DENDRITIC CELL MEDIATED MODULATION OF IMMUNE RESPONSES BY MYCOBACTERIUM VACCAE

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DECLARATION

I, Nina Le Bert, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

The contemporary hygiene hypothesis suggests that certain microorganisms that were present throughout human evolution modulate the host immune system to reduce allergy associated T helper 2 (Th2) responses and inflammatory diseases by augmenting regulatory T cells. The prototypic environmental mycobacterium, *M. vaccae* has been used in mouse models of asthma to support this hypothesis, but data from human models and possible mechanisms are very limited. In view of the role of dendritic cells (DCs) in shaping adaptive T cell responses, the effect of innate immune interactions between human DCs and *M. vaccae* on allogeneic and antigen specific DC-dependent polarisation of T cells was tested. *M. vaccae* can stimulate cellular activation via Toll-like receptor 2 (TLR2) and therefore was compared to a specific TLR2 ligand (Pam_3CSK4) and alternative stimulation with a TLR4 ligand (LPS). *M. vaccae* alone induced DC-dependent inhibition of Th2 responses, in contrast to Pam_3CSK4, which had the opposite effect and LPS, which had no polarising effect. Comparison of DC maturation, genome-wide transcriptional response, and cytokine production in response to each stimulus did not correlate with the specific functional effects. In particular, directly comparable DC transcriptional responses to *M. vaccae* and Pam_3CSK4 suggested that TLR2-mediated transcriptional regulation was not sufficient for inhibition of Th2 responses. Exclusive transcriptional responses to *M. vaccae* implicated a role for CREB1-dependent gene expression and analysis of signalling events confirmed selective early activation of the CREB pathway by *M. vaccae*. Collectively, this work has established that *M. vaccae* interaction with DCs does inhibit human Th2 responses and that further study of the CREB pathway in this model may provide novel insight into the molecular mechanisms of DC-dependent T cell polarisation. The final chapter of results presents development and validation of a novel approach for using short interspersed elements (SINEs) as a tool for normalisation of RT-qPCR data.

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ABBREVIATIONS

7-AAD	7-Amino-Actinomycin D
ACTB	Beta-actin
ADA	Adenosine deaminase
AF	Alexa-Flour
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
AMP/cAMP	Adenosine 5'-monophosphate/ cyclic AMP
AP-1	Activator protein 1
APC	Allophycocyanin
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
Ara-LAM	Arabinofuranosyl-terminated lipoarabinomannan
AREG	Amphiregulin
ARID3A	AT rich interactive domain 3A (BRIGHT-like)
ASC	Apoptosis-associated speck-like protein containing a CARD
ATF	Activating transcription factor
ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide
ATP5B	ATP synthase subunit
AXUD1	Cysteine-serine-rich nuclear protein 1
B2M	Beta-2 microglobulin
BAL	Bronchoalveolar lavage
BBS	Borate-buffered saline
BCG	Bacille Calmette-Guérin
BCL2L14	B-cell CLL/lymphoma 2-like 14 (apoptosis facilitator)
BHLHE40	Basic helix-loop-helix family, member e40
BSA	Bovine serum albumin
BTG1	B-cell translocation gene 1, anti-proliferative
BZRAP1	Benzodiazapine receptor (peripheral) associated protein 1
C18orf1	chromosome 18 open reading frame 1
C9orf3	chromosome 9 open reading frame 3 (aminopeptidase O)
CACNA1E	Calcium channel, voltage-dependent, R type, alpha 1E subunit
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CANX	Calnexin
CARD	Caspase-recruitment domain
CBP	CREB binding protein
CCL	Chemokine (C-C motif) ligand
CCR	CC-chemokine receptor
CD163	Hemoglobin scavenger receptor
cDC	Conventional DC
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDP	Common DC precursor
CFB	Complement factor B
CFU	Colony forming units
ChIP	Chromatin immunoprecipitation

CLDN1	Claudin 1
CLEC10A	C-type lectin domain family 10, member A
CLR	C-type lectin receptor
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CpG	Cytosine guanine dinucleotide
cpm	Counts per minute
CREB1	cAMP responsive element binding protein 1
CREM	cAMP responsive element modulator
CRTC	CREB-regulated transcription coactivator
CSF	Colony stimulating factor
CT	Threshold cycle
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
CV	Coefficient of variation
CX3CR1	CX3C chemokine receptor-1
CXCL	Chemokine (C-X-C motif) ligand
CYC1	Cytochrome c-1
CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1
CYTIP	Cytohesin 1 interacting protein
DAMP	Danger associated molecular pattern
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule 3-grabbing nonintegrin
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethylsulfoxide
DNA/cDNA	Deoxyribonucleic acid/complementary DNA
DNAJA4	DNAJ (Hsp40) homolog, subfamily A, member 4
DUSP	Dual specificity phosphatase
EAE	Experimental autoimmune encephalomyelitis
EAR	Expressed ALU repeat
EBI3	Epstein-Barr virus induced 3
EDTA	Ethylenediaminetetraacetic acid
EFNA1	Ephrin-A1
EGR3	Early growth response 3
EHD1	EH-domain containing 1
EIF4A2	Eukaryotic translation initiation factor 4A2
ELF5	E74-like factor 5 (ets domain transcription factor)
ELISA	Enzyme linked immunoadsorbant assay
ELK1	Member of ETS oncogene family
ELK4	ETS-domain protein (Serum response factor accessory protein 1)
EREG	Epiregulin
ERK1/2	Extracellular signal-regulated kinases 1 and 2
ERRFI1	ERBB (epidermal growth factor) receptor feedback inhibitor 1
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum

FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide
FITC	Fluoresceine isothiocyanate
FLT1	Vascular endothelial growth factor receptor 1
FOS	FBJ murine osteosarcoma viral oncogene homolog (c-FOS, part of transcription factor complex AP-1)
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
FOSL2	FOS-like antigen 2
FOXD1	Forkhead box D1
FoxP3	Forkhead box P3
GADD45B	Growth arrest and DNA-damage-inducible, beta
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBP4	Guanylate binding protein 4
G-CSF	Granulocyte colony stimulating factor
GEM	Guanosine-5'-triphosphate binding protein overexpressed in skeletal muscle
GM-CSF	Granulocyte macrophage colony-stimulating factor
GO	Gene ontology
GPR	G protein-coupled receptor
GRASP	General receptor for phosphoinositides 1-associated scaffold protein
GSK3	Glycogen synthase kinase 3
H89	*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide
Hand-Tcfe2a	Heterodimer: heart and neural crest derivatives expressed / transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)
HAV	Hepatitis A virus
HBEGF	Heparin-binding epidermal growth factor-like growth factor
HBSS	Hank’s buffered salt solution
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HKLM	Heat-killed *Listeria monocytogenes*
HLA	Human leukocyte antigen
HLF	Hepatic leukemia factor (bZIP transcription factor)
HMGB1	High mobility group box 1 protein
HO-1	Hemoxygenase-1
HRP	Horseradish peroxidise
HSP	Heat-shock protein
ICAM-1	Inter-cellular adhesion molecule-1
IDO	Idoleamine 2,3-dioxygenase
IER2	Immediate early response 2
IFI	IFN alpha-inducible protein
IFI44L	IFI 44-like
IFIT	IFI with tetratricopeptide repeats
IFITM1	IFI transmembrane protein 1 (9-27)
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin

INHBA	Inhibin, beta A
iNOS	Inducible nitric oxide synthase
IP10	IFN inducible protein 10 (CXCL10)
IQCG	IQ motif containing G
IQR	Interquartile range
IRF	IFN regulatory factor
IRX3	Iroquois homeobox 3
ISG20	IFN stimulated exonuclease gene 20kDa
ITIM	Immunoreceptor tyrosine-based inhibitor motif
iTreg	Inducible Treg
IκB	Inhibitory NF-κB
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LAD1	Ladinin 1
LAG-3	Lymphocyte activation gene-3
LB	Lysogeny broth
LC	Langerhans cell
LPS	Lipopolysaccharide
LRRC25	Leucine rich repeat containing 25
LTA	Lipoteichoic acid
M value	Average expression stability value
Man-LAM	Mannose-capped lipoarabinomannan
MAP2K3	Mitogen-activated protein kinase kinase 3
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MCP	Monocyte chemotactic protein
MDA	Melanoma differentiation associated protein
MDM	Monocyte-derived macrophage
MDP	Macrophage/DC precursor
MEK1	Mitogen-activated protein kinase kinase 1
MHC	Major histocompatibility complex
MIG	Monokine induced by IFN-γ
MIP	Macrophage inflammatory protein
MKP	MAPK phosphatase
MLB	Mannose-binding lectin
MLN	Mesenteric lymph nodes
MMP	Matrix metallopeptidase
MR	Mannose receptor
MRS	Macrophage scavenger receptor
MS	Multiple sclerosis
MS4A7	Membrane-spanning 4-domains, subfamily A, member 7
MSK1/2	Mitogen- and stress-activated protein kinases 1 and 2
MT1M	Metallothionein 1M
MTF1	Metal-regulatory transcription factor 1
MV	*M. vaccae*
MV10	10 µg/ml of MV

MyD	Myeloid differentiation primary response
NALP	NACHT, leucine rich repeat and pyrin domain containing
NEK6	NIMA (never in mitosis gene a)-related kinase 6
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NF	Normalisation factor
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
NFE2	Nuclear factor (erythroid-derived 2), 45kDa
NFKB1	NF-κB p105 subunit
NFKBIZ	NF-κB inhibitor, zeta
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NLR	Nod-like receptor
NLRC4	NLR family, CARD domain containing 4
NLS	Nuclear localisation signal
NO	Nitric oxide
NOD	Nonobese diabetic
Nod	Nucleotide-binding and oligomerization domain, leucine-rich repeat
NR4A	Nuclear receptor subfamily 4, group A
NTC	No template control
nTreg	Natural Treg
OASL	2'-5'-oligoadenylate synthetase-like
OD	Optical density
OSM	Oncostatin M
OVA	Ovalbumin
p90RSK	p90 ribosomal S6 kinase
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PC	Principal component
PCA	PC analysis
PCERA-1	Synthetic phosphor-ceramide analogue-1
PCR/qPCR	Polymerase chain reaction/quantitative real time PCR
pDC	Plasmacytoid DC
PE	Phycoerythrine
PER1	Period homolog 1 (Drosophila)
PFA	Paraformaldhyde
PGE2	Prostaglandin E2
pH	Potentia Hydgrogenii
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PILAM	Phosphatidyl-*myo*-inositol capped lipoarabinomannan
PIM	Phosphatidylinositol mannoside
PKA	Protein kinase A

PKI	Protein kinase inhibitor
PLAT	Plasminogen activator, tissue
PMA	Phorbol myristate acetate
PP	Peyer's patches
PPBP	Pro-platelet basic protein (CXCL7)
PPD	Purified protein derivative
PRR	Pattern recognition receptor
PS	Lyso-phosphatidylserine
PTX3	Pentraxin 3, long
ra	Receptor antagonist
REL	V-rel reticuloendotheliosis viral oncogene homolog (c-REL)
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (p65)
RELT	RELT tumor necrosis factor receptor
RGS	Regulator of G-protein signaling
RIG-I	Retinoic acid-inducible gene-I
RIN	RNA integrity number
RLR	RIG-I like receptor
RM	Repeated measures
RNA/mRNA	Ribonucleic acid/messenger RNA
RNASE6	RNase A family, k6
RPL13A	Ribosomal protein L13a
RPMI	Roswell Park Memorial Institute
RPS4Y	Ribosomal protein S4, Y-linked
RSAD2	Radical S-adenosyl methionine domain containing 2
RSV	Respiratory syncytial virus
RT	Reverse transcription
RV	Human rhinovirus
RXRA-VDR	Heterodimer: retinoid X receptor, alpha /vitamin D (1,25-dihydroxyvitamin D3) receptor
s.c.	Subcutaneous
S100A8	S100 calcium binding protein A8
SCHIP1	Schwannomin interacting protein 1
SD	Standard deviation
SDC4	Syndecan 4
SDHA	Succinate dehydrogenase complex, subunit A
SDS	Sodiumdodecylsulphate
SDS-PAGE	SDS-polyacrylamide-gel electrophoresis
SEA	Soluble egg antigen
SERINC5	Serine incorporator 5
SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
SGK1	Serum/glucocorticoid regulated kinase 1
SINE	Short interspersed repetitive element
siRNA	Small interfering RNA
SPIB	Spi-B transcription factor (Spi-1/PU.1 related)
SPP1	Secreted phosphoprotein 1
SQSTM1	Sequestosome 1

STAT	Signal transducer and activator of transcription
SYNPO2	Synaptopodin 2
TAE	Tris-acetate-EDTA
TANK	TRAF family member-associated NF-κB activator
TB	Tuberculosis
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TCR	T cell receptor
TES	*Toxocara canis* excretory-secretory
TFBS	Transcription factor binding site
TGF	Transforming growth factor
Th cell	T helper cell
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-1	T cell Ig and mucin-1
Tip-DC	TNF-α and iNOS-producing DC
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TM7SF4	Transmembrane 7 superfamily member 4
TMB	Tetramethylbenzidine
TMEM88	Transmembrane protein 88
TNF	Tumor necrosis factor
TNFAIP3	TNF alpha-induced protein 3
TNFR1	TNF-alpha receptor 1
TORC2	Transducer of regulated CREB activity
TP53	Tumor protein p53
Tr1 cell	T regulatory 1 cell
TRADD	TNFR1-associated death domain
TRAF1	TNF receptor-associated factor 1
TRAM	TRIF-related adaptor molecule
Treg cell	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-beta
TT	Tetanus toxoid
UBC	Ubiquitin C
UBD	Ubiquitin D
UV	Ultraviolet
v/v	Volume to volume ratio (%)
v/w	Volume to weight ratio (%)
VEGFA	Vascular endothelial growth factor A
VMO1	Vitelline membrane outer layer 1 homolog (chicken)
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
XIST	X (inactive)-specific transcript (non-protein coding)
XLAAD	X-linked autoimmunity allergic dysregulation syndrome
YWHAZ	Phospholipase A2
ZBTB10	Zinc finger and BTB domain containing 10
ZC3H12A	Zinc finger CCCH-type containing 12A
ZFAT	Zinc finger and AT hook domain containing

1 INTRODUCTION

1.1 Origins of the hygiene hypothesis

Since the mid 20th century, there has been a dramatic increase in the number of people suffering from allergic disorders such as atopic dermatitis (Williams, 1992), hay fever (Upton et al., 2000) and asthma (Woolcock and Peat, 1997). Epidemiological studies demonstrated that this significant rise of allergic diseases over the last decades is especially prevalent in countries with so-called "Westernised" life styles, such as Australia, Europe and North America (Redd, 2002; Sears, 1997; Smyth, 2002), with substantially lower frequency in developing countries (ISAAC_Steering_Committee, 1998).

It is well established that genetic factors play an important role in the prevalence of atopic diseases and many gene loci associated with prevalence or protection of developing allergic diseases have been identified (Daniels et al., 1996). Single gene mutation may only have mild phenotypes, but major phenotypic effects become evident when various genetic hits occur together. The numerous susceptibility genes can generally be separated into the following groups: innate immune response, T helper 2 (Th2) cell differentiation and effector function, genes expressed in epithelial cells and smooth muscle (Vercelli, 2008).

However, genetic factors alone cannot explain the recent steep increase in atopy and asthma. For example, a study in Greenland reported a 90 % increase in the prevalence of atopy over 11 years, between 1987 and 1998, amounting to an increase of 8 % per year (Krause et al., 2002). This must have been caused by environmental changes, and indeed, Greenland's population has undergone a major lifestyle transformation within a short period of time. Until recently, a rural society living from

traditional hunting and fishing, most people now have modern city lives with employment in service, administration and trade (Krause et al., 2002). Epidemiological observations in populations that have migrated between countries with different prevalence rates of atopy also support of the view that environmental factors must contribute significantly to the increase in allergic diseases. For example, the risk of developing asthmatic symptoms for people that migrated from low prevalence areas to Australia was associated with the length of time since arrival (Gibson et al., 2003). Other data suggest a more complex relationship; a recent study in the United States showed that the incidence for current asthma was lower in Mexican Americans born in Mexico (1.4 %) compared to those born in the United States (7.0 %) (McHugh et al., 2009), suggesting that environmental factors present early in life may be responsible for protection or susceptibility. Studies comparing populations in former East and West Germany are also interesting. These populations clearly have a common genetic ancestry, having been separated for only 40 years. Yet, adults in West Germany were significantly more likely to suffer from bronchial hyperresponsiveness and atopy (Nowak et al., 1996). Since reunification an increase of asthma, hay fever and atopic eczema has occurred in children from Eastern Germany. In particular, children born after 1990 have an increased prevalence of strong allergic sensitisation and atopic eczema (Heinrich et al., 2002).

These German studies contradict speculation that air pollution in the industrialised world is responsible for the increase of atopy and asthma. Between 1990 and 1991 most industrial plants were closed and gas heating replaced former lignite (brown coal) stoves (Ebelt et al., 2001). As a consequence pollution with sulphur dioxide and particulate matter decreased (Ebelt et al., 2001). In addition, air quality has generally improved during the epidemic of asthma in the developed world, further suggesting pollution is not the key determinant for these changes. Nonetheless,

pollution is likely to contribute to pathogenesis or symptoms, since visits as short as one week to environments with better air quality are advantageous for children suffering from asthmatic symptoms (Renzetti et al., 2009).

An environmental factor that the epidemiologist Strachan observed was an association between large families and a reduced risk of developing hay fever (Strachan, 1989). The birth order had a strong effect with first born children being more susceptible than later born siblings. To explain his findings, Strachan developed the "Hygiene Hypothesis", which gained widespread publicity. He proposed that the increased risk of allergic rhinitis in firstborn or only children was due to a lack of infections during early childhood, usually transmitted by unhygienic contact with older siblings (Strachan, 1989). Later studies confirmed his findings, showing that the numbers of siblings as well as the birth order were linked with the prevalence of developing asthma and atopy (Ball et al., 2000; Matricardi et al., 1998). Moreover, attending day-care for children from small families was also shown to be protective against the development of allergies and asthma later in childhood (Ball et al., 2000; Kramer et al., 1999), further supporting Strachan's hypothesis that frequent contact with other children and consequent cross-infections during early childhood may have a protective effect.

Epidemiological studies looking for a direct relationship between childhood infections and allergy gave contradictory results (Illi et al., 2001; Matricardi et al., 2000; Nja et al., 2003). The observations that older siblings or day-care attendance provided protection against allergy, led to the assumption that classical childhood virus infections would be important. However, a large study including more than 13,000 children, showed that infectious diseases occurring within the first 6 month of age were not protective, but that the risk of atopic dermatitis amplified with every infectious disease (Benn et al., 2004). Another study came to similar conclusions, showing that infections

with measles, mumps, rubella, chickenpox, cytomegalovirus, and herpes simplex virus type 1 did not reduce the risk of developing atopic diseases (Matricardi et al., 2000). Moreover, infection with respiratory viruses, including respiratory syncytial virus (RSV) and human rhinovirus (RV), were even able to initiate or worsen the development of childhood asthma (Kotaniemi-Syrjanen et al., 2003; Wenzel et al., 2002). Protective effects of infections with regards to the development of allergic diseases have been found with orofecal and foodborne infections, namely *Toxoplasma gondii, Helicobacter pylori*, *Salmonella* and hepatitis A virus (HAV) (Linneberg et al., 2003; Matricardi et al., 2002; Matricardi et al., 2000; Pelosi et al., 2005; Reibman et al., 2008).

Fewer studies focused on the role of bacterial infections during childhood and the development of allergies. In a Norwegian population, children with atopic parents had a reduced risk of allergic sensitisation when they had a history of otitis media (Nja et al., 2003). The relative role of bacteria or viruses was not assessed in these infections and the findings were inconsistent with an earlier study with German children (Illi et al., 2001). A Danish study showed that exposure to two or three intestinal bacterial pathogens, namely *Clostridium difficile*, *Campylobacter jejuni*, or *Yersinia enterocolitica*, was associated with a higher prevalence of atopy (Linneberg et al., 2003). Infection with *Mycobacterium tuberculosis* and vaccination with *M. bovis* Bacille Calmette-Guérin (BCG) received particular attention with respect to the hygiene hypothesis. A primary study of Japanese schoolchildren, all vaccinated with BCG, found a strong association between positive responders to tuberculin skin tests and reduced asthma and allergies (Shirakawa et al., 1997). This was supported by a large international study, which found that high tuberculosis notification rates were inversely associated with the frequency of asthma (von Mutius et al., 2000). A more recent study in South Africa also affirmed these observations, showing that children with a positive

tuberculin skin test were less likely to develop atopic rhinitis (Obihara et al., 2005). Not all studies support these findings however, and the confounding variables that may have affected the outcomes require further consideration (Matricardi and Yazdanbakhsh, 2003). Also the question of the protective effect of vaccination with BCG, usually associated with a scar, remains unanswered, with contradictory results from different studies (Aaby et al., 2000; Alm et al., 1997; da Cunha et al., 2004; Gruber et al., 2002; Marks et al., 2003; Strannegard et al., 1998; Townley et al., 2004). Where BCG vaccination has been associated with a protective effect, it has been in individuals with a family history of atopic diseases (da Cunha et al., 2004; Marks et al., 2003). In contrast to the human data, BCG is consistently associated with protection in mouse models of allergy (Choi et al., 2005; Erb et al., 1998; Herz et al., 1998; Hopfenspirger and Agrawal, 2002).

The increased prevalence of atopic diseases in Westernised populations was also mirrored by increased prevalence of autoimmune or idiopathic inflammatory diseases, such as type-1 diabetes (EURODIAB_ACE_Study_Group, 2000; Gale, 2002), multiple sclerosis (MS) (Poser et al., 1989; Rosati et al., 1988), and inflammatory bowel disease (Barton et al., 1989; Farrokhyar et al., 2001). These were largely ignored in the original hygiene hypothesis, although similar evidence existed for the contribution of environmental factors as those that were used to support the hygiene hypothesis. This included increased risk of type-1 diabetes (Bodansky et al., 1992; Staines et al., 1997) and MS (Hammond et al., 2000; Leibowitz et al., 1973) with migration from low to high prevalence areas. In addition, as with atopy and allergy, attending day-care centres was also associated with reduced risk of type-1 diabetes (McKinney et al., 2000). The differences in the immunopathogenesis of atopy and allergy to those of autoimmune or inflammatory diseases have become focussed on the role of different T lymphocytes.

1.1.1 T cell subsets in immunopathogenesis of inflammatory and allergic disease

T cell progenitors develop in the bone marrow and migrate to the thymus where they mature. Here the rearrangement of antigen-receptor genes takes place allowing the development of T cells with distinct specificity for numerous heterogeneous antigens. Cells expressing T cell receptors (TCRs) compatible with self-major histocompatibility complex (self-MHC) molecules survive, while those that strongly interact with self-antigen are removed from the repertoire, ensuring their ability of self tolerance. At an early stage of T cell development, the progenitors give rise to two different lines of T cells differentiated by TCR heterodimer subtypes. The major lineage express α:β TCRs and a minority population express γ:δ TCRs. The γ:δ T cells do not upregulate CD4 and CD8 expression, but divide into two subsets. One resides in epithelial tissue and displays TCRs of very limited diversity. These cells are thought to play a role in wound healing (Jameson et al., 2002; Sharp et al., 2005) and their natural targets are poorly defined self-antigens expressed during times of tissue injury (Havran et al., 1991). The second subset of γ:δ T cells show more diverse TCR specificity and circulate. Although their role is not fully understood, it has been shown that they do not require antigen-processing and presentation to recognise their target antigens (Schild et al., 1994; Sciammas et al., 1994; Weintraub et al., 1994), which do not have to be proteins and can be of host and foreign origin (Constant et al., 1994; Schoel et al., 1994; Tanaka et al., 1995; Tanaka et al., 1994).

The major α:β T cell linage gives rise to two functionally distinctive T cell subsets, CD4 and CD8 T cells, and also to Natural killer T (NKT) cells that are derived from $CD4^+/CD8^+$ double-positive cells with TCRs of very limited diversity, which recognise CD1d molecules instead of MHC molecules (Bendelac et al., 2007; Godfrey et al., 2010).

Mature $CD4^+$ and $CD8^+$ T cells that leave the thymus and circulate between the blood stream and peripheral lymphoid organs are called naive T cells as long as they have not encountered their specific antigen. In contrast to immunoglobulin expressed by B cells, the TCR does not bind to antigens directly, but recognises pathogen-derived peptide fragments bound to MHC molecules on antigen-presenting cells and their circulation allows them to make contact with numerous antigen-presenting cells throughout the body. The TCR functions as antigen recognition molecule but to establish an efficient connection the molecules CD8 and CD4 are also required to bind to MHC class I or MHC class II molecules respectively. Fully activated naive T cells undergo clonal expansion, thus proliferate and give rise to many T cells with identical antigen specificity that can act to clear infections. During this phase the T cells differentiate into effector T cells, and while $CD8^+$ T cells become cytotoxic, the fate of $CD4^+$ T cells is more flexible. $CD4^+$ T cells can differentiate into a number of different effector T cells with a variety of functions. Some of these usually short-lived $CD4^+$ and $CD8^+$ effector T cells differentiate further into long-lived T cells, so-called memory T cells, carrying the specific TCR and providing an enhanced response if they encounter the same antigen again, and so offering long-term protection (Harrington et al., 2008). Memory T cells can respond to re-infection more efficiently, since they occur in larger numbers than naive T cells recognising the same antigen, and because their activation arises more rapidly (Berard and Tough, 2002; Swain et al., 2006). When $CD8^+$ T cells recognise peptides presented by MHC class I molecules, typically viral antigens, they will kill any cell that they specifically recognise. The main role of effector $CD4^+$ T cells however is to help or to activate other cells of the immune system, and they are therefore referred to as T helper (Th) cells. Th cells have a central role in the regulation of adaptive immunity, as they augment activation of B cells and consequent immunoglobulin production, and can also augment antigen-specific $CD8^+$ T cell

effector mechanisms or non-antigen-specific effector mechanisms in mononuclear phagocytic cells. Functionally heterogeneous populations of $CD4^+$ T cells have been established that can be distinguished by the cytokines which they produce (Figure 1-1). These subsets were originally described in mice but data are also accumulating to support their existence in man.

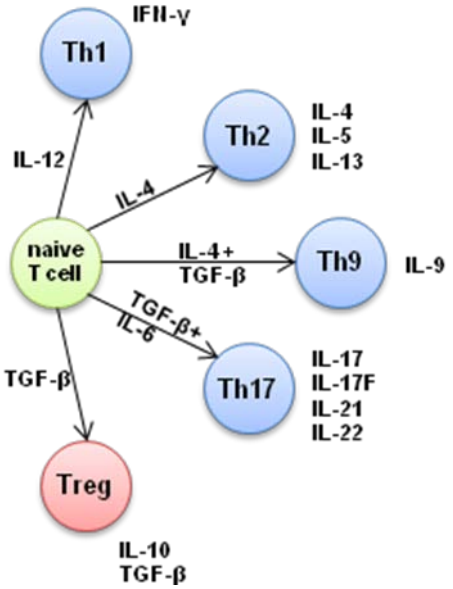

Figure 1-1: Differentiation of $CD4^+$ T cell subtypes and their characteristic cytokines (adapted from (Deenick and Tangye, 2007)).

In the presence of specific cytokines or cytokine combinations, naive $CD4^+$ T cells differentiate into functionally distinctive effector subtypes that can be distinguished by the cytokines which they produce.

1.1.1.1 Th1 cells

A well described $CD4^+$ T cell subset are Th1 cells, which are characterised by their ability to produce interferon-γ (IFN-γ) and interleukin-2 (IL-2) in response to cellular activation and are thought to develop under the influence of the transcription factor T-bet (Lighvani et al., 2001). A main role of Th1 effector cells and their cytokines is to control intracellular bacterial and viral infections. During activation, the cytokine microenvironment of the naive $CD4^+$ T cells may help to determine their differentiation profile, and IL-12 produced by innate immune cells polarises the

differentiation into Th1 cells (Hsieh et al., 1993). IFN-γ exerts several major biological functions through binding to its specific receptor and activation of the transcription factor signal transducer and activator of transcription 1 (STAT1) (Aaronson and Horvath, 2002). IFN-γ primes mononuclear phagocytic cells, such as monocytes, macrophages and dendritic cells (DCs) to be activated by low levels of pathogen associated molecular patterns (PAMPs) in innate immune responses (Nathan et al., 1984; Totemeyer et al., 2006). It has been demonstrated that IFN-γ can upregulate the expression of pattern recognition receptors (PRRs) on macrophages, and thereby preparing these cells to recognise and be activated by pathogens (Bosisio et al., 2002). Th1 cells activate macrophages by cell contact and secretion of IFN-γ, and so enhance their capacity of phagocytosis and killing of intracellular microbes and their expression of IL-12 and IFN-γ. In addition, Th1 cells play a role in helping $CD8^+$ T cells. IFN-γ producing $CD4^+$ T cells can enhance the expression IL-12 by antigen-presenting cells, which enhances the cytotoxic function of $CD8^+$ T cells (Agarwal et al., 2009b; Schmidt and Mescher, 2002; Xiao et al., 2009), and establishes a paracrine feedback loop by stimulating increased expression of IFN-γ (Hsieh et al., 1993; Manetti et al., 1993). Although potent primary $CD8^+$ T cell responses can be induced in the absence of $CD4^+$ T cells, $CD4^+$-deficient mice were unable to generate a robust secondary $CD8^+$ T cell response to a pathogen (Belz et al., 2002; Janssen et al., 2003; Khanolkar et al., 2004; Shedlock and Shen, 2003; Sun and Bevan, 2003), probably due to the lack of IL-2 provided by $CD4^+$ T cells (Williams et al., 2006).

By promoting activation of macrophages and $CD8^+$ T cells, Th1 responses are said to support cell-mediated host defence against intracellular pathogens and their physiological importance is cogently illustrated by increased risk of mycobacterial infections in individuals with IFN-γ receptor (Dorman et al., 2004) or IL-12 deficiency (Altare et al., 1998).

Besides promoting cell-mediated host defence, $CD4^+$ T cells play a role in driving B cell differentiation and the humoral immune response. The clearance of viral infections such as influenza is mediated by cytotoxic $CD8^+$ T cells (Bender et al., 1992) and B cells (Mozdzanowska et al., 1997). However, both cell types alone are only sufficient in clearing infections with the help from $CD4^+$ T cells (Mozdzanowska et al., 1997; Mozdzanowska et al., 2000; Topham and Doherty, 1998). IFN-γ secreted by Th1 cells leads to STAT1 signalling that induces T-bet expression in naive B cells, which activates the Iγ2a germline transcription (Xu and Zhang, 2005). Thus IFN-γ enhances the induction of class-switching to immunoglobulin (Ig)G2a (Finkelman et al., 1988; Snapper et al., 1988) that neutralise infective viral particles (Coutelier et al., 1987; Markine-Goriaynoff and Coutelier, 2002; Schlageter and Kozel, 1990). Severe influenza virus infection is associated with IgG2 deficiency (Gordon et al., 2010) and may be due to decreased expression of IFN-γ (Inoue et al., 1995; Kondo et al., 1997).

A pathogenetic role for Th1 responses has also been proposed in organ-specific autoimmunity (Charlton and Lafferty, 1995), including diseases that occur more frequently in developed countries. One example is type-1 diabetes, where the Th1 cytokines IFN-γ and IL-2 are considered to play an important role in destruction of insulin-producing beta cells in the pancreas probably by activating autoreactive $CD8^+$ T cells (Karlsson et al., 2000; Karlsson and Ludvigsson, 1998; Rapoport et al., 1998; Yoon et al., 1998). Contribution of Th1 cells in mediating diabetes has been supported by T-bet deficient nonobese diabetic (NOD) mouse models that were protected (Esensten et al., 2009), and genetic studies in Japanese type-1 diabetes patients also found associations with T-bet polymorphisms that resulted in augmented transcriptional activity from the IFN-γ promoter, suggesting a role for Th1 cells (Sasaki et al., 2004).

Evidence for contribution of Th1 cells in MS and its animal model of experimental autoimmune encephalomyelitis (EAE) has also been shown. So have T cell clones derived from cerebrospinal fluid of MS patients a Th1 profile with increased levels of tumour necrosis factor (TNF)-α, IFN-γ and IL-2 (Benvenuto et al., 1991), and augmented levels of IL-12 have been found in specimens of MS plaques from the central nervous system (CNS) (Windhagen et al., 1995). A widely prescribed treatment for MS is IFN-β, which probably functions by blocking IL-12 production by antigen-presenting cells, and thereby reducing the induction of Th1 cells (Bartholome et al., 1999; Byrnes et al., 2002; Heystek et al., 2003; McRae et al., 1998; Nagai et al., 2007).

Th1 cells have been implicated as the main mediators of Crohn's disease, a prototypic inflammatory bowel disease. This is based on increased production of IL-12 by isolated macrophages (Liu et al., 1999; Monteleone et al., 1997; Parronchi et al., 1997) and high levels of activated T-bet found in T cells from inflamed Crohn's disease lesions (Neurath et al., 2002). Moreover, T cells isolated from areas with active disease have further characteristics of Th1 cells, as they express increased levels of the IL-12 receptor β2 chain and (Parrello et al., 2000) augmented amounts of IFN-γ (Fuss et al., 1996; Neurath et al., 2002; Parronchi et al., 1997). A key role for IFN-γ has also been shown in a murine transfer model of colitis in which transfer of T cells from IFN-γ knockout mice failed to induce disease (Ito and Fathman, 1997).

1.1.1.2 Th2 cells

An alternative $CD4^+$ T cell subset, the Th2 cells that are indispensable for host immunity to extracellular parasites, such as pathogenic worms (Bancroft et al., 1998; McKenzie et al., 1999; Urban et al., 2000b), was originally described in mouse models.

Specific cytokine secretion patterns and distinct modes of B cell help found in mouse $CD4^+$ T cell clones led to the separation of $CD4^+$ T cells into Th1 and Th2 subclasses (Mosmann et al., 1986). In contrast to Th1 cells that are characterised by their expression of IFN-γ, Th2 cells produce different cytokines, including IL-4, IL-5 and IL-13. The existence of distinct $CD4^+$ T cell subsets in humans with similar cytokine profiles as found in mice was for the first time clearly demonstrated with antigen-specific T cell clones for purified protein derivative (PPD) of *M. tuberculosis* or *Toxocara canis* excretory-secretory (TES). While the PPD-specific T cell clones were stable producers of IFN-γ and IL-2 but not IL-4 and IL-5, the TES-specific clones produced IL-4 and IL-5 but no IFN-γ or IL-2 (Del Prete et al., 1991).

Naive $CD4^+$ T cells differentiate into Th2 cells in the presence of paracrine IL-4 and IL-2 (Le Gros et al., 1990; Swain et al., 1990). Binding of IL-4 to its receptor activates Janus kinase 1 (JAK1) together with JAK3 and leads to phosphorylation of STAT6 (Kelly-Welch et al., 2003), which upregulates expression of the principal Th2 master transcriptional regulator GATA-3 (Ho et al., 2009). However, a recent murine *in vivo* study has demonstrated that Th2 development can occur independently of IL-4/STAT6 signalling, but GATA-3 remains essential (van Panhuys et al., 2008). Similar to IFN-γ and Th1 cells, IL-4 secretion provides a positive feedback loop for Th2 cell development.

The differentiation into Th1 or Th2 cells has been long considered to be irreversible and many studies showed that those differentiated effector cells have a stable phenotype (Grogan et al., 2001). However, recently it was demonstrated that Th2 cells can be reprogrammed and turn on T-bet expression by maintaining GATA-3, thus become $GATA\text{-}3^+/T\text{-}bet^+$ cells that produce IL-4 and IFN-γ (Hegazy et al., 2010), showing that those cells can remain some plasticity.

In contrast to Th1 cells, whose main role is to support cell-mediated host defence against intracellular infections, Th2 cells play an important role in encouraging antibody production by B cells, especially IgE to clear extracellular parasites. IL-4 is responsible for activation of STAT6 that induces antibody heavy chain class-switching in antigen-specific B cells to promote IgE production (Shimoda et al., 1996). IgE antibodies bind their high affinity receptor FcεRI on mast cells and basophils leading to their degranulation. Consequently inflammatory mediators such as histamine are released by those innate immune cells together with several cytokines including IL-4, IL-13, and TNF-α (Kawakami and Galli, 2002).

Next to promoting humoral immune responses, Th2 cells can also activate mononuclear phagocytes. Macrophages that get activated in the presence of Th2 cytokines are so-called alternatively-activated macrophages, since they have distinct characteristics to those activated by Th1 cells and IFN-γ. It has been shown in mice that while IFN-γ supports microbial destruction though inducible nitric oxide synthase (iNOS) and consequently nitrogen intermediates like nitric oxide (NO), IL-4 and IL-13 induce arginase that converts L-arginine to L-ornithine (Munder et al., 1998). Alternatively-activated macrophages are thought to play a role in wound healing, since L-ornithine can be metabolised to proline and polyamines that are important for collagen production and cell proliferation (Hesse et al., 2001; Wynn, 2004).

Other innate immune cells that play a role in Th2-mediated immune responses are eosinophils whose differentiation in the bone marrow is critically dependent on IL-5 (Coffman et al., 1989; Collins et al., 1995; Foster et al., 1996; Hogan et al., 1997). Eosinophils can exhibit anti-helminthic activity by releasing lipid mediators and granule-stored cationic proteins that are directly toxic to the parasites (Gleich and Adolphson, 1986). Recent evidence suggests eosinophils may also be involved in the initiation, regulation and maintenance of Th2 immunity, since human eosinophils

produce and secrete large quantities of T cell-polarising cytokines including IL-4 (Spencer et al., 2009).

Th-2 responses are also thought to have a pathogenic role in asthma and allergy (Larche et al., 2003). Allergic reactions to normally harmless antigens, such as house dust mite, pollen or food allergens, are associated with allergen-specific B cells producing IgE (Gould and Sutton, 2008). As with immune responses to helminths, IgE antibodies trigger mast cell and basophil degranulation in allergic responses. Moreover, eosinophil infiltration to the sensitised organs occurs and their role in allergic airway remodelling events has been confirmed with the development of eosinophil-deficient mice (Humbles et al., 2004; Lee et al., 2004).

The role of Th2 cells in allergic responses has been clearly demonstrated in mouse models of induced allergies (Hogan et al., 1998) and many studies suggest a similar role in humans. Augmented levels of Th2 cytokine production in atopic disease compared to controls has been demonstrated repetitively (Lagier et al., 1995; Leonard et al., 1997; Tang et al., 1995; Till et al., 1997) and for example *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) with grass pollen allergen expands T cells showing a Th2 profile in atopic donors, but allergen-specific T cells in non-atopic donors are Th1-like (Imada et al., 1995). Moreover, Th2-like cells accumulate in the target organs of allergic patients. Patients with atopic asthma have higher levels of Th2 cytokines in bronchoalveolar lavage (BAL) samples compared to healthy controls, and higher expression levels of IL-4 and IL-5 associated with T lymphocytes (Robinson et al., 1992), further increased following allergen challenge (Thunberg et al., 2010). Although it is suggested that asthma in humans is a heterogeneous disease (Anderson, 2008), increased numbers of GATA-3-expressing cells are present in bronchial biopsies of all asthmatic patients (Nakamura et al., 1999). A large group of asthmatic patients

show elevated Th2 cytokine expression in bronchial biopsies and augmented airway hyperresponsiveness, serum IgE, blood and airway eosinophilia, subepithelial fibrosis, and airway mucin gene expression (Woodruff et al., 2009). Finally corticosteroids, that provide effective treatment for allergic disorders, have been shown to suppress Th2 cytokines in the airways of allergic rhinitis patients (Erin et al., 2005; Malmhall et al., 2007). Recent data suggested that corticosteroids achieve their effects by inhibiting the nuclear translocation of GATA-3, the key regulator of Th2 cytokine expression (Maneechotesuwan et al., 2009).

1.1.1.3 Th17 cells

A more recently described $CD4^+$ T cell lineage are the Th17 cells that characteristically secrete IL-17, and whose development is suppressed by IL-4 and IFN-γ (Harrington et al., 2005; Park et al., 2005). These cells are mainly found at barrier surfaces, especially at the mucosa of the gut and function in the resistance to extracellular bacteria and fungi that invade though the epithelium (Khader et al., 2009). It was initially shown in mice that naive Th cells can be induced to differentiate into this cell type by the presence of transforming growth factor-β (TGF-β) in combination with the proinflammatory cytokine IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). First studies in man suggested that instead of TGF-β, IL-1β was important for Th17 development in human (Acosta-Rodriguez et al., 2007a; Wilson et al., 2007). However, it has now become clear that low doses of TFG-β are required, while high doses suppress their differentiation (Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008). Yet, a very recent study in mice showed that a different cytokine combination of IL-1β, IL-6 and IL-23 can induce differentiation of an alternative Th17 cell type (Ghoreschi et al., 2010). In addition to the retinoid orphan nuclear receptor RORγT/RORC2 (mice/humans), which is the master transcription factor of Th17 cells

(Crome et al., 2009; Ivanov et al., 2006; Manel et al., 2008), these IL-23 induced Th17 cells express T-bet (Ghoreschi et al., 2010). In mice IL-6 and IL-21 induce STAT3 phosphorylation which upregulates RORγT (Yang et al., 2007). Differentiated Th17 cells secrete IL-17A, IL-17F, IL-21 and IL-22 (Dong, 2008). After the initial differentiation is induced, IL-21 develops a positive feedback loop that is important for the amplification of Th17 cells (Bettelli et al., 2007), whereas IL-23 produced by DCs is required to stabilise the Th17 cell subset (Veldhoen et al., 2006). IL-17A induces an upregulation of IL-6, IL-8, granulocyte colony stimulating factor (G-CSF), as well as prostaglandin E2 (PGE2) expression (Fossiez et al., 1996; Yao et al., 1995).

Th17 cells are thought to play an important role in preventing fungal infection, as suggested by IL-23 and IL-17 receptor (IL-17R) knockout models of oropharyngeal candidiasis (Conti et al., 2009), and the finding that human memory T cells specific for *Candida albicans* are mostly of the Th17 subset (Acosta-Rodriguez et al., 2007b; Milner et al., 2008). IL-17R knockout mice showed in addition evidence for a crucial role of Th 17 cells in the immune response to extracellular bacterial infections of mucosal tissues by *Neisseria gonorrhoeae* (Feinen et al., 2010), *Kleibsiella pneumonia* (Ye et al., 2001) and *Porphyromonas gingivalis* (Yu et al., 2007). Th17 cells execute their antifungal and antibacterial function by recruitment and activation of neutrophils (Conti et al., 2009; Feinen et al., 2010; Kolls and Linden, 2004; Yu et al., 2007).

Besides the role of Th17 cells in clearing bacterial and fungal infections, those cells are also involved in mediating autoimmune and inflammatory diseases (Bettelli et al., 2008; Dong, 2008; Reiner, 2009). For many diseases, including Crohn's disease, that had previously been associated with a Th1 cytokine profile, data are now emerging that Th17 cells may also be responsible (Brand, 2009). Studies with murine colitis models found a role for IL-23 (Hue et al., 2006; Kullberg et al., 2006; Yen et al., 2006),

albeit IL-17A showed a protective function in T cell-mediated intestinal inflammation (O'Connor et al., 2009). In addition, IL-17 cells may also play a role in EAE mouse models (Cua et al., 2003; Murphy et al., 2010), and augmented IL-23 levels have been described in monocyte-derived DCs from MS patients compared to healthy donors (Vaknin-Dembinsky et al., 2006). Moreover, cerebrospinal fluid from MS patients shows higher frequency of Th17 cells compared to controls, especially during relapse or acute symptoms (Brucklacher-Waldert et al., 2009). However, the relative contribution of Th1 and Th17 cell subsets to autoimmune diseases remains unclear and requires further study.

1.1.1.4 Th9 cells

Lately a new $CD4^+$ T cell type has been described in mice and man, the Th9 cell, induced by a combination of IL-4 and TGF-β to secrete IL-9 (Dardalhon et al., 2008; Veldhoen et al., 2008; Wong et al., 2010). In mice these cells do not express any transcription factor of the other $CD4^+$ T cell subtypes like T-bet, GATA-3, RORγT or forkhead box P3 (FoxP3) (Veldhoen et al., 2008), suggesting that they indeed represent a different subset but so far no specific transcription factor that is uniquely expressed by Th9 cells has been discovered. In man however, IL-4 and TGF-β generated IL-9 expressing cells express GATA-3, but reduced levels of Th2 cytokines IL-4, IL-5 and IL-13 (Wong et al., 2010). The physiological role of Th9 cells is not clearly understood, however IL-9 has long been described as a Th2 cytokine as it was found together with Th2 cytokines IL-4, IL-5 and IL-13 in mesenteric lymph nodes (MLN) during immune responses against murine parasitic infections and serves as a mast cell growth factor (Faulkner et al., 1998). Mice with defective TGF-β signalling, which is likely to be required for Th9 cell differentiation *in vivo*, were unable to expel *Trichuris muris* infection. Those mice had reduced levels of IL-9 and showed defective recruitment

and/or survival of mast cells, supporting a potential role for Th9 cells in the immune response against parasites (Veldhoen et al., 2008). Additionally IL-9 induces mucin production in epithelial cells during allergic reactions, thus playing an important role in airway remodelling in the pathogenesis of asthma in mouse and man (Longphre et al., 1999; Reader et al., 2003; Vermeer et al., 2003). Interestingly, a potential pathogenic role for Th9 cells has been recently described in mouse models of autoimmune diseases. Adoptive transfer of *in vitro* generated myelin-specific Th1, Th2, Th17 and Th9 cells demonstrated that all $CD4^+$ T cell subsets apart from Th2 cells could induce EAE (Jager et al., 2009). Moreover, Th9 cells have been implicated in promoting inflammation in colitis mouse models (Dardalhon et al., 2008).

1.1.1.5 Regulatory T cells

$CD4^+$ T cells that display anti-inflammatory properties have become known as regulatory T (Treg) cells. For the last four decades there has been interest in T cell populations with immunosuppressive properties, involved in limiting inflammation and preventing autoimmunity (Gershon and Kondo, 1971). In 1995 a population of $CD4^+$ T cells constitutively expressing the IL-2 receptor-α (IL-2Rα) chain (CD25) was found to suppress autoimmune diseases (Sakaguchi et al., 1995). However, only with the discovery of the transcription factor FoxP3 (Bennett et al., 2001; Brunkow et al., 2001) and the subsequent demonstration of its essential role in both the development and function of Treg cells (Fontenot and Rudensky, 2005), was it possible to study this population in more detail. There are two types of Treg cells, natural Treg (nTreg) cells and inducible Treg (iTreg) cells. No phenotypical differences between nTreg and iTreg cells have been found that could serve as markers to distinguish their functions and proportions in lymphoid tissue. They both are $CD4^+/CD25^+$ T cells expressing FoxP3 and TGF-β and have suppressive activity (Huter et al., 2008). In the presence of IL-2

and TGF-β, nTreg cells develop in the thymus from $CD4^+$ T cell precursors, characterised by stable expression of the transcription factor FoxP3 (Fontenot et al., 2005). TGF-β-deficient mice develop normal numbers of nTreg cells in the thymus, but TGF-β is required for maintaining expression of Foxp3 and suppressor activity (Marie et al., 2005). The second type, iTreg cells, develops in the periphery from naive $CD4^+/CD25^-$ T cells (Kretschmer et al., 2005; Siewert et al., 2008). Like Th-17 cell differentiation, their development is promoted by TGF-β, but in the absence of the proinflammatory cytokines like IL-6 (Bettelli et al., 2006). Natural Treg cells exhibit substantial plasticity of phenotype. Thus in the presence of IL-6 and absence of exogenous TGF-β they can downregulate FoxP3 and be subverted into the Th17 differentiation programme resulting in expression of IL-17 (Xu et al., 2007). However iTreg cells are resistant to stimulation with IL-6 and maintain FoxP3 expression and suppressor activity (Zheng et al., 2008). In addition to TGF-β, IL-2 is required for the generation of mouse iTreg cells *in vitro*, and their differentiation is supported by retinoic acid (Mucida et al., 2007). The conversion of human naive $CD4^+$ T cells into iTreg cells is also dependent on TGF-β. Contrary to what is seen in mice, priming with exogenous TGF-β is not required, but it has been suggested that endogenous TGF-β plays a role (Amarnath et al., 2007). Development of Treg cells is mediated by signalling through STAT5A/B (Burchill et al., 2007; Yao et al., 2007), which directly induces the upregulation of the transcription factor FoxP3 (Burchill et al., 2007; Yao et al., 2007; Zorn et al., 2006) and expression of CD25 (Nakajima et al., 1997).

The mechanism of how Treg cells mediate their immunosuppressive activity is not fully understood. Expression of the immunoregulatory cytokines IL-10 and TGF-β have been linked to the suppressive role of Treg cells (Asseman et al., 1999; Green et al., 2003), on the other hand blocking experiments have revealed that those cytokines are not necessarily required (Thornton and Shevach, 1998). In mice nTreg cells can also

express the cytokine IL-35 that is important for their maximal suppressive activity (Collison et al., 2009; Collison et al., 2007). IL-35 is not expressed at detectable amounts by human nTreg cells (Bardel et al., 2008), but naive T cells can be induced by DCs primed with human rhinovirus to secrete IL-35 (Seyerl et al., 2010). Several studies demonstrated the importance of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) expressed on Treg cells that can interact with B7 molecules (CD80 and CD86) expressed on activated T cells and DCs (Kolar et al., 2009; Schmidt et al., 2009; Sojka et al., 2009). One study demonstrated that Treg cells lose their ability to suppress B7-deficient effector T cells (Paust et al., 2004). Reverse signalling through CTLA-4 may be important for suppression, since CD28 (also a receptor for B7)-deficient Treg cells can still mediate suppression (Takahashi et al., 2000). More recently it was shown that CTLA-4 on Treg cells can downregulate CD80 and CD86 on DCs, so that they cannot activate effector T cells, which consequently prevented fatal T cell-mediated autoimmune disease and hyperproduction of IgE in mice (Wing et al., 2008). Moreover, Treg cells can modulate the function of DCs via the surface molecule T cell immunoreceptor with immunoglobulin (Ig) and immunoreceptor tyrosine-based inhibitor motif (ITIM) domains (TIGIT), which is highly expressed on Treg cells and induces the expression of IL-10 and TGF-β by DCs (Yu et al., 2009), or the transmembrane molecule lymphocyte activation gene-3 (LAG-3) that interacts with MHC II and inhibits DC activation (Liang et al., 2008).

In addition to the classical $CD4^+/CD25^+/FoxP3^+$ Treg cells other $CD4^+$ T cells with regulatory properties are known, including Th3 and T regulatory 1 (Tr1) cells. Th3 cells are antigen-specific cells that produce large amounts of TGF-β but little IL-10 and IL-4 and no IFN-γ or IL-2 (Chen et al., 1994). In the absence of inflammation the secreted TGF-β can induce FoxP3 expression in activated T cells, which will differentiate into iTreg cells (Carrier et al., 2007). Th3 cells also express FoxP3 but

remain CD25 negative. Tr1 cells have been described as antigen-specific $CD4^+$ T cells with regulatory properties that arise in the presence of IL-10, secrete high levels IL-10 and are $CD25^{-/low}$ and $FoxP3^-$ (Groux et al., 1997; Roncarolo et al., 2006). The expression of IL-10 by Treg cells is also independent of FoxP3 (Gavin et al., 2007), and not always required for their suppressive activity (Thornton and Shevach, 1998). However it is not clear if Tr1 cells can be classified as a separate $CD4^+$ T cell lineage, since also Th1, Th2 and Th17 cells have been shown to express IL-10 under certain conditions (Anderson et al., 2007; Jankovic et al., 2007; McGeachy et al., 2007).

1.2 The immunology of the hygiene hypothesis

Allergies and asthma are hypersensitivity reactions characterised by Th2 cell activity and IgE antibody production against usually harmless environmental allergens, such as those derived from house dust mite and pollen. In contrast, the presence of inflammatory danger signals, such as bacterial or viral infections, triggers IFN-γ producing Th1 cells. Since it had been shown that IFN-γ can reduce Th2-associated IgE production (Paul, 1989), Strachan's observations of the inverse association of siblings or attending day-care early in life with the prevalence of allergic diseases, lead to the hypothesis that cross-infection may provide protection against allergic disease. Strachan's hypothesis, which originated from epidemiological studies only, was given a biological explanation. The immunological concept was that a lack in Th1 stimulation due to diminished childhood infections, would lead to increased Th2 activity, and result in an increased prevalence of Th2-driven diseases like atopy and asthma (Cookson and Moffatt, 1997; Holt et al., 1997).

However, despite some exceptions like infection with HAV (Linneberg et al., 2003; Matricardi et al., 2002; Matricardi et al., 2000), *Salmonella* (Pelosi et al., 2005)

and *H. pylori* (Reibman et al., 2008), epidemiological data could not prove consistently that there is a direct link between viral or bacterial childhood infections and reduced risk of atopy. Moreover, children attending day-care centres that effectively prevented common respiratory tract and enteric infections during their early infancy by implementing a broad infection prevention programme reaching from intensified hand-washing to encouragement to take sick leave (Uhari and Mottonen, 1999) did not show increased allergic morbidity rates later in life (Dunder et al., 2007). That HAV infection was repeatedly shown to be negatively associated with atopic diseases (Linneberg et al., 2003; Matricardi et al., 2002; Matricardi et al., 2000) may have had a different underlying cause than the induction if a missing Th1 stimulus. HAV uses the cellular receptor T cell Ig and mucin-1 (TIM-1), to infect human cells (Feigelstock et al., 1998). This is a protein that is expressed on activated naive $CD4^+$ T cells during their development into Th2 cells (Umetsu et al., 2005), and which has also been linked with regulation of Th17 and Treg cells (Degauque et al., 2008). It has been demonstrated that TIM-1 is associated with protection against atopy (McIntire et al., 2003), and it is likely that HAV interrupts the interaction of TIM-1 with its ligand TIM-4, which is expressed on DCs, and thus limits the development of Th2 responses (Meyers et al., 2005; Umetsu et al., 2005) and augments Treg cells (Degauque et al., 2008). Nevertheless, this is specific for infections with HAV and there is no evidence that early infections with other viruses protect against allergic diseases (Benn et al., 2004; Dunder et al., 2007; Matricardi et al., 2000). The augmentation of Treg cells might be also of importance in the mechanism of *H. pylori* infections prevent allergic disorders (Beswick et al., 2007; Lundgren et al., 2005; Oderda et al., 2007; Raitala et al., 2006).

The original hygiene hypothesis implicated a predominance of Th2 responses over Th1 responses in early infancy and that the cytokine response to early infections reprogrammed the immune system towards more Th1-dominated responses. In the

developed world however, it was hypothesised, that with improved hygiene, vaccination and the use of antibiotics, predisposition of allergy results from insufficient Th1 stimulation that cannot compensate the Th2 dominance. This was supported only by a few murine (Adkins and Hamilton, 1992; Chen et al., 1995a) and human (Vigano et al., 1999) studies that showed more Th2 biased responses in newborns compared to older children and adults. However, a larger and more recent study demonstrated that a general Th2 bias is most likely not evident at birth (Halonen et al., 2009). IL-4 and IFN-γ production were both ~10-fold lower than in adults, but IL-13 was only reduced by 50 %, leading to an IFN-γ:IL-13 ratio that supports an early childhood Th2 bias, but one that is restricted to IL-13 only (Halonen et al., 2009).

Another observation questioned the immunological concept, that providing a Th1 stimulus could prevent allergic diseases. Murine studies showed that passive transfer of antigen-specific Th1 cells into a mouse model of allergy was not protective and resulted in increased inflammation (Hansen et al., 1999; Randolph et al., 1999).

Yet, the examples described above showed already some weaknesses of the original hygiene hypothesis that was based on a linkage between infections and reduced allergy. This simple Th1 versus Th2 model was fatally challenged by two contradicting findings:

First, as mentioned above, the epidemiological evidence that the increase of Th2-mediated immune diseases like atopy and asthma was also accompanied by a rise of Th1- or Th17-mediated autoimmune diseases in the more aseptic developed world (Bach, 2002; Black, 2001; Stene and Nafstad, 2001). Epidemiological data showed for example that the incidences of allergic disorders (Th2) and of type-1 diabetes (Th1) correlate closely within and outside Europe (Stene and Nafstad, 2001). Moreover, Th1 and Th2 type diseases can coexist within one individual and there are even some studies showing, that people suffering from allergies have an increased risk to develop

autoimmune diseases (Hemminki et al., 2010; Kero et al., 2001; Sheikh et al., 2003; Simpson et al., 2002), albeit other studies found the opposite (Cardwell et al., 2003; Rabin and Levinson, 2008; Tirosh et al., 2006). Therefore, even in a single individual the occurrence of those chronic immune diseases cannot be explained by a Th1 or Th2 bias and those diseases must have similar underlying reasons.

Second, the assumption that allergies result from a Th1/Th2 imbalance, is inconsistent with the inverse epidemiological association between allergies and Th2-inducing helminth infections (Araujo et al., 2000; Cooper et al., 2003; Hagel et al., 1993; Huang et al., 2002; Lynch et al., 1983; Medeiros et al., 2003; Nyan et al., 2001; Scrivener et al., 2001; van den Biggelaar et al., 2000). Although helminth-infected individuals showed Th2-biased immune responses compared to uninfected controls (Yazdanbakhsh et al., 1993), allergies are less frequent in endemic areas of infection (ISAAC_Steering_Committee, 1998). The role of helminths in reducing allergic diseases was directly shown in anti-helminth treatment studies. Children that received long-term treatment were afterwards more likely to show positive mite skin pick test reactivity (Lynch et al., 1993; van den Biggelaar et al., 2004), whereas shorter medication had no effect (Cooper et al., 2006). Since helminths are inducers of strong Th2 responses, one would expect infected people to be more likely to suffer from Th2-driven allergic diseases, if the balance of Th1 and Th2 cells was crucial. However, the overall immune response to helminth infections is not only characterised by polarised Th2 cells, but is generally downregulated during chronic infection, manifested by reduced antigen-specific proliferation and IFN-γ and IL-5 production (Grogan et al., 1998). The suppression of the immune response during helminth infection is obviously beneficial for the parasites to allow their long-term survival. However, when chronic infection is established, a suppressed immune response is also advantageous for the host to limit pathology (Bahia-Oliveira et al., 1992; Colley et al., 1986; Tweardy et al.,

1987). Interestingly, a mouse model showed that the immune response to helminth infections varies with the time of infection. Egg production during the infection is accompanied by a strong Th2 response, but later during chronic infection the Th2 cytokine production declines (Grzych et al., 1991). Recently it was demonstrated that chronic infection, but not acute infection, reduces the development of antigen-specific allergic airway inflammation in mice (Smits et al., 2007), which supports the view that only chronic and intense infection protects humans against allergic diseases.

The first study showing a mechanism on how helminths may suppress allergies was published in 2000. The authors demonstrated that parasite-induced cytokine IL-10 reduced the risk of skin test reactivity to mite allergens (van den Biggelaar et al., 2000). IL-10 and TGF-β, both regularly present in helminth-infected individuals (Grogan et al., 1998; van den Biggelaar et al., 2000), are anti-inflammatory cytokines capable of inhibiting effector T cell proliferation. Those cytokines can either play a direct role in dampening the immune response or maybe they are more relevant as inducers of Treg cells. It was shown that the transfer of helminth-induced Treg cells from IL-10 knockout mice, suppressed allergic airway inflammation in allergen challenged sensitised recipient mice (Wilson et al., 2005). FoxP3 expressing Treg cells have the ability to suppress the proliferation of all effector Th cell types, including Th1 and Th2 cells. Supporting the role of Treg cells in prevention of atopic diseases was a study that showed a reduced ability of $CD4^+/CD25^+$ T cells expressing FoxP3 from atopic donors to suppress proliferation and Th2 cytokine production by autologous allergen-stimulated $CD4^+/CD25^-$ T cells compared to non-atopic donors (Ling et al., 2004). It is proposed that the suppressive network induced by chronic helminth infection is not stringently antigen-specific, and consequently also protects against hypersensitivity reactions towards allergens (Smits and Yazdanbakhsh, 2007). This ability of helminth-induced Treg cells to provide bystander suppression is supported by a recent study showing that

$CD4^+/CD25^{high}/FoxP3^+$ T cells from helminth-infected children suppress also the immune response to antigens of mycobacteria and plasmodia (Wammes et al., 2010). In addition to providing bystander suppression, helminth infection can also have a Treg cell adjuvant effect, thus support the development of Treg cells with specificity for a different antigen (Correale and Farez, 2007).

Due to the findings that Th1-driven autoimmune diseases accompanied the rise of allergic diseases in the developed world and that Th2-inducing helminth infections are negatively associated with the risk of atopic diseases, the original hygiene hypothesis, which was based on a simple Th1 versus Th2 model, had to be revised. The prevailing view is that modern living conditions lead to a defective maturation of regulatory networks. Instead of Th1 versus Th2 being the critical balance, more evidence suggests that the crucial factor is the balance between effector and regulatory immune responses (Rook and Stanford, 1998; Wills-Karp et al., 2001; Yazdanbakhsh et al., 2002; Yazdanbakhsh et al., 2001). This model provides an explanation for the rise of Th1- and Th2-mediated chronic immunological disorders, and the fact that individuals suffering from allergy (Akdis et al., 2004; Karlsson et al., 2004; Perez-Machado et al., 2003), autoimmune diseases (Kriegel et al., 2004; Viglietta et al., 2004) and inflammatory bowel diseases (Duchmann et al., 1995; Kraus et al., 2004) show defects of immunoregulation supports the view that insufficient stimulation of the anti-inflammatory network is causing augmentation of those diseases in the industrialised world. The importance of immunoregulation with regards to atopy and autoimmunity becomes apparent in people with genetic defects of the transcription factor FoxP3, which is important for function and development of Treg cells (Di Nunzio et al., 2009). They suffer from X-linked autoimmunity allergic dysregulation syndrome (XLAAD),

with symptoms of allergy, autoimmunity and enteropathy (Bennett and Ochs, 2001).

The recent lifestyle changes in the developed world led not only to reduction of infection with helminths, but also to diminished exposure to many harmless microorganisms. Epidemiological studies described differences in the frequency of asthma, hay fever and allergic sensitisation within one country between urban and rural areas. This is true in the developed and developing world. A study in Ethiopia showed that asthma was more common in metropolitan areas then in remote rural areas (Yemaneberhan et al., 1997). Also children from Northern America, Northern Europe and New Zealand that grew up in farming environments were less likely to suffer from asthma and atopy compared to children less exposed to farming activities (Elliott et al., 2004; Ernst and Cormier, 2000; Klintberg et al., 2001; Leynaert et al., 2001; Portengen et al., 2002; Rennie et al., 2002; Riedler et al., 2001; Von Ehrenstein et al., 2000). Factors like contact with hay, animal sheds and consumption of unpasteurised cow's milk have been shown to be protective (Riedler et al., 2001), and there is evidence that farm children are more heavily exposed to bacterial endotoxin and muramic acid (Braun-Fahrlander et al., 2002; van Strien et al., 2004). The non-pathogenic bacterium *Acinetobacter lwoffii* F78 isolated from cowshed dust showed protective properties in an adult allergy mouse model (Debarry et al., 2007) and maternal exposure could protect offspring from the development of asthma (Conrad et al., 2009). The view that non-invasive organisms might play a role is supported by data that found an inverse correlation between pet ownership and the prevalence of allergic diseases (Hesselmar et al., 1999). Despite the fact that reviewing studies from the last nine years gave contradictory results (Chen et al., 2010), exposure to dogs at early infancy consistently showed a protective effect from developing sensitisation against diverse aeroallergens, especially outdoor allergens (Chen et al., 2010).

In addition to helminth infections, the hygiene hypothesis has now expanded to include non-invasive microorganisms that were constantly present in the environment during mammalian evolution and appear to have developed an essential immunoregulatory role (Rook, 2007). It has been postulated that beside helminths, organisms in mud and untreated water like saprophytic environmental mycobacteria, lactobacilli present in fermenting vegetables and drinks, and also bowel flora must be tolerated by humans and therefore drive regulatory pathways (Mazmanian et al., 2008; Rook, 2007). In addition to specific suppression, that generates tolerance to these organisms, bystander or non-specific suppression may protect the host from atopy and autoimmunity. Therefore, their depletion as a result of modern urbanisation may be at least partly responsible for the increasing incidences of chronic inflammatory disorders (Rook, 2007). Importantly, infection or treatment with Th1-inducing mycobacteria (Mutis et al., 1993), or with Th2-inducing helminths has been shown to be protective for both, Th1-driven autoimmune diseases (Harada et al., 1990; Zaccone et al., 2003) and Th2-driven atopy (Li and Shen, 2009; Wilson et al., 2005; Zuany-Amorim et al., 2002b). A common feature of organisms that correlate negatively with the development of allergies and autoimmunity is their ability to induce regulatory responses in animal models (Smits et al., 2005; Wilson et al., 2005; Zuany-Amorim et al., 2002b), and not a universal set of effector Th cells.

The organisms mentioned above can also be effective in some human clinical trials. Allergic children, when compared to healthy controls, are reported to be less colonised with lactobacilli (Bjorksten et al., 1999), but clinical trials administering different organisms and strains as probiotics have various outcomes (Betsi et al., 2008). The same observation is true of the use of probiotics shown to induce Treg cells in animal models (Smits et al., 2005). Children that received supplementation with

Lactobacillus rhamnosus HN001 early in life showed reduced prevalence of eczema, whereas a *Bifidobacterium* strain was not effective (Wickens et al., 2008). Also patients suffering from allergic rhinitis that received *Lactobacillus casei* Shirota showed reduced levels of allergen-induced cytokines and IgE (Ivory et al., 2008). Several small human studies on inflammatory bowel disease showed also some positive effects of prebiotics that support the growth of indigenous lactobacilli and bifidobacteria (Looijer-van Langen and Dieleman, 2009). Clinical trials with helminth therapy have also given contradictory results. A study in Argentina found that natural helminth infection treated MS and induced myelin-specific regulatory cells (Correale and Farez, 2007). In addition, *Trichuris suis* is reported to have a therapeutic effect on inflammatory bowel disease (Summers et al., 2005), but not on allergic rhinitis (Bager et al., 2010). Some human trials with environmental mycobacteria that had been demonstrated to protect against airway inflammation by inducing Treg cells in allergic mouse models (Zuany-Amorim et al., 2002b) were also successful (Arkwright and David, 2001; Camporota et al., 2003), but other studies showed contrasting outcomes (Arkwright and David, 2003; Berth-Jones et al., 2006; Brothers et al., 2009; Shirtcliffe et al., 2003).

Taken together, organisms have been identified that are relevant for the hygiene hypothesis and animal models show clearly that they can induce regulatory pathways that can suppress allergic and autoimmune diseases. However, a better understanding of the mechanism by which they influence immune responses might improve design and success of their clinical applications.

1.3 Immunomodulation by *M. vaccae*

The genus *mycobacterium* consists of over 80 different species, from which only a few are infectious and cause serious diseases like tuberculosis and leprosy in man. The

vast majority are harmless saprophytes, which are associated with water and soil. They are rod-shaped aerobic bacteria characterised by their unusual thick and hydrophobic cell wall. The mycobacterial cell wall has a unique structure, which is particularly rich in lipids that compose 30 – 60 % of their dry weight, and consists of three parts: the plasma membrane, the cell wall, and the capsule (Daffe and Draper, 1998). Until recently it was thought that mycobacteria lack an outer cell membrane. Hence, they are classified as Gram-positive albeit difficult to stain. Recently new techniques of tomography and microscopy revealed a bilayer structure of an outer membrane (Hoffmann et al., 2008; Zuber et al., 2008).

In the developing world, saprophytic mycobacteria are often present in a quantity of 1 mg or more per litre in untreated water supplies (Rook et al., 2007). Since the introduction of clean supermarket food and the regular purification and chlorination of drinking water in industrialised countries, the contact between humans and saprophytic mycobacteria has become less frequent. Although harmless mycobacteria do not replicate in humans, their inevitable and continuous presence in water and food during mammalian evolution could have led to a role comparable to that of symbiotic bacteria in the gut flora, that are required for directing normal immune system development (Macpherson and Harris, 2004).

1.3.1 *M. vaccae*'s effects in animal models and human clinical trials

The subject of this thesis is the environmental mycobacterium that has received most attention: *M. vaccae*. In 1998, it was reported for the first time that injection of heat-killed *M. vaccae* into ovalbumin (OVA)-pre-immunised mice suppressed serum IgE levels and the expression of IL-5 by splenocytes following OVA re-stimulation *in vitro* (Wang and Rook, 1998). Since then, *M. vaccae*'s activity in murine asthma

models was confirmed in a number of studies, in which *M. vaccae* has repeatedly been shown to be effective in therapeutic and prevention applications (Hopfenspirger et al., 2001; Ozdemir et al., 2003; Smit et al., 2003; Zuany-Amorim et al., 2002a; Zuany-Amorim et al., 2002b). Heat-killed preparations of *M. vaccae* injected into mice either during OVA-sensitisation or later during challenge, when allergy had already been established, reduced airway hyperresponsiveness, airway eosinophilia and IL-5 production following OVA-challenge (Smit et al., 2003).

A number of clinical trials in man suffering from asthma or atopic dermatitis showed positive effects of *M. vaccae* (Arkwright and David, 2001; Camporota et al., 2003). In the first trial with children suffering from moderate to severe atopic dermatitis, interdermal injections of heat-killed *M. vaccae* reduced the affected surface area as well as the dermatitis severity score significantly compared to placebo controls (Arkwright and David, 2001). Later, *M. vaccae* also showed promising effects in small double-blind randomised trial with adults suffering from atopic asthma. Here, treatment with a single dose of *M. vaccae* led to a decreased late phase response and a reduced expression of serum IgE and *in vitro* secretion of IL-5 (Camporota et al., 2003). However, others could not replicate these findings (Arkwright and David, 2003; Berth-Jones et al., 2006; Brothers et al., 2009; Shirtcliffe et al., 2003).

More recently, *M. vaccae* was effective in a clinical trial to cure mild to moderate atopic dermatitis in dogs (Ricklin-Gutzwiller et al., 2007). After a single intradermal injection the dogs were observed for three months. After that time clinical symptoms of atopic dermatitis were significantly reduced (Ricklin-Gutzwiller et al., 2007), for which it is now being developed by Novartis Animal Health. Additionally, *M. vaccae* might have a possible therapeutic efficacy in tuberculosis (TB) (Dlugovitzky et al., 2006; Hernandez-Pando et al., 2000; Xu et al., 2009), and recently showed

efficacy in a large (2,013 patients) phase III clinical trial for prevention of TB in human immunodeficiency virus (HIV)-positive subjects in Tanzania (von Reyn et al., 2010).

The mechanisms, by which *M. vaccae* exerts the reported effects in animal models of allergic disease or indeed a possible therapeutic effect in TB, are not clearly understood. The prevailing view is that *M. vaccae* changes the polarisation of antigen-specific Th cell responses by downregulating Th2 (Rook et al., 2004).

1.3.2 *M. vaccae* modulates T cell responses in mice

In various asthma and allergy models the treatment effect of *M. vaccae* has been associated with reduced Th2 responses, increased Treg responses, possibly increased Th1 responses as well as the induction of cytotoxic $CD8^+$ T cells (see Table 1-1).

The role of Treg cells has been particularly highlighted by the findings in mice where *M. vaccae* caused the induction of an allergen-specific population of $CD4^+/CD45RB^{low}$ Treg cells (Zuany-Amorim et al., 2002b). A single dose of *M. vaccae* given subcutaneously either before Th2-inducing immunisation with OVA, or after the first two of four such immunisations, was able to reduce symptoms of airway inflammation elicited by a subsequent allergen challenge (Zuany-Amorim et al., 2002b), an effect that lasted for twelve weeks after its administration (Zuany-Amorim et al., 2002a). Intravenous transfer of splenocytes or purified $CD4^+/CD45RB^{low}$ T cells from those treated mice to OVA-immunised recipients that had not been given *M. vaccae* reduced the Th2-mediated airway eosinophilia and bronchial hyperresponsiveness following airway challenge (Zuany-Amorim et al., 2002b). The regulatory cells were specific for antigens present during their induction, but once triggered by their specific antigen, they could exert bystander suppression of Th2 responses to unrelated antigens

(Zuany-Amorim et al., 2002b). The suppression of allergic responses in the recipients depended upon release of TGF-β and IL-10 (Zuany-Amorim et al., 2002b) but not IFN-γ (Zuany-Amorim et al., 2002a), which supports further the view that Treg cells were involved.

Table 1-1: Selected references describing *M. vaccae* effects in animal models

***M. vaccae* effects in animal models**
M. vaccae induced $CD8^+$ T cells, which killed macrophages infected with *M. tuberculosis* (Skinner et al., 1997)
Recombinant *M. vaccae* is a Th1 adjuvant for antigens expressed within it (Abou-Zeid et al., 1997; Hetzel et al., 1998)
M. vaccae injection into antigen-preimmunised mice suppressed serum IgE and antigen induced IL-5 production by spleen cells (Wang and Rook, 1998)
New-born mice treated with *M. vaccae* showed reduced allergic responses to allergen (Ozdemir et al., 2003; Tukenmez et al., 1999)
M. vaccae immunisation s.c. of OVA-sensitised pregnant mice reduced IL-5 and enhanced IFN-γ secretion from placenta and splenocytes of offspring on the 2^{nd} and 28^{th} day of life (Akkoc et al., 2008)
Recombinant *M. vaccae* expressing an epitope from potent allergen induced a shift from Th2 to Th1 (IFN-γ) in immunised mice (Janssen et al., 2001)
M. vaccae administered with antigen induced antigen-specific $CD8^+$ T cells (Skinner et al., 2001)
Reduced bronchoconstriction, airway hyperresponsiveness and eosinophilia when *M. vaccae* was given both before and after sensitising injections of antigen (Hopfenspirger et al., 2001)
Single injection of *M. vaccae* prior or after immunisation limited allergic responses by induction of antigen-specific $CD25^+CD45RB^{low}$ Treg in a IL-10 and TGF-β dependent way (Zuany-Amorim et al., 2002a; Zuany-Amorim et al., 2002b)
Intranasal *M. vaccae* into antigen-preimmunised mice suppressed airway hypersensitivity and eosinophilia (Hopfenspirger and Agrawal, 2002)
M. vaccae treatment of antigen-preimmunised mice during antigen challenge suppressed airway hypersensitivity, airway eosinophilia and IL-5 production (Smit et al., 2003)
M. vaccae induced pulmonary $CD11c^+$ cells with increased levels of IL-10, TGF-β and IFN-α mRNA (Adams et al., 2004)
M. vaccae administered orally increased production of IL-10 and IFN-γ by MLN and splenocytes (Hunt et al., 2005)
Intradermal injection of *M. vaccae* was effective in treating mild to moderate cases of atopic dermatitis in dogs (Ricklin-Gutzwiller et al., 2007)

Interestingly, administration of *M. vaccae* by the gastrointestinal route in naive mice, to simulate natural water-borne exposure to this organism, was also shown to increase the release of IL-10 by MLN and IL-10 and IFN-γ by splenocytes (Hunt et al., 2005). Treatments of OVA-sensitised mice with oral and subcutaneous *M. vaccae* were

equally effective at reducing the total cellular infiltrate into the lungs following allergen challenge (Hunt et al., 2005). In addition to a reduction of eosinophils, no increase in inflammatory cells like macrophages and neutrophils was observed (Hunt et al., 2005). The view that oral *M. vaccae* executes its effects by inducing regulatory rather than Th1 responses was supported by the observation that *M. vaccae* treated mice had elevated IL-10 levels in the BAL fluid and supernatant of splenocytes, whereas IFN-γ levels did not differ (Hunt et al., 2005). Additionally, in a murine TB model, oral *M. vaccae* modulated the expression of hemoxygenase-1 (HO-1), idoleamine 2,3-dioxygenase (IDO), FoxP3 and TGF-β, all known to be important for immunoregulation (Hernandez-Pando et al., 2008).

The only hint as to how *M. vaccae* might induce Treg cells was published in 2004. The study confirmed previous observations that *M. vaccae*, when injected in an OVA-induced allergy model of BALB/c mice, caused reduced IL-4 messenger ribonucleic acid (mRNA) levels in the lungs and BAL fluid, while Th1 cytokine expression of IFN-γ and IL-12 mRNA did not increase (Adams et al., 2004). Moreover, increased protein levels of IL-10 were detected in the BAL fluid of animals that had received *M. vaccae* (Adams et al., 2004), again suggesting that the reduced Th2 response was not the result of a switch to a Th1 response, but rather due to the induction of a regulatory cytokine environment. Furthermore, the study demonstrated that following treatment with *M. vaccae* a population of $CD11c^+$ cells in the lungs showed increased expression of the regulatory cytokines IL-10, TGF-β and IFN-α mRNA (Adams et al., 2004), possibly implicating a key role for DCs.

Nothing is known about the mechanism by which *M. vaccae* may modulate human immune responses and no studies of *M. vaccae* interactions with DCs have been reported.

1.4 Role of dendritic cells in immunoregulation

DCs are mononuclear phagocytes, specialised in antigen-presentation. They capture and process antigens in the periphery, migrate to lymphoid organs, the spleen and the lymph nodes, and secrete cytokines essential to the initiation of innate and adaptive immune responses. DCs are characterised by their ability to support naive and memory $CD4^+$ T cell activation and proliferation as well as to stimulate cytotoxic T cells, both in a MHC-dependent manner, thus they control the mediators of adaptive immunity. In addition to their role in T cell activation, DCs may also contribute to so-called peripheral T cell tolerance to self-antigens (Cools et al., 2007). In their resting state, DCs are described as immature because they express relatively low levels of both, costimulatory molecules and MHC class II that are required for $CD4^+$ T cell activation, but exhibit good phagocytic capacity.

1.4.1 Dendritic cell subtypes

Conventional DCs (cDCs) are highly migratory cells that can move from tissues to lymphoid organs and are also present in the peripheral blood. They are characterised by their CD11c expression and originate from pre-cDCs found in bone marrow, blood, spleen and lymph nodes (Liu et al., 2009). They migrate through the blood to lymphoid tissues by entering the lymph nodes through high endothelial venules from where they distribute themselves and become differentiated cDCs (Liu et al., 2009).

The plasmacytoid DC (pDC) population is characterised phenotypically in humans as $CD11c^-/CD123^{high}$ (Liu, 2001; MacDonald et al., 2002). They circulate in blood and are present in the bone marrow, spleen, thymus, lymph nodes, and the liver. They are thought to have a key role in viral infections because they have receptors to sense viral nucleic acids and produce large amounts of type I IFN (Fonteneau et al.,

2003). Moreover, pDCs can also act as antigen-presenting cells and initiate T cell responses (Colonna et al., 2004).

These two DC subtypes are short-lived and have to be continuously regenerated from hematopoietic stem cells in the bone marrow. Contrary to initial predictions, lymphoid and myeloid committed progenitor cells retain the ability to give rise to both pDCs and cDCs (Chicha et al., 2004; Shigematsu et al., 2004). The macrophage/DC precursors (MDPs) differentiate into monocytes and common DC precursors (CDPs) that are proliferating cells in the bone marrow. CDPs then give rise to pre-cDCs and pDCs that leave the bone marrow and enter the blood stream (Liu et al., 2009; Naik et al., 2007; Onai et al., 2007). It is not clear if lymphoid progenitor cells also contribute substantially to the rise of pDCs and cDCs (Geissmann et al., 2010).

Two distinct DC populations characterised by the expression of $CD103^+$ and CX3-chemokine receptor-1^+ ($CX3CR1^+$) respectively are resident in the non-lymphoid tissues such as lung, dermis and intestinal lamina propria. In the lamina propria it has been shown that $CD103^+$ DCs also originate from pre-cDCs whereas $CX3CR1^+$ DCs derive from monocytes (Bogunovic et al., 2009; Varol et al., 2009). It was demonstrated that the balance of the two DC subsets is critical for gut homeostasis (Varol et al., 2009) but only pre-cDC derived $CD103^+$ DCs migrate to the lymph nodes for T cell activation (Schulz et al., 2009).

Langerhans cells (LCs) are another DC subtype that is found in the epidermis. Contrary to most DC subsets, LCs and also microglia cells of the CNS are independent of bone marrow-derived progenitors for their regeneration (Ajami et al., 2007; Merad and Manz, 2009). Recently it was shown that LCs renew from the slow proliferation of differentiated LCs resident in the epidermis, which can be enhanced massively in response to inflammation (Chorro et al., 2009).

Another DC subset is called TNF-α and iNOS-producing (Tip)-DCs, which probably derives from monocytes and is characterised by the production of inflammatory mediators such as TNF-α, nitric oxide and reactive oxygen species. These Tip-DCs have been shown to play an important role during clearance of *Listeria monocytogenes* infection, but also contribute to the development of trypanosome infection-associated tissue injury in mice models (Guilliams et al., 2009; Narni-Mancinelli et al., 2007; Serbina et al., 2003).

1.4.2 Experimental model: monocyte-derived dendritic cells

Circulating DCs are rare and comprise approximately 1 % of circulating PBMCs. Since it is difficult to isolate sufficient numbers for experimental studies, most work is done with DCs that are generated *in vitro*. Human monocytes isolated from peripheral blood are cultured in the presence of IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) and differentiate into DCs (Sallusto and Lanzavecchia, 1994). These monocyte-derived DCs are potent antigen-presenting cells and have many features of primary DCs (Pickl et al., 1996; Sallusto and Lanzavecchia, 1994). During their differentiation these cells lose the expression of typical monocyte markers such as CD14 and FcγRI, and acquire DC morphology. Similar to immature DCs they express CD1 molecules, FcγRII, CD11c and DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and moderate levels of MHC class I and II and costimulatory molecules CD80, CD86 and CD40, and inter-cellular adhesion molecule-1 (ICAM-1) (Geijtenbeek et al., 2000; Sallusto and Lanzavecchia, 1994). Following stimulation the monocyte-derived DCs mature, which leads to a loss of FcγRII expression and reduced antigen-capturing capacity, but highly upregulated expression of MHC and costimulatory molecules, ICAM-1 and of the so-called maturation DC marker CD83 (Sallusto and Lanzavecchia, 1994; Zhou and Tedder,

1996). In addition to their ability to process and present antigens, they are potent inducers of autologous and allogeneic T cell proliferation and have the DC-specific capacity to stimulate naive T cells (Sallusto and Lanzavecchia, 1994).

1.4.3 Activation of DCs

Innate immunity describes immunological responses dependent on host-pathogen interactions between germline encoded PRRs and PAMPs. A number of cell associated PRRs subsets exist. These include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding and oligomerisation domain, leucine-rich repeat (Nod)-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) (see Table 1-2). Their activation sequentially induces recruitment of adapter proteins, kinase signalling cascades and activation of transcription factors that cause wide ranging gene expression changes (Jenner and Young, 2005; Medzhitov, 2001).

TLRs are the best characterised group of PRRs. They are transmembrane molecules whose intracellular C-terminal domain, known as the Toll/IL-1 receptor (TIR) domain, homologous with that of the IL-1 receptor, associates with adaptor molecules to activate downstream signalling pathways. Stimulation of all TLRs, with the exception of TLR3, recruits myeloid differentiation primary response (MyD) 88. Activation of TLR3 recruits the adaptor molecule TIR-domain-containing adapter-inducing interferon-beta (TRIF), which can also associate with the TIR domain of TLR4 through the TRIF-related adaptor molecule (TRAM). Thus only TLR4 stimulation can induce both the MyD88-dependent and TRIF-dependent signalling pathways. The adaptor-dependent signalling cascades lead to the activation of transcription factors like nuclear factor-κB (NF-κB) and IFN regulatory factors (IRFs) (see 1.4.5), and also to activation of mitogen-activated protein kinases (MAPKs) such as p38, c-Jun N-terminal

kinase (JNK) and extracellular signal-regulated kinases 1 and 2 (ERK1/2), which activate the activator protein 1 (AP-1) transcription factor.

Table 1-2: Better known pattern recognition receptors expressed in human cells

Group	PRR	Localisation	Ligand
TLRs	TLR2	plasma membrane	lipoglycans, lipoteichoic acids, peptidoglycans
	TLR1/2	plasma membrane	triacylated lipoproteins
	TLR2/6	plasma membrane	diacylated lipoproteins
	TLR3	endosomal membrane	double-stranded RNA
	TLR4	plasma membrane	lipopolysaccharide
	TLR5	plasma membrane	flagellin
	TLR7	endosomal membrane	single-stranded RNA
	TLR8	endosomal membrane	single-stranded RNA
	TLR9	endosomal membrane	CpG DNA
	TLR10	plasma membrane	?
CLRs	Dectin-1	plasma membrane	β-glucans
	DC-SIGN	plasma membrane	viruses, mycobacteria, *Candida*, *Leishmania*
	MBL	plasma membrane	mannose, N-acetylglucosamine
RLRs	RIG-I	cytoplasm	double-stranded RNA
	MDA-5	cytoplasm	double-stranded RNA
NLRs (>20 members)	Nod1	cytoplasm	peptidoglycan (iE-DAP)
	Nod2	cytoplasm	peptidoglycan (MDP)
	NALP1	cytoplasm	MDP and anthrax toxin
	NALP3	cytoplasm	whole bacteria, bacterial RNA, purine-like compounds, uric acid crystals, extracellular ATP, pore-forming toxins
	NLRC4	cytoplasm	flagellin
	NAIP5	cytoplasm	flagellin

CLRs can interact with a variety of pathogens, including viruses, bacteria, mycobacteria, helminths and fungi, as they recognise largely mannose, fucose and glucan carbohydrate structures. Interestingly the activation of some CLRs like DC-SIGN has been shown to induce signalling cascades that modulate TLR-induced gene expression, but may not induce gene expression alone (Gringhuis et al., 2007). However, triggering of other CLRs such as dectin-1 and dectin-2 can induce gene

transcription independently of other PRRs (Gross et al., 2006; Rogers et al., 2005; Sato et al., 2006).

The RLRs RIG-I and melanoma differentiation associated protein 5 (MDA-5) recognise double-stranded RNA, like TLR3, and also trigger the expression of type-I IFNs (Yoneyama and Fujita, 2007). However RIG-I and MDA-5 seem to play a major role in virus recognition in cDCs and macrophages, while TLR3 might be more important in pDCs (Kato et al., 2005).

Nod1 and Nod2 recognise specific motifs of peptidoglycan from bacterial cell walls (McDonald et al., 2005) and have been shown to trigger the activation of NF-κB and MAPKs (Inohara et al., 2000; Kobayashi et al., 2005). The NLR members NLR family containing a caspase-recruitment domain (CARD) containing 4 (NLRC4), NACHT leucine rich repeat and pyrin domain containing 1 (NALP1) and NALP3 form protein complexes with the adaptor apoptosis-associated speck-like protein-CARD (ASC) and caspase-1, known as inflammasomes, which convert proIL-1β and proIL-18 into active cytokines (Mariathasan and Monack, 2007).

In addition to the recognition of PAMPs, TLRs (Park et al., 2004; Tian et al., 2007; Vabulas et al., 2002; Warger et al., 2006) and NLRs (Hornung et al., 2008) also play a role in the recognition of danger associated molecular patterns (DAMPs), which can also activate immune cells and trigger signalling-mediated transcriptional changes. DAMPs are patterns of host intracellular molecules that have emerged in the extracellular milieu from dying host cells, alerting the immune system about necrotic cell death. The first host molecules that have been shown to activate DCs and augment immune responses were heat-shock proteins (HSPs) (Basu et al., 2000; Binder et al., 2000; Feng et al., 2003), although it cannot be excluded that microbial contamination was responsible for the effects (Bausinger et al., 2002). Another DAMP is the high mobility group box 1 protein (HMGB1), which binds deoxyribonucleic acid (DNA) and

stabilises the nucleus and can boost immune response to antigens possibly by triggering activation of DCs (Rovere-Querini et al., 2004). Moreover, crystals formed by monosodium urate activate NALP3 (Hornung et al., 2008). How immune cells distinguish between PAMPs and DAMPs that are recognised by the same receptors, is not fully understood. One model suggests that binding of DAMPs initiates CD24 binding to Siglec G/10, resulting in reduced cytokine responses (Chen et al., 2009).

Although various cell types can express PRRs, they are mainly present on DCs, macrophages and endothelial cells. Innate immune stimulation of DCs induces their so-called maturation, characterised by increased expression of MHC class II molecules, T cell costimulatory molecules CD80 and CD86, adhesion molecules ICAM1 and CD58, and the lymph node homing CC-chemokine receptor 7 (CCR7), all of which serve to augment DC-T cell interactions (Banchereau and Steinman, 1998; Forster et al., 1999).

1.4.4 Dendritic cells can activate and polarise naive T cells

There is substantial interest in the role of DCs in influencing the functional polarisation of Th cell subsets (Kapsenberg, 2003) and in particular the mechanisms by which innate immune stimulation of DCs may modulate adaptive immune responses (van Riet et al., 2009). Following stimulation of PRRs expressed by DCs, they mature while migrating towards the lymph nodes where they can interact with naive Th cells. Only fully activated DCs express all the signals required for their interaction with pathogen-specific naive Th cells to drive the initiation of effector T cells (Kapsenberg, 2003). The ligation of the TCR with antigenic peptide presented by MHC class II molecules on the surface of DCs is the first signal that is essential for Th cell activation

(Davis and Bjorkman, 1988). However, TCR binding alone fails to induce complete Th cell activation, which requires a second or "costimulatory" signal (Jenkins et al., 1988). This costimulatory signal is provided by the binding of B7 molecules CD80 and CD86 that are expressed and upregulated on mature DCs, to their ligand, CD28, which is found on the surface of resting and activated T cells. Moreover, it is thought that a third signal is critical to determine the class of the Th cell immune response. In addition to the process of DC maturation, processing and presentation of antigenic peptides and the upregulation of costimulatory molecules, innate immune responses by DCs include the production of a number of cytokines that are associated with early polarisation of Th cell responses (Kapsenberg, 2003). For example IL-12 secretion is important for Th1 polarisation (de Jong et al., 2002) and IL-10 is associated with Treg responses (Hart et al., 2004). Similarly, IL-1 and IL-23 that promote induction of Th17 cells can also be produced by DCs (Khamri et al., 2010; van Beelen et al., 2007). It is less clear which cytokines drive Th2 responses, since IL-4 (major Th2-inducing cytokine)-deficient DCs can still induce the development of Th2 immune responses (MacDonald and Pearce, 2002) and several studies suggest that an important factor might be the reduced levels of IL-12 expressed by DCs that induce Th2 (de Jong et al., 2002; Lamhamedi-Cherradi et al., 2008). Therefore, the establishment of a defined cytokine environment by DCs is likely to be crucial for the initiation of the appropriate type of immune response. While DCs have been shown to secrete large quantities of cytokines, other adjoining innate immune cells, such as natural killer (NK) cells, NKT cells, γ:δ T cells, mast cells, eosinophils and basophils might also influence the cytokine microenvironment of the DC-T cell interaction, and thus direct the fate of the T cell response.

In addition it is increasingly evident that alternative innate immune stimuli induce different DC responses. Depending on the type of microorganism that is recognised by PRRs, the DC phenotype develops to drive Th1, Th2, Th17 or Treg

polarisation (Ahrens et al., 2009; Cervi et al., 2004; de Jong et al., 2002; Khamri et al., 2010; Smits et al., 2005). In particular whole genome microarrays to study gene expression changes make it possible to identify the regulation of common and distinct sets of genes activated by different TLR ligands (Jenner and Young, 2005). For example, the transcriptional response to TLR4 ligands is generally greater than the response to TLR2 stimulation and includes the upregulation of IFN-stimulated genes (Jenner and Young, 2005). Therefore stimulation of different PRRs may be expected to modulate Th cell polarisation. However, experimental data suggest much more complicated regulation of Th polarisation. For example, *Escherichia coli*, which stimulates TLR4, but also heat-killed *Listeria monocytogenes* (HKLM), which activates TLR2 have both been shown to induce Th1 responses, whereas TLR2 stimulation with helminth-derived PAMPs promote Th2 responses (van Riet et al., 2009). However more recent studies suggest that the induction of Th2 responses by *Schistosoma mansoni* is independent of TLR stimulation. It was shown that human monocyte-derived DCs primed with the glycoprotein omega-1 isolated from soluble egg antigen (SEA) from *S. mansoni* drive Th2-polarised responses from naive human $CD4^+$ T cells (Everts et al., 2009). Although the receptor of omega-1 is not known, this protein's functions are independent of MyD88 and TRIF signalling (Steinfelder et al., 2009) and it activates DCs most likely by binding to C-type lectin receptors (Meevissen et al., 2010). The molecular mechanisms responsible for differential Th polarisation are still not fully understood (MacDonald and Maizels, 2008; van Riet et al., 2009).

1.4.5 Activation of transcription factors

The activation of TLRs and other PRRs leads to signalling events that result in the expression of immunomodulatory factors such as cytokines and chemokines. This response is orchestrated by the activation of transcription factors, whose differential

activation might play a role in directing the fate of the immune response. A group of transcription factors, such as the NF-κB, IRFs or cyclic adenosine 5'-monophosphate (cAMP)-responsive element binding protein 1 (CREB1), that are constitutively expressed by many cell types including DCs play an important role in the initiation of an immune response. In many cases, these transcription factors are present in the cytoplasm in resting cells but are translocated into the nucleus following receptor-mediated activation.

The most studied and important transcription factors responsible for the expression of proinflammatory cytokines in response to TLR signalling are the NF-κB/Rel family of proteins. These consist of NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), RelB and c-Rel proteins which can form homo- and/or heterodimers. The NF-κB1/RelA (p50/p65) heterodimer is the most abundant in mammalian cells and is often referred to as simply NF-κB. In unstimulated cells, NF-κB is bound to inhibitory NF-κB (IκB) proteins that mask its nuclear localisation signals (NLSs) and inhibit its translocation into the nucleus (Huxford et al., 1998; Jacobs and Harrison, 1998). Moreover, IκB-α proteins contain a nuclear export signal (NES) that is also responsible for the mainly cytosolic pool of NF-κB–IκBα complexes in resting cells (Tam et al., 2000). Although various signals can cause nuclear translocation of NF-κB, generally all pathways converge on the IκB kinase (IKK) complex that is a key molecular switch in the activation of NF-κB. Members of the IKK family, such as IKKα, IKKβ, TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and inducible IκB kinase (IKKε), are serine threonine kinases responsible for the phosphorylation of key molecules that control the entry of a variety of transcription factors into the nucleus. The IKK protein complex, consisting of the essential regulatory subunit IKKγ, also called NF-κB essential modulator (NEMO), and the two protein kinases IKKα and IKKβ, is responsible for the

activation of NF-κB (DiDonato et al., 1997; Mercurio et al., 1997; Rothwarf et al., 1998; Zandi et al., 1997). Following stimulation with TNF-α that engages with TNF-α receptor 1 (TNFR1) and recruits the adaptor molecule TNFR1-associated death domain (TRADD) or stimulation with IL-1 or TLR ligands in conventional DCs that activate the MyD88 or TRIF pathways, IκB gets phosphorylated by the IKK family member IKKβ. The phosphorylation of IκB leads to its polyubiquitination and proteasomal degradation (Chen et al., 1995b; DiDonato et al., 1996; Lin et al., 1995; Scherer et al., 1995). Consequently, NF-κB is no longer bound in the cytoplasm and can shuttle into the nucleus where the Rel subunit, which contains a transactivation domain, can drive the transcription of over 400 target genes (www.nf-kb.org). Despite its strong homology to IKKβ, IKKα is not essential for the rapid NF-κB activation by proinflammatory signals that occurs within minutes (Hu et al., 1999). IKKα activates a different form of NF-κB dimers (NF-κB2/RelB) by controlling the processing of p100, which requires several hours and plays an important role in the formation of secondary lymphoid organs (Senftleben et al., 2001).

Additionally, stimulation of TLR3 and TLR4 in conventional DCs recruits the TRIF adaptor protein and can also lead to the activation of the IKK-related proteins TBK1 and IKKε. TBK1 and IKKε form heterodimers and phosphorylate IRF-3 (Fitzgerald et al., 2003; Hemmi et al., 2004; Sharma et al., 2003), which dimerises and translocates into the nucleus where it induces IFN-β expression (Yoneyama et al., 1998). It is interesting to note that RIG-I like receptor stimulation also activates TBK1 and IKKε, which then phosphorylate IRF-3 and also IRF-7, thereby inducing the expression of IFN-α and IFN-β (Kato et al., 2005).

In pDCs that only express TLR7 and TLR9, the stimulation of these receptors and the recruitment of MyD88 can lead to the activation of IKKα or IKKβ. Similar to the events in cDCs, IKKβ activation results in IκB degradation and NF-κB

translocation, while IKKα plays a critical role in the induction of IFN-α via phosphorylation of IRF-7 (Hoshino et al., 2006). Thus type I IFN expression in pDCs is primarily TLR and IKKα-mediated, while in conventional DCs RIG-I followed by TBK1 and IKKε activation is important (Kato et al., 2005).

Another transcription factor that is constitutively and ubiquitously expressed and has important roles in the immune response is CREB1. In contrast to NF-κB and IRFs that are mainly cytosolic in unstimulated cells, inactive CREB1 is also located in the nucleus. CREB1 has been studied mainly in the field of neurology, as it seems to play a critical role in the development of long-term memory (Silva et al., 1998) and in the therapeutic effect of antidepressants (Blendy, 2006), but lately CREB1 has attracted attention as an important transcription factor in the early innate immune response (Wen et al., 2010). Though CREB1 has also been associated with proinflammatory responses, like the induction of IL-6 (Spooren et al., 2010) or the upregulation of NALP1 (Sanz et al., 2004) that plays an important role in inflammasome mediated processing and activation of IL-1β (Faustin et al., 2007; Martinon et al., 2002), most of the literature suggests that it is rather involved in limiting proinflammatory signals (Alvarez et al., 2009; Ananieva et al., 2008; Hu et al., 2006; Martin et al., 2005; Park et al., 2008) and its activation can prevent endotoxic shock in mice (Ananieva et al., 2008; Martin et al., 2005).

Following stimulation, CREB1 becomes phosphorylated at serine 133 (p-CREB1), which triggers the recruitment of the coactivators CREB-binding protein (CBP) and its paralogue p300 (Arias et al., 1994; Chrivia et al., 1993), which strongly enhances CREB1-dependent transcription (Hagiwara et al., 1993). Originally it was shown that liberation of the catalytic subunits of cAMP-dependent protein kinase A (PKA) can provoke the phosphorylation of CREB1 in the nucleus (Gonzalez and Montminy, 1989). Additionally, various other protein kinase pathways have been

described that can induce phosphorylation of CREB1 Ser133: Ca^{2+} influx can trigger Ca^{2+}/calmodulin-dependent protein kinases II and IV (CaMKII/IV) (Matthews et al., 1994; Sun et al., 1994); ERK1/2 activation can phosphorylate p90 ribosomal S6 kinase (p90RSK) (Ginty et al., 1994; Xing et al., 1996); phosphorylated p38 MAPK can activate mitogen- and stress-activated protein kinases 1 and 2 (MSK1/2) (Wiggin et al., 2002); and phosphatidylinositol 3-kinase (PI3K) can trigger Akt/PKB, which can either directly phosphorylate CREB1 (Du and Montminy, 1998; Kato et al., 2007) or inhibit glycogen synthase kinase 3 (GSK3) that is an inhibitor of CREB1 (Martin et al., 2005). In response to Ca^{2+} influx, additional phosphorylation at Ser142 and Ser143 can also be selectively activated in neurons (Kornhauser et al., 2002). This triple phosphorylation disrupts the engagement of CREB1 with its cofactor CBP and so may activate a specific gene expression profile independent of CBP (Kornhauser et al., 2002).

It is likely that stimulation of different PRRs activates diverse signalling pathways that can result in activation of CREB1. In primary macrophages from mice it was shown that triggering of TLR2, 4 or 9 by Pam_3CSK4 (a synthetic bacterial lipopeptide), lipoteichoic acid (LTA), LPS or cytosine guanine dinucleotide (CpG) DNA respectively, led to phosphorylation of CREB1, which was reduced by a combination of inhibitors of p38 and ERK1/2 (Ananieva et al., 2008). Knockout experiments demonstrated that the kinases MSK1 and MKS2 that are activated downstream of p38 MAPK and ERK1/2 were responsible for the induced phosphorylation of CREB1 (Ananieva et al., 2008). Moreover, macrophages of MSK1/2 double knockouts produced increased amounts of proinflammatory cytokines TNF, IL-6 and IL-12, but reduced expression of IL-10 (Ananieva et al., 2008). Chromatin immunoprecipitation (ChIP) experiments found p-CREB1 and/or phosphorylated activating transcription factor 1 (p-ATF-1; which is related to CREB1 and cannot be distinguished by antibodies) bound to the promoter of *il-10*, and substitution of

serine 133 in CREB1 resulted in reduced expression of the CREB1 target gene *nur77* and also in impaired IL-10 expression in response to LPS (Ananieva et al., 2008). Thus MSK1- and MSK2-mediated activation of CREB1 in response to TLR signalling might acts as a negative feedback loop to control TLR-driven inflammation.

A different study with human monocyte-derived macrophages (MDMs) demonstrated that IFN-γ can inhibit TLR2 mediated IL-10 production and increase levels of TNF-α (Hu et al., 2006). Here, IFN-γ inhibited phosphorylation of p38 and ERK1/2 and so reduced activation of CREB1 (Hu et al., 2006). Moreover IFN-γ also suppressed the phosphorylation of Akt and increased activity of GSK3, which is a negative regulator of CREB1 (Hu et al., 2006). The importance of the PI3K-Akt pathway in inhibiting GSK3 and thus activation of CREB1-mediated IL-10 expression had also been highlighted in response to TLR2, TLR4, TLR5, and TLR9 stimulation (Martin et al., 2005). Moreover it was demonstrated that inhibition of GSK3 reduced the production of proinflammatory cytokines IL-1β, IFN-γ, IL-12p40 and IL-6. Small interfering RNA (siRNA) experiments targeting CREB1 confirmed that the instruction of pro- or anti-inflammatory cytokine production was dependent on GSK3-regulated activity of CREB1. Inhibition of GSK3 increased DNA-binding properties of p-CREB1 and enhanced its association with the coactivator CBP, while reducing interactions between NF-κB p65 and CBP. Despite the fact that the coactivator proteins CBP and p300 associate with numerous transcription factors (Vo and Goodman, 2001), evidence suggests that there is only a limited amount of them present in each cell (Hottiger et al., 1998; Petrij et al., 1995). Since these proteins participate in the effects of both CREB1 and NF-κB it is likely that they have to compete for them in the nucleus.

The role of GSK3 in regulating pro- or anti-inflammatory cytokine responses via differential activation of CREB and NF-κB was also seen in a study with *Francisella tularensis* LVS infection of murine peritoneal macrophages (Zhang et al., 2009).

A recent study using human monocyte-derived DCs demonstrated that the expression of the immunoregulatory cytokine IL-10 in response to zymosan stimulation was also regulated by CREB1 (Alvarez et al., 2009). Binding of p-CREB1 to the *il-10* promoter in response to zymosan stimulation, was dependent on PKA activity (Alvarez et al., 2009). Moreover treatment with the cAMP inducer PGE2 or directly 8-bromo-cAMP, which activates PKA, enhanced the expression of IL-10 (Alvarez et al., 2009). Interestingly blockage of NF-κB increased the production of IL-10 (Alvarez et al., 2009), supporting the view that the balance between NF-κB and CREB1 activity might play an important role in shaping the cytokine response of DCs.

1.4.6 Role of dendritic cell-mediated immunomodulation in the context of the hygiene hypothesis

With regards to the hygiene hypothesis, many studies have focused on the immune responses of DCs following interaction with probiotics, helminths and mycobacteria, which are associated with induction of regulatory immune responses. A common result of these studies is that different species seem to have different effects.

Amongst studies with probiotics, one showed that *Bifidobacterium* strains but not lactobacilli augmented IL-10 expression in $CD11c^+$ and $CD11c^-$ DCs enriched from human blood or intestinal lamina propria (Hart et al., 2004). Moreover, only DCs primed with individual strains, *B. longum* and *B. infantis*, reduced IFN-γ expression in cocultured T cells (Hart et al., 2004). Other studies showed that monocyte-derived DCs primed with specific lactobacilli species can drive the development of Treg cells that produced increased levels of IL-10 and inhibited the proliferation of bystander T cells in an IL-10-dependent fashion (Smits et al., 2005). These active strains were found to bind the C-type lectin receptor DC-SIGN, and generation of Treg cells was DC-SIGN

dependent (Smits et al., 2005). A recent study with mice selected probiotic strains according to their ability to induce high IL-10/IL-12 production ratios and enhanced FoxP3 expression in freshly isolated MLN cells (Kwon et al., 2010). A mixture of the most effective strains was fed to mice, which upregulated FoxP3 expression in $CD25^-$ T cells and enhanced suppressor activity of nTreg cells. The FoxP3 expression was shown to depend on augmented TGF-β, cyclooxygenase-2 (COX-2) and IDO secretion by $CD11c^+$ DCs (Kwon et al., 2010).

Infections with helminths are usually associated with a Th2 response, and several studies have shown that stimulation of murine or human DCs *in vitro* with antigen mixtures or single molecules from helminths yields populations of DCs that have the potential to drive naive T cells towards a Th2 type (van Riet et al., 2007). However, chronic infection with parasitic worms can lead to an anti-inflammatory response with enhanced production of IL-10 and suppressed T cell proliferation. Fractions of schistosomes capable of exerting such effects have been identified. Whereas a water-soluble extract of SEA modulated DCs towards a Th2-driving type, the lipid lyso-phosphatidylserine (PS) extracted from schistosome eggs and adult worms led to the development of DCs that promote IL-10 expressing Treg cells (van der Kleij et al., 2002). As in the case of some *Lactobacillus* strains (Foligne et al., 2007), stimulation of TLR2 on DCs by PS was crucial for this effect (van Riet et al., 2007).

1.5 Mycobacterial interactions with dendritic cells

Activation of the innate immune system by mycobacteria can involve a range of PAMP-PRR interactions shown in Table 1-3. In mammalian cells several mycobacterial products and whole live mycobacteria predominantly induce innate immune cellular

activation via TLR2 (Heldwein and Fenton, 2002; Krutzik et al., 2003), although some species specificity has been reported (Lien et al., 1999).

Table 1-3: PPR recognition of mycobacterial components

PPR	Mycobacterial Components	Species and References
TLR1/2	LpqH lipoprotein	*M. tuberculosis, M. bovis* (Brightbill et al., 1999; Drage et al., 2009; Noss et al., 2001)
TLR1/2	PhoS1 lipoprotein	*M. tuberculosis* (Drage et al., 2009; Jung et al., 2006)
TLR1/2	LprG lipoprotein	*M. tuberculosis, M. bovis* (Drage et al., 2009; Gehring et al., 2004)
TLR2/2	LprA lipoprotein	*M. tuberculosis, M. bovis* (Drage et al., 2009; Pecora et al., 2006)
TLR2	27 kDa lipoprotein	*M. tuberculosis* (Hovav et al., 2004)
TLR2	33 kDa lipoprotein	*M. leprae* (Krutzik et al., 2003)
TLR2	Ara-LAM	*M. smegmatis* (Wieland et al., 2004)
TLR2	Glycopeptidolipid	*M. avium* (Sweet and Schorey, 2006)
TLR2	Lipomannan	*M. tuberculosis, M. bovis* (Quesniaux et al., 2004)
TLR2	PE_PGRS33	*M. tuberculosis* (Basu et al., 2007)
TLR2	PIM2, PIM6	*M. tuberculosis, M. bovis, M. smegmatis* (Gilleron et al., 2003)
TLR2	PILAM	*M. smegmatis* (Heldwein and Fenton, 2002)
TLR2	Soluble tuberculosis factor	*M. tuberculosis* (Means et al., 1999)
TLR4	PhoS1 lipoprotein	*M. tuberculosis* (Jung et al., 2006)
TLR2/TLR4	HSP70	*M. tuberculosis* (Bulut et al., 2005)
TLR4	HSP65	*M. tuberculosis* (Bulut et al., 2005)
TLR9	DNA	*M. bovis, M. tuberculosis, M. paratuberculosis* (Kiemer, 2009; Pott et al., 2009)
Mannose receptor	Man-LAM	*M. bovis, M. tuberculosis* (Nigou et al., 2001; Quesniaux et al., 2004)
Mannose receptor	PIMs	*M. tuberculosis* (Torrelles et al., 2006)
DC-SIGN	Man-LAM	*M. bovis, M. tuberculosis,* but not *M. smegmatis* (Maeda et al., 2003)
DC-SIGN	alpha-glucan	*M. tuberculosis* (Geurtsen et al., 2009)
DC-SIGN	PIMs	*M. tuberculosis* (Torrelles et al., 2006)
Dectin-1		*M. avium, M. tuberculosis, M. abscessus* (Rothfuchs et al., 2007; Shin et al., 2008; Yadav and Schorey, 2006)
Nod1	meso-diaminopimelic acid	*M. paratuberculosis* (Pott et al., 2009)
Nod2	Peptidoglycan-derived muramyl dipeptide	*M. tuberculosis* (Coulombe et al., 2009)
Nod2		*M. paratuberculosis* (Ferwerda et al., 2007)
CCR5	HSP70	*M. tuberculosis* (Floto et al., 2006; Whittall et al., 2006)

Most work on mycobacterium-DC interactions has been performed with *M. tuberculosis* or *M. bovis* BCG, which showed inconsistent findings suggesting induction of Th1-like, Th2-like, Th17-like and also Treg-like immune responses.

Monocyte-derived DCs that were matured with LPS with or without live BCG showed different phenotypes upon additional stimulation with CD40 ligand. BCG lowered the levels of IL-12p70 and increased cytokine secretion of IL-10 in a dose-dependent manner (Madura Larsen et al., 2007). Interestingly with regards to T cell polarisation, BCG-treated DCs were cocultured with naive T cells and decreased the expression of both, Th1 (IFN-γ) and Th2 (IL-4) cytokines but showed increased levels of IL-10, which is associated with a regulatory phenotype (Madura Larsen et al., 2007). Another study suggesting the induction of immunoregulatory properties was performed with macrophages that, when infected with *M. tuberculosis* or BCG, showed IL-10-dependent reduction in expression of MHC class II molecules (Sendide et al., 2005).

Many other studies showed that DCs derived from monocytes and treated with *M. tuberculosis* (Mariotti et al., 2002), BCG (Gagliardi et al., 2005; Gagliardi et al., 2004) or mycobacterial compounds like mannose-capped lipoarabinomannan (Man-LAM) (Johansson et al., 2001) had reduced expression levels of IL-12 and increased levels of IL-10. Another report suggested differences between macrophages and DCs: DCs infected with *M. tuberculosis* secreted IL-12 and supported Th1 type T cells, whilst IL-10 production by infected macrophages inhibited the production of IL-12 (Giacomini et al., 2001).

A recent study compared the effect of various BCG strains on bone marrow-derived mouse DCs stimulated with OVA and showed an increased production of IL-10 and IL-12 (Ahrens et al., 2009). Subsequent coculture experiments with allergen-specific T cells led to reduced IL-5, IL-13 and IFN-γ expression but more IL-10 and

FoxP3$^+$ T cells (Ahrens et al., 2009). However, when allergic mice were treated with BCG *in vivo*, they showed enhanced Th1-associated neutrophilic airway inflammation in association with reduced Th2 and enhanced Treg responses (Ahrens et al., 2009).

DCs inducing a Th2-like response were generated with human monocytes, which were infected with BCG prior to their differentiation into DCs with IL-4 and GM-CSF (Martino et al., 2004). The DCs had a matured phenotype, secreted proinflammatory cytokines and IL-10, but not IL-12, and stimulated allogeneic T cells. Upon coculture with cord blood mononuclear cells they produced the Th2 associated cytokine IL-4, but no IFN-γ (Martino et al., 2004).

More recently it was shown that *M. tuberculosis* treated monocyte-derived DCs induced the secretion of IFN-γ and IL-17 by CD4$^+$ Th cells (Zenaro et al., 2009). The authors demonstrated that the generation of Th17-polarising DCs was dependent on binding of the dectin-1 receptor, whereas engagement with DC-SIGN or the mannose receptor inhibited Th17 and augmented Th1 generation (Zenaro et al., 2009).

In addition, comparisons with other mycobacterial species, such as *M. leprae,* highlight differences in their effects on DCs. Whereas *M. tuberculosis* and BCG induced upregulation of costimulatory markers in immature monocyte-derived DCs in a dose-dependent fashion, *M. leprae* failed to induce maturation and the resulting cytokine profile was similar to that of immature DCs (Murray et al., 2007).

No data are available on DCs interactions with *M. vaccae*. However it was suggested that *M. vaccae* modulates T cell responses via DCs to suppress unwanted effector T cell responses responsible for allergies and autoimmune diseases (Figure 1-2).

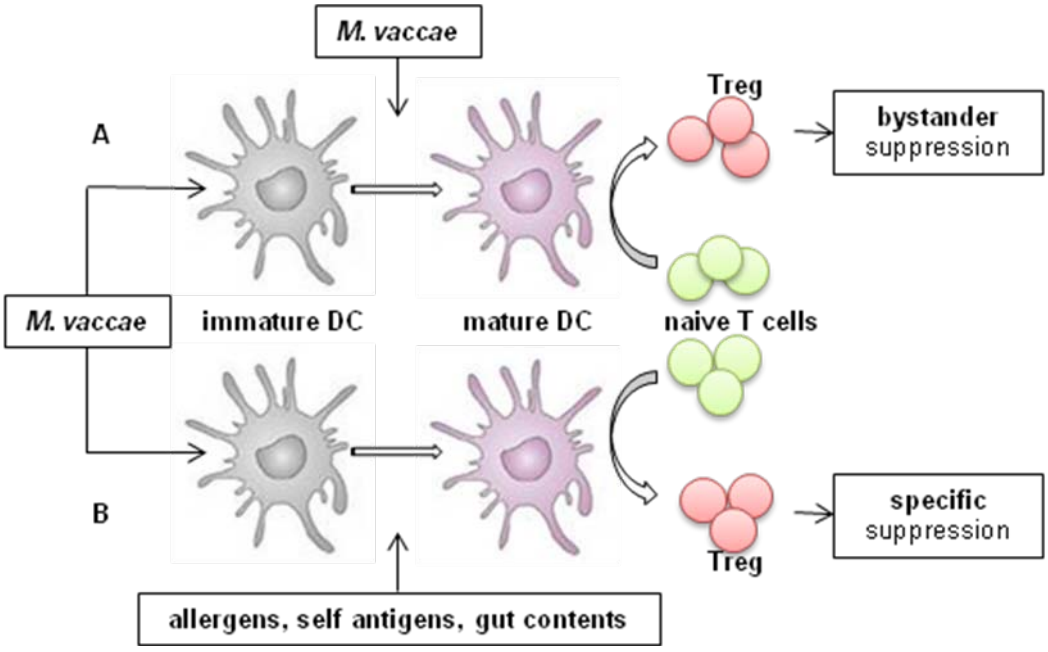

Figure 1-2: The prevailing view of *M. vaccae*'s mode of action (adapted from (Rook, 2007)).

M. vaccae is detected by PRRs on DCs that mature and drive regulatory T cell responses to the antigens of *M. vaccae* **A**: The regular presence of *M. vaccae* in untreated food or drinking water leads to permanent background release of regulatory cytokines from these Treg cells, generating bystander suppression of other immune responses. **B**: The increased number of Treg-polarising DCs can also process allergens, self-antigens and gut content antigens. Consequently, there are increased numbers of Treg cells specifically triggered by these antigens, which downregulate allergies, autoimmunity or inflammatory bowel diseases.

1.6 Real time quantitative PCR normalisation using SINEs

Transcriptional responses are a major component of innate immune responses by DCs. These are commonly quantified by reverse transcription real time quantitative polymerase chain reaction (RT-qPCR). In this methodology, expression levels of genes of interest are typically normalised to so-called housekeeping genes, which should have constant levels of gene expression. Previous reports have suggested that mycobacteria may induce changes in expression levels of several conventional reference genes that confound assessment of target genes (Dheda et al., 2004). The present gold standard for normalising gene expression data is to use multiple reference genes (Vandesompele et al., 2002b), which is resource- and labour-intensive. In the context of studying DC responses to *M. vaccae*, the opportunity to assess a novel normalisation method for

qPCR was considered. In place of conventional reference genes, short interspersed repetitive element (SINE) sequences are amplified with this approach. SINEs derived from retrotransposons and have integrated frequently into mammalian genomes (Lander et al., 2001; Waterston et al., 2002). Many of them occur also in untranslated regions of mRNA and get therefore transcribed together with numerous genes (Hasler et al., 2007). The number of expressed SINEs is due to their abundance expected to be constant, which makes them excellent candidates for stable reference sequences.

1.7 Aims

In vivo mouse studies and clinical studies in man find that *M. vaccae* downregulates Th2 immune responses, increases regulatory T cells and may increase Th1 type responses. As a result there has been extensive interest in its therapeutic application for allergic or inflammatory diseases and augmentation of cell mediated immunity, but the mechanisms for these effects are not known. In view of the pivotal role of DCs in induction and regulation of T cell responses, the principle aims of my thesis are to:

1. Characterise innate immune responses by DCs to stimulation with *M. vaccae*, at the level of innate immune receptors, intracellular signalling, transcriptional and protein responses.
2. Test the hypothesis that *M. vaccae*-primed DCs support Treg responses and reduce Th2-polarised responses.
3. Investigate the mechanism of *M. vaccae* effects on DC-mediated modulation of T cell responses.
4. Test the use of SINEs for normalisation of RT-qPCR analyses.

2 MATERIAL AND METHODS

2.1 Buffers and solutions

Table 2-1: Summary of buffers and solutions

Abbreviated Name	Composition
BBS	3.63 mg/ml sodium tetraborate, 5.25 mg/ml boric acid, 6.19 mg/ml sodium chloride, 0.0005 % v/v polysorbate 80, pH 8.0
FACS buffer	PBS pH 7.4, 1 % FBS, 0.02 % sodium-azide
Fixation buffer	PBS pH 7.4, 3.7 % paraformaldhyde
Freezing mix	FBS, 10 % DMSO
PBS (10x)	80g NaCl, 2.0g KCl, 14.4g Na_2HPO_4, 2.4g KH_2PO_4, adjust to pH 7.4 and to 1 l with dH_2O
PBS/Tween	PBS pH 7.4, 0.05% v/v Tween-20
Running buffer	250 mM Glycine, 25 mM Tris-base, 0.1 % (w/v) SDS
Sample buffer (4x)	2.4 ml 1 M Tris-HCL pH 6.8, 0.8g SDS, 4 ml 100 % Glycerol, 0.01 % bromphenol blue, 1 ml % β-mercaptoethanol, 2.8 ml dH_2O
Stripping buffer	20 ml 10 % SDS, 12.5 ml 0.5 M Tris-HCl pH 6.8, 67.5 ml dH_2O, 0.8 ml β-mercaptoethanol
TAE buffer (50x)	242 g Tris-base, 100 ml of 0.5 M EDTA pH 8.0, adjust to 1 l with dH_2O
TBS (10x)	24.23 g Tris, 80.06 g NaCl, adjust to 1 l with dH_2O and to pH 7.6 with HCl
TBS/Tween	TBS pH 7.6, 0.05% v/v Tween-20
Transfer buffer	800 ml Running buffer, 200 ml methanol

2.2 Mycobacterium vaccae and TLR ligands

M. vaccae strain NCTC 11659 was manufactured by Eden Biodesign (Liverpool, UK), as a preparation containing 319 mg/ml of heat-killed *M. vaccae*. 1 mg of *M. vaccae* equals 10^9 colony forming units (CFU). *M. vaccae* was grown for 14 days in a fermenter in liquid Sauton Synthetic Medium, autoclaved and suspended in borate-buffered saline (BBS). The material (MV06 and MV07) was manufactured under good manufacturing practice guidelines. To test TLR9 activation, a sample of *M. vaccae* was sonicated for 2 minutes on ice using a sterilised probe sonicator (Jencons Scientific, Leighton Buzzard, UK) at 10 mA amplitude and 40 Hz.

Lipopolysaccharide (LPS) from *Salmonella enterica* serotype typhimurium (L6143/045K4056) was obtained from Sigma Aldrich (Dorset, UK). Pam_3CSK4 (synthetic tripalmitoylated lipopeptide), heat-killed *Listeria monocytogenes*, FSL-1, Poly(I:C), Flagellin, Gardiquimod, CL075 and OND2006 were purchased from InvivoGen (Toulouse, France).

2.3 Media

2.3.1 Medium for human primary cells

Roswell Park Memorial Institute (RPMI)-1640 was purchased from GIBCO (Invitrogen, Paisley, UK). Complete RPMI-1640 was made by supplementing with 10 % foetal bovine serum (FBS) (Biosera, East Sussex, UK), 100 U/ml penicillin and 100 U/ml streptomycin (both Invitrogen).

2.3.2 Medium for cell lines

To obtain complete Dulbecco's modified eagle's medium (DMEM, Invitrogen), it was supplemented like RPMI-1640 with 10 % FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and in addition with 100 µg/ml normocin (InvivoGen).

2.3.3 Medium for bacteria

Lysogeny broth (LB) medium contained 10 g tryptone, 5 g yeast-extract and 5 g NaCl per 1 l medium (all Sigma Aldrich). Ampicillin (Sigma Aldrich) was added at a concentration of 100 µg/ml. Solid medium plates were generated by adding 15 g/l agar-agar (Sigma Aldrich) to the liquid medium.

2.4 Cells

All tissue culture work was performed in class II safety cabinets, using sterile technique. Long and short term cell culture incubations were carried out at 37 °C with 5 % CO_2. All tissue culture plates, flasks and petri dishes were purchased from TPP (Trasadingen, Switzerland).

2.4.1 TLR reporter cell lines

Engineered human embryonic kidney (HEK) 293 cells stably transfected with the CD14 gene alone or together with the TLR2 or TLR4 gene, respectively, were a gift from Professor M. Yazdanbakhsh (Department of Parasitology, Leiden University Medical Centre). Plasmid containing cells were selected by adding 5 µg/ml puromycin (InvivoGen) to the complete DMEM culture medium. HEK 296 cells expressing TLR1/2, TLR2/6, TLR3, TLR5, TLR7, TLR8, or TLR9 (InvivoGen) were selected by supplementing complete DMEM with 10 µg/ml of blasticidin (InvivoGen).

2.4.1.1 Passaging of cell lines

HEK 293 transfected cell lines were grown in 75 cm^2 flasks and passaged just before reaching confluency, usually two times per week. The medium was aspirated and the cell layer washed once with phosphate-buffered saline (PBS) (Invitrogen). Then, 0.5 ml Trypsin-EDTA (Sigma Aldrich) was added and the flasks were returned to the incubator for 2-3 minutes. Cells were split 1:5 into the appropriate medium.

2.4.1.2 Freezing and thawing of cell lines

HEK 293 transfected cells for freezing were harvested by trypsinisation, spun down and re-suspended at a density of 3-5 x 10^6 cells/ml in growth medium supplemented with 10 % dimethylsulfoxide (DMSO) (Sigma Aldrich) and transferred to cryovials (Nunc, Roskilde, Denmark). Vials were stored for 24-48 hours at -80 °C in a

freezing container (Nunc) and then transferred into liquid nitrogen for long time storage. Cells to be thawed were incubated at 37 °C in a water bath and then transferred to a 50 ml tube containing 15 ml of pre-warmed growth medium. Cells were centrifuged, re-suspended and plated in the appropriate medium in 25 cm^2 flasks.

2.4.2 Generation of human monocyte-derived dendritic cells

A volume of 120 ml peripheral blood was drawn into heparinised tubes from healthy adult volunteers who had given informed consent. PBMCs were isolated on a density gradient with Lymphoprep (Axis-Shield, Oslo, Norway) by centrifugation at 400 x g for 20 minutes. After repeated washing with Hank's buffered salt solution (HBSS) (Invitrogen), monocytes were isolated by magnetic separation using CD14 MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. The $CD14^+$ cells were cultured in complete RPMI-1640 supplemented with 100 ng/ml human recombinant GM-CSF and 50 ng/ml IL-4 (both gifts from Schering-Plough Research Institute, Kenilworth, NJ, USA). After 4-day incubation these cells were harvested and immediately used as immature DCs. The $CD14^-$ population of PBMCs was cryopreserved in FBS containing 10 % DMSO (Sigma Aldrich) and stored in liquid nitrogen at a concentration of 10^7 cells/ml.

2.4.3 Isolation of naive $CD4^+$ T cells

The cryopreserved $CD14^-$ population of PBMCs was rapidly thawed in a water bath of 37 °C and washed in complete RPMI-1640. Naive $CD4^+$ T cells were isolated using the Naive $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec) according to manufacturer's instructions. This method depleted $CD8^+$, γ:δ, memory and activated T cells, B cells, monocytes, NK cells and macrophages, using the following cocktail of antibodies: CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56,

CD123, anti-TCRγ/δ, anti-HLA-DR, and CD235a (glycophorin A). The negatively selected naive T cells were used immediately in coculture experiments with DCs.

2.4.4 Isolation of total $CD4^+$ T cells

Cryopreserved $CD14^-$ PBMCs were rapidly thawed in a water bath of 37 °C and washed in complete RPMI-1640. Total $CD4^+$ T cells were isolated by negative selection with the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec) according to manufacturer's instructions. Isolated $CD4^+$ T cells were used immediately in coculture experiments with DCs.

2.5 Analysis of TLR activation

TLR-transfected HEK 293 reporter cells were seeded at 35000 cells/well into 96-well microtiter plates. After a resting period of 24 hours, the cells were stimulated overnight with optimised concentrations of positive control TLR ligands (InvivoGen) (see Table 2-2), a dilution series of *M. vaccae* (MV) or 125 ng/ml of TNF-α. For stimulation of HEK 293-CD14/TLR4 cells, 12.5 % supernatant of MD-2 transfected cells (a kind gift from Professor M. Yazdanbakhsh) was added. The activation of the cells was assessed by measuring IL-8 secretion in the supernatants via ELISA (R&D Systems, Minneapolis, MN, USA) (see 2.12.1).

Table 2-2: Optimised concentrations of dilution series for positive control ligands for TLR stimulation

Expressed gene(s)	Positive ligand	Optimised concentration of dilution series for positive ligands			
TLR1/2	Pam_3CSK4	62.5 ng/ml	125 ng/ml	250 ng/ml	500 ng/ml
TLR2/CD14	Heat-killed *Listeria monocytogenes*	1.25*10^7 bac/ml	2.5*10^7 bac/ml	5*10^7 bac/ml	1*10^8 bac/ml
TLR2/6	FSL-1	25 ng/ml	50 ng/ml	100 ng/ml	200 ng/ml
TLR3	Poly(I:C)	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
TLR4/CD14	LPS	0.1 ng/ml	1 ng/ml	10 ng/ml	100 ng/ml
TLR5	Flagellin	0.5 µg/ml	1 µg/ml	2 µg/ml	4 µg/ml
TLR7	Gardiquimod	0.625 µg/ml	1.25 µg/ml	2.5 µg/ml	5 µg/ml
TLR8	CL075	5 µg/ml	10 µg/ml	20 µg/ml	40 µg/ml
TLR9	OND2006	0.3125 µM	0.625 µM	1.25 µM	2.5 µM

2.6 Stimulation of immature dendritic cells

Day 4 purified DCs were harvested and washed in complete RPMI-1640 and seeded at 10^6 cells/well in 6-well plates. DCs were stimulated by adding LPS (100 ng/ml), Pam_3CSK4 (1 µg/ml) or *M. vaccae* (10 µg/ml (MV10), 100 µg/ml (MV100), or 1000 µg/ml (MV1000)) for 0.5-48 hours. As a negative control, DCs were treated with media only (unstimulated DCs). For antigen-specific coculture experiments with autologous $CD4^+$ T cells, 10 µg/ml of tetanus toxoid (National Institute for Biological Standards and Control, UK) was added in addition to the innate immune stimuli. For signalling pathway inhibitor experiments, DCs were treated 2 hours prior to innate immune stimulation with the different inhibitors indicated in Table 2-3.

Table 2-3: Signal transduction pathway inhibitors

Inhibitor	Target	Concentration	Manufacturer
H89	PKA	1.5 – 50 µM	Sigma Aldrich
PKI-(6-22)-amide	PKA	1 – 10 µM	Biomol
LY294002	PI3K	25 µM	Calbiochem
SB203580	p38	25 µM	Calbiochem
SQ22536	adenylate cyclase	100 µM	Calbiochem
PD98059	MEK1	25 µM	Calbiochem

2.7 Coculture of dendritic cells and allogeneic naive $CD4^+$ T cells

DCs that were primed in 6-well plates for 24 hours with different stimuli (see 2.6) were harvested, washed and seeded into round-bottomed 96-well microtiter plates at 0, 10, 100, and 1000 cells/well. 10^5 allogeneic naive $CD4^+$ T cells were added to the DCs. Unstimulated control DCs were analysed after 24 hours for maturation by flow cytometry to verify that handling of the cells did not induce maturation. After 3 day coculture the cells were assed for proliferation (2.14), intracellular cytokine production (2.11.3.2) and IL-10 secretion (2.12.2), and after 6 day coculture for IL-10 secretion (2.12.2) and CD25 and FoxP3 expression (2.11.3.1). The experimental paradigm is summarised in Figure 2-1.

2.8 Coculture of dendritic cells and autologous CD4$^+$ T cells

DCs, which were stimulated for 24 hours with different innate immune stimuli together with tetanus toxoid (TT) (see 2.6) were harvested, washed and seeded into round-bottomed 96-well microtiter plates at 0, 10, 100, 1000 and 10000 cells/well. 10^5 autologous total CD4$^+$ T cells were added to the DCs. Control DCs primed only with TT were analysed after 24 hours for maturation by flow cytometry to verify that handling of the cells did not induce maturation.

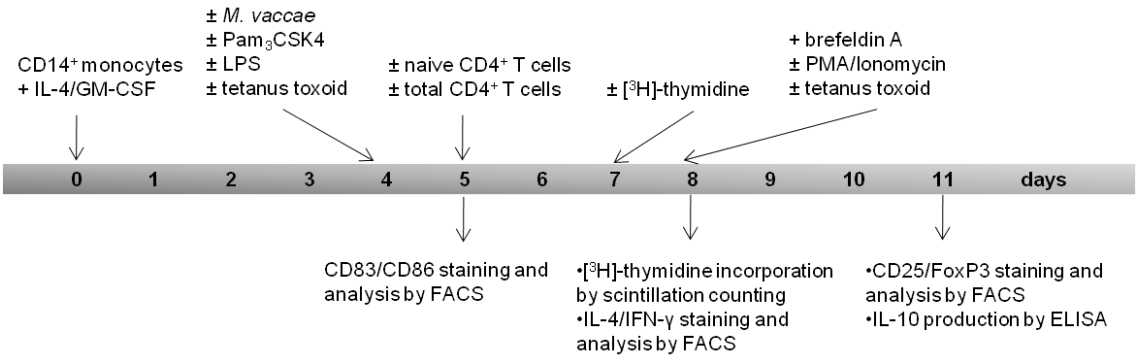

Figure 2-1: Outline of the study protocol of dendritic cell / T cell coculture experiments

PBMCs sorted for CD14$^+$ monocytes where cultured with IL-4 and GM-CSF to generate DCs. On day 4, DCs were stimulated and analysed for maturation after 24 hours. On day 5, T cells were added. On day 7, [^{3}H]-thymidine was added to measure T cell proliferation 18 hours later. On day 8, T cells were re-stimulated with PMA/Ionomycin or tetanus toxoid (TT) together with brefeldin A. After 4 hours, the T cells were analysed for cytokine production by intracellular immunofluoresence staining. On day 11, the supernatants were analysed for IL-10 secretion by ELISA and the T cells were stained for CD25 and FoxP3 expression.

2.9 Culture of naive CD4$^+$ T cells with conditioned media

A volume of 50 µl mouse anti-human CD3 antibody (clone SK7, eBioscience) at a 1:1000 dilution in HBSS was added to flat-bottom 96-well tissue culture plates. After 24 hours of incubation at 4 °C, the plates were washed once with HBSS. 10^5 naive CD4$^+$ T cells were added to each well in 100 µl complete RPMI-1640. Stimulated DC cultures (see 2.6) were spun down and 100 µl of the supernatants together with 1:1000 diluted mouse anti-human CD28 antibody (clone CD28.6, eBioscience) was added to

the allogeneic naive $CD4^+$ T cells. Cells were analysed after 3 days for IL-4 and IFN-γ production by intracellular flow cytometry (see 2.11.3.2).

2.10 Coculture of fixed dendritic cells with allogeneic naive $CD4^+$ T cells

DCs those were primed in 6-well plates for 24 hours with different stimuli (see 2.6) were harvested and washed in HBSS. Half of the cells were re-suspended in 500 µl HBSS and incubated for 30 seconds with 0.05 % glutaraldehyde (final concentration, Sigma Aldrich). The fixation was stopped by adding 2 ml of complete RPMI-1640. To remove excess glutaraldehyde, fixed DCs were washed twice and seeded into round-bottomed 96-well microtiter plates at 0, 10, 100, and 1000 cells/well. 10^5 allogeneic naive $CD4^+$ T cells were added to the DCs. Cells were analysed after 3 days for IL-4 and IFN-γ production by intracellular flow cytometry (see 2.11.3.2).

2.11 Flow cytometry

2.11.1 Antibodies for flow cytometry

Table 2-4: Antibodies for cell staining and detection by flow cytometry

Antibody (host/isotype)	Fluorophor	Clone	Dilution	Manufacturer
TLR2 (mouse IgG2b)	none	383936	≤ 20µg/ml	R&D systems
TLR2 (mouse IgG2a, κ)	none	TL2.1	≤ 20µg/ml	eBioscience
mouse IgG2b isotype	none		≤ 20µg/ml	R&D systems
mouse IgG2a, κ isotype	none	eBM2a	≤ 20µg/ml	eBioscience
anti-mouse IgG (rabbit)	FITC	polyclonal	1:50	DakoCytomation
CD83 (mouse IgG1, κ)	FITC	HB15e	1:10	BD Biosciences
CD86 (mouse IgG1, κ)	PE	2331 (FUN-1)	1:50	BD Biosciences
CD209 (mouse IgG2b, κ)	APC	DCN46	1:50	BD Biosciences
CD4 (mouse IgG1, κ)	PE	L200	1:20	BD Biosciences
CD25 (mouse IgG1, κ)	PE-CY7	M-A251	1:20	BD Biosciences
FoxP3 (mouse IgG1)	AF-674	259D/C7	1:15	BD Biosciences
IL-4 (mouse IgG1, κ)	PE	8D4-8	1:50	BD Biosciences
IFN-γ (mouse IgG1, κ)	APC	B27	1:50	BD Biosciences
IL-10 (rat IgG2a)	APC	JES3-19F1	1:5	BD Biosciences
IL-17A (mouse IgG1, κ)	PE	SCPL1362	1:5	BD Biosciences
annexin-V (mouse IgG2a, κ)	APC	unknown	1:20	eBioscience

All antibodies were used at pre-tested titrated quantities or diluted as recommended by the manufacturer. FITC: fluoresceine isothiocyanate; PE: phycoerythrine; APC: allophycocyanin; AF: Alexa-Flour.

2.11.2 Cell surface immunofluorescence staining

Cells were harvested, washed once in PBS and re-suspended in fluorescence activated cell sorting (FACS) buffer. If not indicated differently, cells were spun down for 5 minutes at 4 °C and 400 x g. Cells were blocked for 30 minutes using 10 % goat serum (Sigma Aldrich) at 4 °C. Cells were stained with the antibodies listed in Table 2-4 diluted in FACS buffer. Briefly, directly conjugated antibodies were added at the appropriate concentrations and incubated for 30 minutes at 4 °C in the dark and washed twice. When the primary antibody was unconjugated, the procedure was the same, but there was an additional incubation with the secondary antibody for 30 minutes at 4 °C in the dark. After two washes the cells were fixed in 3.7 % paraformaldehyde (PFA) and examined by flow cytometry within 24 hours using a FACScan flow cytometer or a FACSArray Bioanalyzer System (both BD Biosciences, San Jose, USA). Data were analysed with FlowJo software (Tree Star, Inc, Ashland, USA).

2.11.3 Intracellular immunofluorescence staining

2.11.3.1 FoxP3 staining

Intracellular FoxP3 staining was conducted following surface staining for CD25 and CD4 (see 2.11.2). Cells were spun down for 10 minutes at room temperature and 250 x g. Cells were fixed, permeabilised and stained using the Human FoxP3 Buffer Set (BD Pharmingen) according to manufacturer's instructions. Briefly, following surface staining, cells were washed once in FACS buffer and incubated in Buffer A (fixative) for 10 minutes at room temperature in the dark. Cells were washed once, spun down for 5 minutes at room temperature and 500 x g and permeabilised with Buffer C for

30 minutes at room temperature in the dark. After two washes and spinning as before, cells were stained with anti-FoxP3 (see Table 2-4) for 30 minutes at room temperature in the dark. After two washes, cells were re-suspended in PBS containing 1 % PFA and examined by flow cytometry within 24 hours.

2.11.3.2 Cytokine staining

Intracellular cytokine staining in T cells was conducted after addition of 7.5 µg/ml ionomycin, 125 ng/ml phorbol myristate acetate (PMA) and 25 µg/ml Brefeldin A (all Sigma Aldrich) (or 10 µg/ml TT and 25 µg/ml Brefeldin A in antigen-specific experiments) for the last 4 hours of culture. Supernatants were removed after centrifugation and cells were incubated for 15 minutes at 4 °C with 1:20 diluted human FcR Blocking Reagent (Miltenyi Biotec). Cells were then fixed in FACS buffer containing 3.7 % PFA at 4 °C for 15 minutes. After two washes, cells were either stained immediately or stored in FACS buffer for up to 24 hours at 4 °C.

FACS buffer was removed and cells were permeabilised with BD Perm/Wash buffer (BD Biosciences) for 15 minutes at 4 °C. The fixed and permeabilised cells were incubated with anti-human cytokine antibodies (see Table 2-4) diluted in BD Perm/Wash buffer at 4 °C for 30 minutes. Cells were washed twice with BD Perm/Wash buffer and re-suspended in FACS buffer prior to flow cytometric analysis.

2.11.4 Cell staining for apoptosis and cell death

For measuring potential toxicity of *M. vaccae* on DCs, cells were stained after 48 hours of stimulation with the nucleic acid dye 7-Amino-Actinomycin D (7-AAD) (BD Biosciences) and analysed 10 minutes later by flow cytometry.

Apoptosis and cell death of inhibitor-treated DCs were detected by annexin-V and propidium iodide (PI) staining, using the Annexin-V Apoptosis Detection Kit APC (eBioscience). Briefly, cells were harvested, washed once in PBS and re-suspended in

Binding Buffer to obtain 1-5 x 10^6 cells/ml. A volume of 100 µl was incubated at room temperature for 1-15 minutes with annexin-V antibody (see Table 2-4). Then, cells were washed and re-suspended in 200 µl Binding Buffer substituted with 5 µl PI staining solution and measured immediately by flow cytometry.

2.12 Enzyme-linked immunosorbent assay

Supernatants from cell cultures used for analysis of secreted protein levels by enzyme-linked immunosorbent assay (ELISA) were stored at -80 °C. ELISA plates (Nunc) were coated overnight at 4 °C with 50 µl/well capture antibody diluted in PBS. Plates were washed three times with washing buffer (PBS/Tween). To block any free residues, wells were incubated with 100 µl/well of 2 % (w/v) bovine serum albumin (BSA) (Sigma Aldrich) in PBS for 1 hour at room temperature. Standards were diluted in the same medium as the cell culture supernatants and two-fold serial dilution were performed to produce a standard curve. After washing for tree times in washing buffer, the plates were incubated with duplicates of standards and samples (50 µl/well) for 2 hours at room temperature. The plates were washed three times and incubated with the biotinylated detection antibody diluted in washing buffer (50 µl/well) for 1 hour at room temperature. After tree more washes in washing buffer the wells were incubated with 50 µl of 1:250 diluted Streptavidin-horseradish peroxidise (Streptavidin-HRP) (eBioscince) for 30 minutes at room temperature. After five more washing steps HRP presence was detected by adding 100 µl/well tetramethylbenzidine (TMB) (eBioscience) substrate for about 10 minutes in the dark. To stop the reaction, 50 µl 2N HCl were added. Colorimetric reactions were quantified immediately on the MRX Revelation plate reader at 450 nm, using Revelation v4.21 software (Dynex Technology, West Sussex, UK) for analysis.

2.12.1 IL-8 ELISA

To test for TLR-dependent cellular activation, supernatants from stimulated TLR-transfected HEK 293 cells (see 2.5) were analysed for IL-8 secretion with an ELISA kit from R&D Systems. The capture antibody was used at 4 μg/ml, the highest standard concentration was 2000 pg/ml, and the secondary antibody was used at 20 ng/ml. Sample dilutions reached from 1:2 to 1:10.

2.12.2 IL-10 ELSIA

Supernatants from 3 or 6 day cocultures of stimulated DCs and allogeneic naive $CD4^+$ T cells were used to measure the amount of secreted IL-10 (see 2.7). Samples were diluted 1:2. The capture antibody (clone JES3-9D7, eBioscience) was used at 2 μg/ml and secondary antibody (clone JES3-12G8, eBioscience) at 1 μg/ml. The highest standard concentration of recombinant human IL-10 (eBioscience) was 5000 pg/ml.

2.13 Multiplex analysis of cytokines

Stored supernatants (-80 °C) of stimulated DC cultures (see 2.6) were used at 1:2 dilutions to quantify the production of 31 different human cytokines, chemokines and growth factors using human Biosource multiplex bead immunoassay kits according to manufacturer's instructions (Invitrogen). The 30-plex assay allows the simultaneous measurement of the following proteins: epidermal growth factor (EGF), Eotaxin, fibroblast growth factor (FGF)-basic, G-CSF, GM-CSF, hematopoietic growth factor (HGF), IFN-α, IFN-γ, IL-1Ra, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, IP-10, monocyte chemotactic protein-1 (MCP-1 or CCL2), monokine induced by IFN-γ (MIG or CXCL9), macrophage inflammatory protein (MIP)-1α (or CCL3), MIP-1β (or CCL4), RANTES (or CCL5), TNF-α and

vascular endothelial growth factor (VEGF). IL-1α was measured using a separate Biosource multiplex kit. The multiplex analysis was performed using Luminex 2001S platform (Luminex, Austin, TX, USA). Concentrations were determined using LuminexIS software 2.3 (Luminex).

2.14 T cell proliferation assay

For quantification of T cell proliferation, 1 µCi of [^{3}H]-thymidine (MP Biomedicals, Irvine, CA, USA) was added on day 2 of DC/T cell coculture (see 2.7 and 2.8) to each well. T cell proliferation was measured after further 18 hours of incubation by harvesting the cells and measuring the incorporation of radioactivity into DNA using a cell harvester (Tomtec, Hamden, CT, USA) and a Microbeta TriLux Scintillation Counter (Perkin Elmer, Waltham, MA, USA). Results were expressed as counts per minute (cpm).

2.15 Quantitative confocal immunofluorescence analysis of NF-κB nuclear translocation

Immature DCs were cultured on 13 mm (No 1.5) glass coverslips (VWR, Batavia, IL, USA) using 2×10^5 cells/cover slip, which had been coated overnight with fibronectin (Sigma Aldrich). After 3 hours at 37 °C, adhered DCs were stimulated for 30 minutes or 2 hours (see 2.6), fixed with 3.7 % PFA (15 minutes, room temperature) and washed with Tris-buffered saline (TBS). Cells on coverslips were first permeabilised with 0.2 % Triton-X100 (Sigma Aldrich) in TBS for 10 minutes, non-specific antibody binding was blocked with 10 % goat serum in TBS, and cells were incubated with primary rabbit polyclonal anti-NF-κB p65 RelA (sc-372) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody overnight at 4 °C. Cells were washed

and stained with secondary antibody Alexa-Fluor (AF)633-conjugated goat anti-rabbit IgG (Invitrogen) together with mouse anti-CD13 PE-conjugated antibody (clone WM15; BD Biosciences) for 1 hour at room temperature. After staining the nuclei for 5 minutes at room temperature with 4,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich), the coverslips were mounted onto glass slides (VWR) using Vectashield hardset mounting medium (Vector Laboratories, Peterborough, UK).

All fluorescence images were captured on a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) with a pin hole of 1 Airy (114.5 µm), scan speed of 400 Hz, and four-frame averaging. Image analysis was performed with Metamorph software v7.17 (Molecular Devices, Sunnyvale, CA, USA) to quantify nuclear:cytoplasmic ratios of NF-κB RelA staining.

2.16 Transcriptional profiling by microarray

10^6 DCs were stimulated in 6-well plates with different stimuli as described above (see 2.6). After 4 hours and 24 hours, the supernatant was collected and the cells were lysed in RLT-buffer (Qiagen, Crawley, UK) and stored at -80 °C. RNA was isolated using the RNeasy kit from Qiagen, according to manufacturer's instructions. DNase treatment was performed in 50 µl reactions with Ambion's (Austin, TX, USA) TURBO DNase kit.

RNA quantity and integrity were measured electrophoretically (Agilent RNA 6000 Nano assay/Agilent 2100 Bioanalyzer, Santa Clara, CA, USA). The Agilent Quick Amp Labeling Kit was used to generate firstly cDNA and subsequently Cy3-labeled cRNA in an amplification step. Purification, labelling-intensity and RNA concentration were verified using a NanoDrop ND-1000 UV-VIS Spectrophotometer (Thermo Fisher

Scientific, Waltham, MA, USA). cRNA was then hybridised onto Agilent 4 x 44K whole human genome cDNA microarrays, according to manufacturer's instructions.

Array images were acquired with Agilent's dual-laser microarray scanner G2565BA (5-µm resolution), and signal data were collected with dedicated Agilent Feature Extraction software (v9.5.1). Log_2 transformed data were then subjected to LOESS normalisation (Chain et al., 2010) and compared by paired T-tests ($p<0.05$) using MultiExperiment Viewer v4.4.1. Genes with significant ($p<0.05$) >2 fold changes, for which refseq accession numbers were available, were selected for further analysis. The DAVID bioinformatics database was used to perform functional annotation cluster analysis (Dennis et al., 2003). Principle component analysis was performed using the R-project (http://www.r-project.org/) to obtain a global overview of gene expression data. The open-access bioinformatics tool oPOSSUM was used to identify overrepresented transcription factor binding sites in selected gene lists (Ho Sui et al., 2005).

2.17 SDS-polyacrylamide-gel electrophoresis (SDS-PAGE)

Cells were harvested and lysed by adding 50 µl or 100 µl of denaturating and reducing sample buffer. To shear the genomic DNA, the samples were sonicated at 40 Hz using an ultrasonic processor (Jencons Scientific) for 20-30 seconds on ice and then heated for 5 minutes at 98 °C. A volume of 18 µl of the samples and 3 µl of PageRuler protein marker (Fermentas, York, UK), used as a protein size reference, were loaded into the pockets of a precast 4-12 % bis-tris gradient gel (Invitrogen). Gels were run in Running buffer at 100 V in the XCell SureLock Mini-Cell electrophoresis system (Invitrogen).

2.18 Western blotting

2.18.1 Antibodies for Western blotting

Table 2-5: Antibodies used for Western blot analysis

Antibody (host)	Conjugate	Clone	Dilution	Manufacturer
IκB-α (rabbit)	none	44D4	1:1000	Cell Signaling
p-p38 (rabbit)	none	3D7	1:1000	Cell Signaling
p-Erk1/2 (rabbit)	none	197G2	1:1000	Cell Signaling
p-CREB (rabbit)	none	87G3	1:1000	Cell Signaling
β-actin (mouse)	none	AC-15	1:10000	Abcam
anti-rabbit-HRP (swine)	HRP	polyclonal	1:2000	DakoCytomation
anti-mouse-HRP (goat)	HRP	polyclonal	1:2000	DakoCytomation

2.18.2 Western blotting

SDS-PAGE gels were equilibrated in Transfer buffer for 15-30 minutes. Protein transfer onto Amersham Hybond ECL nitrocellulose membranes (GE Healthcare, Little Chalfont, UK) was performed in Transfer buffer using the XCell SureLock Mini-Cell electrophoresis system with the blot module (Invitrogen) for 1 hour at 100 V. Membranes were incubated for 1 hour in TBS/Tween containing 1-5 % (w/v) skimmed milk powder (Marvel, Premier Foods, St Albans, UK) to saturate non-specific binding sites and afterwards washed briefly in TBS/Tween. The primary antibody was diluted in TBS/Tween containing 1 % (w/v) skimmed milk powder (Marvel) to a given concentration (Table 2-5) and the membranes were incubated overnight at 4 °C. After being washed three times for 10 minutes with TBS/Tween, the membranes were incubated with secondary antibody conjugated with HRP (Table 2-5) in TBS/Tween containing 1 % (w/v) skimmed milk powder (Marvel) for 1 hour at room temperature. Subsequently the membranes were washed three times as described above, followed by two washes in TBS without Tween. Membrane-bound secondary antibody was detected by chemiluminescence using ECL plus Western blotting detection reagent (GE Healthcare). Chemiluminescence was measured by autoradiography using ECL

Hyperfilm (GE Healthcare). Membranes were incubated for 45 minutes at 60 °C in Stripping buffer for removal of primary and secondary antibodies and then washed intensively in PBS and TBS/Tween before the blocking step and re-probing for a different target.

Western blots were quantified by densitometry analysis using ImageJ version 1.42q (http://rsbweb.nih.gov/ij/).

2.19 Animals for RNA sample preparation

Five to six week old female BALB/c mice were treated by oral gavage with four different formulations of *M. vaccae* and with sterile water as a control (detailed in Table 2-6 below). The first and second group of mice were sacrificed on day 4 and day 30 after treatment, respectively. The remaining mice received a second treatment on day 30 and were sacrificed on day 50. Each time point and treatment was tested in triplicates (N=3), thus the study included a total of 45 mice. Study design and implementation were performed by Professor Oya Alpar's group at the University of London's School of Pharmacy and samples were kindly provided as a gift for validation of the SINE normalisation approach. To protect the RNA from degradation, one half of each spleen and the Peyer's patches were stored for 24 hours at 4 °C in 1 ml of RNA*later* (Ambion). Next, after the RNA stabilisation solution had permeated the tissue, it was removed and the samples were kept at -80 °C until the extraction of RNA.

Table 2-6: Different formulations of *M. vaccae* used for oral treatment

Treatment
100 µl sterile water
100 µg *M. vaccae* / 100 µl sterile water
Sonicated sample (100 µg *M. vaccae* / 100 µl) with 0.05 % m/v Tween 20 (10 amplitude, 2 min in phosphate buffer 7.4)
Sonicated sample (100 µg *M. vaccae* / 100 µl) with 0.05 % m/v Tween 20 + chitosan (10 amplitude, 2 min in phosphate buffer 7.4)
Freeze-dried preparation of 100 µg *M. vaccae* / 100 µl sterile water

2.20 RNA extraction

RNA was isolated from mice spleens and Peyer's patches using the RNeasy Mini kit (Qiagen). 600 µl RLT buffer were added to the tissues that were disrupted and homogenised using a rotor-stator homogeniser (Omni International, Kennesaw, GA, USA) for approximately 30 seconds. As the spleen is a very condense tissue containing many cells, each sample was then divided into three aliquots and refilled with RLT buffer to a total volume of 600 µl. Two aliquots were stored at -80 °C. RNA extraction was carried out following the manufacturer's instructions without conducting the optional on-column DNase digestion. RNA of spleens and Peyer's patches was eluted in 80 µl and 50 µl water, respectively. The nucleic acid concentration was measured using the NanoDrop spectrophotometer (Thermo Scientific) and the RNA was stored at -80°C.

2.21 DNase treatment and RNA cleanup

2.21.1 Method one: using microcon-100 column purification

4 µg RNA from spleens were DNase treated using one unite of RQ1 RNase-free DNase enzyme per 1 µg RNA (Promega) for 30 minutes at 37 °C in 40 µl reactions flowing the manufacturer's instruction. The reaction was split in two for comparison with method two (see 2.21.2 below).

Then the DNase was inactivated by incubation at 65 °C for 10 minutes. 180 µl RNase-free water (Invitrogen) was added to the 20 µl reaction mixture and transferred onto Microcon/Ultracell YM-100 membrane columns (Millipore, Billerica, MA, USA). The columns were centrifuged for 8 minutes at 2500 x g at room temperature. The samples were washed by adding 200 µl RNase-free water onto the columns and spinning as before. Then, 14 µl of RNase-free water was added onto the columns and let

stand for 5 minutes. To collect the purified RNA samples from the columns, the sample reservoir was inverted and spun for 3 minutes at 1000 x g at room temperature. RNA quality and was compared to purification using method two (see 2.21.2 below) via agarose gel electrophoresis (see 2.25).

2.21.2 Method two: using guanidinium thiocyanate phenol chloroform purification

15 µg RNA from spleens and 20 µg RNA from Peyer's patches were DNase treated using one unite of RQ1 RNase-free DNase enzyme per 1 µg RNA (Promega) for 30 minutes at 37 °C in 60 µl and 80 µl reactions, respectively. The reactions contained 5 µl of RnaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen) to prevent degradation. RNase-free water (Invitrogen) was added to a total volume of 80 µl and mixed with 1 ml of TRI Reagent (Sigma Aldrich). After 5 minutes incubation at room temperature, 200 µl of chloroform (Sigma Aldrich) was added and well mixed by hand for 15 seconds and again incubated at room temperature for 3 minutes. Following centrifugation at 4° C at 12,000 x g for 15 minutes, the upper aqueous phase was transferred into a fresh tube. For RNA precipitation, 500 µl isopropyl alcohol (Fulka) was added and incubated for 10 minutes at room temperature. The RNA was pelletised by centrifugation at 4 °C at 12,000 x g for 15 minutes and washed with 1 ml of 75 % ethanol. After centrifugation for 5 minutes at 4 °C and 12,000 x g, the supernatant was removed and the pellet air-dried. The RNA was dissolved in 34 µl RNase-free water (Invitrogen). Two aliquots of 2 µl were used for RNA quality and quantity assessment with the NanoDrop (Thermo Scientific) and BioAnalyzer (Agilent), and all remaining RNA with a RNA integrity number (RIN) > 8 was stored at -80°C until reverse transcription into cDNA.

2.22 Reverse transcription

1 µg RNA was used for all reverse transcription (RT) reactions, which were performed following the manufacturer's instructions using SuperScript III reverse transcriptase and oligo$(dT)_{12-18}$ primer (both Invitrogen). The resulting cDNA was 1:10 diluted by adding 180 µl RNase-free water to the 20 µl reaction. Aliquots of 6 µl were stored at -80 °C until real-time PCR reaction.

2.23 Primer design and qPCR assay optimisation for human and mice SINEs

The consensus sequences of repetitive elements were found on the electronic database Repbase Update (http://www.girinst.org) (Jurka, 2000). Primers were designed for B1- and B2-element consensus sequences, and for the ALU-J family (Table 2-7).

Table 2-7: Primers designed for SINE amplification by qPCR

SINE	Forward primer	Reverse Primer
ALU-J	5'CAACATAGTGAAACCCCGTCTCT	5'GCCTCAGCCTCCCGAGTAG
B1F1/B1R1	5'TGGCGCACGCCTTTAATC	5'GCTGGCCTCGAACTCAGAAAT
B1F2/B1R2	5'GTGGCGCACGCCTTTAAT	5'GCTGGCCTCGAACTCAGAAA
B2F1/B2R1	5'CAATTCCCAGCAACCACATG	5'ACACCAGAAGAGGGCATCAGA
B2F2/B2R2	5'CAATTCCCAGCAACCACATG	5'ACACACCAGAAGAGGGCATCA

For the genes of interest in mice, whose expression was normalised with the B-elements, probe-based assays were designed (Table 2-8), using the primer 3 web site (http://frodo.wi.mit.edu/). Primer and probes were controlled for their melting temperature and secondary structure with NetPrimer (http://www.premierbiosoft.com/netprimer/). In order to ensure probe binding prior to primer extension, which would block the probe binding site, the annealing temperature of the probe was always higher than the one of the primer (Holland et al., 1991). All primer and probes were synthesised by Sigma Aldrich.

Table 2-8: Primers and probes designed for gene of interest expression analysis in mice tissue by RT-qPCR

Gene	Forward primer	Reverse primer	Probe
FoxP3	5'CAATAGTTCCTTCCCAGAGT	5'ATAAGGGTGGCATAGGTG	[6FAM]TCCACAACATGGACTACTTCAAGT[3BQ1]
HO-1	5'CAGGTGATGCTGACAGAGGA	5'GCCAACAGGAAGCTGAGAGT	[6FAM]CCTCTGACGAAGTGACGCCATCT[3BQ1]
IFN-γ	5'GCGTCATTGAATCACACCTG	5'CTGGACCTGTGGGTTGTTG	[6FAM]CTTCTTCAGCAACAGCAAGGCGAA[3BQ1]
IL-5	5'AATGCTATTCCAAAACCTGT	5'ACTCATCACACCAAGGAACT	[6FAM]CTCCTCGCCACACTTCTCTTTT[3BQ1]
IL-10	5'CCAAGCCTTATCGGAAATG	5'ACTCTTCACCTGCTCCACTG	[6FAM]AGGCGCTGTCATCGATTTCTCC[3BQ1]
TGF-β	5'CAGAGAAGAACTGCTGTGTG	5'GGGTTGTGTTGGTTGTAGA	[6FAM]AACCCAGGTCCTTCCTAAAGTC[3BQ1]
TLR2	5'GGTGTCTGGAGTCTGCTGTG	5'GCTTTCTTGGGCTTCCTCTT	[6FAM]CCCTTCTCCTGTTGATCTTGCTCGTAG[3BQ1]

2.24 Plasmid preparation for standard curves

Human and mouse cDNA was used as template in qPCR reactions to amplify the different target sequences. The amplicons of interest were cloned into pCR4-TOPO vectors using the TOPO TA Cloning Kit for Sequencing (Invitrogen) following the manufacturer's instructions. After ligation of the PCR product into the plasmid vector it was transformed via heat-shock (30 seconds at 42 °C) into TOP10 *E. coli* cells (Invitrogen) following manufacture's instruction. Colonies were grown over night at 37 °C on LB-agar plates with 100 µg/ml ampicillin (Sigma Aldrich). Single clones were grown over night (37 °C, 200 rpm) in 5 ml LB-medium supplemented with 100 µg/ml ampicillin and plasmids were isolated according to manufacturer's instruction with a miniprep kit (Qiagen). Plasmids were linearised with PST-I (Promega) for 3 hours at 37 °C prior to re-purification using the PCR cleanup kit (Qiagen) and linearisation was verified by agarose gel electrophoresis. Plasmids were sequenced (Wolfson Institute for Biomedical Research, UCL), and quantified with a NanoDrop spectrophotometer (Thermo Scientific). Stocks of 10^8 copies/µl were stored at -20 °C and later used for assay optimisation and as standards for gene expression quantification.

2.25 Agarose gel electrophoresis

Gel concentrations varied between 1.5-3 % agarose (Sigma Aldrich) depending on the nucleic acid fragment size to be visualised. Gels, containing 50 µg/ml ethidium bromide (Sigma Aldrich), were poured and run in 1x Tris-acetate-EDTA (TAE) buffer. Samples were diluted in 6x loading buffer (Thermo Fisher Scientific). Size standards (Thermo Fisher Scientific) were run with every gel to estimate nucleic acid size. Gels were run at varying field strengths for a duration of 45 minutes to 1 hour. Samples were visualised and photographed using an ultraviolet (UV) transilluminator (Ultra-Violet Products, Cambridge, UK).

2.26 Sequencing of clones containing qPCR products

PCR products were ligated into the pGEM-T easy vectors (Promega) in a 5 µl reaction for 1 hour at room temperature following manufacturer's instructions. As described above, TOP10 competent *E. coli* cells (Invitrogen) were transfected with 5 µl of the ligation reaction and later transferred onto LB-plates containing 100 µg/ml ampicillin and 50 µg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) (both Sigma Aldrich). Positive colonies were individually selected to inoculate 3 ml liquid LB cultures and grown overnight at 37 °C and 180 rpm. Using T7-specific primers, sequencing was performed at EMBL (Heidelberg, Germany).

2.27 qPCR assay optimisation

For qPCR assay optimisation, 10-fold dilution series from 5×10^7 to 5 copies/reaction were generated from the plasmids containing the amplicon of interest. SINE assays were designed with qPCR kits containing SYBR green dye, as variations

in the target sequences were expected; QuantiTech SYBR (Qiagen) was used for ALU-J and SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich) for B1 and B2 reactions. JumpStart Taq ReadyMix (Sigma Aldrich) was used for all reactions amplifying genes of interest. All PCR reaction efficiencies were optimised to close to 100 % by varying primer, probe and $MgCl_2$ concentrations, as well as annealing temperature and time (Table 2-9). All assays were conducted in 12.5 µl volumes of which 5 µl were DNA template using a Rotorgene 6000 thermocycler (Corbett Research). PCR product sizes were checked on a 3 % agarose gel. To test for the presence of contaminating DNA, RNAse/DNase-free H_2O in the place of cDNA and reverse transcriptase (RT-) negative samples were analysed.

Table 2-9: Optimised qPCR assay parameters

Assay	F-primer	R-primer	Probe	$MgCl_2$	Annealing Temp.	95°C	Anneal	72°C
ALU-J	600nM	600nM	-	2.5mM	58°C	10 sec	20 sec	30 sec
B1F1/B1R1	600nM	600nM	-	3.5mM	58°C	10 sec	30 sec	30 sec
B1F2/B1R2	600nM	600nM		3.5mM	58°C	10 sec	30 sec	30 sec
B2F1/B2R1	600nM	600nM	-	3.5mM	58°C	10 sec	30 sec	30 sec
B2F2/B2R2	600nM	600nM		3.5mM	58°C	10 sec	30 sec	30 sec
FoxP3	200nM	400nM	100nM	6.0mM	50°C	10 sec	20 sec	30 sec
HO-1	200nM	600nM	100nM	6.0mM	58°C	10 sec	20 sec	30 sec
IFN-γ	200nM	400nM	100nM	6.0mM	58°C	10 sec	20 sec	30 sec
IL-5	400nM	600nM	100nM	5.0mM	50°C	10 sec	20 sec	30 sec
IL-10	400nM	600nM	100nM	6.0mM	58°C	10 sec	20 sec	30 sec
TGF-β	600nM	200nM	100nM	6.0mM	50°C	10 sec	20 sec	30 sec
TLR2	200nM	600nM	100nM	6.0mM	58°C	10 sec	20 sec	30 sec

2.28 Exposure experiment

A volume of 1 ml DNase/RNase-free distilled water (Invitrogen) was added to 63 1.5 ml non-stick microtubes (Alpha Laboratories, Eastleigh, UK). The tubes were placed at three different locations: a UV-sterilized/hepa-filtered PCR hood; a bench in the pre-PCR laboratory; and on a desk at the entrance to an open-plan office shared by 14 people. Three tubes were left open for each time-point: 0 minutes, 5 minutes, 15 minutes, 30 minutes, 60 minutes, 360 minutes (6 hours), and 1440 minutes (24 hours). At the end of each time point, aliquots of the samples were made for all

subsequent reactions and stored at -20 °C for no more than two weeks. Aliquots of 5 µl of the exposed water were added to the respective real-time PCR reactions.

2.29 GeNorm analyses to identify optimal reference genes

The geNorm Housekeeping Gene Selection Kit (PrimerDesign) was used following the manufacturer's instructions to evaluate twelve commonly used housekeeping genes shown in Table 2-10. In order the use the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/), threshold cycle (CT) values were transformed into relative quantities. CT data were imported into Excel and transformed into ΔCT by subtracting the CT of the most expressed sample (smallest CT) from all samples. The most expressed sample will have a value of zero. Then the ΔCT values were transformed into relative copy numbers: $2^{(-\Delta CT)}$. The software automatically calculates the gene-stability measure *M*, which is an average pairwise variation of a particular gene with all other control genes (Vandesompele et al., 2002b). I used the three genes that showed the most stable expression and calculated the normalisation factor based on the geometric mean (Vandesompele et al., 2002b).

Table 2-10: Mice housekeeping genes used for geNorm analysis

Gene
beta-actin (ACTB)
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
ubiquitin C (UBC)
beta-2 microglobulin (B2M)
phospholipase A2 (YWHAZ)
ribosomal protein L13a (RPL13A)
calnexin (CANX)
cytochrome c-1 (CYC1)
succinate dehydrogenase complex, subunit A (SDHA)
18S rRNA gene
eukaryotic translation initiation factor 4A2 (EIF4A2)
ATP synthase subunit (ATP5B)

2.30 RT-qPCR data presentation and calculations

Copy numbers of genes of interest were obtained using a standard curve. They were normalised by: copy number/normalisation factor (NF). NF of three-best reference genes was their geometric mean of relative expression levels; NF of B-elements was geometric mean of copy number of B1 and B2; NF of β-actin was relative expression level of β-actin. For better comparison of the data after normalisation with the different normalisation methods, data are shown as proportions of expression level from untreated mice on day 4. Therefore all copy numbers were divided by the median copy number of the three water-treated animals from day 4, which equals afterwards 1.

3 *M. VACCAE* ATTENUATES TH2 RESPONSES VIA DENDRITIC CELLS

3.1 Introduction

The role of DCs in shaping adaptive immune responses has been subject to extensive research with the aim of therapeutic modulation of the immune system (Kapsenberg, 2003). Epidemiological evidence that underpins the hygiene hypothesis suggests that host microbial interactions may have a strong influence on the nature of adaptive immunity and investigation of the mechanisms which underlie this effect may provide new opportunities for therapeutic interventions (Rook, 2007). To this end, I have studied the interaction between the environmental saprophyte *M. vaccae* and human DCs. *In vivo* animal studies showed that *M. vaccae* can reduce allergic responses possibly by modulating Th cells by reducing Th2-, increasing Treg- and also augmenting Th1-type responses (Rook et al., 2007). As a result there has been widespread interest in its therapeutic application for allergic diseases and augmentation of Th1 immunity (Dlugovitzky et al., 2006; Ricklin-Gutzwiller et al., 2007), but the mechanisms for these effects are not known. As DCs have a key role in induction and regulation of T cell responses (Kapsenberg, 2003), the innate immune response of monocyte-derived human DCs to stimulation with *M. vaccae* and downstream modulation of allogeneic and autologous T cell responses were explored. In this study, the heat-killed *M. vaccae* used was similar to preparations that have been applied in the animal and human trials to date.

3.2 Objectives

- Test if *M. vaccae* can induce maturation of DCs.
- Study which TLRs are activated by *M. vaccae*.

- Compare DC responses to *M. vaccae* to stimuli for TLRs which are shared or distinct from those of *M. vaccae*.
- Test if DC priming with *M. vaccae* augments T cell proliferation in cocultures.
- Test the hypothesis that *M. vaccae*-primed DCs reduce Th2-polarised responses and investigate if this is due to induced Treg responses.
- Compare if conditioned media from stimulated DC cultures or fixed DCs can replicate the effects of live DCs on T cells.

3.3 Results

3.3.1 *M. vaccae* induces dose-dependent maturation of dendritic cells

Following challenge with microbial or inflammatory stimuli, DCs mature and gain the capacity to stimulate naive Th cells. During the maturation process they upregulate molecules such as CD83 and CD86. CD86 functions as costimulatory signal to initiate primary T cell responses (Johansson et al., 2007). In this study DCs were generated from $CD14^+$ monocytes and differentiated with IL-4 and GM-CSF for four days; a protocol that consistently generated ≥ 95 % DC-SIGN positive cells (Figure 3-1A) with typical DC morphology (Figure 3-1B). The ability of *M. vaccae* to induce maturation of DCs was assessed. Overnight (24 hours) stimulation of DCs with *M. vaccae* increased expression of the maturation markers CD83 and CD86 in a dose-dependent manner that became saturated at reagent concentrations ≥ 100 µg/ml (Figure 3-2A). In addition, the preparation of *M. vaccae* was assessed for potential toxic effects on DCs. Therefore DCs were stimulated with increasing concentrations of *M. vaccae* for 48 hours and stained with 7-AAD, which can only penetrate cell membranes of dying or dead cells to intercalate into double-stranded nucleic acids. This

analysis showed that 100 µg/ml *M. vaccae*, the dose at which maturation of DCs became saturated, was not toxic and the percentage of 7-AAD$^+$ cells was comparable to unstimulated DCs. However, 10-fold higher concentrations of *M. vaccae* (1 mg/ml) led to augmented 7-AAD$^+$ DCs (Figure 3-2B).

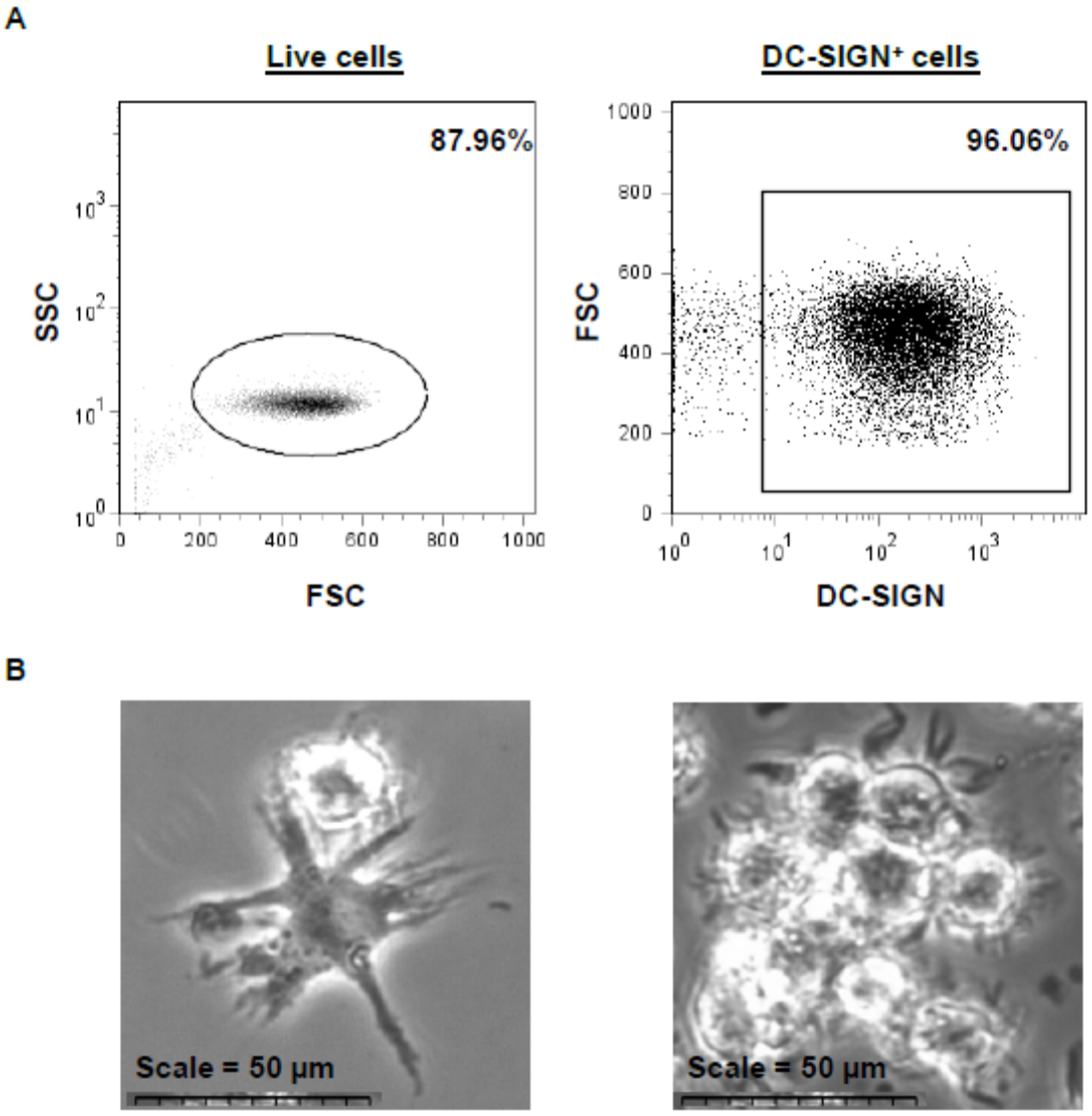

Figure 3-1: Phenotypic analysis of monocyte-derived dendritic cells

Monocyte-derived DCs were prepared as described in *Material and Methods* and analysed by flow cytometry and light microscopy. **A**: The forward and side scatter (FSC/SSC) profile of day 4 DCs is shown (left panel). Subsequent analysis is always gated on the DC-SIGN positive population (right panel). **B**: Photomicrographs on day 4 show DCs adhered to the plastic of the cell culture plate (left), and a group of day 4 DCs floating in the supernatant of the cell culture – note the presence of small dendrites (right).

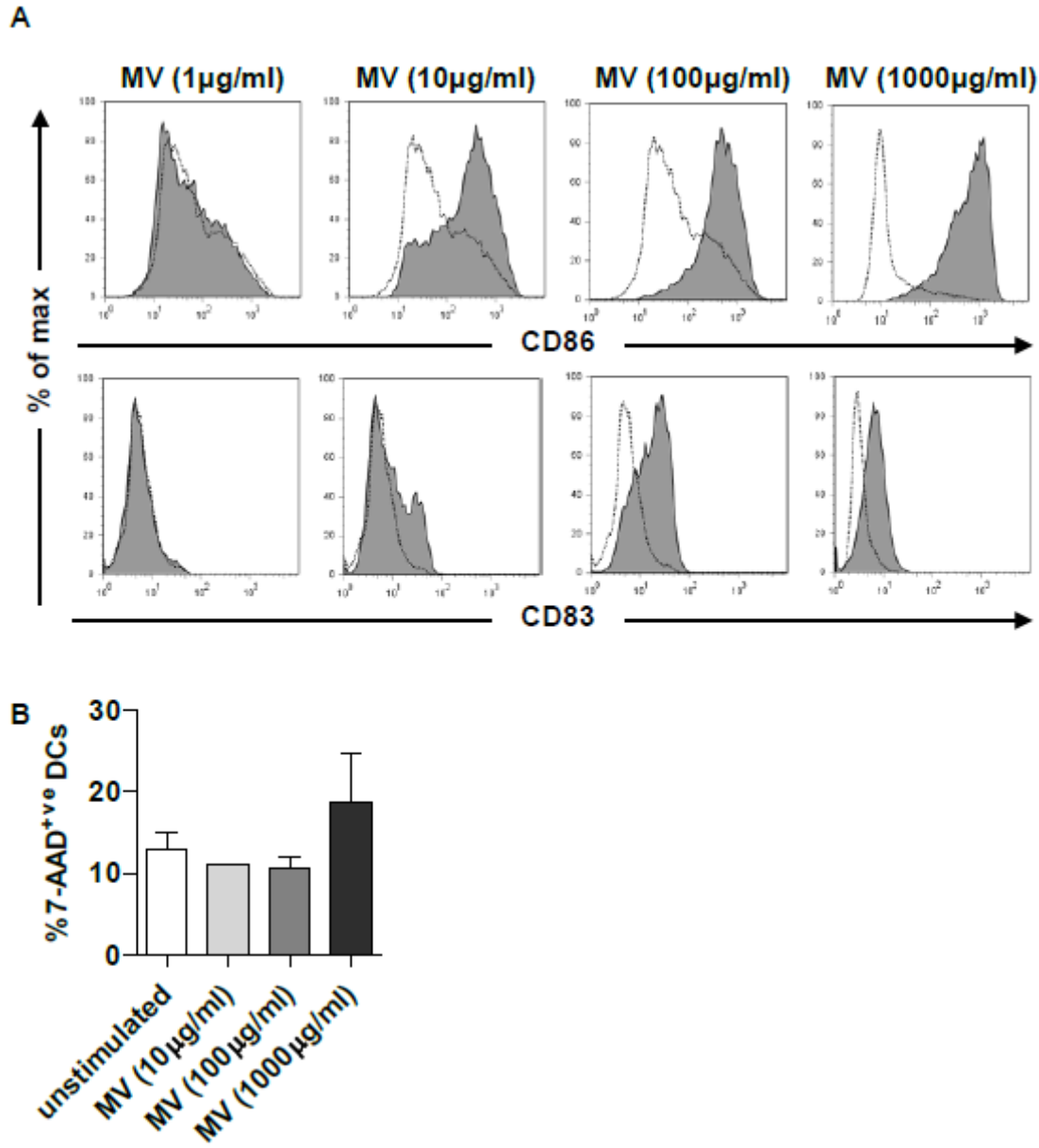

Figure 3-2: *M. vaccae* induces dose-dependent maturation of dendritic cells

A: Flow cytometric analysis of immunofluorescence staining for cell surface CD83 and CD86 expression by DCs showed a dose-dependent response to 24 hours stimulation with *M. vaccae*. White histograms show staining of unstimulated DCs. Flow cytometry histograms show representative data from multiple experimental replicates. **B**: DCs were stimulated with different concentrations of *M. vaccae* for 48 hours and cell viability was analysed by staining with 7-AAD. Data show mean (±SD) of two independent experiments.

3.3.2 *M. vaccae* can stimulate TLR2-dependent cellular activation

TLRs have been identified as a family of homo- or heterodimeric transmembrane molecules that function as innate immune PRRs (Akira and Hemmi, 2003; Beutler, 2009). DCs are known to express a number of TLRs (Medzhitov, 2001)

and TLR-dependent innate immune cellular activation by mycobacteria has been established (Jo et al., 2007). Therefore, the hypothesis that *M. vaccae* may also stimulate TLR-dependent cellular activation was tested. HEK 293 cells expressing specific TLR combinations were used as reporter cell lines by measuring IL-8 release into the culture supernatants consequent upon TLR-dependent cellular activation in response to stimulation with *M. vaccae* or specific control TLR ligands. In line with the existing literature on TLR2 interactions with mycobacteria (Drage et al., 2009; Krutzik et al., 2003; Sweet and Schorey, 2006), *M. vaccae* activated cells expressing either TLR2 alone or TLR1/2 and TLR2/6 heterodimers, in a dose-dependent manner (Figure 3-3). TLR-dependent cytokine secretion in response to *M. vaccae* was not detected with any of the other cell lines transfected with other TLR genes. It had been described that DNA from *M. tuberculosis* can act as a ligand for intracellular TLR9 in human macrophages (Kiemer, 2009). The inability of the preparation of whole *M. vaccae* to induce cellular activation of TLR9 expressing HEK 293 cells was maybe due to failure of uptake and breakdown of the whole bacterium by these cells. Therefore the preparation of *M. vaccae* was sonicated and tested for its ability to activate TLR9-dependent gene expression. However, stimulation of HEK 293 cells expressing TLR9 was also not evident with this preparation of *M. vaccae* (Figure 3-4).

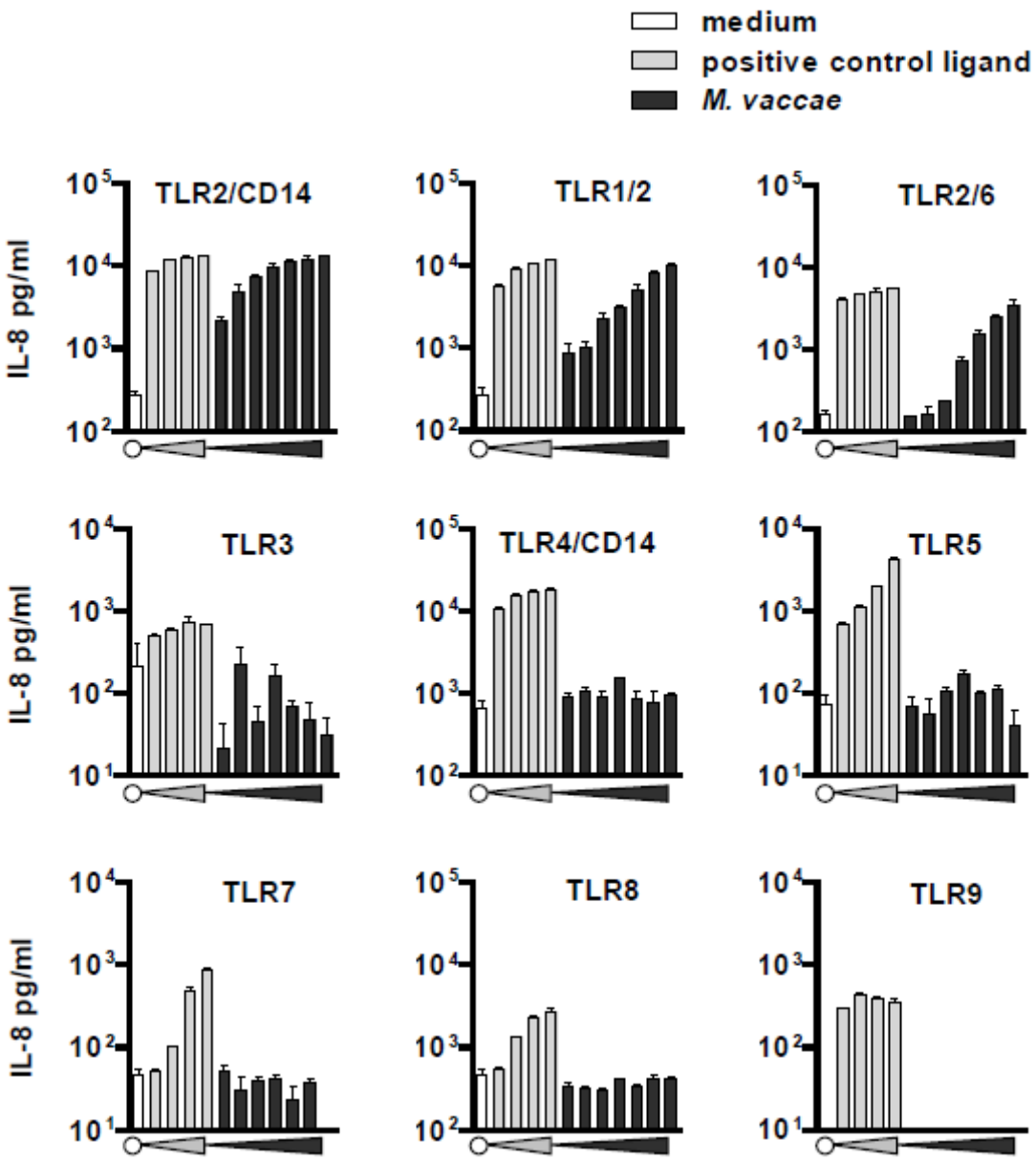

Figure 3-3: *M. vaccae* activates TLR2-dependent gene expression

HEK 293 cells stably transfected with plasmids expressing the TLR genes ±CD14 as indicated, were treated with medium (white), increasing concentrations of positive control ligands (grey) or *M. vaccae* (black). TLR2/CD14: heat-killed *Listeria monocytogenes* (10^7-10^8 /ml), TLR1/2: Pam_3CSK4 (62.5-500 ng/ml), TLR2/6: FSL-1 (25-200 ng/ml), TLR3: poly(I:C) (25-200 µg/ml), TLR4/CD14: LPS (0.1-100 ng/ml), TLR5: Flagellin (0.5-4 µg/ml), TLR7: Gardiquimod (0.625-5 µg/ml), TLR8: CL075 (5-40 µg/ml), TLR9: OND2006 (0.3-2.5 µM) and *M. vaccae* (1-1000 µg/ml). TLR-dependent cellular activation was measured after 24 hours by ELISA quantifying IL-8 concentrations in the cell culture supernatants. Data show mean (±SD) of triplicate cultures. Data are representative of at least two independent experiments.

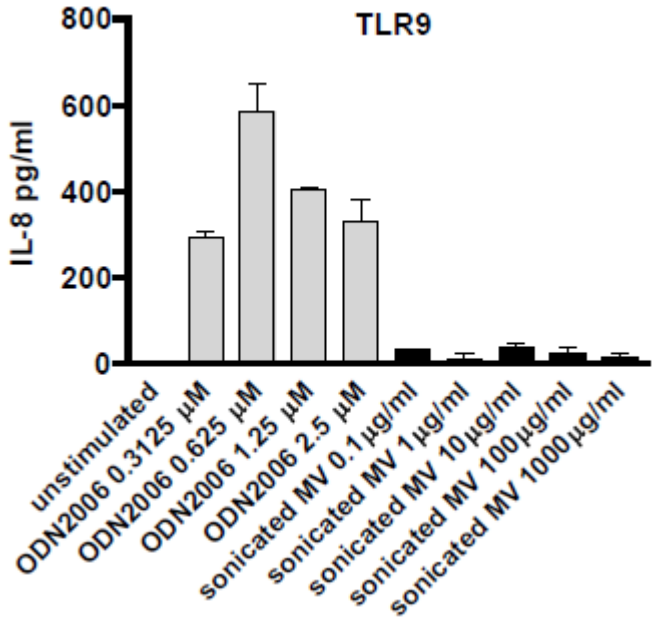

Figure 3-4: Sonicated *M. vaccae* does not activate TLR9-dependent gene expression

HEK 293 cells transfected with plasmids stably expressing TLR9 were treated with medium (white), dilution series of OND2006 (0.3125-2.5 µM) as a control TLR9 ligand (grey), or serial dilutions (0.1-1000 µg/ml) of sonicated *M. vaccae* (black). Cellular activation was measured after 24 hours by ELISA quantifying IL-8 concentrations in the cell culture supernatants. Data show mean (±SD) of three independent experiments.

3.3.3 Blocking of TLR2-dependent cellular activation failed

Although the TLR reporter cells clearly demonstrated that *M. vaccae* induces cellular activation via TLR2, it is likely that it activates also other innate immune receptors, including DC-SIGN, CCR5, dectin-1, Nod1, Nod2 or the mannose receptor (Ferwerda et al., 2007; Floto et al., 2006; Geurtsen et al., 2009; Nigou et al., 2001; Pott et al., 2009; Torrelles et al., 2006; Yadav and Schorey, 2006), for which reporter systems were not available to me. However, blocking of TLR2-dependent cellular activation with specific monoclonal antibodies would have allowed studying TLR2-independent effects of *M. vaccae* on DCs.

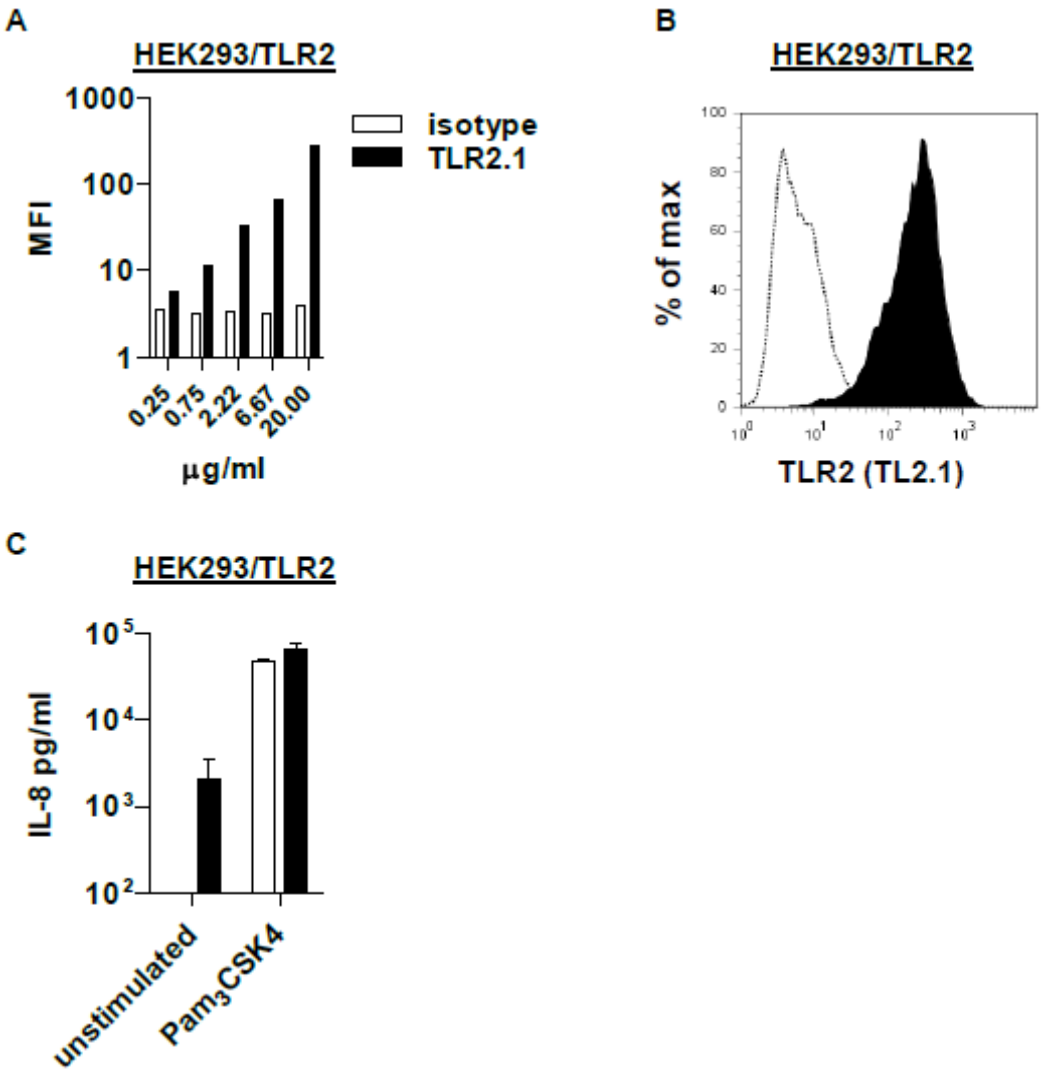

Figure 3-5: TLR2 antibody TL2.1 does not block activation of TLR2-transfected HEK 293 cells

A: HEK 293 cells expressing TLR2 were surface stained with a titration of anti-TLR2 antibody (TL2.1) or isotype control. **B**: Histogram of TLR2 expressing HEK 293 cells surface staining with anti-TLR2 antibody clone TL2.1 (black) and an isotype control (white). **C**: HEK 293 cells expressing TLR2 were treated with anti-TLR2 antibody clone TL2.1 (black) or isotype control (white) for 30 minutes prior to stimulation with Pam_3CSK4 (500 ng/ml). Cellular activation was measured after 24 hours of culture by quantifying IL-8 concentration in the supernatants by ELISA.

Staining of TLR2-transfected HEK 293 cells with a titration of anti-TLR2 antibody clone TL2.1 did not reach saturation even with the highest dose of antibody (Figure 3-5A). When TLR2 expressing HEK293 cells were treated with the highest dose of this antibody prior to simulation with the TLR2 ligand Pam_3CSK4, no reduction in TLR2-dependent secretion of IL-8 was found (Figure 3-5C). The titration suggested that

TLR2 is very highly expressed in these reporter cells, and therefore the antibody was next tested for its ability to block TLR2-dependent activation of DCs.

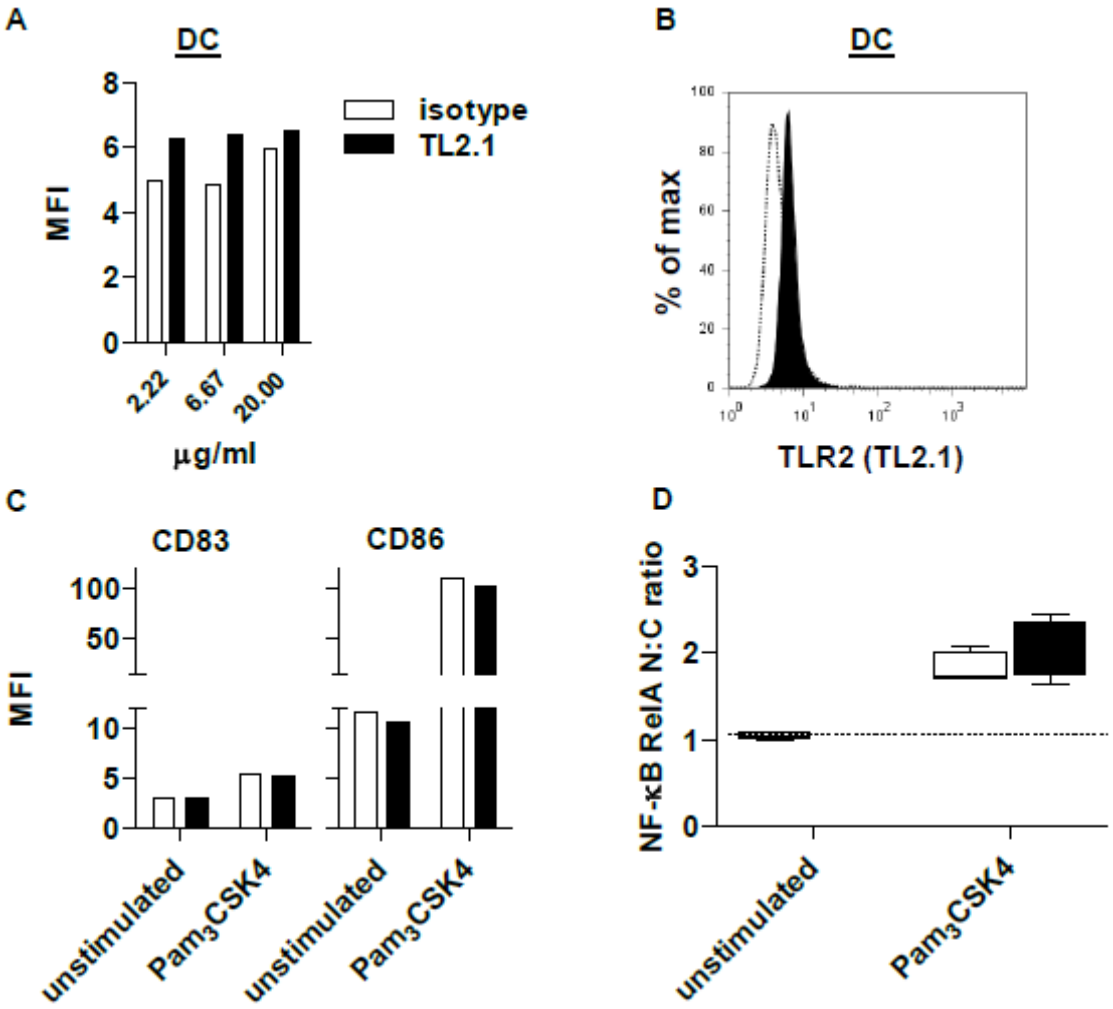

Figure 3-6: TLR2 antibody TL2.1 does not block TLR2-dependent cellular activation of dendritic cells

A: DCs were stained with a titration of anti-TLR2 antibody clone TL2.1 and an isotype control. **B**: Histogram of DC surface staining with anti-TLR2 antibody clone TL2.1 (black) and an isotype control (white). **C**: DCs were treated with TL2.1 or isotype control for 30 minutes prior to stimulation with Pam_3CSK4 (500 ng/ml). Expression of surface maturation markers CD83 and CD86 was measured by flow cytometry. **D**: Ratio of RelA staining in the nucleus (N) and the cytoplasm (C) in DCs treated with an isotype control (white) or anti-TLR2 antibody clone TL2.1 (black) for 30 minutes prior to stimulation for 1 hour with Pam_3CSK4 (1 µg/ml). Box and whisker plots represent median, and range of data from approximately 500 single cell measurements.

Expression of TLR2 on the surface of monocyte-derived DCs, serving as experimental models, was confirmed with the same monoclonal TLR2 antibody (Figure 3-6B). Here the titration of the antibody clone TL2.1 reached saturation and a higher concentration would not have led to increased staining of surface TLR2

(Figure 3-6A). However, DCs that were pre-treated with anti-TLR2 before priming with Pam_3CSK4 showed no reduction of the level of maturation (Figure 3-6C). To determine whether the inability to block TLR2-dependent cellular activation was due to potential turnover of newly expressed TLR2 during the 24 hours of incubation, DCs were treated with the anti-TLR2 antibody clone TL2.1 and then stimulated for only one hour with Pam_3CSK4 and fixed. These DCs were analysed for nuclear translocation of NF-κB, occurring upon innate immune activation of DCs. Here also, there was no reduction of nuclear translocation (Figure 3-6D).

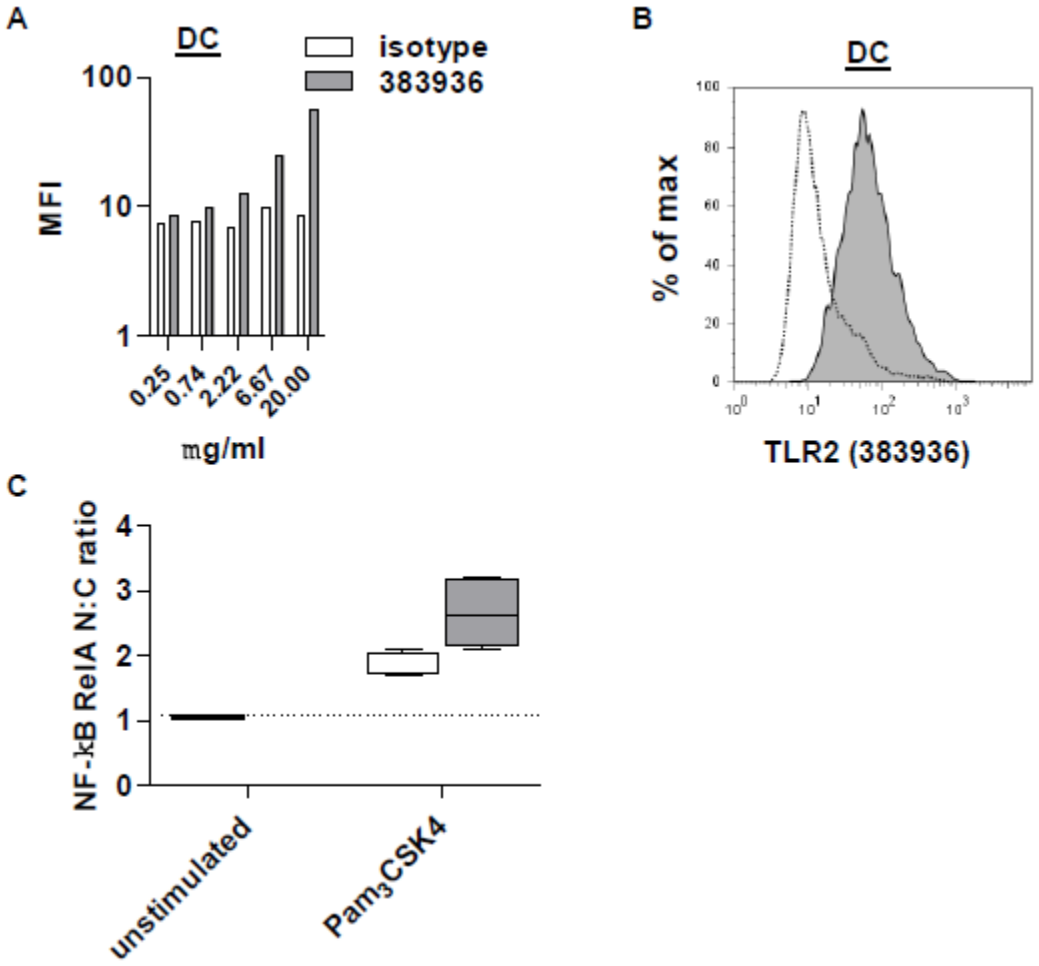

Figure 3-7: TLR2 antibody 383936 does not block TLR2-dependent cellular activation of dendritic cells

A: DCs were stained with a titration of anti-TLR2 antibody clone 383936 and an isotype control. **B**: Histogram of DC surface staining with 383936 (grey) and an isotype control (white). **C**: Ratio of RelA staining in the nucleus (N) and the cytoplasm (C) in DCs treated with an isotype control (white) or 20 µg/ml of anti-TLR2 antibody clone 383936 (grey) for 30 minutes prior to stimulation for 1 hour with Pam_3CSK4 (1 µg/ml). Box and whisker plots represent median, and range of data from approximately 500 single cell measurements.

A second monoclonal anti-TLR2 antibody was therefore tested. Titration of this clone on DCs did not reach saturation (Figure 3-7A). The manufacturer showed 50 % neutralisation when using this antibody at a concentration of 0.03 μg/ml and 100 % with 2 μg/ml. However, pre-treatment of DCs with 20 μg/ml prior to stimulation with Pam_3CSK4 did not neutralise or reduce innate immune activation of DCs tested by measuring nuclear translocation of NF-κB (Figure 3-7C).

3.3.4 *M. vaccae* induces greater maturation of dendritic cells than specific TLR2 stimulation with Pam_3CSK4

In order to develop insight into the specific consequences of DC priming by *M. vaccae*, comparisons were made to other innate immune stimuli with cellular activation pathways that were common or distinct from those of *M. vaccae*. The lack of TLR4 stimulation confirmed the absence of LPS contamination in this preparation of *M. vaccae*, and allowed comparisons of the effects of *M. vaccae* on DCs, to those of LPS stimulation and specific TLR2 stimulation with Pam_3CSK4 (Figure 3-8A). At concentrations of *M. vaccae,* LPS and Pam_3CSK4 that induced maximal increase in CD83 and CD86 surface expression, LPS and *M. vaccae*-induced maturation was significantly greater than that induced by Pam_3CSK4 (Figure 3-8B). Therefore a 10-fold lower concentration of *M. vaccae* (10 μg/ml), which induced comparable maturation to Pam_3CSK4 was also included in the experimental paradigm. In addition to the upregulation of surface maturation markers, stimulated DCs formed characteristic clumps in contrast to unstimulated DCs, whereby the number of clumps in each culture correlated with the magnitude of CD83 and CD86 expression (Figure 3-8C), though this parameter could not be objectively quantified.

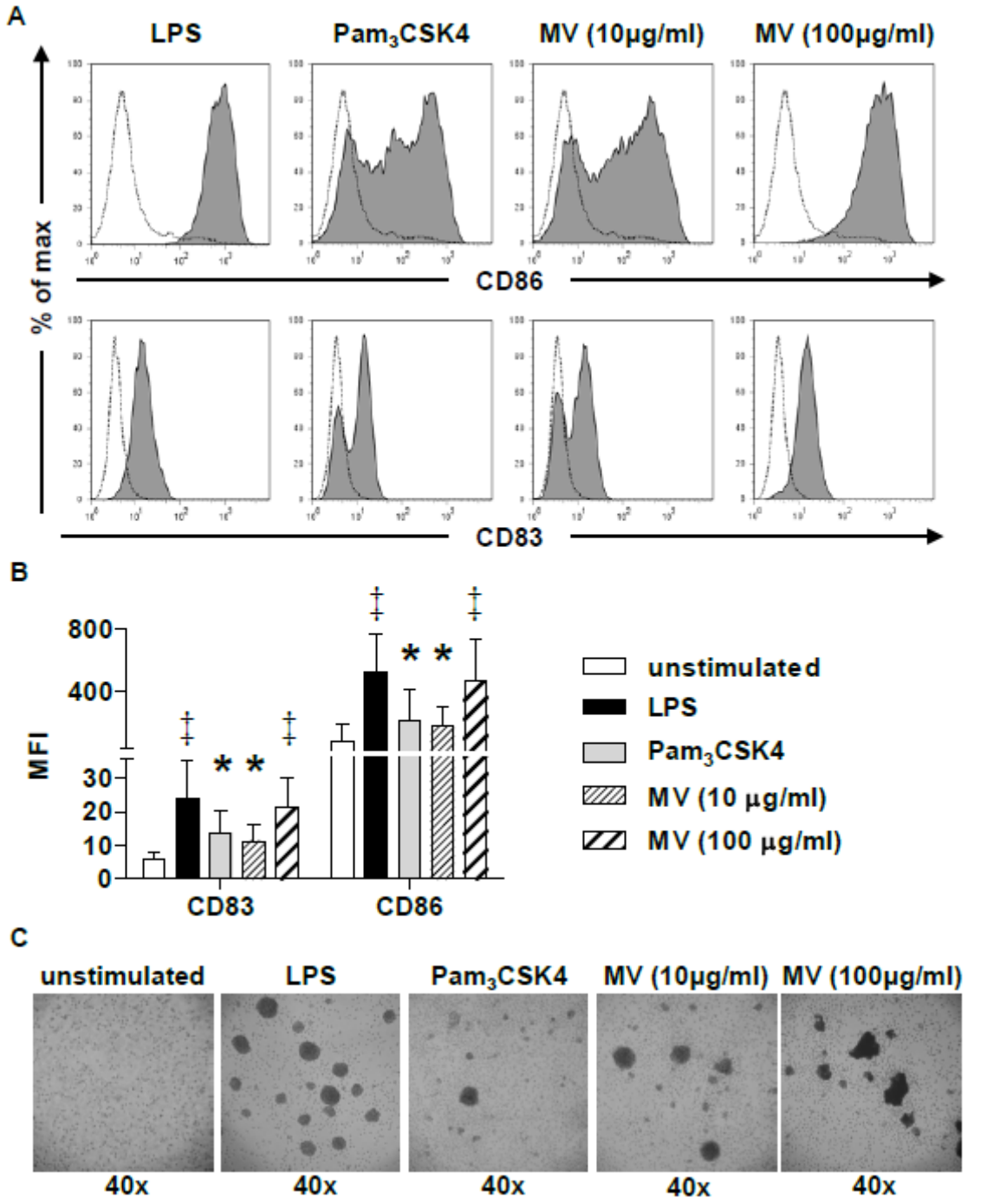

Figure 3-8: *M. vaccae* induces stronger maturation of dendritic cells than TLR2-specific stimulation

A: Flow cytometric analysis of immunofluorescence staining for cell surface CD83 and CD86 expression in DCs after 24 hours of stimulation with LPS (100 ng/ml), Pam_3CSK4 (1 µg/ml) and *M. vaccae* (10 and 100 µg/ml). White histograms show staining of unstimulated DCs. (Flow cytometry histograms show representative data from multiple experimental replicates.) Comparison of mean fluorescence intensity (MFI) for each of these markers **(B)** suggested that the maximum upregulation of CD83 and CD86 expression in response to *M. vaccae* (100 µg/ml) was comparable to that of LPS (100ng/ml) and significantly greater than the response to Pam_3CSK4 (1 µg/ml). Stimulation of DCs with a 10-fold lower concentration of *M. vaccae* (10 µg/ml) generated comparable DC maturation to Pam_3CSK4. (Bars represent mean ±SD of 14 separate experiments. * denotes significant differences to unstimulated cells, and ‡ denotes significant differences to stimulation with Pam_3CSK4 or *M. vaccae* (10 µg/ml), $p<0.001$ by paired t-tests). **C:** Representative photomicrographs of unstimulated and stimulated DC cultures (40x magnification).

3.3.5 Priming of dendritic cells with *M. vaccae* enhances T cell proliferation

Mixed leukocyte cultures with allogeneic T cells provide a valuable approach for study of DC-T cell interactions. This strategy was used to study the effect of innate immune priming of DCs with *M. vaccae* on subsequent T cell proliferation and differential polarisation of Th cell subsets.

The effect of priming DCs with *M. vaccae* (10 μg/ml or 100 μg/ml), LPS (100 ng/ml) or Pam_3CSK4 (1 μg/ml) on T cell proliferation was tested. Immature DCs were treated with each of these innate immune stimuli for 24 hours before addition of naive allogeneic T cells at increasing DC:T cell ratios. Naive $CD45RA^+/CD4^+$ T cells were used in this series of experiments to exclude cells which may harbour memory for mycobacterial antigens and to minimise confounding variability in pre-existing T cell memory from different donors. Since DCs evolve a mature phenotype in prolonged cell culture that may mask the effect of innate immune priming, pilot experiments were initially conducted to establish the earliest time point at which proliferative T cell responses were detectable. This was evident by day 3 of coculture with DCs and was measured by incorporation of $[^3H]$-thymidine added during the last 18 hours of culture. In multiple allogeneic DC-T cell combinations at this time point, T cell proliferation was principally related to the number of DCs (Figure 3-9A). However, considerable variability was also evident in different allogeneic cultures. Therefore repeated measures (RM) 2-way ANOVA was used to test the effect of innate immune priming independently of the number of DCs within matched DC-T cell allogeneic pairs. As expected, this showed a significant effect of DC number (Figure 3-9A) but innate immune priming of DCs with each of the stimuli under study was also associated with significantly enhanced T cell proliferation compared to unstimulated DCs (Figure 3-9B-E). In keeping with the data on DC maturation (Figure 3-8B), this effect was statistically more powerful when DCs were primed with LPS (Figure 3-9B) or

100 µg/ml *M. vaccae* (Figure 3-9E) than with Pam_3CSK4 (Figure 3-9C) or 10 µg/ml *M. vaccae* (Figure 3-9D).

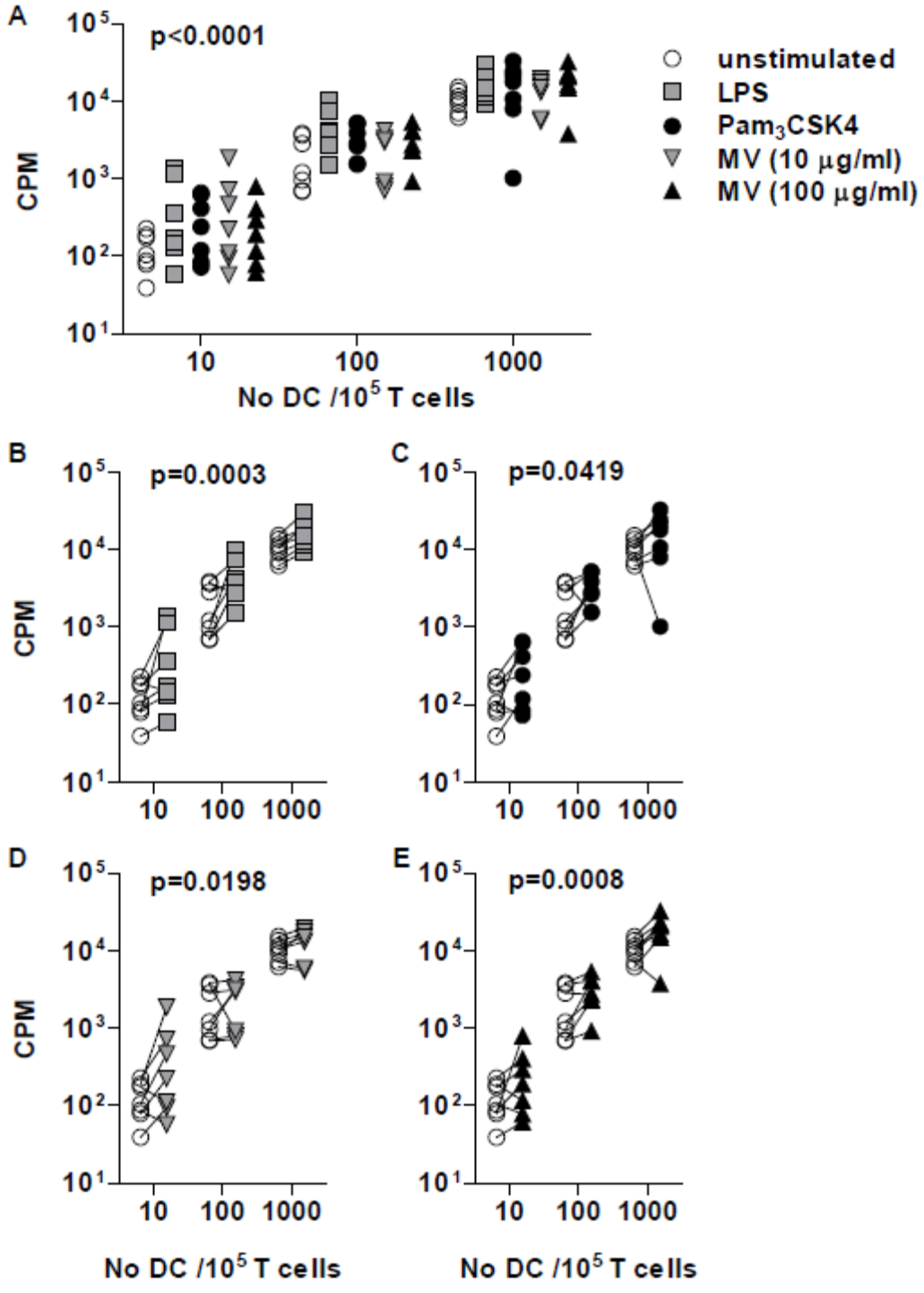

Figure 3-9: Priming of dendritic cells with *M. vaccae* enhances allogeneic T cell proliferation

Naive $CD4^+$ T cell proliferation was assessed by thymidine incorporation (CPM) after 3 days stimulation with allogeneic DCs. The effects of DC number and DC priming with the stimuli indicated were assessed by RM-2-way ANOVA. Increasing DC:T cell ratios were associated with significantly increased T cell proliferation **(A),** and in comparison to unstimulated cells, DCs primed with each of the stimuli also significantly increased proliferation **(B-E)**. There were no significant differences between the stimuli. Each experiment is represented by paired data points.

3.3.6 Pre-treatment of dendritic cells with *M. vaccae* attenuates Th2 responses

To test the hypothesis that *M. vaccae*-primed DCs reduce Th2-polarised responses, the phenotype of allogeneic T cells was analysed after three days of coculture with DCs, which had been pre-treated with each stimulus. Th1/Th2 differential responses were analysed in these experiments, using intracellular staining for IFN-γ and IL-4 as markers for Th1 and Th2 responses respectively, after re-stimulation of cytokine production with PMA and ionomycin. Flow cytometric detection of intracellular cytokines was used to distinguish the cytokines produced by T cells rather than DCs. Moreover it allows quantitation of percentages of cells with Th1 and Th2 responses and measurement of multiple cytokines simultaneously in individual cells.

Increasing numbers of DCs were independently associated with increasing proportions of IFN-γ positive cells (Figure 3-10A) and decreasing proportions of IL-4 producing cells (Figure 3-11A). Therefore Th1 and Th2 responses across the range of DC:T cell ratios were tested to assess the effect of innate immune priming of DCs. LPS, Pam_3CSK4 and *M. vaccae* stimulation of DCs did not significantly affect the relationship between DCs and Th1 responses (Figure 3-10B-F), but differential effects were clearly evident on Th2 responses (Figure 3-11B-F). While LPS had no effect, Pam_3CSK4 priming of DCs was associated with sustained Th2 responses, reducing the inverse relationship between number of DCs and proportion of IL-4 producing T cells (Figure 3-11B). In contrast, DC-priming with *M. vaccae* augmented this negative relationship significantly (Figure 3-11B). Greater inhibition of IL-4 producing T cells by 100 μg/ml *M. vaccae* compared to 10 μg/ml *M. vaccae* suggested a dose-response association for this effect.

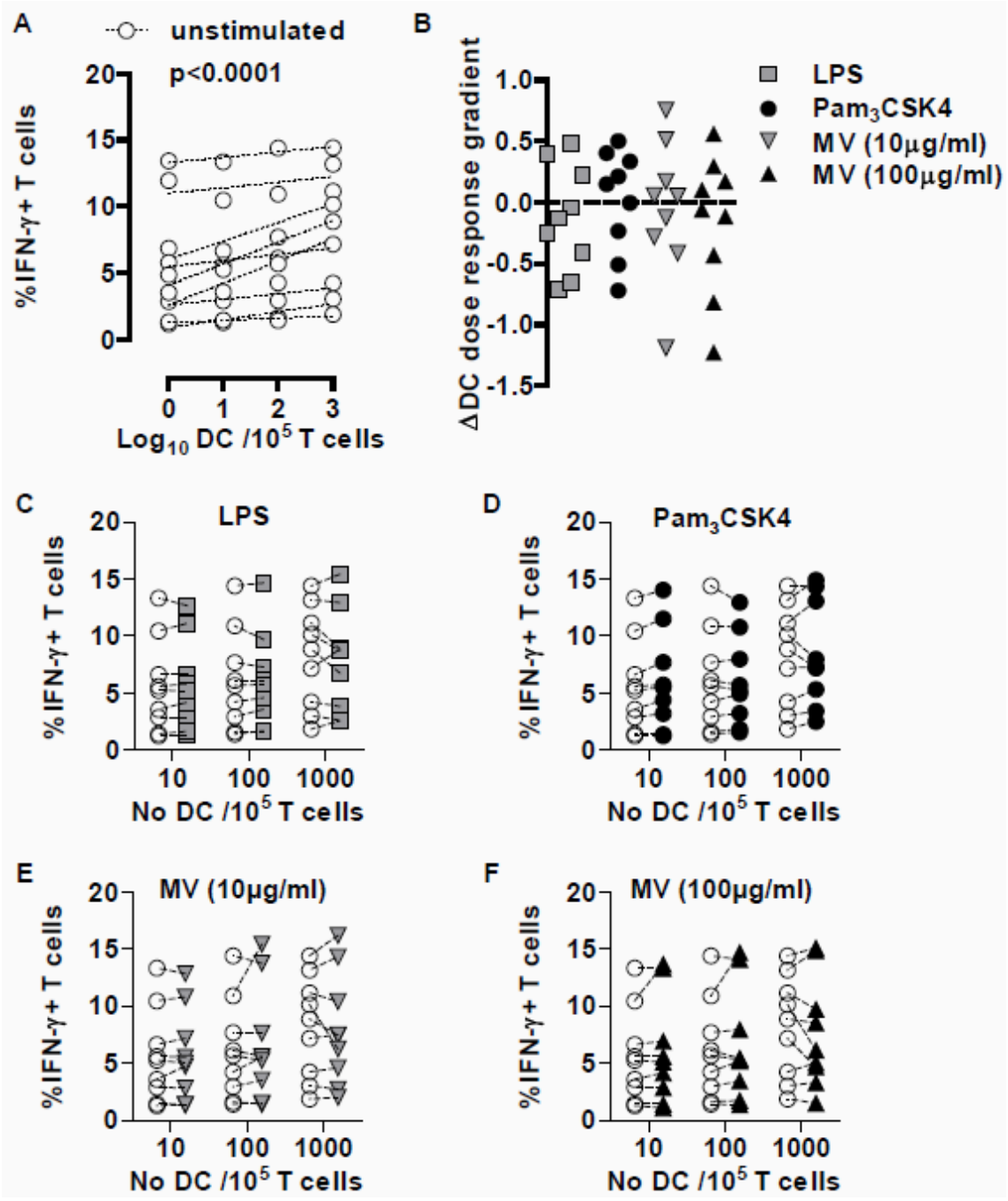

Figure 3-10: Priming of dendritic cells with *M. vaccae* does not affect Th1 polarisation

In 3 day allogeneic cocultures of DCs with naive $CD4^+$ T cells, IFN-γ^+ and IL-4^+ producing T cells were enumerated by intracellular immunofluorescence staining and flow cytometry, after PMA/ionomycin stimulation. The effects of DC number and DC priming with the stimuli indicated were assessed by RM-2-way ANOVA. Increasing DC:T cell ratios were associated with increased proportions of IFN-γ^+ cells ($p<0.0001$) **(A)**. In order to assess the effect of DC priming in this model, the regression relationship between DC:T cell ratio and proportions of IFN-γ^+ cells was determined for each experiment (dotted line) and the gradient of these relationships in unprimed DCs was compared to those of primed DCs **(B)**. DC priming had no significant effect on DC-dependent IFN-γ polarisation of T cells. **C-F**: Percentage of IFN-γ^+ T cells after culture with DCs primed with the stimuli indicated at increasing DC-T cell ratios compared to culture with unstimulated DCs. Each experiment is represented by paired data points.

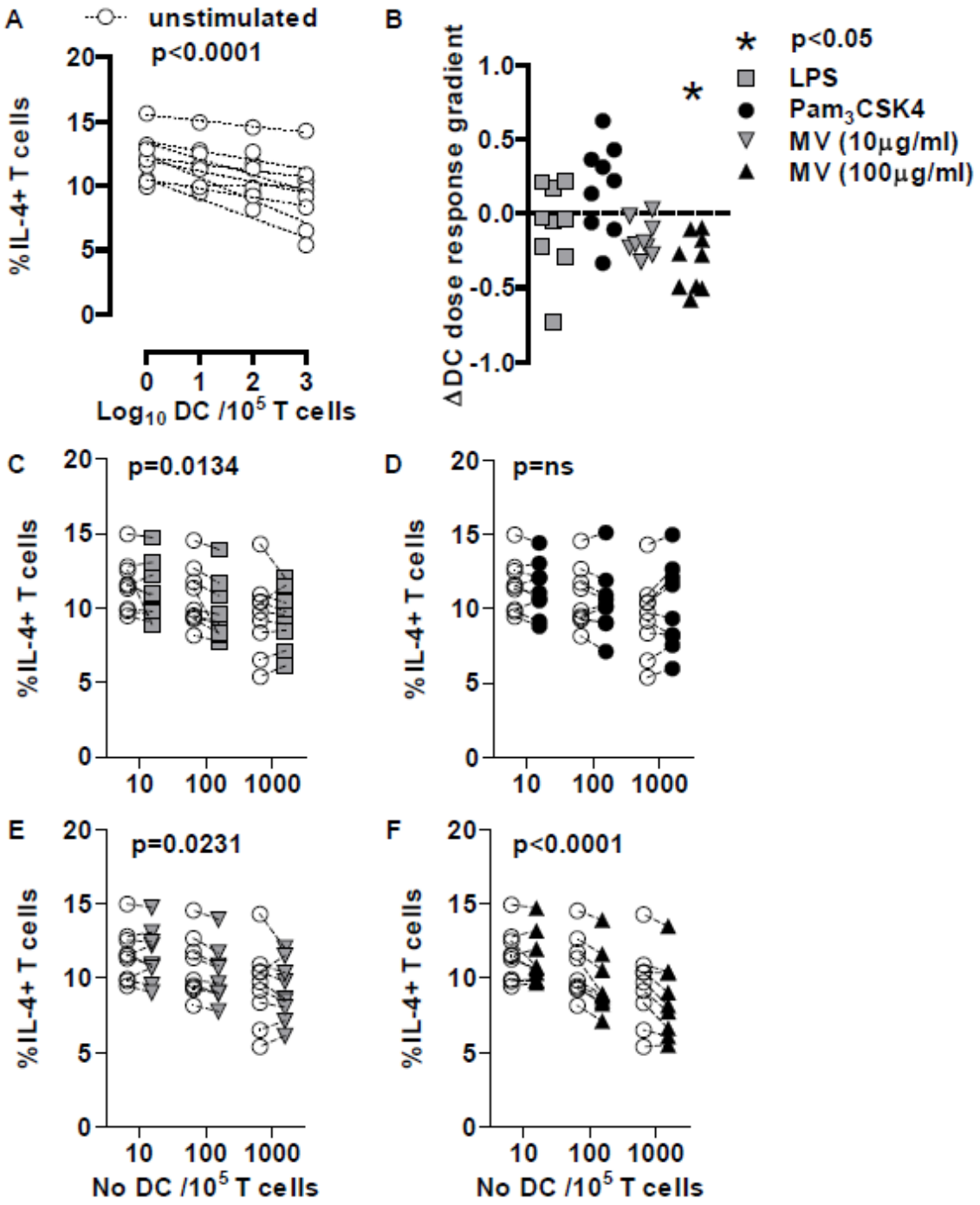

Figure 3-11: Priming of dendritic cells with *M. vaccae* attenuates Th2 responses

In 3 day allogeneic cocultures of DCs with naive $CD4^+$ T cells, IFN-γ^+ and IL-4^+ producing T cells were enumerated by intracellular immunofluorescence staining and flow cytometry, after PMA/ionomycin stimulation. The effects of DC number and DC priming with the stimuli indicated were assessed by RM-2-way ANOVA. Increasing DC:T cell ratios were associated with decreased proportions of IL-4^+ cells ($p<0.0001$) **(A)**. In order to assess the effect of DC priming in this model, the regression relationship between DC:T cell ratio and proportions of IL-4^+ cells was determined for each experiment (dotted line) and the gradient of these relationships in unprimed DCs was compared to those of primed DCs **(B)**. DC priming did differentially affect IL-4 polarisation of T cells. Priming with *M. vaccae* significantly enhanced DC-dependent reduction of IL-4^+ producing T cells in comparison to unstimulated DCs ($p<0.05$, RM-1-way ANOVA). **C-F**: Percentage of IL-4^+ T cells after culture with DCs primed with the stimuli indicated at increasing DC-T cell ratios compared to culture with unstimulated DCs; RM-2-way ANOVA was used to analyse the effect of DC priming. Each experiment is represented by paired data points.

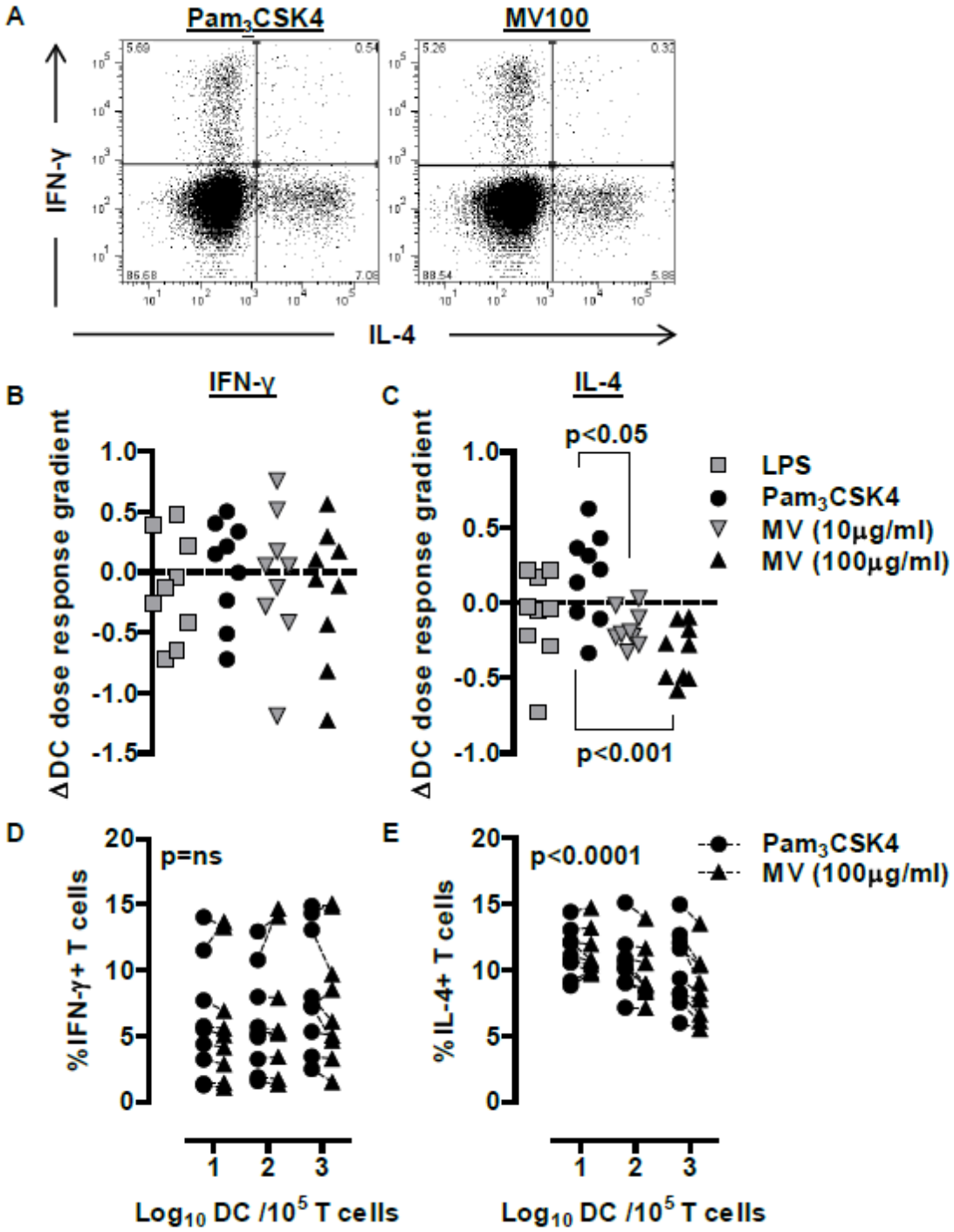

Figure 3-12: Comparison of the effects of dendritic cell priming with *M. vaccae* or TLR2-specific stimulation with Pam_3CSK4 on T cell polarisation

In 3 day cocultures of DCs with allogeneic naive $CD4^+$ T cells, the percentage of IFN-γ^+ and IL-4^+ T cells was measured by intracellular flow cytometry. **A**: Flow cytometry plots of T cells after culture with Pam_3CSK4 (left) or *M. vaccae* (right) primed DCs. The regression relationship between DC:T cell ratio and proportions of IFN-γ^+ or IL-4^+ cells was determined for each experiment (dotted lines) and the gradient of these relationships in unprimed DCs was compared to those of primed DCs **(B-C).** Priming with *M. vaccae* significantly enhanced DC-dependent reduction of IL-4^+ producing T cells in comparison to priming with Pam_3CSK4, which had the opposite effect (paired t-test). Direct comparison, showed significant reduction (RM-2-way ANOVA) of IL-4^+ T cells with increasing numbers of DCs primed with *M. vaccae* compared to those primed with Pam_3CSK4 **(E)**. No differential effects on IFN-γ^+ T cells were evident **(D)**. Each experiment is represented by paired data points.

Detailed analysis showed the divergent effects of specific TLR2 stimulation and *M. vaccae*-priming of DCs on Th2 responses (Figure 3-12). Significant differences between 10 μg/ml *M. vaccae*- and Pam_3CSK4-priming of DCs were also evident despite comparable levels of DC maturation (Figure 3-12C). In addition, LPS-priming of DCs did not significantly attenuate Th2 responses despite inducing similar levels of DC maturation to priming with 100 μg/ml *M. vaccae*. Taken together, these findings suggest that enhanced DC-dependent inhibition of Th2 responses were specific to priming DCs with *M. vaccae* independently of levels of DC maturation.

3.3.7 Dendritic cells primed with *M. vaccae* induce $CD25^{high}/FoxP3^{high}$ T cells

Previous reports from animal models suggested that inhibition of Th2 responses may be the result of enhanced Treg responses in mice receiving *M. vaccae* (Zuany-Amorim et al., 2002a; Zuany-Amorim et al., 2002b). In order to test this hypothesis in the present model, the effects of DC priming on induction of $CD25^{high}/FoxP3^{high}$ T cells in an allogeneic response was assessed (Figure 3-13). Like the other phenotypes studied, there was a clear relationship between the number of DCs and induction of $CD25^{high}/FoxP3^{high}$ cells (Figure 3-13A). In addition, priming of DCs with LPS or 100 μg/ml *M. vaccae* significantly enhanced this induction, but this effect was not evident with Pam_3CSK4 or 10 μg/ml *M. vaccae* (Figure 3-13B-E). These findings did not demonstrate a consistent correlation with effects of DC priming on Th2 responses or inhibition of T cell proliferation. In addition, no evidence of IL-10 production by these cells using intracellular cytokine staining was found (Figure 3-14A) and ELISA of cell culture supernatants showed only augmented IL-10 production in cocultures with LPS-primed DCs (Figure 3-14C). Therefore the $CD25^{high}/FoxP3^{high}$ phenotype may be a feature of T cell activation rather than Treg differentiation (Merlo et al., 2008; Tran et al., 2007; Wang et al., 2007).

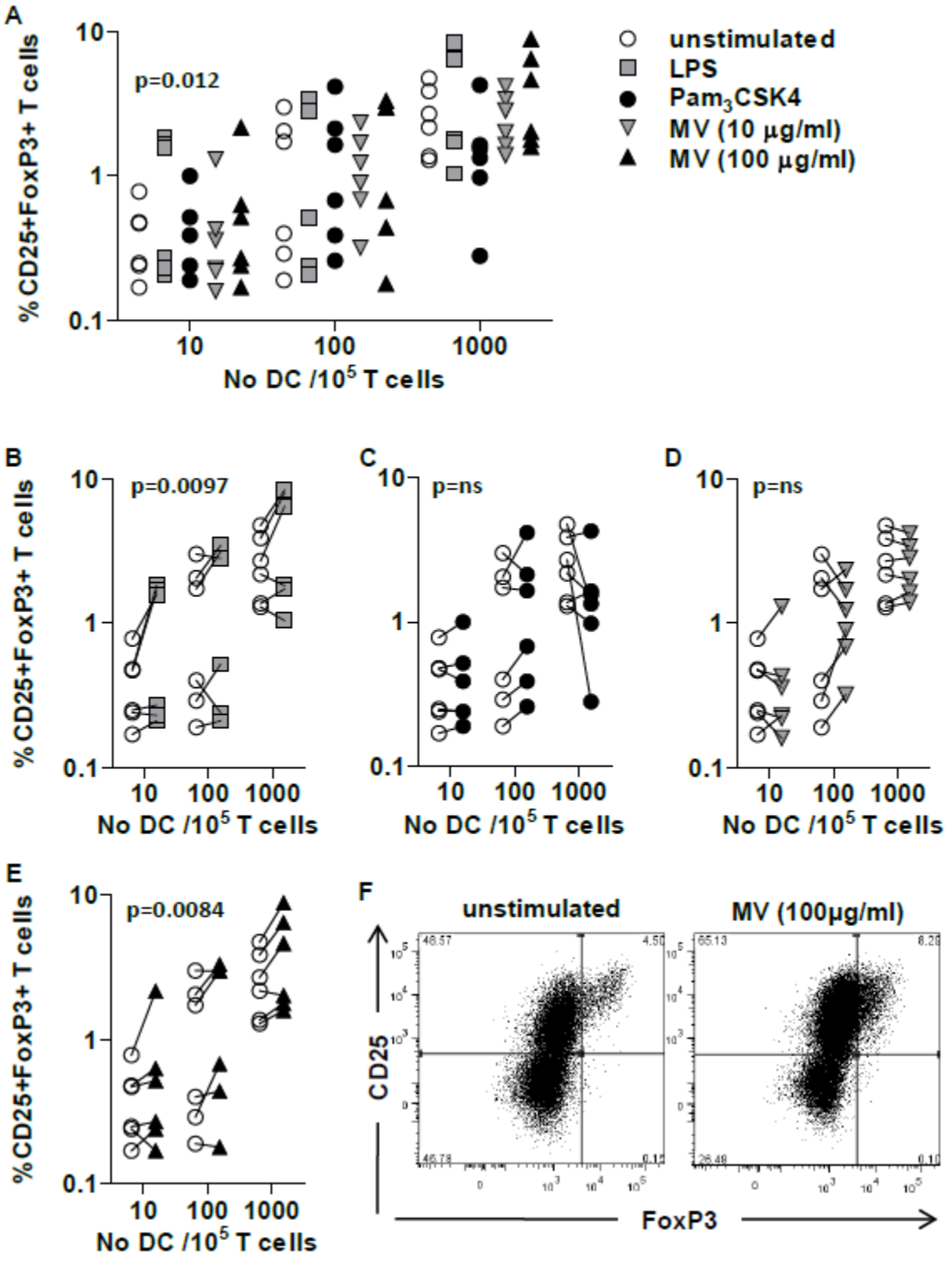

Figure 3-13: Dendritic cells primed with *M. vaccae* induce CD25^{high}/FoxP3^{high} T cells

In 6 day allogeneic cocultures of DCs with naive CD4$^+$ T cells, CD25$^+$/FoxP3$^+$ T cells were enumerated by intracellular immunofluorescence staining and flow cytometry. The effects of DC number and DC priming with the stimuli indicated were assessed by RM-2-way ANOVA. Increasing DC:T cell ratios were associated with increased proportions of CD25$^+$/FoxP3$^+$ T cells **(A)**. In comparison to unstimulated cells, DCs primed with LPS or *M. vaccae* (100 µg/ml) also significantly increased CD25$^+$FoxP3$^+$ T cells **(B, E)**. Each experiment is represented by paired data points. **F**: Representative flow cytometry plots of T cells stained for CD25 and FoxP3 after coculture with unstimulated or *M. vaccae*-primed DCs.

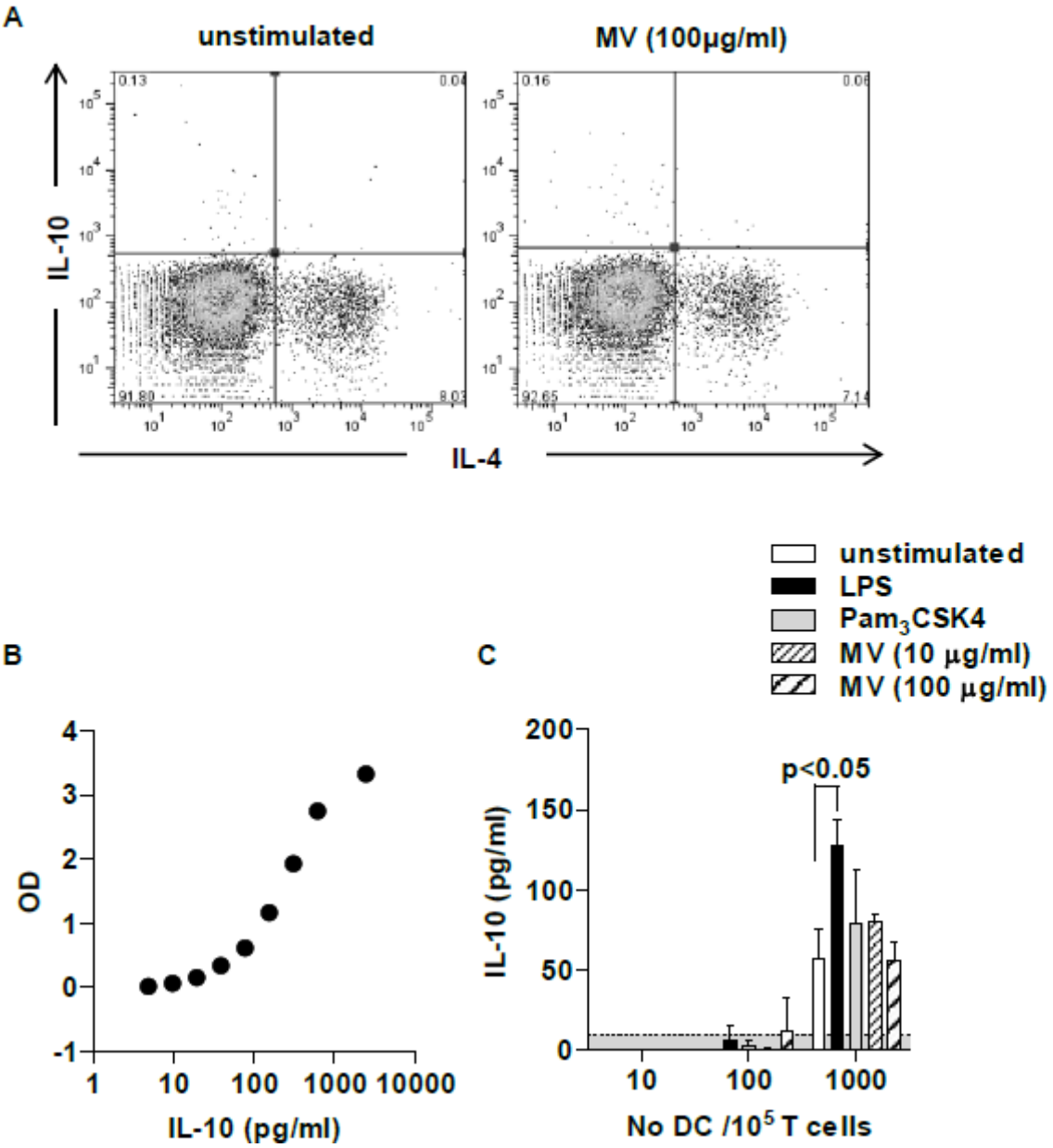

Figure 3-14: T cells from cocultures with *M. vaccae* primed dendritic cells do not produce augmented levels of IL-10

In 3 and 6 day cocultures of DCs and allogeneic naive $CD4^+$ T cells, the production of IL-10 was analysed. **A**: Analysis of IL-10 positive T cells by intracellular flow cytometry did not detect any IL-10 positive cells. The supernatants of DC-T cell cocultures were analysed for IL-10 production by ELISA compared to a standard curve **(B)**. IL-10 above the detection limit was only produced by 6 day cultures with a 1:100 DC:T cell ratio **(C)**. Only priming with LPS significantly augmented IL-10 levels compared to unstimulated DC (t-test). Data show mean (±SD) from three independent DC-T cell experiments.

3.3.8 Dendritic cells primed with *M. vaccae* enhance T cell proliferation, attenuate Th2 responses and induce $CD25^{high}/FoxP3^{high}$ T cells also in antigen-specific cultures

Next I sought to establish whether the effects of *M. vaccae*-primed DCs on naive T cells observed in allogeneic cultures were also evident in antigen-specific responses by the whole population of autologous $CD4^+$ T cells, including memory T cells ($CD45RO^+$). Therefore DCs, generated from blood donors who had previously received tetanus toxoid, were treated with 10 μg/ml tetanus toxoid in addition to each innate immune stimulus used.

Tetanus toxoid alone did not cause maturation of unstimulated DCs (Figure 3-15A) and assessment of proliferation of autologous $CD4^+$ T cells in cocultures confirmed antigen-specific memory (Figure 3-15B).

Similar to the data from allogeneic cultures, T cell proliferation measured on day three of coculture was again principally related to the number of DCs (Figure 3-16A). Priming of DCs with *M. vaccae* or Pam_3CSK4 further augmented T cell proliferation in contrast to priming with LPS (Figure 3-16B-E). Clearly the effect of *M. vaccae*-priming may in part have resulted from the presence of T cells that harbour memory for mycobacterial antigens.

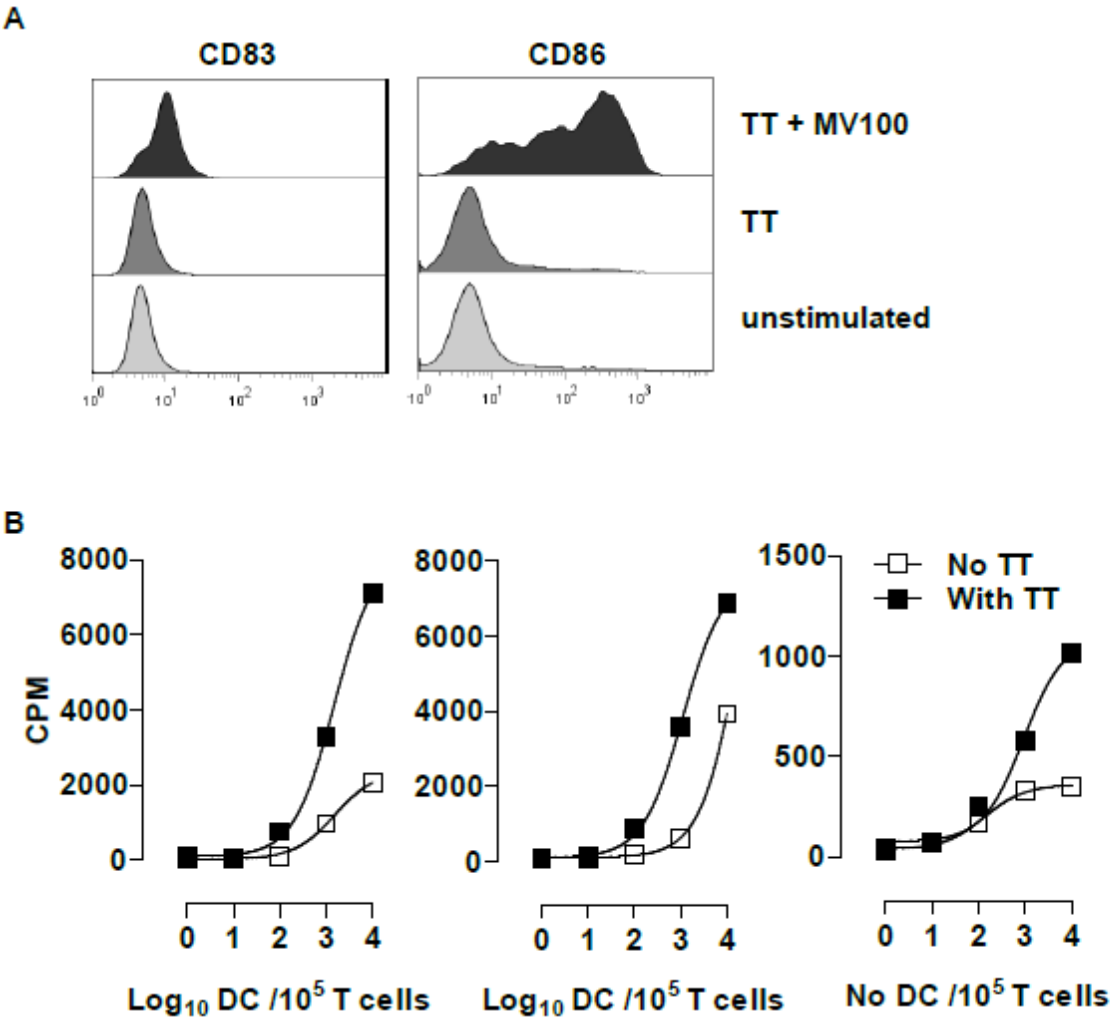

Figure 3-15: Dendritic cells primed with tetanus toxoid do not mature but augment T cell proliferation in autologous cocultures

A: DCs cultured with ± tetanus toxoid (TT) and ± *M. vaccae* for 24 hours were analysed for CD83 and CD86 expression by flow cytometry. TT alone did not upregulate CD83 and CD86 expression on DCs. **B:** T cell proliferation stimulated by 3 day culture with autologous DCs primed for 24 hours ± TT and measured by thymidine incorporation (CPM) showed TT-dependent memory responses in cultures from three separate donors.

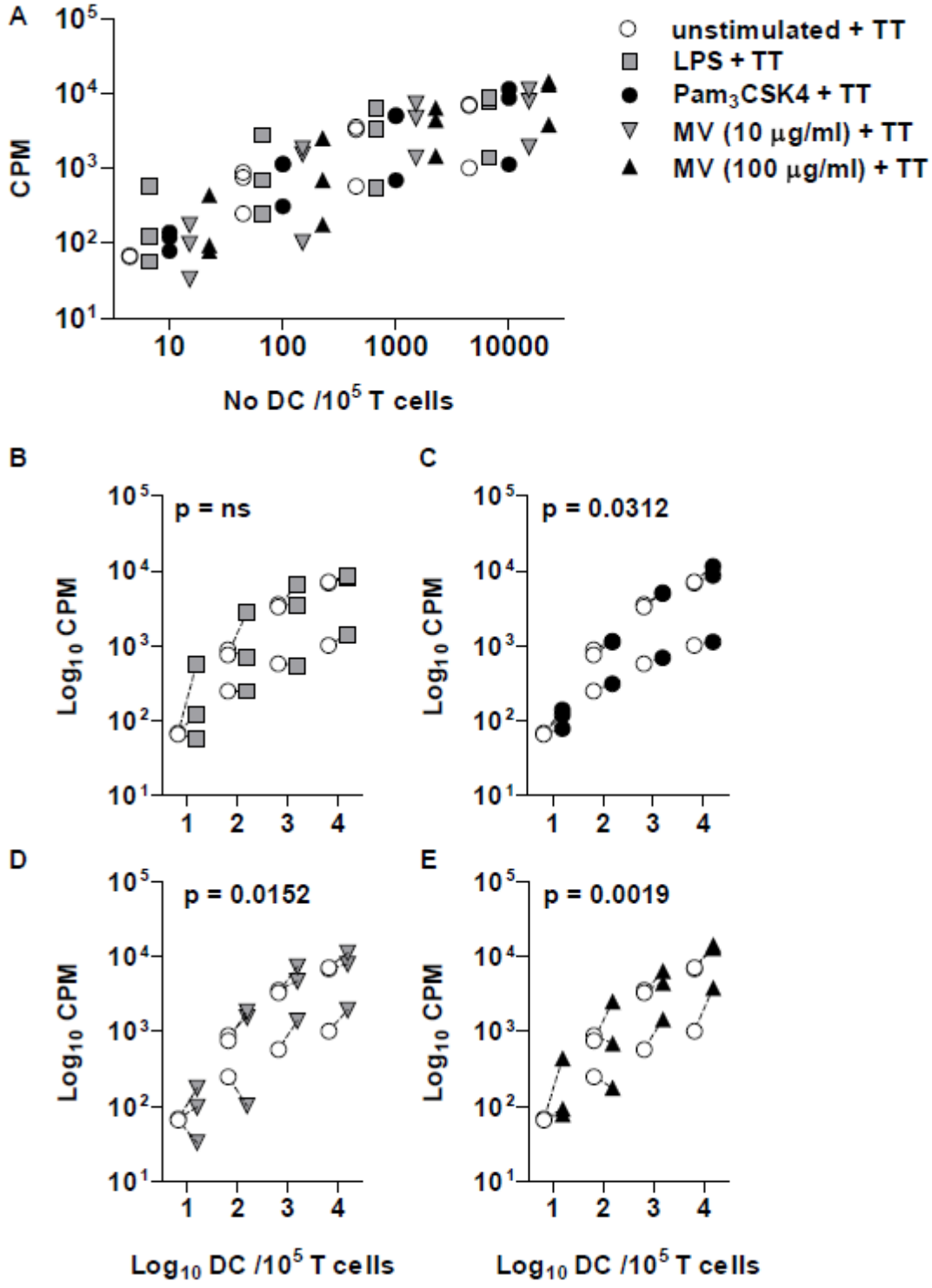

Figure 3-16: Dendritic cells primed with *M. vaccae* enhance T cell proliferation in antigen-specific cocultures

T cell proliferation stimulated by 3 day culture with autologous DCs primed for 24 hours with tetanus toxoid (TT) ± innate immune stimuli showed DC-dependent responses in cells from three separate donors **(A)**. RM-2-way ANOVA showed a significant increase in proliferative responses associated with the priming of DCs with Pam_3CSK4 **(C)** and *M. vaccae* **(D-E)**. Data points represent individual experiments and lines link paired data from the same donor /experiment.

Analysis of Th1 and Th2 cytokine production by total $CD4^+$ T cells in response to autologous DCs primed with tetanus toxoid in addition to each innate stimulus mirrored the observations from earlier allogeneic cocultures. Intracellular staining for IFN-γ and IL-4 was performed on day three of coculture after transient re-stimulation for cytokine production with tetanus toxoid. Similarly in this antigen-specific experimental model, increasing numbers of DCs were independently associated with increasing proportions of IFN-γ producing cells (Figure 3-17A) and decreasing proportions of IL-4 positive cells (Figure 3-17B). The effect of innate immune priming of DCs on Th1 and Th2 responses was again assessed across a range of DC:T cell ratios. Since the T cell proliferation response was smaller in those autologous cocultures compared to earlier allogeneic cocultures at similar DC:T cell ratios, a higher DC:T cell ratio was included into the experiments. The relationship between DCs and Th1 responses was not significantly affected by any of the stimuli under study (Figure 3-17C), but differential effects were evident on Th2 responses (Figure 3-17D). Mirroring the data from allogeneic DC-T cell cocultures, *M. vaccae* augmented the reduction of Th2 responses and greater inhibition of IL-4 producing T cells by 100 μg/ml *M. vaccae* compared to 10 μg/ml *M. vaccae* was again in keeping with a dose-response relationship for this effect. Although priming of DCs with Pam_3CSK4 did not reverse the negative relationship of DC number and Th2 cells like in allogeneic responses, comparison of the effects of *M. vaccae* and specific TLR2 stimulation of DCs showed that they were significantly different (Figure 3-17D, F).

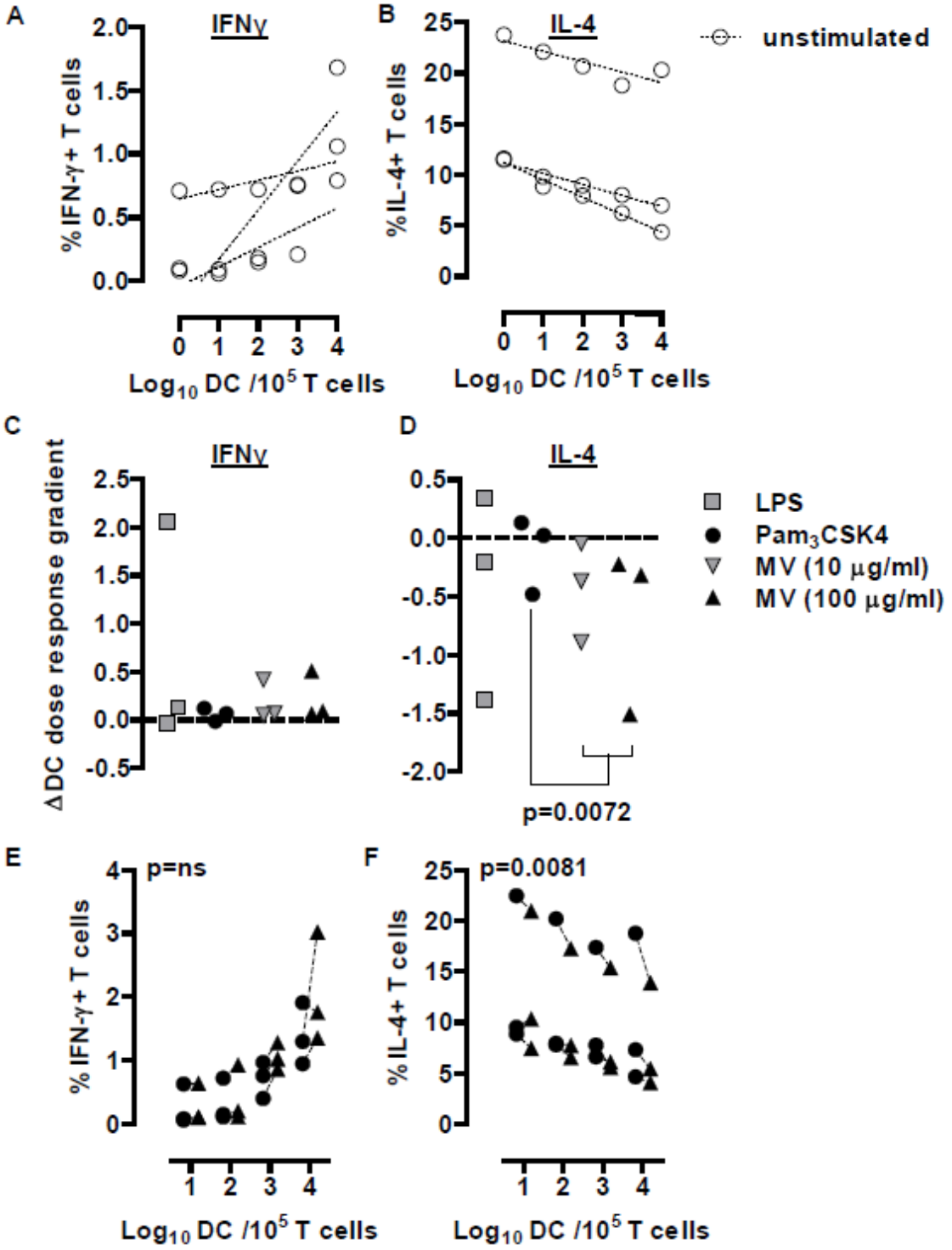

Figure 3-17: Dendritic cells primed with *M. vaccae* reduce Th2 responses in contrast to Pam_3CSK4 in antigen-specific cocultures

In antigen-specific responses to tetanus toxoid, in order to assess the effect of DC priming on Th polarisation, the regression relationship between DC:T cell ratio and proportions of IFN-γ^+ or IL-4^+ cells was determined after 3 days and the gradient of these relationships in unprimed DCs compared to those of primed DCs **(A-B)**. DC priming had no significant effect on DC-dependent IFN-γ polarisation of T cells **(C)**, but did differentially affect IL-4 polarisation of T cells **(D)**. Priming with *M. vaccae* significantly enhanced DC-dependent reduction of IL-4^+-producing T cells in comparison to priming with Pam_3CSK4 (paired t-test). Direct comparison, showed significant reduction (RM-2-way ANOVA) of IL-4^+ T cells with increasing numbers of DCs primed with *M. vaccae* compared to those primed with Pam_3CSK4 **(F)**. No differential effects on IFN-γ^+ T cells were evident **(E)**. Each experiment is represented by paired data points.

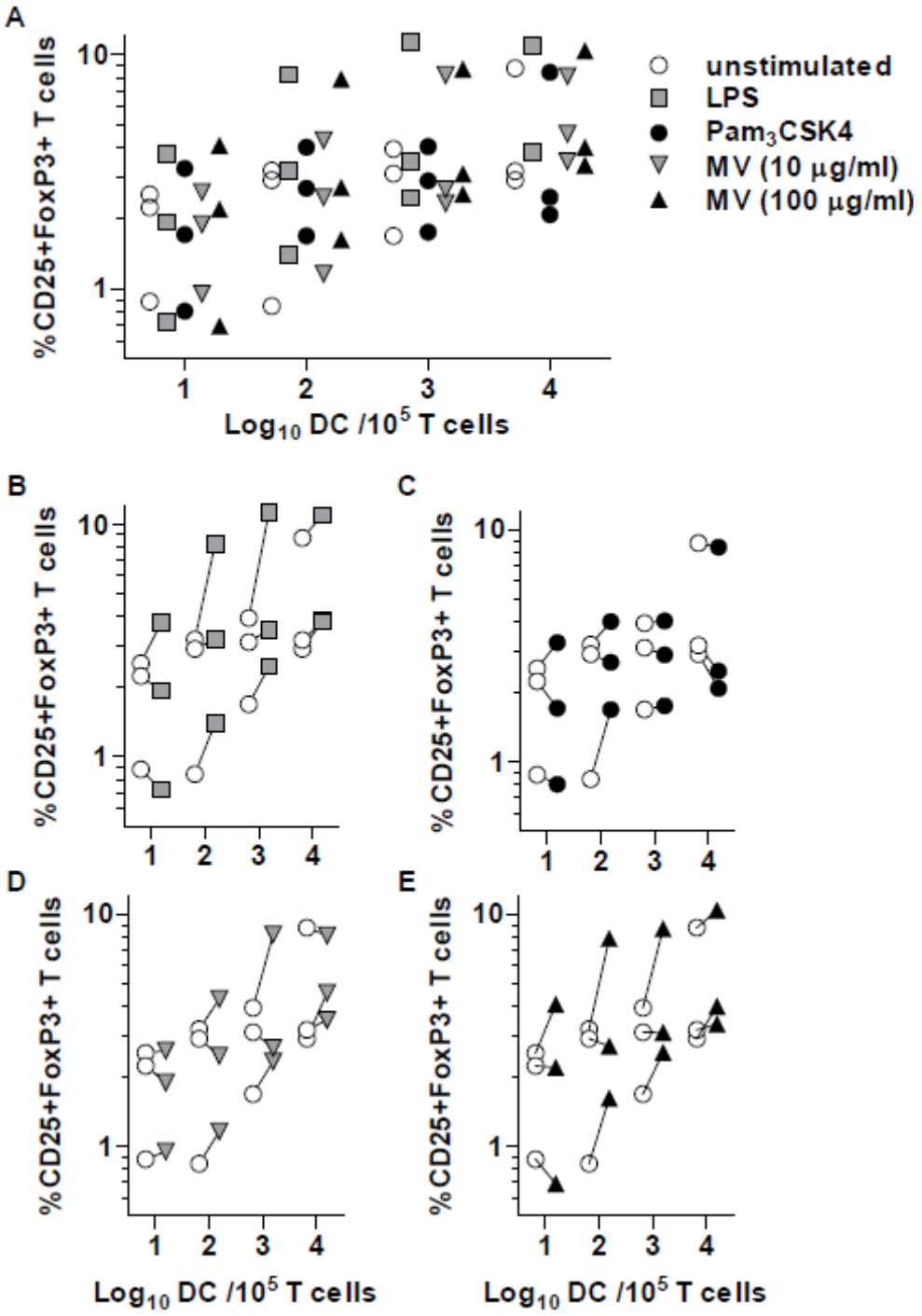

Figure 3-18: Dendritic cells primed with *M. vaccae* induce CD25^{high}/FoxP3^{high} T cells in antigen-specific cocultures

In 6 day antigen-specific cocultures of DCs with autologous CD4$^+$ T cells, CD25$^+$/FoxP3$^+$ T cells were enumerated by intracellular immunofluorescence staining and flow cytometry. The effects of DC number and DC priming with the stimuli indicated were assessed by RM-2-way ANOVA. Increasing DC:T cell ratios were associated with increased proportions of CD25$^+$/FoxP3$^+$ T cells **(A)**. In comparison to unstimulated cells, DCs primed with LPS **(B)** or *M. vaccae* **(D-E)** increased CD25$^+$/FoxP3$^+$ T cells in contrast to Pam_3CSK4 **(C)**. Each experiment is represented by paired data points.

In line with the data from allogeneic cocultures, the percentage of $CD25^{high}/FoxP3^{high}$ T cells was also principally associated with the number of DCs in the antigen-specific DC-T cell coculture experiments (Figure 3-18A). Additional priming of tetanus toxoid treated DCs with LPS and the two doses of *M. vaccae* augmented the percentage of cells with this phenotype in contrast to priming with Pam_3CSK4 (Figure 3-18B-E). Although this effect was not statistically significant as one DC-T cell donor pair behaved differently, diverse effects of *M. vaccae*-treated and Pam_3CSK4-treated DCs are clearly evident.

3.3.9 Conditioned medium from cultures of dendritic cells primed with *M. vaccae* is not sufficient to attenuate Th2 responses

Upon microbial stimulation DCs take up, process and present antigens. In addition to upregulation of MHC and costimulatory molecules, the activated cells produce and secrete cytokines and chemokines, which are thought to play an important role in the polarisation of T cell responses (Kapsenberg, 2003). Therefore, I tested the hypothesis that inhibition of Th2 responses by *M. vaccae*-primed DCs was mediated by such cytokine responses.

A 1:2 dilution of conditioned media from DC cultures primed for 24 hours with *M. vaccae* or the other innate immune stimuli were used to supplement T cell cultures stimulated with plate bound anti-CD3 and soluble anti-CD28 as a surrogate for antigen-presenting cells. After three days, the qualitative effect of supernatants from innate immune primed DCs on T cell responses was assessed by intracellular staining for IFN-γ and IL-4. This demonstrated that cytokines and chemokines present in the supernatants of stimulated DCs together with antibody binding to CD3 and CD28, were

not sufficient to replicate the effect of *M. vaccae*-primed DCs in inhibiting Th2 responses (Figure 3-19A).

3.3.10 Fixed dendritic cells primed with *M. vaccae* do not replicate the effects of live cells

Since using conditioned media from DCs primed with *M. vaccae* did not replicate the effect of coculturing T cell with primed antigen-presenting cells, I then tested the hypothesis that cell surface interactions between DCs and T cells would be sufficient for *M. vaccae*-mediated inhibition of Th2 responses. DCs were stimulated with *M. vaccae*, LPS or Pam_3CSK4 for 24 hours and then fixed with glutaraldehyde. Fixed DCs, which retain cell surface antigen presentation and expression of costimulatory molecules, were cocultured for three days with allogeneic T cells.

As seen before, in allogeneic cultures with live DCs it was clearly evident that increasing numbers of DCs, independent of innate immune priming, were associated with decreasing proportions of IL-4 producing cells (Figure 3-19B). This association was not found in cocultures with fixed DCs. Here the number of DCs did not affect the proportion of Th2 cells (Figure 3-19C). Moreover, priming of DCs with any of the innate immune stimuli prior to their fixation had also no effect on the quantities of IL-4-producing cells (Figure 3-19E).

Taken together, this demonstrated that live DCs primed with *M. vaccae* were required for their ability to inhibit Th2-polarised responses, and that neither antigen presentation nor soluble factors alone were sufficient to replicate the effects.

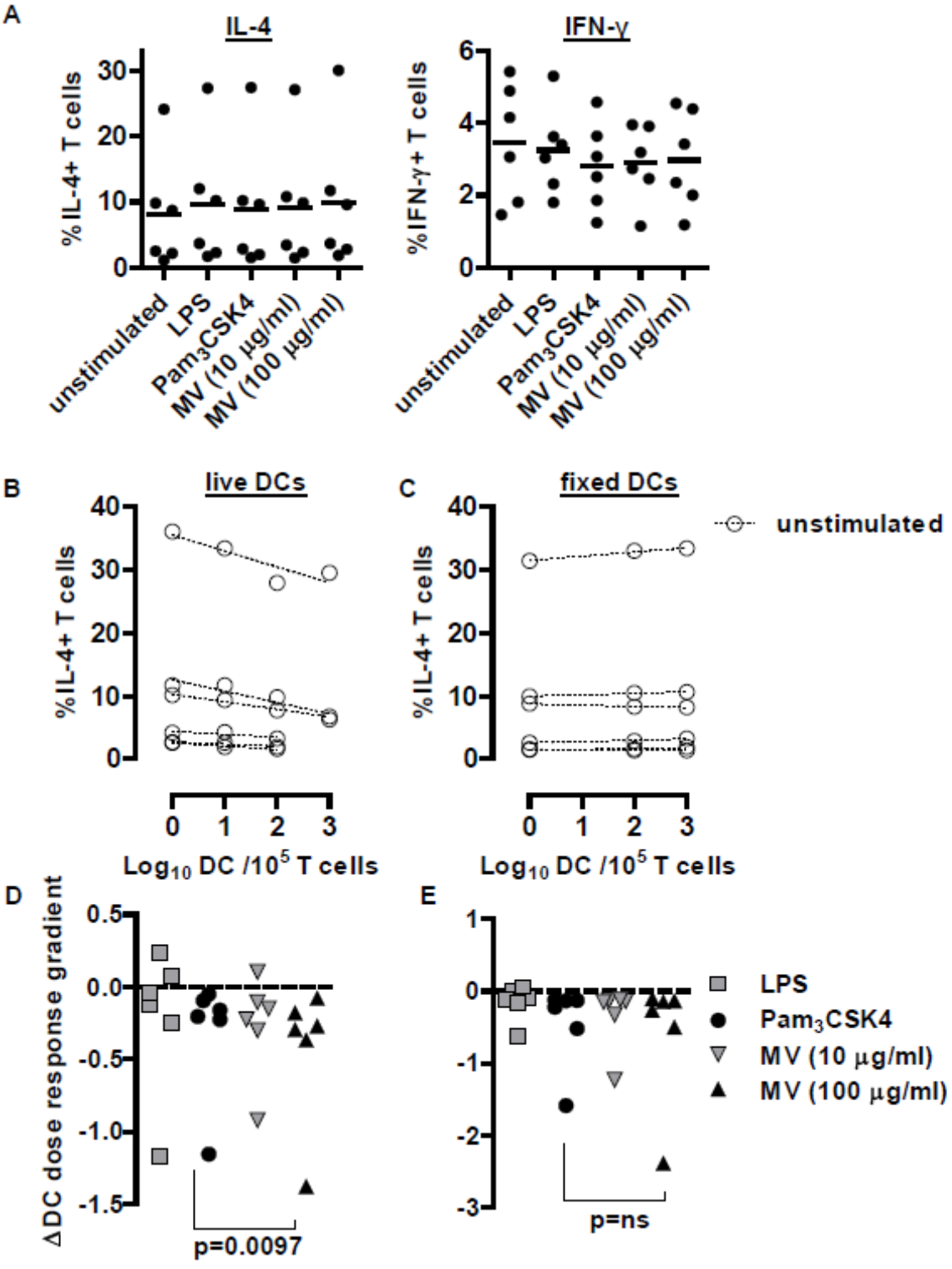

Figure 3-19: Conditioned media from dendritic cells primed with *M. vaccae* and fixed dendritic cells do not attenuate Th2 responses

After 3 day cultures of naive $CD4^+$ T cells in the presence of anti-CD3 and anti-CD28 in conditioned media from allogeneic DCs primed for 24 hours with the different stimuli, the percentage of IFN-γ^+ and IL-4^+ cells was measured by flow cytometry. **A**: The conditioned media from differentially primed DCs did not affect the number of IFN-γ^+ and IL-4^+ T cells. **B**: In contrast to live DCs (left) the number of fixed DCs (right) did not reduce the percentage of IL-4^+ T cells. **C**: Priming of DCs with *M. vaccae* that reduced the Th2 response compared to Pam_3CSK4 when using live DCs (left), had no effect when using fixed DCs (right).

3.4 Discussion

3.4.1 *M. vaccae* induces dose-dependent maturation of dendritic cells

Subsequent to stimulation, DCs mature while migrating to the secondary lymphoid organs. A hallmark of DC maturation is the upregulation of costimulatory molecules including CD83 and CD86 that enable them to activate naive T cells (Johansson et al., 2007). Dose-dependent maturation of DCs by *M. vaccae* was clearly demonstrated. The question of whether this effect is in any way mycobacterium species specific was not addressed here. Interestingly, some mycobacteria, such as *M. leprae* may not have the capacity to induce maturation of DCs (Murray et al., 2007), but this observation may be a dose effect (Hashimoto et al., 2002) and potentially confounded by the limitations in *in vitro* culture of *M. leprae*.

3.4.2 *M. vaccae* induces cellular activation via TLR2

DCs, like a number of other cells, recognise microorganisms with PRRs that identify PAMPs commonly found on bacteria, fungi or viruses. Consequently, DCs can also recognise mycobacteria via innate immune receptors and TLR-dependent activation of DCs had been reported (Jo, 2008). The data presented here showed that *M. vaccae* is able to activate TLR2, which is in line to previous reports of TLR2 activation by mycobacteria (Drage et al., 2009; Krutzik et al., 2003; Sweet and Schorey, 2006). *M. vaccae* induced innate cellular activation of HEK 293 reporter cell lines that expressed combinations with TLR2, TLR1/2 and TLR2/6, but TLR2 alone was also sufficient. The possibility that TLR1 or TLR6 expression alone is also sufficient was not addressed, but so far neither of these receptors has been reported to function as homodimer. It has been reported that DNA from attenuated *M. tuberculosis* and BCG strains might act as a ligand for intracellular TLR9 in human macrophages though the authors reported an extremely low expression of TLR9 measured by qPCR (Kiemer,

2009), and it is often found that CpGs do not stimulate human macrophages. Even after sonication of the whole bacterium preparation of *M. vaccae*, no stimulation of HEK 293 cells expressing TLR9 was evident. Although TLR4-dependent cellular responses to *M. tuberculosis* (Bulut et al., 2005; van de Veerdonk et al., 2010) and *M. ulcerans* (Lee et al., 2009) had been reported, *M. vaccae* did not activate TLR4 expressing HEK 293 cells, suggesting potential differences among mycobacterial species, and confirmed that the preparation under study was free of LPS contamination. This allowed comparisons of the effects of *M. vaccae* to a specific TLR2 ligand (Pam_3CSK4) and to alternative stimulation with a TLR4 ligand (LPS). In addition to TLRs, other PRRs may also be involved in inducing an immune response to *M. vaccae*. These include receptors such as DC-SIGN, CCR5, dectin-1, Nod1, Nod2 or the mannose receptor, which have been demonstrated to interact with other mycobacteria (Ferwerda et al., 2007; Floto et al., 2006; Geurtsen et al., 2009; Nigou et al., 2001; Pott et al., 2009; Torrelles et al., 2006; Yadav and Schorey, 2006). It would have been attractive to test if *M. vaccae* stimulates cells also via one of those potential receptors and to include specific ligands into the experimental paradigm, but reporter systems were not available to me.

3.4.3 Inhibition of TLR2-dependent signalling

In order to study TLR2-independent effects of *M. vaccae* on DCs and T cell polarisation, it was attempted to block and neutralise TLR2-dependent signalling with monoclonal antibodies. First the antibody clone TL2.1 was tested on TLR2 expressing HEK 293 cells. Even very high concentrations of this antibody did not saturate staining of surface TLR2 on those cells, and pre-treatment could not reduce cellular activation induced by stimulation with Pam_3CSK4. As this might result from very high and rapid expression of TLR2 in this reporter cell line, the antibody was tested to block TLR2-dependent activation of the DC model used in these studies. Although the antibody clone TL2.1 detected TLR2 on the surface of DCs and was used at saturated

concentrations, it did not reduce maturation of DCs stimulated with the TLR2 ligand Pam_3CSK4. This was maybe due to rapid turnover of TLR2 molecules on the cell surface during the 24 hours incubation time. On the other hand, neither this anti-TLR2 antibody nor a second clone tested, blocked nuclear translocation of the transcription factor NF-κB, which was measured by confocal microscopy only 1 hour post treatment with the TLR2 ligand Pam_3CSK4. Other reports using the same concentration of clone TL2.1 (20 μg/ml) succeed in blocking NF-κB activation in TLR2 expressing HEK 293 cells (Ariza et al., 2009), and the manufacturer of clone 383936 (R&D systems) showed nearly 100 % neutralisation of TLR2-mediated responses to Pam_3CSK4 at a 10-fold lower concentration of this antibody.

3.4.4 Comparison of dendritic cell maturation in response to differential innate immune stimulation

Quantitative comparison of CD83 and CD86 upregulation by stimulation with concentrations of *M. vaccae*, LPS or Pam_3CSK4 that induced maximal maturation showed that *M. vaccae* and LPS provoked similar and higher responses than Pam_3CSK4. It had been suggested that the strength of TCR stimulation, including the dose of antigens and the maturation status of DCs, may influence T cell polarisation (Boonstra et al., 2003; Brandt et al., 2002; Constant et al., 1995; Hosken et al., 1995; Langenkamp et al., 2000; Steinfelder et al., 2009). *In vitro* stimulation with low doses of priming antigen was repeatedly associated with the induction of IL-4 production in cultures with naive $CD4^+$ T cells, while large doses of antigen favoured the development of Th1 cells (Boonstra et al., 2003; Brandt et al., 2002; Constant et al., 1995; Langenkamp et al., 2000). Consequently it is possible that different expression levels of MHC class II and/or costimulatory molecules, thus different levels of DC maturation, change the effective dose of antigen presented by the DCs to the T cells. To control for potential effects resulting from different levels of DC maturation, a lower

dose of *M. vaccae* leading to similar upregulation of CD83 and CD86 on DCs than stimulation with Pam_3CSK4 was included in the experimental paradigm. Potential differences in the upregulation of other markers of DC maturation in response to priming with *M. vaccae* or the other innate immune stimuli were not addressed. Certainly it would be of interest to study the effect of *M. vaccae* treatment on the expression of CD40 and CD80. *Bifidobacterium* strains, which induced regulatory DCs expressing increased amounts of IL-10, showed decreased levels of CD40 and CD80 expression, but upregulated CD83 and had no effect on CD86 when compared to untreated DCs (Hart et al., 2004). If downregulation of CD80 and CD40 is a general phenotype of Treg-inducing DCs, one could have expected the same phenotype might be seen in DCs stimulated by *M. vaccae*.

3.4.5 Effects of *M. vaccae*-primed dendritic cells on T cell responses

3.4.5.1 *M. vaccae* augments T cell proliferation

To test the hypothesis that *M. vaccae*-primed DCs support Treg cells and reduce Th2-polarised responses, mixed leukocyte cultures with allogeneic naive T cells were used to study DC-T cell interactions. Increasing numbers of DCs augmented T cell proliferation proportionally. This was expected in an allogeneic response and had been previously reported (Banchereau and Steinman, 1998; Felix and Allen, 2007; Pollara et al., 2003). In line with the contemporary hypothesis that innate immunity augments adaptive immune responses, innate immune priming of DCs with LPS, Pam_3CSK4 and *M. vaccae* all augmented subsequent proliferation of naive T cells significantly, compared with unstimulated DCs. Although consistent, this effect seemed quantitatively modest in the experimental model used here. Potent alloreactivity that was observed even with immature DCs may in part mask the effect of innate immune maturation of DCs and is in line with other reports that also described significant but only modest

differences between immature and mature DCs in their capacity to support allogeneic T cell proliferation (Alderman et al., 2002).

3.4.5.2 *M. vaccae* reduces Th2 responses

In human allogeneic cultures of mature monocyte-derived DCs with naive $CD4^+$ T cells it had been reported that the stimulator / responder ratio can play a role in the fate of T cell polarisation; a ratio of 1:300 favoured Th2 responses, while very high ratios of 1:4 drove the development of mixed Th1/Th2 effectors (Tanaka et al., 2000). Various DC:T cell ratios were included in studying the effect of DC-priming on the differentiation of naive T cells. The data show that increasing DC:T cell ratios favour Th1 and reduce Th2 cell differentiation independently of innate immune stimulation of DCs.

Although T cell proliferation correlated with the magnitude of DC maturation, the different DC stimuli induced divergent effects on the phenotype of these T cells. The finding that *M. vaccae*-primed DCs compared with unprimed DCs decreased IL-4 producing T cells with no significant effect on IFN-γ producing T cells is consistent with earlier reports in other models that *M. vaccae* reduced Th2-polarised responses but does not switch to Th1-dominated responses (Zuany-Amorim et al., 2002b), and also consistent with Th1-dominant adaptive immune responses to mycobacteria generally (Bhatt and Salgame, 2007; Flynn and Chan, 2001). The different doses of *M. vaccae* as well as the different DC-T cell ratios did not result in divergent T cell differentiation, but increasing numbers of DCs or higher concentration of *M. vaccae* augmented the Th2 reducing effect.

Interestingly, specific TLR2 stimulation of DCs with Pam_3CSK4 had an effect opposite to that of both concentrations of *M. vaccae*. Pam_3CSK4 treatment of DCs reduced the DC-dependent attenuation of IL-4 responses and suggested that the effects

of *M. vaccae* are not mediated through activation of TLR2. In line with these data others have also shown that TLR2-dependent priming of human monocyte-derived DCs induces Th2 polarisation of naive T cells (Agrawal et al., 2003). This is further supported by murine studies showing that Pam_3CSK4 administration together with OVA augmented IL-4, IL-5 and IL-13 cytokine production by antigen-specific OT-1 and OT-2 T cells (Dillon et al., 2004). In contrast, Pam_3CSK4 in mouse allergy models has been associated with an increase of IFN-γ producing Th1 cells (Patel et al., 2005; Zhou et al., 2008), and with the induction of a Th1/Treg profile (Lombardi et al., 2008). Another mouse study suggested that treatment with Pam_3CSK4 reduced both Th1 and Th2 cells by inducing $CD4^+$ T cell apoptosis (Fukushima et al., 2006). In human blood mononuclear cells from mite-sensitised individuals, Pam_3CSK4 reduced Th2 responses (Taylor et al., 2006), and in whole blood cultures from nematode-infected children Pam_3CSK4 was shown to have IL-10 inducing capacity (Retra et al., 2008). The context specific effects of TLR2 stimulation with Pam_3CSK4 on T cell responses therefore require further study.

Moreover, the magnitude of DC maturation did not correlate with Th1/Th2-polarising effects. Comparable upregulation of CD83 and CD86 in DCs primed with LPS or *M. vaccae* (100 μg/ml) and in DCs primed with Pam_3CSK4 or *M. vaccae* (10 μg/ml) did not translate into comparable effects on Th2-polarised responses. Therefore differences in DC maturation as judged by these markers are not sufficient to account for DC-mediated inhibition of Th2 responses.

3.4.5.3 *M. vaccae* augments $CD25^+FoxP3^+$ T cell populations

Previous reports from animal models suggested that inhibition of Th2 responses may be the result of enhanced Treg responses in mice treated with *M. vaccae* (Zuany-Amorim et al., 2002a; Zuany-Amorim et al., 2002b). T cells cocultured with DCs that were pre-stimulated with *M. vaccae* or LPS significantly increased DC-dependent

upregulation of $CD25^{high}/FoxP3^{high}$ T cells. This finding was consistent with previous studies using autologous reactions where naive T cells upregulated their expression of CD25 and FoxP3 upon coculture with LPS-matured DCs (Verhasselt et al., 2004). Although FoxP3 has been described as the master regulator in the development and function of Treg cells, using its expression as a marker for human induced Treg cells has recently become controversial (Merlo et al., 2008; Tran et al., 2007; Wang et al., 2007). A study demonstrated that after one week of stimulation with anti-CD3 and anti-CD28, most human $CD4^{+}/CD25^{-}$ T cells upregulated FoxP3 and CD25 expression, but did not obtain suppressive ability (Wang et al., 2007). Moreover, upregulation of CD25 and FoxP3 was described following coculture of naive T cells with flagellin-primed allogeneic DCs, but in functional assays these cells lacked regulatory properties (Merlo et al., 2008). This may be confounded by the definition of positive FoxP3 staining that may range from intermediate to high levels. In the results presented here, only $CD25^{high}/FoxP3^{high}$ populations were analysed, however even high expression levels of FoxP3 may not correlate with inhibitory abilities and is insufficient to define human Treg cells (Tran et al., 2007). LPS and the high dose of *M. vaccae*, the two stimuli that induced significantly upregulated $CD25^{high}/FoxP3^{high}$ T cells, also induced significantly enhanced T cell proliferation that was statistically more powerful than with Pam_3CSK4 or 10 μg/ml *M. vaccae*, suggesting that those cells had no suppressive function. Moreover, only *M. vaccae* but not LPS reduced Th2 responses, suggesting that the $CD25^{high}/FoxP3^{high}$ T cells alone were not responsible for this effect. Analysis of IL-10 production that is a feature of some Treg cells was not detectible by intracellular flow cytometry analysis, and IL-10 levels in the supernatants of cocultures showed no correlation with numbers of $CD25^{high}/FoxP3^{high}$ T cells. Taken together it is uncertain that the $CD25^{high}/FoxP3^{high}$ T cells induced by *M. vaccae*-primed DCs were responsible for the reduction of Th2 responses and it is also possible that the upregulation of those

molecules was a feature of T cell activation rather than Treg differentiation. To determine whether these cells have immunoregulatory properties, it would have been possible to sort them and compare their ability to attenuate immune responses with $CD25^-$/$FoxP3^-$ T cells and natural occurring Treg cells.

3.4.5.4 *M. vaccae*'s effect are also evident in antigen-specific T cell responses

Although the observed variations in the phenotype of T cells cocultured with differentially primed DCs were only small, the findings were very consistent and similar to the magnitude of enhanced proliferative responses seen as a result of innate immune priming discussed earlier. To gain confidence in the data and to test the possibility if greater differences in T cell phenotypes could be generated, similar experiments were conducted using autologous DC-T cell cocultures. Here an antigen-specific response to tetanus toxoid was induced and the phenotype of the resulting T cells was compared. These experiments confirmed the findings from allogeneic cocultures, thus *M. vaccae* reduced Th2 responses via DCs in contrast to the specific TLR2 ligand Pam_3CSK4 and none of the stimuli affected associations between DC number and Th1 responses. In these antigen-specific responses, LPS and both concentrations of *M. vaccae* induced $CD25^{high}$/$FoxP3^{high}$ T cells contrarily to Pam_3CSK4, which again did not correlate with a reduction of T cell proliferation.

It was not possible to replicate the effects of *M. vaccae*-primed DCs when using fixed DCs, or by using conditioned media from *M. vaccae*-DC cultures and plate bound anti-CD3 and soluble anti-CD28 as substitute for antigen-presenting cells in cultures with allogeneic naive T cell. This is in line with previous reports that emphasised the importance of TCR activation, costimulation and secretion of soluble factors by DCs on naive T cell differentiation (Kapsenberg, 2003).

3.5 Conclusions

- *M. vaccae* induces maturation of DCs in a dose-depended manner.
- *M. vaccae* induces cellular activation via TLR2, either alone or in combination with TLR1 or TLR6.
- *M. vaccae* and LPS induce significantly greater maturation of DCs than Pam_3CSK4.
- DC priming with *M. vaccae* augments T cell proliferation in cocultures.
- In contrast to specific TLR2 stimulation, *M. vaccae*-primed DCs reduce Th2-polarised responses and augment the percentage of $CD25^{high}/FoxP3^{high}$ T cells in allogeneic and antigen-specific cocultures.
- Live DCs are required for the effects of *M. vaccae* on the T cell phenotype, as they could not be replicated, neither by using conditioned media from stimulated DC cultures and anti-CD3/anti-CD28 as a substitute for antigen-presenting cells, nor by using fixed DCs.

4 TRANSCRIPTIONAL RESPONSES OF DENDRITIC CELLS TO *M. VACCAE*

4.1 Introduction

In the first results chapter it was established that *M. vaccae* can modulate Th cell responses via DCs. Priming of DCs with *M. vaccae* led to reduced numbers of IL-4-producing Th2 cells in mixed leukocyte cultures, while it augmented the number of T cells showing a $CD25^{high}$/$FoxP3^{high}$ phenotype.

Previous studies have also reported that T cell responses to microbial antigens can be partly mediated by innate immune microbial interactions with DCs (Colonna et al., 2006). For example DCs primed with *Bordetella pertussis* promoted mixed Th1/Th17 polarisation (Fedele et al., 2010), DCs primed with omega-1 protein from schistosome eggs induced Th2 cells (Everts et al., 2009; Steinfelder et al., 2009), and priming of DCs with probiotics increased Treg responses (Kwon et al., 2010). Yet, the mechanisms by which DCs adopt a phenotype that promotes different T cell responses are not well established (MacDonald and Maizels, 2008; van Riet et al., 2009) and completely untested in *M. vaccae*-stimulated cells.

A major component of innate immune cellular activation of DCs is to trigger signalling cascades that activate various transcription factors. These transcription factors control immune response gene expression, including the upregulation of cell surface molecules and the induction of immunomodulatory factors such as cytokines and chemokines, which contribute to DC-T cell interactions (Kapsenberg, 2003). In one recent study the expression profile of selected genes was analysed and demonstrated that monocyte-derived DCs primed with Th1 inducing microbes *Escherichia coli* and heat-killed *L. monocytogenes* expressed augmented levels of IL-12 p40, IL-12 p35 and IL-23 p19, whereas the expression of those genes was reduced in DCs primed with

phosphatidylserine containing preparations of Th2 inducing *S. mansoni* and *Ascaris lumbricoides* (van Riet et al., 2009). Genome-wide transcriptional profiling of DCs suggests that innate immune stimulation induces changes to ≥ 1000 genes (Huang et al., 2001). These studies identified core responses that were common to different innate immune stimuli and stimulus specific responses (Huang et al., 2001; Jenner and Young, 2005). Therefore, this strategy was adopted to identify differences between transcriptional responses of DCs to LPS, Pam_3CSK4 and *M. vaccae*, and then bioinformatics approaches could be used to discover potential determinants of *M. vaccae*-specific responses both upstream and downstream of gene expression changes. These may provide candidate molecular mechanisms that mediate downregulation of Th2 responses and upregulation of $CD25^{high}/FoxP3^{high}$ T cells by *M. vaccae*-stimulated DCs.

4.2 Objectives

- Compare transcriptional responses of DCs to *M. vaccae* with responses to specific TLR2 stimulation with Pam_3CSK4.
- Analyse the DC cytokine response to innate immune stimulation with LPS, Pam_3CSK4 and *M. vaccae* and make comparisons with the transcriptional data.
- Identify specific transcriptional and/or cytokine responses of DCs to stimulation with *M. vaccae*.

4.3 Results

4.3.1 Dendritic cells and macrophages have distinctive transcriptional profiles

Baseline gene expression profiles derived from whole genome expression microarrays of the DC model used in this study showed characteristic features associated with DCs (Figure 4-1). In comparison to existing data from our group (Tsang et al., 2009) derived from expression profiles in monocyte-derived macrophages (MDMs), DCs showed higher expression levels of CCR7, CD1A, DC-SIGN (CD209) and the MHC class II molecules. By contrast, CD14, CD68, macrophage receptor with collagenous structure (MARCO) and the macrophage scavenger receptor (MRS1) showed higher expression in MDMs. Of interest, the expression levels of candidate PRRs for the innate immune stimuli under study were also assessed. Expression of TLR1, TLR2, TLR4 and TLR6 were all detectable in DCs. Expression of TLR9, reported to detect *M. tuberculosis* DNA in human macrophages (Kiemer, 2009), was not detectable above background. Of the other putative PRRs for mycobacteria, CCR5, dectin-1, DC-SIGN, Nod1, Nod2 and the mannose receptor (MR) were all expressed by DCs (Ferwerda et al., 2007; Floto et al., 2006; Geurtsen et al., 2009; Nigou et al., 2001; Pott et al., 2009; Torrelles et al., 2006; Yadav and Schorey, 2006).

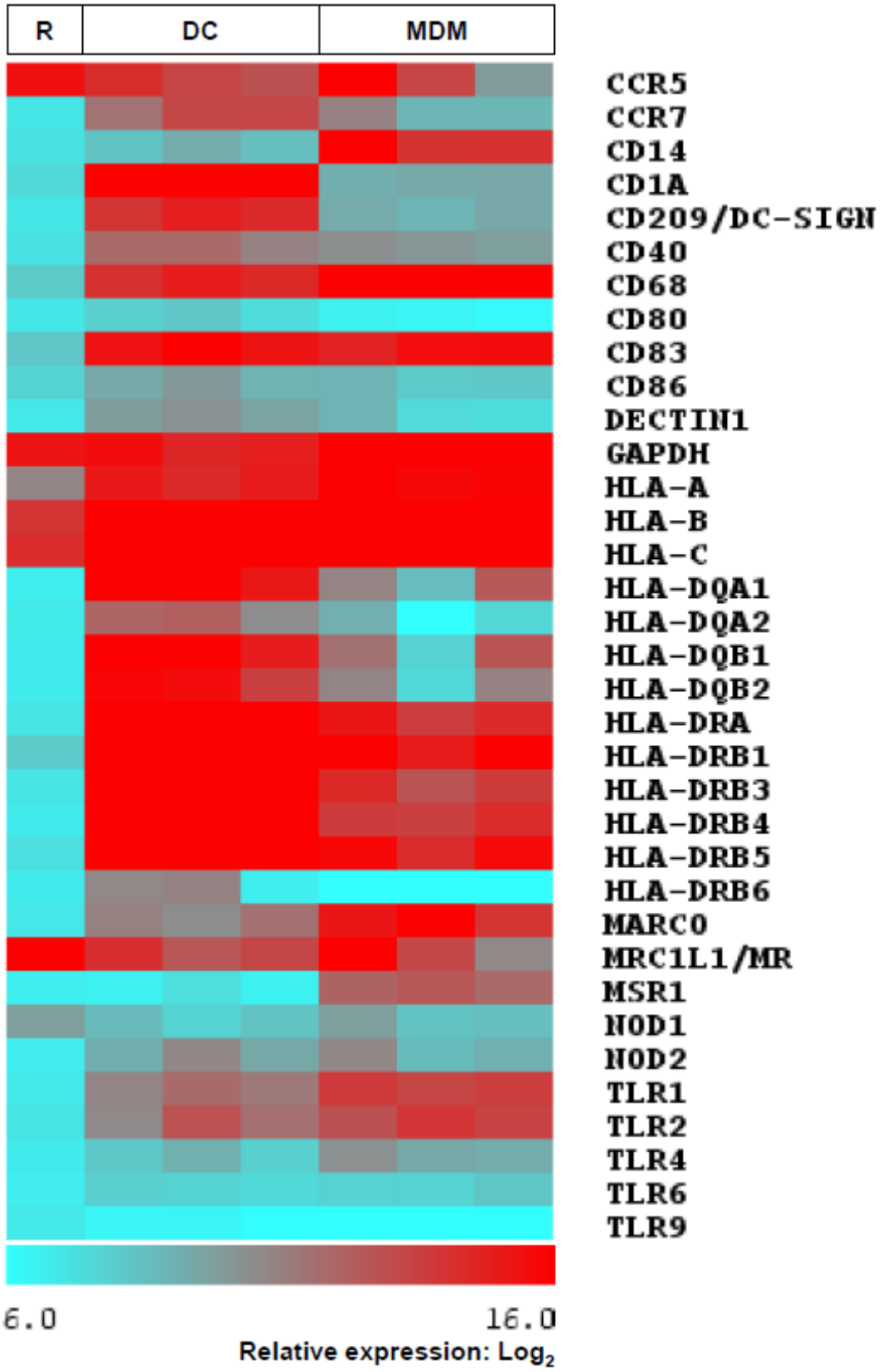

Figure 4-1: Baseline expression of selected genes in dendritic cells

Analysis of baseline gene expression profiles (three separate donors) of selected genes associated with the phenotype of DCs and MDMs and PRRs involved in recognition of mycobacteria compared to a reference (R) of ten different human cell lines.

4.3.2 Stimulated dendritic cells upregulate expression of maturation markers

When DCs were stimulated with LPS, Pam_3CSK4 or *M. vaccae*, the expression level of the surface molecules CD40, CD80, CD83, CD86, CD54 (ICAM-1), CD58 and CCR7 was augmented after 4 hours and 24 hours (Figure 4-2). The upregulation of these classical DC maturation markers validated the flow cytometry data from the previous chapter of results (Figure 3-8) and confirmed that the DCs used for the microarray analysis responded as expected.

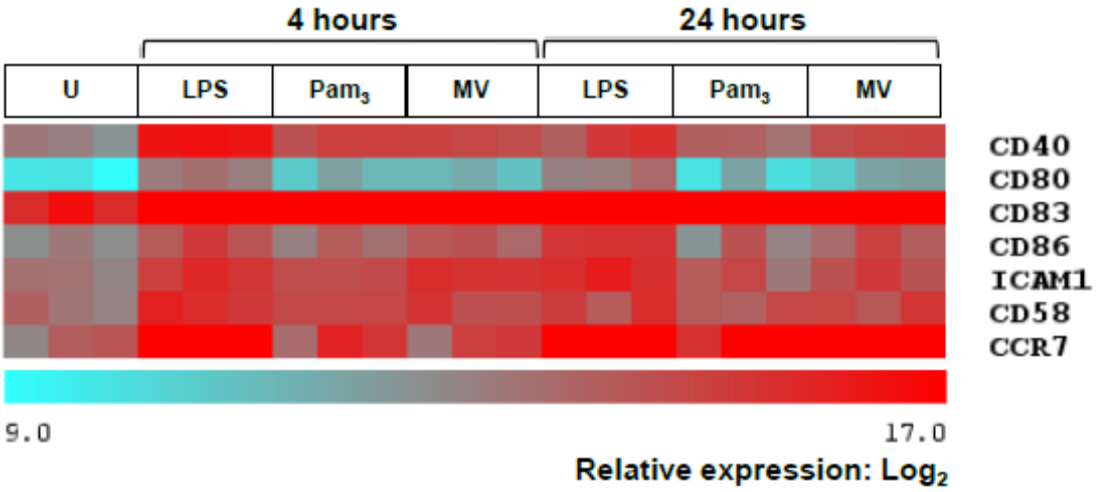

Figure 4-2: Expression of selected genes in dendritic cells that are upregulated when the cells mature

Analysis of gene expression profiles (three separate donors) of selected genes associated with the maturation of DCs following 4 h and 24 h of stimulation with LPS (100ng/ml), Pam_3CSK4 (Pam_3 1 μg/ml) or *M. vaccae* (MV 100 μg/ml) and unstimulated control DCs (U).

4.3.3 Functional annotation of genes upregulated by *M. vaccae*

In view of the principal interest under study, to characterise the effect of *M. vaccae* on DCs, functional annotation clustering analysis was performed on *M. vaccae* induced gene expression changes to identify the gene ontology (GO) associations. The analysis of the early upregulated genes (4 hours) showed that the most enriched functionally related gene clusters were involved in extracellular factors with cytokine and chemokine activity (Table 4-1), while genes upregulated at the 24 hours

time point were mainly associated with signal transduction pathways and lymphocyte activation (Table 4-2).

Table 4-1: Gene ontology (GO) terms from top five enriched groups of genes identified by functional annotation clustering analysis of genes that show >2-fold upregulation in DCs stimulated with *M. vaccae* for 4 hours

Gene ontology term	P value	No of genes	% of gene list	Fold enrichment
GO:0005125~cytokine activity	3.25^{-20}	35	8.71	7.54
GO:0009611~response to wounding	4.85^{-19}	56	13.93	4.06
GO:0006954~inflammatory response	2.98^{-14}	38	9.45	4.49
GO:0043066~negative regulation of apoptosis	8.67^{-14}	39	9.70	4.23
GO:0005615~extracellular space	1.19^{-12}	49	12.19	3.19
GO:0044421~extracellular region part	6.35^{-12}	58	14.43	2.69
GO:0008009~chemokine activity	4.85^{-10}	13	3.23	11.87
GO:0006935~chemotaxis	9.85^{-10}	22	5.47	5.28
GO:0042379~chemokine receptor binding	1.08^{-09}	13	3.23	11.15
GO:0043065~positive regulation of apoptosis	2.51^{-08}	34	8.46	3.04

Table 4-2: GO terms from top five enriched groups of genes identified by functional annotation clustering analysis of genes that show >2-fold upregulation in DCs stimulated with *M. vaccae* for 24 hours

Gene ontology term	P value	No of genes	% of gene list	Fold enrichment
GO:0001775~cell activation	1.95^{-09}	35	5.41	3.29
GO:0046649~lymphocyte activation	5.09^{-09}	28	4.33	3.79
GO:0043066~negative regulation of apoptosis	3.82^{-08}	37	5.72	2.82
GO:0043122~regulation of NF-κB cascade	3.76^{-07}	18	2.78	4.53
GO:0043065~positive regulation of apoptosis	1.62^{-06}	38	5.87	2.38
GO:0009611~response to wounding	6.41^{-06}	42	6.49	2.14
GO:0016477~cell migration	3.50^{-05}	26	4.02	2.54
GO:0042110~T cell activation	6.42^{-05}	16	2.47	3.42
GO:0006954~inflammatory response	7.76^{-05}	28	4.33	2.32
GO:0009967~positive regulation of signal transduction	6.46^{-04}	24	3.71	2.19

4.3.4 Comparison of functional annotation of genes downregulated by the different stimuli

Many genes were also downregulated in DCs following treatment with the different stimuli (Figure 4-3). Functional annotation clustering analysis of the early downregulated genes in DCs stimulated with *M. vaccae*, LPS or Pam_3CSK4 showed that they encode for proteins that are mainly involved in cellular housekeeping processes, negative regulation of transcription and bind to the DNA (Table 4-3).

Table 4-3: GO terms from top three enriched groups of genes identified by functional annotation clustering analysis of genes that show >2-fold downregulation in DCs stimulated with LPS, Pam_3CSK4 or *M. vaccae* for 4 hours

Stimulus	Gene ontology term	P value	No of genes	% of gene list	Fold enrichment
LPS	GO:0031974~membrane-enclosed lumen	1.86^{-17}	183	16.74	1.83
	GO:0005739~mitochondrion	1.80^{-10}	109	9.97	1.86
	GO:0006364~rRNA processing	4.00^{-06}	19	1.74	3.59
	GO:0008033~tRNA processing	3.54^{-04}	14	1.28	3.20
Pam3-CSK4	GO:0042802~identical protein binding	0.01	11	7.80	2.43
	GO:0042579~microbody	0.04	4	2.83	5.22
MV100	GO:0005739~mitochondrion	7.47^{-05}	26	12.15	2.35
	GO:0003677~DNA binding	4.78^{-04}	42	19.63	1.68
	GO:0010629~negative regulation of gene expression	0.02	12	5.61	2.15

Comparison of the GO associations of genes downregulated at the 24 hours time point showed that in DCs stimulated with all stimuli under study at least parts of the inflammatory response are already switched off. However, this seems more significant in *M. vaccae*-stimulated DCs, where only GO associations of genes involved in immune response and cytokine production were downregulated at this later time point (Table 4-4).

Table 4-4: GO terms from top three enriched groups of genes identified by functional annotation clustering analysis of genes that show >2-fold downregulation in DCs stimulated with LPS, Pam_3CSK4 or *M. vaccae* for 24 hours

Stimulus	Gene ontology term	P value	No of genes	% of gene list	Fold enrichment
LPS	GO:0005739~mitochondrion	5.87^{-05}	100	9.17	1.48
	GO:0042611~MHC protein complex	3.59^{-05}	14	1.28	3.94
	GO:0005764~lysosome	2.80^{-04}	28	2.75	2.13
	GO:0006954~inflammatory response	6.89^{-04}	36	3.30	1.82
Pam_3-CSK4	GO:0004969~histamine receptor activity	6.08^{-04}	3	3.26	77.28
	GO:0006954~inflammatory response	0.01	7	7.61	4.10
MV100	GO:0006954~inflammatory response	5.29^{-07}	23	6.59	3.56
	GO:0001819~positive regulation of cytokine production	2.04^{-03}	8	2.29	4.47

4.3.5 The predominant transcriptional responses of dendritic cells to *M. vaccae* and specific TLR2 stimulation are comparable

In order to investigate the differential effects of *M. vaccae* and Pam_3CSK4 on DC-mediated inhibition of Th2 responses, transcriptional responses in DCs primed with 100 ng/ml LPS, 1 μg/ml Pam_3CSK4 and 100 μg/ml *M. vaccae* were compared. RNA samples were collected 4 hours and 24 hours after stimulation. Marked changes to gene expression were detectable in comparison to unstimulated DCs (Figure 4-3). The frequency distribution of significantly (>2-fold) upregulated and downregulated genes suggested that LPS had the greatest effect on gene expression, followed by *M. vaccae* and then Pam_3CSK4 at both time points.

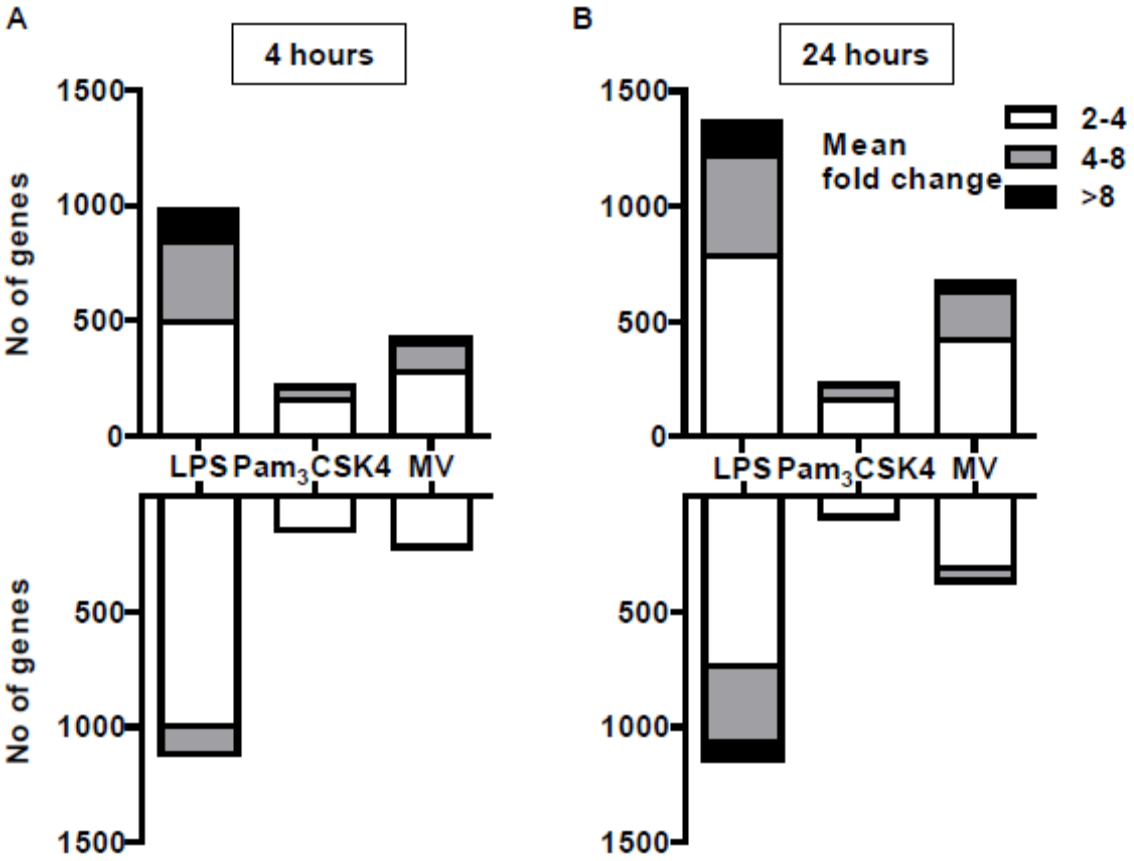

Figure 4-3: Quantitative comparison of gene expression changes in dendritic cells in response to innate immune stimulation

Quantitative comparison of >2-fold up- and downregulated gene expression changes in DCs after 4 h (**A**) and 24 h (**B**) of stimulation with LPS (100 ng/ml), Pam_3CSK4 (1 μg/ml) or *M. vaccae* (100 μg/ml). White: 2-4 fold change, grey: 4-8 fold change, black: > 8 fold change compared to baseline gene expression of unstimulated DCs. Data are derived from the mean of three separate experiments using cDNA microarray gene expression profiling.

In addition, qualitative comparison of >2-fold gene expression changes suggested core and stimulus-specific transcriptional responses at the 4 hours and 24 hours time points (Figure 4-4).

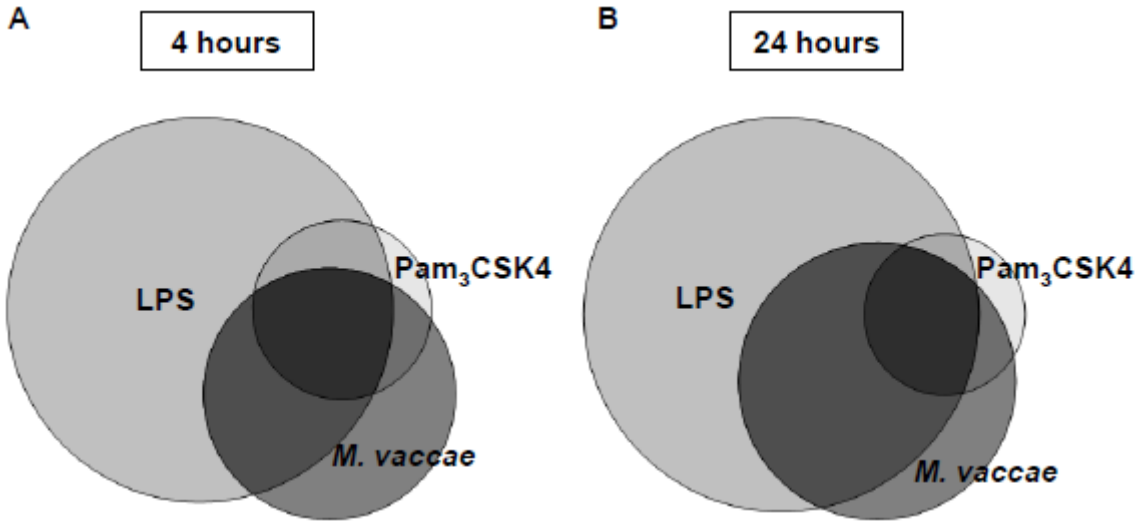

Figure 4-4: Qualitative comparison of upregulated gene expression in dendritic cells in response to innate immune stimulation

Qualitative Venn diagram comparison of >2-fold upregulated genes in DCs after 4 h (**A**) and 24 h (**B**) of stimulation with LPS (100 ng/ml), Pam_3CSK4 (1 µg/ml) or *M. vaccae* (100 µg/ml). Data are derived from the mean of three separate experiments using cDNA microarray gene expression profiling.

Subsequently, principal component analysis (PCA) of transcriptional profiles was used to compare components of the data that are responsible for the greatest gene expression differences, to compare groups of genes that changed the most. The principle component (PC) scores for the four PCs which exhibit the most variance in gene expression data are presented in Figure 4.5, and the relative expression levels for the top 20 genes responsible for the variance in each component are presented in Figure 4.6. In this analysis, stimulation of DCs with LPS induced the greatest gene expression changes in PC1 and PC2. Comparison of expression profiles from 4 hours and 24 hours stimulated DCs shows a pattern of gene expression changes in PC2 at 4 hours which returns to baseline levels at 24 hours. This is in contrast to PC1, which shows gene expression changes at 4 hours that are further augmented at 24 hours. In these

components of the data, gene expression profiles in DCs primed with Pam_3CSK4 or *M. vaccae*, show the same pattern of transcriptional responses, albeit quantitatively less than responses to LPS. PC3 and PC4 show a different pattern of gene expression changes in stimulated DCs. In PC3, LPS stimulation causes transcriptional changes at 4 hours and 24 hours that are divergent to those of DCs stimulated with Pam_3CSK4 or *M. vaccae*. PC4 shows comparable transcriptional changes associated with all three stimuli at 4 hours, but divergent responses at 24 hours, when LPS stimulated cells approximate to unstimulated DCs and cluster away from data points representing DCs stimulated with Pam_3CSK4 or *M. vaccae*.

Taken together, quantitative, qualitative and time course assessment of genome-wide transcriptional responses by PCA suggests that the major transcriptional responses in Pam_3CSK4- and *M. vaccae*-stimulated DCs are comparable.

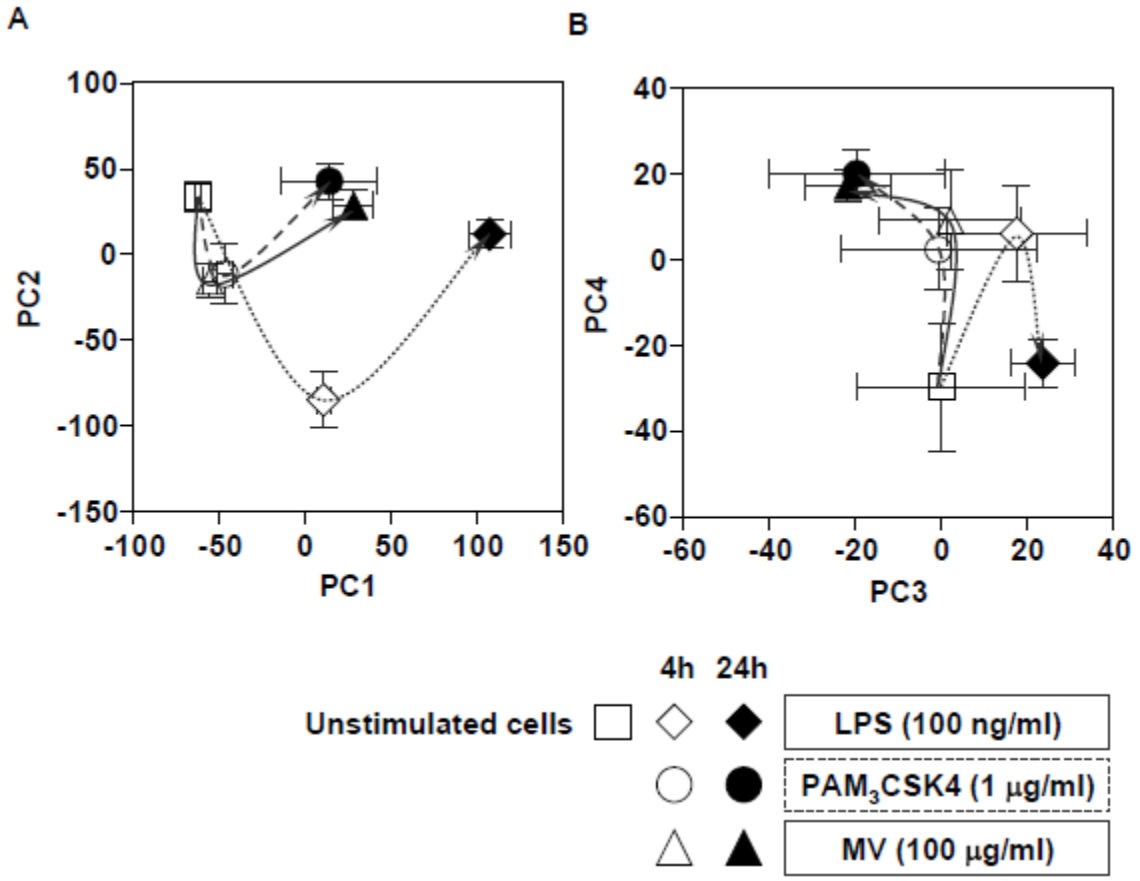

Figure 4-5: The predominant transcriptional responses to *M. vaccae* and specific TLR2 stimulation are comparable

Principal component analysis (PCA) of transcriptional profiling differences in DCs stimulated for 4 h or 24 h with LPS (100 ng/ml), Pam_3CSK4 (1 μg/ml) or *M. vaccae* (100 μg/ml) and control unstimulated DCs. Data points show mean (±SEM) PCA scores for three independent experiments. In **A** and **B**, lines and arrows indicate vector of transcriptional responses to each stimulus with time. PC1 and PC2 show common transcriptional changes to all stimuli, which are quantitatively greatest as a result of LPS stimulation. PC3 and PC4 show divergent responses in LPS stimulated cells compared to Pam_3CSK4 or *M. vaccae*. Transcriptional profiles in *M. vaccae* and Pam_3CSK4 stimulated cells are closely aligned in each component.

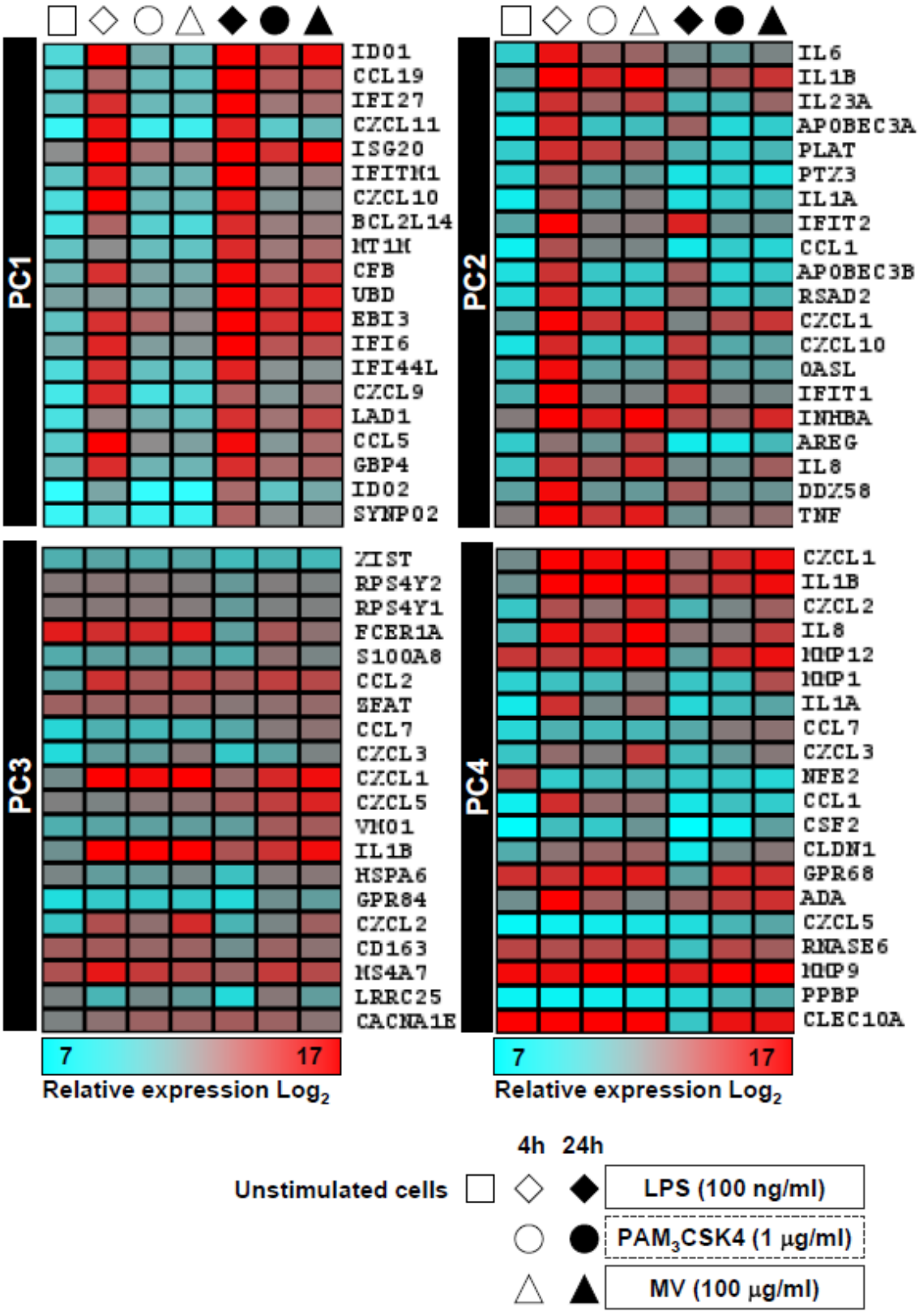

Figure 4-6: Relative expression levels for gene expression differences in differentially stimulated dendritic cells

Heat map representation of relative gene expression levels for the top 20 genes that are responsible for the greatest variance in the first four principle components (PC) of gene expression differences in DCs stimulated with LPS (100 ng/ml), Pam_3CSK4 (1 µg/ml) or *M. vaccae* (100 µg/ml). Data are derived from the mean of three separate experiments using cDNA microarray gene expression profiling. For gene names see list of abbreviations (pages 16-22).

4.3.6 The predominant cytokine responses of dendritic cells to *M. vaccae* and specific TLR2 stimulation are also comparable

Transcriptional responses are not always faithfully translated to protein responses. The functional annotation clustering analysis (4.3.3) had shown that early gene expression changes induced by *M. vaccae* were largely implicated in extracellular factors with cytokine and chemokine activity. Hence, to validate the expression profiling analysis and look for discordance between transcriptional and protein responses, multiple cytokine measurements were made in supernatants of differentially stimulated DCs (Figure 4-7A).

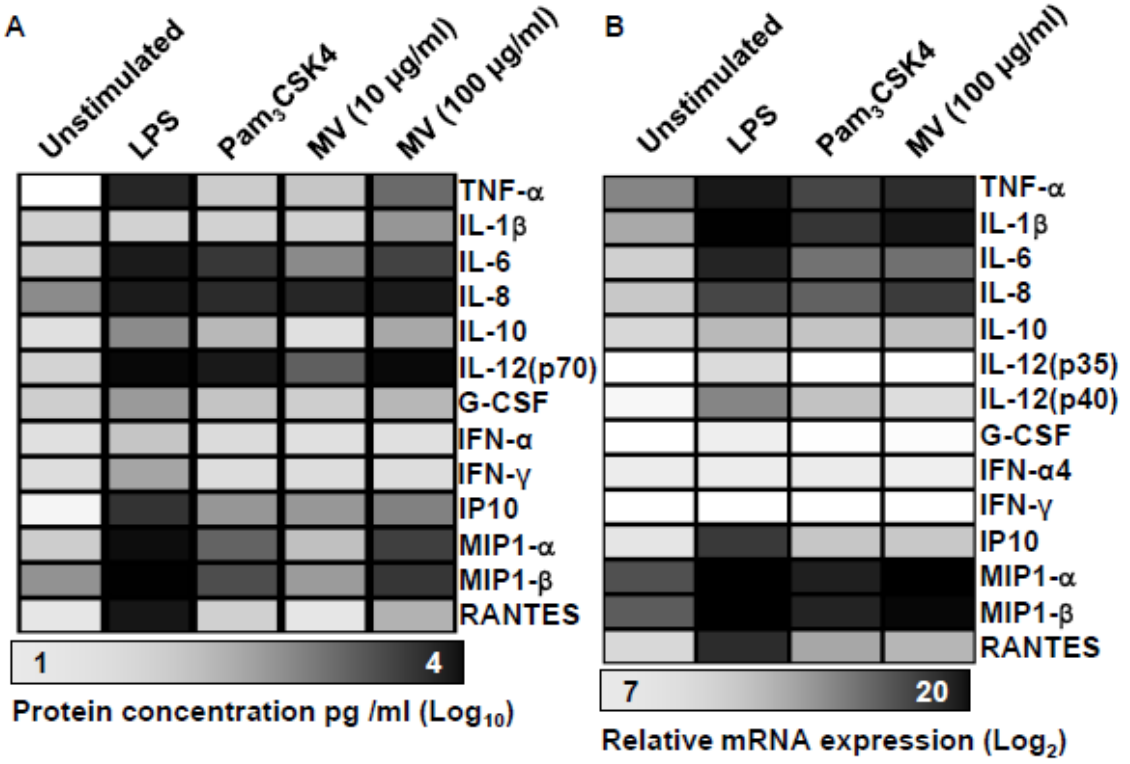

Figure 4-7: Dendritic cell responses to *M. vaccae* and specific TLR2 stimulation are comparable at protein level

A: Selected cytokine and chemokine levels in the supernatants of DCs stimulated for 24 h with LPS (100 ng/ml), Pam_3CSK4 (1 µg/ml) or *M. vaccae* (10 µg/ml or 100 µg/ml) and control unstimulated DCs are presented in a heat map. **B**: For comparison, mRNA expression of the same cytokines and chemokines after 4 h of stimulation. In general, this showed highest levels in LPS-stimulated cells and comparable levels in Pam_3CSK4- and *M. vaccae*-stimulated cells. Data represent mean values from three separate experiments.

These data closely mirrored the expression profiling analysis (Figure 4-7B), accordingly the greatest responses were evident in LPS stimulated DCs and more modest responses in Pam_3CSK4 or *M. vaccae* stimulated DCs were directly comparable.

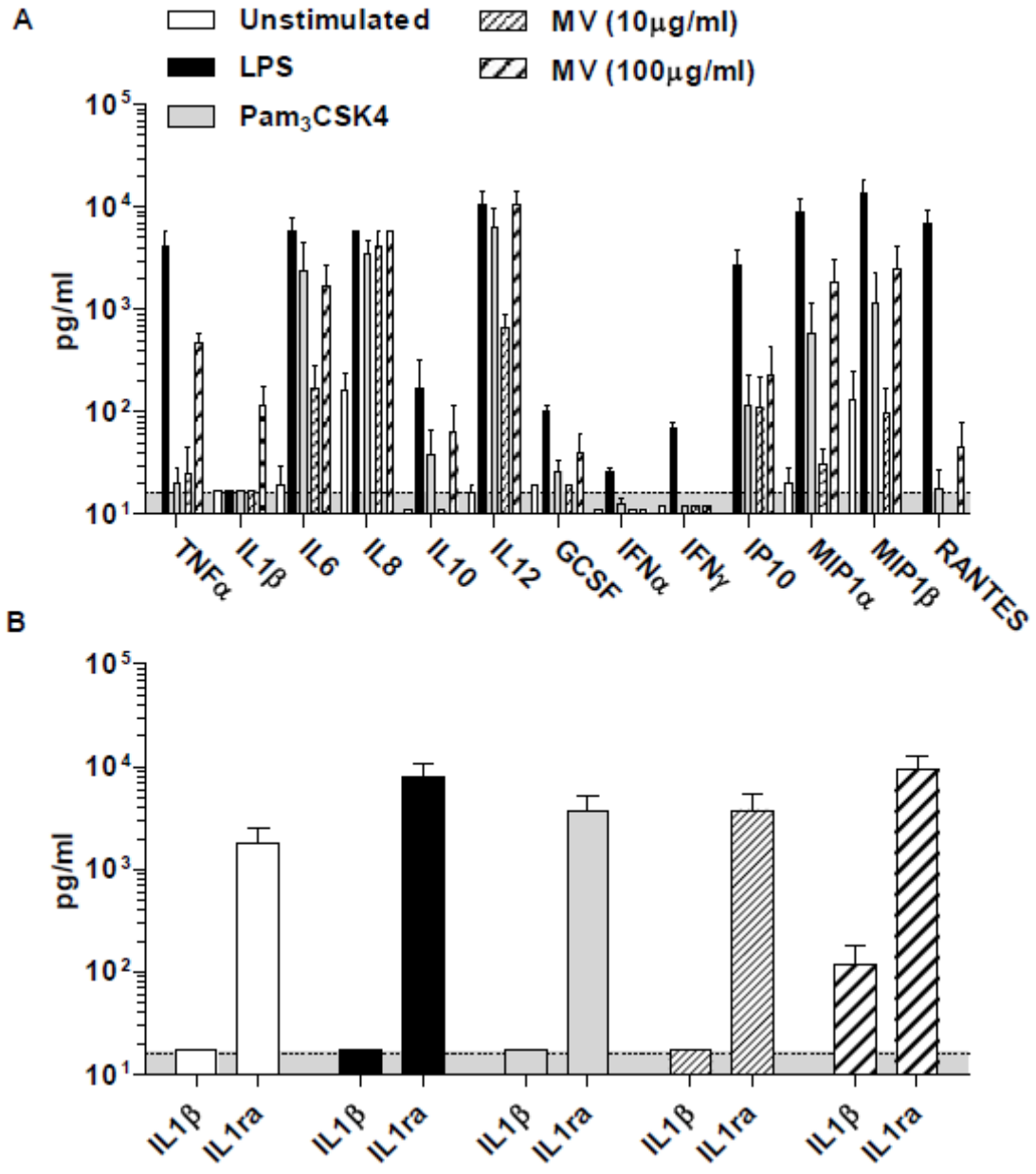

Figure 4-8: IL-1β is secreted by dendritic cells stimulated with *M. vaccae*

A: Concentrations of selected cytokines in cell culture supernatants of DCs stimulated for 24 h with LPS (100 ng/ml), Pam_3CSK4 (1 µg/ml) or *M. vaccae* (10 µg/ml or 100 µg/ml); **B**: focusing on IL-1β and IL-1 receptor antagonist (ra). Data show mean (±SD) of three independent experiments.

Interestingly, increased IL-1β secretion was only detected in DCs stimulated with 100 µg/ml *M. vaccae*, suggesting activation of the inflammasome pathway that is

required for IL-1β release (Martinon et al., 2002). In view of the role of this pathway in augmentation of adaptive immune responses (Martinon et al., 2009), the possibility was considered that IL-1β may contribute to DC-dependent costimulation of T cells that is responsible for the inhibition of Th2 responses associated with *M. vaccae*-priming of DCs. However, it was also found that the homeostatic regulator of IL-1β activity, IL-1-receptor antagonist (ra) was also present in cell culture supernatants at high concentrations that were likely to negate any biological activity of relatively small increase in IL-1β concentration (Figure 4-8B). These data suggest that induction of IL-1β release and by implication, activation of the inflammasome is not responsible for the anti-Th2 effect of DCs primed with *M. vaccae*. This is further supported by the data in the previous chapter showing that DC supernatants did not reproduce the effect of *M. vaccae*-primed DCs (Figure 3-19A).

4.4 Discussion

In the first chapter of results it was established that *M. vaccae* can induce innate immune cellular activation via TLR2. However, priming of DCs with Pam_3CSK4, a specific ligand for TLR2, did not have the same effects on T cell polarisation in allogeneic and antigen-specific mixed leukocyte cultures as priming of DCs with *M. vaccae*. Given that specific TLR2 stimulation has different effects to *M. vaccae*, comparison of transcriptional responses to each stimulus may give greater insight into the molecular mechanisms involved and differences in upstream signalling pathways.

4.4.1 Baseline transcriptional profile of the dendritic cell model studied

First the expression profile of unstimulated DCs was compared to that of a reference, an average expression profile of universal human reference RNA composed of total RNA from ten human cell lines, and to unstimulated MDMs. This verified that

the DC model used in this study expressed genes characteristic for DCs. In comparison to the reference and to MDMs, the DCs showed as expected increased transcription of the surface molecules CD1A, CD40, CD80, CD83, CD86, CCR7 and DC-SIGN (CD209) (Forster et al., 1999; Geijtenbeek et al., 2000; Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996). Moreover, MHC class I molecules that are expressed by all nucleated cells were detected in DCs, MDMs and also in the reference cell lines, whereas MHC class II molecules were as expected only expressed above background in the antigen-presenting DCs and MDMs. In line with the literature (Sallusto and Lanzavecchia, 1994; Yanagihara et al., 1998; Zhou and Tedder, 1996) and the flow cytometry data presented in the previous chapter, the expression of the surface molecules CD40, CD80, CD83, CD86, ICAM-1, CD58 and CCR7 was upregulated when DCs were treated with the different innate immune stimuli, confirming the accuracy of the microarray data. In comparison to DCs, MDMs showed higher expression of the macrophages receptors CD14, MARCO and MSR1, which is supported by the literature (Kraal et al., 2000; Sallusto and Lanzavecchia, 1994; Tomokiyo et al., 2002). Since the stimuli under study, *M. vaccae*, Pam_3CSK4 and LPS are ligands for the TLRs 1/2, 2, 2/6 or 4, and mycobacterial recognition has in addition previously been associated with TLR9, DC-SIGN, CCR5, dectin-1, Nod1, Nod2 or the mannose receptor (Ferwerda et al., 2007; Floto et al., 2006; Geurtsen et al., 2009; Kiemer, 2009; Nigou et al., 2001; Pott et al., 2009; Torrelles et al., 2006; Yadav and Schorey, 2006), the expression of these PPRs was analysed in the DC model. All PPRs were expressed above background except TLR9, which is consistent with previous reports showing that TLR9 expression in humans is restricted to pDCs and B cells (Bernasconi et al., 2003; Hornung et al., 2002; Rothenfusser et al., 2002).

4.4.2 Transcriptional responses of dendritic cells to differential innate immune stimulation

Next, the expression profiles of DCs after 4 hours and 24 hours of innate immune stimulation with the different stimuli under study were compared. Quantitative changes in gene expression showed that LPS caused the greatest response at both time points and the level of these changes in expression was similar to that of previous reports (Huang et al., 2001). The number of up- and downregulated genes was more similar between Pam_3CSK4- and *M. vaccae*-primed DCs. Yet, stimulation with *M. vaccae* provoked slightly more changes than the specific TLR2 ligand, supporting the likelihood that *M. vaccae* is recognised by alternative PRRs in addition to TLR2 (Ferwerda et al., 2007; Floto et al., 2006; Geurtsen et al., 2009; Nigou et al., 2001; Torrelles et al., 2006; Yadav and Schorey, 2006).

Qualitative comparison of gene expression changes in response to LPS, Pam_3CSK4 and *M. vaccae* showed common and stimulus-specific responses. This is in line with previous reports showing that DCs stimulated with different pathogens perform a shared core response and pathogen-specific gene expression programmes (Huang et al., 2001; Jenner and Young, 2005). Remarkably, PCA of these data showed that the major gene expression changes induced by *M. vaccae* and Pam_3CSK4 were extremely alike. Moreover, the response to LPS was in PC1 and PC2 qualitatively similar, but more pronounced. TLR signalling leads to shared activation of transcription factors like NF-κB (Beutler, 2004) and therefore converge on a common set of genes. It was previously shown that activation of TLR2 and TLR4 is both inducing inflammatory/chemotactic cytokine genes, which is seen as the common host response (Huang et al., 2001; Jenner and Young, 2005). In macrophages it was shown that the time course of the core response to TLR2 and TLR4 stimulation was similar, but the

median gene induction was about 2-fold larger in response to LPS (Jenner and Young, 2005), therefore it is likely that PC1 and PC2 are representing the core response.

The similarity of the major gene expression changes induced by Pam_3CSK4 and *M. vaccae* suggests that the main transcriptional responses to *M. vaccae* are mediated via TLR2, and that these are not responsible or at least not sufficient for inhibition of Th2 responses. This is interesting, as in addition to *M. vaccae*, other microorganisms associated with the hygiene hypothesis, including helminths (van der Kleij et al., 2002) and lactobacilli (Foligne et al., 2007) were also shown to signal via TLR2. However, for certain lactobacilli it was also demonstrated that recognition by Nod2 (Foligne et al., 2007) or DC-SIGN (Smits et al., 2005) plays a critical role in the suppression of Th2 responses. Both receptors have been shown to recognise other mycobacteria (Ferwerda et al., 2007; Geurtsen et al., 2009), and therefore may also play a role in the effects of *M. vaccae* on T cell polarisation. An effect of co-receptor usage is seen with the TLR2 ligand zymosan, which also engages dectin-1 and drives the development of IL-10 producing DCs and TGF-β producing macrophages (Dillon et al., 2006). Since BCG, *M. tuberculosis*, *M. smegmatis*, *M. phlei*, *M. avium*, *M. abscessus* and *M. ulcerans* are inducing cellular activation via dectin-1 (Lee et al., 2009; Shin et al., 2008; Yadav and Schorey, 2006), it might be likely that *M. vaccae* also engages this receptor and co-signalling with TLR2 could play a role for the effects of *M. vaccae*. However, *M. vaccae*-stimulation of DCs induced only little production of IL-10 in one donor at protein level and a modest 2.4-fold upregulation at mRNA level. As a result this hypothesis was not tested specifically.

4.4.3 Functional annotation clustering analysis

Functional annotation clustering analysis of genes upregulated in *M. vaccae*-primed DCs showed, as expected in innate immune cells, enrichment for genes involved in inflammatory responses, including cytokine and chemokine activity. At the later time

point gene clusters involved in T cell activation and the NF-κB signalling pathway were also expressed. This is different to stimulation with LPS or Pam_3CSK4, which induce genes involved in T cell activation and NF-κB signalling already at the 4 hours time point. As expected, analysis of the early downregulated genes showed that they are mainly involved in housekeeping and negative regulation of transcription. Interestingly, components of inflammatory responses are downregulated at the 24 hour time point. This is especially evident in *M. vaccae* stimulated genes. Given that the major genes upregulated by *M. vaccae* and Pam_3CSK4 were closely comparable, it is possible to speculate that differences in duration of inflammatory responses may contribute to DC-T cell interactions and consequently differential Th cell polarisation. This hypothesis was not specifically addressed in this thesis.

4.4.4 *M. vaccae* induces secretion of IL-1β

GO terms associated with genes upregulated in *M. vaccae*-primed DCs showed induction of genes involved in chemokine and cytokine activity. The pattern of transcriptional responses assessed by cDNA microarrays was mirrored in measurements of selected cytokines and chemokines in cell culture supernatants, except for increased secretion of IL-1β in *M. vaccae* (100 μg/ml)-primed DCs compared to other stimuli. IL-1β secretion is tightly regulated by activation of the inflammasome and caspase-1 (Martinon et al., 2002). In view of the role of this pathway as a bridge between innate and adaptive immunity (Martinon et al., 2009), the possibility that IL-1β is involved in inhibition of Th2 responses by *M. vaccae*-primed DCs was considered, but the substantial concentrations of IL-1ra in the same samples shed doubt on the biological significance of modest increases in IL-1β. In addition, previous reports showed impaired Th2 immune responses in absence of the NALP3, ASC and caspase-1 (Eisenbarth et al., 2008). The authors showed that alum triggers NALP3 and induces inflammasome activation and IL-1β production. Alum is used to induce Th2-mediated

inflammation in allergy/asthma mouse models, but this required a functional NALP3 inflammasome, as knockout mice had decreased airway eosinophilia and IL-5 production. This suggests that inflammasome activity and IL-1β production rather contribute to increasing Th2 differentiation than inhibiting it, which is in contrast to the effect of *M. vaccae*. More recently it was demonstrated that enhanced inflammasome activity in antigen-presenting cells augmented Th17 cells (Brydges et al., 2009; Meng et al., 2009), hence a shift from Th2 towards Th17 responses might explain the reduction of IL-4^+ T cells in cocultures with *M. vaccae*-primed DCs. Yet, in my mixed leukocyte experiments no IL-17^+ cells were detectable by intracellular flow cytometry (data not shown). Therefore this hypothesis was not assessed any further here. Instead I focussed on gene expression differences that were exclusively induced by *M. vaccae* and on identification of upstream signalling pathways involved.

4.5 Conclusions

- Quantitative, qualitative and time course assessments of genome-wide transcriptional responses showed that the predominant responses in Pam_3CSK4 and *M. vaccae* stimulated DCs are comparable.
- Cytokine secretion by DCs in response to stimulation with *M. vaccae* and Pam_3CSK4 is similar and comparatively smaller than responses to LPS, which closely mirrored the transcriptional data.

5 *M. VACCAE* SELECTIVELY STIMULATES EARLY ACTIVATION OF THE CREB PATHWAY

5.1 Introduction

In the first results chapter I demonstrated that *M. vaccae* is recognised by TLR2. Yet, in contrast to stimulation of DCs with a specific TLR2 ligand (Pam_3CSK4), priming of DCs with *M. vaccae* reduced the Th2 response in mixed leukocyte cultures and augmented the number of $CD25^{high}$/$FoxP3^{high}$ T cells. Microarray analysis showed in line with the literature (Huang et al., 2001; Jenner and Young, 2005) that stimulation of DCs with specific TLR2 and TLR4 ligands or with *M. vaccae* resulted in shared-core and stimulus-specific responses. PCA identified that the major transcriptional responses in DCs to specific TLR2 stimulation with Pam_3CSK4 and stimulation with *M. vaccae* were comparable. Since those two stimuli did not show the same effects on T cell polarisation, I attempted to identify differences in the transcriptional responses that could give insight into the molecular mechanisms involved and differences in upstream signalling pathways.

Stimulation of cellular immune receptors induces signal transduction cascades leading to the activation of transcription factors and consequently altered gene expression. The induction of signalling pathways in response to stimulation of DCs was first studied in mice models. The first investigation of signalling pathways activated in LPS-stimulated DCs showed that NF-κB translocation was required to induce maturation of DCs in terms of upregulation of MHC and costimulatory molecules (Rescigno et al., 1998). LPS-dependent activation of ERK1/2 was not essential for DC maturation but necessary to prevent apoptosis of activated DCs (Rescigno et al., 1998). Later studies on mice DCs stimulated with various TLR ligands showed that the MAPK

pathways p38, ERK1/2 and JNK are involved in DC maturation and production of cytokines (Dowling et al., 2008).

Mycobacteria have been shown to block or activate NF-κB and MAPK pathways. A study showing a positive effect of freeze-dried BCG preparations in mouse asthma models concluded that this effect was at least partially due to blocked activation of NF-κB and p38 MAPK in lung extracts following OVA challenge (Lagranderie et al., 2010). However, infection of human monocytes with *M. tuberculosis* or live BCG has been shown to activate p38 and ERK1/2 pathways (Gagliardi et al., 2009).

The MAPK pathways are activated in murine and human macrophages following infection with virulent and avirulent strains of *M. avium* and they seem to play a critical role in the activation of an immune response and the ability of mycobacteria to replicate (Blumenthal et al., 2002; Klug et al., 2010; Tse et al., 2002). While ERK1/2 phosphorylation is thought to be essential for TNF-α production and reduction of mycobacterial replication, anti-inflammatory IL-10 was shown to depend on p38 MAPK, whose activation promotes bacterial growth (Bhattacharyya et al., 2002; Blumenthal et al., 2002; Klug et al., 2010; Reiling et al., 2001; Tse et al., 2002). This is in line with a recent study showing that human monocyte cultures treated with heat-killed *M. tuberculosis* rapidly activate the p38 MAPK pathway, which was shown to be involved in *il-10* gene expression and interfered with DC differentiation (Remoli et al., 2010). Two recent reports on *M. tuberculosis*-derived proteins showed that those antigens are recognised by TLR2 and can induce maturation of human monocyte-derived DCs. The authors demonstrated that DC maturation and cytokine secretion in response to those *M. tuberculosis*-derived proteins was reduced by pharmacological blocking of ERK1/2, p38 or NF-κB, whereby NF-κB translocation had the biggest effect (Bansal et al., 2010a; Bansal et al., 2010b).

No data are available on signalling pathways activated by *M. vaccae*. To identify transcription factors that are activated in *M. vaccae*-stimulated DCs, which could give insight into upstream signalling pathways, the open-access bioinformatics tool oPOSSUM was applied (Ho Sui et al., 2005). OPOSSUM allows analysing lists of genes for association with overrepresented transcription factor binding sites (TFBSs).

5.2 Objectives

- Identify transcription factors and signalling pathways that are activated by DCs stimulated with *M. vaccae*.
- Compare if *M. vaccae*, LPS and Pam_3CSK4 induce activation of classical NF-κB signalling and the MAPK pathways p38 and ERK1/2.
- Block *M. vaccae*-induced activation of specific signalling pathways in order to study their role in DC-mediated effects of *M. vaccae* on T cell polarisation.

5.3 Results

5.3.1 Sets of genes activated by *M. vaccae* have distinct transcription factor binding sites

In order to investigate the mechanism of differences in gene expression responses, putative TFBSs associated with upregulated gene lists for each stimulus were identified using oPOSSUM. This analysis identified statistically overrepresented TFBS within the gene list of interest compared to the background genome (Table 5-1 and Table 5-2) (Ho Sui et al., 2005).

Table 5-1: Overrepresented TFBSs in DCs stimulated for 4 hours with *M. vaccae*, LPS or Pam_3CSK4. The oPOSSUM programme was used for analysis. For names of transcription factors see abbreviations (pages 16-22).

TFBSs in DCs stimulated with *M. vaccae* (100 μg/ml)	Z-Score	TFBSs in DCs stimulated with LPS (100 ng/ml)	Z-Score	TFBSs in DCs stimulated with Pam_3CSK4 (1 μg/ml)	Z-Score
RELA	37.78	IRF2	30.50	RELA	29.96
REL	36.84	RELA	29.44	REL	28.33
NF-kappaB	30.53	NF-kappaB	23.77	NF-kappaB	26.90
NFKB1	30.41	STAT1	22.32	NFKB1	24.76
CREB1	23.56	REL	21.78	TP53	23.58
SPIB	20.55	NFKB1	19.10	ELK4	10.91
ELF5	17.39	IRF1	17.20	ELK1	10.29
ELK1	16.14	ELK1	12.06		
FOS	14.93	FOS	10.15		
HLF	13.81				
Hand1-Tcfe2a	13.24				
SP1	12.10				
STAT1	11.79				
ELK4	10.40				

Table 5-2: Overrepresented TFBSs in DCs stimulated for 24 hours with *M. vaccae*, LPS or Pam_3CSK4. The oPOSSUM programme was used for analysis. For names of transcription factors see abbreviations (pages 16-22).

TFBSs in DCs stimulated with *M. vaccae* (100 μg/ml)	Z-Score	TFBSs in DCs stimulated with LPS (100 ng/ml)	Z-Score	TFBSs in DCs stimulated with Pam_3CSK4 (1 μg/ml)	Z-Score
RELA	23.06	IRF2	21.00	IRF1	17.66
REL	20.35	RELA	16.96	RELA	16.12
NF-kappaB	17.48	NFKB1	12.02	IRF2	15.92
IRF2	13.86			Fos	14.64
NFKB1	13.67			HLF	13.39
FOXD1	10.44			NFKB1	12.80
				NF-kappaB	11.87

Venn diagram analysis of the significantly overrepresented TFBSs (Z-score ≥ 10 and Fischer score ≤ 0.1) identified for each gene list was then used to look for common and for stimulus-specific pathways upstream of gene expression (Figure 5-1).

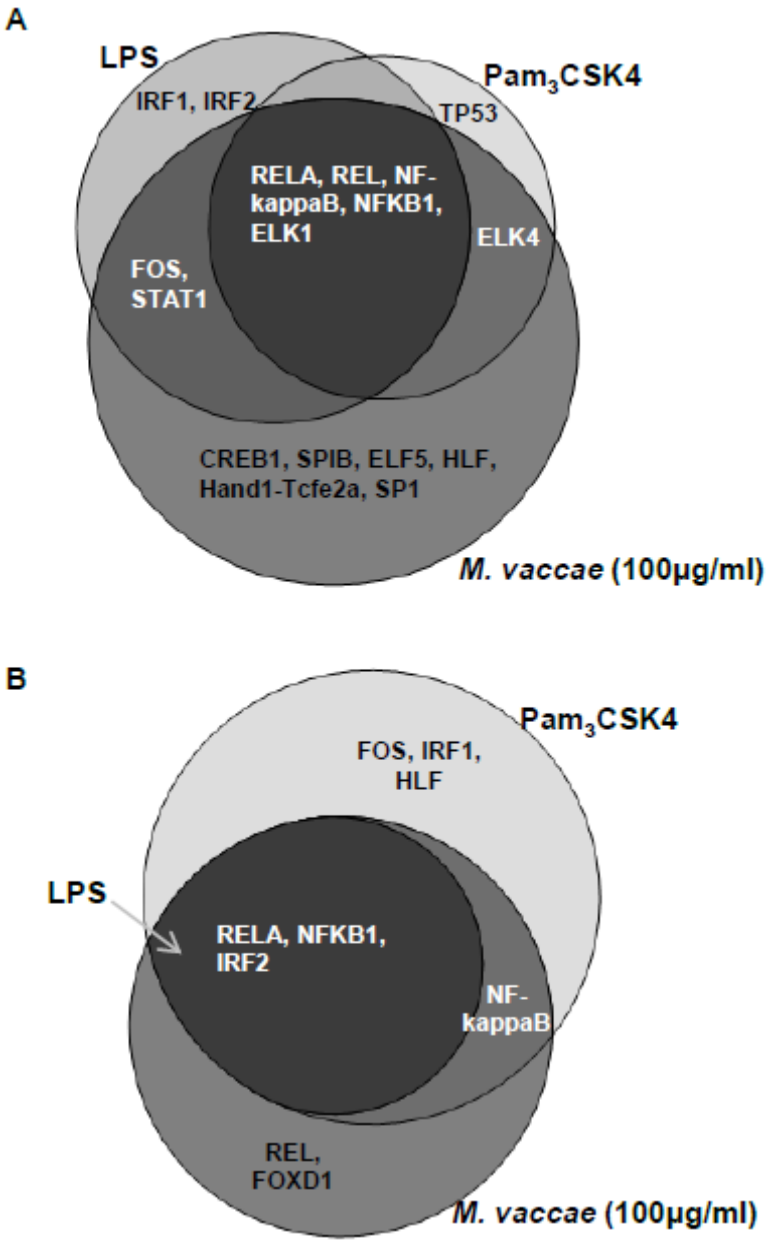

Figure 5-1: Overrepresented TFBSs associated with upregulated genes

Analyses of overrepresented TFBSs associated with genes that are upregulated in DCs in response to 4 h (**A**) and 24 h (**B**) stimulation with *M. vaccae* (100 µg/ml), LPS (100 ng/ml) or Pam_3CSK4 (1 µg/ml), using the online bioinformatics tool oPOSSUM. The Venn diagram shows overrepresented TFBSs that are shared and exclusive in response to the three different stimuli. For transcription factor names see list of abbreviations (pages 16-22).

As expected, this showed enrichment for components of the NF-κB transcription factor family in the common response for all stimuli. At the 4 hours time point, *M. vaccae* activated expression of genes that are enriched with 14 different TFBSs, considerably more than the specific TLR ligands LPS and Pam_3CSK4, which were enriched for nine and seven transcription factors respectively. Moreover, six TFBSs were enriched exclusively in the *M. vaccae* upregulated gene list, CREB1, SPIB, ELF5, HLF, Hand1-Tcfe2a and SP1. At the early time point, only LPS induced overrepresented induction of genes associated with IRFs, whereas the list of upregulated genes in response to Pam_3CSK4 was exclusively enriched for the binding site of TP53. Genes upregulated at the 24 hours time point were enriched for less TFBSs than genes induced at the early time point.

Similar analysis of genes that were exclusively upregulated by stimulation with *M. vaccae* at the 4 hours time point identified CREB1 as the most enriched transcription factor (Table 5-3).

Table 5-3: Overrepresented TFBSs in genes exclusively upregulated in DCs stimulated for 4 hours with *M. vaccae*. The oPOSSUM programme was used for analysis. For names of transcription factors see abbreviations (pages 16-22).

TFBSs in DCs stimulated with *M. vaccae* (100 μg/ml) versus all	Z-Score
CREB1	19.98
SP1	18.93
HLF	17.30
REL	16.06
RELA	14.82
NF-kappaB	11.35
RXRA-VDR	10.09

There are 168 genes upregulated in DCs after 4 hours of stimulation with *M. vaccae* that are associated with a TFBS for CREB1. From this list all genes that are more than 5-fold upregulated are shown in Figure 5-2. Moreover, there are 33 genes with TFBSs for CREB1 that are exclusively upregulated in DCs that were stimulated for 4 hours with *M. vaccae*, but are not induced by LPS or Pam_3CSK4 (Figure 5-3).

Figure 5-2: Genes with TFBSs for CREB1 that are more than 5-fold upregulated in *M. vaccae*-stimulated dendritic cells

Genes that are more than 5-fold upregulated in response to stimulation with *M. vaccae* (100µg/ml) and that are associated with the transcription factor CREB1. Expressions in LPS- and Pam_3CSK4-stimulated and unstimulated (U) DCs are aligned for comparison. For gene names see list of abbreviations (pages 16-22).

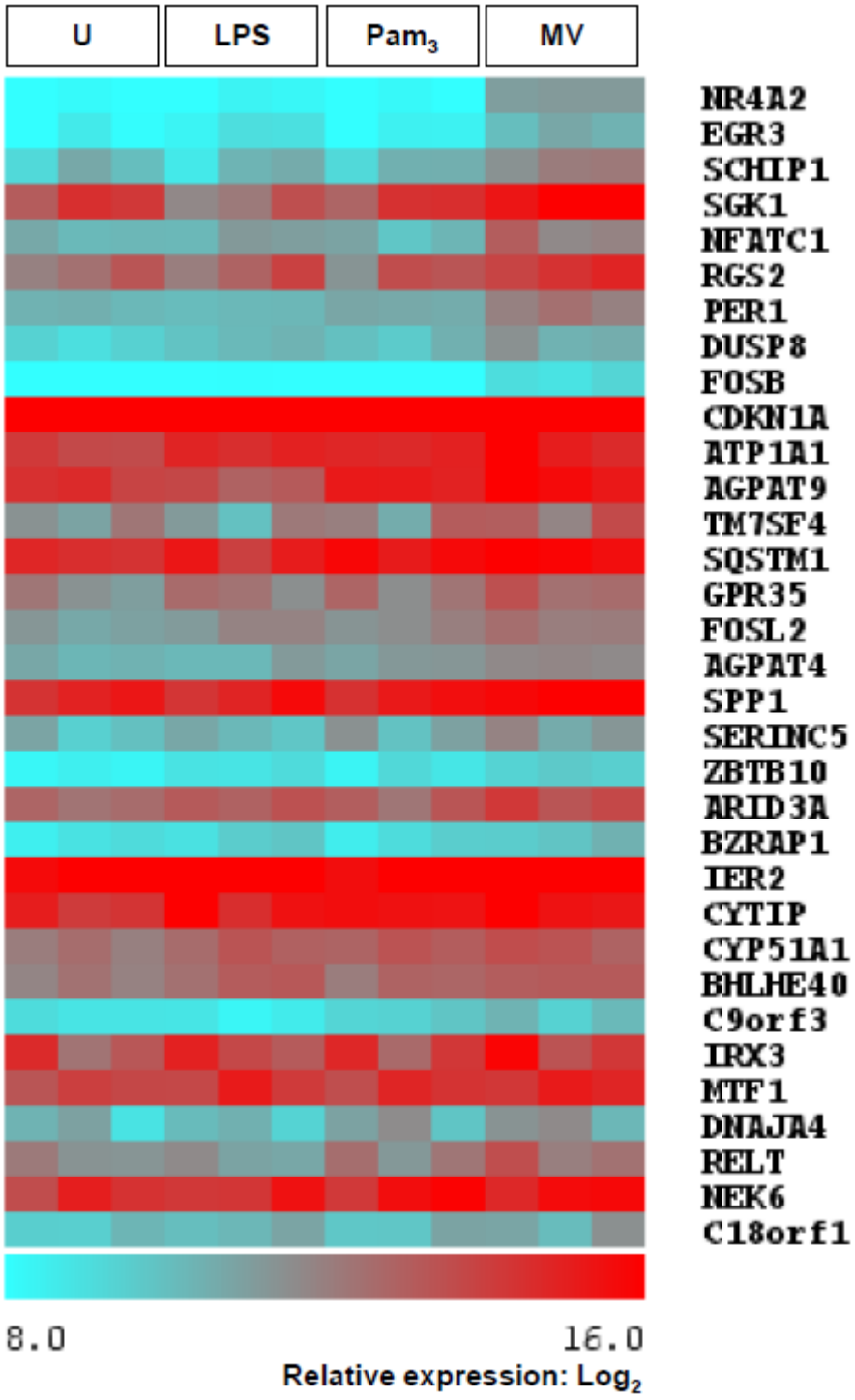

Figure 5-3: Genes with TFBSs for CREB1 that are exclusively upregulated in *M. vaccae*-stimulated dendritic cells

Genes that are exclusively upregulated by DCs in response to 4 h stimulation with *M. vaccae* (100 µg/ml) and are associated with the transcription factor CREB1. LPS- and Pam_3CSK4-stimulated and unstimulated (U) DCs are aligned for comparison. For gene names see list of abbreviations (pages 16-22).

Functional annotation clustering analysis identified that the list of exclusively upregulated genes by stimulation with *M. vaccae* that are associated with CREB1 is significantly enriched for transcriptional regulation (Table 5-4).

Table 5-4: GO terms from top three enriched groups of genes identified by functional annotation clustering analysis of genes that are exclusively upregulated in DCs following 4 hours of stimulation with *M. vaccae* and that are associated with CREB1.

Gene ontology term	P value	No of genes	% of gene list	Fold enrichment
GO:0003700~transcription factor activity	2.44^{-4}	9	27.27	4.79
GO:0030528~transcription regulator activity	4.39^{-3}	9	27.27	3.09
GO:0003677~DNA binding	1.87^{-2}	10	30.30	2.23
GO:0006355~regulation of transcription	5.71^{-3}	10	30.30	2.73
GO:0051252~regulation of RNA metabolic process	6.62^{-3}	10	30.30	2.66
GO:0008654~phospholipid biosynthetic process	1.75^{-2}	3	9.09	14.21

OPOSSUM analysis of genes exclusively upregulated by *M. vaccae* at 24 hours were only enriched for hepatocyte nuclear factor 4 alpha (HNF4A) (Z-score = 13.77) and REL (Z-score = 11.65).

5.3.2 *M. vaccae* selectively stimulates activation of the CREB pathway

The oPOSSUM analysis had identified that TFBSs for CREB1 were the most overrepresented in the list of early upregulated genes in *M. vaccae* stimulated DCs. Moreover, this analysis suggested upregulated expression of genes with TFBSs for members of the NF-κB family as a shared core response induced by all innate immune stimuli under study. However, this bioinformatics analysis only theoretically implicated a role for CREB1-dependent gene expression in *M. vaccae*-stimulated DCs and showed NF-κB-activity in all stimulated DCs. In order to validate this analysis, specific innate immune signalling events were compared in differentially stimulated DCs.

CREB1, as described in more detail in the introduction (1.4.5), is constitutively and ubiquitously expressed and therefore already present in unstimulated cells and does not have to be newly synthesised to induce transcription of genes. Briefly,

phosphorylation of the transcription factor CREB1 on Ser-133 is commonly associated with its activation (Gonzalez and Montminy, 1989), which triggers the recruitment of the coactivators CBP and its paralogue p300 (Arias et al., 1994; Chrivia et al., 1993) and strongly enhances CREB1-dependent transcription (Hagiwara et al., 1993).

Due to the important roles of NF-κB and MAPK pathways in DC maturation and cytokine production, which both might contribute to their ability to polarise T cell responses (Boonstra et al., 2003; Kapsenberg, 2003), and the bioinformatics data suggesting activation of the CREB pathway in *M. vaccae*-stimulated DCs, I sought to compare activation of those pathways in LPS-, Pam_3CSK4- and *M. vaccae*-primed DCs by Western blotting. To study activation of the classical NF-κB signalling pathway cell extracts were analysed for IκB-α degradation. Activation of the MAPK pathways p38 and ERK1/2 was detected by using antibodies specific for their phosphorylated state. Activation of CREB1 was detected by using a monoclonal antibody that recognises endogenous levels of CREB1 only when phosphorylated at Ser-133.

Stimulation of DCs with the TLR4 ligand LPS activated all pathways under study, thus IκB-α was degraded, and ERK1/2, p38 and CREB1 were phosphorylated at the 30 minutes time point (Figure 5-4). The specific TLR2 ligand Pam_3CSK4 had the same effect as LPS. All signalling pathways were turned on at the early time point, but none of them remained activated 120 minutes after stimulation. In stark contrast, *M. vaccae* activated only the CREB pathway at the early time point and in comparison to the other stimuli exhibited sustained activation of this pathway at the late time point (Figure 5-4). Activation of the NF-κB signalling pathway or the MAPK cascade proteins p38 and ERK1/2 in response to stimulation with *M. vaccae* was not detectable by using Western blotting.

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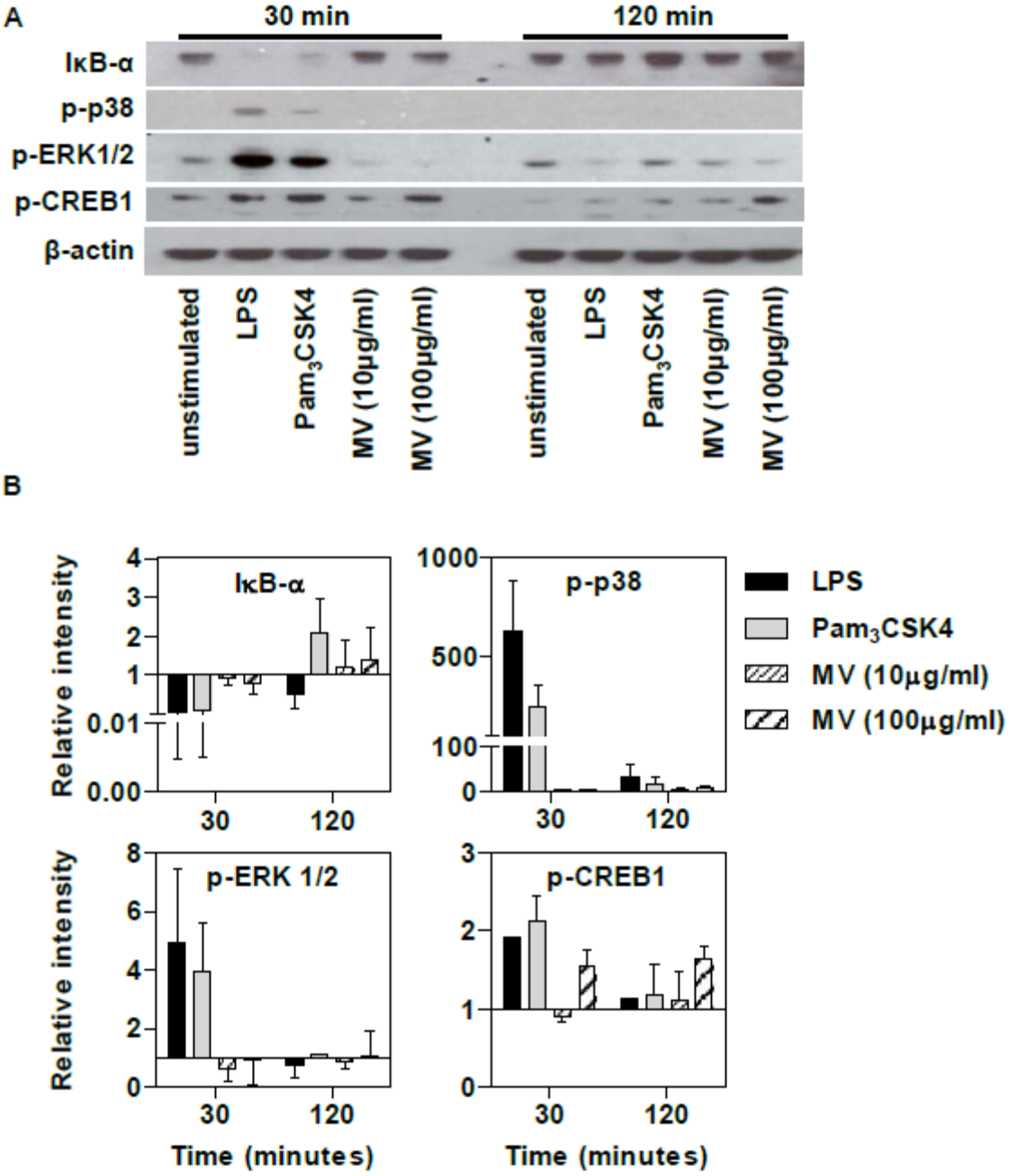


Figure 5-4: *M. vaccae* selectively stimulates activation of the CREB pathway

A: Western blot analysis of candidate innate immune signalling events in DCs shows comparable degradation of IκB-α, and phosphorylation of p38, ERK1/2 and CREB in DCs after 30 min and 120 min of stimulation with LPS (100 ng/ml) and Pam_3CSK4 (1 µg/ml), but selective activation of the CREB pathway by *M. vaccae* (10 µg/ml and 100 µg/ml). **B**: Quantitative densitometry data from three separate experiments are shown. Bars represent mean ±SD.

5.3.3 *M. vaccae* stimulates late activation of the NF-κB pathway

Clearly, the transcriptional profiling data suggest that *M. vaccae* also activates the NF-κB pathway. In resting cells, inhibitory IκB proteins are bound to NF-κB that mask its NLS and inhibit its translocation into the nucleus (Huxford et al., 1998; Jacobs and Harrison, 1998). Moreover, IκB-α proteins contain a NES that is also responsible for the mainly cytosolic pool of NF-κB–IκB-α complexes in resting cells (Tam et al., 2000). In the canonical NF-κB pathway, innate immune activation of cells induces phosphorylation of IκB via the IKK complex, which leads to its polyubiquitination and proteasomal degradation (Chen et al., 1995b; DiDonato et al., 1996; Lin et al., 1995; Scherer et al., 1995). Consequently, NF-κB is no longer bound in the cytoplasm and can shuttle into the nucleus where the Rel subunit, which contains a transactivation domain, can drive the transcription.

Given that degradation of IκB-α was not detected by Western blotting in *M. vaccae*-stimulated DCs, activation of the classical NF-κB pathway was assessed by quantification of NF-κB RelA nuclear translocation by confocal microscopy (Noursadeghi et al., 2008). By using specific antibodies for NF-κB RelA (p65), it was possible to visualise cytosolic and nuclear localisation of NF-κB, which was then quantified using MetaMorph software (Noursadeghi et al., 2008). This method showed that nuclear translocation of RelA was evident after two hours of stimulation with *M. vaccae* (Figure 5-5). In comparison to LPS and Pam_3CSK4, which induced rapid activation of NF-κB that was visibly 30 minutes post-stimulation, activation by *M. vaccae* clearly occurred more slowly.

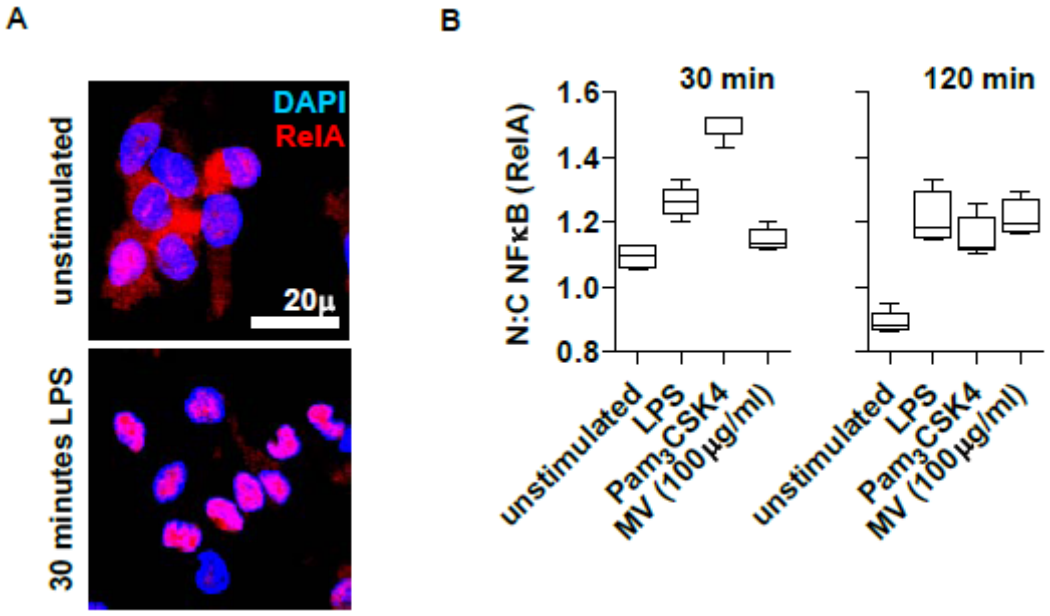


Figure 5-5: *M. vaccae* stimulates late activation of the NF-κB pathway

Quantitative confocal immunofluorescence staining used to detect NF-κB RelA (p65) nuclear translocation in response to innate immune stimulation of DCs (**A**) showed that activation of the classical NF-κB pathway was evident by 30 min in response to LPS or Pam_3CSK4, and by 120 min in response to *M. vaccae* (**B**). Representative data from three separate experiments are shown. Box and whisker plots represent median, and range of data from approximately 500 single cell measurements.

5.3.4 PKA inhibitor H89 blocks *M. vaccae*-mediated phosphorylation of CREB1 in dendritic cells

In order to directly test the role of the CREB pathway in the *M. vaccae*-induced DC-dependent inhibition of Th2 responses, I sought to inhibit this pathway pharmacologically. CREB1 is activated by phosphorylation at serine 133, which can occur via various signal transduction pathways (Figure 5-6). Primarily, PKA has been shown to provoke phosphorylation of CREB1 (Gonzalez and Montminy, 1989). Likewise, various other protein kinase pathways have been described that can induce activation of CREB1, including Ca^{2+} influx that can trigger CaMKII/IV (Matthews et al., 1994; Sun et al., 1994), ERK1/2 activation can phosphorylate p90RSK (Ginty et al., 1994; Xing et al., 1996) and MSK1/2 (Deak et al., 1998; New et al., 1999; Pierrat et al.,

1998), phosphorylated p38 (p-p38) can also activate MSK1/2 (Deak et al., 1998; New et al., 1999; Pierrat et al., 1998; Wiggin et al., 2002), and PI3K can trigger Akt, which can either directly phosphorylate CREB1 (Du and Montminy, 1998; Kato et al., 2007) or inhibit GSK3, an inhibitor of CREB1 (Martin et al., 2005).

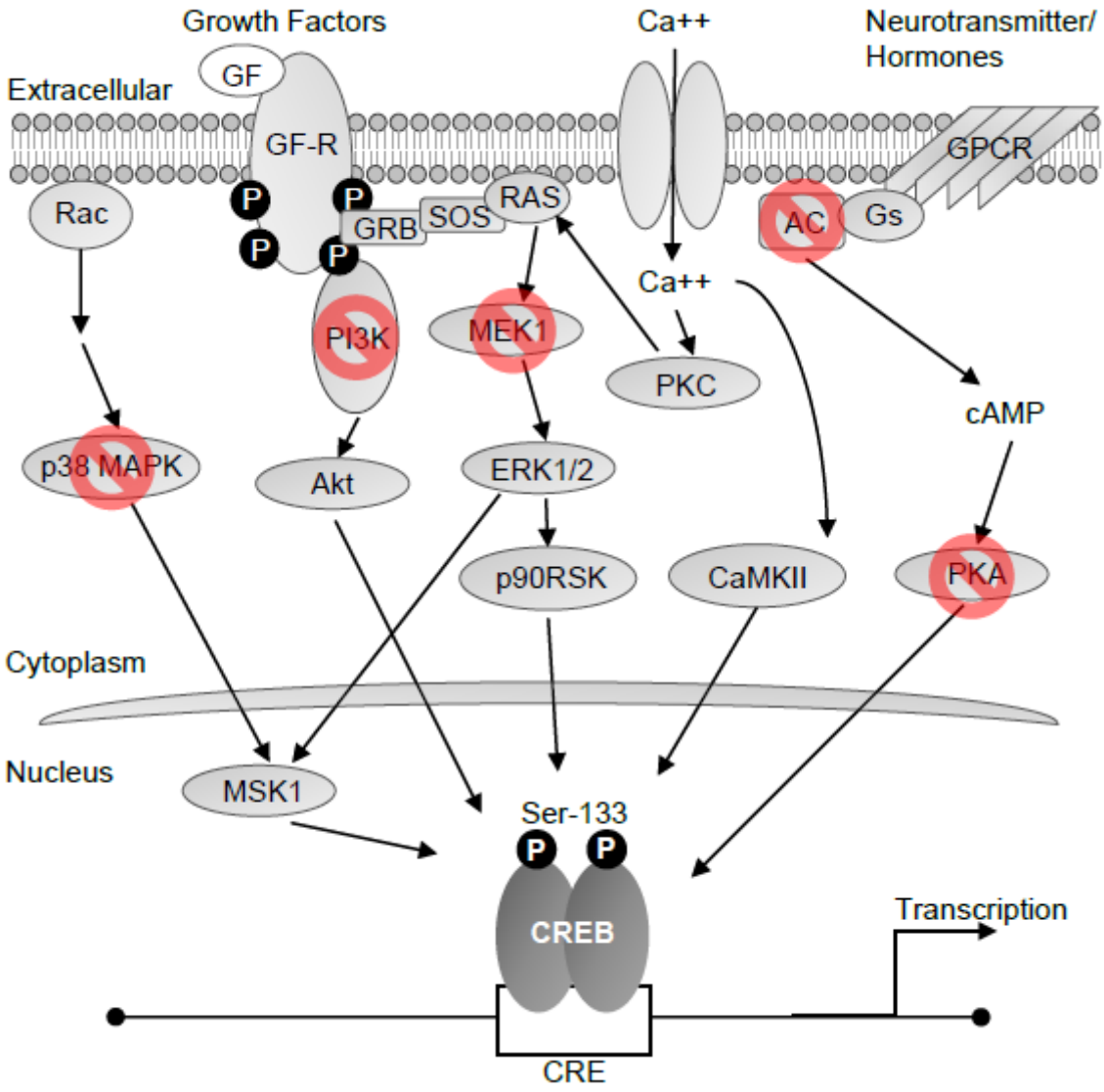


Figure 5-6: Signalling pathways leading to phosphorylation of CREB1 at Ser-133

The transcription factor CREB1 forms homodimers and binds the cAMP response element (CRE) and activates transcription. CREB1 is activated upon phosphorylation of serine 133, which can occur via PKA, CaMKII, p90RSK, Akt and MSK1/2. DCs were incubated 2 h prior to stimulation with a final concentration of 25 µM of the p38 inhibitor SB203580, 25 µM of the PI3K inhibitor LY294002, 25 µM of the MEK1 inhibitor PD98059, 100 µM of the cell-permeable adenylate cyclase (AC) inhibitor SQ22536 or 50 µM of the PKA inhibitor H89.

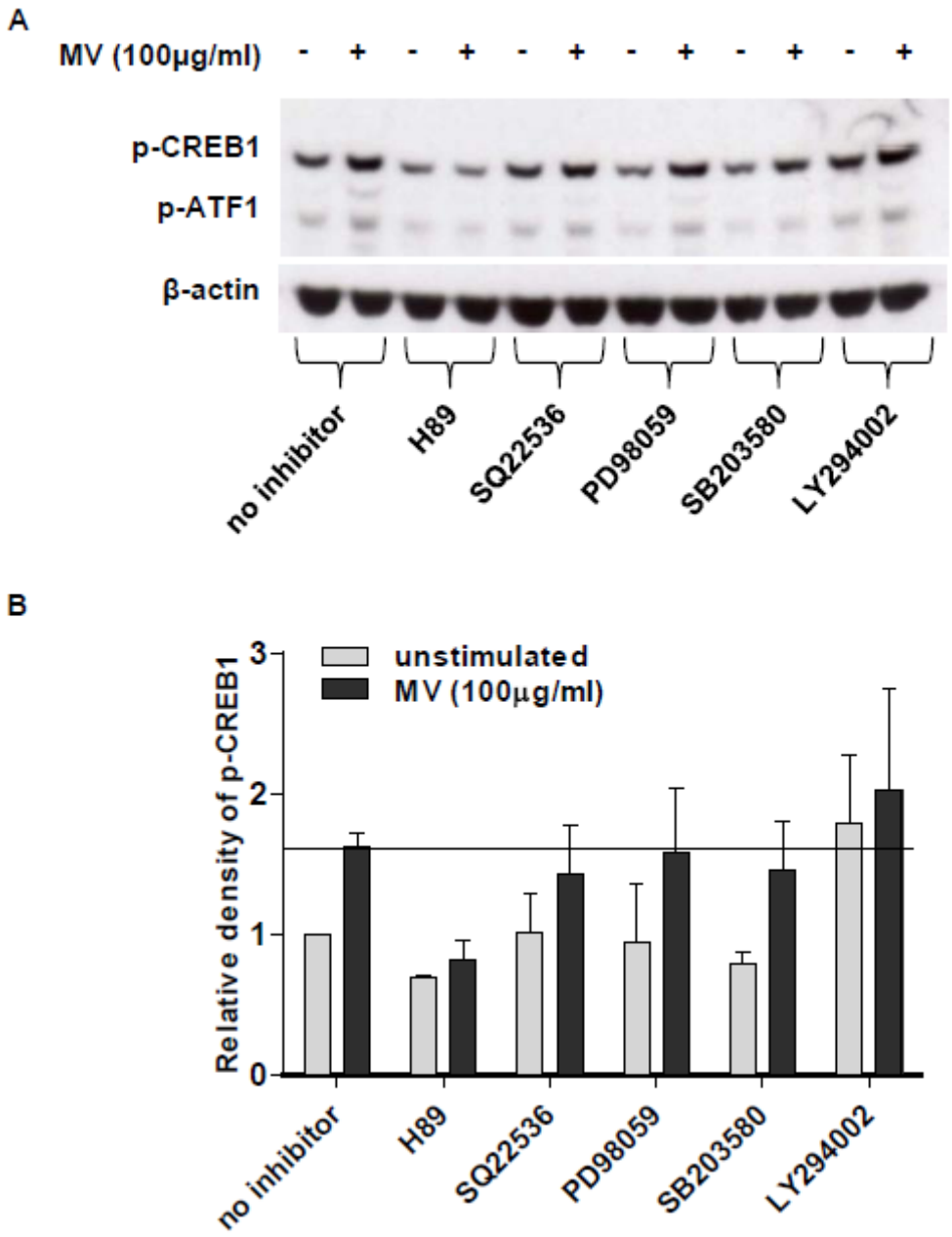


Figure 5-7: PKA inhibitor H89 blocks *M. vaccae*-mediated phosphorylation of CREB1 in dendritic cells

CREB1 phosphorylation in response to *M. vaccae* stimulation of DCs ± preincubation with the various inhibitors, showed attenuated response only in the presence of 50 µM H89 (PKA inhibitor). Representative Western blot analysis **(A)** and densitometry quantitation **(B)** of three separate experiments are shown (mean ±SD).

Amongst the different inhibitors tested, only the common PKA inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H89) blocked *M. vaccae*-mediated phosphorylation of CREB1 in DCs (Figure 5-7). H89 can also block MSK1/2 (Davies et al., 2000), whose main target is CREB1 (Arthur and Cohen, 2000; Wiggin et al., 2002), however blocking of upstream kinases ERK1/2 and p38

with PD98059 or SB203580 respectively did not affect CREB1 phosphorylation. Moreover, treatment of DCs with LY294002, an inhibitor of the PI3K-Akt pathway, did not block *M. vaccae*-induced CREB1 phosphorylation, and rather augmented its phosphorylation in unstimulated DCs. Taken together, pharmacological blocking of various signal transduction pathways that can provoke phosphorylation of CREB1 indicated a role for PKA, independent of adenylate cyclase activity, whose inhibition with SQ22536 had no effect.

5.3.5 PKA inhibitor H89 induces apoptosis and death of dendritic cells

In order to study the role of CREB1 activity in *M. vaccae*-stimulated DCs and its effects on T cell polarisation in mixed leukocyte cultures, DCs were treated with H89 two hours prior to stimulation with LPS, Pam3CSK4 or *M. vaccae*. Following 24 hours of incubation, at the time when T cells would be added, the DCs were analysed for signs of apoptosis and cell death, using annexin-V and PI staining. Annexin-V binds with high affinity to phosphatidylserine that is usually located inside the cell membrane of live cells but is expressed on the cell surface of apoptotic cells. PI can only penetrate the membrane of dying or dead cells and then intercalates into double-stranded nucleic acids. This analysis by flow cytometry showed clearly that the final concentration of 50 μM H89 used for inhibition of *M. vaccae*-mediated phosphorylation of CREB1 caused cell death in the 24 hours of DC culture prior to coculture with T cells (Figure 5-8A+B). The toxicity was caused by H89 and not by DMSO in which H89 was initially dissolved, as a similar concentration of DMSO had no such toxic effect (Figure 5-8A+B).

A serial dilution series of H89 was used to treat DCs for 24 hours in order to assess at which concentration the PKA inhibitor was not toxic (Figure 5-8A+B).

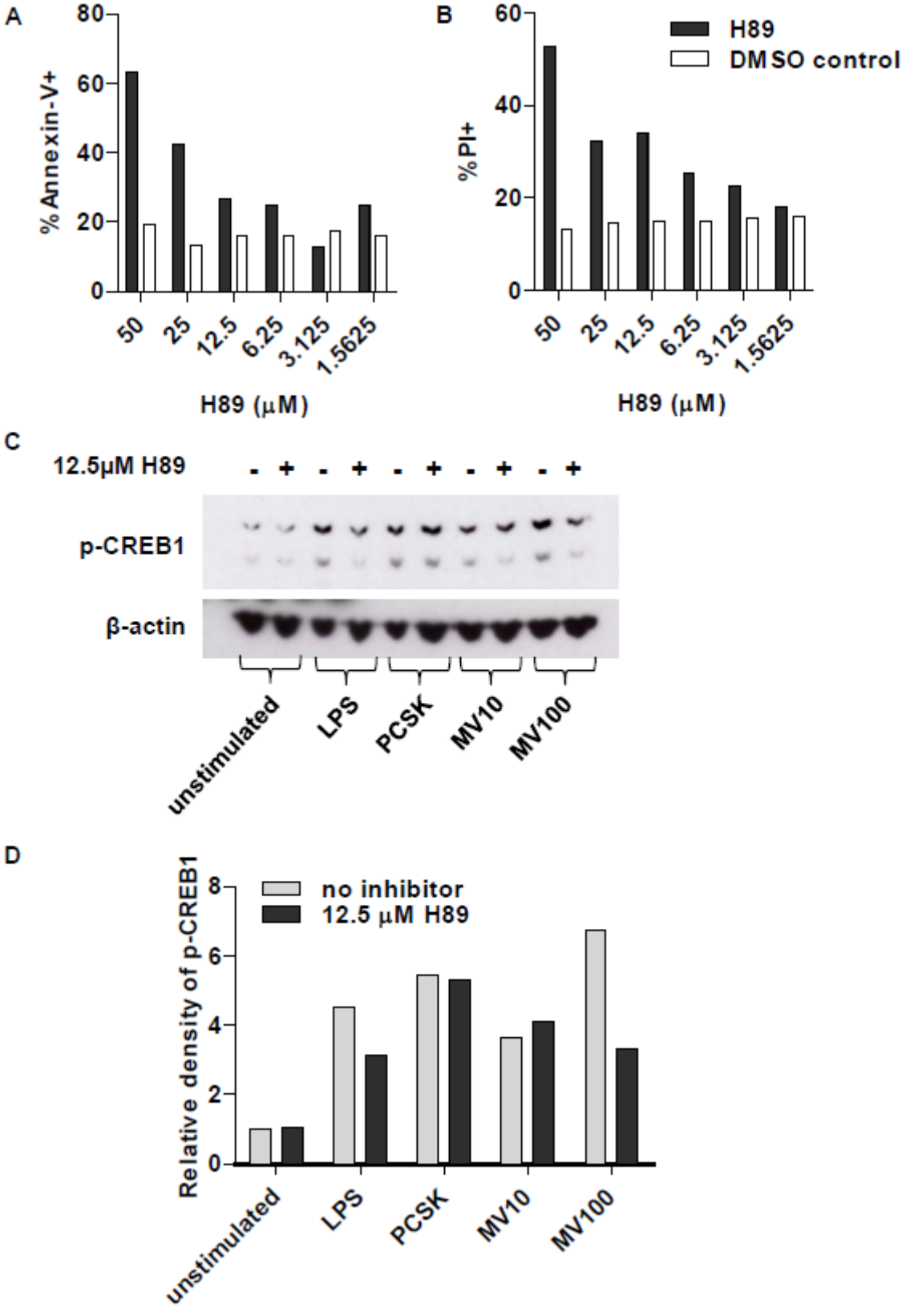


Figure 5-8: PKA inhibitor H89 induces apoptosis and death of dendritic cells

Overnight incubation of DCs with H89, required for functional experiments, shows dose-dependent cytotoxicity reflected in increased annexin-V (**A**) and PI (**B**) staining assessed by flow cytometry. A reduced concentration of only 12.5 µM H89, which showed only modest cytotoxicity, did not block completely *M. vaccae*-mediated phosphorylation of CREB1 assessed by Western blot analysis **(C)** and densitometry quantitation **(D)**.

Subsequently it was tested if a final concentration of 12.5 μM H89, which showed minor toxicity measured by annexin-V and PI staining, would still be able block *M. vaccae*-mediated phosphorylation of CREB1. Western blot and densitometry analysis demonstrated that this four-fold reduced concentration of H89 still inhibited *M. vaccae*-induced phosphorylation of CREB1 by about 50 % (Figure 5-8C+D). Phosphorylation of CREB1 in response stimulation with LPS was only slightly inhibited and that caused by Pam_3CSK4 was not blocked at all by this concentration of H89, suggesting that those TLR ligands activate different or additional signal transduction pathways than PKA, which lead to CREB1 phosphorylation.

Further analysis of DCs after 24 hours of stimulation with the different innate immune stimuli under study demonstrated that H89 did not affect the maturation response of the cells (Figure 5-9). Annexin-V staining showed that less than 12 % of the DCs were apoptotic (Figure 5-10A) compared to above 60 % when 50 μM H89 was used (Figure 5-8A) and the proportion of dead cells was below 2 % (Figure 5-10B) compared to above 50 % with the high concentration of the PKA inhibitor (Figure 5-8B). Nonetheless, DCs treated with H89 were no longer able to induce T cell proliferation when cocultured with allogeneic naive $CD4^+$ T cells (Figure 5-10C+D), probably resulting from the cytotoxic effect of H89.

Overall, it has proved impossible to assess the effect of the PKA inhibitor H89 on the effect of DC priming by *M. vaccae* on Th2 responses, due to significant cytotoxicity compromising the experimental paradigm.

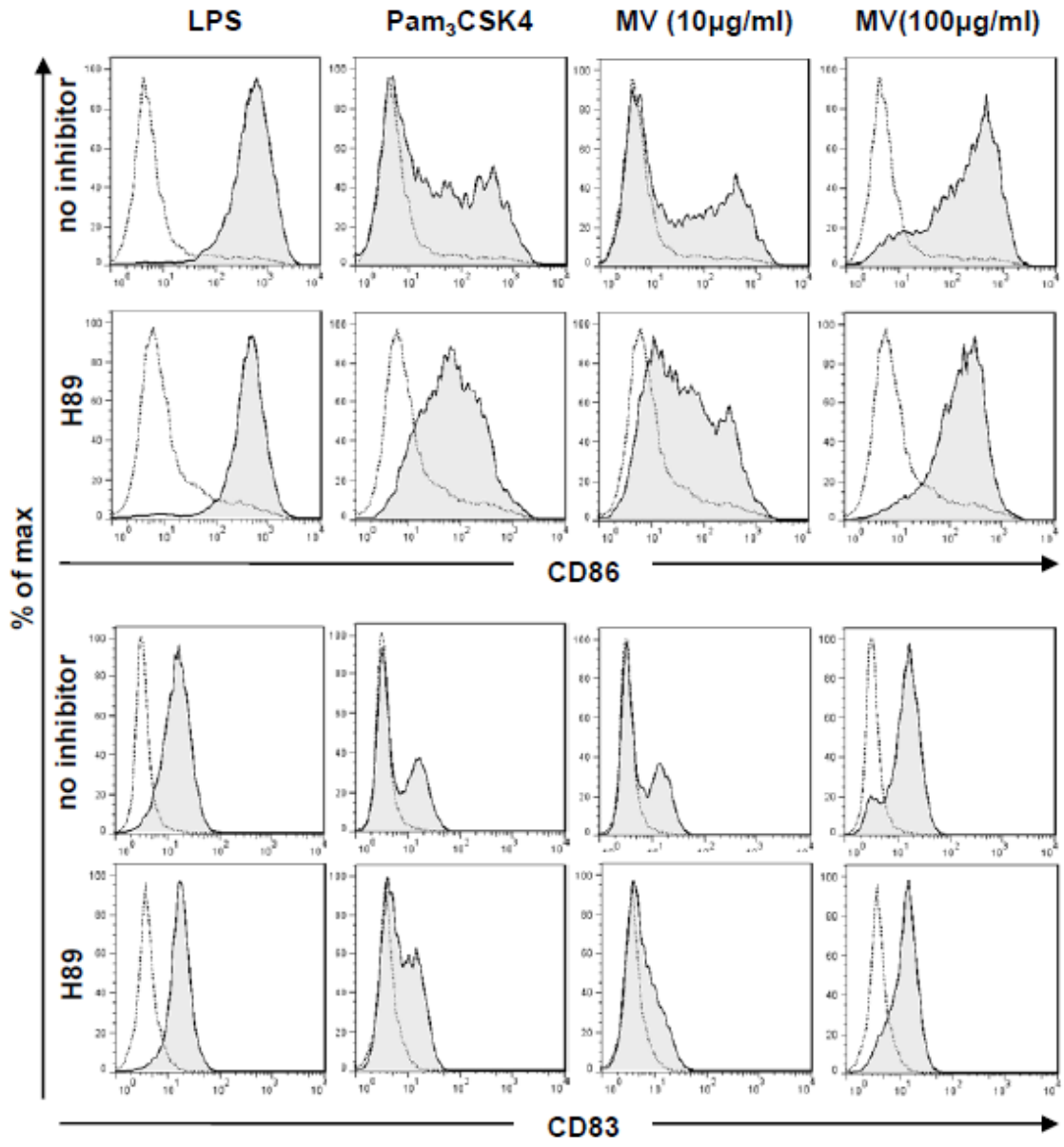


Figure 5-9: Treatment of dendritic cells with 12.5 µM H89 does not influence the maturation

DCs were treated with 12.5 µM H89 for 2 h before addition of LPS (100 ng/ml), Pam_3CSK4 (1 µg/ml) and *M. vaccae* (10 and 100 µg/ml). After 24 h of stimulation, the maturation of DCs was measured by flow cytometric analysis of immunofluorescence staining for cell surface CD83 and CD86 expression. White histograms show staining of unstimulated DCs.

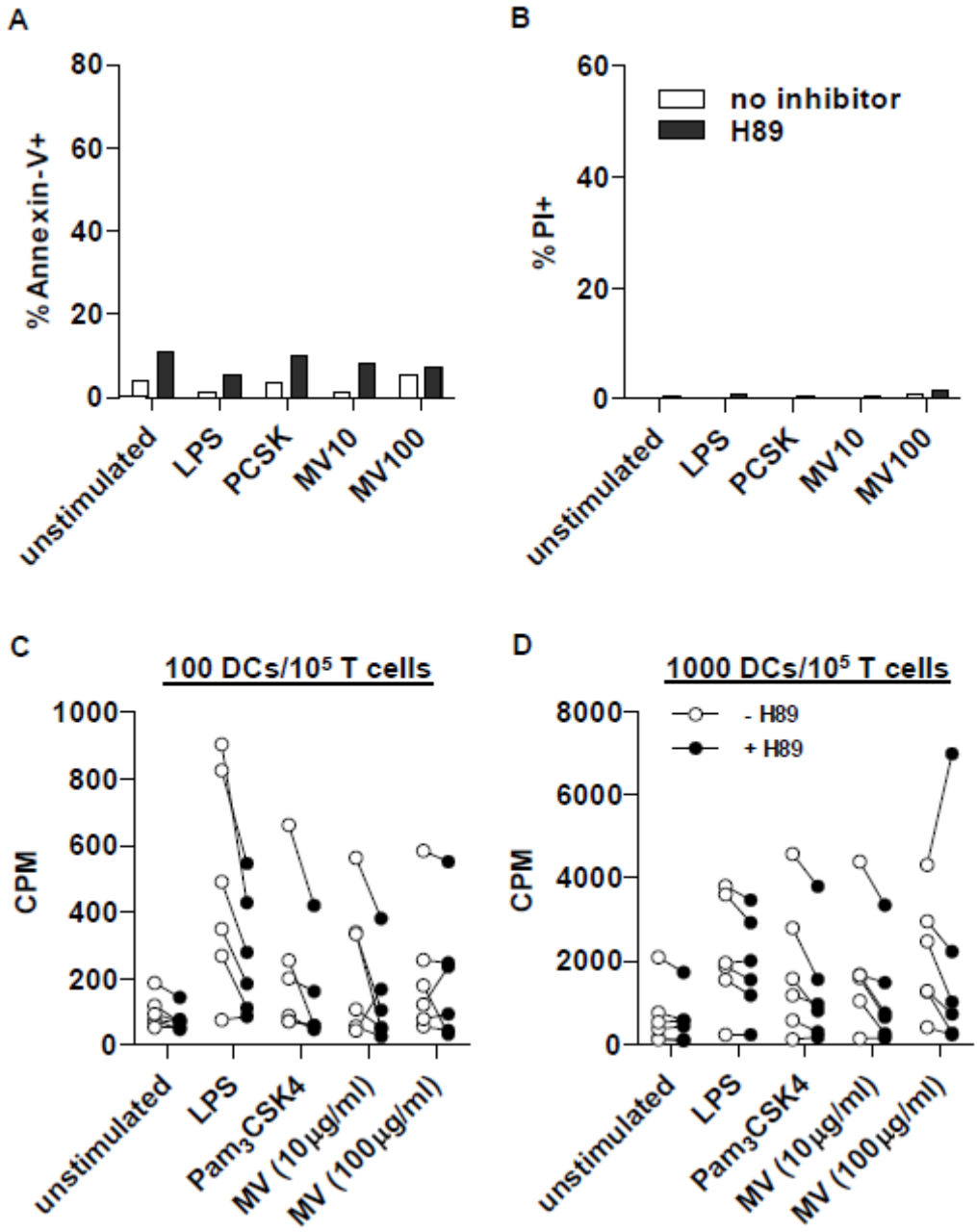


Figure 5-10: Dendritic cells treated with 12.5 µM H89 are no longer able to induce T cell proliferation

DCs were treated with 12.5 µM H89 for 2 h before addition of LPS (100 ng/ml), Pam_3CSK4 (1 µg/ml) and *M. vaccae* (10 and 100 µg/ml). After 24 h of stimulation some DCs were analysed for apoptosis and cell death by annexin-V (**A**) and PI staining (**B**). Remaining DCs were cocultured with naive $CD4^+$ T cells. T cell proliferation was assessed by thymidine incorporation (CPM) after 3 days of stimulation with allogeneic DCs. DCs treated with H89 induced reduced T cell proliferation compared untreated DCs at DC/T cell ratios of 1:1000 (**C**) and 1:100 (**D**). Each experiment is represented by paired data points.

5.4 Discussion

Experiments with TLR-transfected cell lines had shown clearly that *M. vaccae* induces TLR2-dependent cellular activation. However, comparison of *M. vaccae* to specific TLR2 stimulation with Pam_3CSK4 demonstrated different effects on DC priming and subsequent T cell polarisation. The transcriptional response of DCs stimulated with LPS, Pam_3CSK4 or *M. vaccae* was analysed by whole genome microarray to gain insight into possible differences in expression profiles that would allow studying potential molecular mechanisms responsible for the distinct effects seen. However, the general transcriptional response of DCs to stimulation with Pam_3CSK4 or *M. vaccae* was very similar as measured by PCA.

5.4.1 Genes induced by *M. vaccae* are associated with CREB1

Immune response gene expression is regulated by transcription factors whose activation is a key event in innate immune cellular activation of DCs. When analysing overrepresented TFBS up- and downstream of genes upregulated by the three stimuli, it was seen that many of the genes are possibly activated by the classical immune response transcription factor family NF-κB. This is in line with reports stating that NF-κB activation mediates part of a common or core immune response to PRR stimulation (Jenner and Young, 2005). Interestingly, the lists of upregulated genes showed also overrepresented TFBSs specific for each stimulus. LPS induced many genes that are probably regulated by IRF1 and IRF2, transcriptional regulators of type I IFNs and IFN-inducible genes. This is in line with previous data that showed induction of IFN-stimulated genes in responses to TLR4 stimulation but not in response to TLR2 (Jenner and Young, 2005). In mice it was shown that IRF1 is essential for the development of Th1-type immune responses, and its deficiency leads to the induction of Th2- or Treg-type immune responses (Gabriele et al., 2006; Lohoff et al., 1997; Taki et al., 1997). In addition, IRF2 is also involved in the gene expression of IL-12 (p40) and

supports IRF1 in promoting Th1 immune responses (Lohoff et al., 2000). However, genes upregulated in response to stimulation with *M. vaccae* showed only overrepresented TFBSs for IRF2 at the later time point, which might be the reason why DC priming with *M. vaccae* did not augment Th1 responses.

Interestingly, the bioinformatics analysis suggested that stimulation of DCs with *M. vaccae* induced the upregulation of genes that are controlled by a broader range of transcription factors than genes upregulated in response to LPS or Pam_3CSK4. This probably results from the more complex structure of the heat-killed preparation of *M. vaccae* compared to the specific TLR ligands, which is likely to stimulate other PRRs in addition to TLRs. Analysis of all genes upregulated in response to stimulation with *M. vaccae* showed that the most overrepresented TFBSs after those for members of the NF-κB family was for CREB1. Similar analysis of the list of genes exclusively upregulated in response to stimulation with *M. vaccae* found TFBSs for CREB1 the most overrepresented. GO associations identified by annotation clustering analysis demonstrated that the upregulated genes with TFBSs for CREB1 are mainly involved in regulation of transcription and therefore possibly responsible for the different effects of *M. vaccae*-primed DCs on T cell polarisation. For example, the orphan nuclear receptor family NR4A1 (Nur77), NR4A2 (Nurr1) and NR4A3 (NOR-1 or MINOR) has TFBSs for CREB1 and is highly upregulated in *M. vaccae*-treated DCs. This is interesting in view of a previous report on mouse DCs, which showed upregulation of only NR4A3 in activated mouse DCs, while the expression of NR4A1 and NR4A2 remained unchanged. In addition, this study showed a role for NR4A3 in inhibition of IL-12 production by DCs and reduced expression of NR4A3 in DCs augmented T cell proliferation (Wang et al., 2009). In human macrophages overexpression of NR4A1, NR4A2 or NR4A3 reduced expression of IL-1β, IL-6, IL-8, MIP-1α (CCL3), MIP-1β (CCL4) and MCP-1 (CCL2), whereas knockdown of NR4A1 or NR4A3 enhanced

cytokine and chemokine synthesis (Bonta et al., 2006). This was recently confirmed by two studies in mice, one showing via overexpression and knockdown studies on NR4A2 a role for this transcription factor in reduction of inflammatory gene expression, including TNF-α, IL-1β and MCP-1, and in reduction of vascular smooth muscle cell proliferation and inhibition of vascular lesion formation in restenosis models (Bonta et al., 2010). The other showed by overexpression and knockdown that NR4A1 has an anti-inflammatory role as it suppresses TNF-α and MCP-1 (Shao et al., 2010). A mechanism for the anti-inflammatory effects of NR4A1 was suggested in a study on human endothelial cells, where NR4A1 was shown to suppress NF-κB-dependent cellular activation of by direct induction of IκB-α expression (You et al., 2009).

However, the genes upregulated in response to *M. vaccae* at the 24 hours time point were not enriched for TFBSs of any of the CREB1-associated transcription factors upregulated at the 4 hours time point. Therefore I decided to focus on the role of CREB1 and not on downstream transcription factors.

5.4.2 Signalling pathways induced by *M. vaccae*

In order to validate the bioinformatics data, which suggested an activation of the CREB pathway in *M. vaccae*-stimulated DCs, protein immunoblot analysis was performed. Activation of NF-κB was measured by IκB-α degradation, of the MAPKs pathways by measuring phosphorylation of p38 and ERK1/2, and of CREB1 by antibody probing for its phosphorylation at Ser-133.

5.4.2.1 *M. vaccae* activates CREB1

As expected from the microarray data, the high dose of *M. vaccae* activated the CREB pathway at the 30 minutes and 120 minutes time points, but activation of NF-κB and MAPK pathways in *M. vaccae*-stimulated DCs was not detectable by using Western blot analysis. Interestingly, despite marked differences in DC maturation and in

transcriptional and cytokine responses in DCs stimulated with LPS and Pam_3CSK4, the innate immune signalling events assessed here showed a very similar profile. LPS and Pam_3CSK4 stimulation of DCs activated all pathways under study at the early time point including phosphorylation of CREB1, but none of the pathways remained active at the late time point. Phosphorylation of CREB1 by stimulation of cells with LPS (Ananieva et al., 2008; Ardeshna et al., 2000; Avni et al., 2010a; Caivano and Cohen, 2000; Chandra et al., 1995; Eliopoulos et al., 2002) and Pam_3CSK4 (Ananieva et al., 2008) or infection with mycobacteria (Agarwal et al., 2009a; Pathak et al., 2004; Roach et al., 2005), or treatment with killed mycobacteria (Samten et al., 2008) has been described previously. It is established that stimulation with LPS causes rapid phosphorylation of CREB1, which peaks about 30-60 minutes post-treatment (Ananieva et al., 2008; Ardeshna et al., 2000; Avni et al., 2010a; Caivano and Cohen, 2000; Chandra et al., 1995; Eliopoulos et al., 2002). However the literature about the time course of this activated state of CREB1 in mycobacterial treated cells is controversial and very limited. While some authors find p-CREB1 in macrophages 30 minutes (Pathak et al., 2004) or one hour post-infection with mycobacteria but no longer after nine hours (Roach et al., 2005), others find it 16 hours after infection (Agarwal et al., 2009a). The same publication shows that killed mycobacteria do not induce phosphorylation of CREB1 at this late time point, while others demonstrate that treatment of PBMCs with heat-killed *M. tuberculosis* induces phosphorylation of CREB1 after 24 hours, with increased expression at 48-72 hours (Samten et al., 2008). In line with the microarray data, which suggested an early activation of CREB1 in *M. vaccae*-stimulated DCs because already four hours post-treatment genes with TFBSs for CREB1 were significantly upregulated, phosphorylation of CREB1 was detected at 30 minutes and two hours post-treatment.

5.4.2.2 *M. vaccae* does not induce ERK1/2 and p38 MAPKs

Interestingly, Western blot analysis did not detect activation of any other signal transduction pathway under study in DCs stimulated with *M. vaccae*. That ERK1/2 and p38 MAPKs were not phosphorylated in response to *M. vaccae* is different to what has been described for human cells infected with pathogenic and non-pathogenic mycobacteria or treated with BCG (Bhattacharyya et al., 2002; Blumenthal et al., 2002; Cheung et al., 2009; Mendez-Samperio et al., 2005; Reiling et al., 2001). However, a freeze-dried preparation of BCG, which reduced allergy in mice models, was also associated with reduced NF-κB and p38 MAPK activity (Lagranderie et al., 2010). Among the genes with TFBSs for CREB1 that were exclusively upregulated in *M. vaccae*-stimulated DCs was the dual specificity phosphatase 8 (DUSP8) (3.0-fold), and also other MAPK phosphatases (MKPs) like DUSP1 (5.2-fold), DUSP2 (7.4-fold), DUSP4 (6.6-fold) and DUSP5 (4.1-fold) were highly induced by *M. vaccae* albeit not exclusively. Those MKPs have been shown to dephosphorylate MAPKs including ERK1/2 and p38 (Camps et al., 2000), and could be responsible for the inactivity of the MAPK pathways in *M. vaccae*-stimulated DCs. Additionally, the discovery that ERK1/2 and p38 are not activated by *M. vaccae* indicates that downstream signalling from these MAPK is not responsible for *M. vaccae*-induced activation of CREB1.

5.4.2.3 *M. vaccae* induces late activation of the NF-κB pathway

The fact that IκB-α degradation in the classical NF-κB pathway was not detectable in *M. vaccae*-stimulated DCs was surprising, since the oPOSSUM analysis clearly indicated NF-κB-mediated gene expression. Therefore the activity of this pathway was analysed differently by quantifying nuclear translocation of RelA (p65) (Noursadeghi et al., 2008). It became evident that activation of NF-κB occurred more slowly in *M. vaccae*-stimulated DCs compared to its rapid activation in response to treatment with LPS or Pam_3CSK4. At the 120 minutes time point RelA had clearly

translocated into the nucleus where it can drive transcription, nevertheless degradation of IκB-α was not detectable at this time, suggesting that the confocal microscopy assay may be more sensitive than assessment of changes to IκB-α by Western blot.

5.4.2.4 LPS and Pam_3CSK4 induce phosphorylation of CREB1

Western blot analysis of CREB1 phosphorylation demonstrated clearly that in addition to *M. vaccae*, LPS and Pam_3CSK4 were activating this signalling pathway too. Nevertheless, analysis of overrepresented TFBSs in lists of upregulated genes by oPOSSUM did not show evidence that CREB1 induces expression of many genes in response to LPS or Pam_3CSK4 stimulation in DCs. That phosphorylation of CREB1 in LPS- and Pam_3CSK4-stimulated cells was only detectable at the early time point might explain why CREB1 did not induce significant expression. Interestingly, a recent paper, analysing phosphorylation and transcriptional activation of CREB1 in response to LPS in RAW264.7 macrophages, demonstrated in line with my data that LPS-induced phosphorylation of CREB1 is non-functional for transcriptional modulation (Avni et al., 2010a). Moreover, the authors showed that isoproterenol-mediated phosphorylation of CREB1 leads to CRE-regulated transcriptional activity, and they conclude that the functionality of CREB1 for transcription depends on the mechanism that is responsible for the phosphorylation of CREB1 at Ser-133. LPS-induced phosphorylation of CREB1, which is mediated by the p38-MSK1 signal transduction pathway (Ananieva et al., 2008; Caivano and Cohen, 2000; Eliopoulos et al., 2002), was not functional, whereas isoproterenol activates CREB1 by PKA-mediated phosphorylation and induced CRE-dependent luciferase activity in reporter assays (Avni et al., 2010a). Given that *M. vaccae*-dependent phosphorylation of CREB1 could be blocked with the PKA-inhibitor H89, this conclusion is consistent with the data presented here. Additionally, the reduced dose of H89 blocked still above 50 % of *M. vaccae*-mediated phosphorylation of CREB1, but had only a little effect on LPS or Pam_3CSK4-dependent

phosphorylation of CREB1. This suggests in line with the literature (Ananieva et al., 2008; Caivano and Cohen, 2000; Eliopoulos et al., 2002) that LPS- or Pam_3CSK4-mediated phosphorylation of CREB1 is occurring via a different signalling pathway, which in certain circumstances might not lead to functional active CREB1 (Avni et al., 2010a). It has been described that phosphorylation of CREB1 at Ser-133 fails to induce gene transcription if the transcription factor is also phosphorylated at Ser-142, which disrupts secondary structure-mediated interactions between CREB1 and CBP (Parker et al., 1998). However, that this occurs in LPS-induced CREB1 phosphorylation seems unlikely, since thus far MSK1/2 has not been described to phosphorylate CREB1 at Ser-142.

5.4.2.5 CREB1 is activated by other mycobacteria

Activation of the CREB pathway by mycobacteria has been described previously in bone marrow-derived macrophages (Roach et al., 2005). The authors demonstrated that CREB1 was activated in macrophages infected with pathogenic *M. avium* and non-pathogenic *M. smegmatis*. *M. smegmatis* provoked a significantly greater accumulation of phosphorylated CREB1 in the nuclei one hour post-infection, which was quantified by confocal microscopy. Moreover it was shown that CREB1 was responsible for high level TNF-α expression in *M. smegmatis* infected macrophages. The investigators found that the upstream signalling mechanism leading to mycobacterium-mediated CREB1 activation was dependent on PKA and p38, but independent of ERK1/2 or CaMKII activity. In contrast, the data presented here show that *M. vaccae*-mediated activation of CREB1 was only dependent on PKA but not on p38. P38 was not phosphorylated by *M. vaccae*, and the p38 inhibitor SB203580 did not affect phosphorylation of CREB1 in *M. vaccae*-stimulated DCs. This is different to the report described above of *M. avium*- and *M. smegmatis*-infected macrophages (Roach et al., 2005), and may result from differences between using heat-killed and live

organisms, differences between macrophages and DCs or differences between mycobacterial species in their ability to activate CREB1.

5.4.3 The role of CREB1 in immune responses

The role of CREB1 in induction of proinflammatory responses in immunity, including the production of TNF-α, remains highly controversial. Although some studies demonstrate that CREB1 in cooperation with ATF-2 and c-Jun positively regulates IFN-γ production by human T cells in response to *M. tuberculosis* (Liu et al., 2010; Samten et al., 2002; Samten et al., 2005; Samten et al., 2008), other studies on CREB1 show that its activity is rather associated with anti-inflammatory responses, such as generation and development of FoxP3 expressing Treg cells (Kim and Leonard, 2007; Ruan et al., 2009) and IL-10 production in monocytes, DCs and macrophages (Alvarez et al., 2009; Ananieva et al., 2008; Avni et al., 2010b; Hu et al., 2006; Kelly et al., 2010; Martin et al., 2005; Pathak et al., 2004), which has CRE-sites at its promoter (Platzer et al., 1999).

For example a study that showed a role for PKA-signalling and resulting CREB1 activity in anti-inflammatory responses was conducted in RAW264.7 macrophages (Avni et al., 2010b). It was found that the activity of synthetic phosphor-ceramide analogue-1 (PCERA-1), an anti-inflammatory drug, which suppresses TNF-α and upregulates IL-10 (Avni et al., 2009; Goldsmith et al., 2009; Matsui et al., 2002a; Matsui et al., 2002b), was accompanied by augmented cAMP production (Goldsmith et al., 2009) and required PKA-activity that induced phosphorylation of CREB1 (Avni et al., 2010b).

Another study on *M. avium*-infected RAW264.7 macrophages showed that transcription of COX-2, an enzyme required in the biosynthesis of PGE2, which exerts an immunosuppressive function in the context of mycobacterial infection (Rangel

Moreno et al., 2002), partially depended on intact CREB1 binding sites, but also on NF-κB-induced transcription (Pathak et al., 2004). Transcription of COX2 was inhibited by combined pharmacological blocking of p38 and ERK1/2 or by H89. The MAPKs p38 and ERK1/2 are known to phosphorylate MSK1 whose target is CREB1 (Arthur and Cohen, 2000; Deak et al., 1998) and whose activity can also be blocked by H89 (Davies et al., 2000). The authors showed that *M. avium* induced phosphorylation of CREB1 in an MSK1-dependent manner, that p-CREB1 bound to the COX-2 promoter, and that COX-2 expression was inhibited in cells transfected with dominant-negative CREB1. The possibility that MSK1 plays a similar role in *M. vaccae*-mediated activation of CREB1 is unlikely. The data presented here show clearly that neither p38 nor ERK1/2 are activated in *M. vaccae*-stimulated DCs and blocking of those kinases did not reduce *M. vaccae*-mediated phosphorylation of CREB1.

5.4.4 CREB1 may inhibit NF-κB mediated transcription

Recently researchers have shown that IL-10 production by monocyte-derived DCs in response to stimulation with the yeast extract zymosan was dependent on CREB1 activity (Alvarez et al., 2009; Alvarez et al., 2010). ChIP assays demonstrated significant binding of p-CREB1 to the *il-10* promoter, which was also associated with CBP and transducer of regulated CREB activity (TORC2), a CREB1 coactivator also known as CREB-regulated transcription coactivator (CRTC) (Alvarez et al., 2009). Interestingly, the presence of the PKA-inhibitor H89 reduced zymosan-induced IL-10 production, and other studies in human macrophages demonstrated that zymosan-mediated IL-10 production was dependent on calcium signalling (Kelly et al., 2010). In contrast, blockage of NF-κB activity was accompanied by an increase of IL-10 production. Hence the authors concluded that IL-10 production by DCs in response to zymosan is regulated by a fine-tuned balance between CREB1 and NF-κB activity (Alvarez et al., 2009).

It is possible that NF-κB and CREB1 compete for restricted amounts of CBP in the nucleus, a coactivator that participates in the activities of different transcription factors (Vo and Goodman, 2001). It had been shown repeatedly that NF-κB p65 activity is enhanced by interaction with CBP or its paralogue p300 (Sheppard et al., 1999; Zhong et al., 1998), and that association of CREB1 with CBP might suppress NF-κB-mediated transcription (Parry and Mackman, 1997). This study on human endothelial cells showed that elevation of intracellular cAMP and activation of the PKA signalling pathway inhibits NF-κB-mediated transcription, which could be rescued by overexpression of CBP. Moreover, overexpression of CREB1 inhibited p65-mediated transcription, indicating that CREB1 and p65 compete for limiting amounts of CBP (Parry and Mackman, 1997).

Another mechanism by which *M. vaccae* might favour CREB1-mediated gene expression in contrast to LPS or Pam_3CSK4 is that it may disrupt binding of the coactivators CBP or p300 to other transcription factors. It has been shown that *M. tuberculosis* (live bacteria, gamma-irradiated bacteria, and cell wall isolates) could disturb essential protein-protein associations of CBP and p300 with Stat1 and consequently suppresses IFN-γ transcriptional responses in human macrophages (Ting et al., 1999).

That CREB1 activity versus NF-κB activity can influence the polarisation of the immune response has been shown in a publication on human monocytes and PBMCs (Martin et al., 2005). Activation of the PI3K-Akt signalling pathway leads to phosphorylation of the constitutively active GSK3-β on Ser-9, which results in its inactivation. Inactivation of GSK3-β by inhibitors or siRNA suppressed the production of proinflammatory cytokines like IL-12p40, IL-6, IL-1ß, TNF-α, and IFN-γ while augmenting IL-10 expression in response to stimulation with ligands for TLR2, TLR4, TLR5 or TLR9. The authors demonstrated that inactivation of GSK3-β led to enhanced

binding of CBP to p-CREB1 while its binding to NF-κB p65 was reduced. Furthermore, by using siRNA targeting CREB1, the authors verified that the activation of CREB1 was responsible for the switch from inflammatory to anti-inflammatory cytokines. The data presented in this thesis show that *M. vaccae* induces CREB1 activation in DCs and DCs primed with *M. vaccae* reduce Th2 responses. Although here the phosphorylation of CREB1 is not influenced by the PI3K inhibitor LY294002, it is possible that the early activation of the CREB pathway and the comparatively late nuclear translocation of NF-κB p65 are important for the nature of DC-dependent T cell polarisation, however this merits further investigation. Or the other way around, although LPS and Pam_3CSK4 induced phosphorylation of CREB1, this did not lead to significant expression of genes with TFBSs for CREB1. It is a possibility that the simultaneous early activation of NF-κB may bind the majority of CBP, and therefore reducing the transcriptional activity of CREB1 in LPS and Pam_3CSK4 stimulated DCs, and influencing the character of DC-dependent T cell polarisation, yet again, this requires further study.

5.4.5 Inhibitor studies indicate that *M. vaccae* induces phosphorylation of CREB1 via PKA

In order to study if *M. vaccae*-mediated activation of CREB1 in DCs played a role in its ability to decrease Th2 responses and to augment the population of $CD25^+/FoxP3^+$ T cells in allogeneic and antigen-specific cocultures, I aimed to block the phosphorylation of CREB1 pharmacologically. Among the different inhibitors tested, only the widely used PKA inhibitor H89 blocked *M. vaccae*-induced phosphorylation of CREB1. Several studies have raised doubts on the specificity of H89 to inhibit PKA (Murray, 2008). Non-specific effects of H89 that could be important in studying signal transduction in the CREB pathway include inhibition of ERK1/2 phosphorylation (Palacios et al., 2007) or inhibition of MSK1 activity (Davies et al.,

2000), both important in LPS-mediated phosphorylation of CREB1 (Avni et al., 2010a; Caivano and Cohen, 2000; Eliopoulos et al., 2002). However the immunoblot analysis showed no evidence for ERK1/2 or p38 activity in *M. vaccae*-stimulated DCs and blocking of EKR1/2 activation with PD98059 and of p38 activity with SB203580 did not reduce CREB1 phosphorylation, suggesting that H89 prevented *M. vaccae*-mediated phosphorylation of CREB1 by blocking PKA activity. The finding, that the adenylate cyclase inhibitor SQ22536 did not prevent phosphorylation of CREB1 was surprising. In the classical activation pathway of PKA, adenylate cyclase is responsible for the accumulation of cAMP, which binds to the regulatory subunits of PKA causing the release of the active catalytic subunits that can translocate into the nucleus and phosphorylate CREB1 at Ser-133. The finding that PKA activity in *M. vaccae*-stimulated DCs was independent of adenylate cyclase, suggests that an alternative pathway may lead to its activation, although this has not previously been suggested.

Another pathway that was described to induce phosphorylation of CREB1 is initiated by Ca^{2+} influx, which can trigger CaMKII/IV (Matthews et al., 1994; Sun et al., 1994). This pathway was not blocked in the research series presented here, but the CaMKII inhibitor KN-93 should be included in future experiments. The CaM/CaMKII pathway has been shown to be involved in HIV-Tat-induced IL-10 production in human monocytes (Gee et al., 2007). However, here CaMKII did not directly activate CREB1, but induced activation of p38, which was responsible for CREB1-mediated *il-10* expression. The absence of p38 activation by *M. vaccae* makes it very unlikely that this pathway was involved in *M. vaccae*-mediated activation of CREB1. Another report showed that CaMK-II phosphorylates CREB1 also at Ser-142 in addition to Ser-133, which leads to dissociation of the CREB1 dimer that can no longer recruit CBP, hence it inhibits the transcriptional activity of CREB1 (Wu and McMurray, 2001), which is also

unlikely to occur in *M. vaccae*-stimulated DCs, because the transcriptional data showed increased CREB1 controlled transcription.

5.4.6 Cytotoxicity of the PKA inhibitor H89

To study the role of CREB1 phosphorylation in the DC-dependent effects of *M. vaccae* on T cell polarisation, DCs were treated with H89 prior to their innate immune stimulation. However, overnight incubation of DCs with this inhibitor at concentrations that were necessary to inhibit CREB1 phosphorylation also caused significant cytotoxicity compromising the experimental paradigm. The final concentration of 50 μM H89 for blocking PKA activity was chosen after studying the literature cited in the manufacture's recommendations (Azarani et al., 1995; Chijiwa et al., 1990; Muroi and Suzuki, 1993). In addition, a number of publications working with DCs or macrophages used a similar concentration to inhibit PKA activity (Alvarez et al., 2009; Avni et al., 2010a; Avni et al., 2010b). However, other researchers working with human monocyte-derived DCs or with porcine granulosa cells also encountered difficulties due to high toxicity of H89 after 24 and 48 hours of treatment (Gillio-Meina et al., 2005; Wilkin et al., 2001). The attempt to block *M. vaccae*-induced phosphorylation of CREB1 with an alternative PKA inhibitor, the synthetic protein kinase inhibitor peptide (PKI)-(6-22)-amide (Glass et al., 1989), failed (data not shown). The findings that the PKI-(6-22)-amide, which in contrast to H89 is thought to be completely specific for PKA (Murray, 2008), did not inhibit *M. vaccae*-mediated phosphorylation of CREB1, and that the adenylate cyclase inhibitor had also no effect, are raising doubts on the role of PKA.

5.4.7 Future experiments to confirm the role of PKA in *M. vaccae*-dependent activation of CREB1

If CREB1 activity does play a critical role in the effects of *M. vaccae* on DCs that are responsible for the reduction of Th2 responses and augmentation of $CD25^{+}/FoxP3^{+}$ T cells in coculture experiments, it would be desirable to study the signal transduction pathway leading to CREB1 phosphorylation in more detail. To ensure that PKA is indeed responsible for *M. vaccae*-mediated activation of CREB1, it would be useful to test other inhibitors, like (9*R*,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, hexyl ester (KT 5720) or more specific inhibitors such as the alternative protein kinase inhibitor PKI-(Myr-14-22)-amide or R_p-adenosine-3',5'-cyclic monophosphorothioate (Rp-cAMPS) (Murray, 2008). As another strategy to validate if PKA is responsible for *M. vaccae*-induced phosphorylation of CREB1, it would be possible to study PKA activity. Antibodies detecting phosphorylation of the catalytic subunits PKA-α and PKA-β are available as well as phospho-(Ser/Thr) PKA substrate antibodies, which detect peptides and proteins containing a phospho-serine/threonine residue with arginine at the -3 position. However, in addition to PKA, PKC also phosphorylates serine/threonine residues with arginine at the -3 position. Moreover, cAMP levels that bind and activate PKA could also be measured in *M. vaccae*-stimulated DCs using immunodetection.

5.4.8 Future experiments to inhibit NF-κB activity in Pam_3CSK4 stimulated dendritic cells

It is also a possibility that specific TLR2 stimulation with Pam_3CSK4 showed different effects than *M. vaccae* due to the differential time course in the activation of NF-κB. It would be interesting to study the effects of pharmacological inhibition of NF-κB in Pam_3CSK4-stimulated DCs with inhibitors such as *N*-tosyl-l-phenylalanine

chloromethyl ketone (TPCK) (Rescigno et al., 1998; Valentinis et al., 2005) or (E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile (Bay 11-7082) (Bansal et al., 2009). If NF-κB- and CREB1-induced gene transcription is restricted my limited amounts of CPB (Parry and Mackman, 1997), this might lead to enhanced CREB1-mediated gene expression and the DCs might have a similar effect on T cell polarisation than *M. vaccae*-primed DCs.

5.4.9 Future experiments to block CREB1 activity in dendritic cells

Since pharmacological blocking of CREB1 activation was cytotoxic, the role of CREB1 in the effects of *M. vaccae* could be studied by reducing its expression level with siRNA, as it was done to study its role in regulating pro- versus anti-inflammatory cytokine expression in human monocytes (Martin et al., 2005). Although it has been shown that mouse bone marrow-derived immature DCs do not mature when transfected with siRNA/liposome-complexes (Hill et al., 2003), culture of human immature monocyte-derived DCs with siRNA/liposomes induces DC maturation, as emphasised by upregulation of CD83 (Sioud, 2005). An alternative method would be to use electroporation to transfect DCs with siRNA. Using this method it was shown that human monocyte-derived DCs and mouse bone marrow-derived DCs maintain their immature phenotype (Jantsch et al., 2008; Prechtel et al., 2006), probably because the RNA is directly delivered into the cytoplasm, hence avoiding endosomal localisation where it might activate TLR receptors (Sioud, 2005). Lentiviral vectors have been used for transduction and expression of genes in DCs and are therefore being developed for gene therapy purpose. Yet, some authors showed that lentiviral transduction of human and mouse immature DCs induces maturation and production of inflammatory cytokines even with empty vector controls (Breckpot et al., 2007; Breckpot et al., 2010; Tan et al., 2005). One strategy might be to transduce $CD14^+$ monocytes prior to their

differentiation into DCs, which then have been shown to maintain their immature phenotype (He et al., 2005; Rossetti et al., 2010).

5.5 Conclusions

- *M. vaccae*-stimulation of DCs selectively activates the CREB pathway at an early time point, which, in contrast to stimulation with LPS and Pam_3CSK4, is maintained active at the later time point.
- LPS and Pam_3CSK4 induce early activation of the main NF-κB pathway, ERK1/2 and p38 MAPK, and of CREB1.
- *M. vaccae*-mediated phosphorylation of CREB1 can be blocked with the PKA inhibitor H89. However, overnight incubation of DCs with this inhibitor also caused significant cytotoxicity compromising the experimental paradigm.

6 SINEs FOR NORMALISATION OF RT-qPCR DATA

6.1 Introduction

Real-time PCR expression profiling is an important tool for identifying genes involved in regulatory networks that affect a specific phenotype. Compared to microarrays, which due to their high cost can generally only be used to quantify the expression of the whole genome of a restricted number of samples, RT-qPCR provides the simultaneous measurement of gene expression in many different samples, but can only be applied to identify the transcription profile from a smaller number of genes. The technique allows a highly sensitive and reproducible measurement over a large dynamic range of starting target quantities, from one single copy to more than 10^7 copies (Nolan et al., 2006; Palmer et al., 2003). However, the extremely high sensitivity can also be its major weakness. RT-qPCR is a multistep process and at every step error can be introduced, altering the result. In order to differentiate between real changes in gene expression and variations introduced by the operator, it is important to normalise the data obtained by RT-qPCR (Huggett et al., 2005; Wong and Medrano, 2005). Several variables need to be controlled for, including the initial sample amount, RNA recovery and integrity, enzymatic efficiencies as well as the overall transcriptional activity which can vary between samples (Andersen et al., 2004).

To control for all errors introduced between sampling and obtaining the result, an internal control gene, also called reference gene, is typically used as it undergoes the same process as the mRNA of interest. The ideal internal control gene should be stably expressed in all cells or tissues under investigation (Andersen et al., 2004). And even more importantly, the expression of the reference gene should not alter with the experimental treatment. So far, a single gene has not been found that fulfils the requirements in every experiment, consequently it is necessary to validate the

expression of candidate reference genes for all new experimental models (Andersen et al., 2004). Despite the fact that there have been many publications showing that commonly used reference genes like GAPDH or β-actin (ACTB) can vary dramatically in their expression (Bustin, 2000; Schmittgen and Zakrajsek, 2000; Suzuki et al., 2000; Thellin et al., 1999), validation of reference genes is still not always performed. However, using non-validated reference genes for normalisation of RT-qPCR data can potentially lead to false conclusions (Dheda et al., 2005; Tricarico et al., 2002).

Currently the most accurate strategy to control for experimental error and identify true changes in transcription is to validate and exploit the expression of multiple internal reference genes (Vandesompele et al., 2002b). Programmes like geNorm, Bestkeeper and NormFinder rank candidate reference genes according to their expression stability and identify the most suitable for the experiment from which a normalisation factor can be calculated (Andersen et al., 2004; Pfaffl et al., 2004; Vandesompele et al., 2002b). This strategy detects, and can compensate for, error-induced trends. This results in a higher resolution and one can measure smaller changes in gene expression than is possible with a single reference gene. However, identification and measurement of multiple reference genes is labour-intensive, costly and sometimes impossible due to insufficient sample.

To overcome those obstacles, this chapter describes a new strategy, which aims to use a single qPCR assay for normalisation, potentially having the resolution of multiple reference genes. The idea is to use repetitive sequences that have integrated into various sites of the genome and therefore also occur frequently in untranslated regions of expressed mRNA (Hasler et al., 2007). Assays were developed for human and mouse, both targeting SINE sequences.

SINEs are a group of non-autonomous retrotransposons with a length of 80-400 base pairs that are commonly found in mammals. The most prominent and only

active SINE family described in humans are ALU-repeats which, with more than 1 million copies, comprise about 10 % of the human genome (Lander et al., 2001). The mouse genome has five frequently described SINE families B1, B2, B4/RSINE, ID, and MIR. Here, B1- and B2-elements are the most abundant, with 564K and 348K copies respectively (Waterston et al., 2002). Although SINEs are ubiquitously dispersed throughout the genome, ALU and B1-elements can be found highly overrepresented in GC-rich, gene-rich, regions of the DNA (Lander et al., 2001). Therefore they can get transcribed when they are present within regions of mRNAs (Hasler et al., 2007).

The hypothesis is that the overall expression level of SINEs, both ALU- and B-elements, is very stable. They occur so frequently, that expression changes in some of the mRNAs carrying SINEs should not alter the total number of expressed ALU- or B-elements present at a point in a cell. With primers targeting the consensus sequences of ALU-, B1- and B2-repeats, it is possible to reverse transcribe the expressed repeats into cDNA and measure them like a reference gene in RT-qPCR experiments. They represent multiple genes and can be measured in a single assay using only one set of primers. To validate if expressed SINEs could serve as an alternative reference, mouse samples on which various formulations of *M. vaccae* had been tested were kindly provided by the School of Pharmacy, University of London. For comparison, RT-qPCR data of various genes of interest were normalised by using the geNorm algorithm that allows a correct interpretation of the results by calculating the geometric mean of multiple carefully selected stable reference genes, which then functions as the normalisation factor (Vandesompele et al., 2002b). Moreover, the same RT-qPCR data of genes of interest were normalised against mRNA levels of the commonly used single reference gene β-actin (ACTB), which can, similar to GAPDH, dramatically vary in its expression (Bustin, 2000; Schmittgen and Zakrajsek, 2000; Suzuki et al., 2000; Thellin

et al., 1999). Finally the data were normalised against B1- and B2-element expression, and subsequently compared to the other two normalisation techniques.

6.2 Objectives

- Design and optimisation of RT-qPCR assays that target expressed SINEs: ALU-repeats for human and B-elements for mice samples.
- Validate the use of expressed B-elements as a tool for normalisation, by comparing target gene expression data normalised with 1) expressed B-elements, 2) the normalisation factor calculated from multiple validated reference genes, and 3) the commonly used reference gene β-actin.

6.3 Results

6.3.1 Optimisation of SINE qPCR reactions

Human ALU-families and mouse B-elements have consensus sequences, but they are not identical and many variable sequences are present in the genomes (Umylny et al., 2007). In order to detect amplification of as many expressed SINEs as possible the assays had to be SYBR green based, thus independent of sequence specific TaqMan probes (Holland et al., 1991). B-element primers were designed on the consensus repeat sequences from RepBase (http://www.girinst.org/repbase/) to target the most conserved regions in order to enable amplification of as many expressed SINEs as possible within one assay. Two different primer sets for B1-elements (B1F1/B1R1 and B1F2/B1R2) and for B2-elements (B2F1/B2R1 and B2F2/B2R2) were used to amplify cDNA generated from isolated mouse spleen RNA. The RT-qPCR products were cloned into pCR4-TOPO vectors and sequenced. The sequencing data confirmed that the selected

primer pairs amplified a variety of different expressed B-elements with slightly diverse sequences (Table 6-1, Table 6-2, Table 6-3 and Table 6-4). For each set of primers, ten plasmids containing different sequences were mixed together and used as a standard for assay optimisation and efficiency control (plasmids labelled with *).

Table 6-1: CLUSTAL 2.0.12 multiple sequence alignment of B1-elements amplified with the primer pair B1F1/B1R1. (Primer sequences removed; *plasmids selected for standard)

```
 NW_P1_B1_H03.2_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_H05.1_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_G06.2_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_G04.1_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_F06.2_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_F06.1_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_F01.1_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_D06.2_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_C02.1_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_B06.1_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_A04.2_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_A02_UP         ----CCAGCACTC-AGGAGGCAGAGGCAGGCGGG----- 29
 NW_P1_B1_A04.1_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGTGG------ 28
 NW_P1_B1_B04.1_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGTGG------ 28
 NW_P1_B1_C04.1_UP       ----CCAGCACTC-AGGAGGCAGAGGCTGGCGGG----- 29
 NW_P1_B1_H06_UP         ----CCAGCACTC--GGAGGCAGAGGCTGGTGG------ 27
 NW_P1_B1_C03.2_UP       ----CCAGCACTC-AGGAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_B06.2_UP       ----CCAGCACTC-NGGAGGCAGAGGCAGGCGG------ 28
*NW_P1_B1_C05_UP         ----CCAGCACTC-AGGAGGCAGAGGCAGAGGCAGGTGC 34
*NW_P1_B1_G02_UP         ----CCAGTACTC-ATGAAGCAGAGGCAGACGA------ 28
 NW_P1_B1_G03.1_UP       ----CCAGCACTC-GGGAGGCAGAGGCAGACGG------ 28
 NW_P1_B1_E06.2_UP       CCAGCACTC---C-GGGAGGCAGAGGCAGGCGG------ 29
 NW_P1_B1_G04.2_UP       CCATCACTCACTC-GGGAGGCAGAGGCAGACAG------ 32
*NW_P1_B1_D01_UP         ----------CC-AGGAGGCAGAGACAGGCGG------ 21
 NW_P1_B1_E06.1_UP       ----CCAGCACTC-GGGAGGCAGAGACAGGCGG------ 28
*NW_P1_B1_F02_UP         ----CCAGCACTC-GGGAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_F01.2_UP       ----CCAGCACTC-GGGAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_C04.2_UP       ----CCAGCACTC-GGGAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_E03_UP         ----CCAGCACTC-GGGAGGCAT-GGCAGGCGG------ 27
 NW_P1_B1_C02.2_UP       ----CCAGCACTC-GGGAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_B04.2_UP       ----CCAGCACTC-GGGAGGCAGAGGTAGGCGG------ 28
 NW_P1_B1_B05.2_UP       ----CCAGCACTG-GGCAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_B03_UP         ----CCAGCACTT-GGGAGGCAGAGGCAGGTGG------ 28
*NW_P1_B1_G05_UP         ----CCAGCACTT-GGGAGGCAGAG-CTGGTGG------ 27
 NW_P1_B1_D05_UP         ----CCAGCACTT-GGGAGGCAGAGGCAGGTGG------ 28
 NW_P1_B1_E04_UP         ----CCAGCACTT-GGGAGGCAGAGGCAGGTGG------ 28
 NW_P1_B1_G03.2_UP       ----CCAGCACTT-GGGAGGCAGAGGCAGGTGG------ 28
 NW_P1_B1_H05.2_UP       ----CCAGCACTT-GGGAGGCAGAGGCAGGTGG------ 28
 NW_P1_B1_A03_UP         ----CCAGCACTT-GAGAGACAGAGGCAGTTGG------ 28
*NW_P1_B1_F04_UP         ----CCAGCACT--GGGTGGCAGAGGCAGGTGG------ 27
 NW_P1_B1_D02_UP         ----CCAGCACTT-GGGAGGCAG-GGCAGGAGG------ 27
*NW_P1_B1_E02_UP         ----CCAGCACTT-GGGAGGCAG-GGCAGGAGG------ 27
 NW_P1_B1_G01_UP         ----CCAGCACTT-GGGAGGCAGAGGCAGGGGG------ 28
 NW_P1_B1_A05.2_UP       ----CCAGCACTT-GGGAGGCAGAGGCAGGCAG------ 28
 NW_P1_B1_G06.1_UP       ----CCAGCACTT-GG-AGGCAGAGGCAGGCAG------ 27
 NW_P1_B1_A05.1_UP       ----CCAGCACTT-GGGAAGCAGAGGCAGGCAA------ 28
*NW_P1_B1_B01_UP         ----CTAGCACAT-GGGAGGCAGAAGGAGGCG------- 27
 NW_P1_B1_F05_UP         ----CTAGCACAT-GGGAGGCAGAAGGAGGCG------- 27
*NW_P1_B1_A06_UP         ----CTAGCACTT-GGGAGGCAGAGCCAGGCAG------ 28
 NW_P1_B1_D06.1_UP       ----CCAGCACTT-GGGAGGCAGAGGCAGGCGG------ 28
*NW_P1_B1_F03_UP         ----CCAGCACTT-GGGAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_F06.3_UP       ----CCAGCACTT-GGGAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_H03.1_UP       ----CCAGCACTT-GGGAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_C03.1_UP       ----CCAGTACTT-GGGAGGCAGAGGCAGGCGG------ 28
 NW_P1_B1_B05.1_UP       ----CCAGCANNTCGGGAGGCAGAGGCAGGCGG------ 29
```

Table 6-2: CLUSTAL 2.0.12 multiple sequence alignment of B1-elements amplified with the primer pair B1F2/B1R2. (Primer sequences removed; *plasmids selected for standard)

```
 NW_P1_B1_A09_UP        --CCCAGCACTCG-GGAGGCAGAGGCAGAGGCAGAGGCAGTCGGA 42
*NW_P1_B1_E09_UP        --CCCAGCACTCG-GGAGGCCGAGGCAGAGGCAGAGGCAGGCGAA 42
*NW_P1_B1_A07_UP        --CCCAGCACTCA-GGAGGCGGAGGCAGAGGTAGAGGCAGGCAGA 42
 NW_P1_B1_B09.2_UP      --CCCAGCACTCG-GGAGGCAGAGGCAGGGGGA------------ 30
 NW_P1_B1_B08.2_UP      --CCCAGCACTTG-GGAGGCAGAGGCAG--GCAGA---------- 30
 NW_P1_B1_B12.1_UP      -TCCCAGCACTTG-GGTGGCAAAGGCAG--GCAGA---------- 31
 NW_P1_B1_F11.2_UP      --TCCAGCACTTG-GGAAGCAGAAGAAG--GCAGA---------- 30
 NW_P1_B1_E10.1_UP      --CCCAGCACTCG-GGAGGCAGAGGCAG--GCAGA---------- 30
 NW_P1_B1_B08.1_UP      --CCCAGCACTCG-G-AGGCAGAGGCAG--GCAGA---------- 29
 NW_P1_B1_C10.1_UP      --CCCAGCACTCG-GGAGGCAGAGGCAG--GCGGA---------- 30
 NW_P1_B1_F11.1_UP      --CCCAGCACTCG-GGAGGCAGAGGCAG--GCGGA---------- 30
 NW_P1_B1_H07.2_UP      --CCCAGCACTCG-GGAGGCAGAGGCAG--GCGGA---------- 30
 NW_P1_B1_B09.1_UP      --CCCAGCACTCGAGGAGGCAGAGGCAG--GCGGA---------- 31
 NW_P1_B1_H10.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_H12.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_H10.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_H08.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_H07.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_G11.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_G09.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_G09.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_G08.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_G08.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_G07.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_F12.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_F12.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_F08.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_E12.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_E10.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_E08.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_D11.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_D11.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_D10.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_D10.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_D07.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_D07.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
*NW_P1_B1_C12_UP        --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
*NW_P1_B1_C11_UP        --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_C10.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_C07.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_B11.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_B07.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_B07.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_A12.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_A12.1_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGG---------- 30
 NW_P1_B1_C07.1_UP      --CCCAACACTTA-GGAGGCAGAGGCAG--GCGGGT--------- 31
 NW_P1_B1_F10.1_UP      --CCCAGCACTCA-AGAGGCAGAGGCAG--GCGGN---------- 30
 NW_P1_B1_F10.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCGGA---------- 30
 NW_P1_B1_E08.2_UP      --CCCAGCACTCA-GGAGGCAGAGGCAG--GCTGA---------- 30
*NW_P1_B1_A08_UP        --CCCAGCACTCA-GGAGGCAGAGGCAG--GTGGA---------- 30
 NW_P1_B1_E12.1_UP      --CCTAGCACTAA-GGAGGCAGAGGCAA--GCGGA---------- 30
*NW_P1_B1_E11_UP        --CCCAGCACTTA-GGAGGCAGAAGCAG--GTGGA---------- 30
 NW_P1_B1_B11.1_UP      -CCCCAGCACTTGGG-AGGCAGAGACAG--GCGGA---------- 31
*NW_P1_B1_D09_UP        --CCCAGCACTTGGGGAGGCAGAGACAG--GCGAA---------- 31
 NW_P1_B1_B12.2_UP      --CACAGCAATTGGG-AGCTAGAGGCAG--ACGGA---------- 30
*NW_P1_B1_G10_UP        --CACAGCAATTGGG-AGCTAGAGGCAG--GCGGA---------- 30
 NW_P1_B1_G11.2_UP      --CCCAGCACTTGGG-AGGCAGAGGCAG--GCGGA---------- 30
*NW_P1_B1_G12_UP        --CCCAGCACTTGGG-AGGCAGAGGCAG--GCGGA---------- 30
*NW_P1_B1_H09_UP        --CCCAGCACTTGGA-AGGCAGAGGCAG--GCGGA---------- 30
 NW_P1_B1_H08.2_UP      --CCCAGCACTTGGG-AGGCAGAGGCAG--GTGGA---------- 30
 NW_P1_B1_H12.2_UP      --CCCAGCTCTTGGG-AAGCAGAGTCCA--GTGGA---------- 30
 NW_P1_B1_F08.2_UP      ATCCCAGCACTTGGG-AGGCAGAGGCAG--GAGGA---------- 32
 NW_P1_B1_G07.2_UP      --CCTAGCACTTGGG-AGGCAGAGGCAG--GCGGA---------- 30
```

Table 6-3: CLUSTAL 2.0.12 multiple sequence alignment of B2-elements amplified with the primer pair B2F1/B2R1. (Primer sequences removed; *plasmids selected for standard)

```
 NW_P2_B2_C06_UP         GTGGCTCACAACCA----TCCGTAACGAGAC 27
 NW_P2_B2_F06_UP         GTGGCTCAC---CA----TCTGTAACGAGA- 23
 NW_P2_B2_G03_UP         GTGGCTCACATCCA----TCTGTAATGATA- 26
 NW_P2_B2_H02_UP         GTGGCTCACAGCCA----TCTGTAATGAGA- 26
*NW_P2_B2_B01_UP         GTGGCTCACAACCA----TCTGCAATGAGA- 26
 NW_P2_B2_G05_UP         GTGGCTCACAACCA----TCTGCAATGGGA- 26
*NW_P2_B2_B06_UP         GTGACTCACAATCA----TCTGTAATGTGA- 26
 NW_P2_B2_D02_UP         GTGACTCACAACCA----TCTGTAATGAGA- 26
*NW_P2_B2_C02_UP         GTGACTCACAACCA----TCTGTAATGGGA- 26
 NW_P2_B2_H03.2_UP       GTGACTCACAACCA----TCTGTAATGGGA- 26
 NW_P2_B2_C05_UP         GTGTCTTATAACCA----TCTGTAATGGGG- 26
 NW_P2_B2_D01_UP         GTGACTCATAACCA----TCTGTAATGGGA- 26
*NW_P2_B2_A02_UP         GTGGCTCAAAACCA----TCTGTAATGGGA- 26
*NW_P2_B2_B02_UP         GTGGCTCACAAC----TGTCTTTAATGGGA- 26
 NW_P2_B2_D03_UP         GTGGCTCACAACCATCTATCTGTAATGGGA- 30
*NW_P2_B2_B04_UP         GTGGCTCACAAC----CCTCTGTAATGGGA- 26
 NW_P2_B2_E04_UP         GTGGCTCACAAC----CCTCTGTAATGGGA- 26
*NW_P2_B2_A01_UP         GTGGCTCACAAC----CATCTGTAATGGGA- 26
 NW_P2_B2_E01_UP         GTGGCTCACAAC----CATCTGTAATGGGA- 26
 NW_P2_B2_H06_UP         GTGGCTCACAAC----CATCTGTAATGGGA- 26
*NW_P2_B2_A06_UP         GTGGCTCACAACCA----TCTGTATTAGAA- 26
 NW_P2_B2_F03_UP         GTGGCTCACAACCA----TCTGTATTGGAA- 26
 NW_P2_B2_E02_UP         GTGGCTCACAACCA----TCTGTAGTGGAGA 27
 NW_P2_B2_G01_UP         GTGGCTCACAACCA----TCTGTAATGGGG- 26
 NW_P2_B2_E05_UP         GTAGCTCACAACCA----TCTGTAAATGGAA 27
 NW_P2_B2_F05_UP         GTAGCTCACAACCAACCATCTGTAATGGGA- 30
 NW_P2_B2_C04_UP         GTGGCTCACAACCA----CCTGTAATGGAA- 26
*NW_P2_B2_B03_UP         GTGGCTCACAACCA----CCTGTAATGGGA- 26
 NW_P2_B2_E06_UP         GTGGCTCACAACCA----TCTGTAATAGGA- 26
 NW_P2_B2_D06_UP         GTGGCTCACAACCA----TCTGTAATGGGA- 26
 NW_P2_B2_F04_UP         GTGGCTCACAACCA----TCTGTAATGGGA- 26
 NW_P2_B2_G04_UP         GTGGCTCACAACCA----TCTGTAATGGGA- 26
 NW_P2_B2_C03_UP         GTGGCTCACAATCA----TCTGTAATGGGA- 26
 NW_P2_B2_E03_UP         GTGGCTCACAATCA----TCTGTAATGGGA- 26
 NW_P2_B2_D04_UP         GTGCCTCACAGCCA----TCTGTAATGGGA- 26
 NW_P2_B2_D05_UP         GTGGCTCACACCCA----TCTGTAATGGGA- 26
 NW_P2_B2_G02_UP         GTGGCTCACAACCA----TCTGTANTGAGAN 27
*NW_P2_B2_B05_UP         GTGGCTTACAACCA----TCTGTAATGAGA- 26
 NW_P2_B2_H03.1_UP       GTGGCTCATAACCA----TCAATAATGAGT- 26
 NW_P2_B2_H01_UP         GTAGTTCACAACCA----CCCGTAATGAGA- 26
 NW_P2_B2_H05_UP         GTGGCTTATAACCA----CTCGTAATGAGA- 26
 NW_P2_B2_F01_UP         GTGGCTCACAACCA----CCCGTAATGAGA- 26
```

Table 6-4: CLUSTAL 2.0.12 multiple sequence alignment of B2-elements amplified with the primer pair B2F2/B2R2. (Primer sequences removed; *plasmids selected for standard)

```
 NW_P2_B2_E09.2_UP       ATGGTTCATAATGGG--ATC----------------- 18
 NW_P2_B2_F11.2_UP       ATGGTTCATAATGGG--ATC----------------- 18
*NW_P2_B2_A09_UP         GTAGCC-ACAACC----ATCTG---TAATGAGATC--- 27
 NW_P2_B2_D11_UP         GTAGCCCATAACC----ATCTG---GAATGGGATC--- 28
 NW_P2_B2_E10_UP         ATAGCTCATAACC----ATCTG---TAATGTGATC--- 28
 NW_P2_B2_H10_UP         GTAGTTCACAACNC---ATCCG---TAATGAGATC--- 29
 NW_P2_B2_E12_UP         GTGGCTCACAACC----ATCTG---TA-TGGGATC--- 27
 NW_P2_B2_F07_UP         GTGGCTCACAACC----ATCTG---TAATGGGATC--- 28
 NW_P2_B2_A08.2_UP       GTGGCTCGCAACC----ATCTG---TAATGGGATC--- 28
 NW_P2_B2_A07_UP         GTGGCTCACAACC----ATCTG---CAATGGGATC--- 28
*NW_P2_B2_B09_UP         GTGGCTTACAGTC----ATCTG---TAATATGATC--- 28
 NW_P2_B2_F11.1_UP       GTGGCTCACAGGC----ATCTG---TAATGGGATC--- 28
 NW_P2_B2_A08.1_UP       GTGGCTCACAGCC----ATCTG---TAATGGGATC--- 28
 NW_P2_B2_E09.1_UP       GCGGCTTACAACC----CTCTG---TAATGGGATC--- 28
 NW_P2_B2_F12_UP         GTGGCTCACAACC----ATCTG---TAATAGGATC--- 28
*NW_P2_B2_A10_UP         GTGGCTCACAACC----ACCCA---TAACGAGATC--- 28
 NW_P2_B2_F10_UP         GTGGCTCACAACC----ACCCA---TAACGAGATC--- 28
 NW_P2_B2_C10_UP         GTGGCTCACAACC----ACCCA---TAATGAGATC--- 28
 NW_P2_B2_D12_UP         GTGGCTCACAACC----ACCCA---TAATGAGATC--- 28
*NW_P2_B2_B12_UP         GTGGCTCACAAGC----ACCCA---TAATGAGATC--- 28
 NW_P2_B2_H11_UP         GTGGCTCACAACC----ACCCG---TAATGAGATC--- 28
*NW_P2_B2_A11_UP         GTGGCTCAAC-TA----T-CCG---TAATGAGATC--- 26
 NW_P2_B2_D08_UP         GTGGCTCAACGTA----TTCCG---TAATGAGATC--- 28
 NW_P2_B2_C11_UP         GTGGCTCACAACC----ATCCA---TAATGGGATC--- 28
 NW_P2_B2_H08_UP         GTGGCTCTCAACC----ATCCA---TAATGAGATC--- 28
 NW_P2_B2_G08_UP         GTGGCTCACAATT----ATCTA---TGATGGGTTC--- 28
 NW_P2_B2_G09_UP         GTGACTCACAACC----ATCTC---TAATGGGATC--- 28
 NW_P2_B2_F09_UP         GTGGCTCACAACC----ATCAG---TAATGGGATC--- 28
*NW_P2_B2_B10_UP         GTGGCTCACAACC----ATCCG---TAATGAAATC--- 28
 NW_P2_B2_E11_UP         GTGGCTCACAACC----ATCCA---TAATGAAATC--- 28
*NW_P2_B2_A12_UP         GTGGTTCACAACC----ATCTAG--TAATGAAATC--- 29
*NW_P2_B2_B11_UP         ATGGCTCGCAACC----ATCCG---TAACAAAAAAATC 31
 NW_P2_B2_H12_UP         GTGGCTCACAACC----ATCCG---CAACAGAGATC-- 29
 NW_P2_B2_G07_UP         GTGGCTCACAACC----ATCCG---TAACGAAATC--- 28
*NW_P2_B2_B08_UP         GTGGCTCACAACC----ACCCG---TAACGAAATC--- 28
 NW_P2_B2_D10_UP         GTGGCTCACAACC----ATCTG---TAACGAAATC--- 28
 NW_P2_B2_E08_UP         GTGGCTCACAACC----ATCTG---TAACGAAATC--- 28
 NW_P2_B2_H07_UP         GTGGCTCACAACC----ATCTC---TAATGAAATC--- 28
 NW_P2_B2_E07_UP         GTGGCTCACAACC----ATCTG---TAATGGAATC--- 28
 NW_P2_B2_C08_UP         GCGGCTCACGACC----ATCTGT--AATGTGATC---- 28
 NW_P2_B2_D07_UP         GCAGCTCAC-ACC----ATC----------------- 15
 NW_P2_B2_F08_UP         GTGGCTCACAACC----ATCTG---TAATGAGATC--- 28
 NW_P2_B2_G12_UP         GTGGCTCACAACC----ATCTG---TAATGAGATC--- 28
*NW_P2_B2_B07_UP         GTGACTCACAACC----ATCTG---TAATGAGATC--- 28
 NW_P2_B2_C09_UP         GTGGCTCACAACC----ATCTG---TAACGGATC---- 27
 NW_P2_B2_D09_UP         GTGAATCACAACC----ATCTA---TAAAGAGATC--- 28
 NW_P2_B2_C12_UP         GTGACTCTCAAAC----ATTTA---TAATAGGATC--- 28
 NW_P2_B2_G10_UP         GTTCTTCACAACTACTTTTCCAGATCCATGTGATC--- 35
 NW_P2_B2_G11_UP         GTTCTTCACAACTACTTTTCCAGATCCATGTGATC--- 35
```

The plasmids were used for optimisation of the qPCR reactions, whereby various annealing temperatures (58°C, 60°C and 62°C), primer concentrations (200 nM, 300 nM, 400 nM, 500 nM, 600 nM and 700 nM), and magnesium concentrations (2.5 mM, 3.0 mM, 3.5 mM and 4.0 mM) were tested to define optimal assay conditions by comparison of CT values. Standard curves with 10-fold dilution series of the plasmid controls showed that the optimised assays had an efficiency of ≥ 96 % over a range of six orders of magnitude (Figure 6-1A-D). In addition, a qPCR assay targeting human ALU-repeats of the ALU-J family was optimised with plasmid standards, which had an efficiency of 99 % (Figure 6-1E).

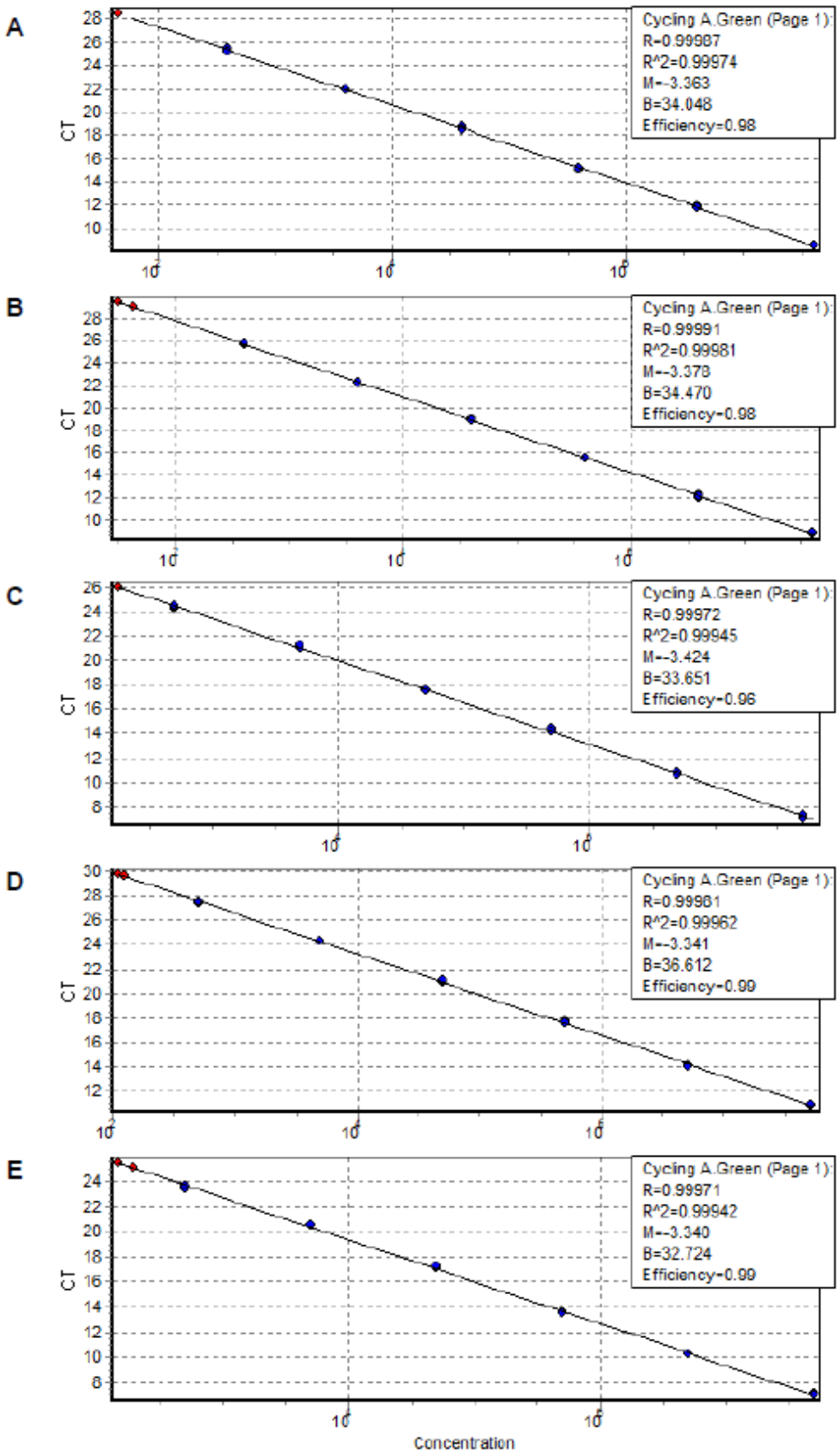


Figure 6-1: Optimised B-element and ALU-J qPCR assays

Optimised qPCR assays preformed with 10-fold dilution series of the standard plasmids. All concentrations were measured in duplicates or triplicates. The assays B1F1/B1R1 (**A**) and B1F2/B1R2 (**B**) had an efficiency of 98%, the B2F1/B2R1 assay (**C**) an efficiency of 96%, and the B2F2/B2R2 assay (**D**) and the ALU-J assay (**E**) an efficiency of 99%. Note: the no template controls (NTC) were positive in all assays (red dots).

6.3.2 Positive no template controls: a problem of contamination?

During the optimisation of qPCR assays for ALU-repeats and B-elements, it was noticed that the no template controls (NTC) were always coming up as positives (Figure 6-1). Even if all reagents were newly purchased and the equipment was UV-treated in a specialised PCR hood in which the reactions were set up, gloves were changed frequently and the experimenter was wearing clean lab coat and face mask, none of these precautions reduced the amplification in the NTCs.

DNase/RNase-free distilled water was distributed in 1.5 ml tubes and exposed to the air for 5 minutes to 24 hours of three different locations: an UV-sterilized/hepa-filtered PCR hood, a bench in the pre-PCR laboratory and on a desk at the entrance to an open-plan office shared by 14 people. ALU-J and B1-element reactions performed with this water produced a product in all time-points, including time 0 (Figure 6-2). When all ALU-J data were compared, there was a mean copy number of 71 copies/reaction, but the data were not distributed normally (Figure 6-2Aa). There was no associated increase in the amount of ALU-J DNA detected with time, although there were two increased results: one at 6 hours from the laboratory bench and one at 24 hours from the open-plan office (Figure 6-2B), which were outside of the 3.09 standard deviation (SD) from the mean (99.8 % contained in interval). If these two outliers are omitted, the distribution becomes normal with a mean of 61 copies/reaction and a coefficient of variation (CV) of 18.79 % (Figure 6-2Ab). When the same samples were analysed using the mouse B1-element primers, all time points yielded a result that had a normal distribution with a mean and CV of 95 copies/reaction and 12.29 %, respectively (Figure 6-2Ac). Furthermore, as with the ALU-J reactions, there was no increase in mouse DNA contamination with time (Figure 6-2C).

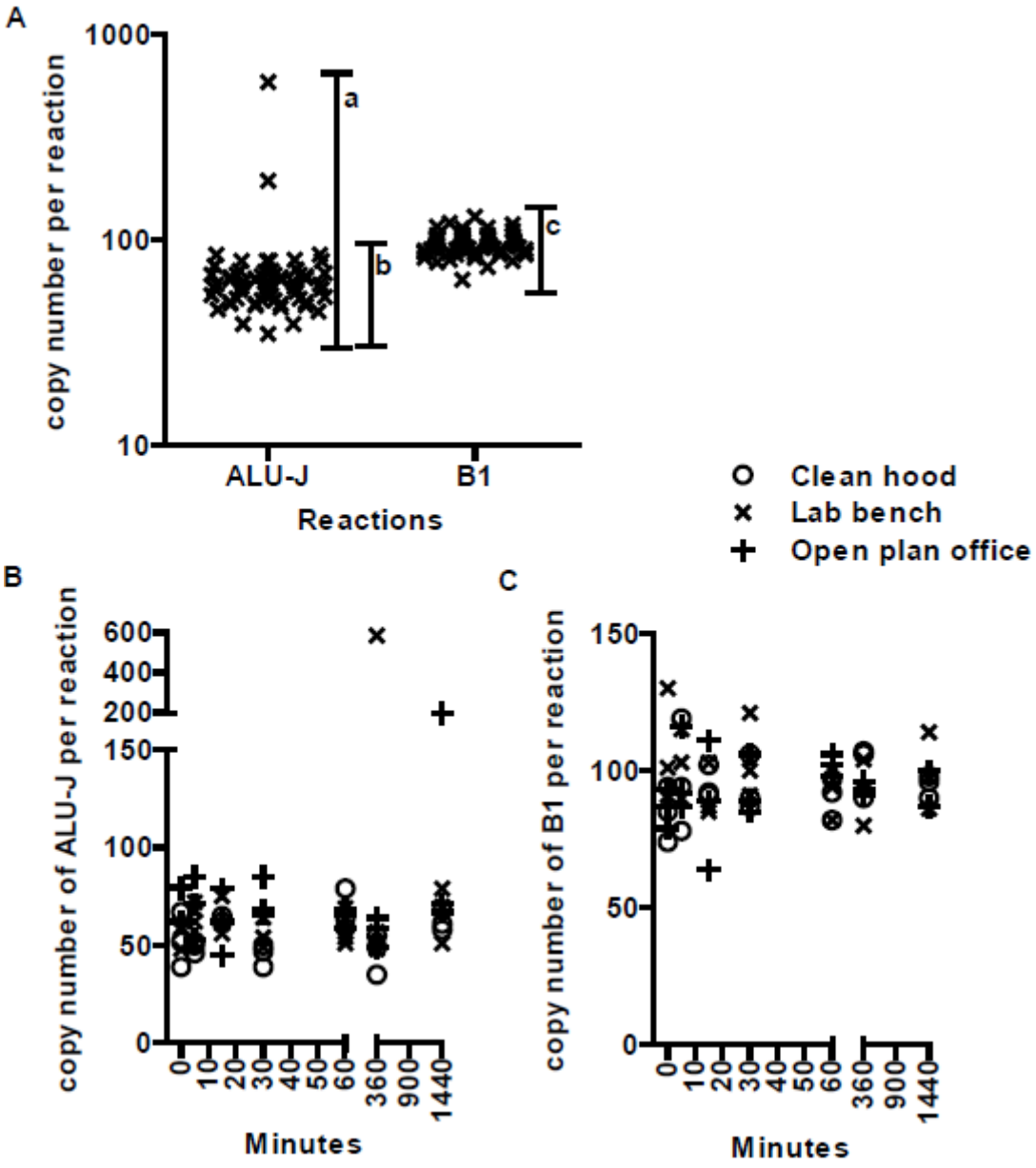


Figure 6-2: Copy number of ALU-J and B1-elements in no template controls exposed to different environments for various periods of time

A: All ALU-J and B1-element experimental data plotted from all exposure experiments. Total spread of ALU-J results (a); spread of ALU-J results minus outliers (b); spread of B1-element results (c). The effect of exposure to air from clean hood (o), laboratory (x), and open-plan office (+) on the detection of (**B**) ALU-J and (**C**) B1-element DNA sequences.

Sequence analysis confirmed that the molecules being amplified by the respective primer sets were ALU-J or B1-element sequences (Figure 6-3). The sequences were slightly different than the plasmids used for the standard curves, thus cross-contamination can be excluded.

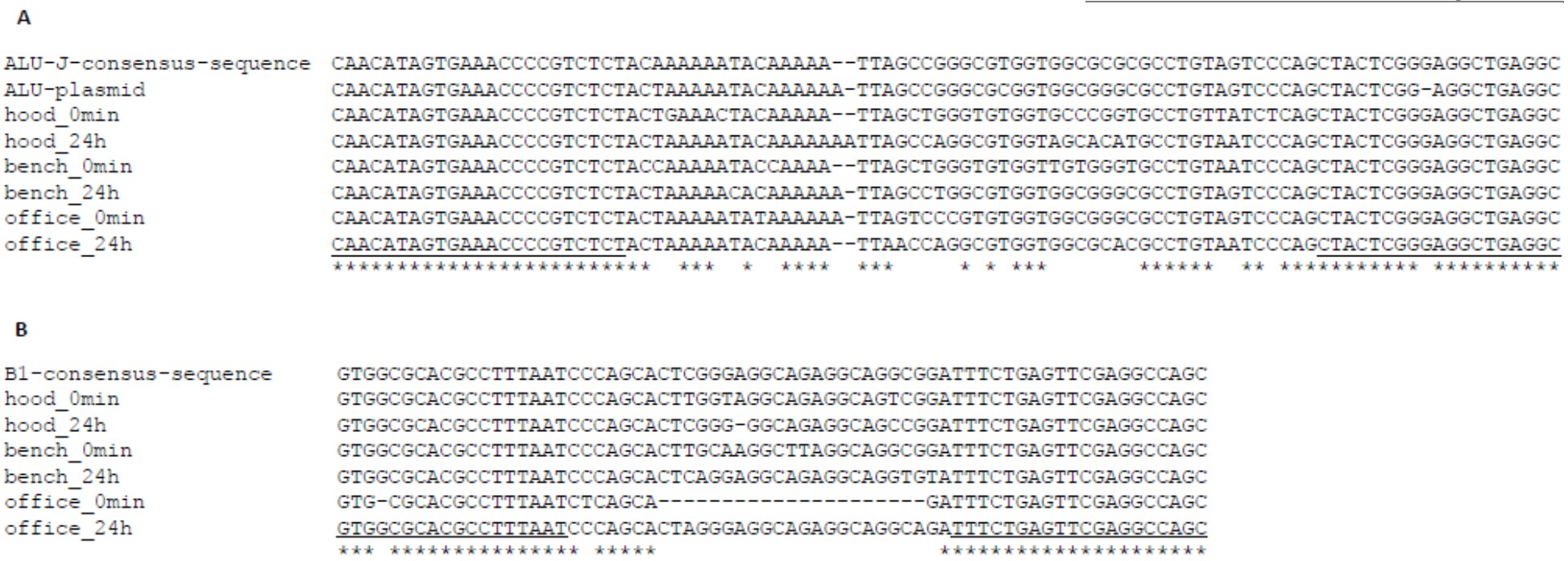


Figure 6-3: No template controls amplify ALU-J and B1-element sequences

Sequences derived from PCR products from 0 minutes and 24 hours exposure reactions with ALU-J (**A**) and B1F2/B1R2 (**B**) primers. Locations of the primers are underlined.

The very constant copy numbers of ALU-J and B1-element DNA measured suggested that the contamination of the assays was already present in the master mix and not introduced during PCR set up. Since the contamination was very little and stable, it should not affect the application of expressed SINEs as a tool for RT-qPCR data normalisation.

6.3.3 Optimisation of sample preparation to obtain good quality RNA

Spleens and Peyer's patches of 43 mice were dissected and stored at -80 °C in RNAlater to prevent RNA degradation. Despite the use of a RNA isolation kit (see *Material and Methods*), the samples were highly contaminated with genomic DNA (Figure 6-4A+B, lane 2). Primers for reference genes and genes of interest can often be designed in a way that the assay spans a large intron such that remaining genomic DNA is not coamplified with cDNA. However this strategy is not recommended as probably about 20 % of the human genes are either single exon genes or have one or more processed pseudogenes (retropseudogene or intronless copy) in the genome (Dunham et al., 1999; Hattori et al., 2000). Therefore it is suggested to treat the RNA sample with RNase-free DNase (Vandesompele et al., 2002a). For the utilisation of expressed SINEs as references for RT-qPCR data normalisation, the removal of contaminating genomic DNA is essential. As there are for example > 1,000,000 copies of ALU-elements present within the human genome (Smit, 1996) and nearly 500,000 copies of B1-elements in the mouse genome (Umylny et al., 2007), even minor contamination could make RT-qPCR data normalisation unfeasible. DNase treatment was first performed according to a protocol used by Professor Vandesompele and colleagues (http://medgen.ugent.be/CMGG/protocols/). Although the residual DNA was no longer visible by electrophoresis using agarose gels (Figure 6-4A, lanes 3+4), this method was not suitable for the removal of genomic DNA contamination, because the RNA was also

degraded (Figure 6-4A, lanes 3+4). This was not a direct result from the DNase treatment, but rather from the subsequent purification method with microcon-100 columns, since the RNA was also degraded in no-DNase controls (Figure 6-4A, lanes 5+6).

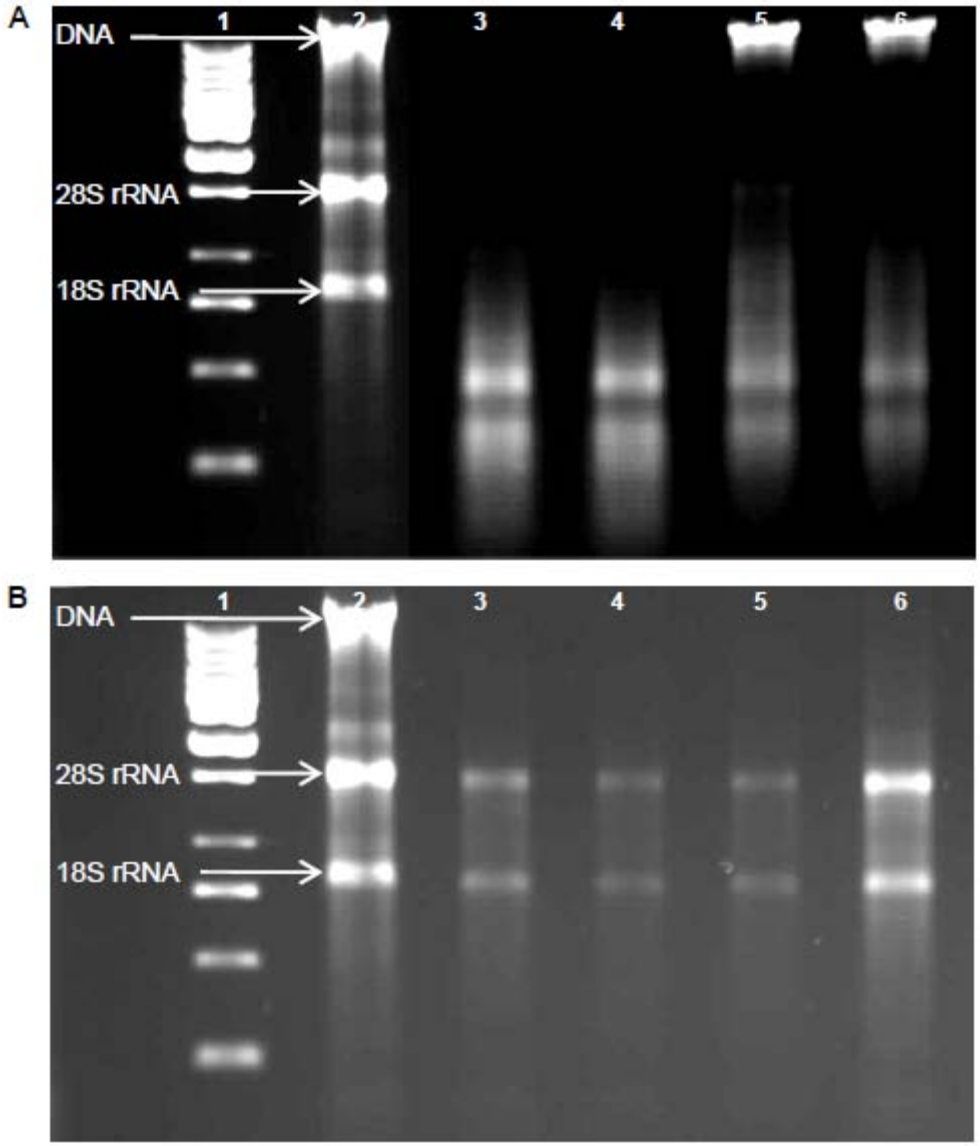


Figure 6-4: DNase treatment of RNA samples

RNA isolated from mice spleens using the RNAeasy kit was DNase treated using two different methods. **A**: Microcon-100 tubes were used for purification. 1: 1kb ladder; 2: RNA sample prior to DNase treatment; 3+4: DNase treatment and microcolumn clean-up; 5+6: no-DNase treatment and microcolumn clean-up. **B**: Guanidinium thiocyanate phenol chloroform was used for purification. 1: 1kb ladder; 2: RNA sample prior to DNase treatment; 3+4: DNase treatment and guanidinium thiocyanate phenol chloroform clean-up; 5+6: no-DNase treatment and guanidinium thiocyanate phenol chloroform clean-up.

Therefore a different method using guanidinium thiocyanate-phenol-chloroform purification was applied for RNA purification subsequent to DNase treatment (Figure 6-4B; for details see *Material and Methods*). Here the residual genomic DNA was no longer visible by agarose gel electrophoresis and the rRNA remained intact (Figure 6-4B, lanes 3+4). Already the purification method alone removed large parts of genomic DNA, which was invisible in no-DNase controls (Figure 6-4B, lanes 5+6).

Furthermore the RNA integrity was measured by using a Bioanalyzer 2100, which confirmed that the RNA was not degraded with RNA integrity numbers (RIN) between 8.1 and 8.4 for all four samples measured. RT-qPCR performance is affected by the RNA integrity, and a RIN higher than five is recommended as good total RNA quality and higher than eight as perfect total RNA for downstream applications (Fleige and Pfaffl, 2006). Therefore, this method of DNase treatment and RNA purification was applied for all 43 mice Peyer's patches and spleen RNA samples that were treated with RNase-free DNase. Subsequent RNA integrity and purity measurements with a Bioanalyzer 2100 and Nanodrop respectively confirmed good quality RNA for all samples (Table 6-5).

Table 6-5: RNA integrity and purity after DNase treatment and guanidinium thiocyanate-phenol-chloroform purification (Peyer's patches (PP); optical density (OD))

ID	Sample	RIN	OD 260/280	Sample	RIN	OD 260/280
1	Spleen, Day 4, M1G1	7.8	1.91	PP, Day 4, M1G1	7.80	1.90
2	Spleen, Day 4, M2G1	8.2	1.98	PP, Day 4, M2G1	8.00	1.91
3	Spleen, Day 4, M3G1	7.8	1.95	PP, Day 4, M3G1	7.90	1.94
4	Spleen, Day 4, M1G2	7.9	1.93	PP, Day 4, M1G2	7.70	1.95
5	Spleen, Day 4, M2G2	9.4	1.96	PP, Day 4, M2G2	7.80	1.94
6	Spleen, Day 4, M3G2	7.3	1.95	PP, Day 4, M3G2	7.20	1.94
7	Spleen, Day 4, M1G3	8.5	1.89	PP, Day 4, M1G3	7.70	1.94
8	Spleen, Day 4, M2G3	7.2	1.93	PP, Day 4, M2G3	8.30	1.96
9	Spleen, Day 4, M3G3	7.7	1.95	PP, Day 4, M3G3	8.50	1.91
10	Spleen, Day 4, M1G4	7.7	1.88	PP, Day 4, M1G4	8.70	1.99
11	Spleen, Day 4, M2G4	7.5	1.85	PP, Day 4, M2G4	8.10	1.95
12	Spleen, Day 4, M3G4	7.6	1.93	PP, Day 4, M3G4	8.50	1.99
13	Spleen, Day 4, M1G5	9.1	1.92	PP, Day 4, M1G5	8.30	1.94
14	Spleen, Day 4, M2G5	9.2	1.91	PP, Day 4, M2G5	7.90	1.88
15	Spleen, Day 4, M3G5	8.9	1.91	PP, Day 4, M3G5	8.50	1.96
16	Spleen, Day 30, M1G1	8.5	1.86	PP, Day 30, M1G1	8.70	1.98
17	Spleen, Day 30, M2G1	8.6	1.96	PP, Day 30, M2G1	8.60	1.87
18	Spleen, Day 30, M3G1	8.9	1.95	PP, Day 30, M3G1	8.40	1.91
19	Spleen, Day 30, M1G2	8.9	1.92	PP, Day 30, M1G2	8.60	1.96
20	Spleen, Day 30, M2G2	8.5	1.93	PP, Day 30, M2G2	7.50	1.91
21	Spleen, Day 30, M3G2	8.4	1.95	PP, Day 30, M3G2	8.00	2.07
22	Spleen, Day 30, M1G3	8.6	1.95	PP, Day 30, M1G3	8.00	1.93
23	Spleen, Day 30, M2G3	9.0	1.94	PP, Day 30, M2G3	8.50	1.89
24	Spleen, Day 30, M3G3	8.6	1.95	PP, Day 30, M3G3	8.70	1.99
25	Spleen, Day 30, M1G4	8.1	1.93	PP, Day 30, M1G4	7.90	1.96
26	Spleen, Day 30, M2G4	8.1	1.95	PP, Day 30, M2G4	8.30	1.96
27	Spleen, Day 30, M3G4	9.5	1.97	PP, Day 30, M3G4	8.10	1.93
28	Spleen, Day 30, M1G5	9.5	1.97	PP, Day 30, M1G5	8.40	1.95
29	Spleen, Day 30, M2G5	9.4	1.97	PP, Day 30, M2G5	8.60	1.93
30	Spleen, Day 30, M3G5	8.4	1.97	PP, Day 30, M3G5	8.40	1.94
31	Spleen, Day 50, M1G1	9.6	1.96	PP, Day 50, M1G1	6.90	1.87
32	Spleen, Day 50, M2G1	9.0	1.94	PP, Day 50, M2G1	8.20	1.94
33	Spleen, Day 50, M3G1	9.3	1.96	PP, Day 50, M3G1	7.30	1.84
34	Spleen, Day 50, M1G2	8.4	1.91	PP, Day 50, M1G2	7.80	1.93
35	Spleen, Day 50, M2G2	9.0	1.88	PP, Day 50, M2G2	8.20	1.92
36	Spleen, Day 50, M3G2	8.3	1.93			
37	Spleen, Day 50, M1G3	7.4	1.80	PP, Day 50, M1G3	8.10	1.93
38	Spleen, Day 50, M2G3	7.9	1.85	PP, Day 50, M2G3	7.80	1.91
39				PP, Day 50, M3G3	6.90	1.91
40	Spleen, Day 50, M1G4	8.2	1.97	PP, Day 50, M1G4	8.30	1.92
41	Spleen, Day 50, M2G4	7.9	1.85	PP, Day 50, M2G4	8.70	1.97
42	Spleen, Day 50, M3G4	7.9	1.92	PP, Day 50, M3G4	7.70	1.95
43	Spleen, Day 50, M1G5	8.8	1.95	PP, Day 50, M1G5	8.10	1.94
44	Spleen, Day 50, M2G5	8.6	1.91	PP, Day 50, M2G5	7.40	1.97

RT-negative controls for all samples were included into the qPCR assays that confirmed successful removal of residual genomic DNA, because the assays became positive at a high CT value similar to the NTCs (Figure 6-5B). QPCR assays with RNA samples that were not treated with DNase and not reverse transcribed demonstrated the importance the DNase treatment. They showed massive contamination with gnomic

B-element DNA (Figure 6-5C) and had a similar CT value as cDNA generated from RNA samples that were DNase treated (Figure 6-5A).

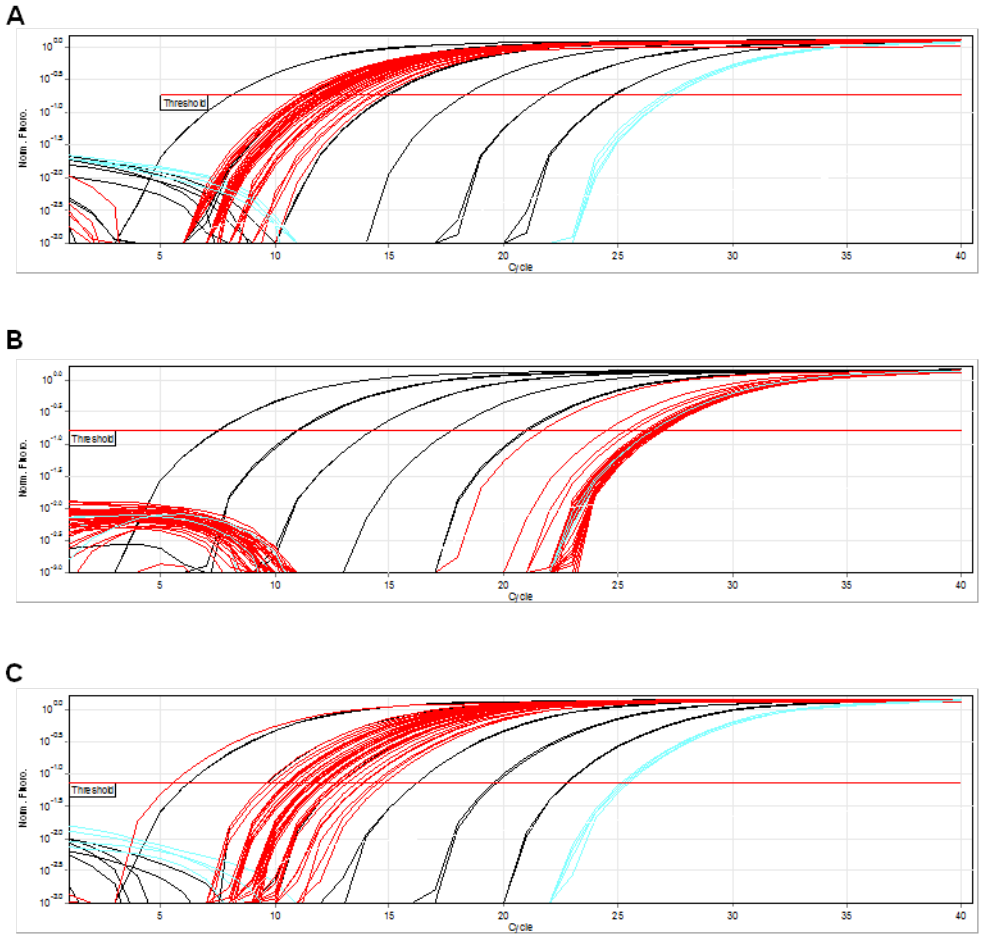


Figure 6-5: DNase treatment removed residual genomic DNA

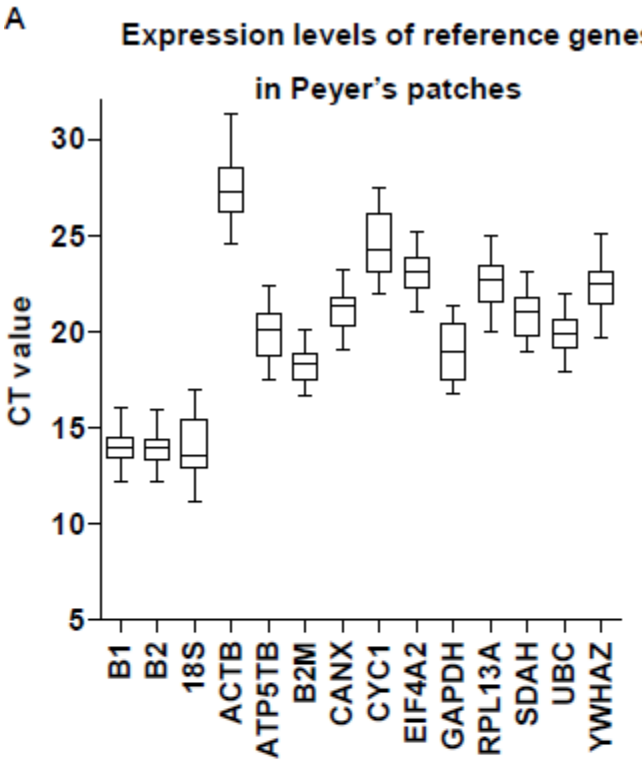
A: RNA samples were treated with DNase, reverse transcribed and analysed for B2-element expression. **B**: RNA samples were treated with DNase and not reverse transcribed (RT-negative controls) and analysed for B2-element DNA contamination. **C**: RNA samples were not treated with DNase (no-DNase controls), not reverse transcribed and analysed for B2-elements content in genomic DNA contamination. Black: standard curve with plasmid controls; red: mice RNA samples; blue: NTCs.

6.3.4 Expression level variability of B-elements in Peyer's patches and spleens of mice is comparatively low

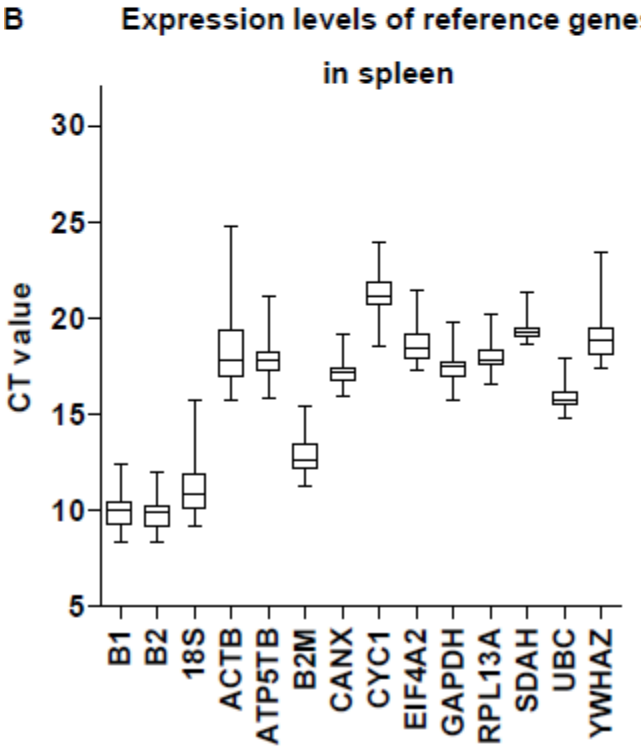
It was aimed to test if SINEs can be used as an alternative to classical control genes, and therefore the expression level of B-elements, B1 and B2, was compared to various common reference genes in spleens and Peyer's patches of mice that had been treated for different periods with diverse pharmacological formulations of *M. vaccae*.

In the total of 43 Peyer's patches and spleens, the B-elements, both B1 and B2, were stably expressed (Figure 6-6). In both tissues the two elements were nearly identical in their expression level and inter-sample variability. Their interquartile range (IQR) was ~1 CT and the maximum range was ~3.8 CTs.

Among the classical reference genes, B2M showed the smallest variability in the Peyer's patches with an IQR of 1.3 CTs (Figure 6-6A). In the spleen samples, the reference gene SDAH was even less variable than the B-elements with an IQR of 0.5 CTs (Figure 6-6B). In both tissues, the frequently used reference gene β-actin showed a considerably greater variability. It had an IQR of 2.2 CTs in the Peyer's patches and of 2.4 CTs in the spleens, and showed large maximal variability of 6.8 CTs (Peyer's patches) and 9.1 CTs (spleens).



Reference gene	CT variation (IQR)
B1	1.05
B2	0.98
18S	2.50
ACTB	2.22
ATP5TB	2.16
B2M	1.27
CANX	1.45
CYC1	3.03
EIF4A2	1.55
GAPDH	2.90
RPL13A	1.83
SDAH	1.94
UBC	1.39
YWHAZ	1.66



Reference gene	CT variation (IQR)
B1	1.10
B2	1.10
18S	1.84
ACTB	2.39
ATP5TB	0.96
B2M	1.25
CANX	0.69
CYC1	1.19
EIF4A2	1.26
GAPDH	0.78
RPL13A	0.72
SDAH	0.53
UBC	0.61
YWHAZ	1.45

Figure 6-6: Expression levels of 12 reference genes and the B1- and B2-elements measured by RT-qPCR

Cycle threshold (CT) values of reference genes expressed in 43 Peyer's patches (**A**) and spleens (**B**) of mice shown as medians (lines), interquartile range (IQR; boxes), and total ranges (whiskers). IQRs of the CT values of the different reference genes measured in 43 samples are shown in the table (right).

6.3.5 Ranking of reference genes according to their expression stability with the geNorm algorithm

The gold standard of normalising gene expression data obtained by RT-qPCR, against which the SINE approach was compared, is not to use the single reference gene which shows the smallest variability, but to generate a normalisation factor based on multiple reference genes. However, the expression stability of all the candidate reference genes has to be validated and only genes that are stably expressed should be included (Vandesompele et al., 2002b). The Microsoft-Excel based tool geNorm calculates average expression stability (M), thus ranks the genes according to the similarity of their expression profiles (Vandesompele et al., 2002b). Genes with higher M values have greater variation in expression, and the threshold proposed for eliminating a gene as unstable was $M \geq 0.5$. The geNorm algorithm was applied to rank twelve reference genes for Peyer's patches (Figure 6-7A) and eleven reference genes for spleens (Figure 6-7B, here EIF4A2 had to be excluded because it did not amplify in one sample). The normalisation factor was calculated with the three reference genes, which showed the lowest variability in expression profiles. The selected genes for the spleen samples were CANX, SDAH and RPL13A and for the Peyer's patches CANX, RPL13A and ATP5TB.

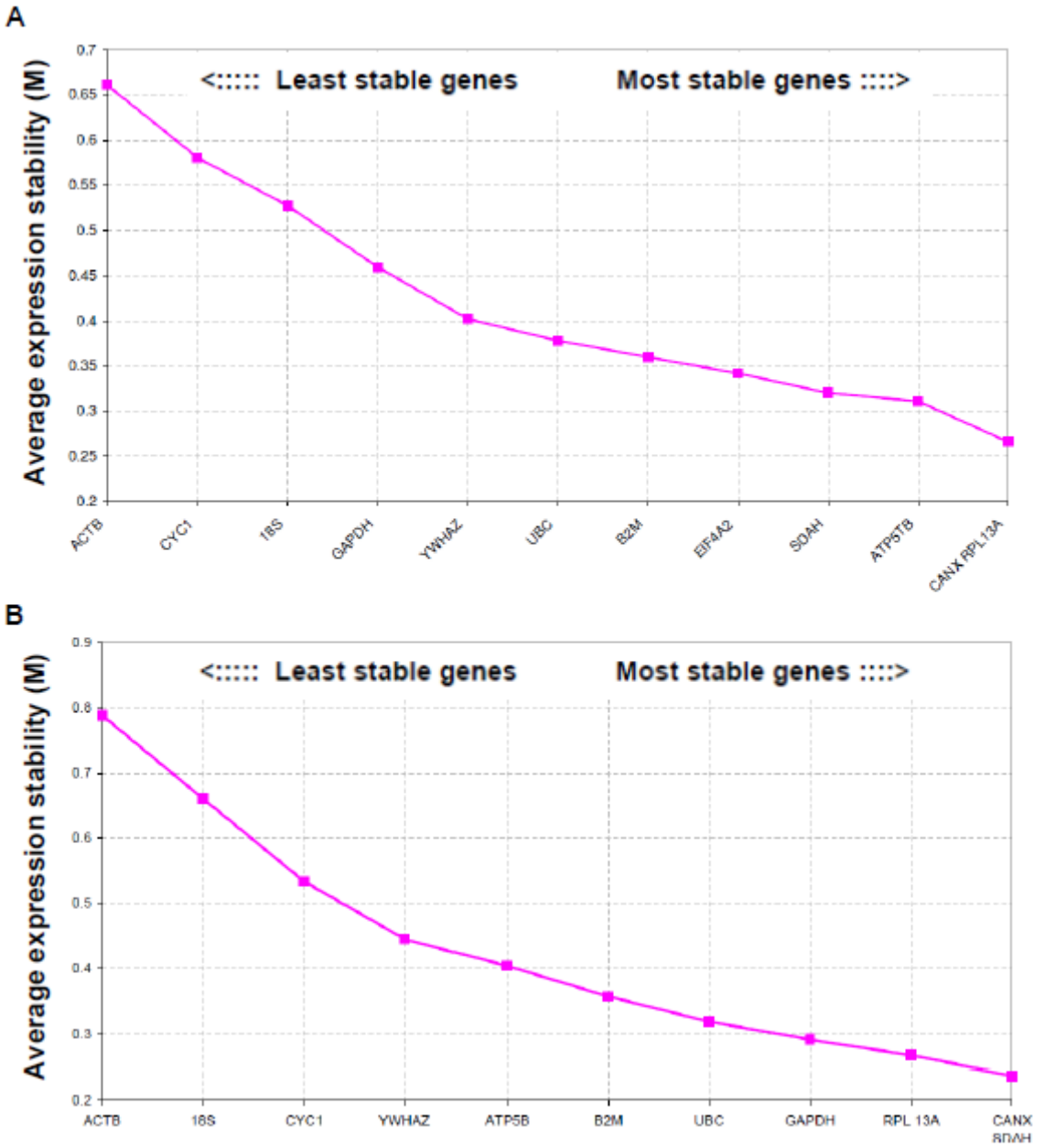


Figure 6-7: Expression stability of candidate reference genes calculated with geNorm

Application of the geNorm algorithm to eleven or twelve genes to identify the three most stable reference genes in Peyer's patches (**A**) and spleens (**B**). Average expression stability values (M) of remaining control genes during stepwise exclusion of the least stable control gene in Peyer's patches and spleen of 43 mice.

6.3.6 Validation: B-elements are suitable for normalisation of RT-qPCR data

Since the generation of a normalisation factor from multiple reference genes is very labour-intensive and problematic if only a small amount of starting material is available, I tested if normalisation with expressed SINEs would be an alternative.

RT-qPCR assays were designed and optimised for six genes of interest, FoxP3, TGF-β, IL-10, IFN-γ, HO-1 and TLR2, which all had an efficiency between 94 % and 100 %. Expression data from those six genes were normalised in Peyer's patches and spleens with the B-elements, the gold standard of three best ranked reference genes, and a single non-validated reference gene, here β-actin (Figure 6-8A, and data not shown). When the data were normalised with the B-elements, the same results were obtained as with the multiple reference gene approach. However, if β-actin was used as a single reference gene, the expression levels of the genes of interest were changing considerably in mice analysed on day 50 after treatment (Figure 6-8A, and data not shown). This was because the expression of β-actin has been actually downregulated in the animals at the later time point, which could be observed by normalising the β-actin expression levels with the multiple reference genes (Figure 6-8B).

These results demonstrate that the expressed B-elements are a suitable tool for normalisation of RT-qPCR data from mice Peyer's patches and spleen samples under the experimental conditions described. Our collaborators in Belgium (Professor J. Vandesompele, Ghent University) and a research group in Italy (Marullo et al., 2010) have validated this novel approach of RT-qPCR data normalisation in various human tissues using ALU-repeats. Therefore the use of expressed SINEs is available for RT-qPCR data normalisation in mice and human samples.

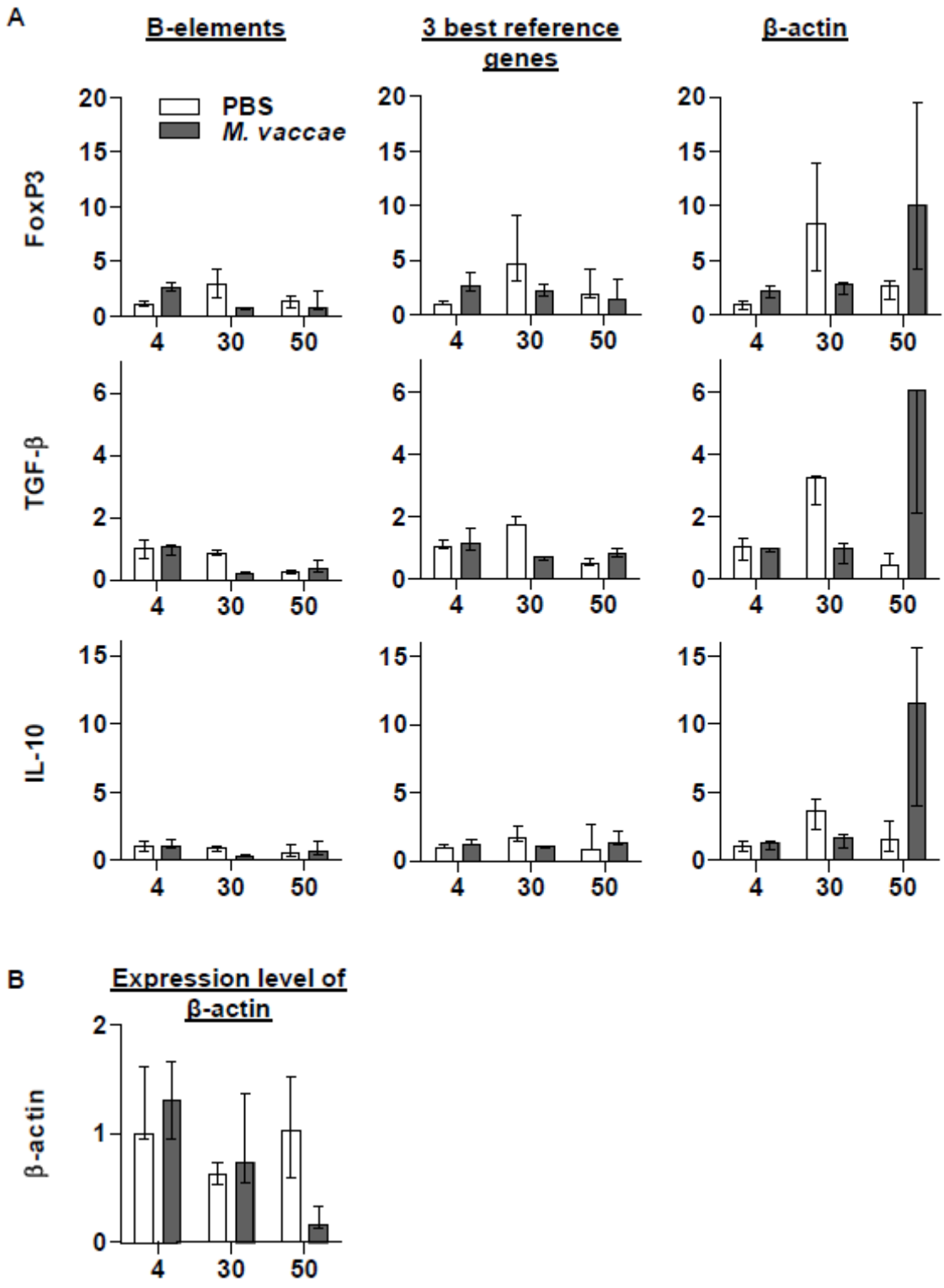


Figure 6-8: Gene expression data normalised with B-elements in comparison to standard methods

A: mRNA levels of FoxP3, TGF-β and IL-10 were normalised to either B-elements (left column), geometric mean of three validated reference genes (middle column) or β-actin (right column). Data are shown as proportions of expression level from untreated mice on day 4, by dividing all copy numbers by the median copy number of the three PBS-treated animals from day 4, which equals afterwards 1. **B**: mRNA quantities of β-actin were normalised with the geometric mean of the three most stable reference genes. Like in A, data are shown as proportions of expression level from untreated mice on day 4.

6.4 Discussion

The optimal reference gene for mRNA expression studies using RT-qPCR should be constantly transcribed in all samples under investigation and should not vary during the experiment. Since it has often been noted that commonly used reference genes can alter their expression profile (Deindl et al., 2002; Glare et al., 2002; Selvey et al., 2001; Zhong and Simons, 1999), more reports appeal for the validation of reference genes before every experiment (Huggett et al., 2005; Radonic et al., 2004). The current gold standard for normalisation of RT-qPCR data is to validate multiple candidate reference genes, select at least three that are stably expressed and use them for calculation of a normalisation factor (Andersen et al., 2004; Pfaffl et al., 2004; Vandesompele et al., 2002b). However, this is very time consuming, especially if one is only interested in a few genes of interest. The aim was therefore to test if expressed SINEs could function for normalisation of RT-qPCR analyses, and may represent an alternative to the resource- and labour-intensive approach of using multiple reference genes.

6.4.1 Possible source of positive no template controls

During the process of optimising the qPCR assay conditions to measure ALU-J and B-element expression, it became evident that amplification of SINEs was also occurring in NTCs. It has been described previously that NTCs appear positive if ALU sequences are targeted by qPCR (Nicklas and Buel, 2006). It has been assumed that the contamination source is the laboratory environment inhabited by humans performing the work (Urban et al., 2000a). To address the influence of the laboratory environment on the level of contamination with human ALU-J and mice B1-element DNA sequences, tubes were exposure to the air of different environments over a 24 hours period. However, there was no associated increase in the amount of B1 or ALU-J DNA detected with time in any of the environments. Therefore it can be concluded that the air

is not the source of contamination. Additionally, the low copy number and low variation observed by the ubiquitous contamination supports further that the contaminating DNA must have been present in one or more components of the master mix. The constant presence of rodent B1-elements in all samples was most likely a result of the hot-start monoclonal mouse antibody used to inactivate the *Taq* polymerase in this PCR assay. This could be further investigated by using a *Taq* polymerase inhibited with magnesium wax beads instead of antibodies or by replacing it with a *Pfu* DNA polymerase that was cloned from *Pyrococcus furiosus* and has minimal polymerase activity at temperatures at or below 50°C and therefore does not require hot-start protection. Identification of the source of human DNA contamination would be a complicated and expensive set of experiments to perform, since there are a vast number of suppliers of various reagents, and any method used to remove contaminating DNA (enzymatic digestion, UV treatment, etc.) will have a detrimental effect on some of the constituent components of the PCR reagents (e.g., primers, dNTPs). Preliminary experiments using UV radiation suggested the molecular grade DNase/RNase-free distilled water was not the source of the observed contamination (data not shown).

Taken together, the contamination with ALU and B-element DNA was very low and constant and should therefore not affect the use of highly expressed SINEs as a tool for normalisation of RT-qPCR data.

6.4.2 SINEs can be suitable tool for normalisation of RT-qPCR data

To validate the use of expressed B-elements for normalisation of RT-qPCR data, mRNA transcription levels of six different genes of interest were measured in Peyer's patches and spleens of mice that had been treated with different formulations of *M. vaccae* during different periods of time. The data were normalised with a) B-elements, b) validated multiple reference genes (geNorm algorithm), and c) the single standard reference gene β-actin (ACTB). Data normalised with the B-elements were

very similar as when they were normalised to the benchmark of multiple reference genes. On the other hand, normalisation with β-actin changed the results. This is important because β-actin is often used as a reference gene, but in this case would have let to the wrong conclusions.

In this study, the expression stability of the B-elements was only tested in two different mouse tissues. Yet, to confirm if they could function as general reference genes, which could be used without any validation, they would need to be tested in more tissues and in association with various treatments or stimuli. This has been done with the ALU-repeats in human tissues by our collaborators (Professor Vandesompele's group, Ghent University), where the assay was included within the set of multiple reference genes, utilised in high throughput scale for hospital diagnostics. A recent publication validated the application of expressed ALU-repeats (EARs) for RT-qPCR data normalisation (Marullo et al., 2010). In line with the data presented here, the authors showed that normalisation with EARs leads to similar data than normalisation with multiple reference genes (geNorm) and that normalisation with a single non-validated reference gene may lead to erroneous quantification of target genes (Marullo et al., 2010).

6.4.3 Considerations when using SINEs as a tool for normalisation of RT-qPCR data

Nevertheless, before the B-elements can be used as a reference for quantitative gene expression normalisation without any validation, one has to be aware that despite their high abundance throughout the mouse genome, there are possibilities that their expression might change. B-elements can not only get expressed as part of untranslated regions of mRNAs, they also harbour an internal B-box containing an RNA polymerase III promoter, which allows them to be expressed independently (Dewannieux and Heidmann, 2005). There are conditions were SINE sequences may

not be suitable for normalisation, since their expression level can be upregulated by cell stress. For example, it was reported that transcripts of B1- and B2-elements in mouse fibroblast cells were increased upon infection with minute virus of mice (MVM) (Williams et al., 2004), and transcripts of human ALU sequences can be increased with the Tat protein of HIV (Jang et al., 1992). Therefore, the expression stability of SINEs has to be further evaluated if they would be applied as a tool for RT-qPCR data normalisation in a different experimental system.

However, in mouse Peyer's patches and spleens, RT-qPCR data normalisation with B-elements can provide a similar accuracy as the multiple reference gene approach, but in a more time-efficient way. For the moment, it cannot be concluded that using B-elements is a better strategy for normalisation than using multiple reference genes, yet the data showed clearly that they can be more accurate than a randomly chosen (non-validated) reference gene.

A downside of using expressed SINEs as a tool for RT-qPCR data normalisation is that this technique requires careful removal of genomic DNA, which is not completely eliminated by regular RNA isolation methods. The data showed that RNA samples that were not treated with DNase contained similar copy numbers of residual genomic B-element DNA as expressed B-element RNA. In comparison, none of the other qPCR assays, for standard reference genes and genes of interest, was positive for RT-negative controls even if those samples were not additionally treated with DNase.

6.5 Conclusions

- RT-qPCR assays designed to target expressed B-elements amplify multiple B-elements with slightly different sequences.

- Assessment of the very constant copy numbers of ALU-J and B-element DNA measured in NTCs suggests that the contamination of the assays was already present in the master mix and not introduced during qPCR set up. Since the contamination is very stable and low compared to the copy number of expressed SINEs, it does not affect the application of expressed SINEs as a tool for RT-qPCR data normalisation.
- Expressed B-elements can be used as a tool for RT-qPCR data normalisation. They deliver similar results as the precise multiple reference approach and can be more accurate than using only a single non-validated reference gene like β-actin.

7 GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 Summary

The environmental mycobacterium *M. vaccae* has been studied in mouse models to test the hypothesis that some non-pathogenic microorganisms can reduce allergy associated Th2 responses and inflammatory diseases by augmenting regulatory T cells. However, data for human models and possible mechanisms are very limited. In this thesis I tested the effect of innate immune interactions between human DCs and *M. vaccae* on DC-dependent T cell responses.

I established an *in vitro* system of allogeneic and antigen-specific mixed leukocyte reactions using primary human cells. Using this system, I could clearly demonstrate that *M. vaccae* can downregulate Th2 cells in human immune responses in a dose-dependent manner. Until now, these effects had only been shown in OVA-induced asthma mouse models. Albeit this effect was small, it was very consistent in a large number of replicate experiments and confirmed by applying the methods to naive T cell polarisation as well as to antigen-specific T cell responses. Several clinical trials in man have so far shown contradictory effects of *M. vaccae* (Arkwright and David, 2001, 2003; Berth-Jones et al., 2006; Brothers et al., 2009; Camporota et al., 2003; Shirtcliffe et al., 2003).Yet, the data presented in this thesis demonstrate that *M. vaccae* clearly has the potential to reduce Th2 responses in humans.

Animal studies had indicated that an augmentation of Treg cells in response to administration of *M. vaccae* was responsible for the reduced Th2 response (Zuany-Amorim et al., 2002a; Zuany-Amorim et al., 2002b). Consistently I also found enlarged $CD25^{+}/FoxP3^{+}$ T cell populations in *M. vaccae*-primed human DC-T cell cocultures. However, production of the anti-inflammatory cytokine IL-10 is a typical feature of

Treg cells, but was not detected by intracellular cytokine staining, or by ELISA assays analysing the supernatants from cocultures. Moreover, LPS also augmented $CD25^+/FoxP3^+$ T cells, and here although IL-10 was detected in coculture supernatants, it had no anti-Th2 effect.

The only hint that DCs might play a role in the effects of *M. vaccae* on T cell responses came from a study showing that *M. vaccae*, when injected in an OVA-induced allergy model of BALB/c mice, caused increased expression of IL-10, TGF-β and IFN-α mRNA in a population of $CD11c^+$ cells in the lungs, which probably represent DCs (Adams et al., 2004). The data in this thesis show unequivocally that *M. vaccae*'s anti-Th2 effects can be mediated via primed DCs.

Further, this study established that *M. vaccae*, similar to other mycobacteria and other organisms associated with the hygiene hypothesis, can induce cellular activation via TLR2. However, specific TLR2 stimulation of DCs had no anti-Th2 effect in mixed leukocyte reactions, but rather augmented the population of $IL\text{-}4^+$ T cells and did not induce $CD25^+/FoxP3^+$ T cell populations. Importantly, whole genome microarray analysis, performed to understand differences in DC responses to *M. vaccae* and specific TLR2 stimulation with Pam_3CSK4, found that the major transcriptional response was very similar. As the two stimuli had clearly opposite effects on DC-dependent T cell responses, it can be expected that TLR2-dependent effects were not sufficient to mediate the effects of *M. vaccae*. Unfortunately, attempts to block TLR2 signalling that would have allowed studying TLR2-independent effects of *M. vaccae* failed. Therefore I focused on other differences in transcriptional responses, which revealed that many of the genes exclusively upregulated by *M. vaccae* are associated with CREB1. Further study of the upstream signalling pathways confirmed *M. vaccae*-induced selective early CREB1 activation and revealed marked differences between *M. vaccae* and the other stimuli on classical NF-κB and MAPK signalling. To

understand the role of CREB1 in *M. vaccae*-mediated effects on T cell polarisation, I aimed to inhibit CREB1 pharmacologically, which led to cytotoxic effects on DCs compromising the experimental paradigm. This thesis has established a basis for future work on *M. vaccae* to study the mechanism of its effects. Beyond that, this study pointed out the complexity and diversity of effects of differential innate immune stimulation of DCs as summarised in Table 7-1. While LPS and *M. vaccae* induced comparable levels of DC maturation, Pam_3CSK4 and *M. vaccae* provoked a similar transcriptional response. Activation of signalling pathways were comparable in LPS- and Pam_3CSK4-primed DCs, and moreover all three stimuli had diverse effects on DC-dependent T cell polarisation.

Table 7-1: Diverse effects of innate immune stimulation of dendritic cells

	LPS	**Pam_3CSK4**	***M. vaccae***
Maturation	+++	+	+++
Transcriptional response	+++	+	+
Signalling pathways	+++	+++	+
T cell polarisation	No effect	Th2 up	Th2 down

The last chapter of this report explored the possible application of expressed SINEs as a tool for normalisation of target gene expression data generated by RT-qPCR. I demonstrated with mouse samples that using SINEs can be as accurate as the very resource- and labour-intensive approach of measuring multiple validated reference genes. Importantly I showed that it can be more precise than using a single non-validated reference gene like β-actin, which can lead to false conclusions. Our collaborators in Belgium and others (Marullo et al., 2010) have shown that this method is also valid when studying human samples, therefore expressed SINEs may simplify accurate RT-qPCR measurements in the future.

7.2 Summary of future work

Future work should focus on the role of CREB1 activity in *M. vaccae*-stimulated DCs and their effects on T cell responses. Since pharmacological inhibition proved difficult, downregulation of CREB1 using siRNA might be an attractive approach. As discussed before, this requires careful experimental design as siRNA-induced activation of DCs would be undesirable. If CREB1 activation in DCs is found important for the effects of *M. vaccae* on T cells, it would be interesting to investigate up- and downstream events of CREB1. This includes analysis of the role of proteins expressed under the control of CREB1. They could be silenced by siRNA too or maybe over-expressed using lentiviral transfection, provided that this could be done without affecting DC responses. Moreover, detailed analysis of the signalling pathway leading to CREB1 phosphorylation would be important. The data in chapter 5 suggested a role for PKA, however, this requires confirmation since adenylate cyclase was found to be dispensable. Study of upstream signalling might give insight into alternative cellular receptors involved in recognition of *M. vaccae*. In chapter 3 it was demonstrated that *M. vaccae* can induce cellular activation via TLR2, but other receptors might be more important for the capacity of *M. vaccae*-stimulated DCs to reduce Th2 responses.

Protein release in response to stimulation with the specific TLR2 ligand and *M. vaccae* was very similar and closely mirrored the gene expression data. Yet, IL-1β was only secreted by *M. vaccae*-stimulated DCs. As the receptor antagonist was also very highly expressed and the literature did not support a role of inflammasome activity for *M. vaccae*'s ability to downregulate Th2 responses, I decided not to pursue this line of data. However, in view of a very recent publication showing on one hand that the inflammasome induces IFN-γ, IL-17A, IL-10, and IL-5 but on the other hand inhibits IL-13 in *S. mansoni*-infected mice (Ritter et al., 2010), it would be interesting to test this experimentally by employing caspase-1 inhibitors, adding recombinant IL-1ra to the

cultures or by adding increasing amounts of IL-1β. In addition, Maizels and colleagues showed lately that excretory-secretory antigens released by helminths mimic TGF-β and induce TGF-βR signalling directly on T cells, leading to induced Treg populations that can suppress Th2 responses in OVA-induced asthma models (Grainger et al., 2010). With regard to this study it would be interesting to analyse TGF-β secretion by *M. vaccae*-stimulated DCs, especially since *M. tuberculosis* was shown to be a potent inducer of TGF-β in human blood monocytes (Aung et al., 2005).

7.3 Publications

Witt, N., G. Rodger, J. Vandesompele, V. Benes, A. Zumla, G. A. Rook, and J. F. Huggett. 2009. *An assessment of air as a source of DNA contamination encountered when performing PCR*. J Biomol Tech 20:236-240.

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