opsonising antibodies. The Th1 cytokine IFN- γ increases the production of IL-12 by DCs and macrophages, and via positive feedback, IL-12 stimulates the production of IFN- γ in Th cells, thereby promoting the Th1 profile [136]. Th2 cells work primarily with B cells, stimulating their proliferation, inducing B cell antibody class switching and increasing neutralising antibody production. IL-4 acts on Th cells to promote the production of Th2 cytokines, while IL-10 inhibits a variety of cytokines including IL-2 and IFN- γ in Th cells and IL-12 in DCs and macrophages [165].

Th17 cells produce cytokines (e.g. IL-22), which stimulates epithelial cells to produce anti-microbial proteins to clear out certain types of microbe. As mentioned earlier Th17 cells primarily produce two main members of the IL-17 family, IL-17A and IL-17F, which are involved in the recruitment, activation and migration of neutrophils

[190]. These cells also secrete IL-21 and IL-22. MHC class I receptors are expressed on all host cells except for some non-nucleated cells e.g. red blood cells. When CD8⁺ T cells bind and recognise pathogenic peptides expressed on the MHC class I receptor i.e. bind to infected cells, the CD8⁺ T cells release several cytotoxins e.g. perforin, granzymes, and granulysin [123]. These function to promote the death of the infected cell via apoptosis. Similarly, apoptosis may be induced via cell-surface interactions between death receptors (e.g. FAS ligand) on the CD8⁺ T cell and the infected cell [123].

1.5 Signalling through GPCRs

1.5.1 G-protein coupled receptors (GPCRs)

GPCRs are the largest family of membrane proteins and mediate most cellular responses to hormones and neurotransmitters, as well as being responsible for vision, smell and taste [217]. As GPCRs are the receptors for hormones, neurotransmitters, ions, photons and other stimuli, they are essential for communication between the inside and outside of the cell. GPCRs couple the binding of agonists to the activation of specific heterotrimeric G proteins, leading to the modulation of downstream effector proteins. The GPCRs are located in the cell membrane and have seven TM-spanning α -helices, an extracellular N terminus, an intracellular C terminus and three interhelical loops on each side of the membrane (Figure 1.11).

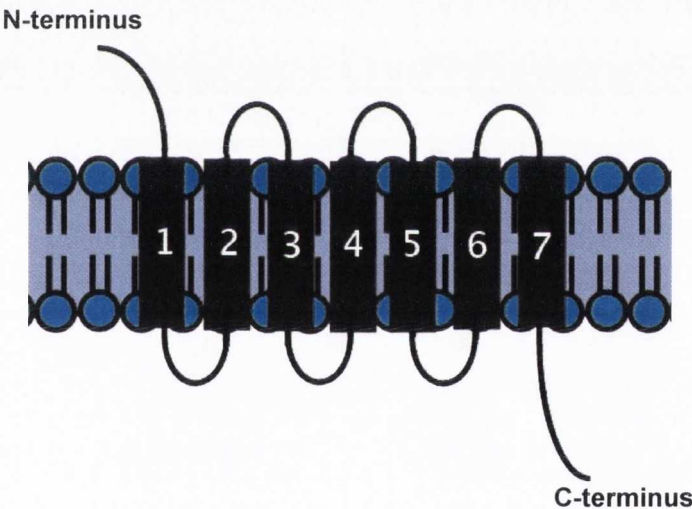


Figure 1.11: The universal structure of G-protein coupled receptors. All GPCRs have an extracellular N-terminal segment, seven transmembrane domains (TM), which form the TM core, three exoloops, three cytoloops, and a C-terminal segment located inside the cytosol. The cytoplasmic tail is responsible for interaction with the downstream components of the signalling pathway. The seven TM structure place the N- and C-terminal segments at opposite membrane surfaces. It allows glycosylation and ligand binding at the N-terminal segment, and phosphorylation and palmitoylation at the C-terminal segment for desensitisation and internalisation.

GPCRs in vertebrates are divided into five families on the basis of structural similarity: rhodopsin, secretin, glutamate, adhesion and frizzled/taste2 [217]. The most significant structural differences lie in the extracellular loops and ligand-binding region. Individual GPCRs have unique combinations of signal-transduction activities involving multiple G-protein subtypes, as well as G-protein independent signalling-

pathways and complex regulatory processes. Extracellularly the GPCR has a ligand-binding region, while intracellularly at the C-terminus lies the effector region of the protein. All GPCRs are thought to operate via a similar molecular mechanism. Binding of the ligand outside the cell to the GPCR causes conformational changes to occur throughout the protein which transduce this signal to the inside of the cell where the receptor associates with distinct classes of heterotrimeric G-proteins [218]. Crystal structures of ligand-activated GPCRs have been solved. These include the human β_2 adrenergic receptor (β_2 AR), the avian β_1 AR and the human A_{2A} adenosine receptor. In the case of the human β_2 AR; adrenaline or noradrenaline binding to cells activates the stimulatory subunit of the heterotrimeric G protein ($G_{\alpha s}$), stimulation of adenylyl cyclase (AC), the accumulation of cyclic AMP (cAMP), the activation of cAMP-dependent protein kinase A (PKA) and the phosphorylation of proteins involved in muscle cell contraction [219].

1.5.2 Heterotrimeric G-proteins

G proteins, short for guanine nucleotide-binding proteins, are a family of proteins involved in second messenger cascades. G proteins are so called because they function as "molecular switches," alternating between an inactive guanosine diphosphate (GDP) and active guanosine triphosphate (GTP) bound state, ultimately going on to regulate downstream cell processes (Figure 1.12). These G-proteins consist of α -subunits bound to $\beta\gamma$ complexes and are attached to the cytoplasmic surface of the plasma membrane. Extracellular stimuli activate these receptors, which then catalyse GTP-GDP exchange on the G protein α -subunit. Heterotrimeric G proteins turn on intracellular signalling cascades in response to the activation of GPCRs and belong to the larger group of enzymes called GTPases.

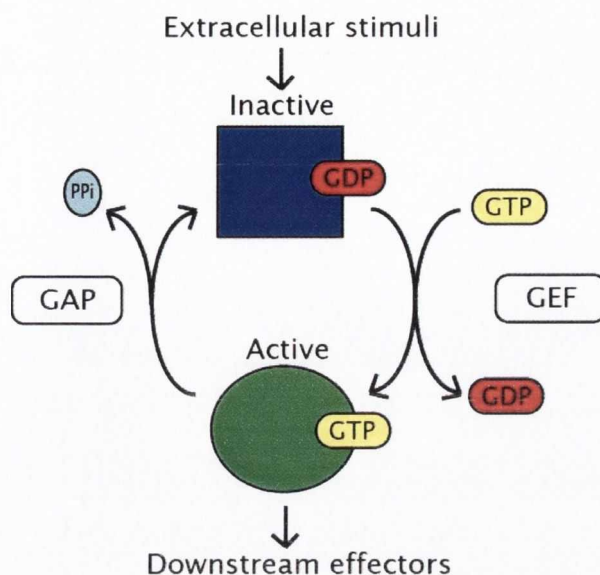


Figure 1.12: The heterotrimeric G-proteins acting as molecular switches. The inactive protein exists bound to a molecule of GDP in the cell. Upon stimulation, the GEF (guanine nucleotide exchange factor) enzyme is switched on and a GTP molecule is exchanged for the GDP molecule on the target protein. This activates the target protein and this is then free to stimulate its downstream effectors. The GAP (GTPase activating protein) enzyme removes the phosphate (PPi) molecule on the GTP, inactivating the target and completing the cycle.

Even though the GPCR superfamily is large and diverse, these proteins interact with a relatively small number of G proteins to initiate intracellular signalling cascades. The switching function of the three G-proteins subunits (α , β , and γ) depends on the ability of the G protein α -subunit ($G\alpha$) to cycle between an inactive GDP-bound conformation that is primed for interaction with an activated receptor, and an active GTP-bound conformation that can modulate the activity of downstream effector proteins [220] (Figure 1.12). In humans, there are 21 $G\alpha$ subunits encoded by 16 genes, 6 $G\beta$ subunits encoded by 5 genes, and 12 $G\gamma$ subunits [221]. Heterotrimers are typically divided into four main classes based on the primary sequence similarity of the $G\alpha$ subunit: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ [222]. Upon ligand binding, there is a conformational change in the cytoplasmic domains of the receptor. Agonist binding to receptors leads to the formation of a receptor-G protein complex. Each member of the G protein family must be able to interact with many different receptors. Furthermore, many receptors can activate multiple G protein signalling pathways. The receptor-G protein interface encodes important information that determines which G proteins can interact with a particular receptor.

Heterotrimeric guanine-nucleotide binding proteins couple the activation of the seven-transmembrane receptors at the cell surface to intracellular responses (Figure 1.13). G proteins are inactive in the heterotrimeric conformation where $G\alpha$ binds GDP and the constitutive $G\beta\gamma$ dimer. Extracellular stimuli, such as hormones, neurotransmitters, chemokines, light and odorants, activate receptors by inducing a conformational change that permits G-protein binding and catalyses GDP release from $G\alpha$, thereby resulting in the formation of a stable, high-affinity complex between the activated receptor and G protein. Binding of GTP to $G\alpha$ destabilises this complex, leading to a structural rearrangement of $G\alpha(\text{GTP})$, $G\beta\gamma$ and the receptor. Both subunits, $G\alpha(\text{GTP})$ and $G\beta\gamma$, go on to interact with downstream effector proteins. The cellular response is terminated when $G\alpha$ hydrolyses GTP to GDP and re-associates with $G\beta\gamma$ thus completing the cycle [223].

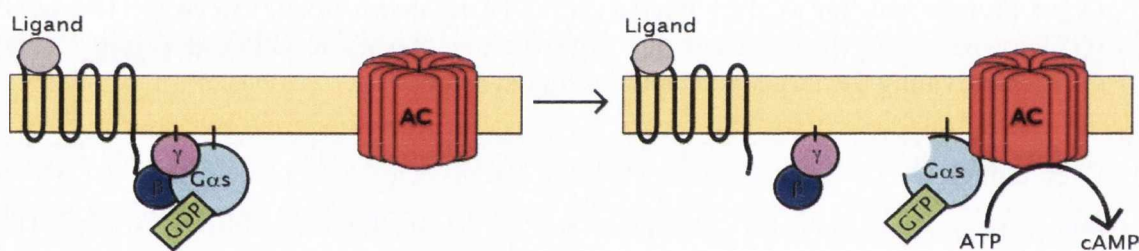


Figure 1.13: Upon ligand binding to the extracellular portion of the GPCR, the heterotrimeric G-proteins transduce the signal to the adenylate cyclase (AC) enzyme located in the plasma membrane. Ligand binding to the extracellular binding site on the GPCR induces a conformational change in the protein, which permits G-protein binding to the cytosolic portion of the GPCR, and catalyses release of GDP from $G\alpha$, resulting in a high-affinity complex between the activated receptor and the G-proteins. Binding of GTP to the $G\alpha$ destabilises this complex and leads to a structural rearrangement of the $G\alpha$ -GTP complex and the $G\beta\gamma$ proteins. The $G\alpha$ stimulatory complex interfaces with the AC enzyme on the plasma membrane inducing cAMP production from ATP stores in the cell.

Physiologically, the receptor-G-protein complex is transient owing to rapid binding of GTP, the cellular concentration of which exceeds that of GDP several-fold. Numerous factors can influence the kinetics of G protein activation, including ligand, receptor and G protein affinities, intracellular protein concentrations and receptor deactivation kinetics.

1.6 Cyclic AMP signalling

1.6.1 Cyclic AMP

Many hormones and neurotransmitters (i.e. first messengers), bind to GPCRs on the cell surface, where their signals are then transduced into the cell via an intracellular second messenger such as cAMP [224]. In the late 1950s, Earl Sutherland discovered that cAMP acts as a second messenger for the hormone adrenaline [225]. He did this by observing that a small heat-stable molecule mediated the effects of adrenaline on extracts of liver cells. This molecule called cAMP, was produced inside cells after hormone treatment and was able to activate the same glycolytic enzymes that were activated by extracellular adrenaline [224].

Second messenger molecules function as activators of specific protein kinases that in turn activate downstream kinase cascades. This leads to the phosphorylation of target proteins. Recently non-kinase effectors of several second messengers have been discovered, for example the Exchange Proteins directly activated by cAMP (Epac1 and Epac2).

Many different ligands are capable of binding to the GPCR on the cell surface and inducing or suppressing cAMP production (Figure 1.14). [226]. The best-known ligands that increase cAMP levels in the cell are adrenaline, noradrenaline, histamine, serotonin, and the cyclooxygenase (COX)-derived prostaglandin, PGE₂. The G α i subunits inhibit AC and production of cAMP. Inhibitors of G α i-coupled ligands include the chemokines CCR1-10 and CXCR1-6 [226].

Following its production by ACs, cAMP is involved in many signalling cascades. It has also been reported that cAMP can be transported outside the cell by members of the multidrug-resistance-associated proteins such as MRP4 [227]. Originally the effects of cAMP within cells were thought to be mediated solely via the activation of PKA, but now two other targets of cAMP are known to mediate cAMP responses. Thus, the effectors of cAMP now are known to include the cAMP-dependent PKA, the guanine nucleotide exchange factor Epac, and cAMP-dependent ion channels. At

the same time, cAMP signalling is stopped by phosphodiesterase (PDE)-catalysed degradation (Figure 1.14).

Cyclic AMP modulates the response of immune cells to many stimuli. Upon stimulation of T cells through their antigen receptors there is a transient increase in cAMP levels in the cell. Persistent high levels of cAMP inhibits many cellular responses and therefore TCR-mediated signalling must be coordinated with the activation of cyclic nucleotide PDEs that degrade cAMP [228]. Elevation of intracellular cAMP inhibits lymphocyte activation and agents that can elevate intracellular cAMP levels have been shown to be immunosuppressive and antiinflammatory [229]. These effects are caused in part by the inhibition of various T cell functions including proliferation, cytokine production and expression of activation markers on the cell surface. Selective inhibitors of cAMP-specific PDEs have been suggested as therapies for the treatment of several diseases especially immune disorders e.g. multiple sclerosis [230] and disorders of the CNS e.g. Alzheimer's disease (AD) [231]. The cAMP signalling pathway can also be used by pathogens themselves to modulate the immune response of the host. *Mycobacterium tuberculosis* bacteria have genes for at least 15 biochemically distinct AC enzymes and its secretion of cAMP into host macrophages alters the host's immune response [232].

Cyclic AMP signalling pathways play a major role in the processing of painful stimuli or nociception [233]. Increased cAMP in neurons is associated with increased nociception whereas agents that inhibit cAMP have analgesic effects. At inflamed sites, PGEs (e.g. PGE₂) sensitise primary afferent nociceptor excitability by activation of ACs through receptor stimulation of the G-protein G_s [234]. Many drugs used as analgesics, e.g. non-steroidal anti-inflammatory drugs (NSAIDs) and opioids target elements of the cAMP sensitisation mechanism. NSAIDs inhibit COX proteins, which convert arachidonic acid to pro-nociceptive PGEs (e.g. PGE₂), which in turn activate ACs through their respective receptors. Opioids, the most powerful pain-relievers known, decrease synaptic transmission partly by inhibiting ACs through receptor stimulation of the inhibitory G-protein G_i. cAMP signalling also has a key role in the neurobiology of learning and memory. Classical anti-obstructive drugs for the treatment of asthma, such as β 2-adrenergic receptor agonists (e.g. fenoterol or

alubutero) act through increased levels of cAMP. For example Ibuprofen is a nonselective inhibitor of COX proteins (NSAID), which decreases cAMP levels and is used to treat inflammation and pain [234].

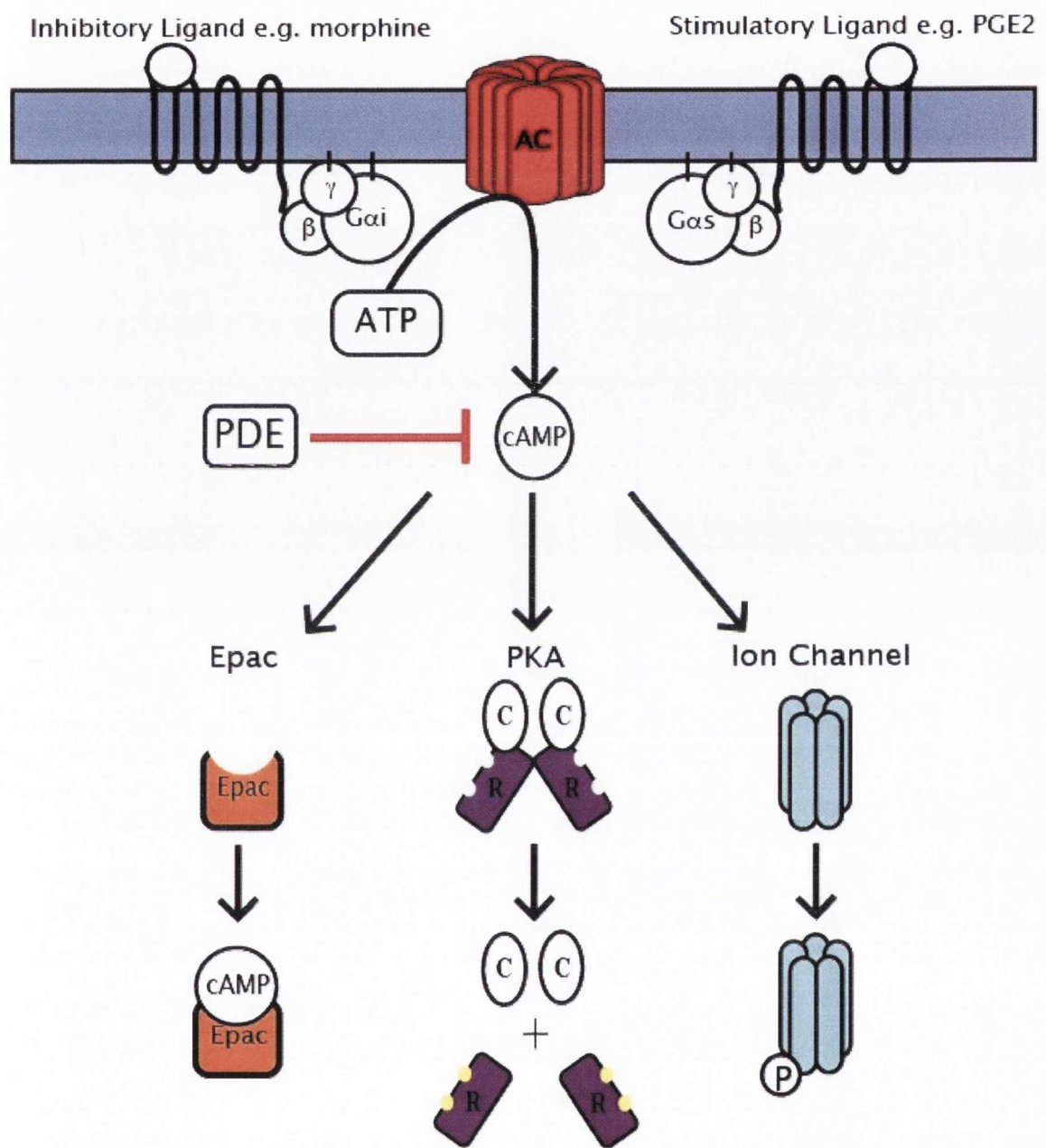


Figure 1.14: The cAMP signalling pathway. Upon stimulatory ligand binding to the GPCR the adenylate cyclase enzyme (AC) is activated by Gαs to produce cAMP from ATP in the cell. In contrast, binding of an inhibitory ligand leads to Gαi subunit interaction with the AC and inhibition of downstream signalling. The cAMP produced can be degraded by phosphodiesterases (PDEs) in the cell. Cyclic AMP has three currently known cellular targets: Epac, PKA and a class of cAMP-gated ion channels.

1.6.2 Adenylyl/adenylate cyclases (ACs)

AC is an ATP-pyrophosphate lyase that converts ATP to cAMP and pyrophosphate, thus inducing an increase in intracellular cAMP (Figure 1.15). The first AC isoform, AC1, was cloned in 1989 [235], and since then there has been much progress in the cloning, characterisation, and structural analysis of the individual AC enzymes. In mammals there are nine membrane-bound ACs, with a tenth ‘soluble’ form that has distinct catalytic and regulatory properties resembling the cyanobacterial enzymes [236]. All of these isoforms have distinct regulation and expression patterns. The AC isoforms are ubiquitously expressed, are crucial for many biological processes and all have individual and clearly distinct physiological functions.

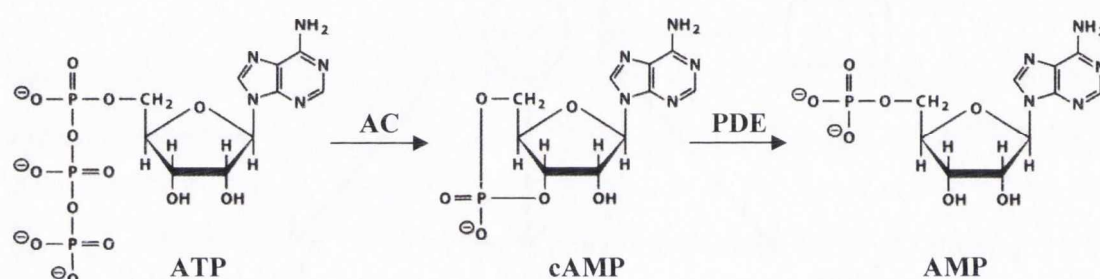


Figure 1.15: cAMP generation from ATP and degradation to AMP. Shown here are the molecular structures of ATP, cAMP and AMP. ATP is the precursor of cAMP in the cells. The adenylyl cyclase enzyme (AC) produces cAMP from ATP upon activation by the stimulatory G-protein. The phosphodiesterases (PDE) degrade cAMP to AMP, thus attenuating the cAMP signal in the cell.

Mammalian TM ACs share a similar structure of a variable N-terminus (and two repeats of a membrane-spanning domain followed by a cytoplasmic domain [235]. Membrane-bound ACs are classified into four different categories based on regulatory properties. Group I consists of Ca^{2+} -stimulated ACs, ACs 1, 3 and 8; group II consists of $\text{G}\beta\gamma$ -stimulated ACs, ACs 2, 4 and 7; group III is comprised of $\text{Gi}\alpha/\text{Ca}^{2+}$ -inhibited AC5 and 6, while group IV contains forskolin-insensitive AC9 [237]. All isoforms of transmembrane ACs are stimulated by the GTP-bound α subunit of Gs ($\text{Gs}\alpha$). The α subunits of Gi (1, 2, 3) can inhibit select AC isoforms [238]. The $\beta\gamma$ subunit of heterotrimeric G proteins can be either stimulatory or inhibitory depending on the AC isoform. $\text{G}\beta\gamma$ is inhibitory for all group I ACs [239].

All AC isoforms are inhibited by high, non-physiological concentrations of Ca^{2+} , via competition for magnesium at the active site. However, AC5 and 6 are inhibited by submicromolar concentrations of free Ca^{2+} , which may have important physiological implications in generating oscillating Ca^{2+} and cAMP signals [240, 241]. Most ACs are regulated by either PKA or PKC. PKA serves as a feedback inhibitor for AC5 and 6 by phosphorylating these isoforms on a serine residue. PKC regulation can be either stimulatory or inhibitory. Several additional regulators and binding partners of ACs exist, including the regulator of G protein signalling (RGS2), the protein associated with Myc (PAM), Snapin, Ric8a, and the A-kinase-anchoring protein (AKAP79).

1.6.3 Phosphodiesterases (PDEs)

PDEs are the enzymes responsible for the degradation of intracellular cAMP, and are therefore critical regulators of cAMP intracellular homeostasis [242]. The PDEs are a large family of enzymes that metabolise cAMP into its inactive 5'-monophosphate [243]. These PDEs are part of a super-family of metallophosphohydrolases that specifically cleave the 3',5'-cyclic phosphate of cAMP. To date, 11 families of PDEs have been identified but there are many isoforms with different expression patterns and functions in mammalian cells [244]. Mammalian PDEs have a common structure with a conserved catalytic domain near the C-terminus, and regulatory domains or motifs mostly at the N-terminus of the protein. One mechanism to increase intracellular cAMP levels is via inhibition of the PDEs. Inhibition of PDEs has been shown to activate specific protein phosphorylation pathways involved in the function of the central nervous system (CNS), [245], in cardiovascular function [246], in inflammatory cells of the immune system [247], in cell adhesion and in metabolic processes [248].

1.7 Protein kinase A (PKA)

1.7.1 Discovery and structure of PKA

The first defined target of cAMP in mammalian cells was PKA. It is only recently that the alternate targets of cAMP have been discovered, and the majority of biological effects of cAMP have been associated with PKA. PKA is a broad-spectrum serine/threonine kinase that can phosphorylate a range of proteins. PKA activation regulates a vast number of cellular processes including metabolism, gene regulation, cell growth and division, cell differentiation, sperm motility and ion channel conductivity [249].

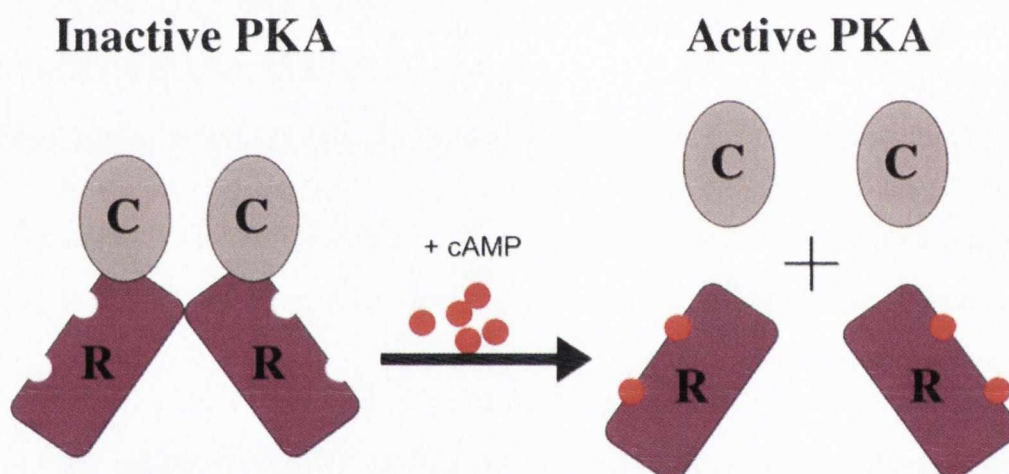


Figure 1.16: Inactive PKA binds cAMP and releases its regulatory and catalytic subunits. In its inactive form PKA exists as a tetrameric holoenzyme consisting of two catalytic (C) subunits bound to two regulatory (R) subunits. cAMP binds to two sites on each R subunit. Upon binding of four molecules of cAMP, the enzyme dissociates into an R subunit dimer with four molecules of cAMP bound, and two free active C subunits that phosphorylate serine and threonine residues on substrate proteins.

In the absence of cAMP, PKA exists as an enzymatically inactive tetrameric holoenzyme consisting of two catalytic (C) subunits bound to a regulatory (R) subunit dimer [250] (Figure 1.16). Cyclic AMP binds to two sites on each R subunit. Upon binding of four molecules of cAMP, the enzyme dissociates into an R subunit dimer with four bound molecules of cAMP, and two free active C subunits that phosphorylate serine and threonine residues on substrate proteins. For PKA, binding of cAMP to defined sites within both R subunits of the tetrameric holoenzyme allows

release and activation of two C subunits and substrate phosphorylation [251]. The C and R subunits of PKA are relatively small (40K and 50-57K respectively) [252]. Both subunits have been crystallised with both substrates and inhibitors [253]. From these structures it has been determined how the cleft opens and closes to grab onto the cAMP molecules at the base of the active site, and how the activating phosphate group is transferred to the protein substrate. Usually the rate of phosphate transfer is quite fast as the catalytic subunit is assembled as a fully phosphorylated and active enzyme that is then kept sequestered in an inactive state by its association with a regulatory subunit so that the active protein is released only upon binding of cAMP.

There are multiple R and C subunits with different biochemical features and activities. When these are assembled together they give rise to a number of PKA holoenzymes with different biological characteristics and activities. Initially there were thought to be only two PKA isozymes, PKAI and PKAII. These contain C subunits associated with two different R subunits called RI and RII. It is now known that there are many more PKA isozymes in the cell [250]. Two RI subunits have been identified, RI α and RI β and two RII subunits RII α and RII β . There are also two C subunits, C α and C β . Splice variants of the C isoforms have also been identified. There is a distinct pattern of expression of different subtypes in different organs and it is known that the subtypes have individual functions. Levels of expression of the different PKA subunits are regulated by hormones acting through GPCRs, mitogenic signals acting through receptors associated with protein tyrosine kinases as well as by steroid hormones [254].

Upon activation of the TCR-CD3 complex in T cells, there is an initial peak of cAMP and PKA activity seen that may serve as an acute negative modulator and a negative feedback of signalling through the TCR/CD3 [254]. Inactive T and B cells contain soluble PKAI and particulate PKAII in a proportion of 3:1. When activated through the antigen receptor, RI α translocates from the cytosol and associates with the antigen complex of both T and B cells [255].

While early models proposed that cAMP was free to diffuse and act throughout the cell, it is now accepted that cAMP can also act locally at sites within cells where

cAMP-effector molecules and either ACs, or PDEs are co-localised. Various studies have shown that PKA can be targeted to selective regions of cells and that cAMP can act selectively at these sites by accessing these targeted effectors [256]. Compartmentalisation of PKA is mediated through binding of the R subunit to subcellular components. PDEs contribute to the establishment of local gradients of cyclic nucleotides by being localised to subcellular compartments and by being recruited into multi-protein signalling complexes. This contributes to the temporal and spatial specificity of cyclic nucleotide signalling by regulating the availability of cAMP/cGMP to their effectors. PDE activity has been found in both the soluble and particulate fractions of the cell. The cAMP signal is delivered to targeted effectors and terminated in a spatially and temporally defined manner by specific PDEs establishing local pools of cAMP close to the effector molecules [257].

1.7.2 PKA and the Immune System

PKA activation has been reported to exert several effects on the immune system. Since PKA regulates many cell processes, acting as a broadly specific serine/threonine kinase, mechanisms conferring specificity on PKA signalling pathways are required. As mentioned previously in lymphocytes, a temporary increase in intracellular cAMP has been reported after antigen receptor stimulation, and, increased cAMP levels in response to PGE₂ and other agents have been reported to cause immunosuppression [258]. Hyperactivation in the cAMP/PKA system has been implicated in the T cell dysfunction associated with HIV infection and a subset of common variable immunodeficiency (CVI) [259]. Conversely inhibition by PKA type I is reduced in patients with the autoimmune disease SLE [260]. There have been several reports indicating that there is a target for PKA in regulation of proximal TCR signalling [261]. All of these examples indicate that PKA has a suppressive function in T lymphocytes as a result of its activation by cAMP. PKA also has an inhibitory effect on macrophages as PKA activation inhibits production of TNF- α , MIP-1 α and leukotriene B₄ while increasing IL-10 and IL-6 production in alveolar macrophages [262]. Cyclic AMP elevation activating PKA in DCs has been found to suppress TNF- α and IL-12 production via the transcription factor c-Fos [263]. Agents that elevate or mimic endogenous cAMP can augment levels of IL-10 [264] and IL-5 [265], but

inhibit IL-4 [266], IFN- γ and IL-2 [265] production by mononuclear cells. The differential regulation of cytokine expression by PKA has been determined to be dependent on the cell type and the environment surrounding the cell. The elaborate regulation (for example the degradation of cAMP by PDEs thus inhibiting PKA activation) and distribution of the PKA pathway components (for example the R and C subunits of activated PKA) is affected by other signalling events, contributing to the differential effects of PKA downstream signalling.

1.8 Exchange protein activated by cAMP (Epac)

1.8.1 Epac Discovery

Epac1 and Epac2 are cAMP-dependent guanine-nucleotide exchange factors (GEFs) for the small GTPases Rap1 and Rap2 [267]. The two isoforms of Epac were discovered in 1998, through a database search of proteins carried out to explain why the cAMP-induced activation of the small GTPase Rap1 was PKA-independent [268]. GEFs are responsible for exchanging an inactive GDP molecule for an active GTP molecule on its target protein. It is a fast and easily reversible method for protein activation in the cell. Epac proteins are important mediators of cAMP signalling, and are frequently interconnected with cAMP signalling through PKA. The Rap GTPases cycle between inactive GDP-bound and active GTP-bound forms. The two isoforms of Epac have GEF activity that converts Rap proteins into their active forms, following which GTPase-activating proteins in the cell then convert Rap to the inactive form [269] (Figure 1.12). Epac and PKA may act independently but are often associated with the same biological process, in which they fulfil either synergistic or opposite effects [270].

The two isoforms of Epac, Epac1 and Epac2, are coded by two distinct genes, RAPGEF3 and RAPGEF4 in mammals. Epac1 and Epac2 are present in most tissues, although with different expression levels. While Epac1 is ubiquitously expressed in all tissues with high levels of expression in blood vessels, kidney, adipose tissue, CNS, ovary, and uterus, Epac2 is detectable most notably, but not exclusively, in the brain, pituitary, adrenal gland and pancreas [271]. Recently a shorter N-terminal splice variant of Epac2 named Epac2B has been identified in the adrenal gland,

suggesting that alternative Epac isoforms may add to the intricacy of Epac effects [272]. Following this discovery, the long isoform Epac2 can be referred to as Epac2A [272]. Epac proteins are expressed within different subcellular compartments such as the nucleus, the cytosol, nuclear and plasma membranes and their localisation varies depending on the cell type and cell cycle [270].

1.8.2 Epac protein structure

The Epac proteins are multi-domain proteins with an N-terminal regulatory region and a C-terminal catalytic GEF region, with each region capable of functioning independently with respect to cAMP binding and GEF activity [267] (Figure 1.17). The regulatory region of Epac1 has two domains, a Dishevelled-Egl-10-Pleckstrin (DEP) domain and a cyclic mononucleotide-binding (CNB) domain as well as an N-terminal extension [273]. The DEP domain is thought to be involved in the localisation of Epac, most probably via interaction with either a lipid or a membrane protein. The Ras-exchange motif (REM) domain in the catalytic region of Epac is conserved in nearly all mammalian GEFs with a CDC25-homology domain (HD) [267]. With Epac, this is involved in connecting the regulatory region with the catalytic region. The entire N-terminal domain of Epac1 may act as a protein-interaction domain as it has been reported that this region directs Epac1 to mitochondria [274]. The GEF activity of Epac is accomplished by an extensive association between the helical hairpin, ionic latch (IL) loop and central core of the CDC25 HD and the nucleotide-binding site of Rap1 [275]. Epac2 also exhibits these domains, along with an additional CNB domain. The critical domain for the regulation of Epac activity by cAMP is the conserved CNB domain immediately N-terminal to the catalytic domain [273]. As the structure of Epac1 is identical to Epac2 except for an extra CNB domain, these crystal structures depicting the conformations of Epac2 are comparable for Epac1.

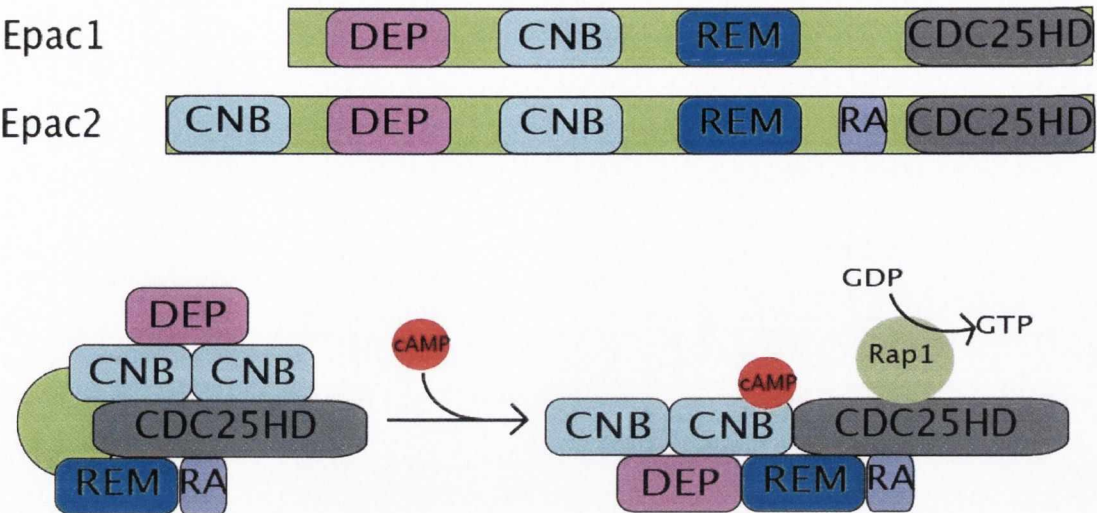


Figure 1.17: The multi-domain structures of Epac 1 and Epac 2. Shown on top is the domain structure of Epac1 and Epac2, including the regulatory region with the cyclic nucleotide-binding domain(s) (CNB) and the catalytic region with the CDC25-homology domain (CDC25HD) responsible for the guanine-nucleotide-exchange activity. The Disheveled-Egl-10-Pleckstrin (DEP) domain is involved in membrane localisation; the Ras exchange motif (REM) stabilises the catalytic helix of CDC25HD and the Ras-association (RA) domain is a protein-interaction motif. In the lower part of this Figure, activation of Epac (shown for Epac2) by cAMP results in the opening of the protein to enable interaction with Rap, and consequently, the conversion of RapGDP to RapGTP.

X-ray crystallography of Epac2A and NMR spectroscopy of Epac1 have provided new information about the molecular mechanism by which cAMP controls Epac activation [276]. The 3-D structure of Epac in the presence of cAMP and in its inhibited state has been shown via X-Ray crystallography [276] (Figure 1.18). The regulatory domain of Epac is auto-inhibitory and is repressed by binding of cAMP. In the absence of cAMP, the proteins are in a closed auto-inhibited conformation, and upon cAMP binding, the active conformation is preferred. Binding of cAMP to Epac induces large conformational changes within the protein and releases the autoinhibitory effect of the N terminus of the protein, leading to Rap activation. From this structural analysis, it is thought that activation of Epac requires binding energy.

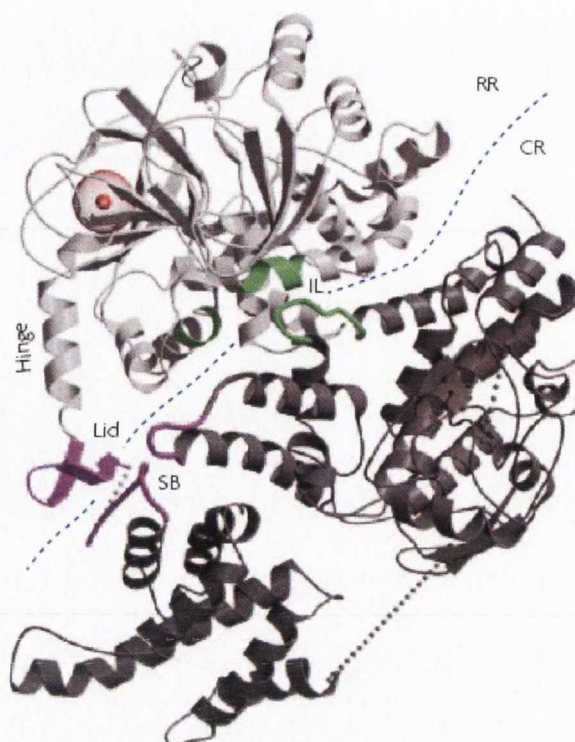


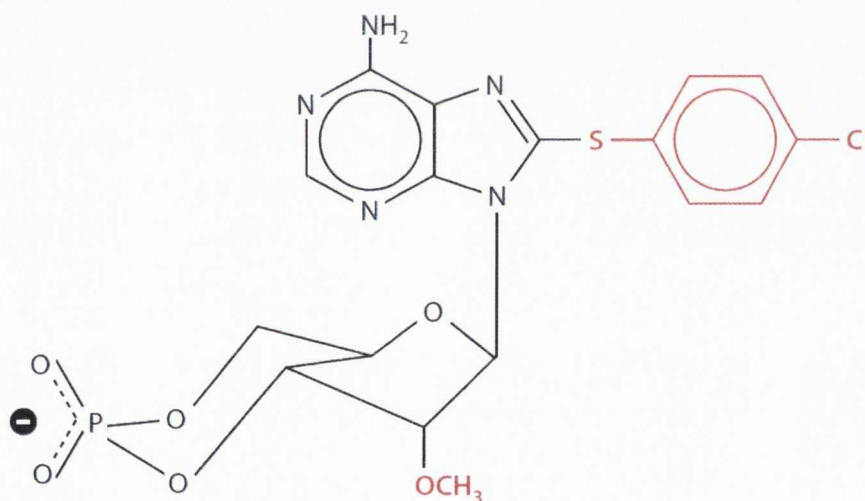
Figure 1.18: The regulation of Epac by cAMP. Epac2 in its inhibited conformation. The structural border between the regulatory region (RR) and the catalytic region (CR) is shown by the dashed blue line. The red sphere shows the cAMP binding site in the second cyclic nucleotide binding (CNB) domain. Figure from: [277]

There are conflicting reports on the binding affinity of Epac and PKA for cAMP. It has been found that the AC_{50} for Epac is higher than that for PKA [278]. AC_{50} is the half maximal activating concentration i.e. the amount of cAMP that activates 50% of its target. This implies that PKA is activated at a lower concentration of cAMP than Epac. However, there has been a conflicting report showing that the binding affinities are similar [279], so it is unclear how PKA and Epac relate to each other with respect to their cAMP affinities. As described in more detail later, spatial regulation of the components of the cAMP signalling pathway affects the results after cAMP elevation. Therefore the differences in cAMP binding affinity for PKA and Epac could be explained by the compartmentalisation of the cAMP signalling pathway constituents.

1.8.3 Epac activation

In order to discriminate between PKA and Epac signalling effects in cells, specific agents to activate these proteins had to be developed. In 2002, a few years after the cloning of Epac, cAMP derivatives capable of specifically activating Epac and PKA were developed [280]. A highly conserved glutamate residue present in the CNBD of

the PKA regulatory subunits was established to be absent in Epac isoforms [280]. This glutamate residue is required for hydrogen bond formation with the 2'-hydroxyl (2'-OH) of the cAMP ribose group of PKA regulatory subunits, but not the Epac proteins [281]. Analogues containing a 2'-O-Me group in the place of this 2'-OH group, selectively bind to the Epac proteins [280]. To increase the ability for these analogues to activate Epac, a parachlorophenylthio (pCPT) group was added at position 8 on the adenine moiety of 2'-O-Me-cAMP. The chemical structure of the Epac activator 8-pCPT-2'-O-Me-cAMP or 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cAMP (8pCPT) is shown in Figure 1.19. The two substituted groups i.e. the 2'-O-Me and the 8'-pCPT, are shown in red. This cAMP analogue, binds both Epac1 and Epac2 proteins with high affinity but has reduced affinity for PKA [280]. 8pCPT activates Rap1 with greater efficiency and potency than cAMP [280, 282]. The Epac agonist activates both Epac1 and Epac2 equally and many studies using the activator have been carried out to establish the role of these proteins in cell function [270].



8-pCPT-2'-O-Me-cAMP

Figure 1.19: The chemical structure of the Epac-selective cAMP analogue 8-pCPT-2'-O-Me-cAMP (8pCPT). The proton of the 2'OH group of the ribose of cAMP has been replaced with a methyl (-CH₃) group, and the proton at the 8' position of the base has been replaced with a 4-chlorophenylthio (pCPT) group (modifications of cAMP to make 8pCPT are indicated in red) to mediate specificity for Epac proteins and increase affinity for Epac1. Figure from [283].

However, it is necessary to be cautious in the use of 8pCPT because it has been demonstrated that depending upon the model or the doses used, 8-pCPT-2'-O-Me-cAMP or its non-hydrolysable form sp8-pCPT-2'-O-Me-cAMP, can non-specifically

activate cAMP/PKA or cGMP/PKG pathways by inhibiting PDE activity [284]. Although 8pCPT is resistant to PDE degradation, it has been reported to bind and thereby inhibit some PDE isozymes, (PDE1, 2, and 6), thereby increasing intracellular cyclic nucleotide levels [284]. It has been suggested that metabolites of 8-pCPT-2'-O-Me-cAMP may regulate gene expression and cell function through unknown signalling pathways [285]. Therefore, it is necessary to prove that any effect of 8pCPT is not due to the activation of targets other than Epac. Currently there is no specific pharmacological antagonist of Epac. siRNA or recombinant mutants of Epac are used to differentiate between Epac- and PKA-mediated effects, and to assess the function of Epac in cells. Interestingly, it has been reported that the Epac2 isoform is a direct target of the widely used antidiabetic drugs, sulfonylureas, an example of which is glybenclamide [286]. This could provide a method to distinguish Epac2 effects from Epac1, depending on the cell type used.

1.8.4 Epac activation effects

Epac proteins have been shown to be involved in a large number of cellular functions such as cell division, cell adhesion and migration, differentiation, secretion and growth. Epac-selective cAMP analogues have helped to reveal cAMP-regulated processes that are mediated via the action of Epac. As described in the following sections, Epac has been found to have effects on cardiac contraction, insulin secretion, vascular permeability, neuronal processes, inflammation and renal function.

1.8.4.1 Epac and the Immune System

As described earlier, cAMP signalling can directly control inflammation by regulating the immune response of leukocytes. Most leukocytes express the Epac1 protein [287], which links part of the cAMP signal to the inflammatory response.

The main function of phagocytic cells in innate immunity is to engulf and kill the pathogen via phagocytosis. Phagocytosis requires increased phagosomal production of reactive oxygen species (ROS), along with the secretion of several inflammatory mediators. PGE₂ is a lipid metabolite generated at sites of inflammation, increases cAMP levels in phagocytes and thus negatively regulates phagocytosis [288]. The

effects of PKA and Epac activation on the inhibition of phagocytosis vary among the different leukocyte cells. FcγR-mediated phagocytosis in alveolar macrophages is inhibited via Epac1 but not via PKA [262]. In contrast to the alveolar macrophages, it is PKA and not Epac that is involved in peripheral blood monocyte phagocytosis [289], whereas in microglia and peritoneal macrophages both of the cAMP effectors inhibit myelin phagocytosis [290]. In liver macrophages, i.e. Kupffer cells, specific Epac activation also suppresses the pathogen-induced production of ROS, in a PKA-independent manner [291].

Early investigations into the suppression of chemokine production following cAMP elevation suggested that it was PKA-dependent [262], but Epac1 activation has been reported to modulate the pathogen-induced production of several inflammatory mediators in various leukocytes [292]. PGE₂ has been found to suppress LPS induced IFN-β production from macrophages in an Epac-dependent manner [293]. At the same time PGE₂ suppresses LPS-induced TNF-α production in these cells in a PKA-dependent but Epac-independent manner. PGE₂ treatment of activated DCs inhibits expression and release of the inflammatory chemokines CCL3 and CCL4 in an Epac-dependent manner [294].

In several cell lines, including lymphocytes, Rap1 has been shown to have a role in integrin-mediated cell adhesion [295], and Rap1 is crucial for chemokine-induced lymphocyte adhesion, polarisation, and transmigration [296]. Therefore, it is possible that Epac may have a proinflammatory role through modulation of leukocyte adhesion and migration. At the site of inflammation, circulating leukocytes bind to the endothelium, and then migrate across the endothelium to the site of infection. Activation of Epac1 in the monocytic cell line U937 results in β-1 integrin activation and adhesion to fibronectin and the vascular endothelium [287]. In addition, 8pCPT enhances polarisation and migration of these U937 cells [287], further suggesting a role for Epac1 in the regulation of leukocyte recruitment.

1.8.4.2 Cardiac Function of Epac

It has long been known that cAMP, via both PKA and cAMP-gated ion channels, is an important regulator of heart function. Depending on the duration of signalling, cAMP can either induce alterations in cardiac contraction by modifying Ca^{2+} levels or induce hypertrophy (enlargement) of the cardiomyocytes and eventually lead to cardiac dysfunction [297]. Epac signalling can increase the contraction of cardiomyocytes and induce cardiomyocyte hypertrophy [283]. It has been reported that Epac has a role in cardiac Ca^{2+} regulation, showing that Epac acts on Ca^{2+} release from the sarcoplasmic reticulum [298].

1.8.4.3 Epac in Insulin Secretion

Exocytosis is a process of cellular secretion or excretion in which substances contained in vesicles are released from the cell by fusion of the vesicular membrane with the outer cell membrane. Epac function has been linked to the regulation of exocytosis. The connection of Epac with exocytosis was first suggested when it was found that Epac2-mediated cAMP-induced Ca^{2+} -dependent secretion of growth hormone from neuronal PC12 cells interacted by interacting with Rim2, a target of the small GTPase Rab3 [299]. There has been some evidence showing that Rap1 may directly link Epac signalling to Ca^{2+} mobilisation through direct protein-protein interactions with the sarco/endoplasmic reticulum Ca^{2+} -ATPase in the endoplasmic reticulum in pancreatic β -cells [300]. In pancreatic β -cells direct activation of Epac with 8-pCPT increased the number of exocytic sites [301] and elevations in intracellular cAMP increased the amount of insulin-containing granules at the plasma membrane, allowing glucose-induced membrane/granule fusion [302]. Epac2, the main Epac isoform expressed in β -cells [303], is needed for the effects of glucose signalling on insulin exocytosis [304]. In addition, specific activation of Epac by 8pCPT induces exocytosis in human β -cells, which requires the presence of glucose [300]. These actions of cAMP seem to have both a PKA-dependent component and an Epac-dependent component.

1.8.4.4 Neuronal effects of Epac

Both Epac1 and Epac2 are expressed in most parts of the nervous system [305]. cAMP elevation, partially via Epac, has been reported to mediate the ability of several neuro-hormones to modulate the release of synaptic vesicles. Epac modulates the downstream signalling of the neuronal hormone γ -aminobutyric acid (GABA) following receptor ligation [306]. GABA reduces cAMP levels and thus inhibits synaptic transmission, which is relieved by 8pCPT [306]. At the neuromuscular junction, Epac activation was shown to increase postsynaptic current following excitation [307]. Epac also enhances exocytosis in neuroendocrine cells [308]. The effects of Epac activation on synaptic transmission can be long-lasting and can affect synaptic plasticity and therefore learning and memory [283]. Together with PKA, Epac contributes to the regulation of neuronal differentiation, neurite outgrowth, and axon regeneration [283].

1.8.4.5 Vascular Function of Epac

The vascular endothelium forms a barrier that controls the exchange of material between the blood and the surrounding tissues and makes up the inner lining of blood vessels [309]. Cell-cell junctions regulate transport between the cells and these junctions are made up of both tight and adherent junctions [309]. Movement of leukocytes from blood to tissues during inflammation depends on the permeability of the endothelial barrier [310]. The increased permeability from inflammatory mediators is reduced by the cAMP pathway's strengthening effect on the endothelial barriers following hormonal signalling. This cAMP effect has been determined to be due to PKA inhibition of actin dynamics [311]. Signalling to Rap via Epac1 contributes to the effects of cAMP-elevating hormones on endothelial function in vascular endothelial cells i.e. it reduces endothelial barrier permeability [312]. The enhanced permeability induced by inflammatory mediators such as thrombin is also counteracted by Epac1 activation [313]. Epac has been found to redistribute junctional molecules (e.g. VE-cadherin) to lateral surfaces where they can then interact with binding partners on adjacent cells and Epac also inhibits cytoskeletal reorganisation which is essential for increased endothelial permeability, thus showing Epac's ability

to reduce vascular endothelial cell permeability [314]. Activation of Epac both stabilises the endothelial barrier and inhibits signalling through the IL-6-STAT3 pathway through induction of SOCS3. Earlier, Epac1 activation in the monocytic cell line U937 increased adhesion to fibronectin and the vascular endothelium (through integrin activation), as well as enhancing the cell's polarisation and migration [287]. This suggests a role for Epac1 in the regulation of leukocyte recruitment through modification of the internal cytoskeletal cell organisation. However, in this instance, there is Epac-mediated inhibition of cytoskeletal reorganisation and integrin modification in the vascular endothelial cells [314]. This contradiction can be explained by the fact that these cell types are different and have dissimilar functions. It suggests that Epac activation has different effects based on the specific cell type where Epac is activated.

1.8.4.6 Epac and cell adhesion and migration

A role for Epac as a key regulator of cell adhesion and migration has been suggested, and the ability of Epac and Rap1 to control the activity of cell surface integrins is a key component. Integrins are a family of heterodimeric transmembrane proteins composed of α and β subunits and are involved in many cell adhesion processes [315]. Integrin activation and their signalling mechanisms are important areas of study, as their ability to control cell adhesion, and therefore processes such as immune cell and tumour cell migration could be used in order to treat inflammatory diseases and cancer.

Rap1 regulates the integrins and a defect in Rap1 activation has been shown to be the basis of an inherited leukocyte adhesion deficiency disease (LAD-III) [316]. Rap1 alters integrins activity by changing both ligand affinity and avidity (i.e. by grouping integrins at the cell surface). Adhesion via integrins of the OvCar3 cell line to fibronectin has been shown to be cAMP-dependent and PKA-independent and over-expression of Epac1 in these cells increased both basal and cAMP-stimulated adhesion [317]. Epac is also involved in the regulation of leukocyte integrin activity. Primary monocyte adhesion via integrins to HUVECs under flow was increased upon Epac activation [287]. Unsurprisingly neither Rap1 nor Epac regulate activation of all

integrin types. The localisation within the cell has been suggested to be a factor in determining whether Epac activation results in integrin activation. In unpolarised, non-migrating cells Epac is usually localised to a perinuclear region [318] and in T cells it has been shown that activation of a pool of Rap1 at the plasma membrane results in integrin activation [319]. From these studies it is clear that Epac plays a role in regulating integrins both in inflammatory processes and in cells not directly involved in inflammation.

1.9 Spatial regulation of cAMP and its effectors in the cell

Although cAMP can rapidly diffuse within the cytosol, there is not a homogenous increase of cAMP within the cell following ligation of a cAMP-elevating hormone to its receptor. In fact, cAMP becomes unevenly distributed and concentrated in local microdomains [320]. PDEs, which are confined to specific subcellular compartments, are the main culprits of this compartmentalisation. These PDEs degrade cAMP levels locally and thus generates cAMP gradients in the cell. In addition to the compartmentalisation of cAMP, the cellular location of the cAMP effectors are also regulated by binding to scaffolding proteins, as has been extensively studied for PKA [321]. A-kinase anchoring proteins (AKAPs) target PKA to distinct subcellular locations and mediate the assembly of large signalling complexes, thereby linking PKA to specific cellular functions. Similarly, Epac proteins are spatially regulated by different anchoring mechanisms, which can discriminate between Epac1 and Epac2.

The sequestration of a signalling enzyme to a specific subcellular environment, ensure that upon activation, the enzyme is near its relevant targets. AKAPs play a central role in cAMP signalling, altering both downstream and upstream events in the cAMP pathway and allowing the second messenger to have localised effects [321]. The correct intracellular targeting of Epac, PKA, PDEs and AC enzymes, may be critical in determining the response of cells to elevations in intracellular cAMP. This suggests that cAMP signalling is spatially and temporally regulated by diverse anchoring mechanisms, which control specific functions of the cAMP effectors by recruitment to distinct subcellular locations.

The AKAP protein family is quite diverse and contains more than 50 members. These proteins share some common features; a PKA-anchoring domain that binds PKA, they bind other signalling enzymes to form multi-protein complexes and they target these signalling complexes to specific subcellular sites through various targeting motifs like lipid modifications and protein-protein interaction domains [322]. AKAPs localise PKA to specific subcellular sites, thus focusing PKA activity towards its relevant substrates. These anchored pools of PKA must then be selectively activated and this means that cAMP is must be available in these discrete areas [323]. The targeting and regulation of PKA-mediated phosphorylation of its substrates is another area where AKAPs play an important role. AKAPs are also capable of forming multi-protein complexes that integrate cAMP signalling with other pathways and signalling events. Various extracellular signals activate cAMP/PKA via binding to GPCRs and therefore the cAMP/PKA pathway is tightly regulated on several levels to maintain specificity. AKAPs and PDEs have been shown to be colocalised in cells. In addition to spatially restricting PKA activity, AKAP-PDE complexes also function to ensure that PKA activity is rapidly quenched by the local degradation of cAMP (Figure 1.20). Physiological increases in cAMP concentration occur in discrete microdomains. Most cAMP/PKA-regulated physiological processes require an anchored kinase. The localised cAMP microdomains require carefully compartmentalised and anchored pools of PDE enzymes.

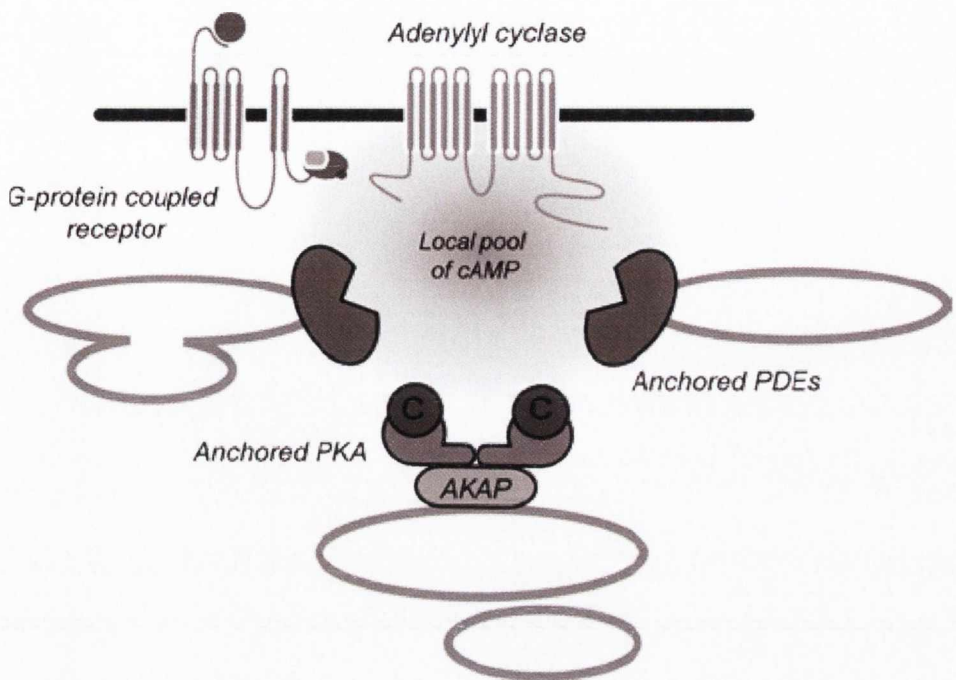


Figure 1.20: Ligand binding to various GPCRs activates adjacent ACs and generates local pools of cAMP. PDEs regulate the local concentration and distribution of cAMP. Particular GPCRs are confined to specific domains of the cell membrane in association with intracellular organelles or components of the cytoskeleton. The subcellular structures may contain specific PKA isoenzymes that, through anchoring via AKAPs, are maintained close to the receptor and the AC. PDEs are also anchored and serve to limit the extension and duration of cAMP signals. These mechanisms serve to localize and limit the assembly and triggering of specific pathways to a defined area of the cell close to the substrate. Figure from [257]

Subcellular localisation of PKA is mainly determined by anchoring of the R subunits (of PKA) by AKAPs, which originally were seen as contaminants of purified PKA [324], and later understood to enhance the efficiency and specificity of the signalling events. The anchoring of PKA by AKAPs confines PKA activity to a relevant subset of potential substrates. At the same time PKA itself is regulated by its two regulatory subunits. The majority of known AKAPs bind specifically to the RII holoenzyme. Although some AKAPs bind to both PKA subtypes [321]. PKA type I is mainly cytoplasmic while PKA type II is typically particulate and confined to subcellular structures and compartments anchored by cell- and tissue-specific AKAPs [322].

Epac1 binding to cAMP induces a conformational change which targets Epac1 to the plasma membrane via its DEP domain [325]. Epac1 targeting to the plasma membrane is needed to induce Rap activation (at the plasma membrane) and for efficient cell adhesion via integrin signalling [325]. Activated Ras proteins bind

Epac2 via its RA domain, thus targeting it to the plasma membrane, but this is not dependent on its conformation [326]. An alternative membrane targeting sequence resides within the N-terminus of Epac2; this sequence is absent in the adrenal gland-specific Epac2B isoform [272]. Both mechanisms of membrane targeting of Epac2 have been associated with Rap-mediated processes at the plasma membrane.

AKAPs have been shown to alter the spatial regulation of Epac. AKAP multiprotein complexes containing Epac have been identified in various cell types [327]. In neuronal cells, Epac2 has been shown to form a complex with the plasma membrane-associated AKAP79/150, PKA and PKB/Akt [328]. In contrast to PKA, direct activation of Epac2 increased phosphorylation of PKB/Akt, a protein known to be involved in neuronal regulation. AKAP150 seems therefore to coordinate PKA and Epac-mediated PKB/Akt phosphorylation. AKAP9 and Epac1 interact together and affect microtubule dynamics, which enhances the endothelial cell barrier [329]. At the plasma membrane, Epac has been shown to be involved in synapse morphology and function when it interacts with the anchoring proteins PSC-95 and/or Neuroligins [330]. Epac1 is also targeted to microtubules in both interphase and mitotic cells [274]. This targeting may be mediated either by direct interactions with tubulin or by the microtubule-associated protein MAP1 [331]. Other reported localisations of Epac1 that may be associated with distinct functions include centrosomes [332], the nuclear pore complex [333], mitochondria [274], macrophagic phagosomes [332], and the apical membrane of renal epithelial cells [334].

1.10 Cyclic AMP effectors and their effects on transcription factors involved in immune responses

Multiple transcription factors are involved in the immune response and function by binding to and inducing activation-specific genes. These transcription factors include members of the NF- κ B/Rel/NFAT, AP-1 and CREB/ATF families [335]. Several of these transcription factors can be modulated by PKA activity. Activation of T cells through the TCR results in redistribution and activation of several kinases and signalling proteins downstream of the TCR.

CREB

One of the principle targets of cAMP signalling is the cAMP response element (CRE)-binding protein (CREB). CREB is a transcription factor involved in immune system gene regulation and PKA plays a major role in this process [336]. Following activation of PKA, some of the C subunits released from the regulatory subunits are able to enter the nucleus from the cytoplasm [337]. The transcriptional activity of CREB is regulated by a single phosphorylation on Ser-133 that leads to complex formation with the coactivator, CBP and binding to CRE elements [338]. CREB is known to be phosphorylated in response to various stimuli, but PKA-mediated phosphorylation of CREB on Ser133 is essential for transcription activation in response to cAMP [339]. Phosphorylation of Ser133 promotes recruitment of the coactivator CBP or its paralogue p300 [338]. In T cells, CRE elements can be found in the TCR and CD3 genes [340, 341], as well as in other genes involved in lymphocyte activation. It is thought that CREB has an essential role in controlling genes involved in cell cycle progression and proliferation.

NFAT

Binding sites for nuclear factor of activated T cells (NFAT) have been identified in several genes involved in T cell activation including the IL-2 gene [342]. In resting cells, NFAT-proteins are retained in the cytoplasm until a nuclear localisation signal is unmasked as a result of dephosphorylation by the Ca^{2+} -dependent phosphatase, Calcineurin. This results in rapid nuclear translocation, association with AP-1 and binding to NFAT sites. PKA inhibition of IL-2 expression has been reported several times. Transfection of the PKA catalytic subunit inhibits IL-2 promoter activity while overexpression of NFAT counteracts the inhibitory effect of PKA [343]. Calcineurin and PKA act as opposing regulators of the NFAT-14-3-3 complex. Dephosphorylation by Calcineurin results in release of NFAT from 14-3-3 and nuclear translocation, while phosphorylation by PKA creates a binding site for 14-3-3 and results in reduced NFAT activity.

NF- κ B

NF- κ B is an inducible transcription factor and a key mediator of both innate and adaptive responses. It regulates a wide variety of cellular and viral genes including several cytokines, cell adhesion molecules and human immunodeficiency virus (HIV) [344]. NF- κ B consists of homodimers or heterodimers belonging to the Rel family of proteins [335]. Most of these contain a PKA consensus phosphorylation site (RRXS) located close to the NLS (nuclear localisation sequence), and there have been several reports of NF- κ B activation as a result of PKA phosphorylation [345]. NF- κ B family members in mammalian cells include c-Rel, Rel A (p65), Rel B, NF- κ B1 (p50/105), and NF- κ B2 (p52/p100). These proteins share a conserved 300 amino acid region known as the Rel homology domain, which is responsible for DNA binding, dimerisation, and nuclear translocation. PKA activation in macrophages was found to induce NF- κ B activation and IL-6 activation [346]

In its resting state, NF- κ B is complexed in the cytoplasm with its inhibitor I κ B that prohibits nuclear translocation and activation. Upon activation by TNF- α , UV or triggering of some Toll-like receptors, I κ B is phosphorylated by I κ B kinase, targeted for degradation and then releases NF- κ B. A portion of the PKA catalytic subunit (PKA-C), but not the PKA regulatory subunit (PKA-R), binds to I κ B and associates with the NF- κ B-I κ B complex [345]. This cAMP-independent portion of PKA-C is bound to I κ B and kept in an inactive state until I κ B is degraded and the active PKA-C is released. Active PKA-C then phosphorylates the p65 Rel subunit of NF- κ B leading to increased activating activity of NF- κ B. Therefore, NF- κ B can be regulated by two different populations of PKA-C. One of these populations is sensitive to cAMP- and PKA-mediated regulation of transcriptional elements, and places PKA in key positions for fine tuning expression of genes involved in immune activation.

LPS activates NF- κ B through TLR4-dependent signal transduction [347]. Stimulation with LPS also results in elevated cAMP in Raw 264.7 murine macrophages and there have been conflicting reports regarding the action of cAMP/PKA on NF- κ B [345] [348]. On the one hand, signals that cause the degradation of I κ B result in activation

of PKA in a cAMP-independent manner and the subsequent phosphorylation of p65. Phosphorylation by PKA both weakens the interaction between the N- and C-terminal regions of p65 and creates an additional site for interaction with CBP/p300. Therefore, PKA regulates the transcriptional activity of NF- κ B by modulating its interaction with CBP/p300 [349]. In contrast, the inhibitory action of the cAMP/PKA pathway on the transcriptional activity of NF- κ B appears to be exhibited by modifying the C-terminal transactivation domain of p65, either directly or indirectly [348]. Although PKA is known to affect NF- κ B activation, there has only been one study carried out to determine if Epac-mediated Rap1 activation, affects NF- κ B activity [350]. This study found that the transcriptional activity of NF- κ B could be elevated by an Epac1-mediated Rap1 pathway in murine macrophages. This suggested that Epac activation is capable of activating NF- κ B through Rap1

Project Aims and Objectives

Current subunit vaccine strategies involve the addition of an adjuvant to antigens in order to make the vaccine more effective. Alum is the most common adjuvant in use however it predominantly drives a Th2 response in recipients. There is a need for adjuvants capable of driving more potent Th1 responses, which are required for protective immunity against certain diseases such as T.B. or malaria. The ultimate aim of this project is to determine if Epac has potential to be used as a Th1 driving adjuvant in vaccine formulations. The widespread expression of the Epac proteins in immune cells and the currently unknown effects of Epac activation on cytokine production suggest that the potential for an Epac activator to act as a Th1 driving adjuvant should be investigated.

The effects of Epac activation on innate and adaptive immunity have not been investigated previously. PKA, on the other hand, has long been recognised as a cAMP effector, although its reported effects on the immune system are contradictory. The dogma indicates that it has an anti-inflammatory role while at the same time it activates NF- κ B and increases IL-6 production. This project aims to determine and compare the consequences of Epac and PKA activation on the innate and adaptive immune systems, and to investigate if there is a potential for manipulating these systems in developing adjuvants for inducing proinflammatory T cell responses.

This project aims to investigate the following questions:

1. Does Epac activation modulate DC maturation and cytokine production?
2. What effects does activation of Epac have on innate immunity *in vivo*?
3. Does Epac activation modulate adaptive immune responses and have potential as adjuvant for driving a pro-inflammatory Th1 or Th17 responses?

Hypothesis:

Activation of the Epac pathway modulates innate and adaptive immune responses and has potential as an adjuvant strategy to promote cell mediated immunity.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Tissue Culture Reagents

Ammonium chloride lysis solution

0.88% w/v NH₄Cl dissolved in endotoxin-free distilled water (dH₂O) filter sterilised. Stored at 4°C.

Cell culture medium

Roswell Park Memorial Institute (RPMI)-1640 medium (Biosera) was supplemented with 8% (v/v) heat inactivated (56°C for 30min) foetal calf serum (FCS, Biosera), 100mM L-Glutamine (Gibco) and 100µg/ml penicillin/streptomycin (Gibco). Complete RPMI (cRPMI) medium was used to culture all cells *in vitro*.

2.1.2 *In vitro* stimulation materials

Table 2.1: PRR agonists used *in vitro*

Ligand	Receptor	Source
Lipopolysaccharide (LPS) <i>E. coli</i> , Serotype R515	TLR4	Alexis Biochemicals (Axxora Platform)
CpG	TLR9	Oligos Etc.
Heat-killed bacteria	Various	N/A

Table 2.2: *In vitro* stimulation

Stimulus	Concentration	Source
Anti-CD3	0.01µg/ml - 1µg/ml	BD Pharmingen
PMA	25ng/ml	Sigma-Aldrich
Ionomycin	200ng/ml	Sigma-Aldrich

Bacteria-derived molecules

The heat-killed bacteria used *in vitro* for the stimulation of cells were derived from *Escherichia coli* (*E. coli* – BL21 strain). An agar plate of Luria-Bertani (LB) broth (Sigma) was streaked with *E. coli* from a glycerol stock in the –80°C freezer. This

was left to grow overnight at 37°C and the next day a single colony was isolated and grown up overnight at 37°C in 10ml LB broth. This overnight culture was transferred into 100ml LB broth and incubated shaking at 37°C for 2 hours. A sample was taken to measure the OD₆₀₀. A further sample was taken to determine the concentration by diluting and then plating onto LB plates and growing up. The cells were heated to 70°C for 15 minutes to kill them, after which they were plated onto LB plates to ensure no growth occurred and the cells were dead. The cells were pelleted and resuspended in phosphate buffered saline (PBS), to a final concentration of 1×10^9 cells/ml. These were aliquotted and stored at -80°C.

2.1.3 Agonists/Inhibitors used for *in vitro* and *in vivo* studies

All reagents are from Sigma unless otherwise stated.

Agonists/Inhibitors

8-(4-Chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT-2'-O-Me-cAMP) is a potent, specific and membrane-permeable activator of the exchange factor directly activated by cAMP (Epac) and was obtained from BioLog Lifescience Institute, Germany. The compound was dissolved in endotoxin-free PBS to make a 5mM stock solution. N⁶-Benzoyladenosine-3', 5'-cyclic monophosphate (N⁶-Benzoyl-cAMP) is a membrane-permeant and site-selective activator of cAMP-dependent protein kinases (PKA) and was obtained from BioLog Lifescience Institute, Germany. The compound was dissolved in endotoxin-free PBS to make a 1mM stock solution. N⁶,2'-O-Dibutyryladenosine-3'-5'-cyclic monophosphate·Na (Dibutyryl-cAMP) is a cell permeable analogue which preferentially activates cAMP-dependent protein kinase and was obtained from Biomol International, LP.

Table 2.3: List of cAMP pathway modulators used *in vitro* and *in vivo*

Inhibitor/Agonist	Specificity	Source
8-pCPT-2'-O-Me-cAMP	Epac agonist	BioLog, Germany
N ⁶ -Benzoyl-cAMP	PKA agonist	BioLog, Germany
Dibutyryl-cAMP	cAMP analogue	BIOMOL International LP
Forskolin	adenylate cyclase activator	BIOMOL International LP
IBMX	non-specific PDE inhibitor	BIOMOL International LP
Glybenclamide	Epac2 agonist/sulfonylurea drug	Sigma

2.1.4 ELISA reagents

Buffers and solutions for ELISA:

Phosphate-buffered saline (PBS) 10X

400g NaCl
58g Na₂HPO₄ or 143.3g Na₂HPO₄.12H₂O
10g KH₂PO₄
10g KCl
Dissolved in 5L of dH₂O and brought to pH 7.2
Diluted to 1x with dH₂O before use.

Wash Buffer: PBS with 0.05% (v/v) Tween-20

1L 10x PBS pH 7.2
5ml Tween-20
Made up to 10L with dH₂O

Reagent diluent

BD Pharmingen kits (IFN- γ , IL-4, IL-6, IL-5, IL-12p40): made up in 1x PBS
R&D Systems Duosets (IL-12p70, IL-23, IL-10, IL-17): made up in 1% (w/v) Bovine Serum Albumin (BSA) made in 1x PBS
Biolegend kits (IL-10, IL-17): 1% (w/v) BSA made in 1x PBS

Block solution

BD Pharmingen: 10% (w/v) milk dissolved in PBS (except IL-6: 3% (w/v) BSA)

R&D Systems Duosets & Biolegend kits: 1% (w/v) BSA, dissolved in 1x PBS

Biolegend kits (IL-10 and IL-17): 1% (w/v) BSA dissolved in 1x PBS

Phosphate Citrate Buffer (1L)

10.19g anhydrous citric acid

36.9g Na₂HPO₄·12H₂O or 14.6g Na₂HPO₄

made up to 1L with dH₂O and brought to pH 5.0

Stop solution (1M H₂SO₄)

26.74ml 18M H₂SO₄

473.26ml dH₂O

Add 20µl per well of 96-well plate to stop enzyme reaction

Carbonate Buffer

4.2g NaHCO₃

1.78g Na₂CO₃

Make up to 500ml with dH₂O and bring to pH 9.5

Table 2.4: ELISA antibodies for cytokine ELISA

Antibody	Source	Test sample dilution
IL-10	R&D Systems	Neat or 1/2
IL-10	Biolegend	Neat or 1/2
IL-12p70	R&D Systems	Neat
IL-23	R&D Systems	Neat
IL-17	R&D Systems	Neat
IL-17	Biolegend	Neat
IL-4	BD Pharmingen	Neat
IL-5	BD Pharmingen	Neat
IL-6	BD Pharmingen	1/50 for DCs & Neat for splenocytes
IFN-γ	BD Pharmingen	Neat
IL-12p40	BD Pharmingen	1/100 or 1/200 for DCs & Neat for splenocytes

All supernatants were diluted as outlined above for cytokine analysis except for *in vivo* studies when the sample supernatants were not diluted.

Table 2.5: ELISA reagents for serum antibody detection.

Antibody	Source	Starting sample dilution		Blocking buffer
		Pre-boost	Post-boost	
IgG	Sigma	1/100	1/2000	10% (w/v) Milk
IgG1	BD Pharmingen	1/100	1/2000	10% (w/v) Milk
IgG2a	BD Pharmingen	1/100	1/100	10% (w/v) Milk
IgG2b	BD Pharmingen	1/100	1/100	10% (w/v) Milk
IgG3	BD Pharmingen	1/100	1/100	10% (w/v) Milk
IgG2c	BD Pharmingen	1/100	1/100	10% (w/v) Milk

Table 2.6: Recombinant cytokines used as ELISA standards

Cytokine	Standard Range	Source
Mouse IL-10	0 – 2000pg/ml	R&D Systems
Mouse IL-12p70	0 – 2500pg/ml	R&D Systems
Mouse IL-17	0 – 1000pg/ml	R&D Systems
Mouse IL-4	0 – 2500pg/ml	BD Pharmingen
Mouse IL-5	0 – 2500pg/ml	BD Pharmingen
Mouse IL-6	0 – 5000pg/ml	BD Pharmingen
Mouse IFN- γ	0 – 10ng/ml	BD Pharmingen
Mouse IL-12p40	0 – 5000pg/ml	BD Pharmingen
Mouse IL-23	0 – 2500pg/ml	R&D Systems

2.2.5 Flow cytometry reagents

FACS buffer

2% (v/v) FCS (Biosera)

0.1% (w/v) sodium azide

made in 1x PBS and stored at 4°C.

FACS blocking buffer

Anti-CD16/CD32 (Fcγ Block; BD Pharmingen).

Add at a concentration of 2.5µg/ml in FACS buffer.

Table 2.7: Antibodies for evaluation of DC activation

Antibody specificity	Clone	Source	Final conc.
CD80-FITC	16-10A1	BD-Pharmingen	1µg (0.2µl/100µl sample)
CD86- PE	GL-1	BD-Pharmingen	0.4µg (0.2µl/100µl sample)
CD40-FITC	3/23	BD-Pharmingen	1µg (0.2µl/100µl sample)
CD40-APC	1C10	eBioscience	0.4µg (0.2µl/100µl sample)
I-A/I-E (MHC class II)-PE	M5/114.15.2	BD-Pharmingen	0.6µg (0.3µl/100µl sample)
I-A/I-E (MHC class II)- APC-eFluor™ 780	M5/114.15.2	eBioscience	0.6µg (0.3µl/100µl sample)
CD11c-APC	HL3	BD-Pharmingen	0.3µg (0.15µl/100µl sample)
CD11c PerCP-Cy5.5	HL3	BD-Pharmingen	0.3µg (0.15µl/100µl sample)

Table 2.8: Antibodies for identification of T cell subsets and intracellular cytokine staining

Antibody specificity	Clone	Source	Final conc.
CD3 FITC	145-2C11	BD-Pharmlngen	1µg (0.2µl/100µl sample)
CD3 APC-Cy7	17A2	BD-Pharmlngen	1µg (0.5µl/100µl sample)
IFN-γ PE	XMG1.2	BD-Pharmlngen	0.6µg (0.3µl/100µl sample)
IL-17 PerCP-Cy5.5	TC11-18H10	BD-Pharmlngen	0.6µg (0.3µl/100µl sample)
CD8α PE-Cy7	53-6.7	BD-Pharmlngen	0.4µg (0.2µl/100µl sample)
CD4 APC	RM4-5	BD-Pharmlngen	0.4µg (0.2µl/100µl sample)
CD197(CCR7) PerCP-Cy5.5	4B12	BD-Pharmlngen	1µg (0.5µl/100µl sample)
CD62L APC	MEL-14	BD-Pharmlngen	1µg (0.5µl/100µl sample)
CD49b FITC	DX5	BD-Pharmlngen	2.5µg (0.5µl/100µl sample)
γδTCR FITC	GL3	BD-Pharmlngen	2.5µg (0.5µl/100µl sample)
CD11c PerCP-Cy5.5	HL3	BD-Pharmlngen	0.3µg (0.15µl/100µl sample)
CD11b Pe-Cy7	M1/70	BD-Pharmlngen	0.4µg (0.2µl/100µl sample)

2.1.6 Real-time PCR reagents

Table 2.9: Primer sets for qPCR: (Source: MWG Operon)

Primer		Primer sequences (5'-3')
β-actin	FP	TCCAGCCTTTCTTGGGT
	RP	GCACTGTGTTGGCATAGAGGTC
IFN-γ	FP	CAGCAACAGCAAGGCGAAA
	RP	CTGGACCTGTGGGTTGTTGAC
T-bet	FP	GCCAGGGAACCGCTTATATG
	RP	GACGATCATCTGGGTCACATTGT
IL-18	FP	AATGACCAAGTTCTCTTCGTTGAC
	RP	ACAGCCAGTCCTCTTACTTCAC
EPAC1	FP	GTGTTGGTGAAGGTCAATTCTG
	RP	GCCACACCACGGGCATCT
IL-12p35	FP	GGTGAAGACGGCCAGAGAAA
	RP	GGCAACTCTCGTTCTTGTGTAG
IL-12p40	FP	GTGTAACCAGAAAGGTGCGTTC
	RP	TCGGACCCTGCAGGGAAC

(FP = forward primer, RP = reverse primer)

2.1.7 Confocal microscopy reagents

Table 2.10: Confocal microscopy reagents and antibodies:

Reagent	Source	Final concentration
Rabbit anti-mouse EPAC1 (H-70 clone)	Santa Cruz Biotechnology	1µg/ml
Alexa Fluor 488-conjugated goat anti-rabbit IgG	Invitrogen	4µg/ml
Alexa Fluor 594-conjugated wheat germ agglutinin (WGA)	Invitrogen	2µg/ml
Bisbenzimidide H 33258 (Hoechst)	Sigma	25µg/ml
Fluorescent Mounting Medium	Dako, Ireland	N/A
Goat-serum	Sigma	5% (v/v)
Alexa Fluor 568-conjugated goat anti-rabbit IgG	Invitrogen	4µg/ml
Alexa Fluor 488-conjugated phalloidin	Sigma	50µg/ml

2.2 Methods

2.2.1 Animals

Specific pathogen-free female BALB/c and C57/BL6 mice were acquired from Harlan UK Ltd (Bicester, Oxon, UK) and maintained in the BioResources unit in TCD according to the regulations and guidelines of the Irish Department of Health and Children and the European Union. Experiments were performed under licence from the Department of Health and with the approval of the Trinity College Dublin BioResources Ethics Committee.

2.2.2 Cell Culture

Cell culture

Cells were cultured at 37°C with 5% CO₂.

Cell counts

Cell viability was determined by trypan blue exclusion. Cells were diluted at either a 1/10 or a 1/50 dilution (depending on starting cell density) in trypan blue (Sigma-Aldrich). 10 µl of the cell suspension was loaded onto a disposable haemocytometer (Hycor Biomedical, USA). The numbers of viable cells were counted using a light microscope. The number of cells per ml was determined using the following formula: number of cells/ml = cell number $\times 10^4 \times$ dilution factor.

2.2.2.1 Culturing of the GM-CSF secreting J558 cell line

The GM-CSF secreting J558 cell line was a gift from Dr. Nathalie Winter, Pasteur Institute, and was generated by cloning the mouse gene for granulocyte-monocyte colony stimulating factor (GM-CSF) into a mammalian expression vector [351], which was subsequently transfected into the J558 cell line. These cells secrete GM-CSF into their growth medium, which can then be quantified and used to grow BMDCs. The cells were retrieved from frozen stock (liquid nitrogen) and the first two passages of the cell line were grown (1×10^6 cells/ml) in selection medium, RPMI supplemented with G418 (Geneticin 1mg/ml, Gibco), at 37°C. After the second

passage in selection medium, the cells (1×10^6 cells/ml) were grown without G418 to a 'medium' density, harvested and washed in RPMI. The cells were re-seeded at 2.5×10^5 cells/ml for future passages. The supernatant from the cell line was harvested after pelleting the cells by centrifugation (1200rpm for 5 minutes). The cells were resuspended in cRPMI and cultured for a further 8 passages. The supernatant was collected at each passage until passage number 9. This supernatant was pooled and stored at -20°C until GM-CSF was quantified by ELISA (R&D Systems) and aliquotted into smaller volumes for DC culture.

2.2.2.2 Murine bone marrow-derived DC isolation and culture

Bone-marrow derived dendritic cells (BMDCs) were generated from BALB/c mice using a method similar to that described by Lutz *et al* [352]. Mice were euthanised and their femurs and tibiae removed and dissected from the surrounding muscle tissue. The bone marrow was flushed out with cRPMI using a 27G needle. The cell aggregates were broken up using a 19G needle and the cell suspension was pelleted by centrifugation (1200rpm for 5min). The pellet was resuspended in 1ml of ammonium chloride (0.88% w/v) lysis solution for 2min to lyse the red blood cells. The cells were then washed in cRPMI, pelleted by centrifugation (1200rpm for 5 min) and resuspended in 10ml of cRPMI. Cell viability was assessed by trypan blue exclusion. Immature BMDC were cultured at 1×10^6 cells/ml in cRPMI supplemented with supernatant from the GM-CSF expressing J558 cell line (20ng/ml GM-CSF). After 3 days incubation, 30ml of fresh cRPMI containing 20ng/ml GM-CSF was added to each culture flask.

On day 6 the flasks were removed from the incubator and cell culture supernatant was carefully removed to eliminate non-adherent cells (e.g. granulocytes) from the culture. 25ml of sterile pre-heated (37°C) PBS (Biosera), was added to each flask and the cells were removed by gentle repeat pipetting whereupon the PBS suspension was transferred into 50ml tubes containing 10ml fresh medium. Sterile pre-heated (37°C) EDTA (25ml 0.02%; Sigma-Aldrich) was added to each flask and the flasks were placed in the incubator for 10 minutes. These flasks were removed from the incubator; the cells were removed by gentle repeat pipetting and transferred to a 50ml tube with 10ml cRPMI. The cells derived from the PBS wash and EDTA wash were pelleted by centrifugation (1200rpm for 5 minutes), pooled together and resuspended in cRPMI. Cell viability was assessed by trypan blue exclusion. Cells were cultured at 1×10^6

cells/ml in cRPMI medium with 20ng/ml GM-CSF. After 2 days incubation (day 8), 30ml cRPMI with 20ng/ml GM-CSF was added to the flasks. After a further 2 day incubation (day 10) the loosely adherent cells were harvested by gentle repeat pipetting. Cell viability was assessed and cells were plated in 96-well plates for DC stimulation experiments (6.25×10^5 cells/ml) in cRPMI containing 10ng/ml GM-CSF. The DCs were left overnight at 37°C and then stimulated the next day.

An immortalised bone-marrow derived macrophage cell line (BMDM) from C57/BL6 mice was used to produce macrophages for confocal microscopy analysis.

2.2.2.3 Culture of murine BMDC in the presence of the Epac agonist

DCs were isolated according to the protocol described above, with the following modification. The Epac agonist 8-pCPT-2'-O-Me-cAMP (0.03pM – 30nM) was added at day 0, day 3, day 6, day 8 and day 10 of culture.

2.2.2.4 Stimulation of BMDCs *in vitro*

The precise conditions used for stimulation of DCs *in vitro* are outlined in each Figure legend. Murine BMDCs were plated at 6.25×10^5 cells/ml in 96 well round bottom plates (Grenier Bio-one). Cells were stimulated (Table 2.3) for 1h before addition of TLR agonists and then left for 24 hours before the supernatants were collected and analysed for cytokines by ELISA (Section 2.2.3.1). In experiments investigating DC maturation, 24hr after stimulation of the DCs, the cells were gently removed, washed and used for immunofluorescence analysis (section 2.2.5.1).

2.2.2.5 Antigen-specific cytokine production by lymph node, peritoneal cells and spleen cells *ex vivo*.

Isolated spleens and draining lymph nodes were homogenised and passed through a 70µm cell strainer (BD Pharmingen) to obtain single cell suspensions. Spleen cells were centrifuged at 1200rpm for 5 minutes and red blood cells were lysed by resuspending cells in 1ml of 0.88% (w/v) ammonium chloride solution for 2 minutes. Cells were washed and resuspended in fresh cRPMI medium. Peritoneal cells isolated via lavage were centrifuged and resuspended in cRPMI for stimulation. Spleen cells (2×10^6 cells/ml), lymph node cells (1×10^6 cells/ml) or peritoneal cells (1×10^6 cells/ml) were cultured in triplicate wells of 96-well 'U-bottomed' microtitre plates at

37°C and 5% CO₂ with various concentrations of antigen, or with the mitogen PMA (25ng/ml, Sigma-Aldrich) and anti-CD3 (0.01 – 0.1µg/ml, BD Pharmingen SanDiego USA) or medium only as positive and negative controls respectively. Supernatants were collected for analysis of cytokine production by ELISA as in section 2.2.3.1.

2.2.3 Analysis of Immune responses

2.2.3.1 Measurement of cytokine production by ELISA

Concentrations of IL-12p70, IL-23, IL-17, IL-10 and GM-CSF were measured using commercially available ELISA kits (R&D Systems). Concentrations of IL-10 and IL-17 were also measured using commercially available kits from Biolegend. Concentrations of IL-6, IL-12p40, IL-4, IL-5 and IFN-γ were measured using commercially available ELISA antibody pairs (BD Pharmingen). The ELISAs were carried out according to the manufacturer's protocols as outlined below.

High binding 96-well plates (Grenier Bio-one) were coated overnight at 4°C or for 2hrs at 37°C with 40µl/well of rat anti-mouse capture antibody specific for the cytokine of interest (Table 2.11) in PBS. After washing, non-specific binding sites were blocked with 100µl/well of blocking solution (see Table 2.4) for 2hrs at room temperature. Plates were washed and then 30µl of test supernatant (diluted as per Table 2.4) or serially diluted standards (Table 2.6) were added to plates and incubated overnight at 4°C, or for 2hrs at 37°C. Plates were then washed and 40µl/well of a specific biotinylated goat anti-mouse IgG antibody, specific for the cytokine of interest, was added (Table 2.11), and the plates left for 2hrs at room temperature. After this incubation, plates were washed and incubated in the dark for 30 minutes with 40µl/well of horseradish-peroxidase (HRP)-conjugated streptavidin (1:200 in 1% (w/v) BSA/PBS for R&D kits, or streptavidin from Sigma-Aldrich at 1/750 dilution for all other kits). Finally plates were washed and 40µl of Ortho-Phenylenediamine (OPD) substrate in phosphate citrate buffer (0.4mg/ml) was added per well, for all cytokines except IL-12p70. IL-12p70 was developed using 3,3',5,5'-tetramethylbenzidine (TMB) (Millipore). The enzyme reaction was quenched by the addition of 1M H₂SO₄. The O.D. values were determined by measuring absorbance at 492nm (or 450nm for IL-12p70) using a microtitre plate reader (Multiscan FC,

Thermo Scientific). Cytokine concentrations for test samples were evaluated using a standard curve prepared using recombinant mouse standards of known concentrations (Table 2.6).

Table 2.11: ELISA antibodies for detection of cytokines.

Capture Antibody	Source	Concentration
IL-10 (purified)	R&D Systems	4µg/ml
IL-10 (purified)	Biolegend	1/200 dilution
IL-23 (purified)	R&D Systems	4µg/ml
IL-12p70 (purified)	R&D Systems	4µg/ml
GM-CSF (purified)	R&D Systems	2µg/ml
IL-17 (purified)	R&D Systems	2µg/ml
IL-17 (purified)	Biolegend	1/200 dilution
IL-4 (purified)	BD-Pharmingen	0.5µg/ml
IL-5 (purified)	BD-Pharmingen	1µg/ml
IFN-γ (purified)	BD-Pharmingen	2µg/ml
IL-12p40 (purified)	BD-Pharmingen	0.25µg/ml
IL-6 (purified)	BD-Pharmingen	1µg/ml
Detection Antibody	Source	Concentration
IL-10 Biotin	R&D Systems	500ng/ml
IL-10 Biotin	Biolegend	1/200 dilution
IL-23 Biotin	R&D Systems	200ng/ml
IL-12p70 Biotin	R&D Systems	400ng/ml
GM-CSF Biotin	R&D Systems	50ng/ml
IL-17 Biotin	R&D Systems	200ng/ml
IL-17 Biotin	Biolegend	1/200 dilution
IL-4 Biotin	BD-Pharmingen	1µg/ml
IL-5 Biotin	BD-Pharmingen	1µg/ml
IFN-γ Biotin	BD-Pharmingen	1µg/ml
IL-12p40 Biotin	BD-Pharmingen	1µg/ml
IL-6 Biotin	BD-Pharmingen	1µg/ml

2.2.3.2 Measurement of antibody production via ELISA

Serum antigen-specific antibody titres of IgG, IgG1, IgG2a, IgG2b and IgG3 and IgG2c were analysed using commercially available antibodies (Table 2.5). Medium-binding 96-well ELISA plates (Grenier, Bio-one) were coated with 50µl per well of OVA at 50µg/ml in Carbonate Buffer and left overnight at 4°C. The plates were washed in PBS-Tween and non-specific binding sites were blocked by incubation of plates with 200µl/well of 10% w/v milk for 2hrs at room temperature. The plates were washed and serum samples were added (50µl/well) at a starting dilution specified in Table 2.5 and then serially diluted 1 in 2 across the plate in PBS and left overnight at 4°C. After sample incubation, plates were washed and biotinylated antibodies specific for IgG, IgG1, IgG2a, IgG2b, IgG2c or IgG3 (Table 2.5) were added to the plates (50µl/well) for 1hr. Plates were washed and incubated in the dark for 30 minutes with 50µl/well of horseradish-peroxidase (HRP)-conjugated streptavidin (Sigma-Aldrich, 1/750). Finally plates were washed and 50µl of Ortho-Phenylenediamine (OPD) substrate in phosphate citrate buffer (0.4mg/ml) was added per well. The enzyme reaction was quenched by the addition of 20µl of 1M H₂SO₄ per well. Absorbance at 492nm was determined using a microtitre plate reader (Multiscan FC, Thermo Scientific). Antibody concentrations were expressed as end point titres calculated by regression of a curve of O.D. values versus reciprocal serum levels to a cut off point of 2 standard deviations above PBS control.

2.2.3.3 Spleen cell stimulation protocol

Specific conditions are outlined in each Figure legend. Spleens isolated from BALB/c mice were homogenised and passed through a 70µm cell strainer (BD Pharmingen) to obtain a single cell suspension. Spleen cells were centrifuged at 1200rpm for 5 minutes and red blood cells were lysed by resuspending cells in 1ml of 0.88% (w/v) ammonium chloride solution for 2 minutes. Cells were washed and resuspended in fresh cRPMI to at 1x10⁶ cells/ml. 96-well 'U-bottomed' microtitre plates were coated with anti-CD3. Plates were incubated with 25µl of anti-CD3 (0.01µg/ml – 0.5µg/ml in PBS) per well, for 2 hrs at 37°C or overnight at 4°C, whereupon the spleen cells (1 x 10⁶ cells/ml) were added to the plate (at 200µl/well). The spleen cells were incubated for 1 hour alone at 37°C and 5% CO₂. After this incubation time, heat-killed bacteria were added to the plates, and these plates were incubated for 6hr - 96hr at 37°C and

5% CO₂. The supernatants were removed and cytokine concentrations were determined by ELISA.

2.2.3.4 Silencing gene expression in splenocytes

1 x 10⁷ splenocytes were resuspended in Amaxa Mouse T cell Nucleofector[®] solution (Lonza) and transfected with either 20nM ON-TARGETplus SMARTpool RAPGEF3 specific for Epac1 (siEPAC) or ON-TARGETplus non-targeting pool control siRNA (i.e. scrambled siRNA (siSCR)) as a negative control for transfection (both from ThermoScientific Dharmacon[®] siGLO[®]). The cells were transfected with the siRNA using the X-001 mouse T cell programme on the Amaxa Nucleofector[®] II Device (Lonza AG). The transfected cells were resuspended in cRPMI and plated at 1 x 10⁶ cells/ml (200µl/well) on anti-CD3 (0.5µg/ml) in a 96 well plate. After a 24hr incubation at 37°C and 5% CO₂, to allow the transfection to take effect, the cells were stimulated with various concentrations of the Epac activator. After 72hrs the supernatant was removed and analysed via ELISA for cytokine production.

2.2.4 Real-time PCR analysis

2.2.4.1 RNA isolation

RNA was isolated using a High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol. Cells were lysed in the Lysis/Binding-buffer (from the Isolation Kit) diluted 1:2 in PBS, and frozen at -80°C until ready for RNA isolation. The cells were then put into a High Pure Filter column (from the Isolation Kit) and incubated with DNase (1 KU/sample). After washing, RNA was eluted and stored at -80°C.

2.2.4.2 cDNA synthesis

For cDNA production, 5µl RNA, isolated as described in section 2.2.4.1, was reverse transcribed. The reaction mixture/master-mix for the generation of cDNA from RNA is shown in Table 2.12. The reverse transcription was performed according to the parameters described in Table 2.13 and carried out in a Thermo Scientific Hybaid Px thermal cycler. To exclude genomic DNA contamination, one control sample was included where the RT was replaced by water. The cDNA was stored at -20°C until required for real-time PCR (RT-PCR).

Table 2.12: Master-mix composition for cDNA synthesis

Reagent	Volume per sample (μl)	Source	Concentration
RNA	5	-	Max. of 1μg
dNTPs	2	Bioline	2.5mM of each nucleotide A,T,G&C
RT Buffer	2	Promega	1x
Random Primers	0.5	MWG Operon	1μg/μl
RNase OUT	0.25	Promega	10U
RT enzyme	0.25	Promega	50U

Table 2.13: Programme outline for cDNA generation.

Temperature (°C)	Time	Cycles
25	10 minutes	1
42	30 minutes	1
95	3 minutes	1

2.2.4.3 Real-time PCR

The real-time PCR was performed in ABGene® 96-well plates with the GoTaq kit according to the protocol provided using the 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction mixture/master-mix for the real-time PCR reaction is shown in Table 2.14. The real-time PCR assay was performed according to the parameters described in Table 2.15 and carried out in a 7500 Fast Real-Time PCR System from Applied Biosystems.

Table 2.14: Master-mix composition for real-time PCR

Reagent	Volume per sample (μl)
SyBr green	2.5
nH ₂ O	5.5
Forward Primer (FP)	0.5
Reverse Primer (RP)	0.5
CXR	0.05
cDNA	1

Table 2.15: Reaction programme for real-time PCR

Temperature (°C)	Time	Cycles
95	2 minutes	1
95	3 seconds	40
60	30 seconds	

2.2.4.4 Analysis of RT-PCR data

The analysis of the real-time PCR data was done with the 7500 Fast System SDS software package.

2.2.5 Flow cytometry:

2.2.5.1 Analysis of BMDC Maturation

BMDC surface marker expression was analysed by flow cytometry (CyAn ADP, Dako, Colorado). The flow cytometer was calibrated using the compensation function in the Summit software, in conjunction with BDTMCompBeads (anti-rat anti-hamster Igκ) (BD Biosciences). All of the following steps were performed in the dark. BMDCs (1x10⁶ cells/ml) were harvested in 200µl of FACS buffer and then incubated with Fc-receptor block (0.5µl per 10⁶ cells, BD Pharmingen) for 10 minutes. Cells were then incubated for 30 minutes on ice with antibodies specific for the DC marker CD11c, and antibodies specific for the co-stimulatory molecules CD80, CD86, CD40 and MHC class II (Table 2.7). After 30 minutes, cells were washed in 2ml of FACS buffer, centrifuged (1200rpm for 5 minutes), resuspended in 300µl of FACS buffer and analysed for immunofluorescence. Samples were acquired using Summit software (Dako, Colarado) and data were analysed using FlowJo software (Treestar, Oregon).

2.2.5.2 Intracellular cytokine staining on spleen cells

Splenocytes (1x10⁶ cells/ml) were cultured and stimulated as in section 2.2.3.3. Four hours before intracellular cytokine staining on the cells, Brefeldin A (10µg/ml) was added to stop export of intracellular cytokines, after which the cells were resuspended in FACS buffer. All of the following steps were performed in the dark. The cells were stained for extracellular markers as outlined in Table 2.8, left for 30 minutes, washed and then fixed and permeabilised according to the manufacturers protocol

described in the Cell Fixation and Permeabilisation kit from AnDerGrub. The cells were resuspended in 40µl of reagent A (to fix the cells) for 15 minutes, washed, and resuspended in 40µl of reagent B (to permeabilise the cells) containing the intracellular staining antibodies, and left for 15 minutes at room temperature. The cells were washed, resuspended in 300µl FACS buffer and analysed for immunofluorescence. Samples were acquired using Summit software (Dako, Colarado) and data were analysed using FlowJo software (Treestar, Oregon).

2.2.5.3 Determination of antigen-specific responses by intracellular cytokine staining

Spleen cells, mediastinal lymph node cells and peritoneal lavage cells were prepared as in section 2.2.2.5, from mice that had been immunised with the OVA antigen. The cells were plated on either cRPMI (as a control) or OVA (500µg/ml) and incubated at 37°C. After 1 hr Brefeldin A was added (10µg/ml) and the cells were incubated for a further 5 hrs at 37°C, whereupon the cells were analysed as in section 2.2.3.3 for antigen-specific cytokine production.

2.2.6 Cell sorting

For isolation and depletion of specific spleen cell populations, spleen cells (100-200 x 10⁶ cells) were stained with antibodies against CD3, CD8α or DX5 (CD49b) and then filtered through a disposable 30µm cell filter from Partec. The cells were analysed and sorted into discrete cell populations based on specific antibody staining using Beckman Coulter (Dako) 3-laser, 8 channel, MoFlo hi-speed cell sorter. As a control, unstained cells were sorted through the machine to account for effects of the sorting process. The isolated cells were plated on anti-CD3 (0.1-1µg/ml) and stimulated as in section 2.2.3.3.

2.2.7 Confocal staining protocol

All confocal microscopy was carried out with the help of Dr. Jim Harris (Adjuvant Research Group, Trinity College Dublin).

2.2.7.1 Confocal analysis of BMDCs

DCs were plated in a volume of 1ml at 6.25×10^5 cells/ml on 19mm coverslips in a 12-well plate. The cells were fixed in 2% paraformaldehyde, left for 30 minutes at room temperature, and permeabilised with 0.1% (v/v) Triton X-100 in 1% (w/v) BSA for 10 minutes. See Table 2.10 for all reagents and reagent concentrations used in confocal experiments. The cells were blocked with 5% (v/v) goat-serum in 3% (w/v) BSA for 30 minutes at room temperature, and then stained with the rabbit anti-mouse Epac1 primary antibody for 1 hour at room temperature protected from the light. After washing with PBS (x5), cells were stained with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated wheat germ agglutinin for 1hr. The cells were stained with Bisbenzimidide H 33258 for 5 minutes to visualise the nuclei, then washed five times with PBS. The coverslips were mounted onto glass slides with Fluorescent Mounting Medium (Dako, Ireland) and analyzed on an Olympus FV1000 laser scanning confocal microscope.

2.2.7.2 Spleen cell cytopins and confocal staining protocol

Splenocytes were sorted according to cell specific markers as described in section 2.2.6. The cells were counted and 200 μ l of cells (at 1×10^6 cells/ml) were centrifuged at 1200rpm for 5 minutes in a Shandon Cytospin 3 cytocentrifuge. The cells were fixed in 2% paraformaldehyde, left for 30 minutes at room temperature and were then permeabilised with 0.1% (v/v) Triton X-100 in 1% BSA for 10 minutes. See Table 2.10 for all reagents and reagent concentrations used in confocal experiments. The Fc receptors were blocked with 5% (v/v) goat-serum in 3% BSA for 30 minutes at room temperature and then stained with the rabbit anti-mouse EPAC1 primary antibody for 1hr at room temperature, protected from light. After washing with PBS (x5), the cells were stained with Alexa Fluor 568-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated phalloidin for 1hr. The nuclei were stained with Bisbenzimidide H 33258 for 5 minutes, then washed five times with PBS. The coverslips were mounted onto glass slides with Fluorescent Mounting medium (Dako, Ireland) and analyzed on an Olympus FV1000 laser scanning confocal microscope.

2.2.8 Removal of LPS from OVA

As commercially available OVA (from Sigma) is contaminated with LPS, the LPS in the OVA used in for vaccinations, was removed via a Detoxi-Gel™ endotoxin removal kit (Thermo scientific) according to the manufacturers protocol. The Detoxi-Gel™ resin column was regenerated by washing the column through completely five times with 1% (w/v) sodium deoxycholate, followed by five washes with pyrogen free H₂O (Baxter) to remove the detergent. Post-regeneration, the OVA solution was added to the columns. The first 900µl of fluid that ran through the column was discarded, as this is the column bed volume with no OVA present. The sample was run through three columns sequentially three times and the eluate was collected. The column was then washed through with pyrogen free H₂O (Baxter) and the first 900µl was collected as this is the column bed volume containing OVA. The OVA sample was tested for protein concentration (BCA assay- Thermo Pierce®) in order to determine the amount of OVA present. The concentration of LPS present following the endotoxin removal process was determined using an endotoxin detection kit (Lonza).

2.2.9 *In vivo* studies

2.2.9.1 Determination of the immunomodulatory effects of i.p. injection of the Epac activator alone or in the presence of TLR agonists

Specific conditions for *in vivo* experiments are outlined in each Figure legend. For the single injection experiments, female BALB/c mice (6-8 weeks old, Harlan UK) were injected intraperitoneally (i.p.) with:

1. PBS (as control),
2. 8pCPT 0.1mmol (0.5mM),
3. LPS (10µg/mouse) or R848 (25µg/mouse) alone, or
4. 8pCPT with either LPS (10µg/mouse) or R848 (25µg/mouse).

24hrs later the mice were sacrificed, whereupon the spleen and mediastinal lymph nodes were removed, the blood collected and the peritoneum washed (i.e. peritoneal lavage collected).

For the five-day acute exposure experiments, BALB/c female mice (6-8 weeks old, Harlan UK) were injected i.p. for five consecutive days with:

1. PBS (as control),
2. 8pCPT 0.1mmol (0.5mM) or N⁶-Bz 0.1mmol (0.5mM),
3. LPS (10µg/mouse) or
4. 8pCPT or N⁶-Bz with LPS.

On the sixth day the mice were sacrificed, whereupon the spleen and mediastinal lymph nodes were removed, the blood collected and the peritoneum washed (i.e. peritoneal lavage collected).

For both *in vivo* experiments, the cells from the spleen, mediastinal lymph nodes and peritoneum were isolated and stimulated *ex vivo* as described in section 2.2.2.5. The spleen (2×10^6 cells/ml), mediastinal lymph nodes (1×10^6 cells/ml) and peritoneal cells (1×10^6 cells/ml) were stimulated with PMA (25ng/ml), Ionomycin (200ng/ml) and anti-CD3 (0.01-0.1µg/ml). After 3 days, supernatants were removed and analysed for cytokines by ELISA (see section 2.2.3.1). The spleen cells, mediastinal lymph node cells and peritoneal cells were also stimulated with Brefeldin A (10µg/ml) with or without PMA and ionomycin, for five hours before intracellular analysis of cytokine production was performed as described in section 2.1.5.2.

2.2.9.2 Immunisation study using OVA antigen in order to investigate the adjuvant potential of the Epac agonist when used in the presence or absence of LPS

Female BALB/c mice (6-8 weeks old, Harlan UK) were injected i.p. with:

1. PBS (as control),
2. OVA (50µg/mouse),
3. OVA and LPS (10µg/mouse),
4. 0.1mmol (0.5mM) 8pCPT with OVA, or
5. 8pCPT and LPS.

These injections were followed 21 days later with an identical booster immunisation. The day before the booster immunisation, blood was taken from the mice via tail bleeds, in order to measure antigen-specific serum antibody concentrations. Seven days after the second immunisation, the mice were sacrificed, and the spleen and

mediastinal lymph nodes were removed. The cells were isolated from these organs and from the peritoneum, and stimulated *ex vivo* as described section 2.2.1. Spleen cells (2×10^6 cells/ml), mediastinal lymph node cells (1×10^6 cells/ml) and peritoneal cells (1×10^6 cells/ml) were stimulated with OVA (500µg/ml). After 3 days, supernatants were removed and analysed for cytokines by ELISA (see section 2.2.3). Antigen-specific T cell responses were also detected by intracellular cytokine staining as described in section 2.2.5.3. Antigen-specific serum antibody titres for both pre- and post-boost immunisations were determined by ELISA (see section 2.2.3.2).

2.2.9.3 Immunisation study using the *Mycobacterium tuberculosis* Hybrid 1 (H1) antigen in order to investigate the adjuvant potential of the Epac agonist 8pCPT in the presence or absence of CpG.

Female C57/BL6 mice (6-8 weeks old, Harlan UK) were injected i.p. with:

1. PBS,
2. H1 (Ag856-ESAT6) antigen (2µg/mouse),
3. H1 and CpG (50µg/mouse),
4. 0.1mmol (0.5mM) 8pCPT with H1, or
5. 8pCPT, H1 and CpG,

These injections were followed 21 days later with an identical booster immunisation. The day before the booster immunisation, blood was taken from the mice via tail bleeds, in order to determine antigen-specific serum antibody titres. Seven days after the second immunisation, the mice were sacrificed, and the spleen and mediastinal lymph nodes were removed. Spleen, mediastinal and peritoneal cells were stimulated *ex vivo* as described in section 2.2.1. Spleen cells (2×10^6 cells/ml), mediastinal lymph node cells (1×10^6 cells/ml) and peritoneal exudate cells (1×10^6 cells/ml) were stimulated H1 antigen (0.4, 2 or 10µg/ml) and anti-CD3 (0.1µg/ml). After 3 days, supernatants were removed and analysed for cytokines by ELISA (see section 2.2.3). Antigen-specific serum antibody titres for both pre- and post-boost immunisations were determined by ELISA (see section 2.2.3.2).

2.2.10 Statistical analysis

Statistical analyses were performed using Graphpad Prism software. Statistical differences in mean cytokine values or antibody titres between experimental groups were determined by student's *t*-test for two group analysis or one-way ANOVA for groups of three or more comparison. P-values of less than 0.05 were considered significant. Where significant differences were found, the Tukey-Kramer multiple comparisons test was used to identify differences between individual groups.

Chapter 3

An investigation of the role of Epac in Dendritic cells

Introduction

The innate immune system plays a vital role in providing the signals that are required to induce an effective adaptive immune response. The nature of the adaptive response induced mainly depends on the initial ‘danger signals’ [84] detected by the innate immune cells following infection (and vaccination). Antigen-presenting cells (APCs) are essential for initiating immune responses by presenting antigen to T cells. Several cell types are capable of presenting antigen including DCs, macrophages and B cells, although DCs are regarded as the most efficient stimulators of naïve T cells and the subsequent establishment of immunologic memory. DCs are the key sentinel APCs for priming naïve T cells [85], and are a fundamental link between the innate and adaptive immune systems. The primary aim of this chapter is to investigate the effects of Epac activation on DCs.

In general, vaccination strategies try to mimic the key characteristics of pathogens, and DCs are considered to play a central role in the induction of adaptive immune responses by vaccination. DCs are found in the T cell areas of lymphoid tissues and exist as minor cellular components in most tissues. In the mouse, the expression of the integrin CD11c distinguishes DCs from macrophages. GM-CSF promotes the expansion/differentiation of bone marrow and blood monocytes into DCs, which can then be matured further with stimuli such as microbial products [87]. In this chapter, this observation was exploited in order to generate DCs and study the effects of Epac activation in DCs.

DCs secrete various cytokines, which vary according to the different stages of DC development and maturation, and the type of immune response. A wide variety of cytokines may be expressed (not necessarily simultaneously) by mature DCs including IL-12, IL-1 α , IL-1 β , IL-15, IL-18, IFN- α , IFN- β , IFN- γ , IL-10, IL-6, IL-16, TNF- α , and MIF [109]. The exact cytokine repertoire expressed will depend on the nature of the stimulus, maturation stage of the DC and the existing cytokine microenvironment. The distinct cytokine patterns released by mature DCs ultimately determine their T helper cell polarising capacities [110]. The interaction of DCs with naïve T cells can lead to different types of effector responses, or to T cell tolerance,

depending on the type of DC and its activation state. DCs are major producers of IL-12 and their production of IL-12 mediates Th1 cell development and IFN- γ production [353]. IL-12 is essential to the generation of a protective immune response to TB, by inducing IFN- γ expression and the activation of antigen-specific Th1 cells [354]. Analysis of IL-12 production following Epac activation should give an indication as to whether Epac activation is capable of altering DC IL-12 production and thereby inducing IFN- γ production.

During their conversion from immature to mature cells, DCs undergo a number of phenotypical and functional changes. Efficient antigen presentation requires high levels of MHC complexes and co-stimulatory molecules at the cell surface. DCs upregulate co-stimulatory molecules such as CD40, CD58, CD80 and CD86 upon maturation [91], as well as surface MHC Class I and Class II .

As described in chapter 1, increased cAMP and PKA activation in DCs and macrophages is known to be immunosuppressive. PKA activation inhibits production of TNF- α , MIP-1 α and leukotriene B₄ while increasing IL-10 and IL-6 production in alveolar macrophages [262]. The increase in cAMP, subsequently activating PKA in DCs, has been found to suppress TNF- α and IL-12 production via the transcription factor c-Fos [263]. Whether or how Epac activation affects DCs has not been defined.

The key aims and objectives of this chapter were to:

- Determine the effects of Epac activation on DC cytokine production
- Determine the effects of Epac activation on the maturation status of DCs
- Determine the effects of culturing DCs with GM-CSF and an Epac activator compared to GM-CSF alone

3.1 Epac1 and Epac2 are expressed in DCs and Macrophages.

In order to determine if Epac1 and Epac2 were expressed in DCs, the cells were isolated and prepared for confocal microscopy. Both forms of Epac were found to be expressed, although Epac1 is much more abundantly expressed than Epac2 (Figure 3.1). Macrophages are also key regulators of immune responses. Therefore, Epac expression in bone marrow-derived macrophages (BMDM) was analysed via confocal microscopy, and Epac1 was found to be widely expressed (Figure 3.2). Stimulation of the cells with LPS, which as a TLR4 agonist activates the DCs and induces maturation, did not alter the expression pattern of the Epac protein (Figure 3.1). This was also the case with the macrophages, where the addition of LPS did not alter Epac expression (Figure 3.2).

3.2 Elevation of intracellular cAMP and PKA activation inhibits IL-12 and increases IL-10 production by DCs.

cAMP is produced, from ATP, by the action of the AC enzyme, while cAMP is degraded by PDEs. In order to determine the effects of elevated cAMP on cytokine production by DCs, several compounds that act to increase intracellular cAMP were used. Since the development of Th1 promoting adjuvants is a priority in vaccine research, IL-12 was specifically investigated, as it is the predominant polarising cytokine for the Th1 response. It is composed of the subunits IL-12p40 and IL-12p35, which dimerise to form IL-12p70, the active form of IL-12 [136]. IL-23 is composed of IL-12p40, which dimerises with the IL-12p19 subunit to form active IL-23 [136], which is an important cytokine for inducing Th17 responses. IL-10 is an anti-inflammatory cytokine produced by many different cells. It has diverse effects, which result in the suppression of immune responses.

Dibutyryl-cAMP (DB-cAMP) is a cell-permeable cAMP analogue so the cAMP generating machinery is not affected. Addition of DB-cAMP resulted in inhibition of IL-12p40 and IL-12p70 at both concentrations used (10 μ M and 100 μ M) (Figure 3.3). IL-10 was increased significantly at the highest concentration of DB-cAMP (100 μ M). Similar results were seen due to increased intracellular cAMP, following addition of

the compounds IBMX and Forskolin. IBMX is a non-specific PDE inhibitor and Forskolin activates the AC enzyme. IBMX at a concentration of 100 μ M decreased IL-12p40 and IL-12p70 while increasing IL-10 production (Figure 3.3). Forskolin inhibited IL-12p40 production at both concentrations used, 1 μ M and 100 μ M (Figure 3.3). Overall, elevating cAMP levels resulted in decreased IL-12p40 and IL-12p70 production and increased IL-10.

One of the downstream targets of cAMP is PKA, so the effects of PKA activation on cytokine production were investigated. In order to confirm the *in vitro* effect of PKA activation on cytokine production by DCs, DCs were incubated with the PKA-specific agonist N⁶-Benzoyl-cAMP (N6-Bz). PKA activation by N6-Bz (0.0156mM, 0.0625mM, 0.25mM and 1mM), inhibited IL-12p40 and IL-12p70, while increasing IL-10 and IL-6 expression upon stimulation with heat-killed *E. coli* (HK) bacteria (Figure 3.4).

3.3 The Epac activator 8pCPT-2'-OMe-cAMP (8pCPT), at picomolar concentrations induces IL-12p40 and IL-12p70 production by DCs.

The *in vitro* effects of Epac activation on DC cytokine production were next determined. The specific agonist 8pCPT-2'-O-Me-cAMP (8pCPT) was used to selectively activate Epac. While 8pCPT is a specific activator of Epac, the Epac-specific effects are concentration dependent and at high concentrations the molecule may activate PKA (outlined in more detail in chapter 1). High concentrations of the Epac activator (0.156mM – 2.5mM) were found to inhibit LPS-induced IL-12p40 production (Figure 3.5), similar to what was seen with nonspecific cAMP elevation and PKA activation. Several dose-response assays were then carried out in order to determine the optimal concentration of 8pCPT to modulate the cytokine response of the DCs. A dose-response curve of the effects of Epac activation on cytokine production by DCs showed that picomolar concentrations of the Epac activator significantly increased IL-12p70 production, and slightly enhanced IL-12p40 production by DCs activated with HK bacteria (Figure 3.6). The dose response outlined the effects of nM amounts of the Epac activator also, but these effects reached a level comparable to the PBS control, until the pM concentrations of the

Epac activator began their effects on IL-12 production (data not shown). Epac activation had no significant effect on IL-6 or IL-10 production (Figure 3.6). As the data here is presented as a representative experiment taken from three separate experiments, three individual experiments showing the IL-12p70 increase following Epac activation is also presented in Appendix Figure B.

The effects of Epac activation on mRNA expression of the IL-12 subunits, IL-12p35 and IL-12p40, was also analysed (Figure 3.7). It was found that 6hours post-stimulation of DCs with the Epac activator, there were increases in the mRNA expression of both IL-12p35 and IL-12p40 subunits (Figure 3.7A). The addition of HK bacteria as a TLR agonist further enhanced this induction (Figure 3.7B), although this was only significant with IL-12p35 and not IL-12p40 when compared to the control. The expression of mRNA for the transcription factor T-bet (important for induction of a Th1 response) and the cytokine IL-18 (a pro-inflammatory cytokine that can interact with IL-12 to induce Th1 responses) were also analysed (Figure 3.8). Six hours post-Epac activation, the expression of T-bet was doubled but IL-18 mRNA expression was unaltered (Figure 3.8). T-bet is characteristically a transcription factor measured in T cells as an indicator of Th1 type responses and not DCs, but it was decided to measure in this instance as T-bet can be an indicator of activation of a Th1 type response in DCs.

3.4 Stimulation of DCs with glybenclamide (GLB) induces pro-inflammatory cytokine production.

Glybenclamide (GLB) belongs to a group of sulfonylurea compounds used to treat type II diabetes in patients and has been found to bind specifically to Epac2 [286]. As Epac activation by 8pCPT results in increased IL-12 production by DCs, it was decided to investigate if this compound could exert a similar effect. A range of concentrations of GLB were tested to examine changes in DC cytokine production. An increase in DC IL-12p70 production was seen, especially at the higher 10 μ M concentration of GLB (Figure 3.9). There was an upward trend in IL-12p40 production, but this was not significant. Neither IL-23 or IL-6 production were affected by treatment with GLB (Figure 3.9).

3.5 Intracellular cAMP elevation, PKA activation and Epac activation all affect DC maturation.

DCs that have not encountered a PAMP or danger signal are in an immature state. Upon encountering one (or both) of these signals the DCs convert from immature to mature cells. During this conversion DCs undergo a number of changes, including upregulation of co-stimulatory molecules, which are important for interaction with T cells.

In order to determine the effect of cAMP elevation on DC maturation marker expression, the cells were incubated with the cAMP analogue DB-cAMP (50 μ M) alone or followed two hours later by HK bacteria (Figure 3.10). The flow cytometry data were analysed in terms of both the percentage of cells positive for the marker and median fluorescence intensity (MFI). MFI values are used to measure the shift in fluorescence intensity of a specific population of cells i.e. it allows the measurement of any variation in the degree of expression of a marker on a cell.

Stimulation of DCs with DB-cAMP with and without HK bacteria resulted in an increase in MHC class II expression as seen by both the percentage of cells positive for MHC class II expression and the MFI values (Figure 3.10). Control cells showed MHC class II expression of 55.18% (both upper quadrants), which increased to 62.27% of DB-cAMP cells (Figure 3.10). There was no increase in the MFI value of MHC class II in control cells (301) when compared to DB-cAMP treated cells (298) (Figure 3.10). MHC class II expression was 62.2% in HK-treated cells, which increased to 68.7% on cells treated with DB-cAMP and HK bacteria (upper right and upper left quadrants). The MFI value of MHC class II in HK-treated cells was 311 and this increased to 462 in DB-cAMP and HK bacteria treated cells (Figure 3.10). CD40 expression was slightly decreased, from 5.28% in control cells to 4.07% on DB-cAMP-treated cells. However, DB-cAMP reduced CD40 expression from 31.34% on HK bacteria-treated cells to 9.38% on cells treated with DB-cAMP and HK bacteria (upper right and lower right quadrants).

Treatment of the DCs with the cAMP elevator had little effect on the percentage of CD86 positive cells either with or without the addition of HK bacteria to the cells (Figure 3.10). Expression of CD80 was decreased upon treatment of the DCs with the cAMP elevator, both in the presence and absence of HK bacteria (Figure 3.10). There were 9.67% CD80⁺ cells in the control group (upper and lower right quadrants), decreasing to 6.91% in DB-cAMP treated cells. Treatment with HK bacteria increased the percentage of CD80⁺ cells to 19.64% which was reduced to 14.57% by treatment with DB-cAMP (Figure 3.10). Overall, analysis of CD80, CD86, CD40 and MHC class II expression showed that cAMP elevation enhances MHC class II while decreasing CD40 and CD80 expression in DCs (Figure 3.10).

The effects of PKA activation on the maturation markers CD80, CD86, MHC class II and CD40 in DCs were subsequently studied. MHC class II expression was increased following PKA activation by N6-Bz (0.1mM and 1mM), while expression of CD40 on HK-treated cells was decreased by the PKA agonist (Figure 3.11). Specifically, the MFI of MHC class II expression was increased by N6-Bz treatment, while the total percentage of cells expressing MHC class II was unchanged. The MFI value for MHC class II was 292 on control cells, which increased to 332 for 0.1mM and 366 for 1mM N6-Bz (Figure 3.11). In order to show MHC class II and CD40 expression individually, a histogram profile was prepared and is shown in Figure 3.12. The data indicates a slight increase in MHC class II⁺ cells at the highest PKA activator concentration (1mM), but a decrease after addition of HK bacteria (Figure 3.12). The percentage of CD40⁺ cells was decreased by activation of PKA with N6-Bz (Figure 3.11 and Figure 3.12). CD40 expression was 62.3% (upper and lower right quadrants) in HK-treated cells, which was reduced to 26.79% in cells treated with 0.1mM N6-Bz and HK bacteria and to 13.10% in cells treated with 1mM N6-Bz and HK bacteria (Figure 3.11). This decrease in CD40 expression after PKA activation in HK bacteria-treated cells mirrors the effects of cAMP elevation seen in Figure 3.10.

Treatment of DCs with the PKA agonist increased the percentage of CD86⁺ cells (Figure 3.11 and Figure 3.13). In the control cells 64.9% of cells were positive for CD86 (upper quadrants), which increased to 78.9% with 0.1mM and 91.47% with 1mM N6-Bz. CD80 expression was also increased, from 70.9% in control cells to 87.9% with 0.1mM and 91.7% with 1mM N6-Bz (Figure 3.11). The percentage of

CD80⁺CD86⁺ cells was also increased, from 50.3% (upper right quadrant) in control cells to 74.5% with 0.1mM and 86.0% with 1mM N6-Bz (Figure 3.11). For cells treated with both HK bacteria and N6-Bz, there was no increase in CD86⁺ cells, with the HK-treated CD86 expression at 95% (upper quadrants), 0.1mM+HK at 91.3% and 1mM+HK at 87.8% (Figure 3.11). There was an increase in CD80 expression after PKA activation in HK bacteria-treated cells, but it was not as great as the increase in CD80 expression in non-HK bacteria-treated cells (Figure 3.11 and Figure 3.13). In cells treated with HK bacteria 88.0% were CD80⁺ (upper and lower right quadrants) increasing to 94.66% with 0.1mM N6-Bz and HK bacteria and to 94.98% with 1mM N6-Bz and HK bacteria treatment (Figure 3.11). Overall, PKA activation increases CD80 and CD86 expression while inhibiting CD40 expression and increasing the intensity of MHC class II expression on DCs. cAMP elevation in DCs using DB-cAMP, mirrors the effects of PKA activation on MHC class II and CD40 expression but not CD80 and CD86 expression.

The effect of Epac activation on DC maturation was next investigated. An increase in MHC class II expression was observed, with the biggest increase at the lowest concentration of the Epac activator (3pM) (Figure 3.14). In control cells, MHC class II expression was 81.7% (upper quadrants) increasing to 87.9% (8pCPT 3pM), 83.6% (8pCPT 30pM) and 83.2% (8pCPT 30μM). The MFI values showed an increase in MHC class II expression, again predominantly at the lowest Epac activator concentration. The MFI values increased from 292 in control cells to 647 (8pCPT 3pM), 362 (8pCPT 30pM) and 326 (8pCPT 30μM). Overall CD40 expression was increased from 1.57% (upper and lower right quadrants) of control cells to 3.2% (8pCPT 3pM), 2.5% (8pCPT 30pM) and 2.6% (30μM). The histograms in Figure 3.14 show that Epac activation increased MHC class II expression but not CD40. The greater effect of lower pM concentrations of the Epac activator on DC maturation mirrors the effects seen earlier on cytokine production by DCs (Figure 3.6 and Figure 3.7). Neither CD80 nor CD86 expression were altered upon addition of the Epac activator, either in the presence or absence of HK bacteria (Figure 3.14).

3.6 Differentiation of DCs in the presence of the Epac activator 8pCPT generates DCs, which produce increased IL-12 in response to TLR ligand stimulation.

DCs derived from a standard GM-CSF culture or a culture in GM-CSF with N6-Bz or 8pCPT were restimulated *in vitro* with HK bacteria to assess cytokine production. In control DCs (i.e. GM-CSF generated), treatment with increasing concentrations of HK bacteria induced increasing levels of IL-12p40, IL-12p70, IL-23 and IL-10 production (Figure 3.16). Differentiation of DCs with N6-Bz (0.1&10 μ M) decreased the HK bacteria-induction of IL-12p40, IL-12p70 and IL-10 (Figure 3.16). The inhibition of IL-12p40 was less marked than the IL-12p70 - particularly at high HK bacteria concentrations. IL-10 was suppressed in N6-Bz-differentiated DCs upon stimulation with the highest concentration of HK bacteria (Figure 3.16). The inhibition of IL-10 by N6-Bz was only seen when DCs were stimulated with 10x HK, while at the lower concentrations of HK bacteria stimulation no inhibition is apparent (Figure 3.16). The inhibitory effect of differentiating DCs in the presence of N6-Bz on IL-12 production mirrors the suppression of IL-12 in GM-CSF cultured DCs upon restimulation with N6-Bz *in vitro*.

In contrast to the effects of the PKA activator, culture of DCs with the Epac agonist (8pCPT), induced a pro-inflammatory cytokine profile following HK bacteria treatment. Differentiation of DCs with 8pCPT (0.03&300pM) increased the HK-induction of IL-12p40, IL-12p70 and IL-23 (Figure 3.17). This is in direct contrast to the effects on cytokine production of differentiating DCs with N6-Bz. IL-10 production was suppressed upon stimulation of 8pCPT-differentiated DCs with the highest concentration of HK bacteria (Figure 3.17).

CpG (a TLR9 agonist) is a strong stimulator of IL-12 production from DCs. The effects of stimulating 8pCPT-differentiated DCs with CpG were investigated (Figure 3.18). CpG-induced IL-12p70 production was significantly higher in 8pCPT-differentiated DCs (Figure 3.18). IL-12p40 and IL-23 production was also enhanced in 8pCPT-differentiated DCs but only following restimulation with the lowest CpG concentration. In contrast, IL-10 production was lower in DCs differentiated in the presence of 8pCPT than in control cells (i.e. DCs differentiated with GM-CSF alone)

(Figure 3.18). The cytokine profile of 8pCPT-differentiated DCs following restimulation with HK bacteria and CpG are similar.

As differentiation of DCs in the presence of 8pCPT significantly altered their capacity to produce cytokines, the maturation state of the DCs was investigated. CD40 expression was increased in the cells treated with the lower 8pCPT concentration. In the control, 37.2% of cells were CD40⁺ (upper and lower right quadrants), while 61.1% of 8pCPT 0.3pM-treated and 37.6% of 8pCPT 300pM-treated cells were CD40⁺ (Figure 3.19). In cells restimulated with HK, CD40 and MHC class II expression was comparable between control and 8pCPT-differentiated cells (Figure 3.19). The percentage of CD40⁺MHC class II⁺ double positive cells was also increased, from 36.1% (upper right quadrant) of control cells to 58.6% (8pCPT 0.3pM), however the overall MHC class II expression was only slightly increased in 8pCPT-differentiated cells (Figure 3.19).

Expression of CD86 but not CD80 was increased in 8pCPT-differentiated cells at the 0.3pM concentration. The percentage of CD86⁺ cells was increased from 34.0% in the control group (upper quadrants) to 45.3% (8pCPT 0.3pM) and 38.4% (8pCPT 300pM). The double positive CD86⁺CD80⁺ upper-right quadrant shows an increase from 29.4% of control cells to 38.5% of 8pCPT 0.3pM-treated cells, and the overall percentage of CD80⁺ cells was increased from 49.3% (upper and lower right quadrants) of the control cells to 52.2% of 8pCPT 0.3pM-treated cells (Figure 3.19). In cells stimulated with HK bacteria the effects of differentiating DCs with 8pCPT were more modest. The MFI value for CD86 expression increased from 396 in control cells to 895 in 8pCPT 0.3pM+HK bacteria-treated cells, while the CD80 MFI value increased from 173 in HK bacteria treated control cells to 230 in 8pCPT 0.3pM+HK treated cells (Figure 3.19). A table summary of the flow cytometry data pertaining to DC stimulation with the cAMP analogue, the PKA activator or the Epac activator is contained in Appendix Figure A.

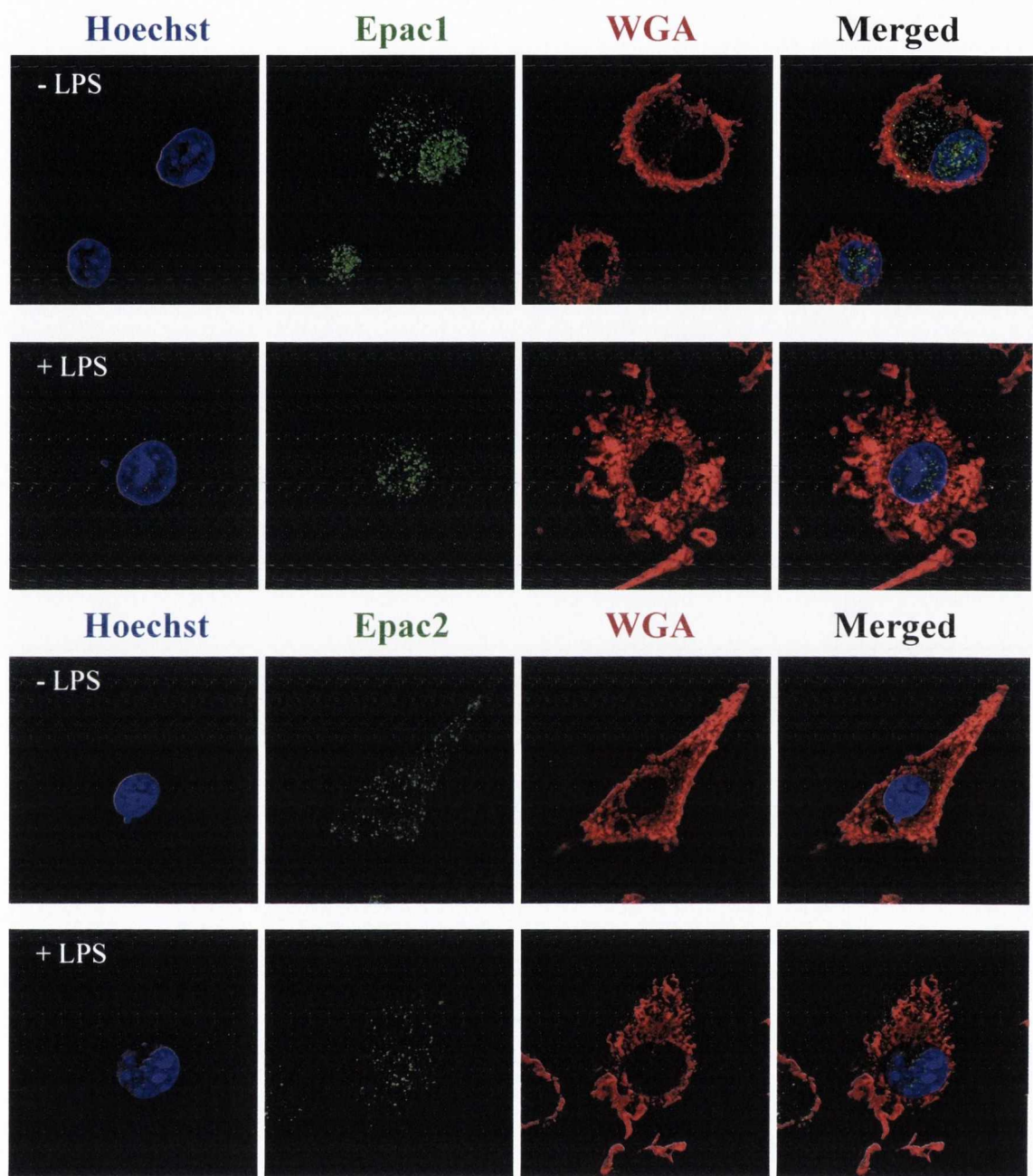


Figure 3.1: Epac1 and Epac2 are expressed in DCs. DCs (1×10^6 cells/ml) were cultured on coverslips and stimulated with or without LPS for 24hours, after which they were fixed with 2% paraformaldehyde and stained for confocal microscopy as described in materials and methods. Blue shows nuclear staining by Hoechst, green shows staining for either Epac1 or Epac2 and red shows membrane-staining by wheat-germ agglutinin (WGA). Data are representative of at least three independent experiments.

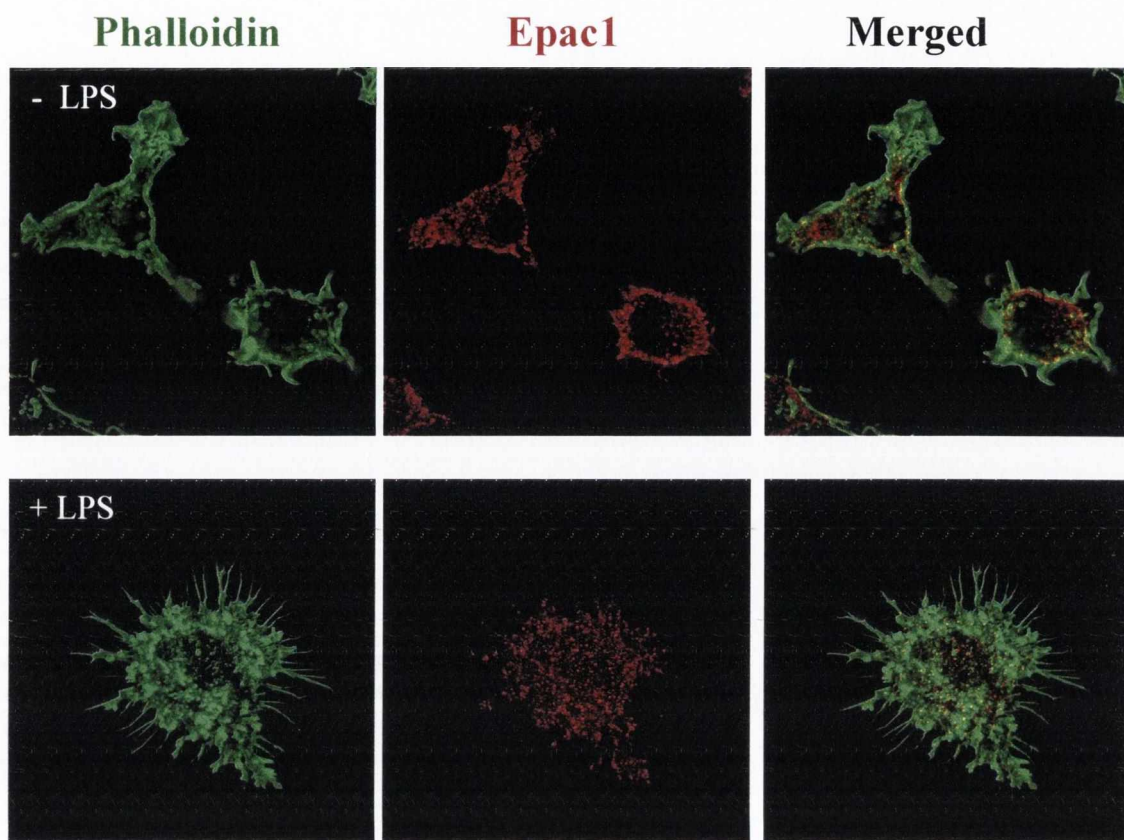


Figure 3.2: Epac1 is expressed in macrophages. Bone marrow-derived macrophages (BMDM) were cultured on coverslips with (bottom row) or without (top row) LPS, fixed with 2% paraformaldehyde and stained for confocal microscopy as described in materials and methods. Red shows staining for Epac1 and green shows membrane-staining by phalloidin. Data are representative of at least three independent experiments.

This confocal staining was carried out by Dr. James Harris

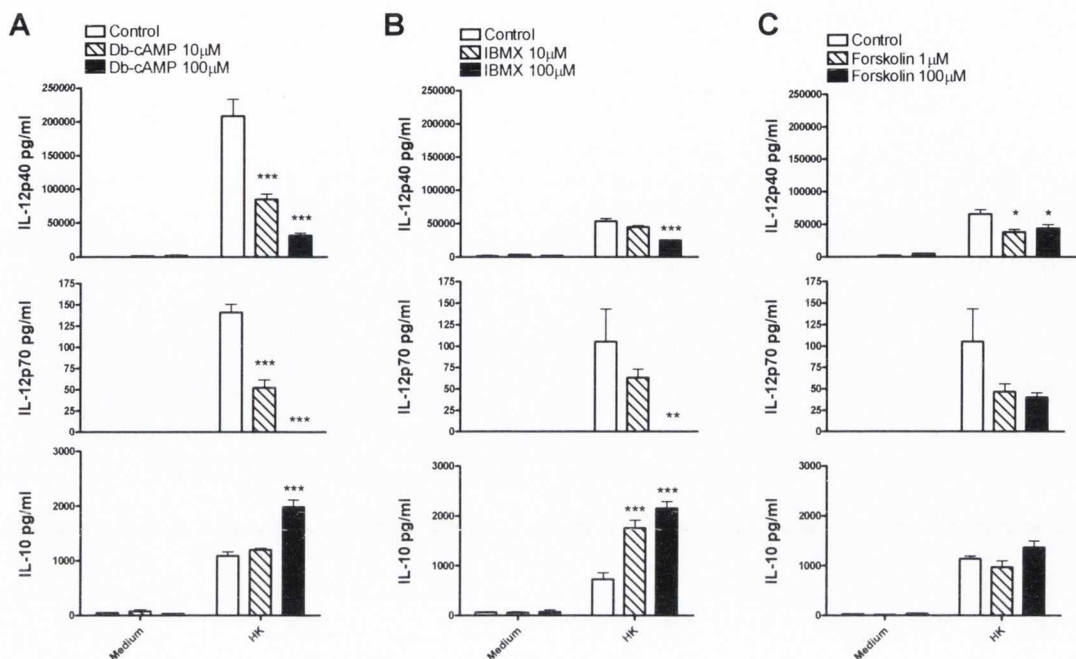


Figure 3.3: Elevation of intracellular cAMP by DB-cAMP, IBMX and Forskolin in DCs inhibits IL-12p40 and IL-12p70 production while increasing IL-10. DCs (6.25×10^5 cells/ml) were incubated with the cAMP analogue Dibutyryl-cAMP (DB-cAMP) (10 & 100 μ M) (A), the non-specific PDE inhibitor IBMX (10 & 100 μ M) (B), the adenylate cyclase activator Forskolin (1 & 100 μ M) (C) or PBS (control), Additional groups were stimulated after 2 hours with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1, 1 or 10 bacteria to 1 DC. After 24 hours the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between HK and treated samples + HK are defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Data are representative of at least three independent experiments with mean $\pm$ S.D. run in triplicate.

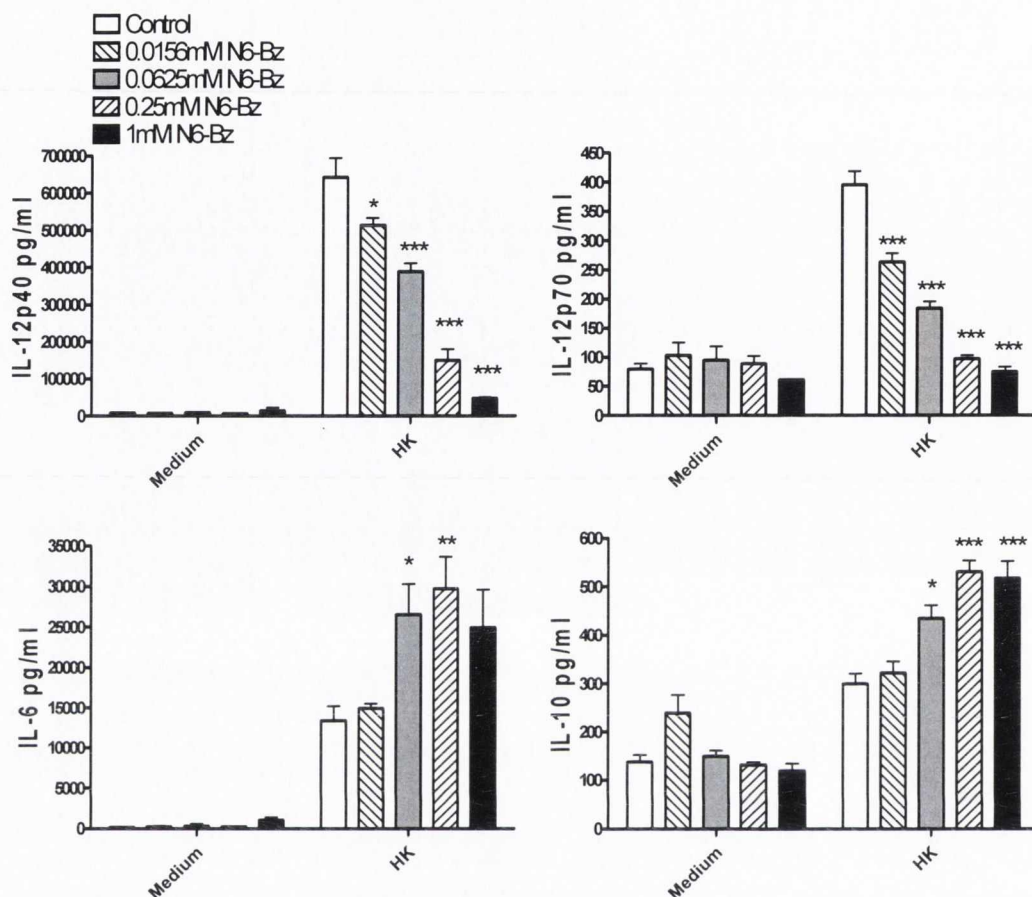


Figure 3.4: Stimulation of DCs with the PKA agonist N6-Bz inhibits secretion of IL-12p40 and IL-12p70 while increasing the production of IL-10 and IL-6 in DCs. DCs (6.25×10^5 cells/ml) from BALB/c mice were incubated with medium or a range of concentrations of the selective PKA activator N6-Benzoyl-cAMP (N6-Bz) or PBS (control), alone, or for 2 hours prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1 bacteria to 1 DC. After 24 hours the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between HK and N6-Bz-treated samples + HK are defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Data are representative of at least three independent experiments with mean $\pm$ S.D. run in triplicate.

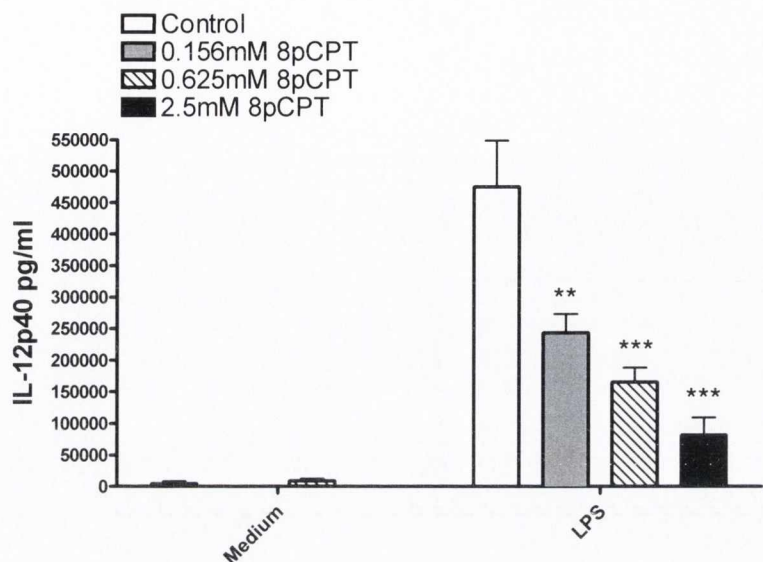


Figure 3.5: Millimolar concentrations of the Epac activator 8pCPT inhibit secretion of IL-12p40 by DCs. DCs (6.25×10^5 cells/ml) from BALB/c mice were incubated with a range of concentrations of the selective Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) or PBS (control) alone, or for 2 hours prior to stimulation with LPS (1ng/ml). After 24 hours, the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between LPS and treated samples + LPS are defined as $p < 0.01$ (**) and $p < 0.001$ (***). Data are representative of at least three independent experiments with mean $\pm$ S.D. run in triplicate.

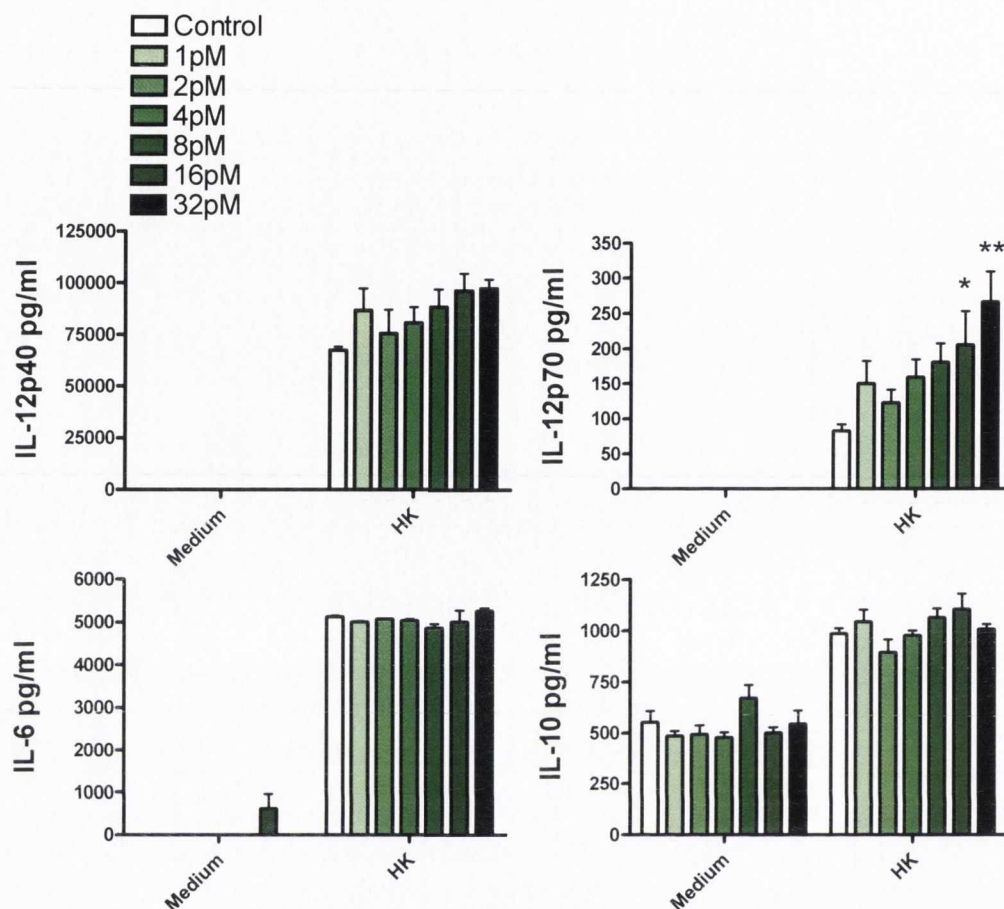


Figure 3.6: Picomolar concentrations of the Epac activator 8pCPT increases IL-12p40 and IL-12p70 secretion in DCs. DCs (6.25×10^5 cells/ml) from BALB/c mice were stimulated with a range of concentrations of the selective Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) or PBS (Control) alone, or for 2 hours prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1 or 1 bacteria to 1 DC. After 24 hours the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between HK and treated samples + HK are defined as $p < 0.05$ (*) and $p < 0.01$ (**). Data are representative of at least three independent experiments with mean $\pm$ S.D. run in triplicate.

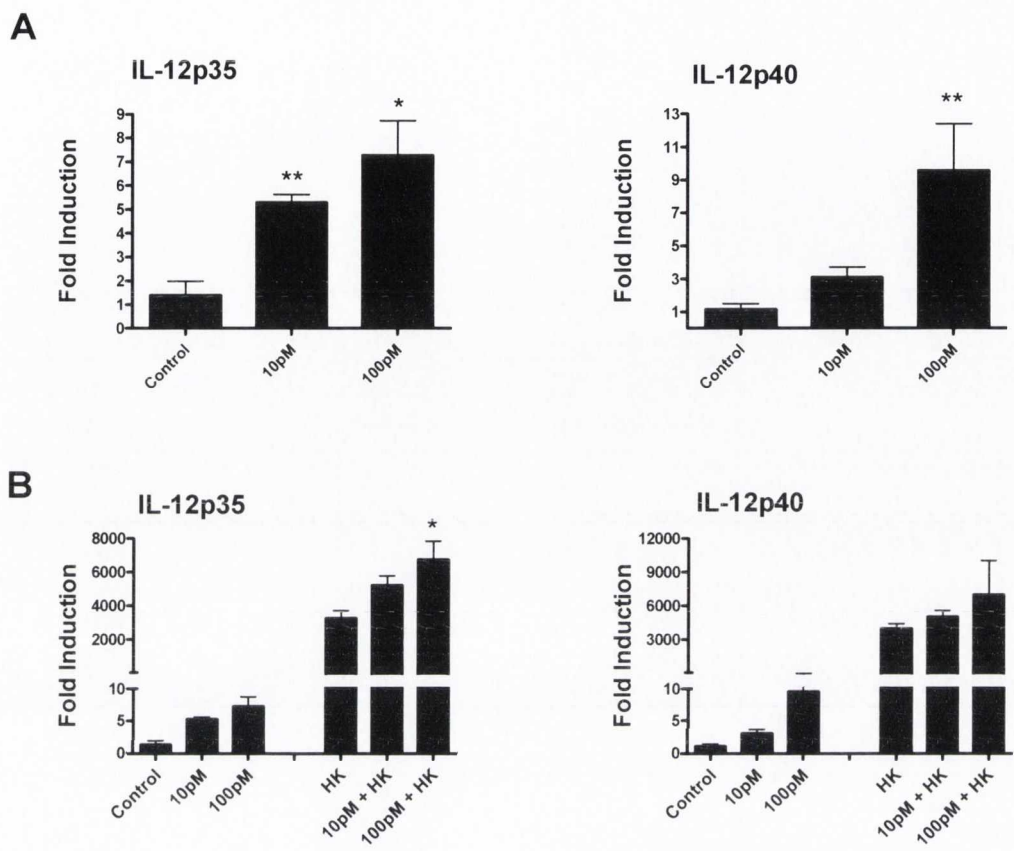


Figure 3.7: IL-12p35 and IL-12p40 mRNA expression are increased six hours after stimulation of DCs with the Epac agonist 8-pCPT. DCs (6.25×10^5 cells/ml) from BALB/c mice were incubated with the selective Epac activator, 8-pCPT-2'-O-Me-cAMP or PBS (control) alone, or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 1 bacteria to 1 DC. After 6 hours the cells were lysed and analysed for mRNA expression of IL-12p40 and IL-12p35 by real-time PCR as described in materials and methods (section 2.2.4). Differences between control and Epac agonist-treated samples are considered significant at $p < 0.05$. Significance levels are defined as $p < 0.05$ (*) and $p < 0.01$ (**). Data are representative of two independent experiments with mean $\pm$ S.D. run in triplicate.

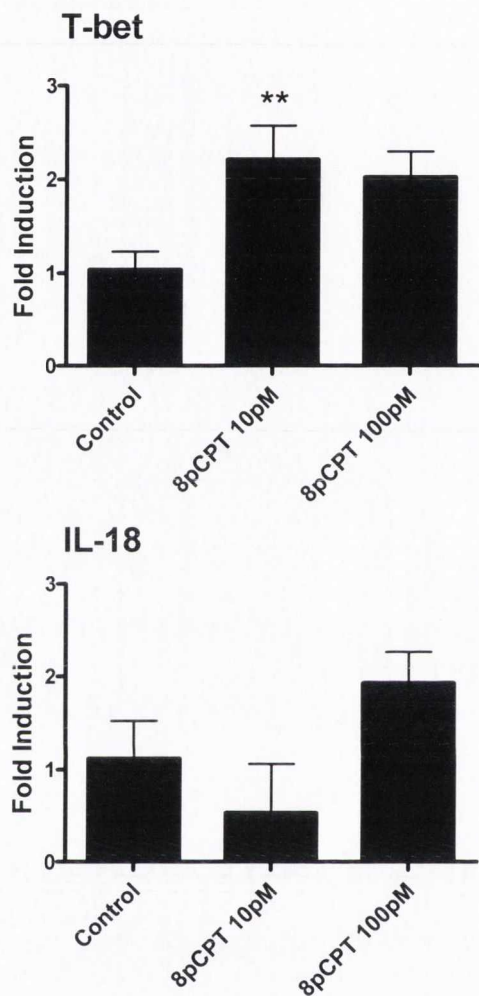


Figure 3.8: Six hours after stimulation of DCs with the Epac agonist 8pCPT, T-bet mRNA expression is increased, while IL-18 mRNA expression is unchanged. DCs (6.25×10^5 cells/ml) from BALB/c mice were incubated with the selective Epac activator, 8-pCPT-2'-O-Me-cAMP (8pCPT) or PBS (Control). After 6 hours the cells were lysed and analysed for mRNA expression of T-bet and IL-18 by real-time PCR as described in materials and methods (section 2.2.4). Significant differences between control and Epac agonist-treated samples are defined as $p < 0.01$ (**). Data are representative of two independent experiments with mean $\pm$ S.D. run in triplicate.

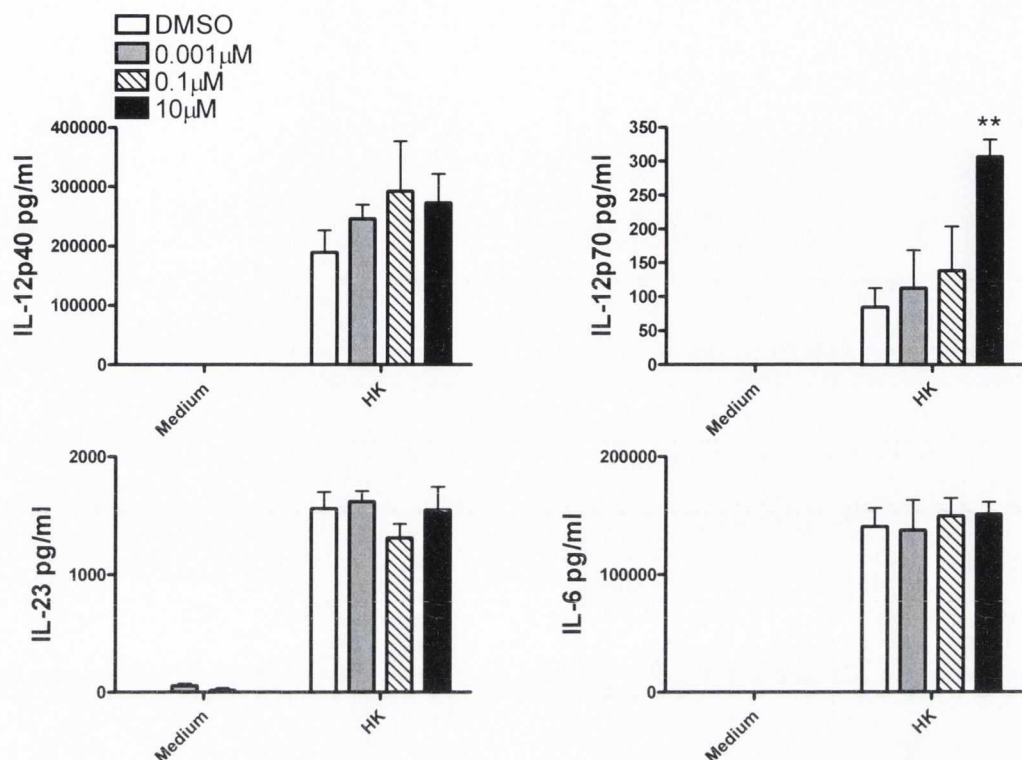


Figure 3.9: Stimulation of DCs with the sulfonylurea Epac2 agonist glybenclamide enhances pro-inflammatory cytokine production. DCs (6.25×10^5 cells/ml) from BALB/c mice were incubated with a range of concentrations of Glybenclamide (0.01 μ M-10 μ M) or with DMSO alone or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1 bacteria to 1 DC. After 24 hours the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between HK and treated samples + HK are defined as $p < 0.01$ (**). Data are representative of three independent experiments with mean $\pm$ S.D. run in triplicate.

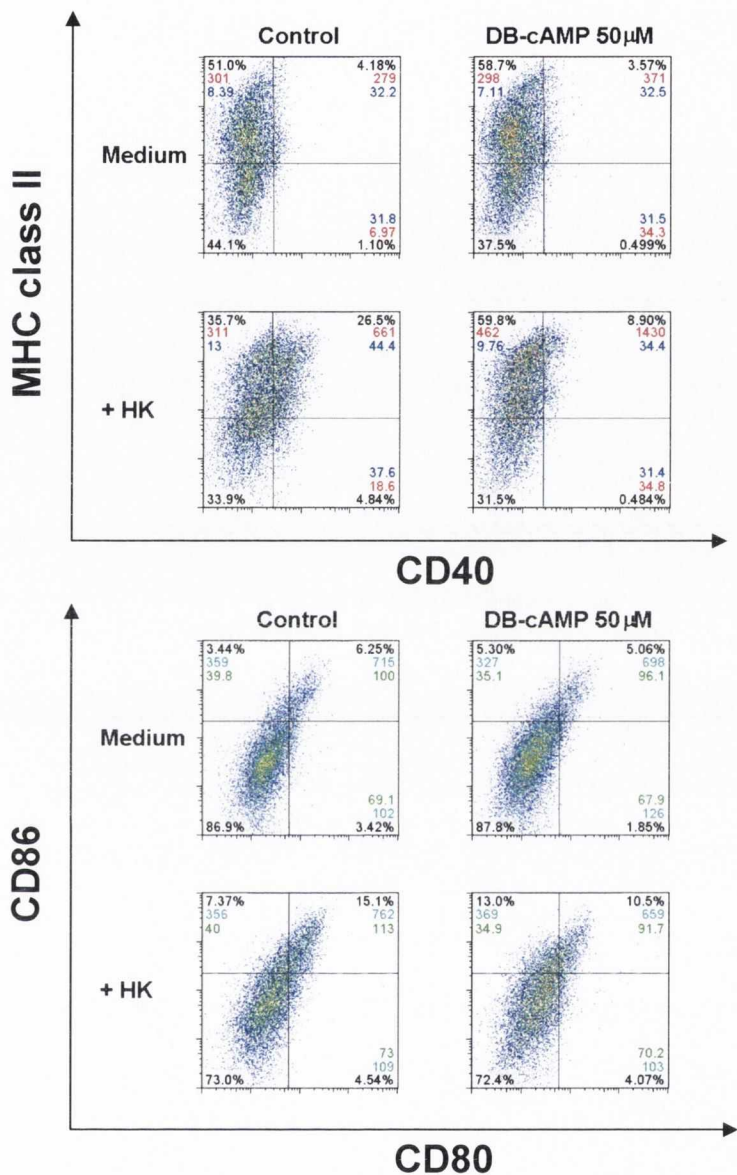


Figure 3.10: Elevation of intracellular cAMP by the cAMP analogue DB-cAMP enhances MHC class II expression while inhibiting CD40 expression on DCs. DCs (1×10^6 cells/ml) from BALB/c mice were stimulated with the cAMP analogue (DB-cAMP: 50μM) or PBS as an untreated control (Control). Additional groups of DCs were treated 2 hours later with Heat Killed *E. coli* (HK) at a ratio equivalent to 1 bacteria cell to 1 DC (1:1). After 24 hours, cells were washed, and immunofluorescence was performed with antibodies specific for CD80, CD86, CD40 and MHC Class II. Staining for CD40 versus MHC class II and CD80 versus CD86 is shown. Total percentages of cells are shown in black in each quadrant. Median fluorescence values (MFI values) are shown for each of the maturation markers in each quadrant; CD40 (blue), MHC class II (red), CD80 (green) and CD86 (cyan). Data are representative of three independent experiments.

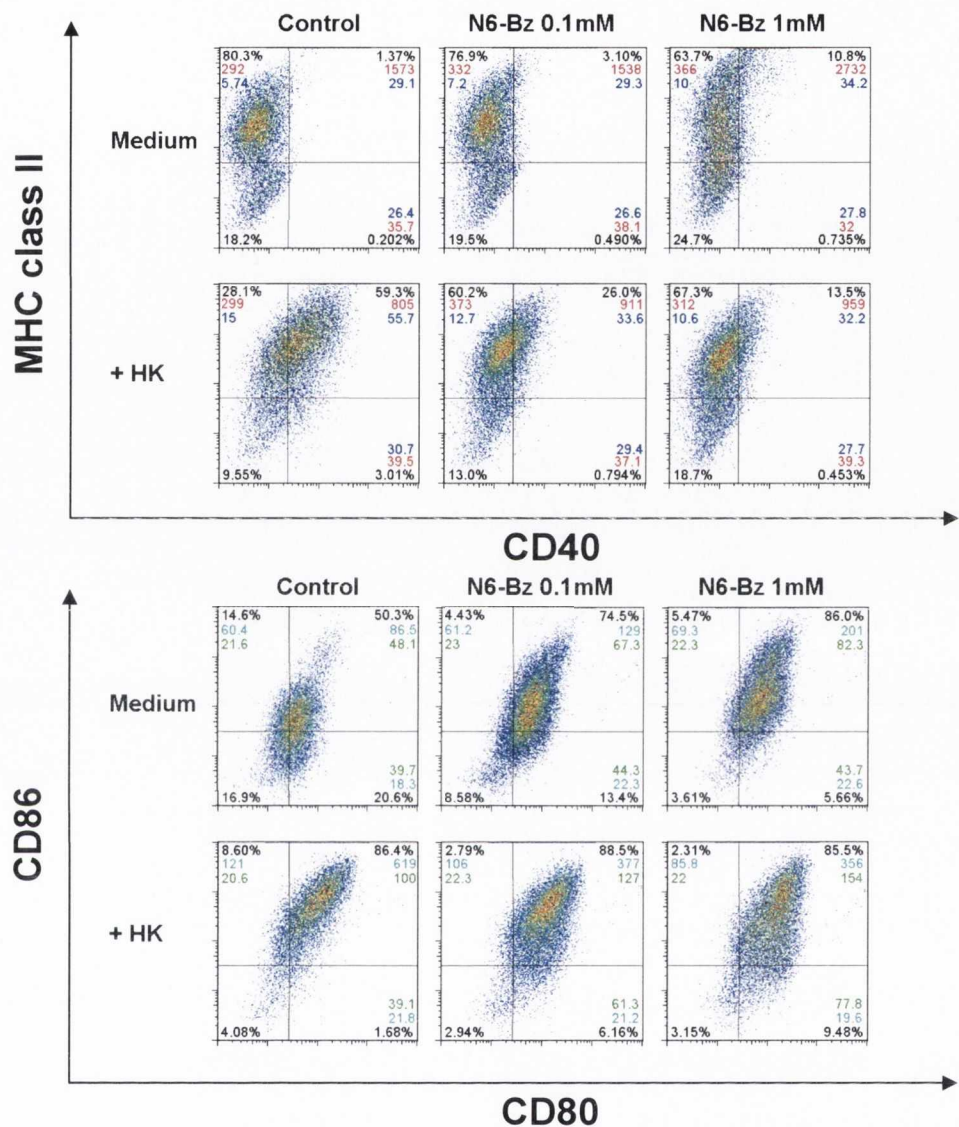


Figure 3.11: Stimulation of DCs with the PKA agonist N6-Bz increases CD86 and CD80 expression while decreasing CD40 expression on DCs. DCs (1×10^6 cells/ml) from BALB/c mice were stimulated with the PKA activator (N6-Bz: 0.1mM & 1mM) or treated with PBS alone (Control). Additional groups of DCs were treated 2 hours later with Heat Killed *E. coli* (HK) at a ratio equivalent to 1 bacteria cell to 1 DC (1:1). After 24 hours, cells were washed, and immunofluorescence was performed with antibodies specific for CD80, CD86, CD40 and MHC Class II. Staining for CD40 versus MHC class II and CD80 versus CD86 is shown. Total percentages of cells are shown in black in each quadrant. Median fluorescence values (MFI values) are shown for each of the maturation markers in each quadrant; CD40 (blue), MHC class II (red), CD80 (cyan) and CD86 (green). Data are representative of three independent experiments.

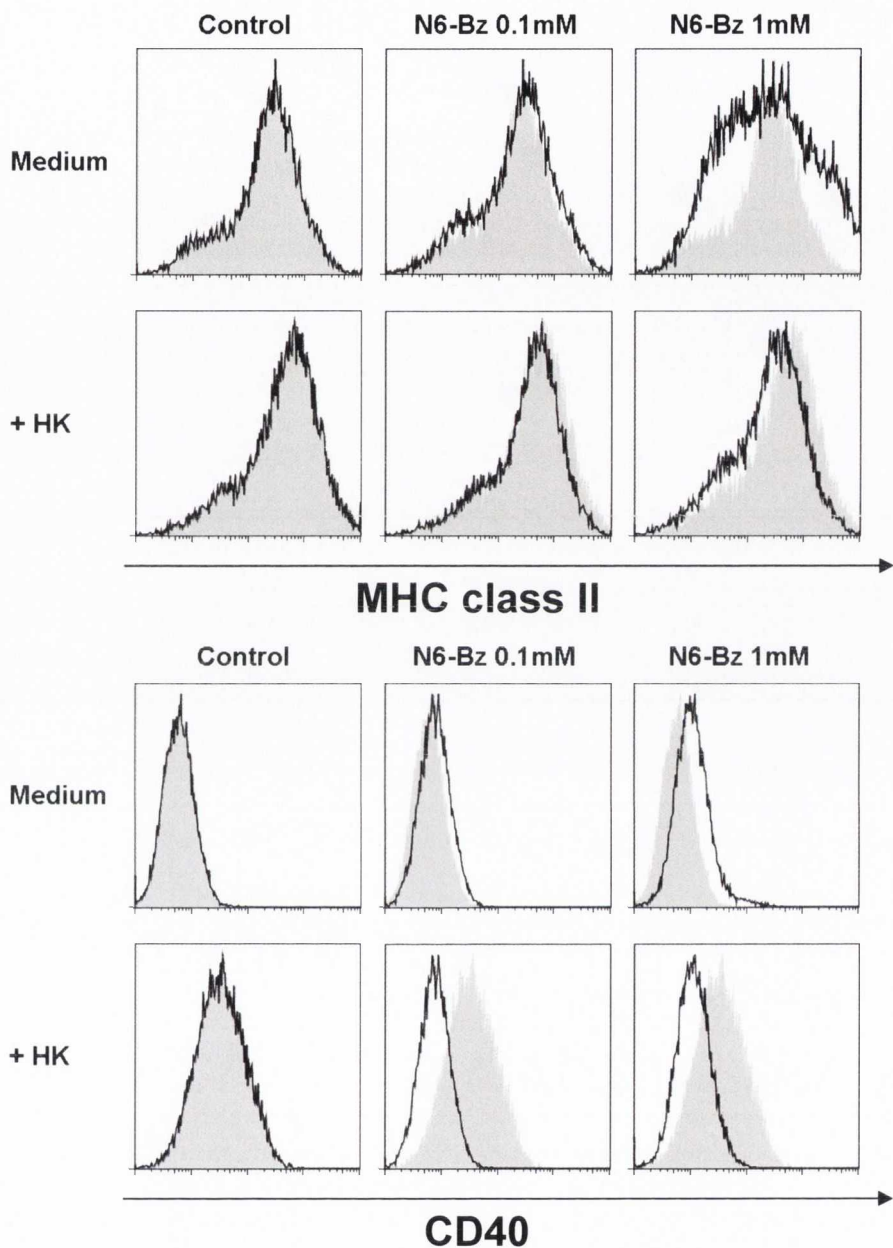


Figure 3.12: Stimulation of DCs with the PKA agonist N6-Bz decreases CD40 expression on HK bacteria-treated DCs. DCs (1×10^6 cells/ml) from BALB/c mice were stimulated with the PKA activator (N6-Bz: 0.1mM & 1mM) or PBS alone as an untreated control (Control). Additional groups of DCs were treated 2 hours later with Heat Killed *E. coli* (HK) at a ratio equivalent to 1 bacteria cell to 1 DC (1:1). After 24 hours, cells were washed, and immunofluorescence was performed with antibodies specific for CD40 and MHC Class II. Staining for CD40 or MHC class II is shown. The grey shaded histogram, indicates the control, while the black line indicates the N6-Bz-treated cells. Data are representative of three independent experiments.

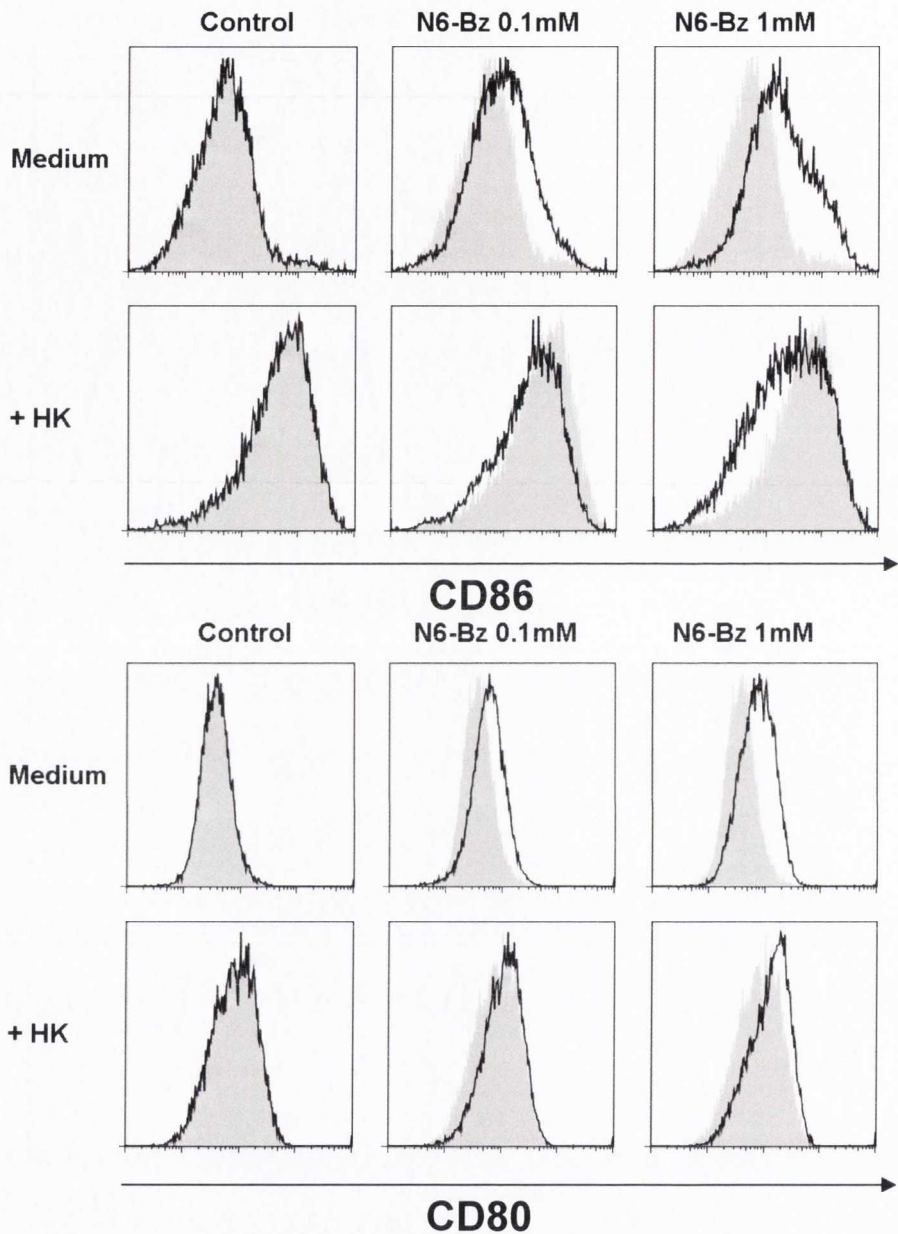


Figure 3.13: Stimulation of DCs with the PKA agonist N6-Bz increases CD86 and CD80 expression on DCs. DCs (1×10^6 cells/ml) from BALB/c mice were stimulated with the PKA activator (N6-Bz: 0.1mM & 1mM) or PBS alone as an untreated control (Control). Additional groups of DCs were treated 2 hours later with Heat Killed *E. coli* (HK) at a ratio equivalent to 1 bacteria cell to 1 DC (1:1). After 24 hours, cells were washed, and immunofluorescence was performed with antibodies specific for CD80 and CD86. Staining for CD80 or CD86 is shown. The grey shaded histogram, indicates the control, while the black line indicates the N6-Bz-treated cells. Data are representative of three independent experiments.

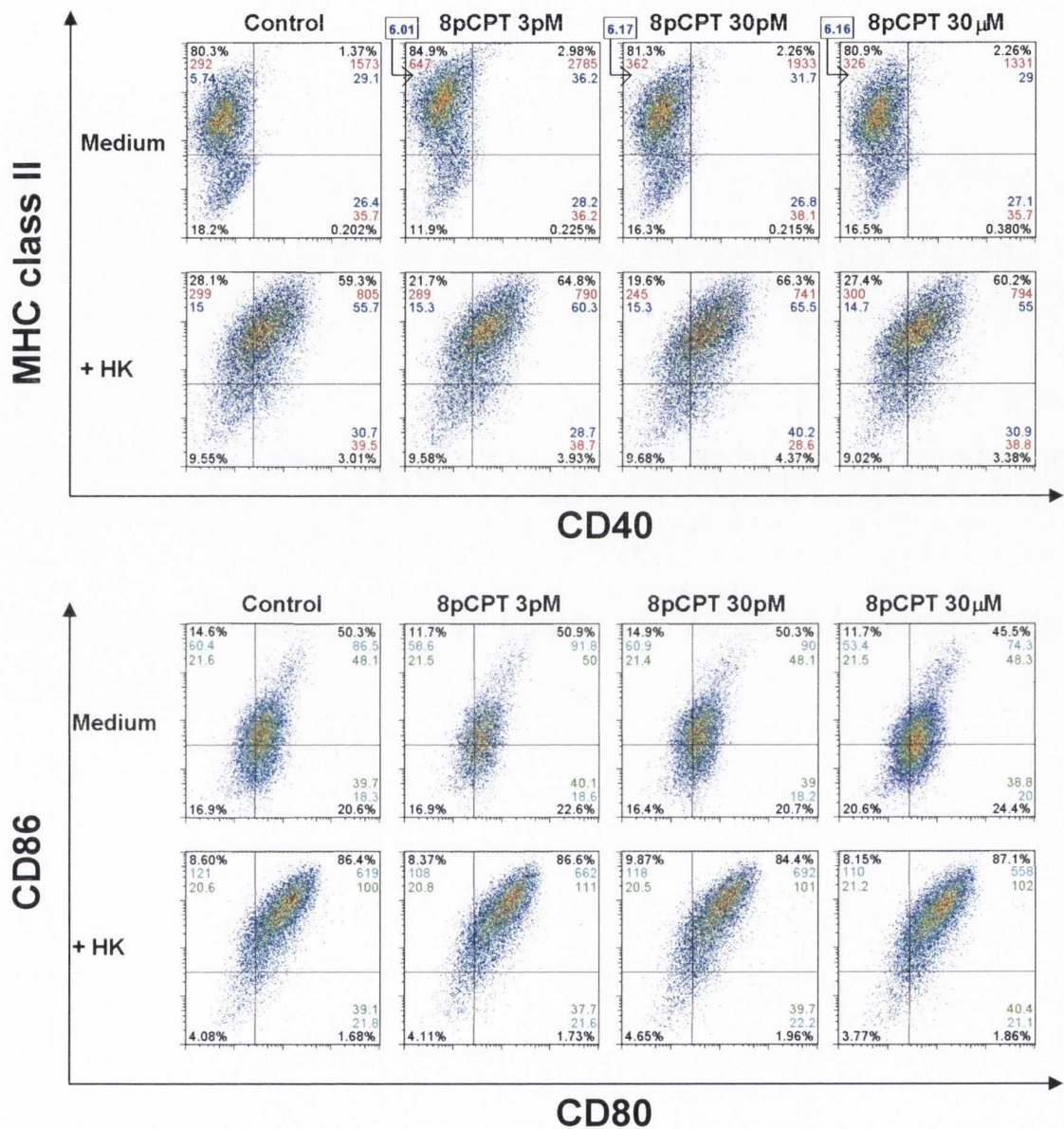


Figure 3.14: Stimulation of DCs with the Epac agonist 8pCPT increases MHC class II expression on DCs. DCs (1×10^6 cells/ml) from BALB/c mice were stimulated with the Epac activator (8pCPT: 3pM – 30µM) or PBS alone as an untreated control (Control). Additional groups of DCs were treated 2 hours later with Heat Killed *E. coli* (HK) at a ratio equivalent to 1 bacteria cell to 1 DC (1:1). After 24 hours, cells were washed, and immunofluorescence was performed with antibodies specific for CD80, CD86, CD40 and MHC Class II. Expression of CD40 versus MHC class II and CD80 versus CD86 is shown. Total percentages of cells are shown in black in each quadrant. Median fluorescence values (MFI values) are shown for each of the maturation markers in each quadrant; CD40 (blue), MHC class II (red), CD80 (green) and CD86 (cyan). Data are representative of three independent experiments.

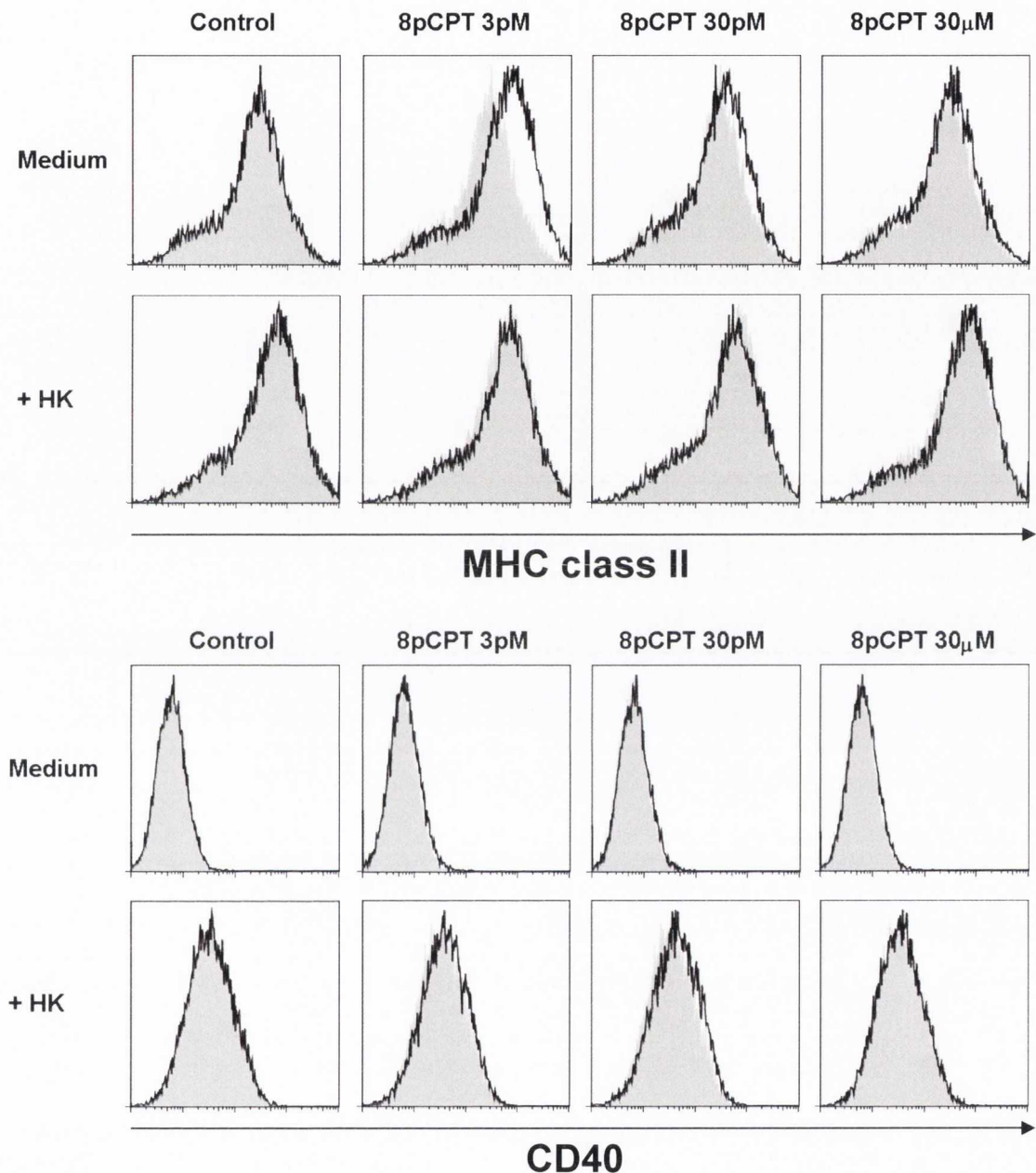


Figure 3.15: Stimulation of DCs with the Epac agonist 8pCPT increases MHC class II expression in DCs. DCs (1×10^6 cells/ml) from BALB/c mice were stimulated with the Epac activator (8pCPT: 3pM – 30μM) or PBS alone as an untreated control (Control). Additional groups of DCs were treated 2 hours later with Heat Killed *E. coli* (HK) at a ratio equivalent to 1 bacteria cell to 1 DC (1:1). After 24 hours, cells were washed, and immunofluorescence was performed with antibodies specific for CD80, CD86, CD40 and MHC Class II. Immunofluorescence of CD40 or MHC class II is shown. The grey shaded histogram, indicates the control, while the black line represents 8pCPT-treated cells. Data are representative of three independent experiments.

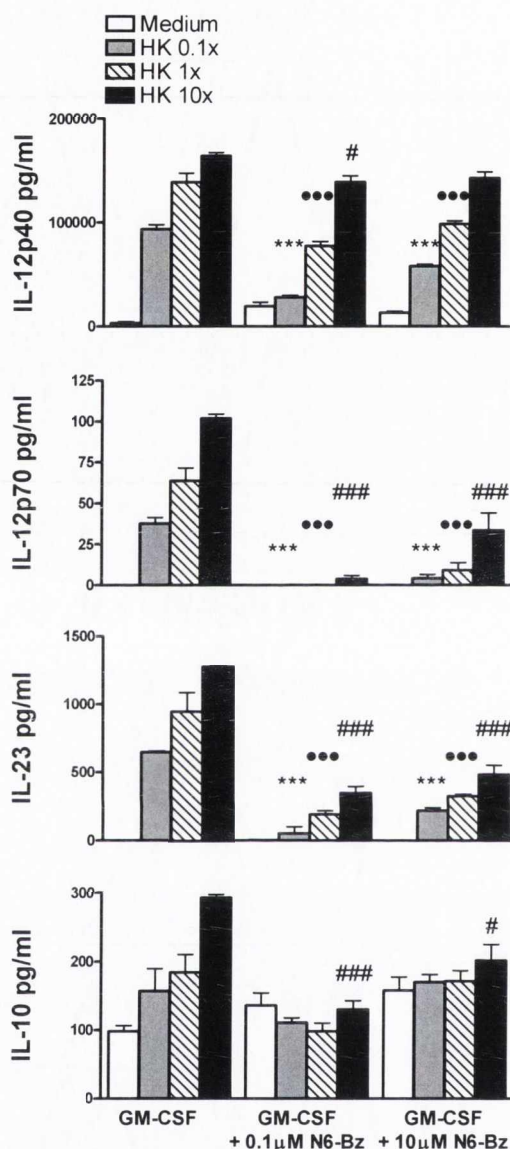


Figure 3.16: Culture of DCs with the PKA agonist N6-Bz inhibits secretion of IL-12p40, IL-12p70 and IL-23 upon re-stimulation with HK bacteria. DCs (6.25×10^5 cells/ml) from BALB/c mice cultured with GM-CSF alone or with GM-CSF and the PKA agonist N6-Benzoyl-cAMP (N6-Bz 0.1-10 μM) were treated with a range of concentrations of Heat Killed *E. coli* (HK) (at ratios equivalent to 0.1, 1 or 10 bacteria cells to 1 DC). After 24 hours the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between HK(0.1x) and treated samples + HK(0.1x) are defined as $p < 0.001$ (***). Significant differences between HK(1x) and treated samples + HK(1x) are defined as $p < 0.001$ (•••). Significant differences between HK(10x) and treated samples + HK(10x) are defined as $p < 0.05$ (#) and $p < 0.001$ (###). Data are representative of three independent experiments with mean $\pm$ S.D. run in triplicate.

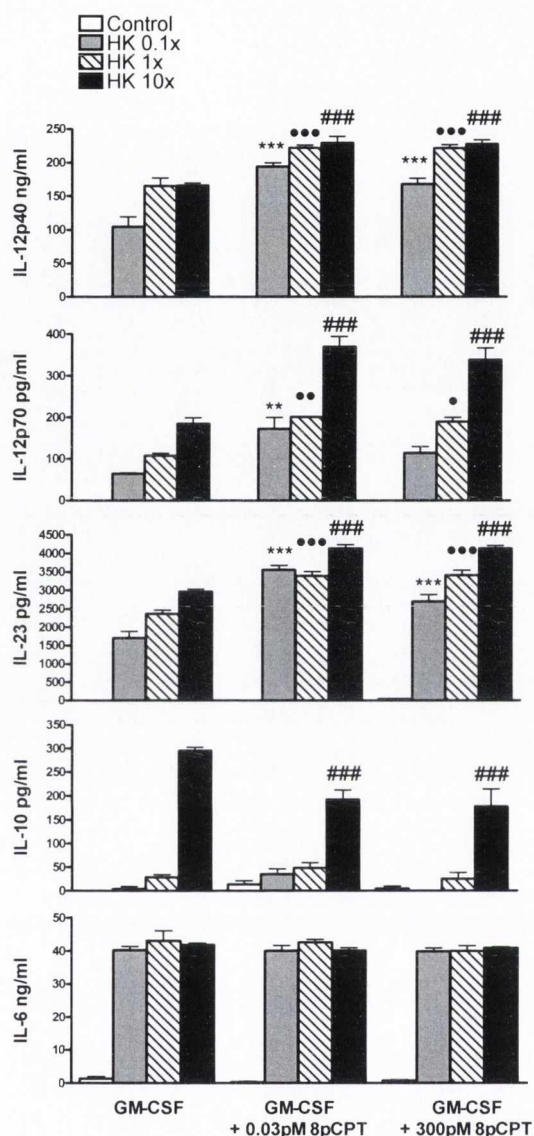


Figure 3.17: Culture of DCs with the Epac agonist 8pCPT increases secretion of IL-12p40, IL-12p70 and IL-23 upon re-stimulation with heat-killed bacteria. DCs (6.25×10^5 cells/ml) from BALB/c mice cultured with GM-CSF alone or with GM-CSF and the Epac agonist 8-pCPT-2'-O-Me-cAMP (8pCPT: 0.03pM – 300pM) were treated with a range of concentrations of Heat Killed *E. coli* (HK) (at ratios equivalent to 0.1, 1 or 10 bacteria cells to 1 DC). After 24 hours the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between HK(0.1x) and treated samples + HK(0.1x) are defined as $p < 0.01$ (**) and $p < 0.001$ (***). Significant differences between HK(1x) and treated samples + HK(1x) are defined as $p < 0.05$ (•), $p < 0.01$ (••) and $p < 0.001$ (•••). Significant differences between HK(10x) and treated samples + HK(10x) are defined as $p < 0.001$ (###). Data are representative of three independent experiments with mean $\pm$ S.D. run in triplicate.

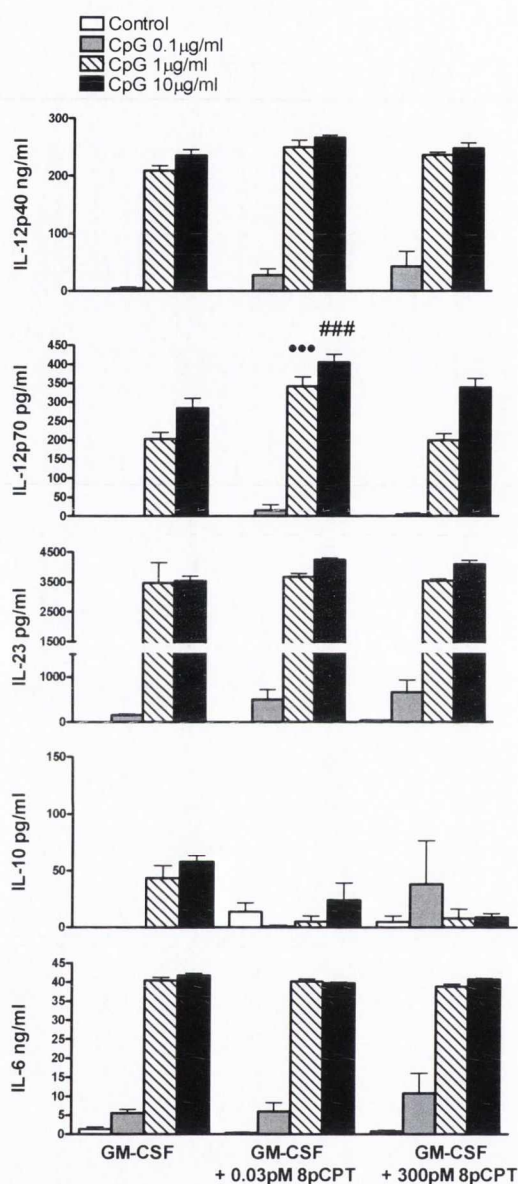


Figure 3.18: Culture of DCs with the Epac agonist 8-pCPT increases secretion of IL-12p40, IL-12p70 and IL-23 upon re-stimulation with CpG. DCs (6.25×10^5 cells/ml) from BALB/c mice cultured with GM-CSF alone or with GM-CSF and the Epac agonist 8-pCPT-2'-O-Me-cAMP (8pCPT: 0.03pM – 300pM) were treated with a range of concentrations of CpG (0.01 $\mu\text{g/ml}$ – 10 $\mu\text{g/ml}$). After 24 hours the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between CpG(1 $\mu\text{g/ml}$) and treated samples + CpG(1 $\mu\text{g/ml}$) are defined as $p < 0.001$ (***). Significant differences between CpG(10 $\mu\text{g/ml}$) and treated samples + CpG(10 $\mu\text{g/ml}$) are defined as $p < 0.001$ (###). Data are representative of three independent experiments with mean $\pm$ S.D. run in triplicate.

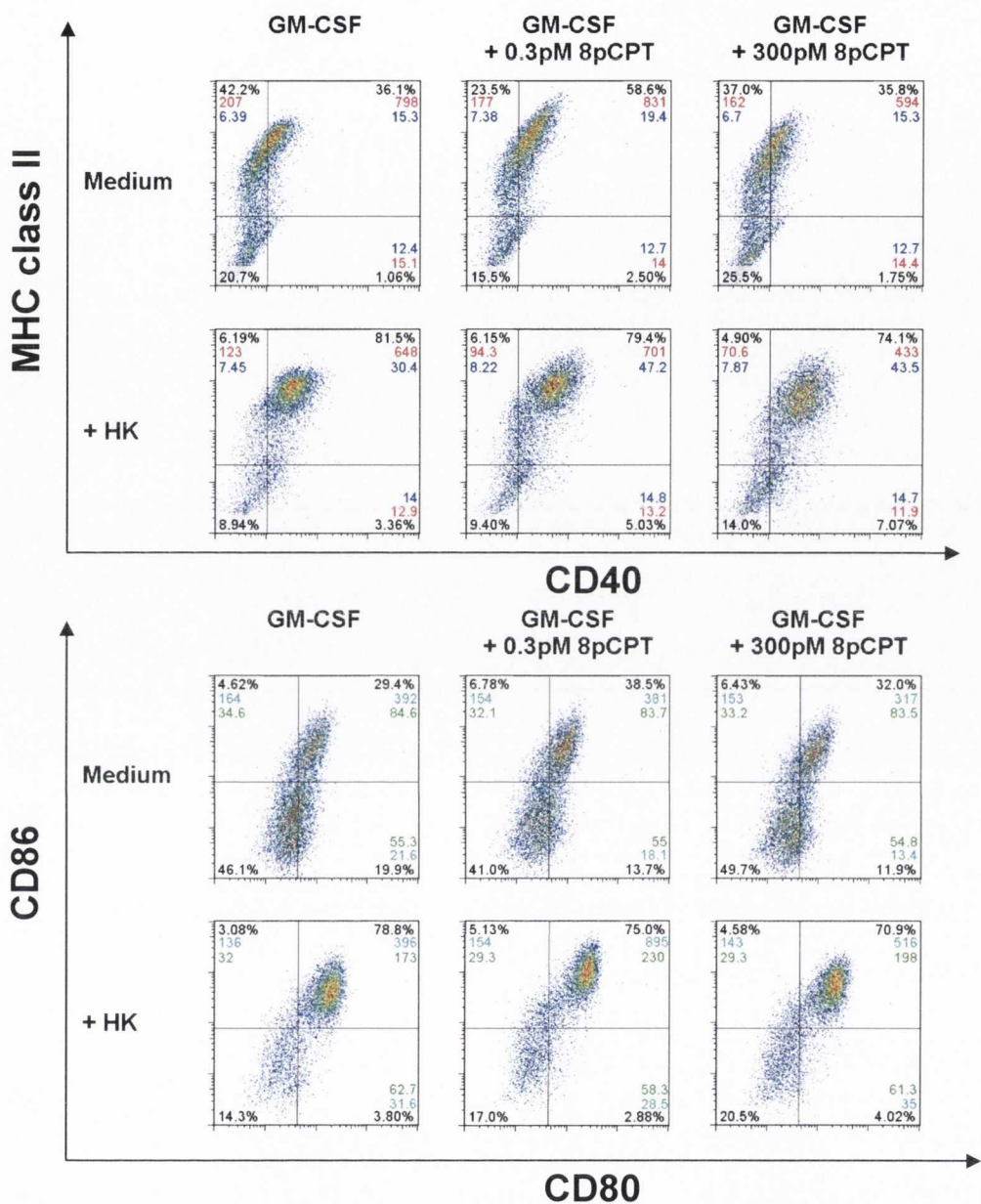


Figure 3.19: Culture of DCs with the Epac agonist 8-pCPT increases CD40 and CD86 expression. DCs (1×10^6 cells/ml) from BALB/c mice were stimulated with the Epac activator (8pCPT: 0.3pM – 300pM) or PBS alone as an untreated control (Control). Additional groups of DCs were treated 2 hours later with Heat Killed *E. coli* (HK) at a ratio equivalent to 1 bacteria cell to 1 DC (1:1). After 24 hours, cells were washed, and immunofluorescence was performed with antibodies specific for CD80, CD86, CD40 and MHC Class II. Expression of CD40 versus MHC class II and CD80 versus CD86 is shown. Total percentages of cells are shown in black in each quadrant. Median fluorescence values (MFI values) are shown for each of the maturation markers in each quadrant; CD40 (blue), MHC class II (red), CD80 (green) and CD86 (cyan). Data are representative of three independent experiments.

Discussion

The anti-inflammatory and immunosuppressive effects of cAMP were first described 35 years ago [355]. However, the effects of cAMP elevation in cells are not always inhibitory. For example, the bacterial enterotoxins, cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT), are very powerful adjuvants and their enhancement of intracellular cAMP has been proposed as a dominant factor in their adjuvant effects *in vivo* [356]. This contradiction indicates that activation of cAMP signalling pathways has diverse effects on the immune system that cannot be simply categorised as immunosuppressive. The recent discovery of the cAMP downstream effector Epac, has further added to the complexity associated with this signalling pathway.

Innate immune responses are crucial to the induction of adaptive immunity; therefore, manipulation of the innate immune response is an important property of effective vaccine adjuvants. DCs are the principal APCs linking the innate and adaptive immune responses [85], and it is therefore important to determine the effects of downstream components of the cAMP pathway, e.g. Epac and PKA, on these cells. Several reports have demonstrated that the elevation of intracellular cAMP modulates cytokine production by DCs. Prostaglandin E₂ (PGE₂) is a potent stimulator of GPCRs leading to increased cAMP. It is produced in abundance at sites of infection and it inhibits key inflammatory functions of macrophages through cAMP-dependent mechanisms. For example, PGE₂ inhibits TNF- α secretion while increasing IL-10 [357, 358] and it has been found to inhibit IL-12 production in human monocytes [359]. However, PGE₂ has been found to increase IL-23 production from DCs [360], which is a key cytokine for the pro-inflammatory Th17 pathway.

Manipulation of the cAMP pathway in humans has been accomplished using various strategies. Current drug therapies that modulate the cAMP signalling pathway include several different classes of PDE inhibitors. The PDEs are the enzymes responsible for the degradation of cAMP in the cell. Inhibition of these enzymes causes intracellular cAMP to accumulate. Caffeine was the first known non-specific PDE inhibitor [361]. PDE4 is the predominant PDE found in airway smooth muscle cells and in inflammatory and immune cells [247]. Several PDE4 inhibitors have been developed, e.g. cilomilast and roflumilast, and are used clinically as bronchodilators [362], to

enhance circulation [363], to treat depression and psychosis [364] and to treat asthma and chronic pulmonary disease (COPD) [365].

This study was performed in order to determine the effects of selective activation of PKA and Epac, the main cellular targets of cAMP signalling, on DC cytokine production. Firstly the effect of non-selective intracellular cAMP elevation on DC cytokine secretion was determined by using the cAMP analogue DB-cAMP, the non-specific PDE inhibitor IBMX and the AC activator Forskolin. Elevation of intracellular cAMP resulted in decreased IL-12p40 and IL-12p70 secretion, while IL-10 secretion was increased. This corresponds with what has been reported in the literature where CT (which elevates intracellular cAMP) inhibited IL-12 production in human monocytes *in vitro* [366]. PKA was long regarded as the only cAMP target in cells and its activation has been reported to decrease TNF- α , MIP-1 α and LTB₄ secretion while increasing IL-10 and IL-6 production in pulmonary alveolar macrophages *in vitro* [367]. Activation of PKA in DCs has been found to suppress TNF- α and IL-12 production via the transcription factor c-Fos [263]. In this study the effects of PKA activation on DC cytokine production were investigated using the PKA specific activator N6-Bz. PKA activation inhibited IL-12p40 and IL-12p70 secretion while increasing IL-10 and IL-6 production in a dose-dependent manner in the presence of HK bacteria. This correlates with the effects measured in response to non-selective cAMP elevators.

Since the first description of Epac in 1998 [268], it has been implicated in various cell functions. A study was carried out to determine if Epac-mediated Rap1 activation affected NF- κ B activity [350] and found that Epac1 activation elevated the transcriptional activity of NF- κ B in murine macrophages. This indicated that Epac is capable of activating NF- κ B through Rap1 and, since NF- κ B is a key mediator of both innate and adaptive responses it suggested a role for Epac in the modulation of immune responses. PGE₂ (a cAMP elevator) suppressed LPS induced IFN- β production from macrophages in an Epac-dependent manner and suppressed LPS-induced TNF- α production in these cells in a PKA-dependent but Epac-independent manner [304]. PGE₂ treatment of activated DCs inhibited expression and release of the inflammatory chemokines CCL3 and CCL4 in an Epac-dependent manner [305].

These results indicate that Epac and PKA can have complimentary or contrasting effects on immune responses.

It has not been established if Epac plays a role in DC cytokine production. In this study, the effects of Epac activation on DC cytokine production were determined using the specific Epac activator 8pCPT. A wide range of concentrations of the activator were used to fully investigate its effects on DC cytokine production (0.008pM – 2.5mM). Interestingly, in contrast to the effects of PKA activation, Epac activation enhanced IL-12p40 and IL-12p70 production by the DCs. There was no effect on either IL-6 or IL-10 production upon activation of Epac, indicating that unlike PKA, Epac is unable to modulate production of these cytokines in DCs. A study has shown however, that Epac activation is able to induce IL-6 production via SOCS3 in vascular endothelial cells [368]. The concentrations of the Epac activator used in the latter paper, however, ranged from 1-200μM, whereas in this project it was found that picomolar concentrations of the activator were able to induce IL-12 but not IL-6. Since PKA has been found to increase the secretion of IL-6, it may be possible that these reported effects of Epac on IL-6 were actually due to the activation of PKA and not Epac, particularly as it has been shown that the Epac activator, if used at high concentrations, may also activate PKA [369]. In the current study, inhibition of IL-12 production by DCs was seen when the Epac activator was used at mM and high μM concentrations. Therefore the concentrations of the Epac, and possibly PKA, activators are extremely important factors when investigating their effects on cytokine production. The demonstration in the current study that Epac activation can promote IL-12 is the first indication that this pathway may promote production of this pro-inflammatory cytokine. A method to determine if PKA or Epac is being activated at the varying doses of PKA and Epac activators, could be devised by measuring the phosphorylation of CREB upon PKA activation or the addition of GTP to Rap1 proteins via Western Blot. Currently there are antibodies available for measurement of the phosphorylation status of CREB once there is sufficient sample to detect an ample signal. There has been a method described where the GTP versus GDP (i.e. activated or not activated) status of Rap1 is measured but currently this is only for the human Rap1 protein and not the mouse, which has some slight differences in the codon sequences which may affect the accuracy of the detection method. A future direction

could be to develop an assay to determine if PKA or Epac is activated by measuring the downstream effectors of these cAMP pathway constituents. As there is no direct method available for measurement of the activation of the Epac protein, measurement of Rap1 activation may not be sufficient as there may be other undiscovered downstream targets of Epac activation.

Glybenclamide (GLB) belongs to a group of sulfonylurea compounds used to treat type II diabetes in patients, and has been shown to bind specifically to Epac2 [286]. DCs treated with GLB showed increased IL-12p70 and IL-12p40 secretion, but no change was observed in IL-23 and IL-6 production. The effects induced by treatment of DCs with the Epac activator are similar to the effects induced by GLB treatment of these cells. As GLB is a treatment for diabetes, this induction of IL-12 is an undesirable side effect of patient treatment with GLB, and further study may need to be carried out to precisely determine if unwanted immune effects are occurring.

DCs exist in two functional states, immature and mature. Immature DCs are efficient in taking up and processing antigens while mature DCs are primed for interactions with naïve T cells. A variety of factors can induce DC maturation including: microbial agonists of PRRs (e.g. LPS), inflammatory cytokines and ligation of cell surface receptors (e.g. CD40). Therefore the DCs can be activated directly, by triggering of PRRs, or indirectly, by exposure to inflammatory signals produced by the immune cells. Mature DCs express increased numbers of cell-surface markers such as MHC molecules, CD40, CD80 and CD86 all of which are important for interaction with naïve T cells [91]. Analysis of the maturation state of DCs following Epac activation allows the determination of whether Epac activation can alter the expression of DC maturation markers. Epac through Rap1 signalling has been shown to modify integrin expression on cells which suggests that Epac signalling could alter cell surface expression of other proteins such as the maturation markers expressed on DCs [370]. It has not been established whether Epac activation in DCs is capable of modifying their maturation.

cAMP elevation, using the cAMP analogue DB-cAMP, resulted in an increase in the expression of MHC class II molecules on the surface of DCs. Upon addition of HK bacteria, the increase in MHC class II expression was further enhanced. DB-cAMP

also increased CD86 while having no effect on CD80 expression, either when added alone or with HK bacteria. Similar to cAMP elevation, MHC class II expression was increased in DCs stimulated with the PKA activator N6-Bz. Upon stimulation with HK bacteria, CD40 expression was strongly inhibited while MHC class II expression was increased. These effects of PKA activation on CD40 expression are similar to those elicited by the nonspecific elevation of intracellular cAMP. The PKA activator upregulated CD80 and CD86 expression in DCs in the absence of PAMP stimulation, though it inhibited HK bacteria-induced expression of CD86. This is in contrast to the effects of cAMP elevation on CD80 and CD86, where CD86 was increased both in the presence and absence of HK *E.coli*. The effects of cAMP elevation in DCs with DB-cAMP correlated with what has been reported with CT and forskolin, both of which increased the expression of CD80, CD86 and MHC class II in human DCs [371].

The effects of Epac activation on DC maturation were also investigated in this study. Several studies have suggested a role for Epac as a key regulator of cell adhesion and migration, central to which is the ability of Epac and Rap1 to control the activity of cell surface integrins. For example, it has been reported that cAMP elevation via activation of Epac-Rap1 can promote integrin-mediated adhesion of Ovar3 cells (a human ovarian carcinoma cell line) [372]. In addition, Epac signalling has been implicated in the regulation of endothelial cell-cell adhesion via the vascular endothelial (VE)-cadherin [314, 373]. In this study, it was found that Epac activation increased MHC class II expression on DCs, similar to the effects of PKA activation and non-specific cAMP elevation. In contrast to PKA activation and cAMP elevation, Epac activation did not affect the expression of CD40, CD86 or CD80 by DCs. This could indicate that while Epac activation does not affect maturation marker expression, it may have effects on integrin marker expression by DCs.

The developmental pathways that lead to the production of DCs are quite complex. There are many specialised subtypes of DCs, and there is a difference between DCs formed in the steady state (in the absence of infection) as opposed to DCs generated during an inflammatory response. Several culture systems are now available for generating DCs, using different precursor-cell populations and driven by different cytokines [374]. Monocytes and early progenitor cells give rise to DCs when cultured

Chapter 4

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effects occurred with the lower concentration of the Epac activator tested. Addition of HK bacteria to these cells did not alter the percentages of cells expressing these markers when compared to the control. However the MFI values indicate that there is still an increase in the intensity of their expression. Overall, culture of DCs with the Epac activator increases IL-12 and IL-23 production while also increasing the expression of MHC class II, CD40, CD86 and CD80 on DCs. This indicates that differentiation of DCs while Epac is activated, induces a proinflammatory DC phenotype, in contrast to what occurs when DCs are cultured with N6-Bz.

PKA and Epac activation have distinct effects on both cytokine production and maturation of DCs. Epac activation in DCs induces IL-12 production, at both an mRNA and protein level. IL-12 is a key cytokine in the promotion of a Th1-type immune response, indicating that Epac activation may have a role to play in this response. Addition of the Epac activator to undifferentiated bone-marrow cells in the presence of GM-CSF promoted the resulting DCs to produce more IL-12, indicating that Epac activation during the differentiation of these cells into DCs polarises the resulting cytokine profile of these DCs towards a Th1 promoting response. As mentioned in Chapter 1, NF- κ B is a transcription factor that regulates genes in both the innate and adaptive immune response. It is possible that the induction of IL-12 following Epac activation is due to interaction with this key transcription factor.

Introduction

In chapter 3, it was established that Epac1 and Epac2 were expressed in DCs and Epac activation increased the transcription and secretion of IL-12 by DCs and the expression of MHC class II on these cells. The spleen is the largest secondary lymphoid organ, containing about one-quarter of the body's lymphocytes [377]. The white pulp of the spleen is composed of lymphocytes, macrophages, DCs, plasma cells, arterioles, and capillaries [378]. It is organized as lymphoid sheaths, with T and B cell compartments around the branching arterial vessels, which closely resemble the structure of lymph nodes [379]. As the spleen has a mixed population of leukocytes and plays an important role in initiating immune responses, the effects of Epac activation on splenocytes were assessed.

As mentioned previously, upon stimulation of T cells through their antigen receptors there is a transient increase in cAMP levels in the cell. Persistent high levels of cAMP inhibit many cellular responses and therefore TCR-mediated signalling must be coordinated with the activation of cyclic nucleotide PDEs that degrade cAMP [228]. Elevation of intracellular cAMP inhibits lymphocyte activation and agents that can elevate intracellular cAMP levels, have been shown to be immunosuppressive and anti-inflammatory [229]. Upon activation of the TCR-CD3 complex in T cells, there is an initial peak of cAMP and PKA activity, that may serve as an acute negative modulator and a negative feedback signalling mechanism through the TCR/CD3 [254]. The PKA catalytic subunit inhibits IL-2 promoter activity (by binding to it), while overexpression of NFAT counteracts this inhibitory effect of PKA [343]. The effects of Epac activation on T cell cytokine production are unknown. As the cAMP pathway, through PKA, is intrinsically linked to T cell cytokine production, it is possible that Epac activation is capable of altering the cytokine profile of these cells.

As described in chapter 3, PKA activation and Epac activation have very different effects on DC cytokine production. Epac activation promotes IL-12 production from DCs while PKA activation inhibits IL-12 production. As described in chapter 1, IL-12 is a polarising

cytokine for development of a Th1 response, whose signature cytokine is IFN- γ . The effects of Epac activation on spleen cell cytokine production are currently unknown, so it was necessary to examine these effects and determine if they may be related to the responses induced in DCs by Epac activation.

The principal aims and objectives of this chapter are to:

- Determine the expression of Epac1 and Epac2 in spleen cells by confocal microscopy
- Determine the effects of Epac activation on spleen cell cytokine production
- Investigate the effects of the sulfonylurea compounds (known Epac2 activators) on spleen cytokine production

4.1 Epac1 is expressed by lymphoid cells in the spleen

In chapter 3, Epac1 and Epac2 were shown to be expressed in DCs, with Epac1 more abundant than Epac2 (Figure 3.1). As described in the introduction to this chapter, the spleen comprises multiple types of lymphoid and myeloid cells. In order to determine expression of Epac in these cell types, spleen cells were stained with antibodies specific for various lymphoid cells in the spleen (Figure 4.1). Antibodies against CD3, CD4 and CD8 were used to identify helper and cytotoxic T cells. CD11b is an integrin expressed on myeloid cells including monocytes, macrophages, and granulocytes. Splenocytes were sorted using a high-speed MoFlo cell sorter and then cytopspun onto coverslips for analysis. In addition to immunofluorescent analysis of the cell types, the morphology of the cells, especially the nuclei, also aids in the identification of cells. The lymphocytes appear as small round cells, with a round nucleus taking up most of the cell. Neutrophils have a characteristic multi-lobed nucleus while basophils and eosinophils are larger cells with bigger single lobed nuclei. All CD3⁺ cells, including both CD4⁺ and CD8⁺ subtypes (Figure 4.1), and CD11b⁺ cells (Figure 4.2) expressed Epac1. Expression was found predominantly, if not exclusively, on the membrane with little or no expression in the nucleus (Figure 4.1 & Figure 4.2). As CD11b⁺ cells represent a mixture of cell populations, four representative pictures are shown, with the different nuclear structures of the different cell types shown (Figure 4.2). In addition NK and NKT cells, i.e. DX5⁺ cells, were found to express Epac1 (Figure 4.1). In contrast, Epac2 was not as widely expressed as Epac1, as was observed in chapter 1 with DCs (Figure 4.3). Incubation of cells with the isotype control and secondary antibody alone shows that the antibodies are not binding non-specifically (Figure 4.4).

4.2 Non-specific elevators of intracellular cAMP inhibit cytokine production by spleen cells.

Having determined the effects of cAMP and its effectors on DCs in chapter 3, we next sought to analyse the effects of Epac and PKA activation on splenocyte cytokine production. The effects of increasing cAMP production in anti-CD3-stimulated spleen

cells were tested using either the cAMP analogue dibutyryl cAMP (DB-cAMP) (Figure 4.5) or the adenylate cyclase activator Forskolin (Figure 4.6). HK bacteria was added to the cells to enhance the induction of splenocyte cytokine production, and upon addition of a cAMP-elevator to these cells to assess whether cAMP elevation could modify the HK-bacteria induced cytokine profile. After 96 hours in culture the supernatants were removed and analysed for cytokine production.

cAMP elevation in spleen cells using DB-cAMP alone resulted in suppression of IFN- γ at the highest concentration of DB-cAMP (100 μ M). Treatment of spleen cells with DB-cAMP and HK bacteria resulted in suppression of HK-induced IFN- γ and IL-17 at all concentrations of DB-cAMP tested (Figure 4.5), and suppression of IL-4 and IL-6 was seen at the highest concentration of DB-cAMP (100 μ M) (Figure 4.5). Stimulation of splenocytes with the lowest concentration of DB-cAMP (10 μ M) induced an increase in HK bacteria-induced IL-6 production, but this was not significant with respect to HK bacteria alone (Figure 4.5). cAMP elevation in spleen cells using Forskolin had a similar effect to DB-cAMP on cytokine production (Figure 4.6). Forskolin treatment alone suppressed anti-CD3-induced IFN- γ and IL-4 production by splenocytes, although this was not significant (Figure 4.6). However, stimulation of splenocytes with both 100 and 500 μ M Forskolin resulted in significant suppression of HK bacteria-induced IFN- γ , IL-4, IL-17 and IL-6 production (Figure 4.6).

4.3 The PKA activator N⁶-Benzoyl-cAMP inhibits cytokine production by spleen cells.

In chapter 3, it was shown that PKA activation in DCs suppressed IL-12p40 and IL-12p70 production from DCs (Figure 3.4). In order to determine the effects of PKA activation on spleen cell cytokine production, spleen cells were incubated with the PKA activator N⁶-Benzoyl-cAMP (N6-Bz) in the presence and absence of heat-killed bacteria (Figure 4.7). PKA activation significantly inhibited HK bacteria-induced IFN- γ , IL-4, IL-17 and IL-6 production by spleen cells (Figure 4.6). In the absence of heat-killed bacteria, there was also some suppression of cytokine production but this was not significant (Figure 4.7). The suppression of IL-6 production in response to PKA activation in

splenocytes is in contrast to the enhancing effects of PKA activation in DCs as seen in chapter 3 (Figure 3.4).

4.4 The Epac activator 8pCPT induces an increase in cytokine secretion by spleen cells

The effects of stimulating splenocytes with the Epac activator were investigated next. Spleen cells were plated on anti-CD3 and incubated in the presence of the Epac activator 8pCPT-2'O-Me-cAMP (8pCPT) for four days at 37°C, after which the supernatant was removed and analysed for cytokine production by ELISA. Epac activation promoted IFN- γ , IL-4, IL-10 and IL-17 production in spleen cells (Figure 4.8). All three concentrations of the activator (1pM, 10pM and 100pM) promoted IFN- γ secretion; the 10pM and 100pM concentrations of the activator induced a significant increase in IFN- γ with respect to unstimulated control cells. The Epac activator (at 10pM or 100pM concentrations) also induced a significant increase in the production of both IL-4 and IL-10. IL-17 was also increased by the Epac activator, but not significantly (Figure 4.8). The fold induction profile (Figure 4.9) showed that although all of the cytokines were induced upon Epac activation, the enhancement of IFN- γ with respect to unstimulated control cells was far greater than the enhancement in IL-17, IL-4 and IL-10. Enhancement of IFN- γ secretion by the Epac activator (at 10pM and 100pM) induced between a 10- and 20-fold increase in IFN- γ secretion, when compared to the control (Figure 4.9). In contrast, treatment of spleen cells with the same concentrations of the Epac activator induced between less than a 5-fold increase in IL-4, IL-17 and IL-10 production (Figure 4.9).

The effects of adding the Epac activator to cells together with HK bacteria were also determined. Stimulation of splenocytes with HK bacteria induced the production of all the cytokines tested, IFN- γ , IL-4, IL-17 and IL-10 (Figure 4.10). The addition of Epac activator at 10pM and 100pM concentrations significantly enhanced the induction of IFN- γ by HK bacteria (Figure 4.10). Stimulation with the Epac activator also induced slight increases in the secretion of IL-4, IL-10 and IL-17, but these were not significant

with respect to cells treated with HK bacteria alone. The fold induction profile again indicated that IFN- γ is the predominant cytokine induced by Epac activation in spleen cells both with and without addition of HK bacteria (Figure 4.11). Epac activation, using 8pCPT at 10 and 100pM enhanced the HK bacteria-induced IFN- γ by at least approximately 2-2.5 fold when compared to HK bacteria alone, much more than the other cytokines (Figure 4.11). As the data presented in Figure 4.10 is important in establishing the ability of Epac to promote increased IFN- γ production from spleen cells, the data from three individual experiments is shown in Appendix Figure C.

4.5 The Epac2 activator Glybenclamide enhanced cytokine production from spleen cells.

Glybenclamide (GLB) belongs to a class of drugs called sulfonylureas. It is currently used to treat type 2 diabetes and acts to increase insulin release from beta cells in the pancreas. Epac2 is highly expressed in the pancreas and these sulfonylureas have been found to specifically activate Epac2. Spleen cells were stimulated with a range of concentrations of GLB, in the same manner as described previously with the Epac activator. Stimulation of spleen cells with GLB alone significantly enhanced IFN- γ and IL-4 production, while IL-17 and IL-6 were also increased following GLB stimulation but not significantly (Figure 4.12). Treatment of spleen cells with GLB in conjunction with HK bacteria promoted significant increases in IFN- γ , IL-4, IL-17 and IL-6 production compared to stimulation with HK bacteria alone (Figure 4.12). IL-10 production was not enhanced in response to GLB treatment in spleen cells (data not shown).

4.6 siRNA knockdown of Epac1 blocks the induction of cytokines by the Epac activator 8pCPT

To determine if the enhancing effects of the Epac activator on spleen cell IFN- γ production were specific for Epac, siRNA knock-down of Epac1 was performed. Spleen

cells were transfected with either a pool of siRNAs specific for Epac1 or with a non-targeting pool of control siRNA (scrambled siRNA (siSCR)). The spleen cells were transfected, plated on anti-CD3, and stimulated with the Epac activator. Two siRNA concentrations (20&100nM) (concentrations derived from established protocols in the literature) were tested to determine their effects on Epac mRNA expression (Figure 4.13). Transfection of splenocytes with 20nM siRNA reduced Epac mRNA expression by approximately 50%, and this concentration of siRNA was used for transfection in subsequent studies.

Both real-time PCR and ELISA were used to analyse the effects of Epac1 knock-down on IFN- γ production following treatment with the Epac activator. Real-time PCR was used to determine IFN- γ mRNA expression 6 hours post-stimulation with the Epac activator (Figure 4.14a). Treatment of control siSCR transfected cells with the Epac activator (10 and 100pM) increased IFN- γ mRNA production. In contrast, IFN- γ expression was significantly reduced in siEPAC-transfected cells stimulated with the Epac activator (Figure 4.14a). The transfection process itself has an effect on the cell's ability to respond to a stimulus. It is known that transfection of a cell causes high amounts of cell death. To combat this unwanted side effect, several steps were taken, including, but not limited to, the use of a transfection buffer and system specially designed for lymphocyte survival and a minimal impact on their function. As the control cells were also transfected, but with a scrambled siRNA sequence, this acts as a good control for the cells transfected with the Epac siRNA. The levels of protein expression of IFN- γ are lower when compared to previous experiments, but both the control siSCR cells and siEPAC cells can be compared to each other.

The suppression in Epac-induced IFN- γ , using siRNA for Epac1, was also seen at the protein level. Stimulation of siSCR-transfected spleen cells with the Epac activator induced increases in IFN- γ secretion. In contrast, spleen cells transfected with siEPAC secreted significantly less IFN- γ upon stimulation with the Epac activator (Figure 4.14b), thus confirming that the ability of the Epac activator to induce IFN- γ is dependent on Epac1. Cells were also treated with PMA and ionomycin. PMA is a mitogen and is frequently used in combination with ionomycin to induce cytokine production and stimulate lymphocytes. Both control siRNA- and Epac1 siRNA-transfected cells secreted

comparable concentrations of IFN- γ in response to PMA and ionomycin stimulation (Figure 4.14b).

The transfection process itself may be stressful to cells, especially to primary non-immortalised cells. To ensure that the reduced IFN- γ by siSPAC1-transfected cells in response to Epac activation was not a result of increased cell death, the cells were isolated and analysed by flow cytometry for cell death by propidium iodide staining (Figure 4.15). Propidium iodide is a fluorescent molecule that is unable to cross the membrane of a live cell, but is able to enter dead cells and therefore can be used to identify dead cells. The percentages of PI-positive (dead) cells in splenocytes transfected with either scrambled control siRNA or Epac1 siRNA are similar, indicating that cell death is not a factor in the suppression of IFN- γ secretion by Epac1 siRNA (Figure 4.15).

4.7 Treatment of splenocytes with the Epac agonist 8pCPT induces a rapid IFN- γ response.

A study was performed to determine the kinetics of IFN- γ secretion by splenocytes stimulated with the Epac activator. Six hours post Epac activation, a significant increase in IFN- γ was observed in splenocytes stimulated with HK bacteria and the Epac activator compared to cells treated with HK bacteria alone, indicating that IFN- γ is produced rapidly (Figure 4.16a). PMA/ionomycin and the TLR agonist R848 were added as positive controls. 8pCPT-treatment of spleen cells induced IFN- γ production at 6 hours and 96 hours and addition of HK bacteria enhanced these effects (Figure 4.16).

IL-12 is known as a major inducer of IFN- γ production [380]. In order to deduce if 8pCPT was inducing IL-12 production which then went on to induce IFN- γ production, or if IFN- γ production occurred first, IL-12p40 levels were analysed in order to determine if and when this was being induced. Epac activation did induce IL-12p40 secretion by splenocytes, although it was not produced until at least 48 hours post stimulation with the Epac activator. However, at 6 hours post 8pCPT stimulation, IFN- γ is detected early on in the culture (Figure 4.17). However, the Epac activator (at 100pM) did induce a significant increase in HK bacteria-induced IL-12p40 production at 6 hours

post stimulation (Figure 4.17). The concentration detected was very low (less than 10pg/ml), (a level which is at the outer detection limit of the assay but still within the standard curve) but it suggests that the presence of HK bacteria does enhance Epac induced IL-12p40 production at an early timepoint. The supernatants were also analysed for IL-12p70 and IL-23 production but these cytokines were not detected (data not shown).

4.8 Epac activation with 8pCPT in spleen cells induces IFN- γ production from CD8⁺ cells.

Having determined that Epac activation induces IFN- γ in splenocytes, it was important to assess which cells were responsible for this increased IFN- γ secretion. IL-17 production by Epac activator-stimulated splenocytes was also analysed to compare to Epac-induced IFN- γ production. Intracellular flow cytometry was performed on the spleen cells with antibodies specific for the cytokines IFN- γ and IL-17, and antibodies specific for CD3, CD4, CD8, $\gamma\delta$ TCR, CD49b (NK cells) and CD11b (myeloid cells). Firstly, the protocol for determining intracellular IFN- γ production was optimised. Brefeldin A (BFA) is commonly used to inhibit secretion of cytokines from the cell but is known to be toxic when used over a prolonged time. A dose-range experiment was performed where splenocytes were stimulated with a range of concentrations of BFA at multiple timepoints to assess inhibition of cytokine secretion and induction of cell death (Figure 4.18). Cells exposed to BFA for between 8 and 24hours showed between 25-50% cell-death as assessed by propidium iodide incorporation, in comparison to unstimulated (control) cells where there was approximately 20% cell death. The percentage of death in cells exposed to BFA for 4 hours was lower (<20%) so this timepoint was chosen in conjunction with a concentration of BFA of 10 μ g/ml, which suppresses IFN- γ secretion from cells without inducing excessive cell death (Figure 4.18), and is comparable to concentrations used in the literature.

Following optimisation of this protocol the spleen cells were plated on anti-CD3, incubated in the presence of the Epac activator and left for four days at 37°C after which the cells were incubated with BFA, PMA and ionomycin for 4 hours. Following this,

immunofluorescence was performed on the cells to determine which cells were secreting IFN- γ in response to Epac activation. While IFN- γ can be produced by both CD4⁺ and CD8⁺ cells, it was found that the primary producer of IFN- γ in response to Epac activation was CD8⁺ T cells (Figure 4.19). $\gamma\delta$ TCR⁺ cells produced small amounts of IFN- γ , but were responsible for the bulk of IL-17 production in response to anti-CD3, which was enhanced by the Epac activator at 10 and 100pM (Figure 4.19). As this is a key Figure showing the effects of Epac activation on IFN- γ production by individual cell types, and is a representative sample from one experiment, three individual experiments were normalised to the control and averaged together in order to summarise the data collectively in one figure as shown in Appendix Figure D.

As NK cells are known to be prime producers of IFN- γ , it was necessary to determine if these cells play a role in the induction of IFN- γ by Epac. Two markers were used to identify NK cells, NKp46 and DX5 (CD49b). NKp46 is a NK-cell specific marker found on both human and mouse cells while DX5 is a marker for NK cells in mice only. NKp46 is incapable of staining NKT cells, while DX5 stains both NK and NKT cells. No increases in either NKp46⁺IFN- γ ⁺ or DX5⁺IFN- γ ⁺ cells were observed after 6 hours stimulation with the Epac activator, indicating that NK cells were not the source of early IFN- γ production (Figure 4.20).

4.9 Stimulation of a splenocyte population depleted of CD8⁺ cells with the Epac activator 8pCPT does not induce an increase in IFN- γ .

In order to determine if the IFN- γ induction by Epac activation in splenocytes was solely from CD8⁺ T cells and not dependent on another cell type, splenocytes were sorted into several populations based on surface marker expression and then stimulated with the Epac activator. Initially CD3⁺ cells were purified and plated on anti-CD3 for 4 days (Figure 4.21). The purified CD3⁺ population contained 94.7% pure CD3⁺ cells, showing a high percentage purity of CD3⁺ cells (Figure 4.21b). Treatment of these purified CD3⁺ cells with the Epac activator, resulted in increased IFN- γ production by CD8⁺ cells, with the unstimulated (control) population containing 12.0% CD8⁺IFN- γ ⁺ cells compared to

14.8% and 17.2% CD8⁺IFN- γ ⁺ cells following stimulation with 10pM and 100pM respectively of the Epac activator (Figure 4.21a). Similarly, stimulation of HK bacteria-treated cells with the Epac activator induced increases in CD8⁺IFN- γ ⁺ cells. R848, a TLR7/8 agonist, is known to induce IFN- γ production and was therefore included as a control (Figure 4.21a). Treatment of the purified CD3⁺ cells with the Epac activator did not result in increased IFN- γ production by CD4⁺ cells and this is shown in Appendix Figure E. As CD8⁺ cells were found to be the main source of Epac-induced IFN- γ a cell sort was performed where these cells were identified and removed from the spleen cell population. In the absence of CD8⁺ cells the Epac-agonist induced IFN- γ increase was significantly reduced, indicating that CD8⁺ cells are necessary for this increase in IFN- γ production (Figure 4.22).

The spleen cell population was also depleted of NK and NKT cells to assess the role of these cells in Epac-induced IFN- γ production. In populations depleted of NK/NKT cells an increase in IFN- γ was still evident following Epac activation, although this was reduced when compared to the non-depleted spleen cells (Figure 4.23). This indicates that NK cells are not the source of the Epac-induced IFN- γ , but that these cells may be important for amplifying the IFN- γ response, which is not surprising as NK cells are very responsive to IFN- γ . It was also observed in the NK cell-depleted population that the IL-4 and IL-6 response following stimulation with the Epac activator or with R848 was virtually unchanged when compared to the undepleted population. However, in comparison to the undepleted population, no IL-17 was detected in the NK depleted population in response to either anti-CD3 stimulation alone or in combination with either the Epac activator or R848 (Figure 4.23). NK cell-depleted splenocytes were stimulated with the 8pCPT and analysed by flow cytometry for IC IFN- γ and increased IFN- γ was found in CD8⁺ cells stimulated with 8pCPT compared to unstimulated cells. (Figure 4.24).

4.10 Epac activation with 8pCPT induces IFN- γ production from CD8⁺ CD44^{lo}, CCR7⁻CD62L⁻ cells.

In 1999 Sallusto *et al.*, demonstrated that memory T cells could be divided into CCR7⁻CD62L⁻CD45RA⁺ or CCR7⁺CD62L⁺CD45RO⁺ cell populations, with the first being designated effector memory cells (Tem) and the latter called central memory T cells (Tcm) [381]. CCR7 is a receptor involved in the migration of lymphocytes to lymph nodes while CD62L is involved in the attachment of immune cells to HEVs [382, 383]. In humans, resting memory cells express CD45RA while upon activation these cells express CD45RO. In mice, CD44 is used as a marker for memory cells as an equivalent marker to CD45RA and CD45RO. Low expression of CD44 (CD44^{lo}) is characteristic of naïve cells and high expression of CD44 (CD44^{hi}) indicates memory cells. Epac activation was shown in the previous section to induce IFN- γ production from CD8⁺ T cells. Intracellular flow cytometry was performed in order to determine the type of CD8⁺ T cell responsible for this Epac-mediated IFN- γ production. Splenocytes were stimulated with the Epac activator and analysed for surface expression of CCR7, CD62L and CD8 in addition to intracellular IFN- γ (Figure 4.25). Treatment of cells with the Epac activator slightly increased CCR7 expression by splenocytes, however there was a dramatic decrease in CD62L expression upon treatment with the Epac activator (both 10 and 100pM) (Figure 4.25). Upon further analysis of the four cell populations outlined in Figure 4.25 (i.e. CCR7⁻CD62L⁻, CCR7⁺CD62L⁻, CCR7⁻CD62L⁺ and CCR7⁺CD62L⁺), it was found that most of the IFN- γ induced by Epac activation was produced from cells negative for both CCR7 and CD62L, and these cells were CD8⁺ (Figure 4.25). Some IFN- γ was produced by CCR7⁻CD62L⁺ cells in response to Epac activation (Figure 4.25), however there were less cells present in this population than in the CCR7⁻CD62L⁻ population indicating that this IFN- γ is a small percentage of the total IFN- γ produced by the CCR7⁻CD62L⁻ population. There was no IFN- γ detected in CCR7⁺ cells (data not shown). Purified CD3⁺ cells were also analysed for CD44 expression. It was found that Epac activation enhances IFN- γ production by CD44^{lo} cells (Figure 4.26). In contrast,

PMA/ionomycin-treated cells had increased CD44 expression and IFN- γ production was predominately from CD44^{hi} cells.

4.11 Transfer of spleen cell supernatants stimulated with the Epac activator 8pCPT onto naïve DCs induces an increase in IL-12 production from these cells.

Having established that Epac activation promotes IFN- γ production from CD8⁺ T cells, and IL-12 production from DCs, the effect of Epac-driven IFN- γ on DC cytokine production was investigated. In order to do this, spleen cells were cultured with the Epac activator and anti-CD3 alone. Cell free supernatants were transferred onto naïve DCs, which were left for 24hrs in culture and the DC cytokine profile was analysed (Figure 4.29). Treatment of DCs with the supernatant from spleen cells stimulated with 8pCPT and anti-CD3 induced an increase in DC IL-12p70 and IL-6 production (Figure 4.27). Addition of HK bacteria to these DCs significantly enhanced the IL-12p70 increase, but both IL-23 and IL-6 production was suppressed (Figure 4.27). The spleen cell supernatant that was used to stimulate the DCs was tested for various cytokines prior to addition to the DCs. No IL-12p70 was detected in the supernatant prior to addition to the DCs, indicating that the DCs are responsible for the observed increase in IL-12p70 (data not shown).

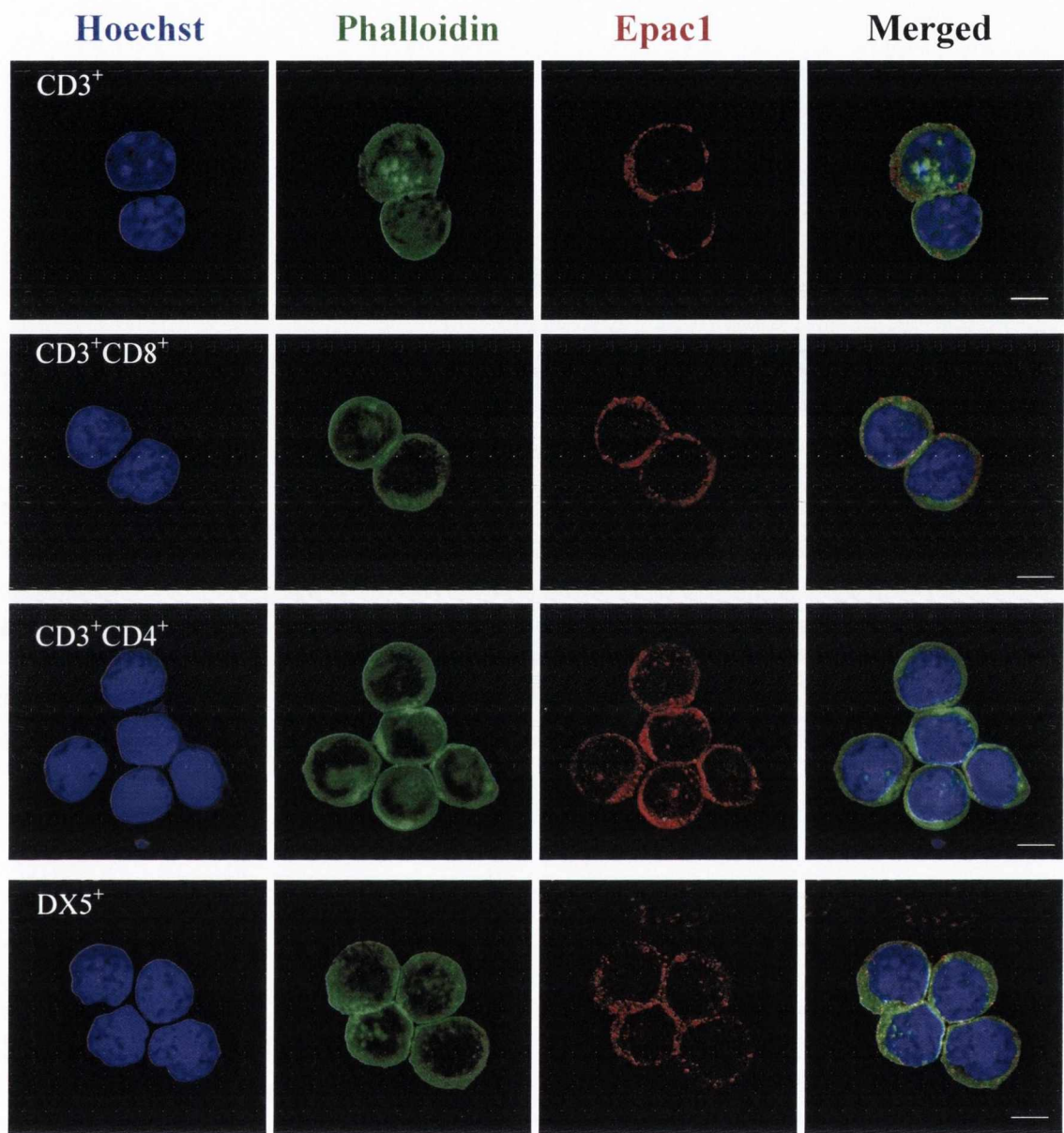


Figure 4.1: Epac1 is expressed in CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ lymphocytes and in DX5⁺ NK cells in the spleen. Immunofluorescence was performed on spleen cells with antibodies specific for CD3, CD4, CD8 and DX5. The use of these antibodies allowed the cells to be sorted into discrete populations by a Beckman Coulter (Dako) MoFlo hi-speed cell sorter. The cells were counted and 200µl of cells (at 1 x 10⁶ cells/ml) were cytopspun onto coverslips, fixed and visualised for Epac1 via confocal microscopy. Data are representative of at least three independent experiments.

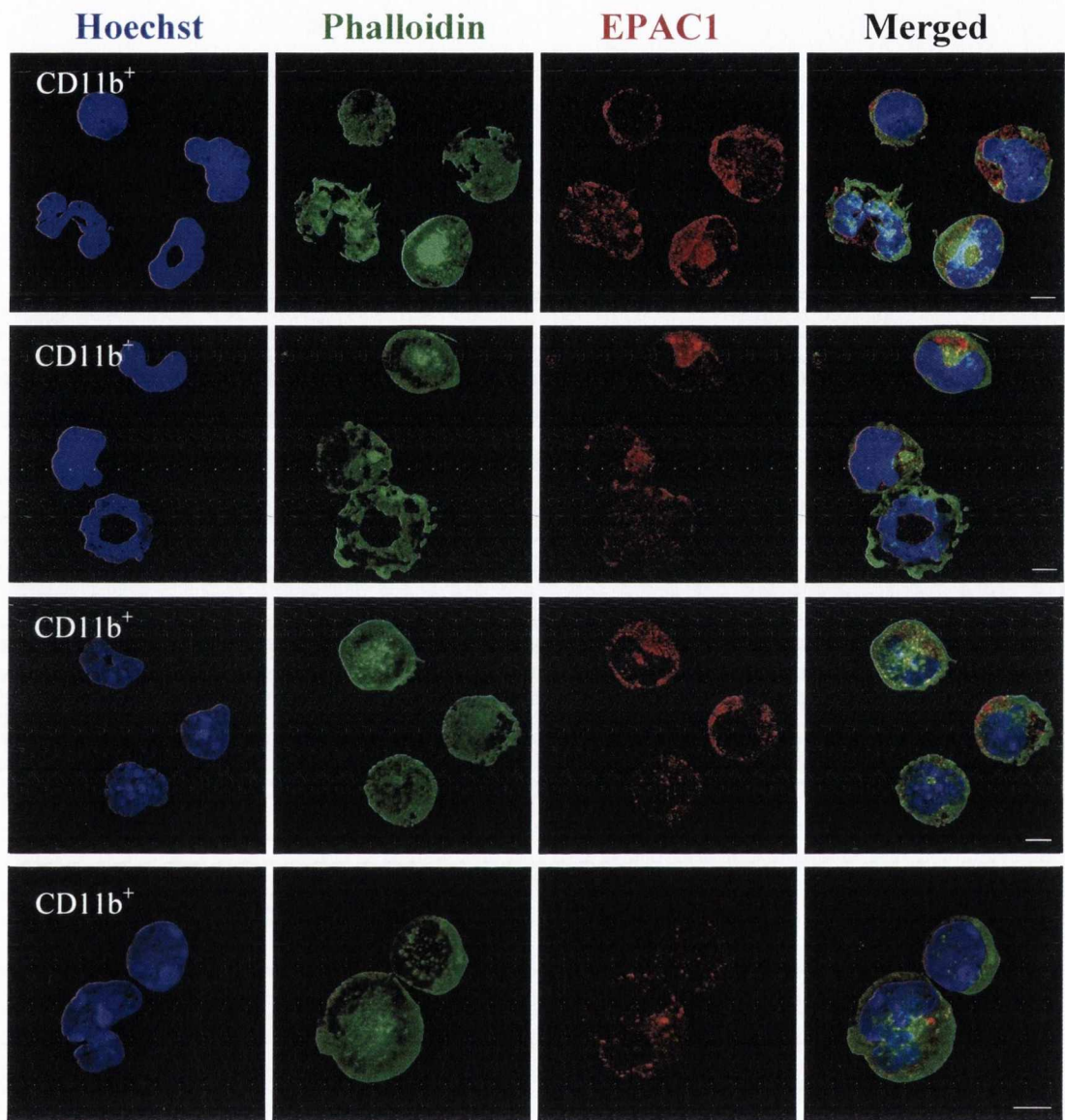


Figure 4.2: Epac1 is expressed by CD11b⁺ cells in the spleen cell population. Immunofluorescence was performed on spleen cells with an antibody specific for CD11b. The cells were sorted into discrete cell populations based on specific antibody staining by a Beckman Coulter (Dako) MoFlo hi-speed cell sorter. The cells were counted and 200µl of cells (at 1 x 10⁶ cells/ml) were cytopun onto coverslips, fixed and visualised for Epac1 via confocal microscopy. Data are representative of at least three independent experiments.

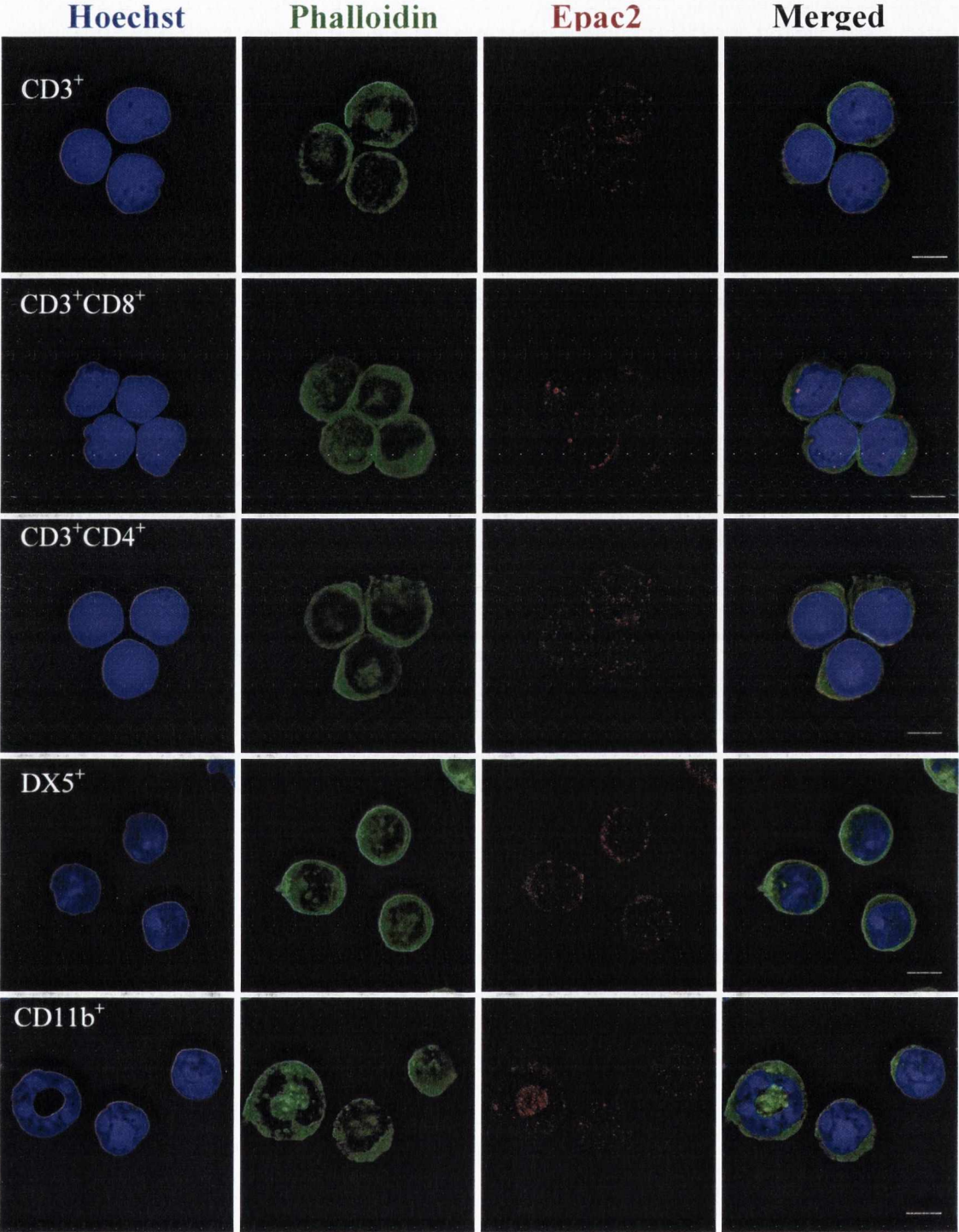


Figure 4.3: Epac2 is expressed by lymphoid cells in the spleen. Immunofluorescence was performed on spleen cells with antibodies specific for CD3, CD4, CD8, DX5 and CD11b. The cells were analysed and sorted into discrete cell populations based on specific antibody staining by a Beckman Coulter (Dako) MoFlo hi-speed cell sorter. The cells were counted and 200µl of cells (at 1×10^6 cells/ml) were cytopspun onto coverslips, fixed and visualised for Epac2 via confocal microscopy. Data are representative of at least three independent experiments.

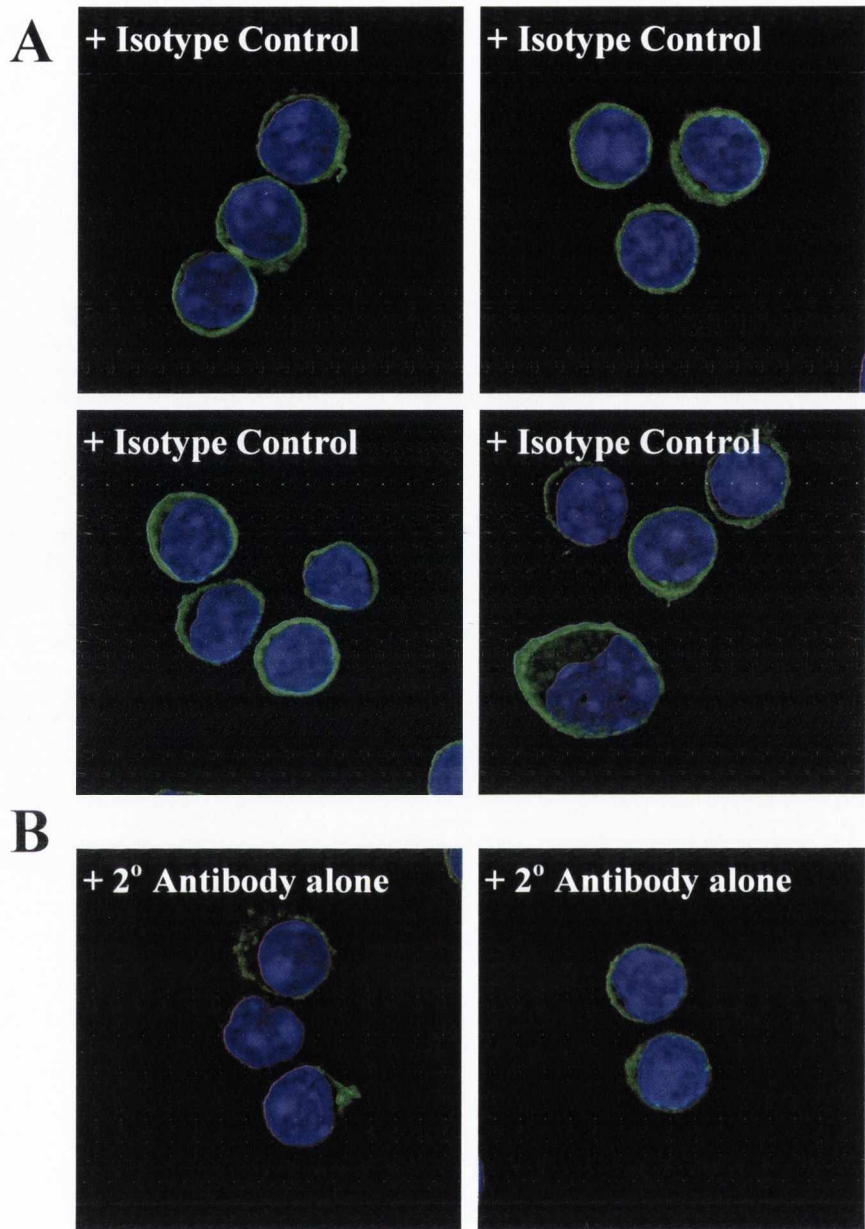


Figure 4.4: The isotype control antibody for Epac1 and Epac2 does not stain the spleen cells. Spleen cells were counted and 200µl of cells (at 1×10^6 cells/ml) were cytospun onto coverslips, fixed and visualised with the isotype control antibody and the secondary antibody (n=4) (A) or the secondary antibody alone (n=2) (B) via confocal microscopy as described in materials and methods. Data are representative of two independent experiments.

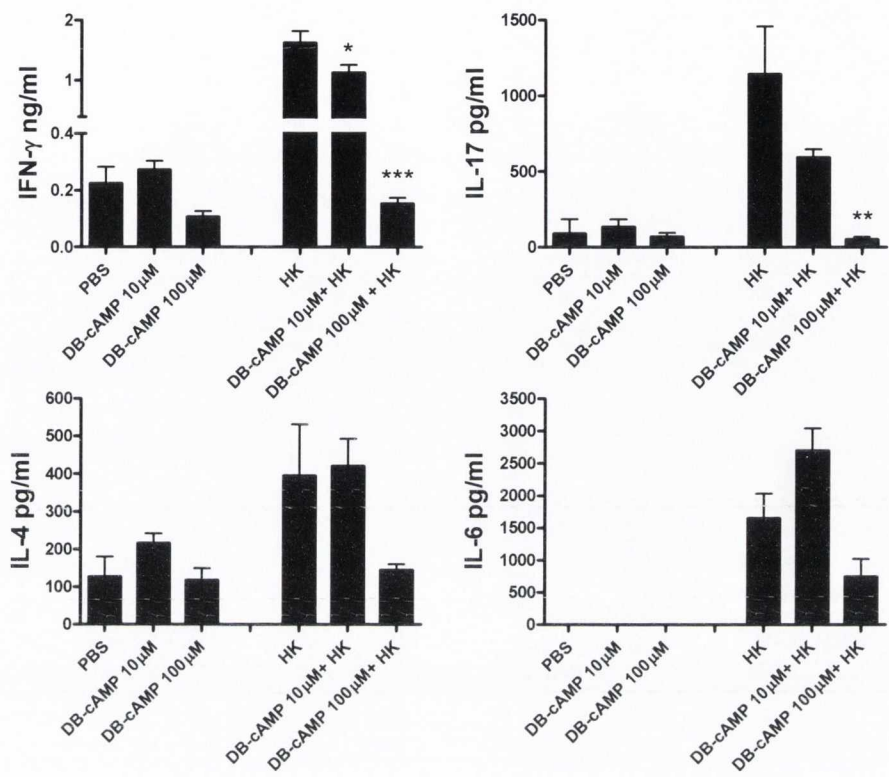


Figure 4.5: Elevation of intracellular cAMP in spleen cells by the cell-permeable cAMP analogue, DB-cAMP, inhibits heat-killed bacteria induced IFN-γ and IL-17 production. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with the cAMP analogue Dibutyryl-cAMP (DB-cAMP) (10 & $100 \mu\text{M}$) alone, or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1 bacteria to 1 spleen cell. After 96 hours, the cytokine concentrations in the supernatants were determined by ELISA. Significance differences between HK and DB-cAMP+HK-treated samples are defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Data are representative of three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

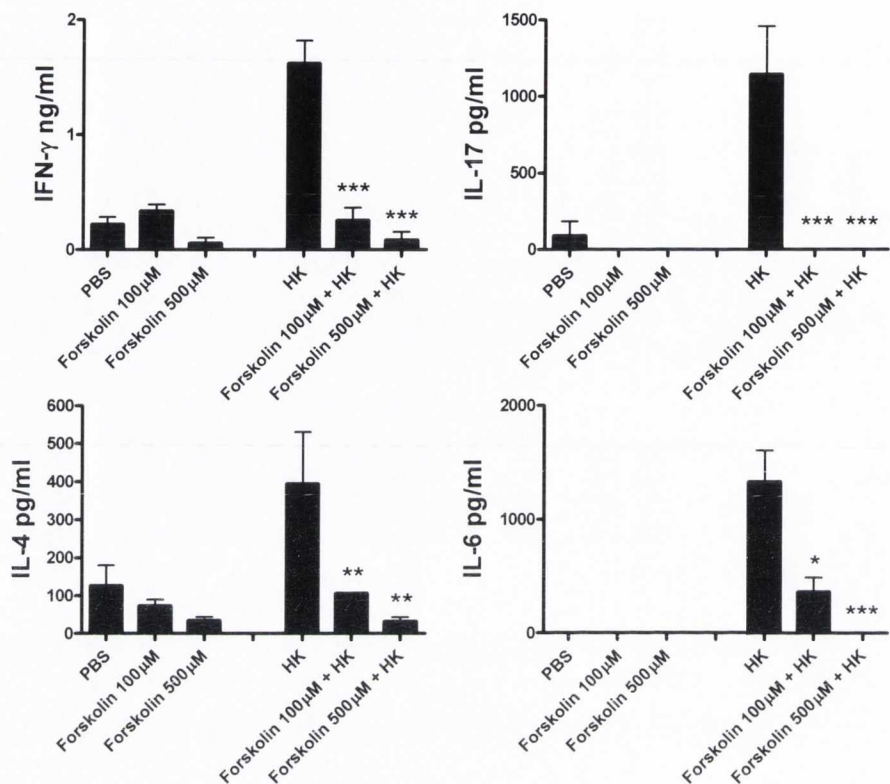


Figure 4.6: Elevation of intracellular cAMP by the adenylate cyclase activator, Forskolin, in spleen cells inhibits IFN- γ , IL-17, IL-4 and IL-6 production. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with the adenylate cyclase activator Forskolin (10 & $100 \mu\text{M}$) alone, or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1 bacteria to 1 spleen cell. After 96 hours, the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between HK and Forskolin+HK-treated samples are defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Data are representative of three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

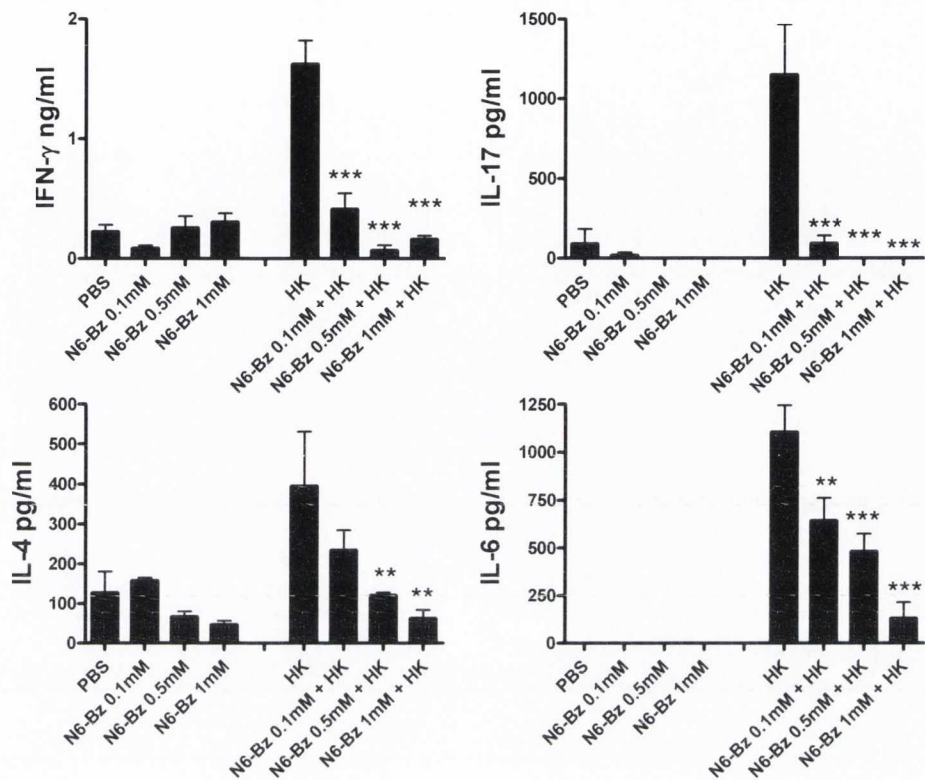


Figure 4.7: Treatment of splenocytes with a selective PKA agonist inhibits heat-killed bacteria-induced IFN- γ , IL-17, IL-4 and IL-6 secretion. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 (0.01 μ g/ml) and incubated with a range of concentrations of the PKA activator N⁶-Bz-cAMP (N6-Bz) alone, or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1 bacteria to 1 spleen cell. After 96 hours, the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between HK and N6-Bz+HK-treated samples are defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Data are representative of three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

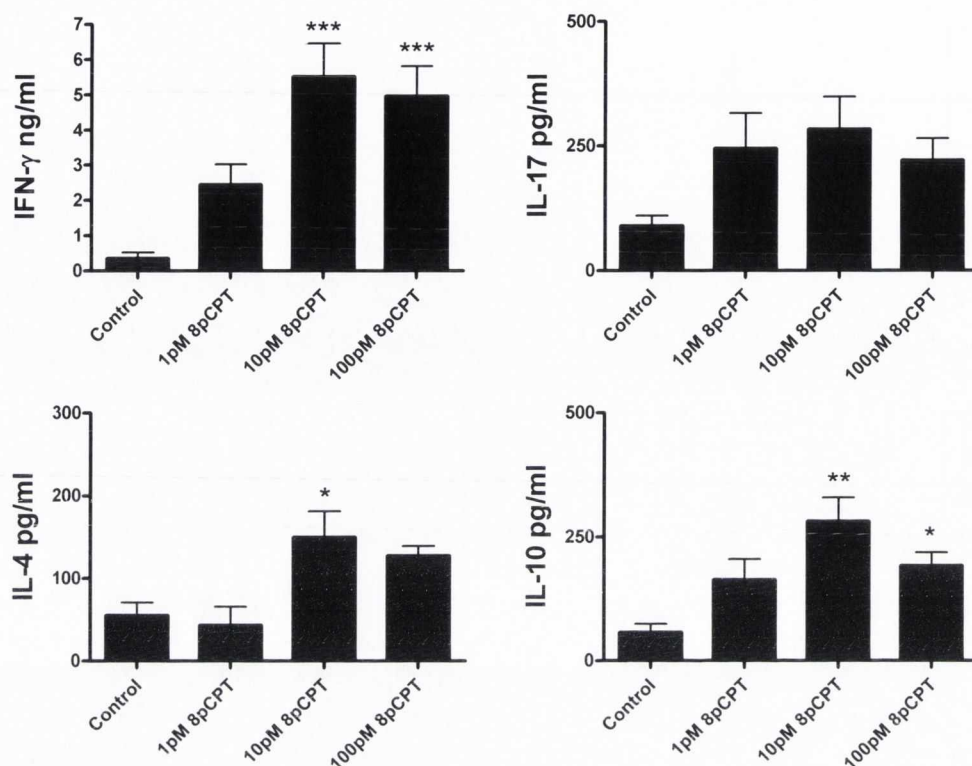


Figure 4.8: Epac activation promotes cytokine production by spleen cells. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT). After 4 days stimulation, the supernatants were removed and the cytokine concentrations in the cell-free supernatants were determined by ELISA. Significant differences between control (medium alone) and 8pCPT-treated samples are defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Data are representative of three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

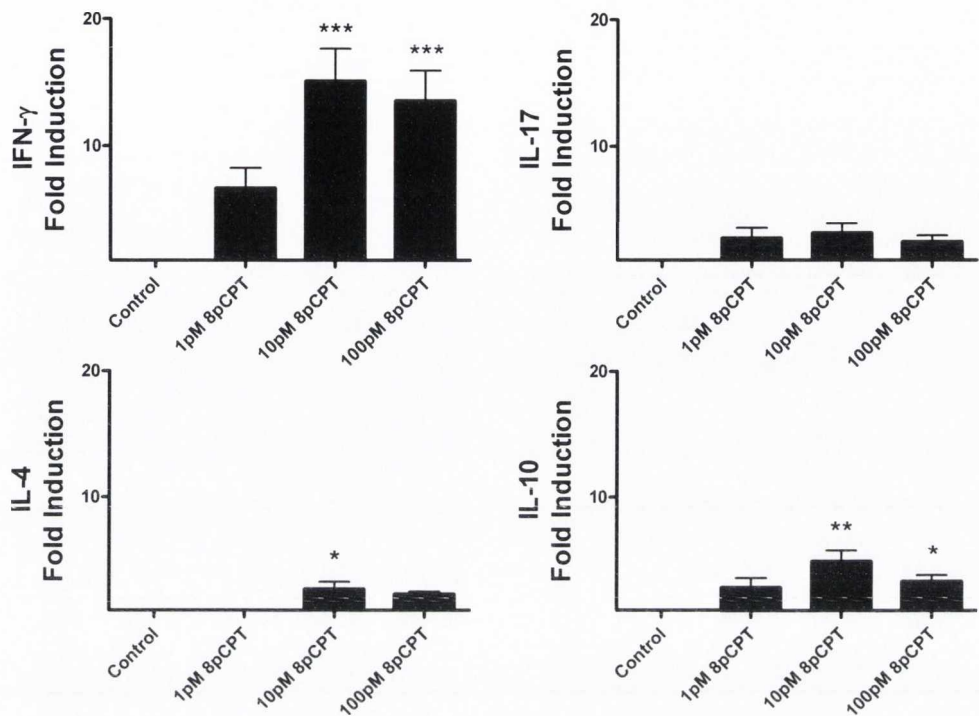


Figure 4.9: Epac activation predominantly promotes IFN- γ production in splenocytes and also enhances IL-4, IL-10 and IL-17 production. Splenic cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT). After 96 hours, the supernatants were removed and the cytokine concentrations in the cell-free supernatants were determined by ELISA. Fold induction of each cytokine compared to the unstimulated control is shown. Significant differences between control and 8pCPT-treated samples are defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Data are representative of at least three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

x

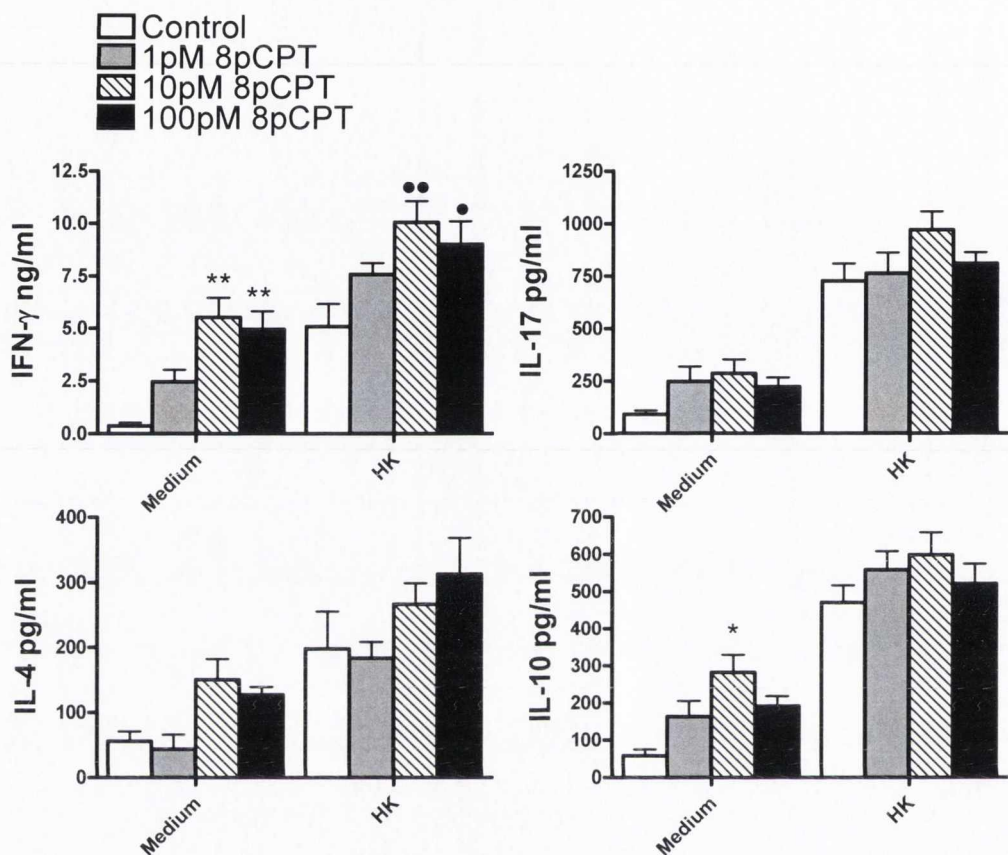


Figure 4.10: Epac activation promotes IFN- γ production from spleen cells in the presence and absence of heat-killed bacteria. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) alone, or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1 bacteria to 1 spleen cell. After 96 hours, the cytokine concentrations in the supernatants were determined by ELISA. Significant differences between unstimulated (control) and 8pCPT-treated samples are defined as $p < 0.05$ (*) and $p < 0.01$ (**). Significant differences between HK and 8pCPT+HK-treated samples are defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Data are representative of three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

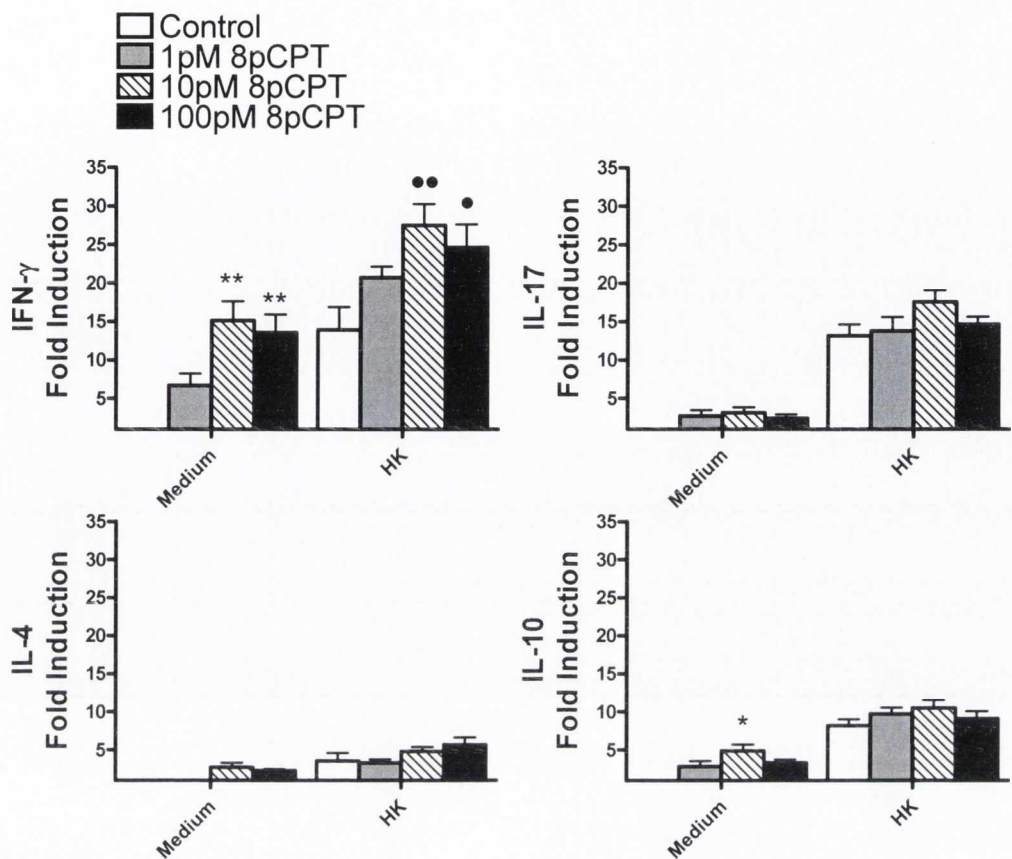


Figure 4.11: Epac activation promotes production of IFN- γ in spleen cells stimulated both with and without heat-killed bacteria. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) alone or for one hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1 bacteria to 1 spleen cell. The spleen cells were incubated at 37°C for 96 hours, after which the supernatants were removed and cytokine concentrations in the cell-free supernatants were determined by ELISA. Fold induction of each cytokine compared to the unstimulated control is shown. Significant differences between control and 8pCPT-treated samples are defined as $p < 0.05$ (*) and $p < 0.01$ (**). Significant differences between HK and 8pCPT- + HK treated samples are defined as $p < 0.05$ (•) and $p < 0.01$ (••). Data are representative of three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

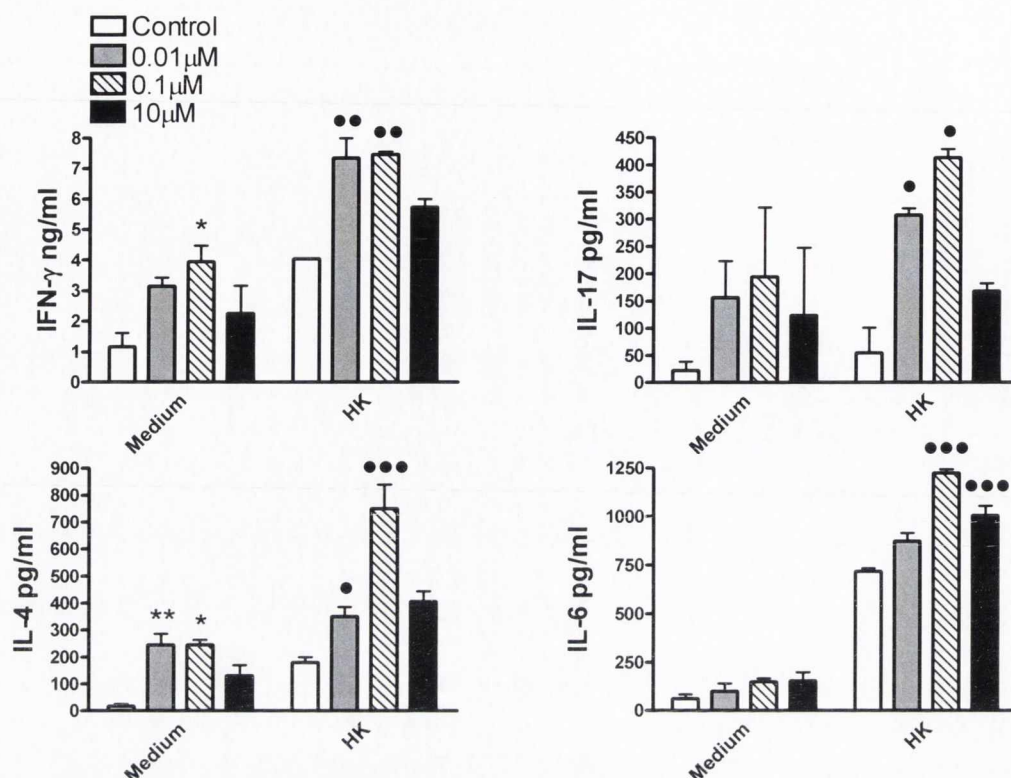


Figure 4.12: Treatment of spleen cells with the sulfonylurea compound Glybenclamide induces cytokine production by spleen cells. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 (0.1 μ g/ml) and incubated with a range of concentrations of Glybenclamide (0.01-10 μ M) alone or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 0.1 bacteria to 1 spleen cell. The spleen cells were incubated at 37°C for 96 hours, whereupon supernatants were removed and the cytokine concentrations in the cell-free supernatants were determined by ELISA. Significant differences between unstimulated (control) and glybenclamide-treated samples are defined as $p < 0.05$ (*) and $p < 0.01$ (**). Significant differences between HK-stimulated and Glybenclamide + HK-treated samples are defined as $p < 0.05$ (•), $p < 0.01$ (••) and $p < 0.001$ (•••). Data are representative of two independent experiments with mean $\pm$ S.D. of samples run in triplicate.

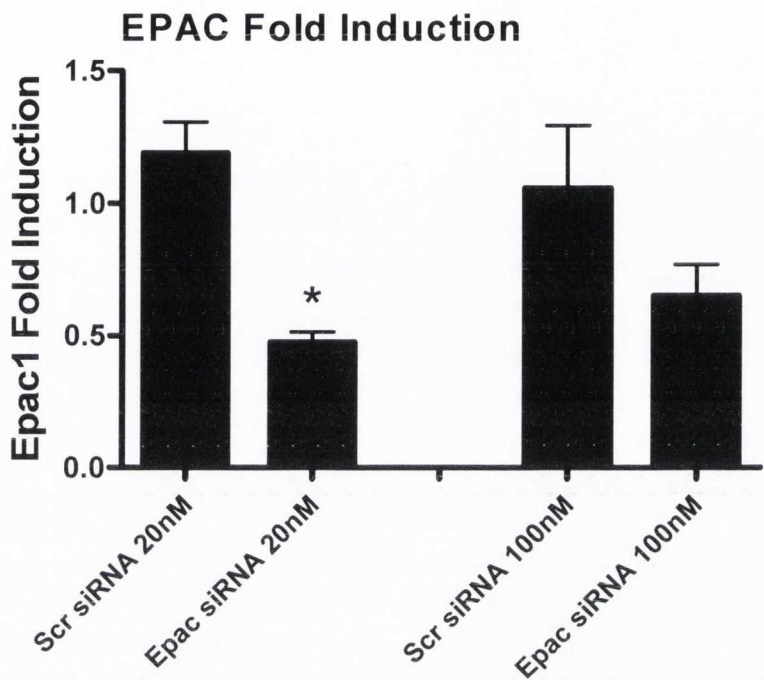


Figure 4.13: Epac1 mRNA expression is reduced by siRNA knockdown of Epac1 in spleen cells. Spleen cells (1×10^7 cells/ml) were transfected with siRNA specific for EPAC1 (Epac siRNA) or control scrambled siRNA (Scr siRNA). Transfected spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.1 \mu\text{g/ml}$). 72hours post transfection the cells were lysed, RNA isolated and real-time PCR was carried out on the cells to determine EPAC1 mRNA expression. Significant differences between cells transfected with Scr siRNA and EPAC siRNA are defined as $p < 0.05$ (*). Data are representative of three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

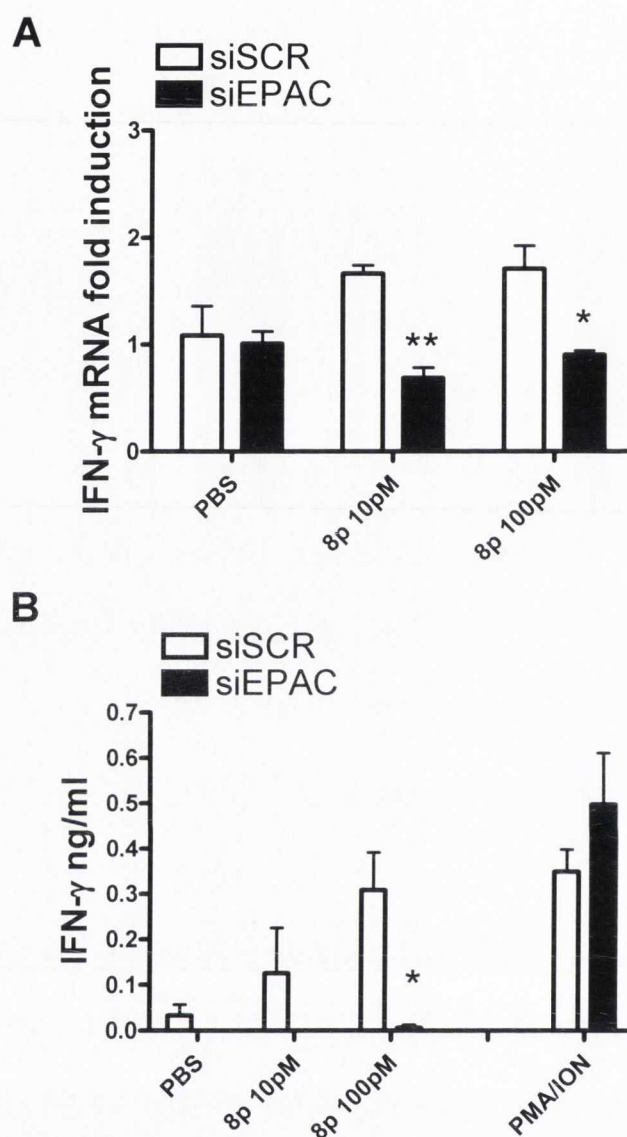


Figure 4.14: Knock-down of Epac1 with siRNA decreases the induction of IFN- γ by the Epac activator. Spleen cells (1×10^7 cells/ml) were transfected with siRNA specific for EPAC1 (EPAC siRNA) or control scrambled siRNA (Scr siRNA). Transfected spleen cells (1×10^6 cells/ml) were plated on anti-CD3 (0.1 μ g/ml), and left for 24 hours. The cells were then stimulated with the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT; 10pM or 100pM), or with PMA/ION and were left for 6 hours at 37°C after which the cells were collected and RT-PCR was carried out for IFN- γ (A) or for 72 hours at 37°C, whereupon supernatants were removed and the cytokine concentrations in the cell-free supernatants were determined by ELISA (B). Significant differences between siSCR (white bar) and siEPAC (black bar) transfected cells are defined as $p < 0.05$ (*) and $p < 0.01$ (**). Data are representative of three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

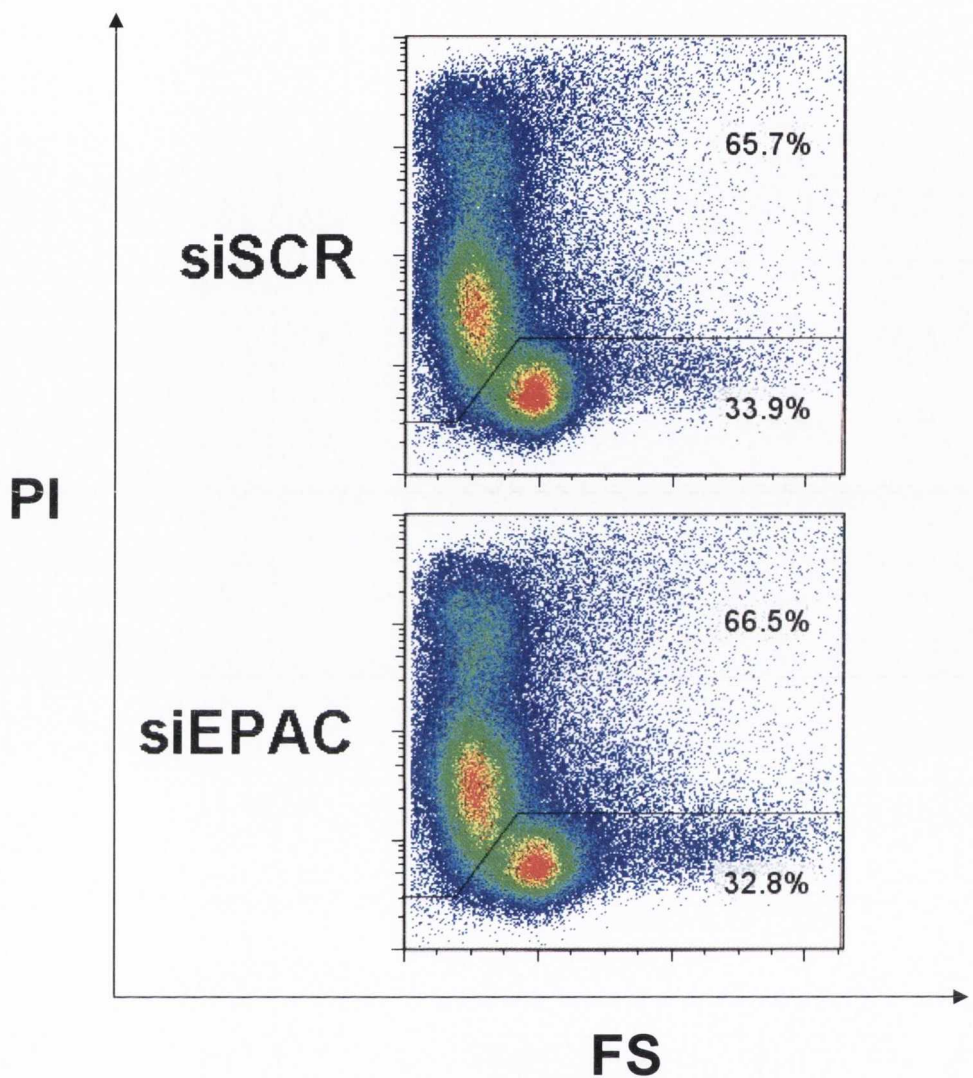


Figure 4.15: Cytotoxicity is comparable between cells transfected with Epac siRNA and Scrambled siRNA 24hours post-transfection. Spleen cells (1×10^7 cells/ml) were transfected with siRNA specific for Epac1 (siEPAC) or control scrambled siRNA (siSCR). Transfected spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.1 \mu\text{g/ml}$). 24hours post transfection, cells were analysed for propidium iodide (PI) incorporation to assess cell death, and analysed by flow cytometry. The dot plots show forward scatter (FS – showing cell volume/size) versus propidium iodide incorporation (PI). PI negative cell percentage values are indicated on the bottom right-hand side while PI positive cell percentage values are indicated on the top right-hand side. Data are representative of three independent experiments.

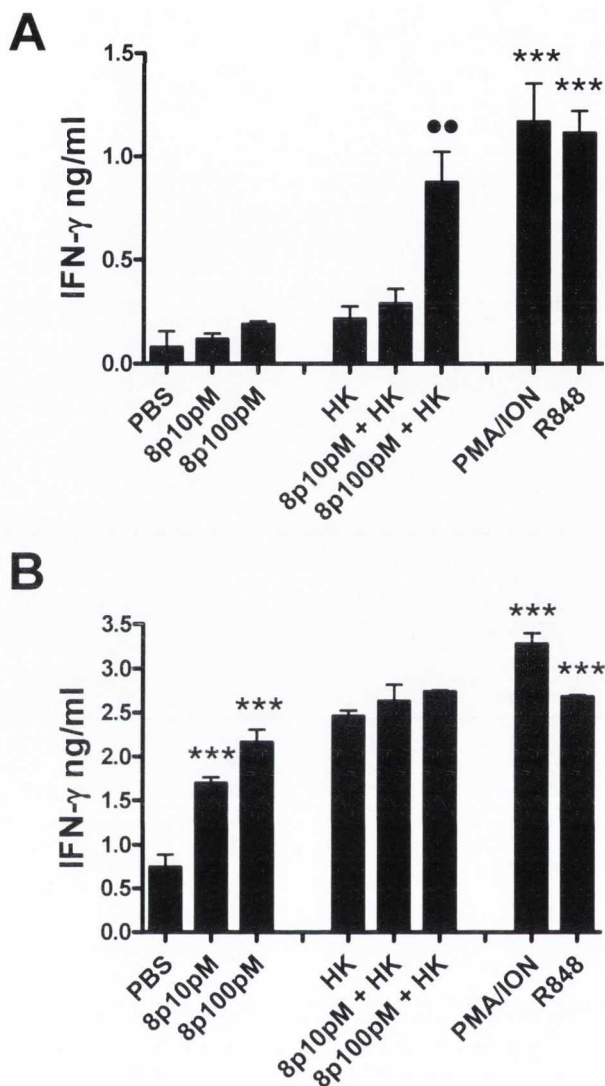


Figure 4.16: The enhancing effect of Epac activation on IFN- γ secretion was detected as early as 6hours after stimulation. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 (0.1 μ g/ml) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) alone or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio of 1 bacterium to one cell. The spleen cells were incubated at 37°C for 6hr (A) or 96hr (B), whereupon the supernatants were removed and cytokine concentrations in the cell-free supernatants were determined by ELISA. Significant differences between unstimulated (control) and 8pCPT-treated/R848/PMA and ionomycin samples are defined as $p < 0.001$ (***). Significant differences between HK and 8pCPT+HK-treated samples are defined as $p < 0.05$ (•), $p < 0.01$ (••) and $p < 0.001$ (•••). Data are representative of at least five independent experiments with mean $\pm$ S.D. of samples run in triplicate.

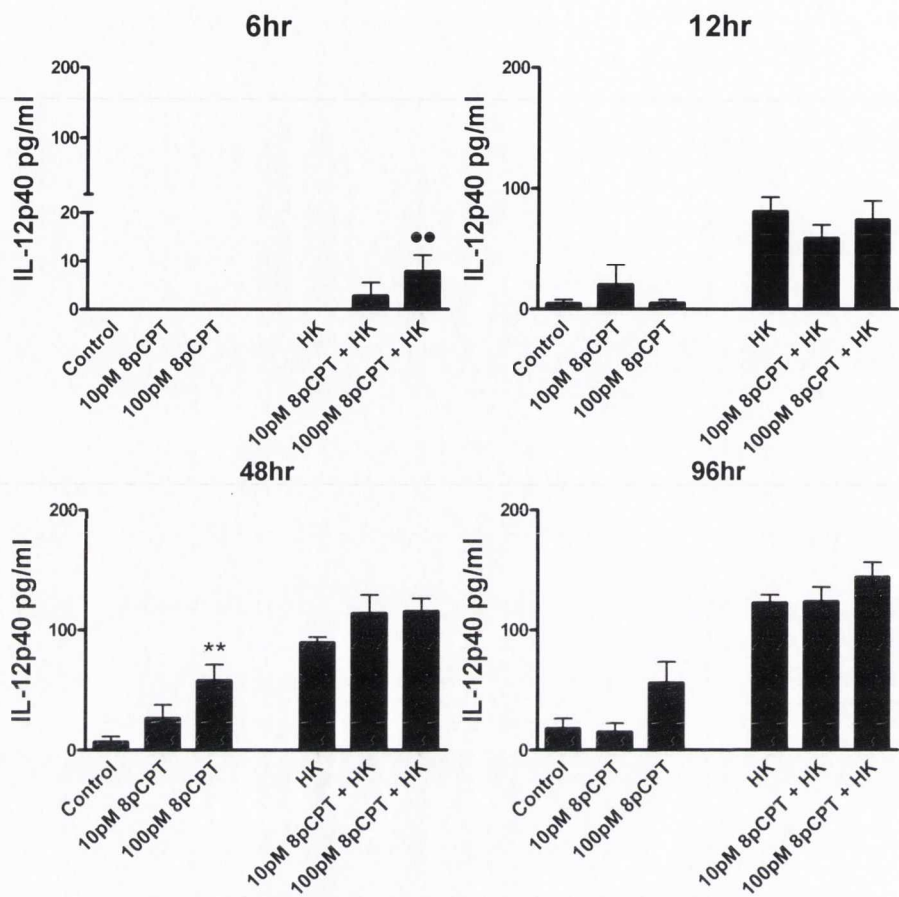


Figure 4.17: Epac activation promotes the secretion of IL-12p40 by spleen cells. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) alone or for 1 hour prior to stimulation with heat-killed *E. coli* at a ratio of 1 bacterium to one cell. The spleen cells were incubated at 37°C for 6hr-96hr, whereupon the supernatants were removed and cytokine concentrations in the cell-free supernatants were determined by ELISA. Significant differences between control and 8pCPT-treated samples are defined as $p < 0.01$ (**). Significant differences between HK- and 8pCPT+HK-treated samples are defined as $p < 0.01$ (••). Data are representative of at least three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

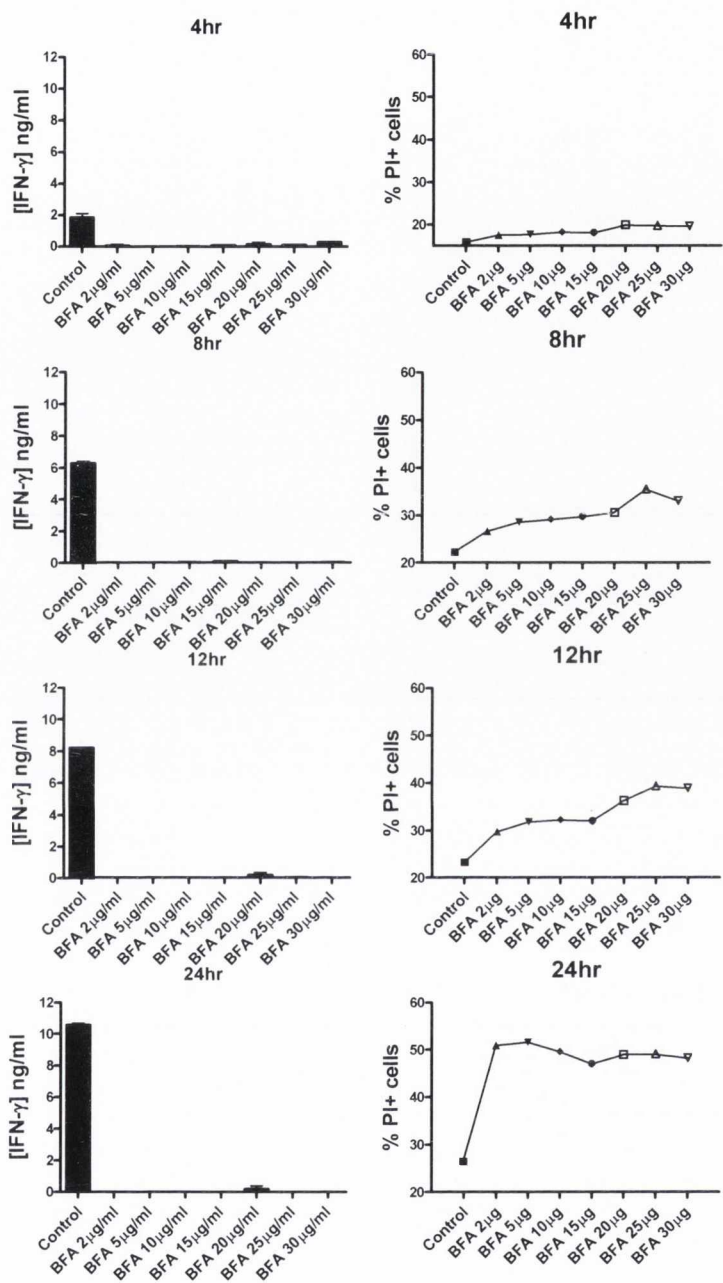


Figure 4.18: Kinetic study showing the suppression of cytokine secretion and the induction of cell death by brefeldin A in splenocytes. Splenocytes (1×10^6 cells/ml) were plated on anti-CD3 (0.01 μ g/ml) and stimulated with Brefeldin A (BFA) at a range of concentrations (2–30 μ g/ml) for several timepoints (4hrs–24hrs) whereupon supernatants were removed and the IFN- γ concentrations in the cell-free supernatants were determined by ELISA. Immunofluorescence was performed where cells were analysed for propidium iodide (PI) incorporation to assess cell death. Data are representative of two independent experiments.

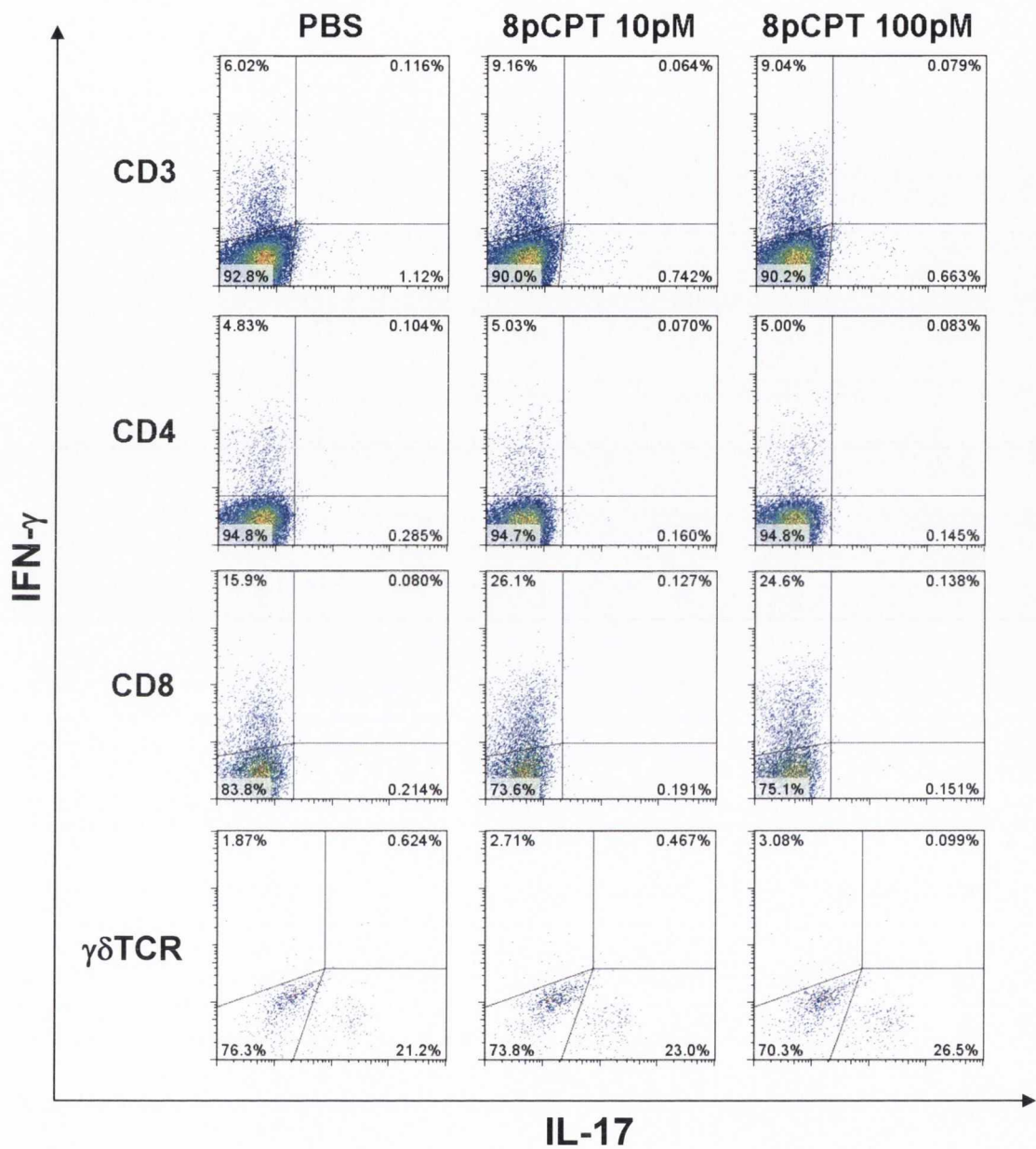


Figure 4.19: Epac activation in spleen cells promotes IFN- γ production from CD3⁺CD8⁺ cells. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) at 10 or 100pM. The spleen cells were incubated at 37°C for 96hours, whereupon cells were stained with antibodies specific for CD3, CD4, CD8 and $\gamma\delta\text{TCR}$, and the cytokines IFN- γ and IL-17. Dot plots show staining for IFN- γ versus IL-17 in the CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ or CD3⁺ $\gamma\delta\text{TCR}$ ⁺ cells. Data are representative of at least three independent experiments.

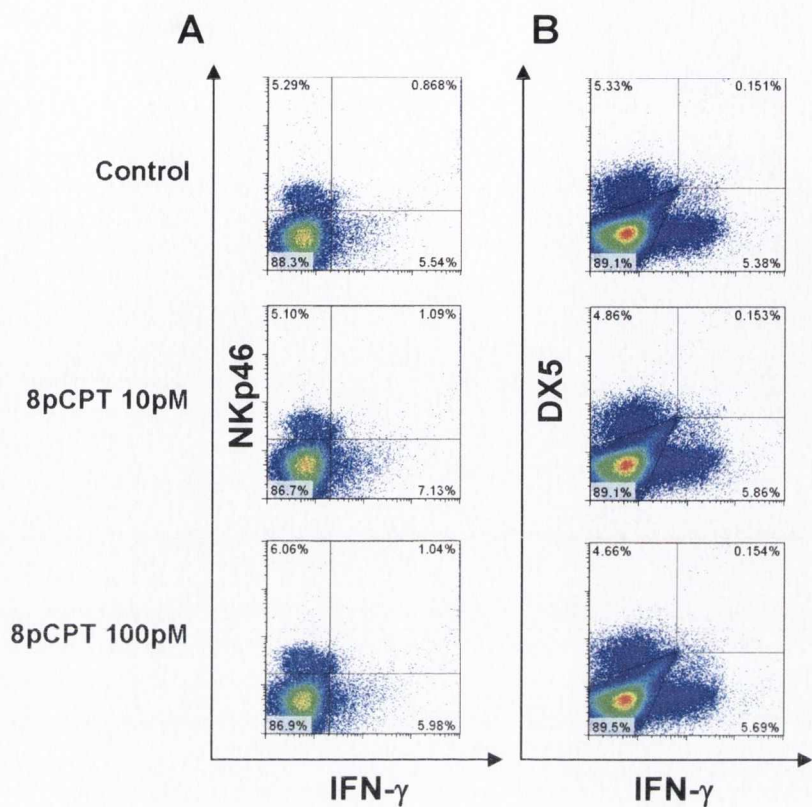


Figure 4.20: Epac activation does not enhance IFN- γ production from NK cells. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT). The spleen cells were incubated at 37°C for 6 hours, whereupon immunofluorescence was performed with antibodies specific for the NK cell markers DX5 or NKp46 and for the cytokine IFN- γ . Dot plots show IFN- γ versus NKp46 (A) or IFN- γ versus DX5 (B). Data are representative of at least three independent experiments.

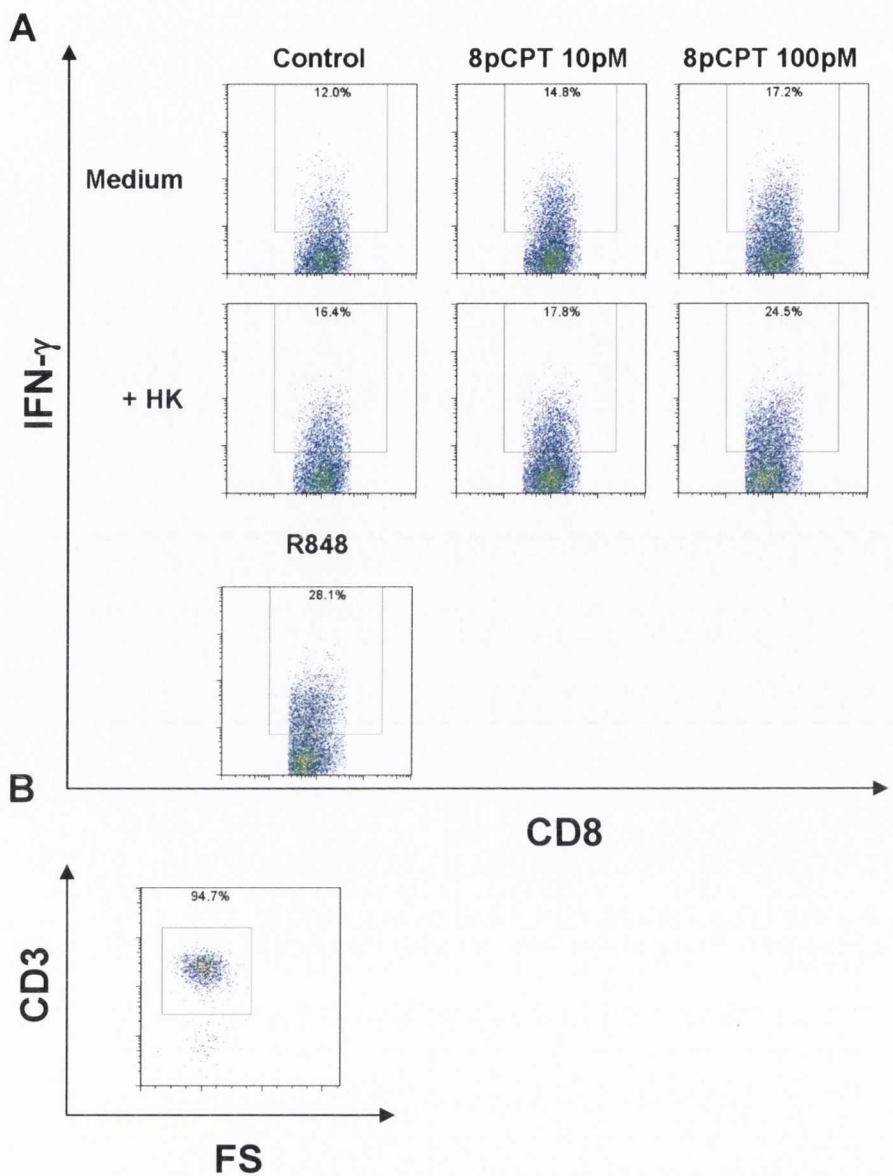


Figure 4.21: The Epac activator can promote IFN- γ production in a purified CD3⁺-population. Flow cytometry was performed on spleen cells with an antibody specific for CD3. The cells were analysed and sorted into a CD3-positive population based on specific antibody staining using a Beckman Coulter (Dako) MoFlo hi-speed cell sorter. CD3⁺ spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.1 \mu\text{g/ml}$) and incubated with either R848 ($10 \mu\text{g/ml}$) or a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) alone or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio of 1 bacterium to one cell. After 96 hours at 37°C , Brefeldin A with PMA/Ionomycin was added for four hours and intracellular flow cytometry was performed. Dot plots show CD8⁺IFN- γ ⁺ cells in the CD3⁺ population (A) and the purity of the CD3⁺ population (B). Data are representative of at least three independent experiments. IFN- γ expression by CD4⁺ T cells in this experiment is shown in Appendix Figure E.

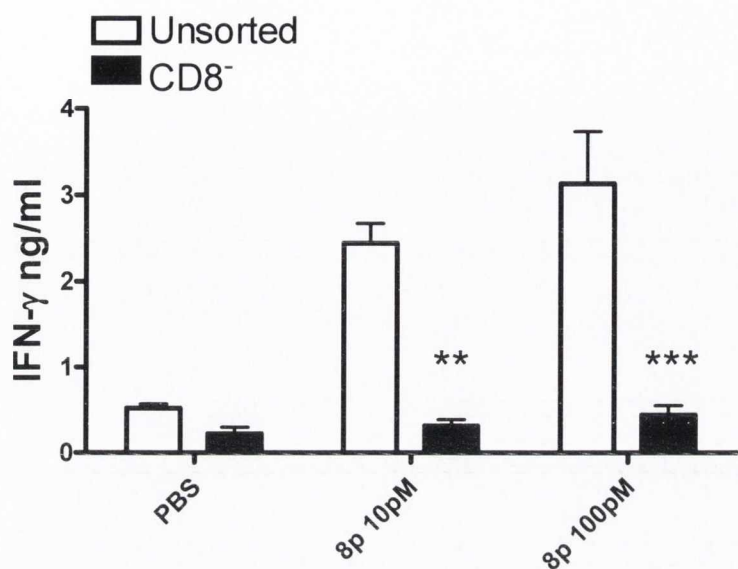


Figure 4.22: Depletion of CD8⁺ T cells abrogates the Epac-mediated enhancement in IFN-γ secretion. Flow cytometry was performed on spleen cells with an antibody specific for CD8. The cells were analysed and sorted into a CD8-negative population based on specific antibody staining using a Beckman Coulter (Dako) MoFlo hi-speed cell sorter. Unsorted spleen cells were used as a control. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($1 \mu\text{g/ml}$) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT). The cells were left for 96 hours at 37°C , whereupon supernatant were removed and the cytokine concentrations in the cell-free supernatants were determined by ELISA. Data are representative of two independent experiments with mean $\pm$ S.D. of samples run in triplicate. . Significant differences between control and 8pCPT-treated samples are defined as $p < 0.01$ (**) and $p < 0.001$ (***).

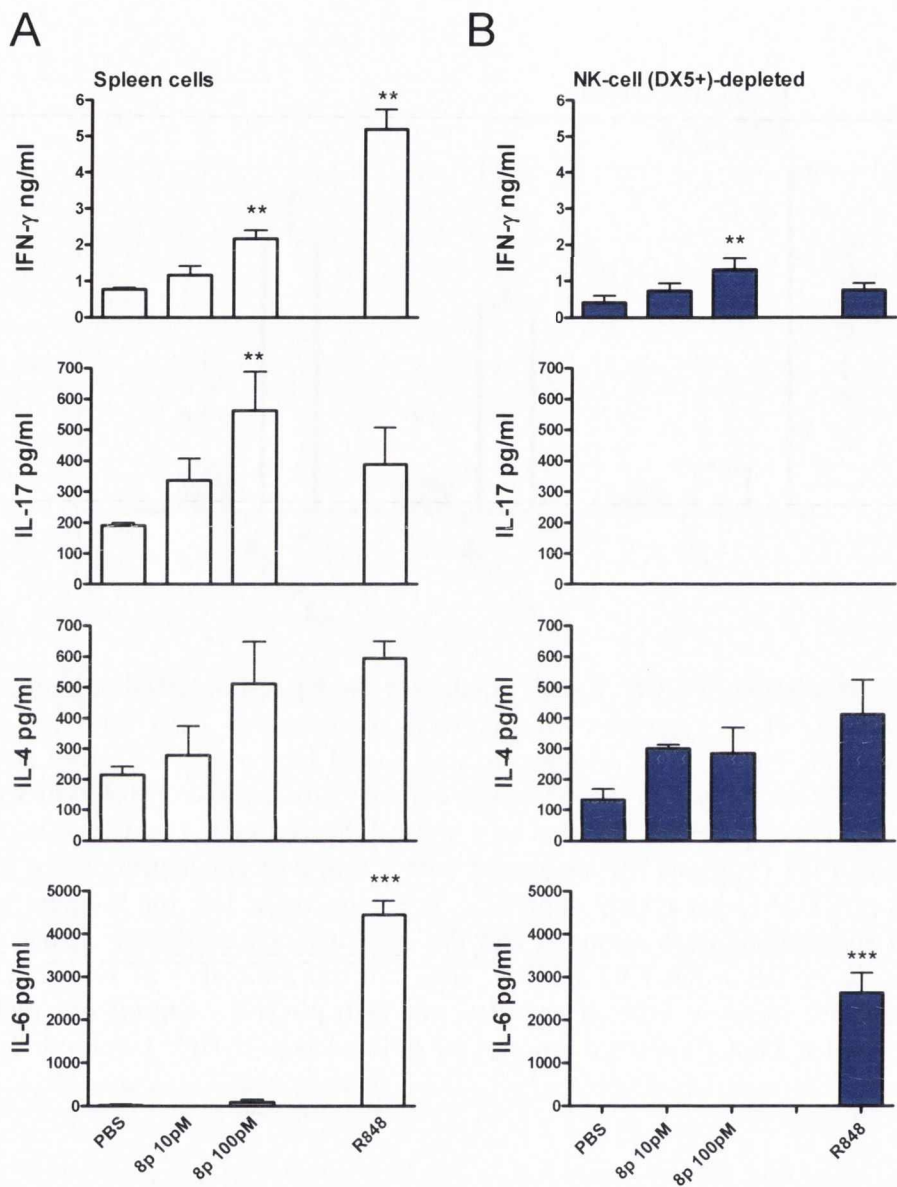


Figure 4.23: Epac activation can promote IFN- γ production in DX5⁺ cell-depleted splenocytes. Flow cytometry was performed on spleen cells using an antibody specific for DX5. The cells were analysed and sorted into a DX5⁻ population based on specific antibody staining by a Beckman Coulter (Dako) MoFlo hi-speed cell sorter. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($1 \mu\text{g/ml}$) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) or R848 ($10 \mu\text{g/ml}$). The cells were left for 96 hours at 37°C , whereupon the supernatant was removed and cytokine concentrations in the cell-free supernatants were determined by ELISA. Cytokine data of unsorted spleen cells (control) are shown in A while DX5⁺-depleted cells are shown in B. Data are representative of two independent experiments with mean $\pm$ S.D. of samples run in triplicate. Significant differences between control and 8pCPT/R848-treated samples are defined as $p < 0.01$ (**) and $p < 0.001$ (***).

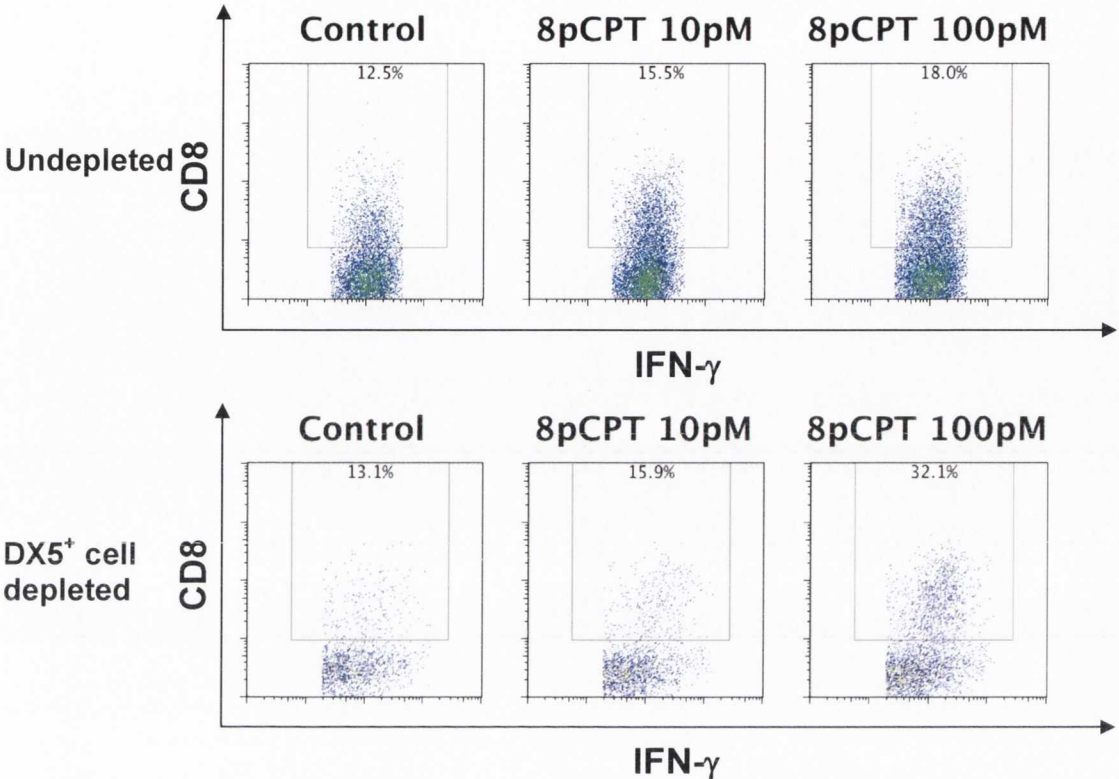


Figure 4.24: Depletion of NK cells does not compromise the Epac induced increase in IFN- γ production from CD8⁺ T cells. Flow cytometry was performed on spleen cells with antibodies specific for DX5. The cells were analysed and sorted into a DX5 negative population based on specific antibody staining using a Beckman Coulter (Dako) MoFlo hi-speed cell sorter. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.5 \mu\text{g/ml}$) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT), for 96hours at 37°C , whereupon Brefeldin A with PMA/Ionomycin was added for four hours and intracellular flow cytometry was performed. Dot plots show the CD8⁺IFN- γ ⁺ cell population. Data are representative of two independent experiments.

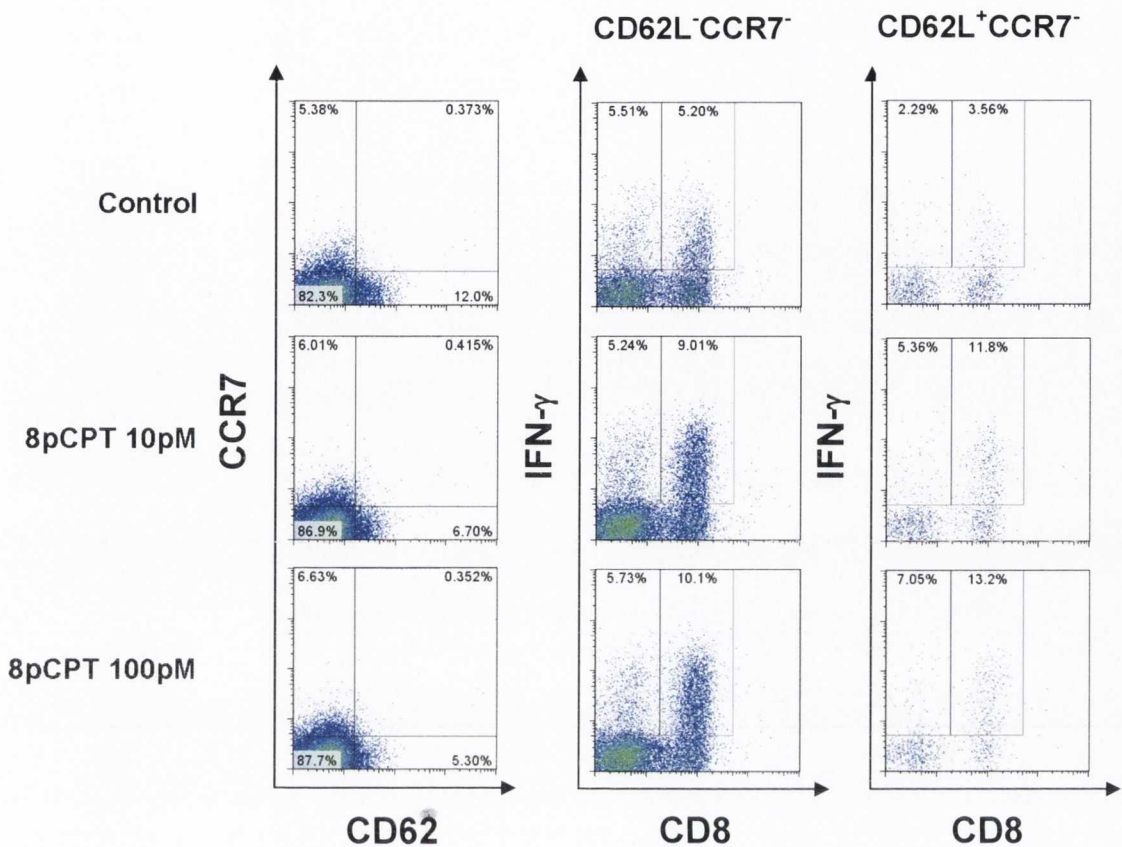


Figure 4.25: Epac activation in spleen cells reduces CD62L expression on spleen cells and induces IFN- γ production from CD62L⁻ CCR7⁻ CD8⁺ T cells. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 (0.1 μ g/ml) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) for 72hours at 37°C, whereupon the supernatant was removed and flow cytometry was performed on spleen cells with antibodies specific for CD62L and CCR7. Data are representative of at least three independent experiments with mean $\pm$ S.D. of samples run in triplicate.

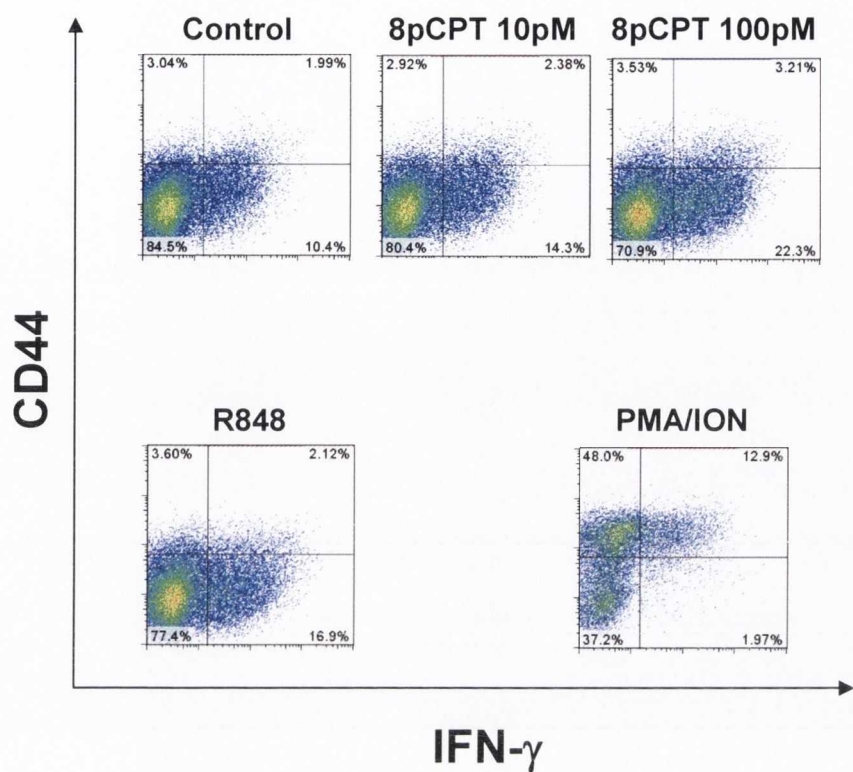


Figure 4.26: The enhancement in IFN- γ production mediated by Epac activation in splenocytes is principally in CD44^{lo} cells. Spleen cells were passed through a CD3⁺ T cell purification column. The negatively-purified CD3⁺ (1×10^6 cells/ml) were plated on anti-CD3 (0.1 μ g/ml) and incubated with the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT; 10pM or 100pM) or with R848 or PMA/ionomycin for 72hours at 37°C, whereupon flow cytometry was performed on spleen cells with antibodies specific for CD44 and IFN- γ . The expression of CD44 versus IFN- γ in CD3⁺ cells is presented. Data are representative of two independent experiments.

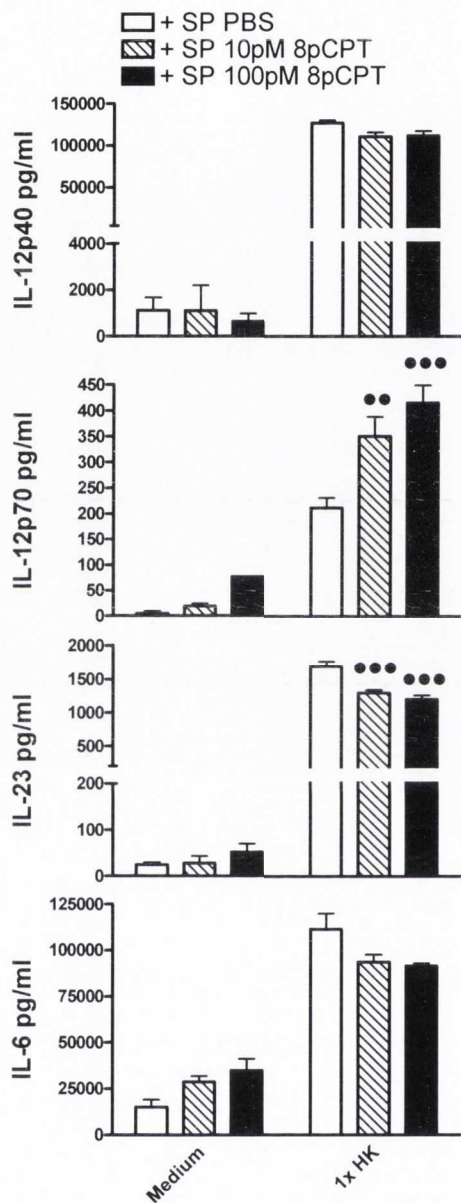


Figure 4.27: Transfer of supernatants from Epac agonist-treated splenocytes onto DCs promotes IL-12p70 production by the DCs. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated with the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) (10-100pM). The spleen cells were incubated at 37°C for 96 hours, whereupon cell-free supernatants were removed and transferred onto naïve DCs. The DCs were left for 24 hours at which point the supernatant was removed and the cytokine concentrations in the cell-free supernatants were determined by ELISA. Data are representative of two independent experiments with mean $\pm$ S.D. of samples run in triplicate. Significant differences between HK- and 8pCPT+HK-treated samples are defined as $p < 0.01$ (**) and $p < 0.001$ (***).

Discussion

The data presented in chapter 3 clearly demonstrated that activating the Epac pathway exerted immunomodulatory effects on DCs and these were strikingly different to the effects of PKA activation. Here a broader approach was adopted to assess the effects of Epac activation in splenocytes. In contrast to the effects of PKA activation or cAMP elevation, Epac activation induced proinflammatory cytokine production, predominantly IFN- γ production, from spleen cells. This Epac-induced IFN- γ production was found to be produced mainly by effector memory CD8⁺ T cells, with a CD44^{lo}CCR7⁻CD62L⁻ phenotype.

Previous studies have shown that elevated cAMP and PKA activation have inhibitory effects on T cell cytokine production *in vitro*. cAMP elevation by both DB-cAMP and PGE₂ reduced IL-4 mRNA expression in activated human T lymphocytes, showing that IL-4 expression is negatively regulated by PKA-dependent signalling *in vitro* [266]. cAMP/PKA has also been found to decrease anti-CD3 monoclonal antibody-induced T cell proliferation and IL-2 production *in vitro* [384]. Activating cAMP signalling by DB-cAMP, Sp-cAMPS and forskolin suppressed ConA-stimulated PBMC (peripheral blood mononuclear cell) proliferation [385]. Agents that elevate or mimic endogenous cAMP have also been reported to increase production of IL-10 [264] and IL-5 [265], but inhibit IL-4 [266] and IL-2 [265] secretion by mononuclear cells. Additionally PKA activation can inhibit antigen-specific T cell proliferation and cytokine production in human PBMCs [386]. PKA activation in these cells inhibited the production of IFN- γ , TNF- α , IL-2 and IL-4 from both CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells *in vitro*. Proliferation of these cells was also reduced but apoptosis was not increased [386]. These findings correlate with the effects of PKA activation found on splenocyte cytokine production here, and further confirm the role of PKA as a negative regulator of immune responses *in vitro*.

In chapter 3, it was established that Epac activation increases IL-12p70 production from DCs, suggesting that it may enhance Th1 responses. Therefore the effect of activating Epac on T cell cytokine production, particularly its effect on IFN- γ production was determined. Since the spleen contains a mixture of cells including T

cells, B cells, DCs and NK cells [387], it provides a useful model to study the effects of Epac and PKA activation on immune cells. Addition of the Epac activator to spleen cells strongly increased IFN- γ production, while also enhancing the production of IL-4, IL-17 and IL-10. When the fold induction of the various cytokines was assessed it was clear that the activation of Epac in these cells predominantly increases IFN- γ production. The consequences of Epac activation in spleen cells were markedly different to PKA activation. PKA activation in splenocytes inhibited the production of IFN- γ , IL-4, IL-17 and IL-10, which correlates with its previously outlined immunosuppressive role [266, 384]. The suppression of cytokines by PKA activation has been linked to its suppressive effects on IL-2 production, as IL-2 is a cytokine responsible for inducing proliferation of T cells. In order to confirm that the enhancement in IFN- γ production by 8pCPT is due to Epac, and not an off-target effect of the activator, an siRNA experiment was carried out on spleen cells. When Epac1 was knocked down, the IFN- γ induction mediated by Epac activation was absent, as shown by the IFN- γ secretion and mRNA profiles. As discussed in the previous chapter, glybenclamide (GLB) is a sulfonylurea compound, used to treat type II diabetes, which binds and activates Epac2 [286] and exerted comparable effects to 8pCPT on DC cytokine production in chapter 3, so the effects of GLB on splenocyte cytokine production were determined. GLB induced production of not only IFN- γ , but also IL-17, IL-4 and IL-6. This indicates that the effects induced by treatment of DCs with the Epac agonist 8pCPT, are replicated with the selective Epac2 agonist GLB.

In order to identify the cells producing IFN- γ and IL-17 in response to Epac activation, intracellular flow cytometry was performed. Epac activation enhanced IFN- γ production from splenic CD3⁺CD8⁺ T cells and also enhanced IL-17 production from $\gamma\delta$ T cells. $\gamma\delta$ T cells are an innate source of IL-17 and can lead to the development of a Th17 response [388]. $\gamma\delta$ T cells have been reported as early producers of IL-17 in the lung during TB infection [389]. *In vivo* during the non-antigen-specific early phase of infection, IFN- γ is believed to be primarily provided by NK and NKT cells in response to pathogen-derived inflammatory mediators, but it has also been reported that CD8⁺ cells are capable of producing IFN- γ in response to PAMP stimulation *in vivo* [390]. IFN- γ was produced by CD8⁺ cells, in response to

Epac activation, as early as 6 hours post-stimulation. As NK cells are a primary source of IFN- γ it was important to determine their role in Epac-driven IFN- γ production. In order to do this, a spleen cell population was prepared where the NK and NKT cells had been removed. IFN- γ production by CD8⁺ cells in the NK and NKT cell depleted populations upon Epac activation was reduced when compared to the control undepleted cells. The control cells were whole unsorted splenocyte cells that had undergone the same treatment as the depleted cell population, i.e. antibody staining and passage through the MoFlo high-speed cell sorter. This was done to ensure that no non-specific effects due to the sorting process affected the resulting cytokine profile. Although the IFN- γ levels were reduced in the NK and NKT cell-depleted populations stimulated with the Epac activator, IFN- γ production was still detected. This indicates that the NK cells are not the primary producers of Epac-induced IFN- γ , but do act to amplify the amount of IFN- γ produced. IFN- γ is known to act in positive feedback loops on cells, where it induces further IFN- γ production once present. Thus, the IFN- γ produced by the CD8⁺ T cells may act on the NK and NKT cells in the culture and enhance IFN- γ productivity by these cells. Purified CD3⁺ cells were also stimulated with 8pCPT and the CD8⁺ cells in this population responded to Epac activation by producing IFN- γ , similar to the response in the whole spleen population. This was a key control as it suggests that Epac activation with purified T cells is sufficient to promote IFN- γ and is not dependent on accessory cells. CD8⁺ cells were also depleted from a whole spleen cell population and the residual CD8⁻ cells were assessed for cytokine production upon Epac activation. It was found that in the absence of CD8⁺ T cells no IFN- γ was produced upon Epac activation, further confirming their important role in IFN- γ production in this context. Overall, the results of these cell depletion experiments indicate that CD3⁺CD8⁺ T cells are the primary source of IFN- γ produced in response to Epac activation and that this IFN- γ is amplified in the presence of NK cells but is not dependent on their presence.

Since Epac activation induces IFN- γ production from CD8⁺ T cells it was necessary to further characterise these cells. Intracellular flow cytometry was performed in order to determine the type of CD8⁺ T cell responsible for this Epac-mediated IFN- γ induction. Migrating naïve T cells interact with antigen-bearing DCs in secondary lymphoid organs, and depending on the nature of the stimulus, can undergo

activation, proliferation and differentiation into effector cells [391]. Naïve T cells migrate from blood to peripheral lymph nodes where CD62L, the chemokine receptor CCR7 and the integrin LFA-1 are needed for rolling, sticking and firm adhesion to the luminal side of high endothelial venules (HEVs) [382, 383]. As naïve T cells differentiate into memory cells, their gene-expression profiles and migration patterns are altered [392]. Effector T cells have a reduced potential for homing to lymph nodes due to decreased expression of lymph-node-homing receptors, e.g. CCR7 and CD62L, and a greater capacity to migrate to inflamed tissues owing to increased expression of chemokine receptors such as CCR5 and CCR2 [393]. In 1999, a model describing subpopulations of memory T cells was outlined, in which ‘central’ memory (T_{cm}), CCR7⁺CD62L⁺CD45RO⁺, and ‘effector’ memory (T_{em}), CCR7⁺CD62L⁺CD45RO⁺, T cells was proposed, based on the role of CD62L and CCR7 in determining the homing properties of T cells [381]. Central memory T cells can rapidly proliferate in secondary responses, express CCR7 and CD62L and are found in lymph nodes, whereas effector memory T cells, which are characterised by immediate effector function, lack CD62L and CCR7 and are found in blood, peripheral tissues and spleen but not in lymph nodes [394, 395].

Epac activation principally enhanced production of IFN- γ from the CD8⁺ CCR7⁺ CD62L⁺ cell population. This indicates that effector memory CD8⁺ T cells are producing the IFN- γ upon Epac activation. These cells do not migrate to lymph nodes but do have an immediate effector function and migrate to the site of inflammation. A primary aim of this thesis was to investigate the possibility of using Epac activation as a vaccine adjuvant strategy, this increased migration of effector memory CD8⁺ T cells to the site of inflammation (or site of vaccine injection) and increased production of IFN- γ at the site of injection could allow for a Th1-type response to the antigen of interest.

CD62L expression was decreased following Epac activation on spleen cells. The expression pattern of CD62L on activated T cells is regulated by the duration of antigenic stimulation. It has three stages of expression; initially, TCR stimulation induces the rapid shedding of CD62L from the T-cell surface, then within 24–48 hours, CD62L is re-expressed, and thirdly if TCR stimulation continues then CD62L expression is suppressed for a longer time [396, 397]. Treatment of spleen cells with the Epac activator alone induced a suppression of CD62L expression, potentially

suggesting that Epac activation may amplify TCR signalling in T cells. This effect is not completely surprising as after activation of the TCR-CD3 complex in T cells, there is an initial peak of cAMP and PKA activity, that may serve as an acute negative modulator and a negative feedback signalling mechanism through the TCR/CD3 [254].

Purified CD3⁺ cells were also analysed for CD44 expression. It was found that the increase in IFN- γ production following Epac activation and R848 stimulation was from CD44^{lo} cells. Treatment of splenocytes with PMA/ionomycin led to upregulation of CD44 expression and IFN- γ production by this stimulus came from CD44^{hi} cells, which has been shown previously [398]. CD44, the hyaluronan receptor, is expressed by both lymphoid and myeloid leukocytes [399, 400]. CD44 has been shown to function primarily by supporting leukocyte rolling at inflammatory sites, but CD44 gene knockout (KO) mice suggest that low expression of CD44 might facilitate lymphocyte homing to peripheral lymph nodes during inflammation [401-403].

The signals involved in IL-12 and IFN- γ production are intrinsically linked, IL-12 induces IFN- γ production while IFN- γ can induce IL-12 production. These pathways are also under self-regulation with both positive feedback loops promoting cytokine induction and negative feedback loops attenuating the cytokine response. Therefore experiments were conducted to investigate if the IFN- γ produced upon Epac activation could affect cytokine production by DCs, particularly if IFN- γ production could enhance IL-12 production from DCs. The addition of spleen cell supernatant from cells stimulated with 8pCPT were added to differentiated DCs and increased IL-12 production upon restimulation with HK bacteria or CpG. The spleen cell supernatant was tested for the presence of IL-12p70 and it was not detected, indicating that this increase in IL-12p70 production by DCs was as a result of the supernatant stimulating the differentiated DCs to produce IL-12.

The results in chapter 3 and in this chapter indicate that activation of Epac and PKA exerts opposing effects on immune responses. Epac activation has the potential to induce a proinflammatory response; particularly IFN- γ secretion while PKA activation and nonselective cAMP elevation exerts an inhibitory effect on cytokine production.

Chapter 5

**An investigation of the immunomodulatory
role of Epac activation *in vivo***

Introduction

The ability of a selective Epac activator to promote the production of proinflammatory cytokines by splenocytes and DCs *ex vivo* was demonstrated in chapters 3 and 4. However, the effects of Epac activation on immune responses *in vivo* have not been investigated previously and in order to address this issue, the potential of an Epac activator as an immunomodulator or vaccine adjuvant was determined.

As mentioned in chapter 1, CT increases cAMP levels and is a potent mucosal vaccine adjuvant, which has been shown to induce Th2 responses in systemic and mucosal tissues [404]. *In vivo*, mice given CT before systemic challenge with LPS had markedly reduced serum levels of IL-12p40 and IFN- γ , showing that CT can inhibit Th1 immune responses, and that mucosally administered CT can enhance Th2-dependent immune responses [404]. While the *in vitro* anti-inflammatory effects of PKA and cAMP elevation are well described (and shown in chapters 3 and 4), their *in vivo* effects are not quite so clear-cut. In fact, PKA activation *in vivo* has been shown to promote IL-4 production from CD4⁺ T cells. The differences between *in vitro* and *in vivo* effects have been suggested to be due to the suppression of IL-2 production by PKA. IL-2 is an important cytokine for proliferation and maintenance of cellular responses. Suppression of IL-2 production could have a larger effect *in vitro* in a closed environment, while *in vivo* there could be compensatory mechanisms. Epac, as outlined in Chapter 1, has been shown to play a role in cell migration, suggesting that Epac activation may regulate cell recruitment *in vivo* [273]. Epac is also involved in the regulation of leukocyte integrin activity. Epac activation increased integrin-mediated adhesion of primary monocytes to HUVECs under flow [276].

Three immunisation protocols were used to test the effects of Epac activation *in vivo*. The first was a five-day schedule where the Epac activator was administered intraperitoneally (i.p.) every day for five days and the mice sacrificed on the sixth day. This method gives an indication of the effects of Epac activation on innate immunity at the site of injection and in the draining lymph node. The i.p. route was chosen for these studies as it allows easy access to cells at the injection site. The

peritoneal cavity was washed with PBS in order to isolate the cells that have migrated to the site of injection. In addition, the mediastinal lymph nodes adjacent to the thymus, as the primary draining lymph nodes following i.p. injection [405], and the spleen were isolated for analysis. The second protocol used was a single injection of the activator followed by analysis of changes in the immune response after 24 hours. This method allows analysis of the effects of Epac activation at an earlier timepoint, and facilitates a determination of whether a single injection of the Epac activator is capable of altering the immune response. The third approach concerned the co-administration of the Epac activator with an antigen to determine if Epac activation could enhance an antigen-specific response. Two different antigens were tested, the poorly immunogenic, model antigen ovalbumin (OVA) and, Hybrid 1 (H1) antigen which is a fusion of two proteins Ag85B and ESAT6 derived from *Mycobacterium tuberculosis*, a bacterium which causes tuberculosis (TB) and requires a Th1-response for clearance. It has been shown that vaccination with a fusion protein consisting of Ag85B and ESAT6 (Hybrid1/H1) promoted a strong immune response, which is highly protective against TB in the mouse and in nonhuman primate models, and this fusion protein is currently in clinical trials [16, 17]. The Epac activator 8pCPT, was tested *in vivo* either alone or in combination with a TLR agonist (the TLR4 agonist LPS, the TLR7/8 agonist R848 or the TLR9 agonist CpG). These TLR agonists are efficient adjuvants, and so the ability of Epac activation to alter TLR-agonist driven innate and adaptive immune responses was studied.

The principal aims of this chapter were to:

- Determine if injection of the Epac activator could modulate innate immunity.
- Determine if Epac can promote antigen-specific cellular and humoral immunity either alone or in the presence of a TLR ligand.

5.1 The innate effects of Epac activation are in contrast to the effects of PKA activation *in vivo*.

In order to compare the effects of Epac and PKA activation on innate immunity, mice were immunised i.p. on five consecutive days with either the Epac activator 8pCPT or the PKA activator N6-Bz and were sacrificed on day 6. The peritoneal cavity was washed in order to determine cell recruitment to the site of injection, and the mediastinal lymph nodes and the spleens were harvested. The mediastinal lymph nodes located near the thymus have been described as the main draining lymph nodes following i.p. injection [405]. Analysis of the cytokine profile from restimulated cells *ex vivo* was carried out to allow a comparison of the effects of injection with the Epac and PKA activators. Initially the effects of injection of the PKA activator and the Epac activator were compared at three doses, 0.002mmol (1µg), 0.02mmol (10µg) and 0.2mmol (100µg) per mouse. This dose range is more (upwards of 1000 times more) than the doses used in the cell-based studies outlined in chapters 3 and 4. The dose range was chosen based on internal laboratory work done, where the effects of different compounds had been tested in *in vitro* cell based studies and then transferred to *in vivo* studies.

Unstimulated mediastinal lymph node cells from mice injected with the Epac activator at doses of 0.02mmol and 0.2mmol showed enhanced IFN-γ production (200-700pg/ml), but not IL-4, compared with cells from mice given PBS as a control (Figure 5.1a). Injection of the PKA activator at doses of 0.002mmol and 0.02mmol led to enhanced IL-4 (50-120pg/ml) production by mediastinal lymph node cells, but failed to promote IFN-γ production (Figure 5.1b). These results indicate that activation of the Epac pathway promotes the production of IFN-γ, a characteristic Th1 cytokine, while PKA activation promotes the production of the Th2-characteristic cytokine IL-4 in the mediastinal lymph nodes. IL-17 was not detected in these unstimulated cells (data not presented).

Based on this study a 0.1mmol dose of each of the activators was chosen, and mice were immunised i.p. with this dose of the activators with or without LPS. Injection of mice with the Epac agonist alone promoted the secretion of IFN-γ in anti-CD3

stimulated mediastinal lymph node cells. In contrast, injection of the PKA agonist significantly inhibited IFN- γ secretion in anti-CD3 stimulated mediastinal lymph node cells (Figure 5.2). LPS injection enhanced the production of IL-17 by mediastinal lymph node cells stimulated with anti-CD3 when compared to mice injected with PBS (Figure 5.2). In comparison to injection with LPS alone, injection of mice with the specific Epac agonist with LPS significantly enhanced the secretion of IFN- γ and IL-17, but not IL-4, by anti-CD3 stimulated mediastinal lymph node cells (Figure 5.2a). In contrast, injection of the PKA agonist with LPS significantly enhanced the secretion of IL-4 and IL-17, while inhibiting IFN- γ , when compared to LPS injection alone (Figure 5.2b). This enhancement in IFN- γ secretion by the Epac activator mirrors the effects seen *in vitro* in chapter 4, where Epac activation promoted IFN- γ production from spleen cells.

5.2 Injection of the Epac activator 8pCPT over 5 days induces IFN- γ production from CD3⁺CD8⁺ cells in the spleen.

Having demonstrated that Epac activation has strikingly different immunomodulatory effects to PKA activation, the effects of PKA activation *in vivo* were not further characterised, as the effects of Epac activation are the main focus of this thesis. BALB/c mice were injected i.p. with the Epac activator, 8pCPT, on five consecutive days and then sacrificed on day 6. The spleen cells were isolated and stimulated with anti-CD3, with or without PMA and ionomycin (Figure 5.3). Injection with the Epac activator induced an increase in IFN- γ production in unstimulated cells and in splenocytes stimulated with anti-CD3 alone or with PMA and ionomycin (Figure 5.3). IL-6 production was also enhanced following injection of the Epac activator, but only in unstimulated cells and cells restimulated with the highest concentration of anti-CD3 and PMA with ionomycin (Figure 5.3). There was no significant increase in IL-17 production in mice injected with 8pCPT, but a decrease in IL-4 production was seen in spleen cells restimulated with 0.01 μ g/ml anti-CD3 with PMA and ionomycin (Figure 5.3). IL-10 production was also determined, but no difference was detected upon injection with the Epac activator (data not presented).

As discussed in chapter 4, the spleen is composed of several different cell types. T cells ($CD3^+$), NK cells ($DX5^+$), NKT cells ($CD3^+DX5^+$) and $\gamma\delta$ T cells ($CD3^+\gamma\delta TCR^+$) are the principal cellular sources of IFN- γ . These markers, together with CD11b, a marker for granulocytes, and CD11c, a marker for DCs were used to determine the effects of Epac activation on the cellular composition in the spleen and the cellular source of IFN- γ upon Epac activation.

Spleen cells were isolated from control (PBS) and 8pCPT-injected mice and were incubated with Brefeldin A was added for 5hours to impede cytokine excretion from the cells. These cells were then analysed for IFN- γ production by the different cell types. An increase in $CD3^+IFN-\gamma^+$ cells from an average of 1.69% in the control to 2.43% in the Epac-treated mice and a significant increase in $CD8^+IFN-\gamma^+$ cells from an average of 2.03% in the control to 3.67% in the Epac agonist-treated mice was observed (Figure 5.4a). The percentage of $CD4^+IFN-\gamma^+$ cells was slightly enhanced, but injection of the Epac activator alone increased the number of IFN- γ -producing $CD8^+$ cells. Injecting LPS alone increased IFN- γ production by splenic $CD8^+$ T cells compared to injection with PBS, and this was not enhanced further by co-injecting the Epac agonist and LPS (Figure 5.4b). LPS injection alone also increased the percentage of NK and NKT cells producing IFN- γ (Figure 5.4b). Co-injection of the Epac activator with LPS significantly enhanced IFN- γ production by NKT cells (Figure 5.4b).

5.3 Activation of the Epac pathway *in vivo* increases the percentage of splenocytes producing IFN- γ and IL-17.

There was an increase in the percentage of IL-17 $^+$ splenocytes after injection of the Epac activator, both in the presence and absence of LPS (Figure 5.5). Injection of 8pCPT led to a significant increase in the $CD3^+IL-17^+$ population, which increased from an average of 0.66% in the control to 0.99% (Figure 5.5a). Injection of LPS alone resulted in an average of 1.41% $CD3^+IL-17^+$ splenocytes and this was increased by co-injection with the Epac activator to an average of 2.06% $CD3^+IL-17^+$ cells (Figure 5.5b). Similarly, there was a significant increase in $CD8^+IL-17^+$ cells from an average of 1.42% in the control to 2.37% in the Epac agonist-treated mice (Figure

5.5a). Injection of LPS alone resulted in an average of 3.17% CD8⁺IL-17⁺ spleen cells compared to 4.51% in mice injected with the Epac agonist and LPS (Figure 5.5b). CD4⁺ cells showed no increase in IL-17 production upon 8pCPT-injection, either with or without co-injection of LPS (Figure 5.5). Since 8pCPT injection resulted in increased IL-17 and IFN- γ production in CD3⁺CD8⁺ cells, co-expression of IFN- γ and IL-17 in these cells was determined. While injection of the Epac activator enhanced the percentage of both IFN- γ ⁺IL-17⁻ and IFN- γ ⁺IL-17⁺ splenocyte populations, the enhanced IL-17 production was almost exclusively seen in cells co-expressing IFN- γ (Figure 5.6). An increase in IFN- γ ⁺ splenocytes from an average of 3.30% in control mice, to 5.28% in the Epac agonist-injected mice was seen (Figure 5.6). There was no difference in the percentage of IFN- γ ⁺ from CD8⁺ cells in mice injected with LPS and the Epac agonist compared to LPS alone (Figure 5.6). The percentage of CD8⁺ cells producing IL-17 was increased from an average of 1.81% in the PBS group to 2.78% in the Epac agonist injected group (Figure 5.6). In contrast to IFN- γ , this increase in IL-17 production from CD8⁺ cells was still apparent in mice immunised with the Epac activator with LPS, with an average of 3.90% IL-17⁺ CD8⁺ cells in the LPS group and 5.26% in the group injected with the Epac activator and LPS (Figure 5.6).

5.4 Injection of the Epac activator 8pCPT increases the percentage of splenic CD11c⁺ and NKT cells

The spleen was analysed by flow cytometry for changes in the percentages of specific cell populations, following injection with the Epac activator over five consecutive days. The dotplots are shown in Figure 5.7a while a bar chart of the percentage values of individual populations are shown in Figure 5.7b. It was found that the percentage of CD11c⁺ cells, a marker used to identify DCs, significantly increased from an average of 0.76% in control mice to 1.00% in mice injected with the Epac activator (Figure 5.7). The percentage of cells positive for the NK cell marker DX5 increased marginally from an average of 5.72% in the control mice to 6.38% in the mice injected with the Epac activator (Figure 5.7). In order to differentiate between NK cells and NKT cells the cells were analysed for co-expression of CD3 marker and DX5 marker. NKT cells (identified as CD3⁺DX5⁺) were significantly increased from an average of 0.76% in the control mice to 1.00% in the Epac activator-treated mice,

while the CD3⁺DX5⁺ cells were only slightly increased from an average of 4.06% in the control mice to 4.54% in the Epac-treated mice (Figure 5.7). In contrast, injection of 8pCPT did not significantly alter the percentage of CD3⁺, CD4⁺ or CD8⁺ cells in the spleen (data not presented).

5.5 A single injection of the Epac activator induces increased IFN- γ production in the spleen and mediastinal lymph nodes, at an early timepoint.

Having determined that injecting 8pCPT daily for 5 days exerts a significant immunomodulatory effect, the capacity of a single i.p. injection of 8pCPT to modulate immune responses was determined.

Twenty-four hours after i.p. injection, spleens were isolated and splenocytes were stimulated with anti-CD3 alone or together with PMA and ionomycin (Figure 5.8). Stimulation of splenocytes from mice injected with 8pCPT with anti-CD3 and with PMA and ionomycin induced significantly increased IFN- γ and IL-17 production compared to splenocytes from mice injected with PBS (Figure 5.8). In contrast, a single injection with LPS did not increase IFN- γ or IL-17 production, and addition of the Epac activator to LPS did not enhance secretion of IFN- γ or IL-17. However, LPS injection did enhance IL-4 production (average of 244pg/ml) in unstimulated spleen cells when compared to PBS control. Co-injection of 8pCPT and LPS decreased the amount of IL-4 produced in unstimulated cells (average of 34pg/ml) when compared to LPS injection alone (Figure 5.8). The spleen cells were analysed via flow cytometry in order to assess any changes in the percentages of specific cell populations (Figure 5.9). The percentage of CD3⁺, CD4⁺ and CD8⁺ lymphocytes were slightly increased in the spleen following immunisation with the Epac activator (Figure 5.9A). Injection of 8pCPT significantly increased the percentage of $\gamma\delta$ T cells in the spleen (Figure 5.9B). Co-injection of the Epac activator with either R848 or LPS did not significantly increase the percentage of CD4⁺, CD8⁺, (data not presented) or $\gamma\delta$ T cells in the spleen (Figure 5.9).

5.6 A single injection of the Epac activator 8pCPT enhances IFN- γ production by peritoneal and lymph node lymphocytes

In order to analyse the effects of a single injection of the Epac activator at the site of injection, peritoneal cells were harvested. The peritoneal cells were stimulated with anti-CD3 *ex vivo* and analysed for cytokine production (Figure 5.10). Injection with the Epac activator induced an increase in IL-17 production by these cells, but no increase in IFN- γ production was observed (Figure 5.10). There was no significant change in either IL-4 or IL-6 production upon 8pCPT injection (Figure 5.10). The lack of a significant increase in IFN- γ production could be the concentration of anti-CD3 used to stimulate these cells being too concentrated. This is indicated by the concentrations of IFN- γ detected, which are quite high (>10ng/ml). Intracellular flow cytometric analysis of IFN- γ expression by peritoneal cells cultured for 6hours *ex vivo* was therefore carried out. An increase in CD8⁺IFN- γ ⁺ cells was seen upon injection of 8pCPT in the peritoneum (Figure 5.11). The dotplot of the CD8⁺ cells isolated from the peritoneum is different in comparison to the spleen cell CD8 cells, which is due to the fact that the cells isolated from the peritoneum were very granular cells i.e. with high side scatter profile. This could be because these cells are at the site of injection and No $\gamma\delta$ T cells or NK cells producing IFN- γ were detected in the peritoneum (data not presented). Peritoneal cells positive for both CD8 and IFN- γ , increased from an average of 9.69% CD8⁺IFN- γ ⁺ cells in the PBS control, to 13.96% CD8⁺IFN- γ ⁺ cells in mice injected with 8pCPT (Figure 5.11). The addition of R848 (TLR7/8 agonist) to the Epac activator did not alter the percentage of CD8⁺IFN- γ ⁺ cells (Figure 5.11), and neither did the addition of LPS (data not presented). This production of IFN- γ by peritoneal CD3⁺CD8⁺ cells mirrors the *in vitro* effects seen in chapter 4, where Epac activation in spleen cells principally promoted IFN- γ production by CD8⁺ T cells.

As the main draining lymph nodes following i.p. injection, the mediastinal lymph nodes were analysed for changes in cytokine production 24hours after injection with 8pCPT. Unstimulated and anti-CD3-stimulated mediastinal lymph node cells from mice injected with 8pCPT secreted significantly higher concentrations of IFN- γ than cells from mice injected with PBS (Figure 5.12a). Co-injection of the Epac activator and LPS induced an upward trend in the IFN- γ response when compared to injection

of LPS alone (Figure 5.12b). However, injection of 8pCPT either alone or with LPS resulted in a decrease in IL-4 production in unstimulated and anti-CD3-stimulated mediastinal lymph node cells (Figure 5.12). This is similar to results observed previously where IL-4 production was suppressed after injection with the Epac activator for five days (Figure 5.3). No IL-17 was detected in these cells.

Flow cytometric analysis was performed on MLN cells to determine the cellular source of the IFN- γ (Figure 5.13). Injection of 8pCPT increased IFN- γ expression by CD3⁺CD8⁺ T cells. Expression increased from an average of 0.96% CD3⁺IFN- γ ⁺ cells to 1.40% in mice injected with 8pCPT (Figure 5.13). There was an increase in CD3⁺CD8⁺IFN- γ ⁺ cells, from an average of 2.15% in the PBS control to 3.73% in mice injected with 8pCPT (Figure 5.13). There was no difference in IFN- γ production by CD4⁺, NK, NKT or $\gamma\delta$ T mediastinal lymph node cells following Epac agonist injection (Figure 5.13). The percentage of specific cell subsets was determined in the MLN of these mice, however no significant differences in the percentage of CD3⁺ lymphocytes, NK, NKT or $\gamma\delta$ T cells present in the mediastinal lymph nodes 24hours post injection were detected (data not presented).

5.7 Epac activation alters cell numbers and increases IFN- γ production in the spleen but not in an OVA-specific response

Having demonstrated that activation of the Epac pathway can exert immunomodulatory effects *in vivo* the adjuvant potential of this approach was investigated. The effects of Epac activation on both antibody production and cell-mediated immunity to the co-administered model antigen, ovalbumin (OVA), were investigated.

Spleen cells from mice immunised with both the Epac activator and OVA did not secrete enhanced antigen-specific cytokines compared to mice injected with antigen alone (Figure 5.14). OVA injection alone induced an antigen-specific IL-4 and IL-6 response, indicating that OVA alone promoted a Th2-type response. Interestingly, co-injection of 8pCPT with OVA resulted in a significantly lower OVA-specific IL-4 response (Figure 5.14), similar to the non-specific suppression of IL-4 production

seen previously after injection of the Epac activator (Figure 5.8 and Figure 5.12). Antigen-specific IL-6 production was also suppressed after injection of the Epac activator, although this was not significant (Figure 5.14). No antigen-specific response was detected in mediastinal lymph node cells (data not presented). The mediastinal lymph nodes were isolated and analysed via flow cytometry for changes in the percentages of cells present. An upward trend in the percentage of CD3⁺CD4⁺ cells present in the mediastinal lymph nodes was found in mice injected with the Epac activator and OVA. This increase was found in unstimulated cells and cells stimulated with the OVA antigen, indicating that this increase is not antigen-specific but rather an effect of injecting the Epac activator (Figure 5.15). In contrast to the effects on CD4⁺ cells, the percentage of CD8⁺ cells present was unaffected while $\gamma\delta$ T cell numbers were slightly reduced following immunisation with the Epac activator and OVA compared to OVA alone (Figure 5.15).

No antigen-specific cytokine response was detected in the peritoneal exudate cells of mice immunised with the Epac activator and OVA (data not presented). The sera of these mice were analysed for OVA-specific antibody production, however no significant difference was found after immunisation with 8pCPT and OVA compared to OVA alone (Figure 5.16). While 8pCPT injection with OVA did not enhance antigen-specific antibody production, there was an indication that IgG2a was enhanced in mice injected with 8pCPT, LPS and OVA compared to injection with LPS and OVA alone (Figure 5.16). Four mice produced increased IgG2a in this group compared to two mice in the LPS with OVA control group. Overall however, co-injection of the Epac activator with OVA did not enhance OVA-specific antibody production or cell-mediated immunity, but did exert non-specific effects on the immune response.

5.8 Epac activation enhances antigen-specific antibody titres and T cell responses to the *Mycobacterium tuberculosis* H1 vaccine antigen.

A similar immunisation study to the OVA-adjuvant study in the previous section was performed, but instead of OVA, the candidate *Mycobacterium tuberculosis* vaccine antigen, H1, was used. This antigen is derived from the causative agent of TB, which

is thought to require a Th1-specific response for protective immunity. Since the *in vitro* data in chapter 4 and the *in vivo* data described earlier in this chapter suggested that the Epac activator induces production of the Th1 cytokine IFN- γ , this makes this antigen a suitable candidate for investigation of 8pCPT as a Th1-driving adjuvant. CpG, a TLR9 agonist, was also used in these experiments. CpG induces Th1 responses [406], and the ability of the Epac activator to enhance the effects of CpG was investigated.

Unstimulated spleen cells from mice immunised with 8pCPT and H1 produced more IFN- γ (351pg/ml) than mice immunised with H1 alone (119pg/ml) (Figure 5.18). The H1-specific IFN- γ was not increased by the Epac activator. However an upward trend, although not significant, in IFN- γ production was apparent in mice injected with CpG. Antigen-specific IL-17 was secreted by restimulated splenocytes from mice immunised with CpG, H1 and 8pCPT but not in mice immunised with 8pCPT and antigen alone, while Epac activation did not significantly enhance IL-4 production (Figure 5.17). Co-injection of 8pCPT with H1 led to enhancement of antigen-specific IL-10 compared to H1 alone, while the Epac activator did not enhance the CpG-induced H1-specific IL-10 response (Figure 5.17).

The mediastinal lymph nodes were analysed for H1-specific cytokine response (Figure 5.18). Injection of the Epac activator and H1 induced increased IFN- γ production in unstimulated mediastinal lymph node cells compared to injection of H1 alone, i.e. a non-antigen-specific increase in IFN- γ production (Figure 5.18). Immunisation with H1 alone induced an average of 374pg/ml IFN- γ by unstimulated spleen cells, which increased to 580pg/ml in cells from mice injected with H1 and 8pCPT. Likewise, in the presence of CpG, co-injection of 8pCPT increased IFN- γ secretion by unstimulated cells from 379pg/ml with CpG alone to 774pg/ml (Figure 5.18). No antigen-specific IFN- γ , IL-17 or IL-4 was detected in any immunised mice (Figure 5.18). An upward trend in IL-10 production was observed at the highest concentration of H1 antigen used for restimulation (10 μ g/ml H1), but the differences between groups were not statistically significant (Figure 5.18).

Peritoneal cells from these mice were also isolated and stimulated *ex vivo*. No antigen-specific increase in cytokine production due to Epac activation was detected. Injection of CpG and H1 with and without 8pCPT induced antigen-specific IFN- γ and IL-10 production but addition of 8pCPT did not enhance this effect (Figure 5.19). However, anti-CD3 restimulation of peritoneal cells from mice injected with the Epac activator and H1 with and without CpG, induced increased IFN- γ production compared to injection without the Epac activator (Figure 5.19). This effect was specific to IFN- γ as the Epac activator did not significantly enhance anti-CD3-induced IL-17, IL-10 or IL-4 production (Figure 5.19).

5.9 The Epac activator 8pCPT enhances antigen-specific antibody responses to co-injected H1.

Sera from vaccinated mice was analysed for antibodies specific for the H1 antigen (Figure 5.20). Enhanced IgG1 and IgG2c production was observed from mice immunised with the Epac activator and H1 antigen compared to the antigen alone post one or two immunisations (Figure 5.20). There was a significant increase in H1-specific IgG2c production in mice immunised with 8pCPT and H1, compared to injection with H1 alone, post two immunisations (Figure 5.20). Injection of mice with H1 antigen and CpG enhanced all subtypes of antibodies analysed (Figure 5.20). Co-injection of CpG and H1 with 8pCPT did not significantly enhance antibody production, possibly due to the strength of the ability of CpG to induce antibody production by itself, however there was an upward trend in all subtypes, particularly IgG1 and IgG2b (Figure 5.20).

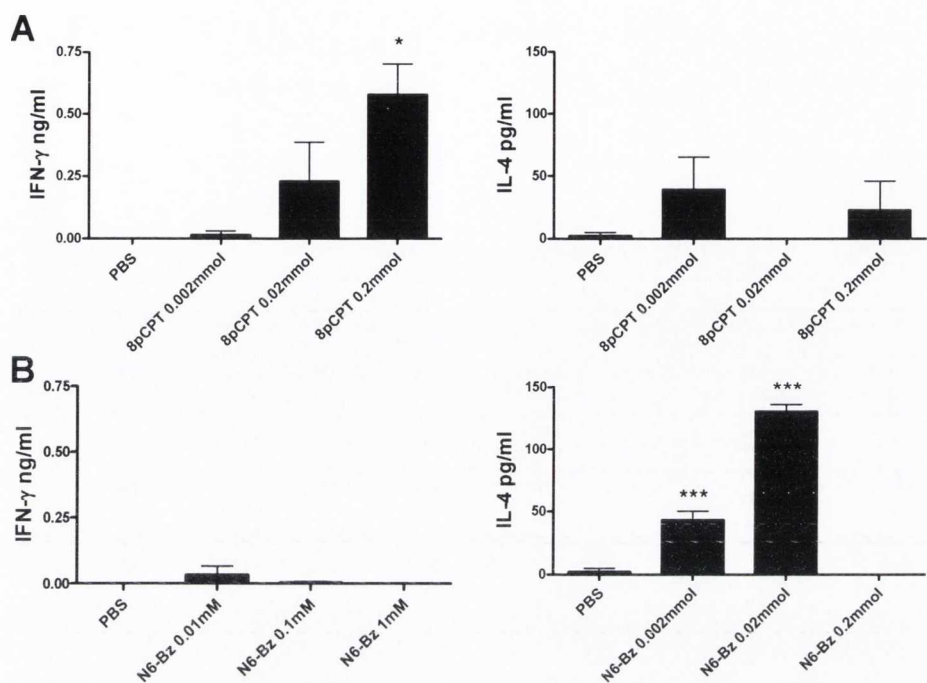


Figure 5.1: Injection of mice with the Epac agonist 8pCPT enhances IFN- γ production, while injection of the PKA agonist N6-Bz enhances IL-4 production by mediastinal lymph node cells. BALB/c mice were immunised i.p. on 5 consecutive days with PBS, or increasing doses (1 μ g/mouse (0.002mmol), 10 μ g/mouse (0.02mmol) and 100 μ g/mouse (0.2mmol)) of the selective Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) (A) or the selective PKA activator N6-Bz-cAMP (N6-Bz) (B). On day 6 the mice were sacrificed. Cells from the mediastinal lymph nodes were isolated, and plated in complete RPMI. After 3 days the cytokine concentrations in the supernatant were determined by ELISA. Significant differences between mice injected with PBS mice and mice injected with the agonist are defined as $p < 0.05$ (*) and $p < 0.001$ (***). A total of 21 mice with 3 mice per treatment group were used in this experiment.

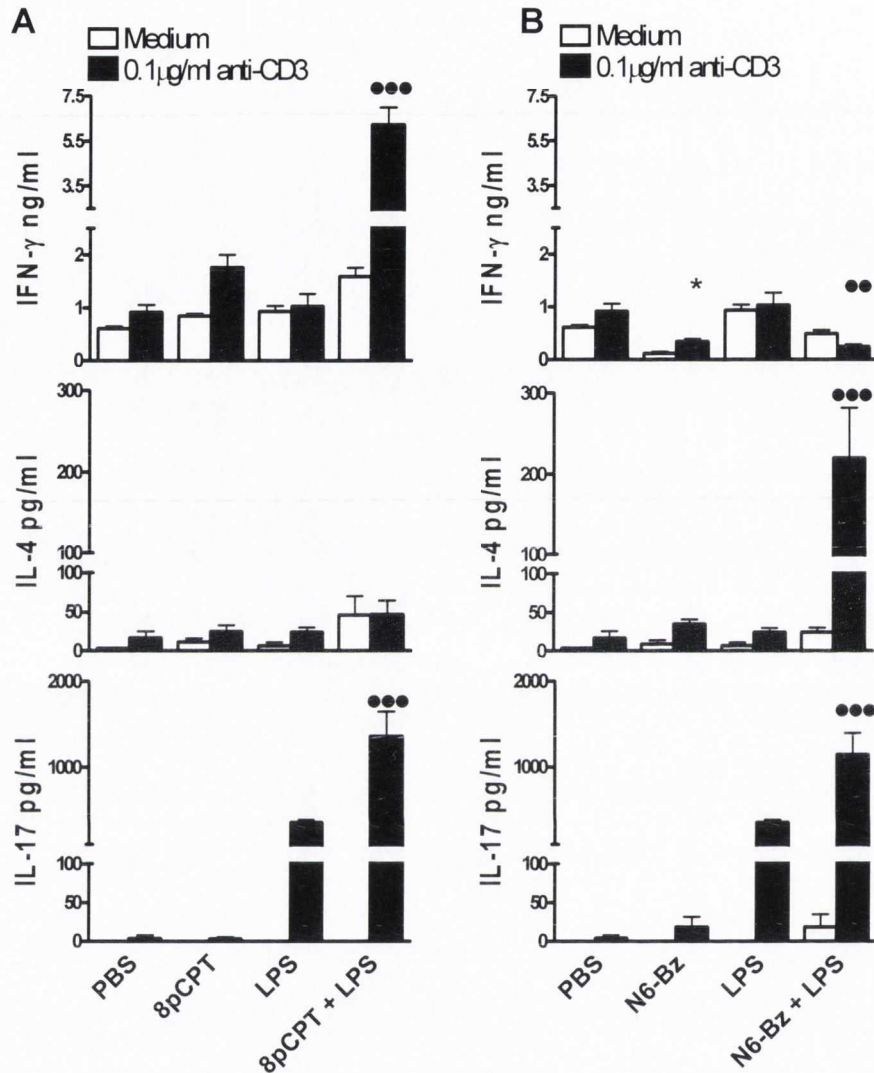


Figure 5.2: Injection of mice with the Epac activator 8pCPT enhances IFN- γ production while injection of mice with the PKA activator N6-Bz enhances IL-4 production by mediastinal lymph node cells. BALB/c mice were injected i.p. on 5 consecutive days with PBS, the selective Epac activator 8pCPT (0.1mmol), the selective PKA activator N6-Bz-cAMP (N6-Bz) (0.1mmol), LPS (10 μ g/mouse), LPS with 8pCPT or LPS with N6-Bz. On day 6 the mice were sacrificed. The mediastinal lymph node cells were isolated, and plated on anti-CD3 (0.5 μ g/ml). After 3 days the cytokine concentrations in the s-upernatant were determined by ELISA. Significant differences between PBS-injected mice and mice injected with either 8pCPT alone or N6-Bz alone are defined as $p < 0.05$ (*). Significant differences between LPS-injected mice and mice injected with either 8pCPT or N6-Bz together with LPS are defined as $p < 0.01$ (**) and $p < 0.001$ (***). A total of 15 mice with 3 mice per treatment group were used in this experiment.

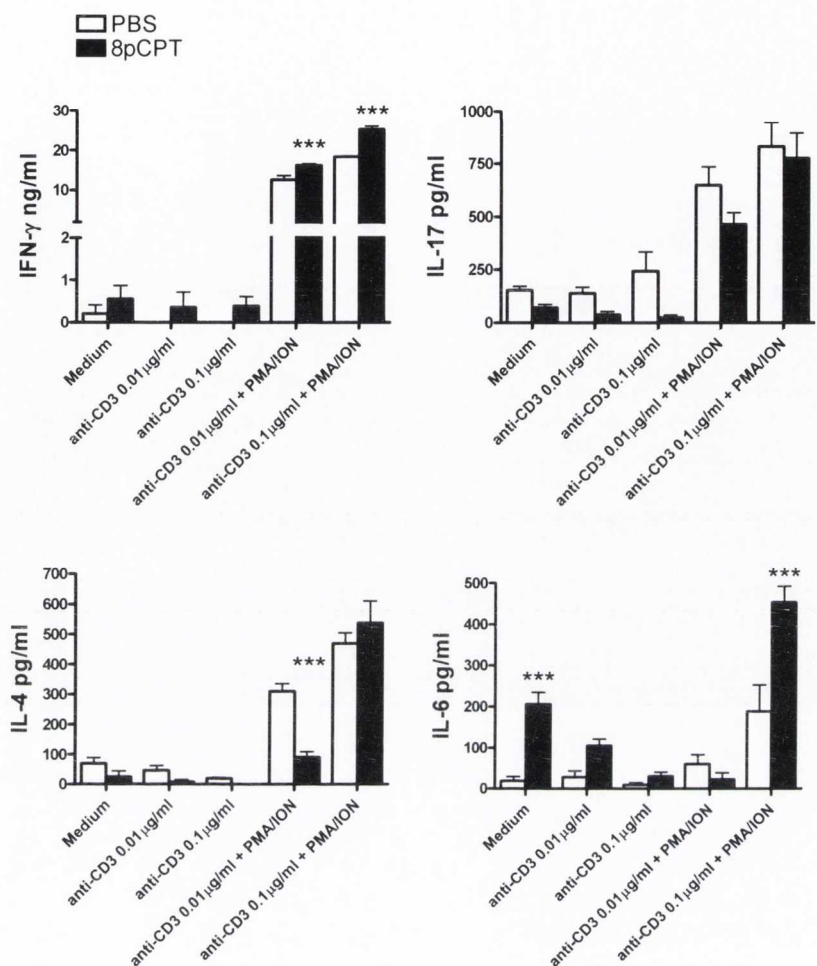


Figure 5.3: Injection with the selective Epac activator 8pCPT on five consecutive days increases IFN-γ and IL-6 production by spleen cells. BALB/c mice were injected i.p. with PBS (white bar) or 0.1mmol 8pCPT (black bar).on 5 consecutive days. On day 6, the mice were sacrificed and the spleen cells were isolated and plated on anti-CD3 (0.01µg/ml and 0.1µg/ml) with and without PMA (40ng/ml) and Ionomycin (200ng/ml). After 3 days the cytokine concentrations in the supernatant were determined by ELISA. Significant differences between PBS-injected mice (white bar) and mice injected with 8pCPT (black bar) are defined as $p<0.001$ (***). A total of 16 mice with 4 mice per treatment group were used in this experiment.

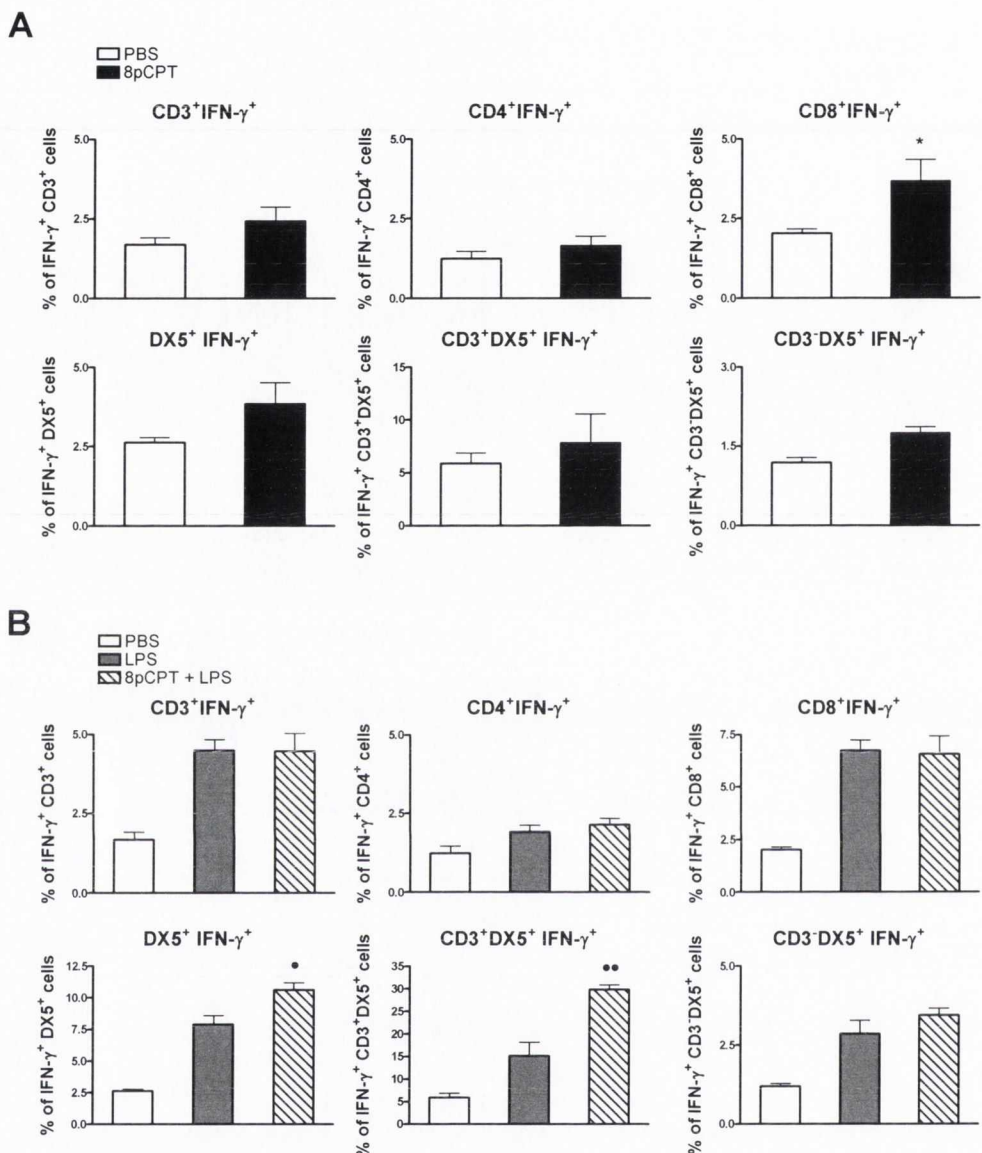


Figure 5.4: Injection with the Epac activator 8pCPT induces an increase in IFN- γ production from CD8⁺ and NKT cells in the spleen. BALB/c mice were injected i.p. with PBS (as control), 8pCPT (0.1mmol), LPS (10 μ g/mouse) or 8pCPT with LPS on 5 consecutive days. The spleen cells were isolated and left for five hours in the presence of Brefeldin A (10 μ g/ml) after which flow cytometry was performed with antibodies specific for CD3, CD4, CD8, DX5 and IFN- γ . Mice injected with PBS or 8pCPT are shown in A, while mice injected with PBS, LPS or 8pCPT+LPS are shown in B. Significant differences between PBS-injected mice and mice injected with 8pCPT are defined as $p < 0.05$ (*). Significant differences between LPS-injected mice and mice injected with 8pCPT and LPS are defined as $p < 0.05$ (●) and $p < 0.01$ (●●). A total of 24 mice with 4 mice per treatment group were used in this experiment.

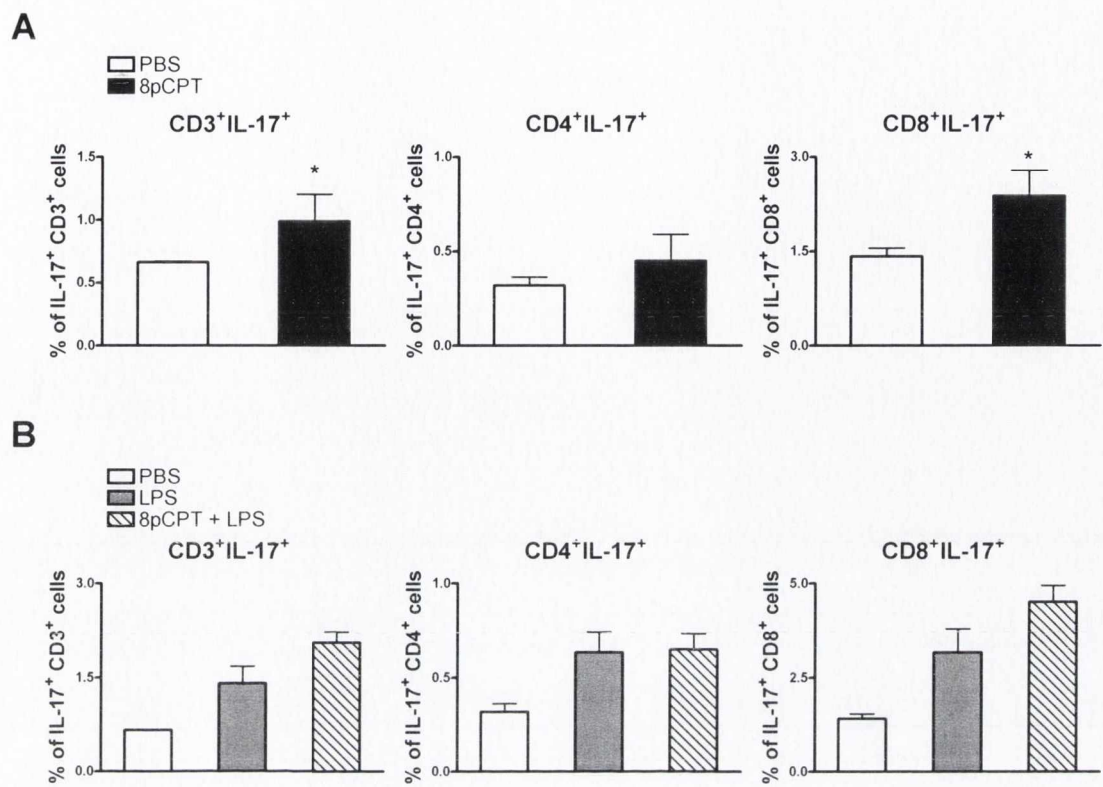


Figure 5.5: Injection with the Epac activator 8pCPT enhances IL-17 production by CD8⁺ cells in the spleen. BALB/c mice were injected i.p. with PBS (as control), 8pCPT (0.1mmol), LPS (10μg/mouse) or 8pCPT with LPS on 5 consecutive days. Spleen cells were isolated and left for five hours in the presence of Brefeldin A (10μg/ml) after which flow cytometry was performed with antibodies specific for CD3, CD4, CD8, and IL-17. Mice injected with PBS or 8pCPT are shown in A, while mice injected with PBS, LPS or 8pCPT+LPS are shown in B. Significant differences between PBS-injected mice and mice injected with 8pCPT are defined as $p < 0.05$ (*). A total of 24 mice with 4 mice per treatment group were used in this experiment.

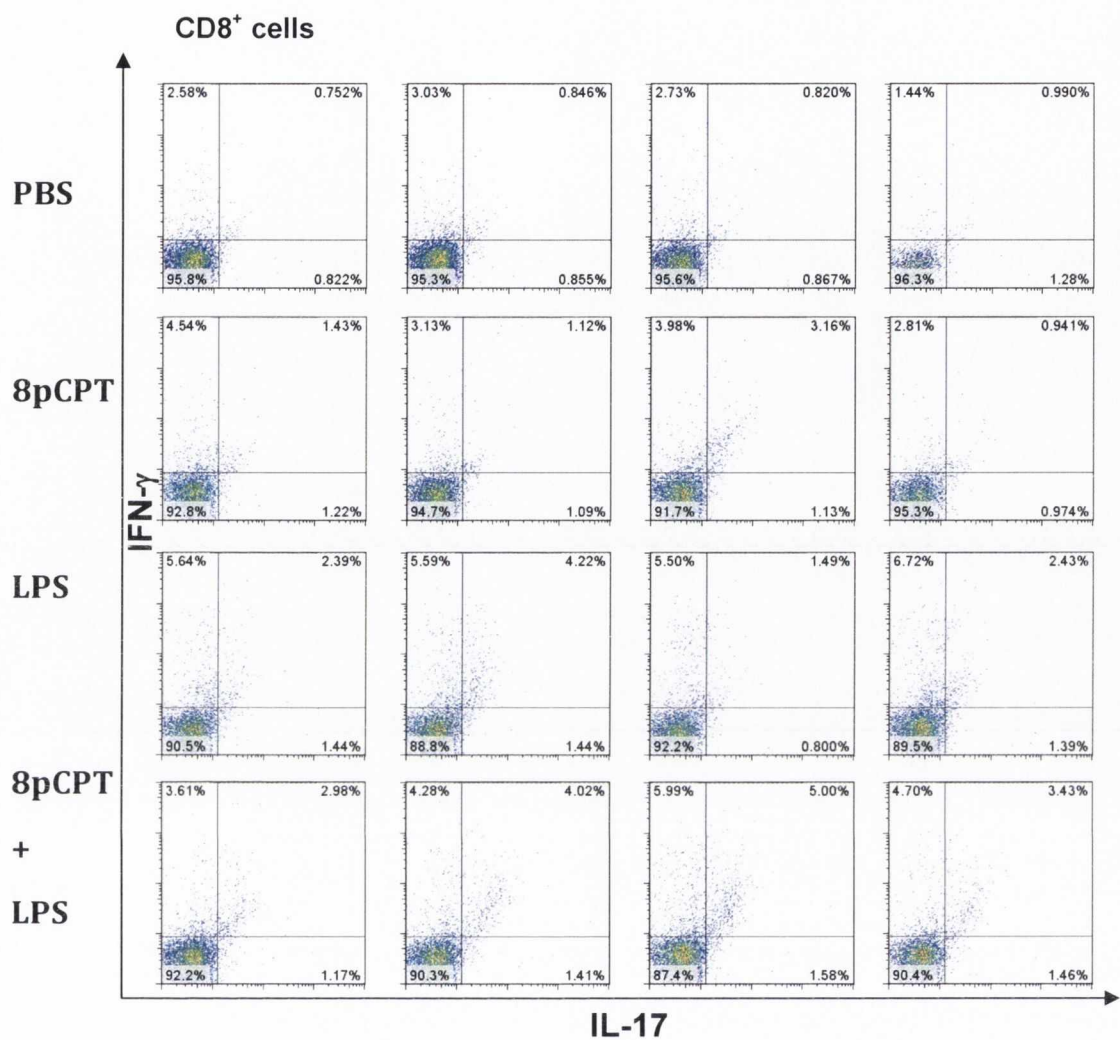


Figure 5.6: The percentages of CD8⁺IFN-γ⁺ and CD8⁺IL-17⁺ cells in the spleen are increased following injection of the Epac activator 8pCPT. BALB/c mice were injected i.p. with PBS (as control), 8pCPT (0.1mmol), LPS (10μg/mouse) or 8pCPT with LPS on 5 consecutive days. Spleen cells were isolated and left for five hours in the presence of Brefeldin A (10μg/ml) after which flow cytometry was performed with antibodies specific for CD3, CD4, CD8, IFN-γ and IL-17. Dot plots show CD8⁺ cells producing IFN-γ and IL-17. The four columns are the dotplots of each individual mouse in the group.

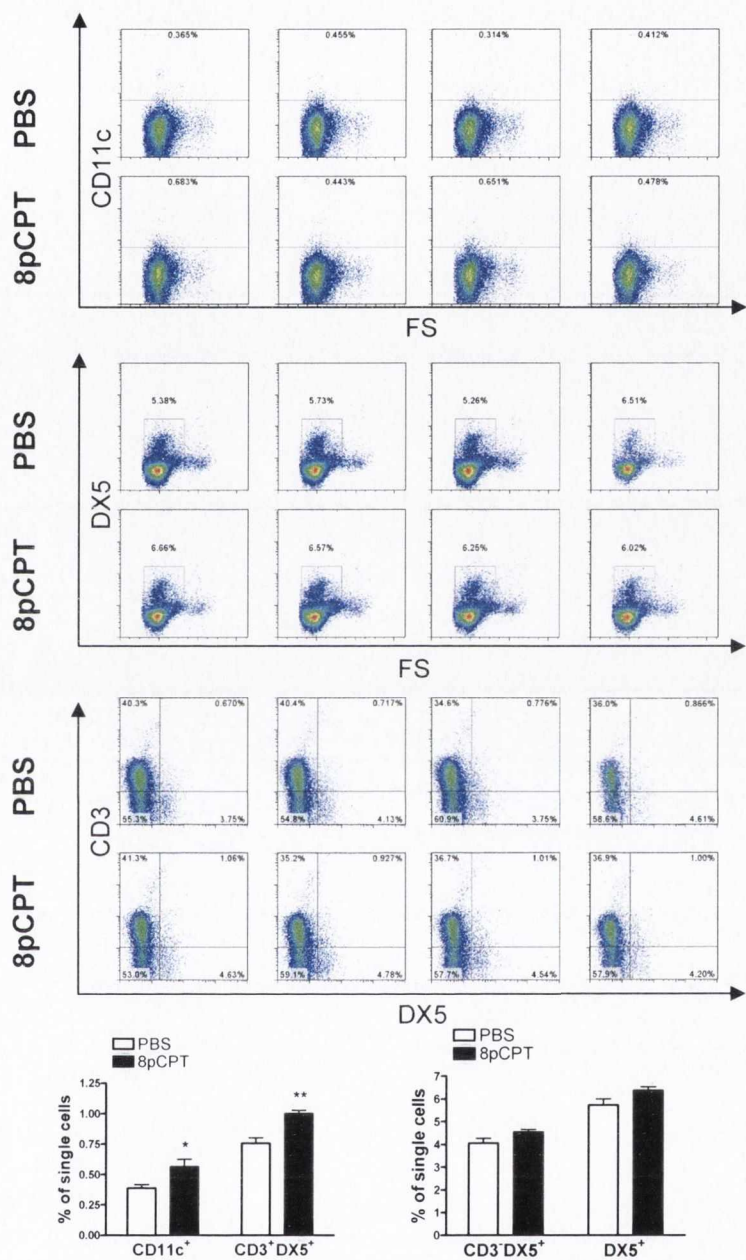


Figure 5.7: The percentage of NKT and CD11c⁺ cells in the spleen are increased following injection with the Epac activator over five days. BALB/c mice were injected i.p. on 5 consecutive days with PBS, or the selective Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) (0.1mmol). On day 6 the mice were sacrificed. Cells from the spleen were isolated, left for five hours in the presence of Brefeldin A (10µg/ml), and flow cytometry was performed on stimulated spleen cells using markers specific for DX5, CD11c, and CD3. Dot plots show CD11c vs forward scatter (FS), DX5 vs FS and CD3 vs DX5 (A). (B) shows the percentages of specific cell subtypes. A total of 24 mice with 4 mice per treatment group were used in this experiment.

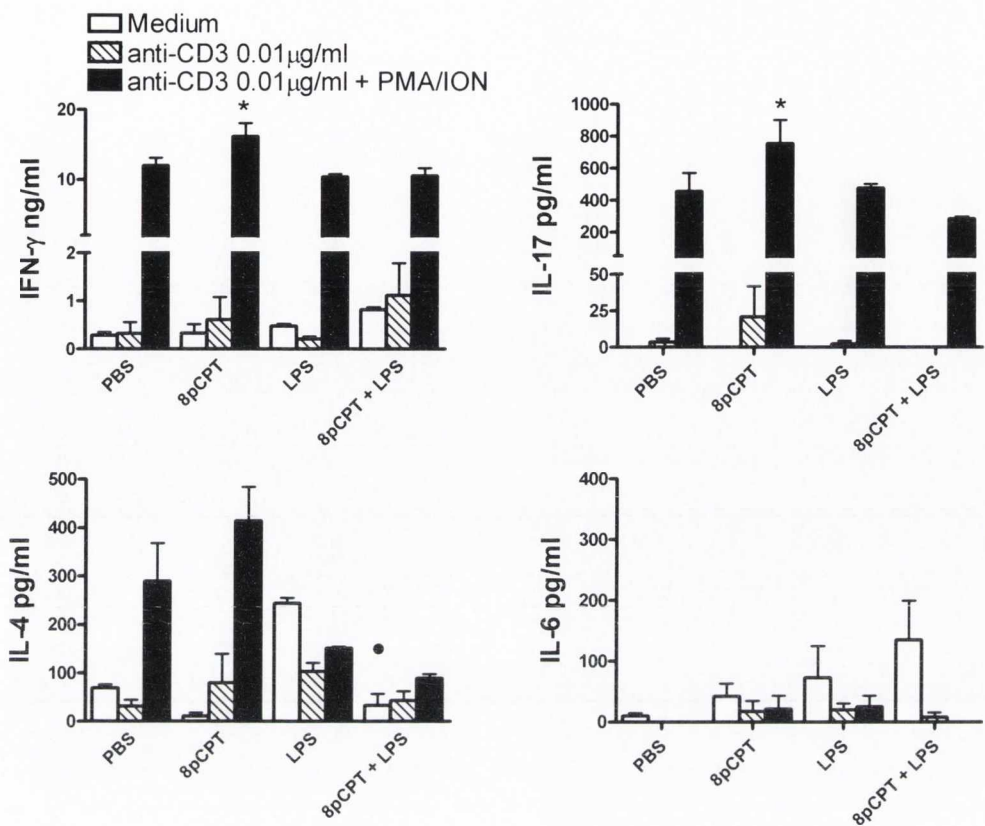


Figure 5.8: A single injection of the Epac activator 8pCPT enhances splenocyte IFN-γ and IL-17 production in response to anti-CD3 stimulation. BALB/c mice were injected i.p. with PBS (as control), 8pCPT (0.1mmol), LPS (10μg/mouse) or 8pCPT with LPS. 24 hours later cells were isolated from the spleen and plated on anti-CD3 (0.01μg/ml) with and without PMA (40ng/ml) and Ionomycin (200ng/ml). After 3 days the cytokine concentrations in the supernatant were determined by ELISA. Significant differences between PBS-injected mice and mice injected with 8pCPT are defined as $p < 0.05$ (*). Significant differences between LPS injected mice and mice injected with 8pCPT+LPS are defined as $p < 0.05$ (●). A total of 24 mice with 4 mice per treatment group were used in this experiment.

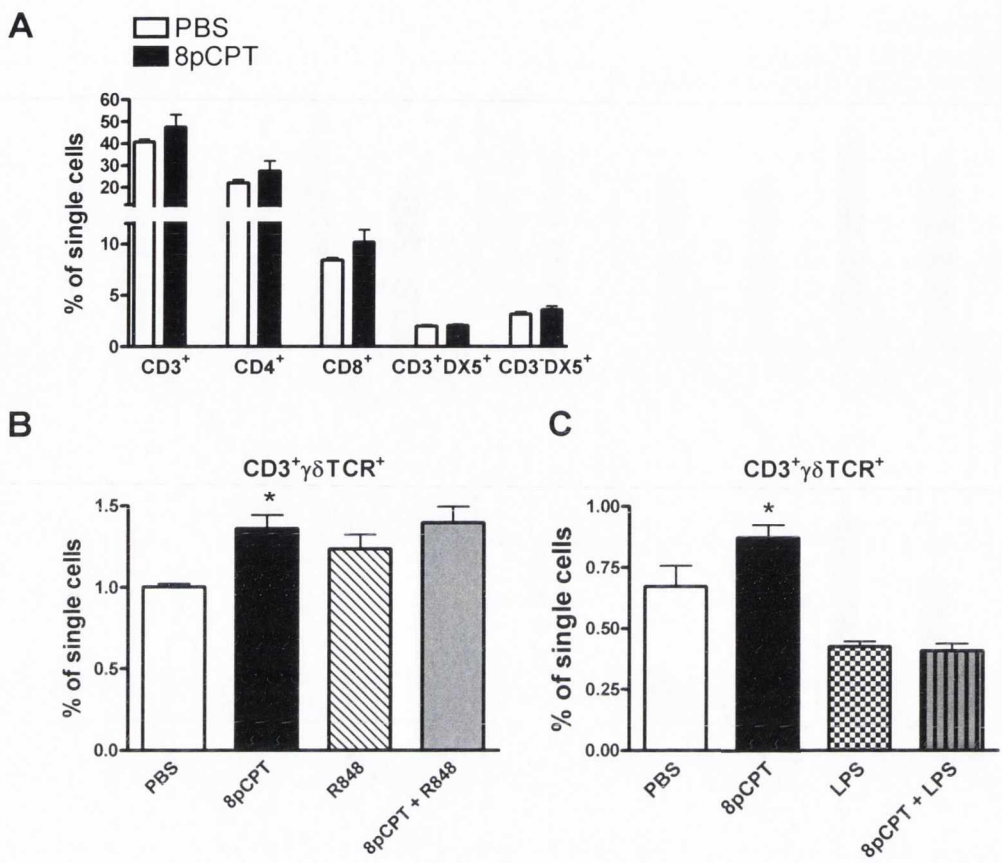


Figure 5.9: A single injection of the Epac activator 8pCPT increases the percentage of $\gamma\delta$ T cells present in the spleen after 24 hours. BALB/c mice were injected i.p. with PBS (as control) or 8pCPT (0.1mmol). 24 hours later cells were isolated from the spleen and flow cytometry was performed with antibodies specific for CD3, CD4, CD8 and DX5 (shown in A) and CD3 with $\gamma\delta$ TCR (shown in B and C). Significant differences between PBS-injected mice and mice injected with 8pCPT are defined as $p < 0.05$ (*). A total of 24 mice with 4 mice per treatment group were used in this experiment.

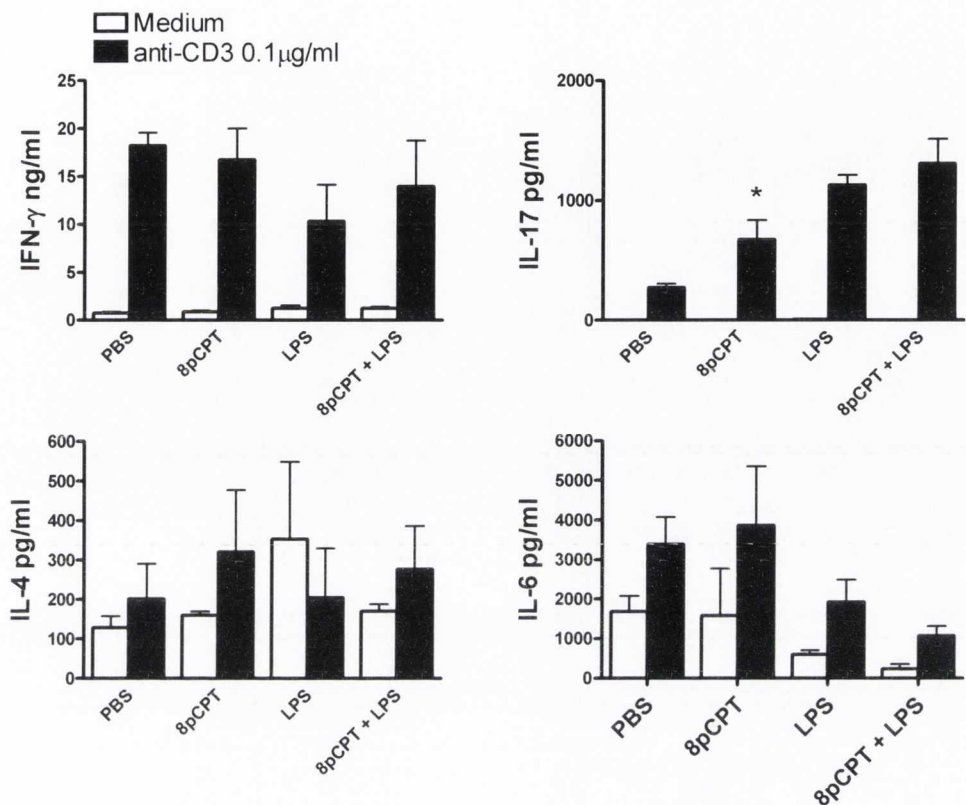


Figure 5.10: A single injection of the Epac activator 8pCPT promotes increased IL-17 production from peritoneal cells. BALB/c mice were injected i.p. with PBS (as control) or 8pCPT (0.1mmol). 24 hours later, cells were isolated from the peritoneum and plated on anti-CD3 (0.1 μ g/ml). After 3 days the cytokine concentrations in the supernatant were determined by ELISA. Significant differences between PBS-injected mice and mice injected with 8pCPT are defined as $p < 0.05$ (*). A total of 24 mice with 4 mice per treatment group were used in this experiment.

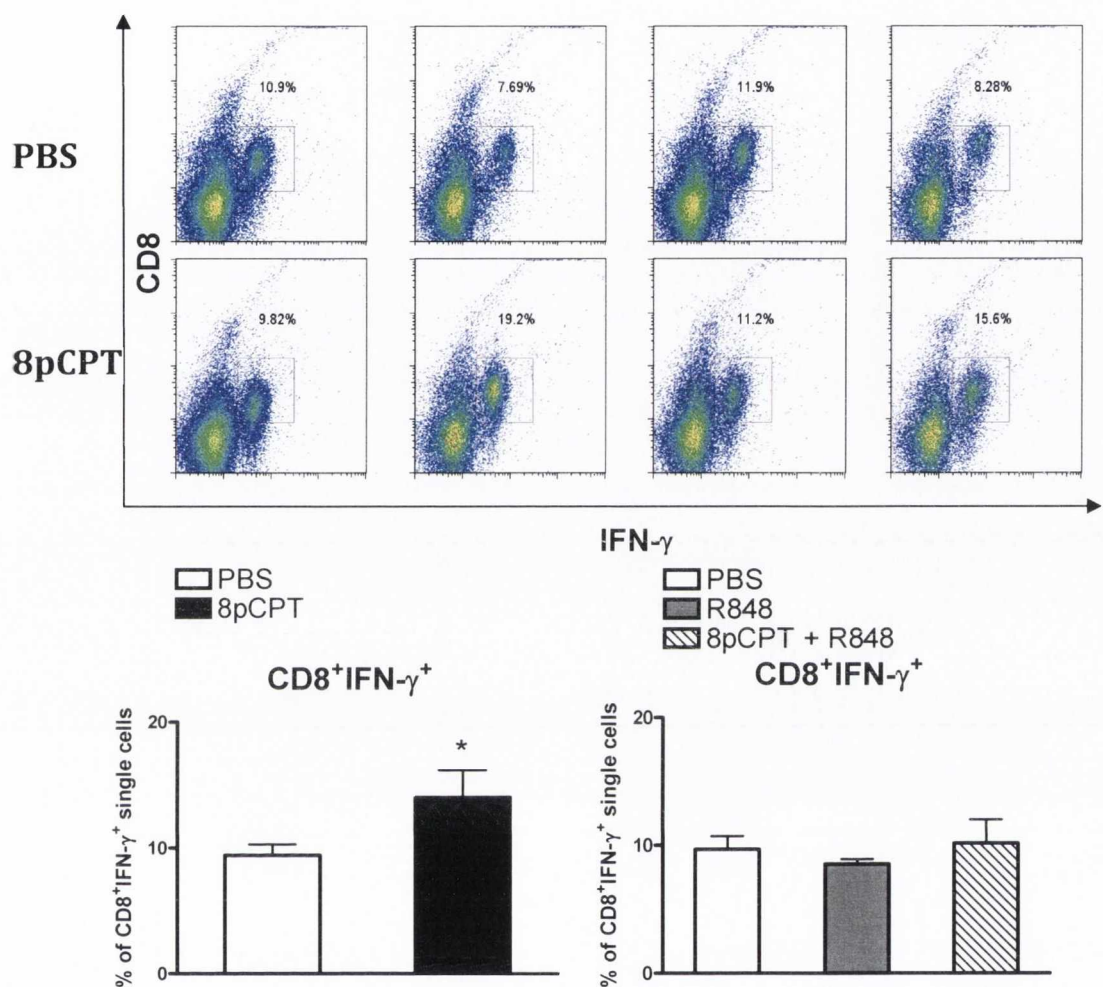


Figure 5.11: A single injection of the Epac activator 8pCPT promotes an increase in IFN- γ ⁺CD8⁺ cells in the peritoneal lavage. BALB/c mice were injected i.p. with PBS (as control), 8pCPT (0.1mmol), R848 (10 μ g/mouse) or 8pCPT with R848. 24 hours later cells were isolated from the peritoneum, left for five hours in the presence of Brefeldin A (10 μ g/ml) and flow cytometry was performed with antibodies specific for CD3, CD8 and IFN- γ . Significant differences between PBS-injected mice and mice injected with 8pCPT are defined as $p < 0.05$ (*). A total of 24 mice with 4 mice per treatment group were used in this experiment.

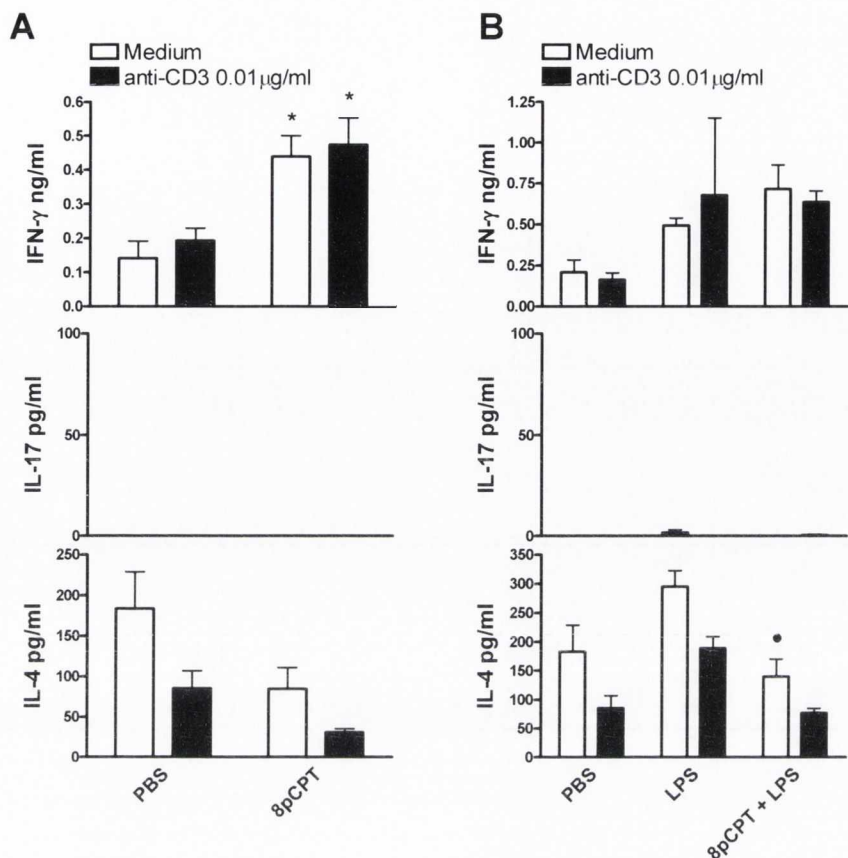


Figure 5.12: A single injection of the Epac activator 8pCPT enhances the production of IFN- γ , but not IL-4 or IL-17, in mediastinal lymph nodes. BALB/c mice were injected with PBS (as control), 8pCPT, LPS (10 $\mu\text{g}/\text{mouse}$) or 8pCPT with LPS i.p. 24 hours later cells were isolated from the mediastinal lymph nodes and plated on anti-CD3 (0.01 $\mu\text{g}/\text{ml}$). After 3 days the cytokine concentrations in the supernatant were determined by ELISA. Significant differences between PBS-injected mice and mice injected with 8pCPT are defined as $p < 0.05$ (*). Significant differences between LPS injected mice and mice injected with 8pCPT+LPS are defined as $p < 0.05$ (●). A total of 24 mice with 4 mice per treatment group were used in this experiment.

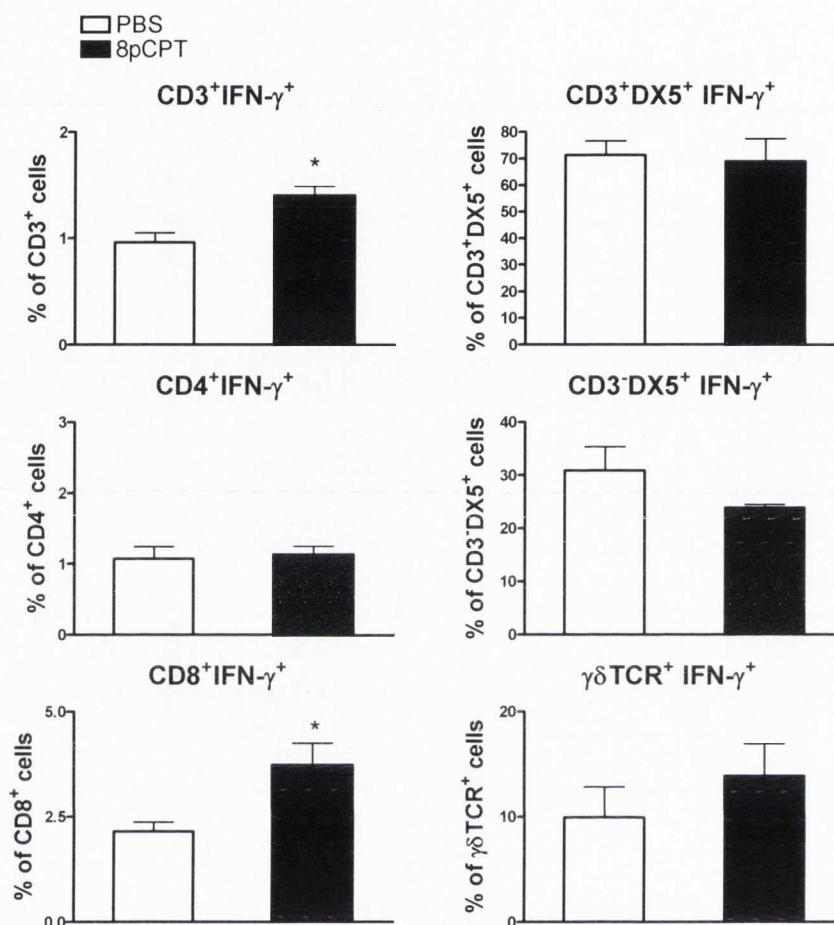


Figure 5.13: A single injection of the Epac activator 8pCPT increased IFN- γ production by CD8⁺ cells in the mediastinal lymph nodes. BALB/c mice were injected i.p. with PBS (as control) or 8pCPT (0.1mmol). 24 hours later, cells were isolated from the mediastinal lymph nodes, left for five hours in the presence of Brefeldin A (10 μ g/ml) and flow cytometry was performed with antibodies specific for CD3, CD4, CD8, DX5, $\gamma\delta$ TCR and IFN- γ . Significant differences between PBS-injected mice and mice injected with 8pCPT are defined as $p < 0.05$ (*). A total of 24 mice with 4 mice per treatment group were used in this experiment.

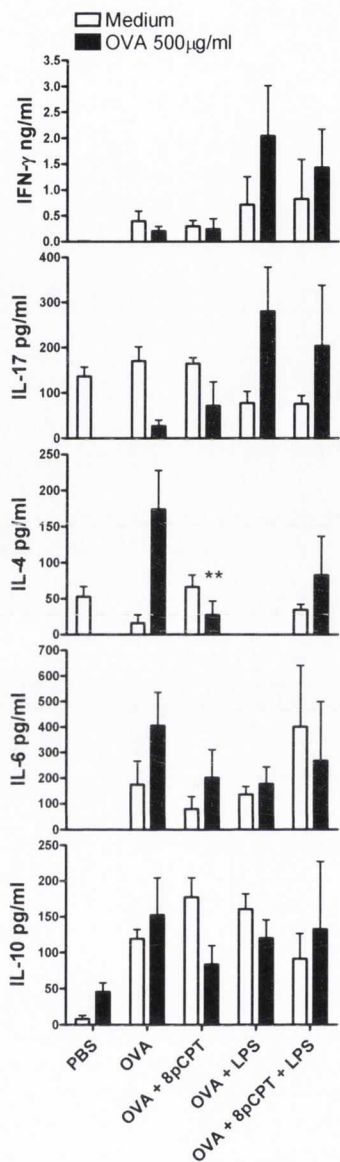


Figure 5.14: Co-injection of the Epac activator 8pCPT with OVA does not result in an increase in antigen-specific cytokine production by spleen cells. BALB/c mice were injected i.p. with PBS, OVA alone or OVA with 0.1mmol of the selective Epac activator (8pCPT), LPS (10 μ g/mouse) or 8pCPT with LPS, followed by an identical boost 21 days later. Seven days later mice were sacrificed. The spleen cells were isolated and plated with medium or OVA (500 μ g/ml). After 3 days the cytokine concentrations in the supernatant were determined by ELISA. Significant differences between OVA-injected mice and mice injected with OVA+8pCPT are defined as p<0.01(**). A total of 35 mice with 5 mice per treatment group were used in this experiment.

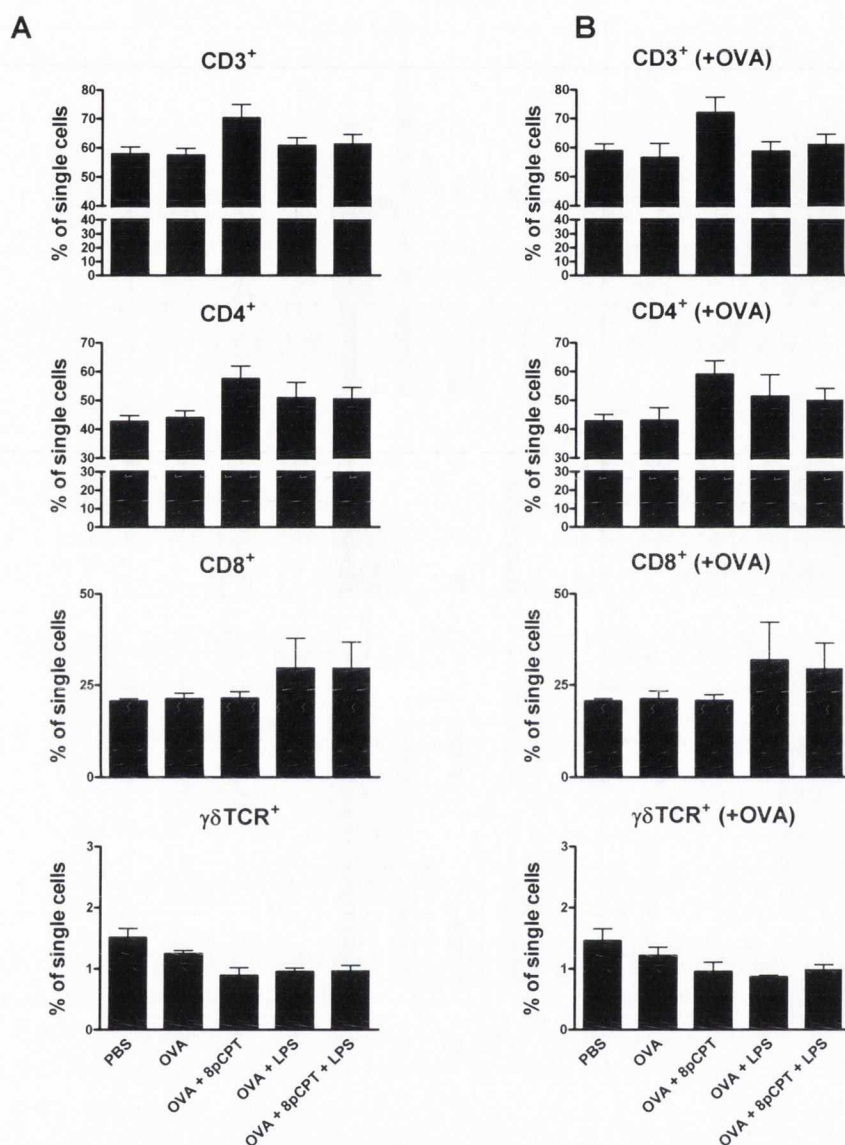


Figure 5.15: Injection of mice with OVA in the presence of the Epac activator 8pCPT results in an increase in the percentage of CD3⁺CD4⁺ cells in the mediastinal lymph nodes. BALB/c mice were injected i.p. with PBS, OVA either alone or with 0.1mmol of the selective Epac activator (8pCPT), LPS (10μg/mouse) or 8pCPT with LPS, followed by an identical boost 21 days later. Seven days later mice were sacrificed. The mediastinal lymph node cells were isolated and plated on RPMI or OVA for one hour followed by the addition of Brefeldin A. Six hours post-stimulation, the spleen cells were analysed via flow cytometry for expression of CD3, CD4, CD8 and γδTCR. A total of 35 mice with 5 mice per treatment group were used in this experiment.

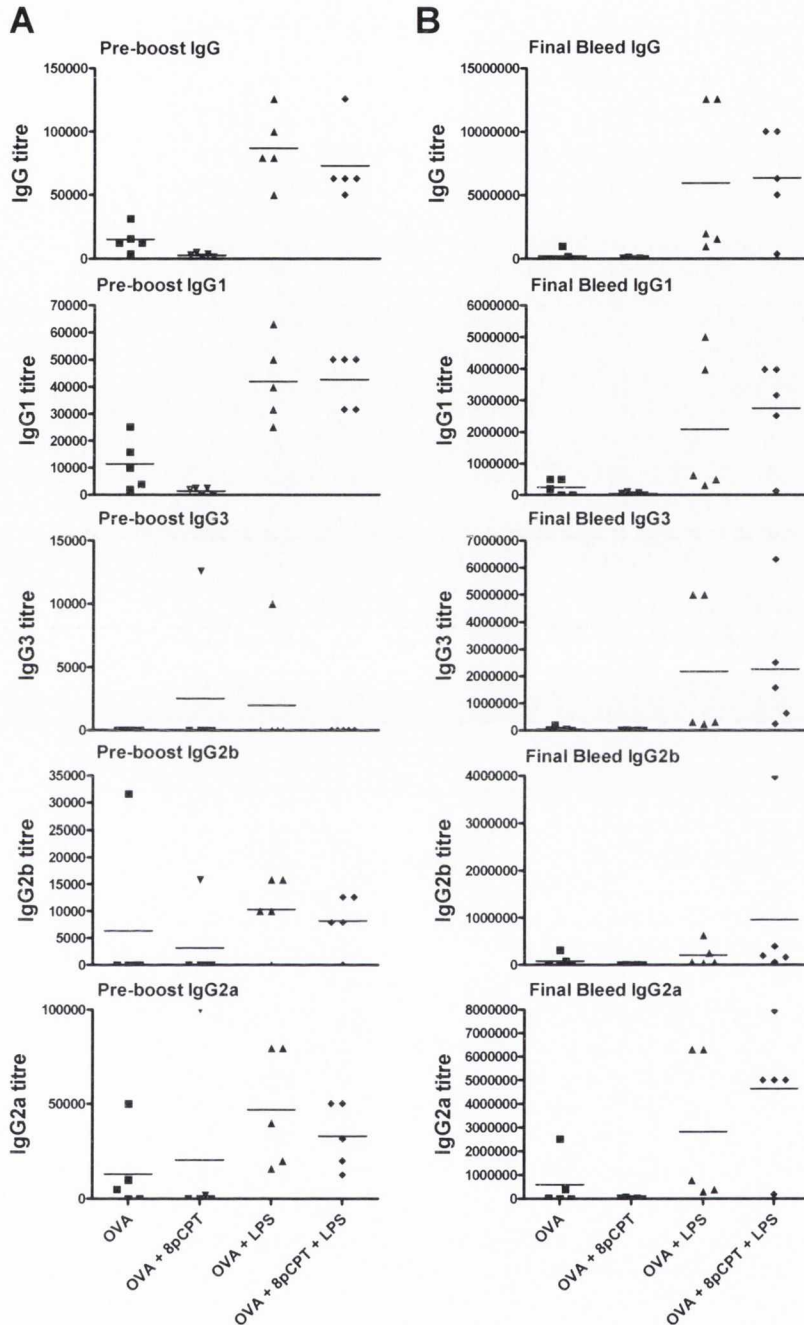


Figure 5.16: Co-injection of the Epac activator 8pCPT with OVA does not enhance antigen-specific antibody responses. BALB/c mice were immunised i.p. with PBS, OVA either alone or with 0.1mmol of the selective Epac activator (8pCPT), LPS (10 μ g/mouse) or 8pCPT with LPS, followed by an identical boost 21 days later. Seven days later mice were sacrificed. Blood serum was analysed for pre-boost (A) and final antibody titres (B). A total of 35 mice with 5 mice per treatment group were used in this experiment.

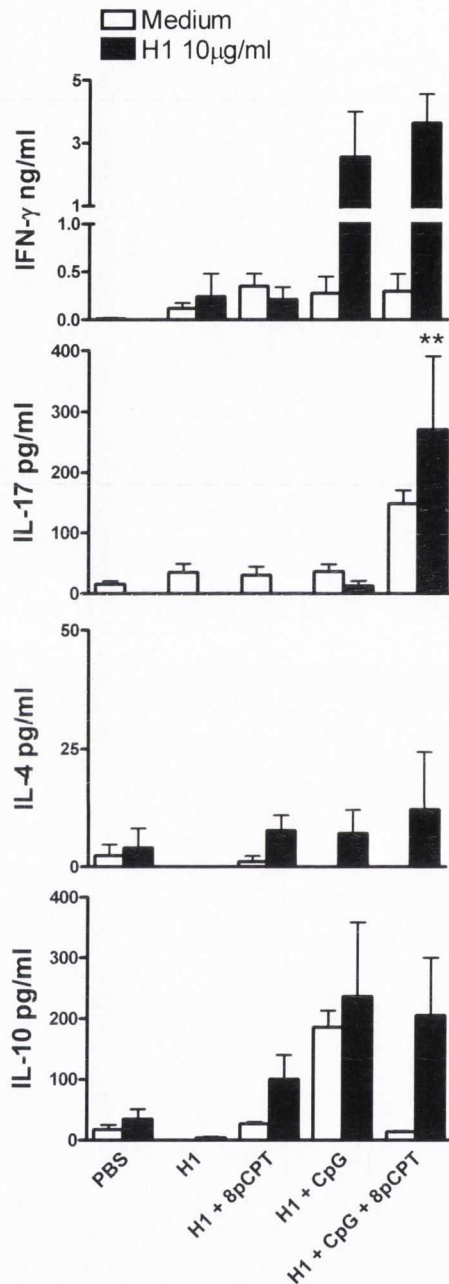


Figure 5.17: Co-injection of H1 with the Epac activator 8pCPT enhances antigen-specific splenic IL-17 and IL-10 responses. C57BL/6 mice were immunised i.p. with PBS, H1 antigen either alone or with 0.1mmol of the selective Epac activator (8pCPT), CpG (10μg/mouse) or 8pCPT with CpG, followed by an identical boost 21 days later. Seven days later mice were sacrificed. The spleen cells were isolated and plated with H1 antigen (10μg/ml). After 3 days the cytokine concentrations in the supernatant were determined by ELISA. Significant differences between H1- and CpG-injected mice and mice injected with H1, CpG and 8pCPT are defined as $p < 0.01$ (**). A total of 35 mice with 5 mice per treatment group were used in this experiment.

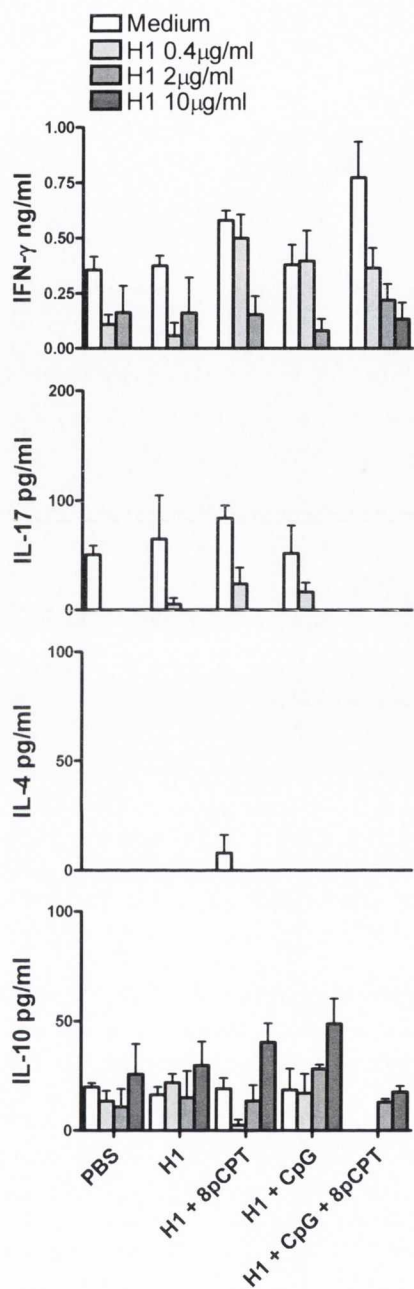


Figure 5.18: Co-injection of the Epac activator 8pCPT with H1 induces a non-specific increase in IFN- γ production by mediastinal lymph node cells. C57BL/6 mice were immunised i.p. with PBS, H1 antigen either alone or with 0.1mmol of the selective Epac activator (8pCPT), CpG (10 μ g/mouse) or 8pCPT with CpG, followed by an identical boost 21 days later. Seven days later mice were sacrificed. The mediastinal lymph node cells were isolated and plated with H1 antigen (0.4, 2 or 10 μ g/ml). After 3 days the cytokine concentrations in the supernatant were determined by ELISA. A total of 35 mice with 5 mice per treatment group were used in this experiment.

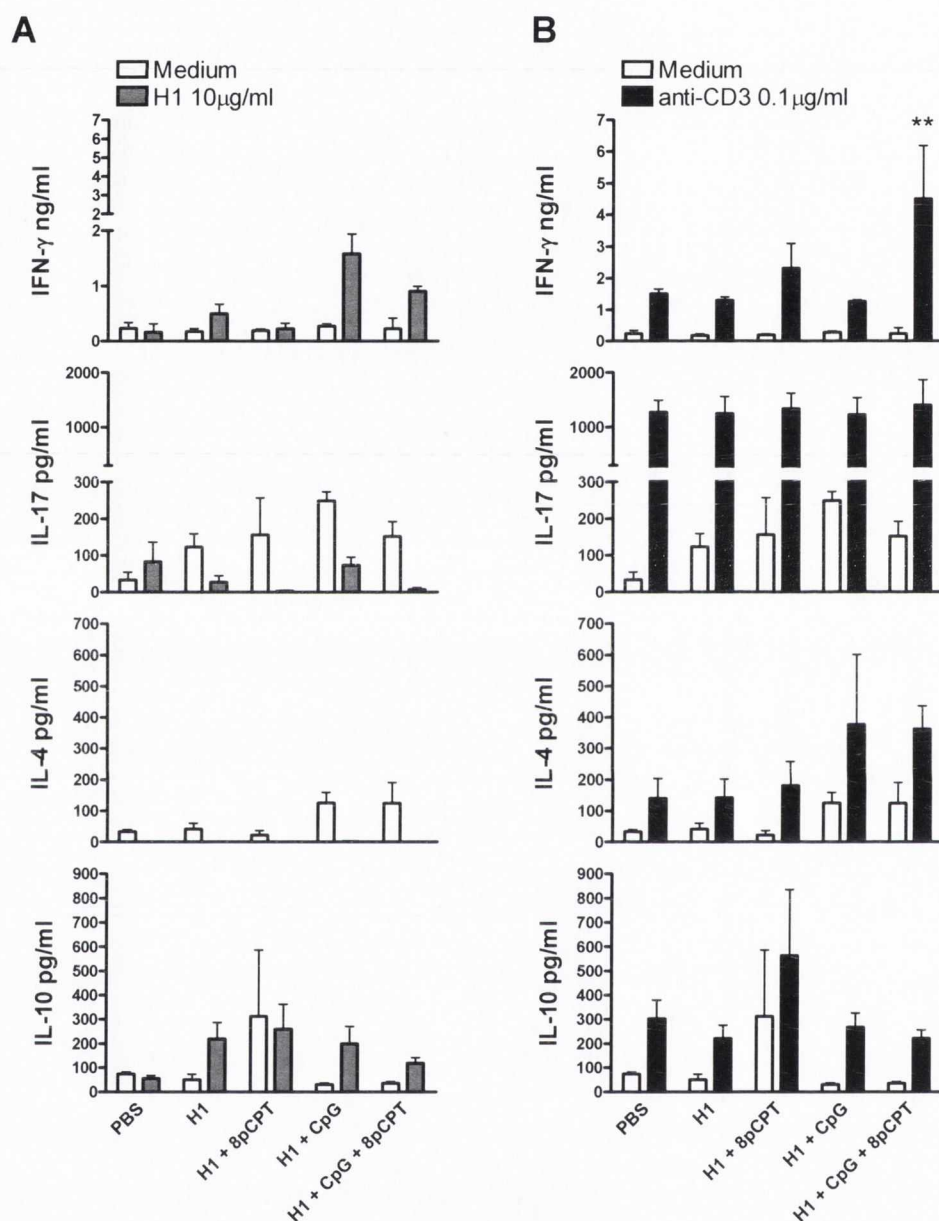


Figure 5.19: Co-injection of the Epac activator 8pCPT with H1 does not enhance antigen-specific cytokine production by peritoneal cells. C57BL/6 mice were immunised i.p. with PBS, H1 antigen either alone or with 0.1mmol of the selective Epac activator (8pCPT), CpG (10 μg/mouse) or 8pCPT with CpG, followed by an identical boost 21 days later. Seven days later mice were sacrificed. The peritoneal cells were isolated and plated on medium, H1 antigen (10 μg/ml) (A) or anti-CD3 (0.1 μg/ml) (B). After 3 days the cytokine concentrations in the supernatant were determined by ELISA. Significant differences between H1- and CpG-injected mice and mice injected with H1, CpG and 8pCPT are defined as $p < 0.01$ (**). A total of 35 mice with 5 mice per treatment group were used in this experiment.

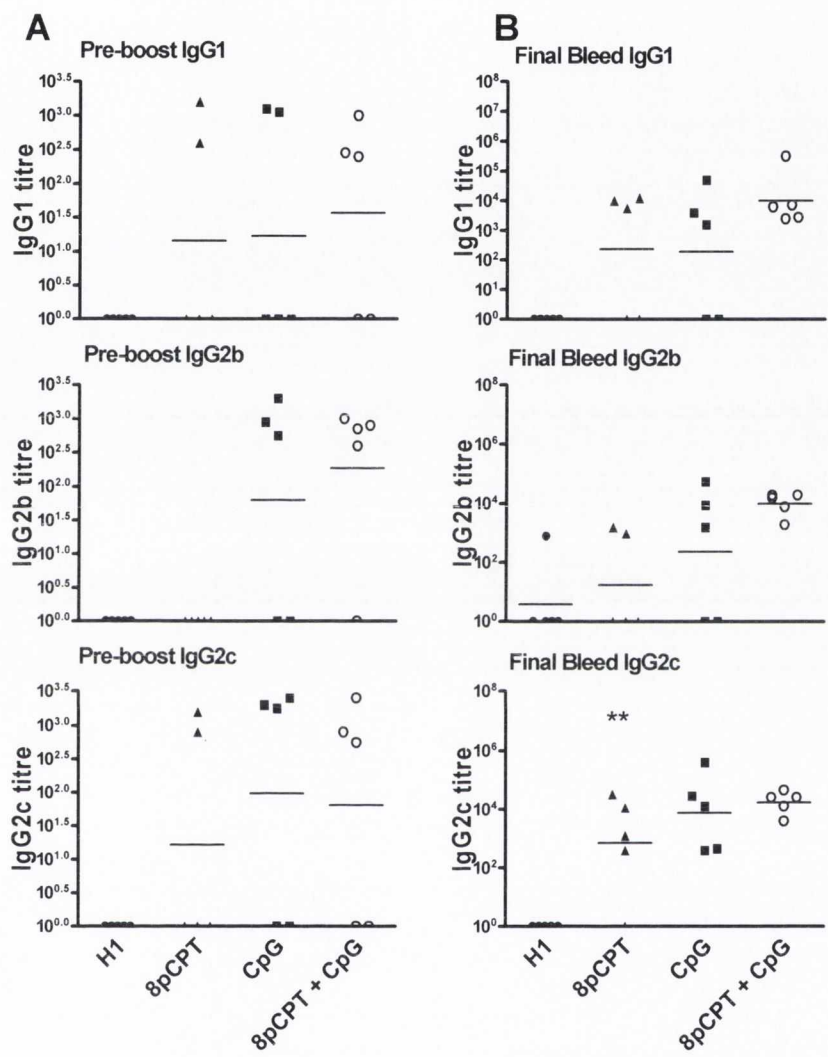


Figure 5.20: Vaccination with H1 in the presence of 8pCPT induces a significantly higher antigen-specific IgG2c response than vaccination with H1 alone. C57BL/6 mice were immunised i.p. with PBS, H1 antigen (2µg/mouse), 8pCPT (0.1mmol), CpG (10µg/mouse) or 8pCPT with CpG, followed by an identical boost 21 days later. Seven days later the mice were sacrificed, and day 20 and day 28 sera were assayed and antigen-specific IgG1, IgG2b and IgG2c antibody titres determined. Shown are the pre-boost antibody titres (A) and the post-boost antibody titres (B). Significant differences between H1-injected mice and mice injected with H1 and 8pCPT are defined as $p < 0.01$ (**). A total of 35 mice with 5 mice per treatment group were used in this experiment.

Discussion

In chapter 3, Epac activation was shown to enhance production of the Th1-promoting cytokine IL-12 by DCs, while in chapter 4 the ability of Epac activation to promote IFN- γ production by splenocytes was demonstrated. These results suggest that activation of the Epac pathway may have the potential to promote IFN- γ producing T cell responses.

CT increases cAMP levels and is a potent mucosal vaccine adjuvant, which has been shown to induce Th2 responses in systemic and mucosal tissues [404]. CT enhances Th2 responses while inhibiting Th1 responses, as mentioned previously, where mice given CT suppressed LPS-induced IL-12p40 and IFN- γ in the serum *in vivo* [404]. As outlined in chapter 1, many hormones and neurotransmitters bind to GPCRs on the cell surface and act to enhance or suppress cAMP production. Adenosine, which upon binding to its receptor A(2A), elevates intracellular cAMP, has been found to modulate effector T cells *in vivo*. It was found that upon ligation of A(2A) CD4⁺ and CD8⁺ antigen-specific cell-proliferation was reduced [407]. Addition of IL-2 *in vivo* reversed the agonist-mediated T cell proliferation inhibition. It has been found that PDE inhibitors (which elevate intracellular cAMP concentrations) suppress macrophage activation and nitric oxide (NO) production *in vitro* and *in vivo* [408]. *In vitro* activation of the β 2-adrenoreceptor increased cAMP levels and led to inhibition of IL-12 production by LPS-stimulated human monocytes and CD40-stimulated DCs [409]. Activation of this receptor was also found to inhibit development of Th1-type cells, but promoted Th2 differentiation, and *in vivo* stimulation of the β 2-adrenoreceptor resulted in decreased IL-12 production by LPS-stimulated blood lymphocytes [409]. PKA is a negative regulator of T cell function, mediated primarily by its control of IL-2 expression in T cells via the transcription factor NF-AT [410]. It has been shown that cAMP elevation via the PKA pathway induces IL-4 production in naïve T cells and promotes a Th2 response *in vivo* [411]. Overall, cAMP signalling via PKA suppresses T cell proliferation while inducing IL-4 production *in vivo*.

The effects of Epac activation on immune responses *in vivo* have not been investigated previously so an initial study was performed to compare the effects of

injecting the PKA activator and the Epac activator. A five-day protocol was used, which allowed analysis of innate responses, and, as there were five separate injections of the activators, it gave the optimal circumstances for detection of changes in the immune response triggered by chronic exposure to the activators. It was found that the Epac and PKA activators had differential effects on cytokine production by the draining lymph nodes. The Epac activator promoted IFN- γ production, while the PKA activator promoted IL-4 production. The induction of IFN- γ by the mediastinal lymph node cells upon injection of the Epac activator mirrors the effects of the Epac-activator on IFN- γ production by spleen cells *in vitro*, and correlates with its ability of Epac activation to drive production of the Th1 cytokine IL-12 by DCs, as described in chapter 3. In chapter 3 it was also established that PKA activation inhibited HK bacteria-induced IL-12 production by DCs. This reduction in the IFN- γ inducing cytokine, IL-12, correlates with the absence of IFN- γ production, as observed here, following PKA activation *in vivo*. Data presented in chapter 4 demonstrated that PKA activation in spleen cells *in vitro* inhibited IL-4 production, which is in contrast to the effects of PKA activation *in vivo*. However, the effects of PKA activation *in vivo* may allow a more accurate representation of the systemic effects of PKA activation.

In chapter 4 it was established that the Epac activator enhanced IFN- γ production by splenic CD8⁺ T cells. Injecting the Epac activator also increased IFN- γ production by spleen cells, which upon further investigation, was found to be produced by CD8⁺ T, NK and NKT cells. At the site of 8pCPT injection CD8⁺ T cells, but not NK or NKT cells, were found to have enhanced IFN- γ production. A proportion of these CD8⁺IFN- γ ⁺ cells were found to be IL-17⁺ also, indicating that the proinflammatory Th17 characteristic cytokine IL-17, is being produced by these cells also. The production of IFN- γ by CD8⁺ cells mirrors the effects seen *in vitro* after Epac activation in the spleen (chapter 4). However, while splenic NK and NKT cells were found to secrete enhanced IFN- γ *in vitro* upon Epac activation, the principal cells responsible for the induction of IFN- γ following Epac activation *in vitro* were CD8⁺ T cells. This could potentially indicate that the CD8⁺ cells are the prime producers of IFN- γ at the site of injection following injection of the Epac activator.

During the non-antigen-specific early phase of infection, IFN- γ is believed to be primarily secreted by NK and NKT cells in response to pathogen-derived inflammatory mediators, but it has been reported that CD8⁺ cells are capable of producing a large proportion of IFN- γ (spleen – 30% and lymph nodes – 70%) in response to PAMP stimulation *in vivo* [390]. In this paper, three hours after LPS injection, IFN- γ production was found in splenic NK, NKT and a significant fraction of CD8⁺ T cells [390]. IFN- γ was also detected in memory CD8⁺ T cells from mice injected with type 1 IFN or poly(IC) (a TLR3 agonist) [390]. These results indicate that memory T cells may add to innate immunity by providing a non-antigen-specific source of IFN- γ . This is very similar to what is observed in chapter 4 upon Epac activation in the spleen *in vitro*, where memory CD8⁺ T cells are responsible for the Epac-mediated IFN- γ increase. It would be worthwhile to determine if the same cell type is responsible for the IFN- γ increase following Epac activation *in vivo* as was determined *in vitro*.

It has been demonstrated that human CD8⁺ T cells are affected by elevation of cAMP and PKA activation. Upon cAMP elevation and PKA activation *in vitro*, using DB-cAMP or PGE₂, in CD8⁺ T cells isolated from PBMCs, both IL-17 and IFN- γ mRNA was suppressed and IL-10 mRNA was enhanced [412]. These results indicate that PKA is involved in the switch of the cytokine production profile of CD8⁺ T cells towards IL-4 production and inhibition of IL-17 production. This data, along with the results shown here, indicate that PKA and Epac have differential effects with regard to the ultimate response put into effect by the body to induce clearance of a specific pathogen. Epac activation enhances the production of the Th1 characteristic cytokine IFN- γ from CD8⁺ T cells, while PKA promotes IL-4 production.

The *in vitro* spleen experiments in chapter 4 indicate that the Epac-activator-induced IFN- γ response is not dependent on NK or NKT cells. These cells act to amplify the IFN- γ response but are not responsible for the increase in IFN- γ production due to Epac activation. The enhancement of IFN- γ production after Epac activation, by NK and NKT as well as CD8⁺ cells *in vivo*, could be explained by the fact that the cells have been exposed to the activator over five days, and that even if the initial IFN- γ response came from a specific cell type (i.e. CD8⁺ cells), after five days other cells

may amplify the IFN- γ response. NK cells are known to be major producers of cytokines such as IFN- γ [413]. They have been shown to be involved in the early control of virus infection, in tumour immunosurveillance, and in the regulation of immune responses [414]. The production of IFN- γ by NK cells helps to shape T cell responses in lymph nodes, possibly by a direct interaction between naïve T cells and NK cells migrating to secondary lymphoid compartments from inflamed peripheral tissues and by an indirect effect on DCs [415]. This indicates that NK cells are known to enhance IFN- γ production, which is what is seen here following Epac activation *in vivo*. Interestingly, co-injection of LPS with the Epac activator did not enhance IFN- γ production by lymphocytes, but did enhance IFN- γ expression in NK and NKT cells, indicating that the second stimulus, i.e. of the TLR ligand, enhanced the Epac-induced IFN- γ response by NK and NKT cells, but not by CD8⁺ cells.

As outlined in chapter 1, Epac has been linked with cell migration via signalling through Rap1 [416]. This could indicate that Epac activation *in vivo* may lead to alterations in cell migration or recruitment following injection of the Epac activator.

A single injection of the Epac activator increased the percentages of splenic CD4⁺, CD8⁺ and CD3⁺ γ δ TCR⁺ cells while it did not affect NK and NKT cells in the spleen. It was found that the numbers of DCs, NK and NKT cells were increased in the spleens of mice injected for five days with the Epac activator. This suggests that initially (i.e. 24hours after Epac activator injection) an increase in the percentages of lymphocytes are seen in the spleen, whereas after 5 days the increased cell populations are different and include DCs, NK and NKT cells. The increased lymphocyte numbers are no longer apparent at this timepoint, indicating that it is the percentages of lymphocytes that are increased first in response to injection with the Epac agonist.

Priming of CD8⁺ T cells by DCs has been described in three stages; the activation phase where there is a stable interaction for the first 8hours in the lymph node between the DC and CD8⁺ T cell, the second phase which lasts for the next 12hours where T cells have a long-lasting stable interaction with the DC, and begin to secrete

IL-2 and IFN- γ , and the third phase is where there is high motility and rapid proliferation of the T cells and occurs 2 days later [417].

DCs, as described in chapter 1 and 3, are important APCs, capable of patrolling to and from the site of injection [85]. NK and NKT cells are prime producers of IFN- γ , and an increase in their cell numbers could indicate an enhancement of their response to the Epac activator *in vivo*. The increase in these splenic populations in response to injection with the Epac activator could indicate an increase in proliferation of these cell types, or that migration was enhanced. Further studies could be carried out to investigate this further: for example, measurement of cell proliferation *in vivo* using CFSE dilution in conjunction with flow cytometry [418] or measurement of migration of cell types *in vivo* using various cell imaging techniques.

A key objective of this work was to determine if the Epac activator could be used as an adjuvant to promote antigen-specific Th1 and CD8 responses, since the *in vitro* and innate *in vivo* data indicate that Epac activation predominantly enhances IFN- γ production.

Alum is the best characterised and predominantly-used adjuvant in vaccine formulations. However, its primary effect is to promote a Th2-type response. It has long been known that a Th1-type response is needed for vaccination against diseases such as TB and malaria. Initially OVA was used as a test antigen with the Epac activator as the adjuvant. However, no enhancement of antigen-specific cytokine or antibody responses was detected following injection of OVA with 8pCPT as adjuvant. OVA alone was found to induce production of antigen-specific IL-4 production. While there was no enhancement of antigen-specific cytokine or antibody responses in response to Epac activation, immunisation with 8pCPT was found to inhibit the OVA-induced antigen-specific IL-4 production. The *in vitro* spleen experiments in chapter 4 did not show 8pCPT suppression of IL-4 production in the spleen. However, a single injection of 8pCPT suppressed IL-4 production, in both the mediastinal lymph nodes and the spleen *in vivo*, while IFN- γ production was enhanced. This indicates that not only is the Epac activator promoting IFN- γ and IL-17 production, it is also suppressing IL-4 production *in vivo*. Suppression of IL-4 was not seen upon

Epac activation in spleen cells *in vitro* (Chapter 4), which may reflect the limitations of the *in vitro* model and the effects of Epac activation *in vivo*, may allow the systemic effects of Epac activation to be observed more accurately.

These results suggest that 8pCPT can inhibit antigen-specific Th2 responses, while mediating its enhancing effects on IFN- γ production. It has been reported that mature mouse CD8⁺ cells, activated in the presence of IL-4 *in vitro*, lose CD8 receptor expression, cytolytic function and IFN- γ production capability [419]. As outlined in chapter 1, there is extensive crosstalk between Th cell lineages and the characteristic cytokines produced to mediate their effects. There is positive feedback by cytokines involved in Th differentiation and cross inhibition of some lineages by other lineages. Both IFN- γ and IL-4 are known to mutually suppress each other's signalling [420]. *In vivo* experiments have shown that endogenous IFN- γ levels offset the IL-4 induction of Th2 cytokines, the downregulation of CD8 expression, and the anti-tumour response in CD8⁺ T cells [420]. TGF- β suppresses both Th1 and Th2 differentiation and both IL-4 and IFN- γ inhibit Th17 differentiation. Therefore, while these OVA experiments did not show enhancement of antigen-specific Th1 responses following immunisation with 8pCPT, this suppression of an antigen-specific Th2 response bodes well for development of 8pCPT as a vaccine adjuvant to promote a Th1 response and perhaps a different candidate antigen would be more suitable for testing this hypothesis.

A second antigen was tested to determine the ability of the Epac activator to induce an antigen-specific response. This antigen was derived from *Mycobacterium tuberculosis*, a bacterium that causes TB and requires a Th1-response for clearance. TB is a high priority target for vaccination strategies, due to its high economic cost and high mortality rates in immunosuppressed individuals, including people with HIV. The existing vaccine against TB, bacillus Calmette-Guérin (BCG), can be effective in preventing severe forms of TB in children but has a very limited efficacy against the establishment of latent TB and reactivation, and its predicted protection against pulmonary TB can vary from 0-80% [421]. Also, new multi-drug resistant TB strains are emerging, and with the spread of HIV and its resultant immunosuppressive effects, there is increased mortality from TB infection. The need for new TB vaccines has led to much research and development in order to engineer new vaccines, which

are capable of either boosting the current BCG vaccine or to replace BCG altogether. The new vaccines being developed, that are in clinical trials, are designed to prevent infection and are based on antigens that are recognised in infected individuals, such as the components from the ESAT-6 (early secretory antigenic target) or the Ag85 family [422]. It has been shown that vaccination with a fusion protein consisting of Ag85B and ESAT6 (Hybrid1/H1) promoted a strong immune response, which is highly protective against TB in the mouse and in nonhuman primate models, and this fusion protein is currently in clinical trials [423]. This fusion protein was used in this study to investigate the effects of the Epac activator on the immune response. Use of this antigen should allow analysis of the effects of 8pCPT on the development of antigen-specific responses.

Co-injection of the Epac activator and the H1 antigen increased IFN- γ production by mediastinal lymph node and peritoneal cells. However, this IFN- γ was not antigen-specific. The Epac activator induced increased antigen-specific IL-17 production in the spleen, but there was no induction of IL-4. These results indicate that two injections of the Epac activator were able to induce predominantly Th1- and Th17-type responses, but not a Th2-type response, in a non-antigen-specific manner. Further studies would need to be carried out to fully investigate the potential for Epac activation to enhance the H1-specific response but these results are promising. These results are similar to the *in vitro* spleen results seen in chapter 4 and the *in vivo* data seen in this chapter, where Epac activation induces IFN- γ and IL-17 production, but does not induce IL-4 production.

The promotion of antigen-specific humoral immunity is a priority for most vaccines. Th2 responses, i.e. high IL-4 and little IFN- γ production results in IgG1 and IgE secreting cells with little IgG2a [424]. In contrast, Th1 responses, with high IFN- γ , results in IgG2a production and not much IgG1 or IgE production [425]. Thus, the IgG2a and IgG2c antibody subtypes are characteristic of a Th1 response (C57BL/6 mice do not carry the IgG2a isotype, but have IgG2c instead) [426]. Immunisation of the Epac activator with H1 induced significantly increased IgG2c production. This indicates that the Epac activator alone is capable of inducing an antigen-specific Th1-type antibody response. While there was no significant antibody response to the OVA antigen, four out of five mice produced increased IgG2a after immunisation with the

Epac activator, OVA and LPS, compared to two out of five mice after immunization with OVA and LPS. The main difference between these two studies is the antigen used. OVA immunisation alone promoted IL-4 production, whereas the H1 antigen did not. The different antigens used could therefore account for the difference in the antibody profile. This shows that there is a strong influence of the antigen type on the response generated.

Overall these studies show that the Epac activator alone can promote IFN- γ secretion by CD8⁺ T cells, non-antigen-specific IFN- γ production and antigen-specific IgG2c antibody production. Further studies could be carried out in order to fully characterise the 8pCPT-mediated effects. A dose-response would be useful in order to determine if alterations in the 8pCPT concentration, or the antigen concentration, could induce stronger responses. This dose response experiment would be important to perform as the Epac-mediated results could be altered depending on dose, and the optimal dose would be necessary to be determined. The route of administration is an important area to characterise, as this can make a difference in the type of response induced. The draining lymph node would change depending on the placement of the initial injection potentially providing different responding cells. In addition, the cell profile at the injection site changes depending on where the injection happens, potentially leading to a different response which should be fully explored. Co-injection of the TLR9 agonist CpG, while a known inducer of IFN- γ and Th1 responses itself [427], with 8pCPT did not result in a synergistic enhancement of IFN- γ production; it would be interesting to study whether addition of another type of PAMP could synergise with 8pCPT to induce a stronger Th1-type response. A time-course experiment to track changes in cell proliferation/migration would be interesting, or analysis of integrin expression on the cells producing Epac-activator-enhanced IFN- γ .

Chapter 6

General discussion

Adjuvants are substances that when added to vaccine formulations, along with a specific antigen induce a stronger immune response than the antigen alone [428]. Development of adjuvants capable of promoting a Th1-type response is a priority in TB and malaria vaccine research [13]. It has been shown that cAMP elevation via the PKA pathway induces IL-4 production in naïve T cells, and promotes a Th2 response *in vivo* [411]. In this project Epac, a downstream component of the cAMP pathway, was investigated for its immunomodulatory potential.

In this study it was found that Epac and PKA exert differential effects on the immune response, where Epac activation using the Epac activator 8pCPT induced primarily Th1-type responses. However, there are still some questions to be addressed. The cAMP pathway is complicated and diverse, and the downstream effectors Epac and PKA exert varied effects depending on the cell type. As cAMP is capable of activating both PKA and Epac in cells, why does a signal to increase intracellular cAMP levels exert such different effects, and what drives cAMP towards the PKA pathway as opposed to Epac?

One explanation for the diversity in cAMP-mediated effects could be due to the variation in the cAMP pathway constituents themselves. These include the AC enzyme, PDEs, and the Epac and PKA proteins, all of which exist in more than one form. Epac has two isoforms, Epac1 and Epac2, and both these isoforms are present in most tissues, although with different expression levels [271]. There are several PKA isozymes in the cell with distinct expression patterns, and these contain C and R subunits, which each have different subunits and splice variants and have characteristic functions [250]. Levels of expression of the different PKA subunits are regulated by hormones acting through GPCRs, mitogenic signals acting through receptors associated with protein tyrosine kinases as well as by steroid hormones [254]. The AC enzyme responsible for cAMP generation has several different isoforms. In mammals there are nine membrane-bound ACs, with a tenth ‘soluble’ form [225]. These AC isoforms have distinct regulation, are ubiquitously expressed, are crucial for many biological processes and all have individual and clearly distinct physiological functions. There have been eleven families of PDEs identified, but there are many isoforms with different expression patterns and functions in mammalian cells [244]. The best-known ligands that increase cAMP levels in the cell are

adrenaline, noradrenaline, histamine, serotonin, and the cyclooxygenase (COX)-2-derived PGE₂, but there are many other ligands capable of altering cAMP levels within the cell. By observing the variation in these components alone, it is clear that the cAMP signalling pathway is a complex network. It suggests that the cAMP pathway is under elaborate control and exerts diverse effects. Elevation of cAMP in one cell type can exert very different effects to elevation in another cell type, which is reflected in the various cell processes, associated with cAMP signalling as outlined in chapter 1.

A further level of regulation could result from different localisation of the cAMP pathway constituents within the cell. Originally it was thought that cAMP was evenly distributed and acted throughout the cell, but it is now believed that cAMP acts locally at sites within cells where cAMP-effector molecules and either ACs, or PDEs are co-localised. PDEs contribute to the establishment of local gradients of cAMP by being localised to subcellular compartments and by being recruited into multi-protein signalling complexes. PDEs establish local pools of cAMP close to the effector molecules [257]. Various studies have shown that PKA can be targeted to selective regions of cells and that cAMP can act selectively at these sites by accessing these targeted effectors [256]. Therefore cAMP changes can occur in microdomains within the cell. This could account for the differential effects of cAMP on Epac or PKA. If cAMP levels are elevated in the vicinity of either of these downstream effectors, one of these could be activated while the other is not i.e. the availability of cAMP to its effectors is regulated. The signalling constituents can be sequestered to a specific subcellular environment, by AKAP complexes, thus ensuring that upon activation, they are near their relevant targets. AKAPs play a central role in the resolution of cAMP signalling, shaping both downstream and upstream events in the cAMP pathway and providing a mechanism by which this second messenger can have localised effects [321]. The correct intracellular targeting of Epac, PKA, PDEs and AC enzymes, may be critical in determining the response of cells to elevations in intracellular cAMP. This suggests that cAMP signalling is spatially and temporally regulated by diverse anchoring mechanisms, which control specific functions of the cAMP effectors by recruitment to distinct subcellular locations.

While the complexity of localisation, expression and distribution of the cAMP pathway constituents could explain why PKA and Epac activation exert such different effects, it does lead to the question as to whether both PKA and Epac are activated at the same time upon cAMP elevation or activated individually? It could be that these proteins are under tight regulation and sequestered away from the cAMP pools, and the type of signal elevating cAMP decides which is activated. However the question remains as to when PKA or Epac are activated after cAMP signalling? The specific activators for PKA and Epac both work when added directly to the cell indicating that the Epac and PKA proteins are available for activation. It could be that both PKA and Epac are activated at the same time and only the effects of one pathway are manifested. The immunomodulatory effects following cAMP elevation observed *in vitro* in this project suggest that it is the PKA effects that are in assent over the Epac-mediated effects. In this project, PKA activation *in vivo* induced IL-4 secretion while the ability to secrete the Th1 polarising cytokine IL-12 was suppressed, indicating induction of a Th2-type response. In contrast, Epac activation enhanced IFN- γ and IL-12 production while IL-4 expression was suppressed. Thus, activation of Epac and PKA exert strikingly different effects on Th1 and Th2 polarisation.

Upon PKA binding to cAMP, phosphorylation occurs and the two regulatory and two catalytic subunits, which then readily diffuse throughout the cell, mediate their effects. Phosphorylation is reversible, post-translational modification of a protein, which adds a phosphate group to a protein thus inducing a conformational change in the protein structure [429]. The Epac proteins are GEFs, which act to exchange GDP for GTP on their target protein. The exchange of GTP for GDP on the target protein complex is transient due to rapid binding of GTP, the cellular concentration of which exceeds that of GDP several-fold. Numerous factors can influence the kinetics of G protein activation, including ligand, receptor and G protein affinities, intracellular protein concentrations and receptor deactivation kinetics. The exact kinetics of PKA and Epac activation of their downstream effectors remains to be fully described. There are several *in vitro* methods to determine PKA and Epac pathway activation, but *in vivo* outcomes are much more difficult to measure. There have been several methods outlined in order to investigate phosphorylation signalling networks *in vivo*, including high-throughput mass spectroscopy to determine their temporal and

subcellular location *in vivo*, and computational methods used to construct phosphorylation signalling networks *in vivo* [430, 431]. These methods could be used to outline the kinetics and locations of PKA and Epac activation *in vivo*.

Epac activation was shown in this project to promote IFN- γ production from splenic CD8⁺ T cells, specifically CD8⁺ effector memory T cells (CCR7⁻CD62L⁺). The effector memory cells reside at the site of inflammation. But why is this IFN- γ induction specific to this cell type? There are many cell types known as major producers of IFN- γ , including NK, NKT cells and Th1-type CD4⁺ T cells [143, , 413]. NK cells are known to be major producers of IFN- γ [413], and have been shown to be involved in the early control of virus infection, in tumour immunosurveillance, and in the regulation of immune responses [414]. During the non-antigen-specific early phase of infection, IFN- γ is believed to be primarily provided by NK and NKT cells in response to pathogen-derived inflammatory mediators [432]. However, it has been reported that CD8⁺ T cells are capable of producing a large proportion of IFN- γ (spleen – 30% and lymph nodes – 70%) in response to LPS stimulation *in vivo* [390]. Differential Epac protein expression in lymphocytes does not appear to be a selective reason for the IFN- γ production in CD8⁺ T cells, as both Epac1 and Epac2 proteins are expressed in all these cell types, and while Epac1 is expressed more abundantly, Epac2 is still present and there does not appear to be differential expression of Epac proteins between CD8⁺ T cells and CD4⁺ or NK cells. It is possible that CD8⁺ cells are more responsive to Epac activation, but there is currently no evidence to confirm this. The effector memory CD8⁺ T cells are supposed to respond and act at the site of inflammation, and they may respond rapidly to Epac activation by producing IFN- γ following injection. The data presented in this project does not deal with whether it is the effector memory CD8⁺ T cell subtype responding *in vivo* as they do *in vitro* to Epac activation, and this would be an interesting avenue to investigate further in the future.

Epac and its downstream effector Rap are known to regulate integrin expression on the cell surface, particularly integrins involved in migration of cell types [370]. Specific Epac activation in CD8⁺ T cells could modulate integrin expression on these cells and therefore change the signalling profile. In chapter 4, it was shown that

CD62L expression was suppressed following Epac activation. CD62L is a selectin necessary for the entry into lymph nodes, and its loss along with absence of CCR7 expression would ensure these cells remain at the site of inflammation. This cell-surface adhesion molecule may not be the only one affected after Epac activation, so further study would need to be carried out in order to investigate the expression profile of adhesion molecules and chemokine receptors on CD8⁺ T cells after Epac activation, and comparison to the other cells at the site of injection.

One leading question is to why the cAMP pathway would have as its downstream effectors two proteins capable of inducing such divergent immune responses. Several pathogens encode toxins, which modulate cAMP signalling, and it could be that this may represent an immune evasion strategy by the pathogens. Cholera toxin (CT) from the bacterium *Vibrio cholerae*, pertussis toxin (PT) from the bacterium *Bordetella pertussis* and the *E. coli* heat-labile toxin (LT) are all known to alter cAMP levels and therefore modulate the cAMP signalling pathway. These toxins have been studied extensively, and have been found to elicit some immunostimulatory effects. *In vivo*, CT is a potent adjuvant capable of inhibiting Th1 responses while enhancing mucosal Th2 and Th17 responses [404] and PT has been reported to induce antigen-specific Th1 and Th2 responses *in vivo* [433], as well as being used to promote experimental allergic encephalomyelitis (EAE), the mouse model of multiple sclerosis, in mice [434]. Even though these toxins show clear immunostimulatory effects, the modulation of cAMP signalling by the pathogens must be in some way beneficial to the pathogen. The role of Epac in these toxin-mediated effects would be worthwhile to study, and it could indicate why these two divergent immune pathways exist downstream of cAMP signalling.

PKA is known to negatively regulate T cell function by controlling IL-2 expression via the transcription factor NF-AT [410], but at the same time promoting IL-4 production *in vivo*. Interaction of the TCR with the peptide-loaded MHC molecules on the APC activates T cells to proliferate and to produce cytokines, including IL-2 [343]. This leads to a complex signalling cascade, which involves hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). DAG activates PKC and IP₃ induces Ca²⁺ mobilisation [435]. TCR signalling is already known to be under cAMP and PKA regulation. As

described in chapter 1, upon activation of the TCR-CD3 complex in T cells, there is an initial peak of cAMP and PKA activity seen that could act as an acute negative modulator and a negative feedback regulator of signalling through the TCR/CD3 [254]. Epac is involved in vesicle release in pancreatic β -cells and there has been some evidence showing that Rap1 may directly link Epac signalling to Ca^{2+} mobilisation through direct protein-protein interactions with the SERCA Ca^{2+} ATPase in the endoplasmic reticulum of the heart [411]. It would be interesting to determine if Ca^{2+} levels are altered in CD8^+ T cells following Epac activation. Future experiments could also assess IL-2 secretion following Epac activation and compare this to the induction of IFN- γ .

In chapter 5, it was shown that the Epac activator IFN- γ production and IgG2c antibody production in response to the mycobacterial H1 antigen but not in response to OVA. This demonstrates that the antigen used for testing exerts a large effect on the response generated. Further studies should be carried out to determine the optimal formulation of the Epac activator in adjuvant formulations, based on these encouraging preliminary data. The H1 antigen could be used again with some of the parameters of the vaccination experiment modified. Alterations in the Epac activator concentration may be beneficial, and potentially a different TLR or NLR agonist could be used. When these parameters were optimised a study into the protective effects of vaccination against *Mycobacterium tuberculosis* would be warranted [436]. In experiments not presented here, immunisation of 8pCPT and chitosan, with and without CpG, enhanced H1-specific IgG2c production as well as increased IFN- γ and IL-17, but not IL-4, production. Chitosan is known to promote Th1 responses [437] and its ability to enhance 8pCPT-mediated effects supports the idea that an alternate formulation of the Epac activator could lead to improved adjuvant responses. It would also be instructive to assess different methods of Epac agonist administration. For instance in these experiments, the agonist was solubilised in PBS and directly injected, however it is known that particulate vaccines exert increased immunomodulatory effects and may improve vaccine efficiency. Conjugation of the Epac activator to particles could allow the Epac activator to be maintained at the site of injection and exert its effects for a prolonged period.

The effects of Epac activation outlined here could lead to its use in several beneficial areas. Epac activation could be used as an adjuvant to promote a Th1 IFN- γ producing defence i.e. particularly against intracellular pathogens causing diseases like TB, malaria or HIV. Cancer vaccination studies have shown that the cytokine profile of long-term cancer-survivors have high IFN- γ levels [438] and it has been reported that patients with advanced cancers have reduced activation of IFN signalling pathways [439]. Vaccines against tumour antigens, including the Epac activator as an adjuvant, could lead to a targeted immune response and protection against the tumour. In addition instances where a Th2 response is undesirable, i.e. in allergic responses [440], Epac activation, inducing IFN- γ production and suppressing IL-4 production, could be used as a preventative vaccine. These diverse, important applications of Epac activation show its central role in adjusting the immune response and its potential for therapeutic use.

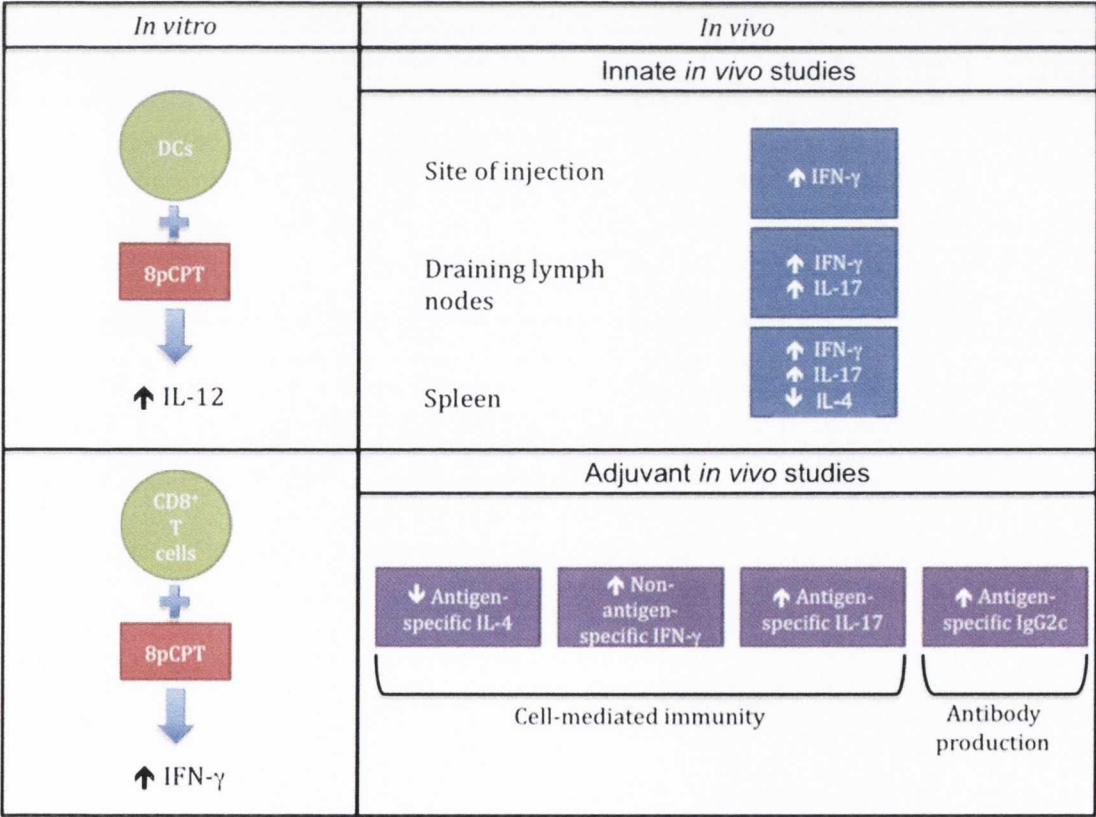


Figure 6.1: Outline of Epac activator 8pCPT-mediated effects *in vitro* and *in vivo*.

Chapter 7

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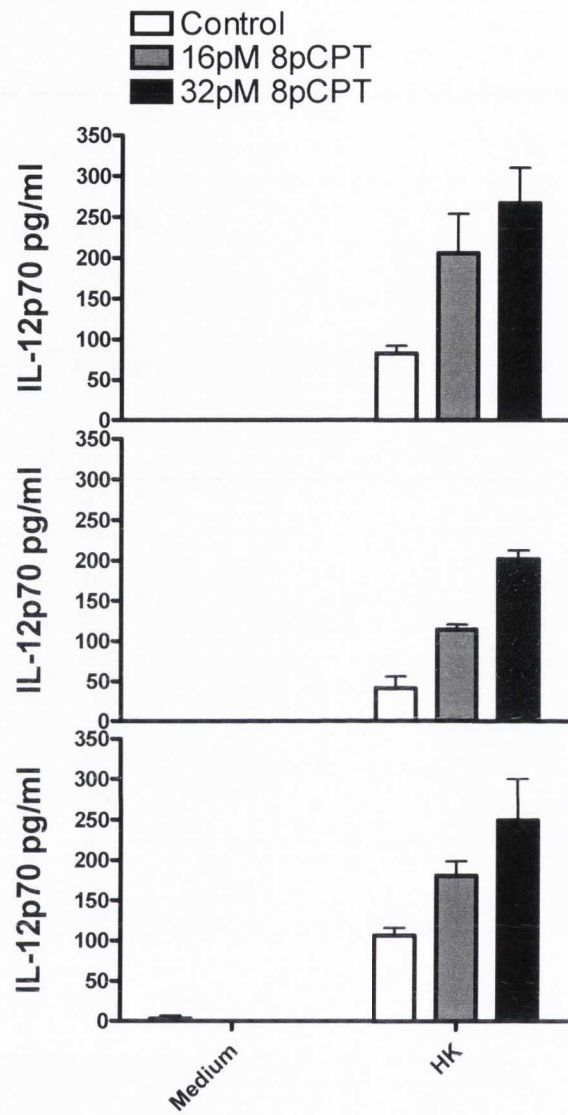
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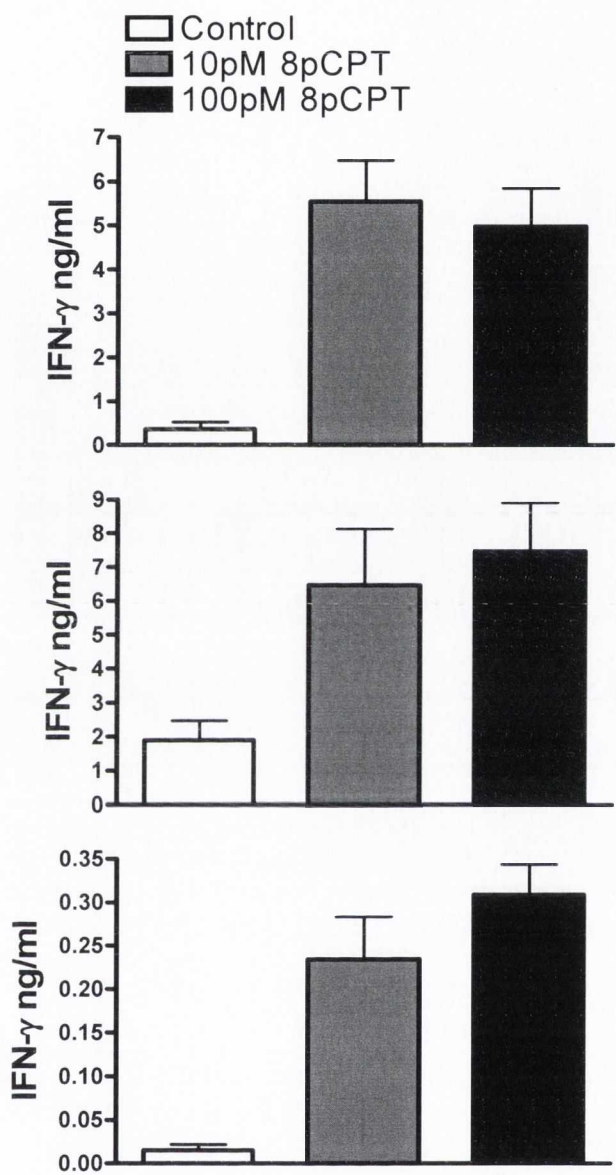
Appendix

(A) DC stimulation			
cAMP elevation + DB-cAMP	↑ MHC class II	↓ CD40	
PKA activation + N6-Bz	↑ CD86	↑ CD80	↓ CD40
Epac activation + 8pCPT	↑ MHC class II		
(B) Co-culture			
+ the PKA activator N6-Bz	↓ IL-12p40	↓ IL-12p70	↓ IL-23
+ the Epac activator 8pCPT	↑ IL-12p40	↑ IL-12p70	↑ IL-23
	↑ CD40	↑ CD86	

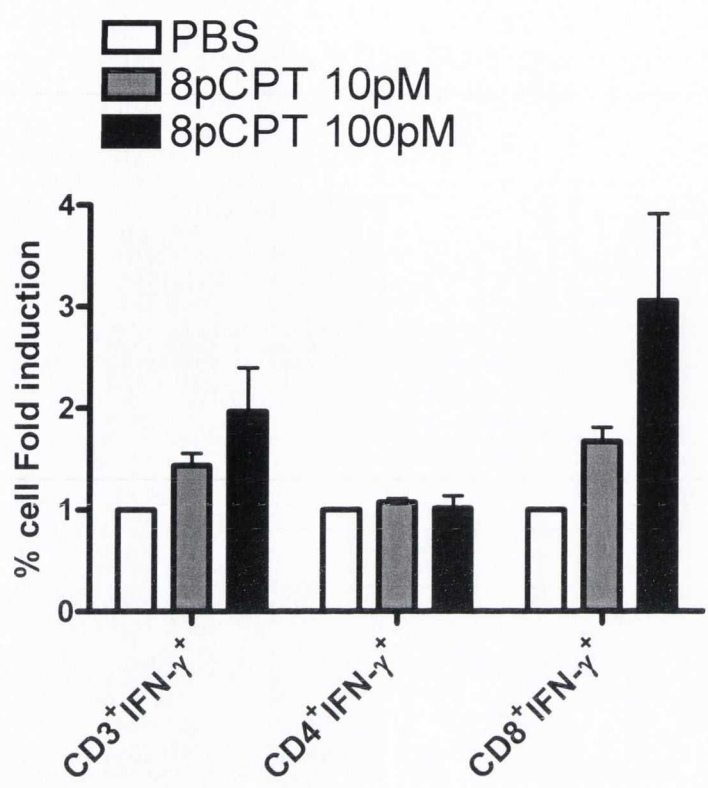
Appendix Figure A: A summary of flow cytometry data presented in Figures 3.10 – 3.19. (A) Shown here is the flow cytometry profile upon DC stimulation with the cAMP analogue DB-cAMP, the PKA activator N6-Bz or the Epac activator 8pCPT. ↑ indicates an increase in cell surface expression while ↓ indicates a decrease. (B) Shown here are the effects of co-culture of DCs with either the PKA activator N6-Bz or the Epac activator 8pCPT. ↑ indicates an increase in cell surface expression or cytokine production while ↓ indicates a decrease.



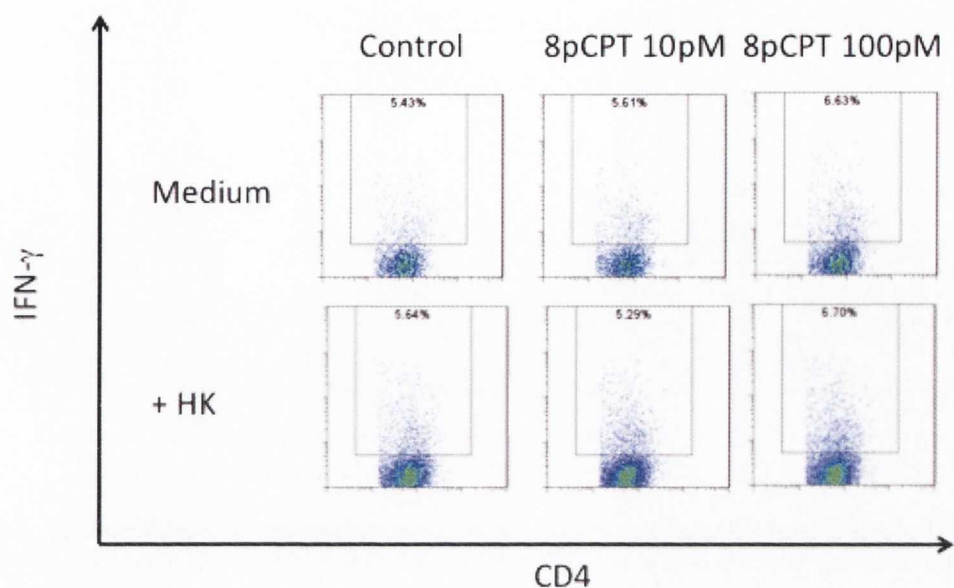
Appendix Figure B: Picomolar concentrations of the Epac activator 8pCPT increases IL-12p70 secretion in DCs. DCs (6.25×10^5 cells/ml) from BALB/c mice were stimulated with the selective Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) or PBS (Control) alone, or for 2 hours prior to stimulation with heat-killed *E. coli* (HK) at a ratio equivalent to 1 bacteria to 1 DC. After 24 hours the cytokine concentrations in the supernatants were determined by ELISA. Each individual graph shows one of three individual experiments showing an increase in IL-12p70 production following Epac activation, as shown in a representative graph in Figure 3.6.



Appendix Figure C: Epac activation promotes IFN- γ production from spleen cells. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 ($0.01 \mu\text{g/ml}$) and incubated the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) or with PBS as the control. After 96 hours, the cytokine concentrations in the supernatants were determined by ELISA. Each graph represents an individual independent experiment with mean $\pm$ S.D. of samples run in triplicate.



Appendix Figure D: Epac activation in spleen cells promotes IFN- γ production from CD3⁺ and CD8⁺, but not CD4⁺, cells. Spleen cells (1×10^6 cells/ml) were plated on anti-CD3 (0.01 μ g/ml) and incubated with the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) at 10 or 100pM. The spleen cells were incubated at 37°C for 96hours, whereupon cells were stained with antibodies specific for CD3, CD4 and CD8, and the cytokine IFN- γ . Data represents the fold induction profile, of three individual experiments, combined together and compared to control unstimulated cells, showing the percentage of IFN- γ producing cells within each indicated population.



Appendix Figure E: The Epac activator does not promote IFN- γ production from CD4⁺ cells. Flow cytometry was performed on spleen cells with an antibody specific for CD3. The cells were analysed and sorted into a CD3-positive population based on specific antibody staining using a Beckman Coulter (Dako) MoFlo hi-speed cell sorter. CD3⁺ spleen cells (1x 10⁶ cells/ml) were plated on anti-CD3 (0.1 μ g/ml) and incubated with a range of concentrations of the Epac activator 8-pCPT-2'-O-Me-cAMP (8pCPT) alone or for 1 hour prior to stimulation with heat-killed *E. coli* (HK) at a ratio of 1 bacterium to one cell. After 96hours at 37°C, Brefeldin A with PMA/Ionomycin was added for four hours and intracellular flow cytometry was performed. Dot plots show CD4⁺IFN- γ ⁺ cells in the CD3⁺ population. Data are representative of at least three independent experiments.