Investigations into the Role of Endogenous Annexin-A1 in Dendritic Cell Biology

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Thesis submission to the University of London (Faculty of Science)
In fulfilment for the Doctorate of Philosophy

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ACKNOWLEDGEMENTS

Firstly, I would like to extend my deepest gratitude and thanks to my supervisors, Dr Fulvio D'Acquisto and Prof. Mauro Perretti for giving me the opportunity to pursue this PhD study and for their continual support and excellent advice and guidance as well as academic and research freedom during my PhD progression.

Secondly, I would like to thank specially my family for their moral support, especially my parents, Steve Huggins and Glynis Huggins as well as my brother, Vincent Huggins, and financial support, particularly from my aunts, Rona Huggins and Bettie Huggins.

Thirdly, I would like to especially thank Rob McKerrell for his endless close personal support, being a confidant and dear friendship during these four years of my PhD work.

Fourthly, I would like to particularly acknowledge my godfather, Anthony Stoute, for his contribution and personal investment in the progress of my academic achievements – especially this pursuit.

Finally, I would like to acknowledge Prof. Roderick Flower, Dr Dianne Cooper, Dr Nikolaos Paschalidis, Dr Andre Sampaio, Dr Magali

Pederzoli-Ribiel and Dr Asif Iqbal for their counsel at the various stages of this PhD study.

I would like to dedicate this work posthumously to my grandparents, George Vincent Huggins and Beryl Enid Huggins.

ABSTRACT

A school of literature has shown that Annexin-A1 (Anx-A1) is an endogenous anti-inflammatory protein that exerts a regulatory control over the innate immune system in order to restore homeostasis after an inflammatory reaction. Surprisingly, recent published works have highlighted that Anx-A1 has an alternate role in the adaptive immune system by positively modulating the strength of TCR signalling and biasing helper-subset differentiation.

Dendritic cells are a class of innate leukocytes, poised at the environmental interface, that are the essential immune cells responsible in the initiation of T-cell driven responses. These findings provided the foundation for this PhD project, the principal aim of which is to provide a link between the disparate effects of Annexin-A1 in innate and adaptive immunity by investigating the role of endogenous Annexin-A1 in dendritic cell biology and its effector function as an antigen-presenting cell towards T cell activation and differentiation. To address this hypothesis, I cultured bone marrow-derived dendritic cells from AnxA1-deficient mice or control littermates and stimulated with LPS (100ng/ml) then compared phenotypic and functional characteristics.

My results demonstrate that Anx-A1$^{-/-}$ bone marrow derived dendritic

cells show an increased number of $CD11c^{+}$ cells expressing high levels of some maturation markers such as CD40, CD54 and CD80 and a decreased capacity to take up antigen compared to control Anx-$A1^{+/+}$ cells. However, analysis of LPS-treated dendritic cells from Anx-$A1^{-/-}$ mice demonstrated a diminished up-regulation of maturation markers, a decreased migratory activity *in vivo* and an attenuated production of the inflammatory cytokines Interleukin (IL)-1β, Tumour Necrosis Factor (TNF)-α and IL-12.

This defect was resultant of an impaired Nuclear Factor (NF)-κB/DNA-binding activity due to lack of Anx-A1 signalling as demonstrated by the reduced activation of Extracellular-signal Regulated Kinase (ERK) 1/2 and protein kinase B (PKB)/Akt compared to cells from control littermates.

As a consequence of these defects, I assessed the antigen-presenting/T-cell activating capabilities of these DC. Anx-$A1^{-/-}$ DC showed an impaired capacity to stimulate T cell proliferation and differentiation in allogeneic mixed leukocyte reaction. To dissect this biologically relevant phenomenon further, I employed an antigen-specific, T-cell restricted model; a co-culture system of chicken ovalbumin peptide-pulsed, LPS-matured bone marrow-derived DC incubated with transgenic TCR T cells from OT-I/RAG-$1^{-/-}$ (OT-I, OT-I/$CD8^{+}$) or OT-II/ RAG-$1^{-/-}$ (OT-II, OT-II/$CD4^{+}$) mice. Peptide-pulsed, LPS-matured Anx$A1^{-/-}$ DC failed to initiate an appropriate T cell activation in both OT-I and OT-II T cells indicated by reduced cell proliferation when compared to T cells co-cultured with peptide-

pulsed, LPS-matured AnxA1$^{+/+}$ DC. Additionally, comparison of peptide-pulsed, LPS-matured AnxA1$^{-/-}$ DC with AnxA1$^{+/+}$ DC counterparts detected severely diminished levels of IL-2 from co-cultures with OT-I T cells and ablated IFN-γ production from co-cultures with both OT-I and OT-II T cells.

In conclusion, AnxA1 seems to act as a positive modulator of immunogenic activation of DC, whereby the AnxA1 signal pathway has a probable synergism with the TLR4 signalling cascade. DC-derived AxnA1 appears to contribute in promoting T cell activation with a larger influence on OT-I/CD8^{+} T cells than OT-II/CD4^{+} T cells. Altogether these findings suggest that inhibition of Anx-A1 expression or function in dendritic cells might represent a useful way to modulate the adaptive immune response and pathogen-induced T cell-driven immune diseases.

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INTRODUCTION

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aa	amino acid
Ac2-26	Annexin-A1 N-terminal peptide, aa-2 – aa-26
Akt/PKB	Protein kinase B
ALX/FPR2	Lipoxin A_4 receptor/Formyl peptide receptor 2
Anx-A1	Annexin-A1
Anx-A1$^{-/-}$	Annexin-A1 null homozygous
Anx-A1$^{+/+}$	Annexin-A1 wild-type
Anx-A2	Annexin-A2
AP-1	Activator protein 1
APC	Antigen presenting cell
APS	Ammonium Persulphate
BALB/c	Laboratory inbred stain of the house albino mouse
BCR	B-cell receptor
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
C57BL/6	Laboratory inbred mouse strain, substrain 6
CCL	cysteine-cysteine chemokine ligand
CD	Cluster of Differentiation
CD40L	CD40 ligand
cDNA	Complementary DNA
CFA	Complete Freund's Adjuvant
CIA	Collagen-Induced Arthritis

COX	Cyclooxygenase
CpG	Single-stranded dinucleotide cytosine-guanine
$cPLA_2$	Cytosolic phospholipase A_2
C-terminus	Carboxyl-terminus
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CXC	cysteine-X-cysteine chemokine ligand
DAG	Diacylglycerol
DAMP	Danger-associated molecular patterns
dATP	Deoxyadenosine triphosphate
DC	Dendritic Cell
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	1, 4-Dithio-DL-threitol
dTTP	Deoxythymidine triphosphate
E. coli	Escherichia coli
ECL	Enzymatic chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate

FPR	Formyl peptide receptor
FPRL	Formyl peptide receptor-like
FSC	Forward scatter
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GPCR	G-protein coupled receptor
HLA	Human leukocyte antigen
hr Anx-A1	Human recombinant Annexin-A1
HRP	Horseradish peroxidase
HSP	Heat shock protein
IB	Immunoblotting
IκB	Inhibitor of nuclear factor-κB
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
IP	Immunoprecipitation
IP-3	Inositol trisphosphate
IP-10	IFN-γ-inducible protein 10
IRAK	IL-1-receptor-associated kinase
ITAM	Immunoreceptor tyrosine activation motif
IRF	IFN response factor
JNK	c-Jun N-terminal kinase

kD	kilo-Dalton
LBP	LPS-binding protein
LC	Langerhans Cell
LPS	Lipopolysaccharide
LFA	Lymphocyte function-associated antigen
Lyso-PS	Lysophosphatidylserine
LRR	Leucine-rich repeat
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage colony-stimulating factor
MD-2	Myeloid differentiation protein 2
mDC	Myeloid dendritic cell
MAPKK	MAPK kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
moDC	Monocyte-derived Dendritic Cell
mRNA	messenger RNA
MyD88	Myeloid differentiation factor 88
MW	Molecular Weight
NEMO	NF-κB essential modifier protein
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear Factor-κB
NOD	Nucleotide-Binding Oligomerization Domain
NOS	Nitric oxide synthase

N-terminus	Amino-terminus
OD	Optical Density
ODN	Oligonucleotide
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PFA	Paraformaldehyde
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol bisphosphate
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLP	Proteolipid Protein
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
pTCR	pre-TCR
PVDF	Polyvinylidene Fluoride
RA	Rheumatoid Arthritis
Rel	Recticuloendotheliosis oncogene
RH	Rel homology
RNA	Ribonucleic Acid
ROR-γ	RAR-related Orphan Receptor-γ

ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Room Temperature
SAA	Serum Amyloid A
SLAM	Signalling Lymphocyte Activation Molecule
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	Side scatter
ssRNA	single-stranded RNA
STAT	Signal transducer and activator of transcription
TAB	TAK-binding protein
TAE	Tris-acetate-EDTA
TAK	TGF-β-activated kinase
TANK	TRAF-family member-associated NF-κB activator
TBK1	TANK-binding kinase 1
TCR	T-cell Receptor
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TGF-β	Transforming Growth Factor-beta
Th	T-helper-cell
TIR domain	Toll/IL-1 receptor domain
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
VCAM	Vascular Cell Adhesion Molecule
VLA	Very Late Antigen

1. INTRODUCTION

1.1. THE INFLAMMATORY RESPONSE

Inflammation is an adaptable response that is sparked by nocuous conditions such as tissue injury and infections. It is generally thought to be a beneficial, controlled process but can become detrimental if dysregulated resulting in patho-physiological events and death. Despite the aetiology, the inflammatory response supposedly evolved as a flexible reaction for restoring homeostasis. Therefore, a successful inflammatory response involves the elimination of the pernicious agents followed by a resolution and repair phase (Medzhitov, 2008).

This process is achieved by the immune system fulfilling its four main duties. Firstly, there is **immunological recognition**; that is the presence of an infection must be detected, which is performed by leukocytes of innate immune system and lymphocytes of the adaptive immune system. The second task is the involvement of **immune effector functions** to contain and possibly eliminate the infection by employing the coordination of complement proteins in the blood, antibodies and the killing capacities of lymphocytes and some

leukocytes. Whilst the destructive nature of these cells is in play, the immune response must be managed as not to cause unnecessary damage to the body. This is the third undertaking referred to as **immune regulation**; the ability of the immune system to self-regulate and thus a very important feature of immunity. Our environment is inundated by microorganisms, many of which cause disease. Yet despite this incessant exposure metazoan hosts only rarely become ill. The fourth facet is to protect against recurring disease due to the same pathogen. This unique feature is **immunological memory** and it is the capability of the adaptive immune system to execute this course of action so once exposed to an infection, an individual will formulate stronger and more immediate response to subsequent exposures creating protective immunity (Murphy et al., 2008).

The inflammatory process, which eradicates pathogens or repairs tissue damage, broadly involves both divisions of the immune system: innate immunity and adaptive immunity to effectively complete its course.

1.1.1. Innate Immunity

The innate immune system is present, in comparable forms, in all organisms of the Eukaryotic domain. From the single-celled Amoebozoa kingdom to the complex multi-cellular Animalia kingdom, this system is a phylogenetically preserved mechanism that provides rapid, generalised (i.e. antigen non-specific) host defence against noxious agents - hence its name. The innate immune system of metazoan hosts consists of several elements, some of which would not traditionally, in an immunological sense, be considered part of the immune system. These elements include anatomical barriers, secretory molecules and cellular components.

There are three aspects to the anatomical barriers (Brodell and Rosenthal, 2008). Firstly, the mechanical wall that include the external epithelial layers, i.e. the skin (Burtenshaw, 1945, Burtenshaw, 1942), and the internal epithelial layer that are armoured with mucosal linings, i.e. the mouth, intestines and anus, as well as equipped with the specialized feature of motile cilia to deflect debris and bacteria, i.e. the broncho-pulmonary tract (Knowles and Boucher, 2002). The second aspect comprises of chemical factors; fatty acids in sweat and its low pH inhibit bacterial growth (Wille and Kydonieus, 2003, Fluhr et al., 2001, Kabara et al.,

1977, Nieman, 1954), lysozyme and phospholipases present in tears, saliva and nasal secretions weaken bacterial membranes (Boman, 1995), and low molecular weight proteins with antimicrobial activity known as defensins (Ganz, 2003, Fellermann and Stange, 2001). Thirdly are the biological factors that originate from the cooperation between beneficial organisms and their host. The normal flora of commensals living in host skin and gastrointestinal tract prevent colonisation of opportunistic pathogens by competitive survival for resources and by secreting toxic substances (Cheesman and Guillemin, 2007).

Another constituent of innate immunity are secretory molecules, often triggered when the anatomical barriers are compromised and pathogen incursion or tissue abrasion occurs. It entails the complement system, the coagulation system and other molecules. The complement system is a group of small pro-proteins mainly produced and secreted by the liver into the circulation. They are the major non-specific humoral defence mechanism and once activated can lead to increased vascular permeability (oedema), recruitment of phagocytes (Jose, 1987) and lysis and opsonisation of bacteria and parasites (Jose, 1987, Mackay et al., 2001). The inflammatory relevance of the coagulation system's involvement chiefly depends on the severity of the tissue damage (Cicala and Cirino, 1998, McGilvray and Rotstein, 1998). Some products of coagulation contribute to increased vascular permeability (Lee et al., 1996) and as

chemoattractants for phagocytes (Bar-Shavit et al., 1984, Bar-Shavit et al., 1983a, Bar-Shavit et al., 1983b). Moreover, some coagulation products have direct antimicrobial activity such as platelet-derived β-lysin, which acts as a cationic detergent in the killing of Gram-positive bacteria (Donaldson and Tew, 1977, Donaldson et al., 1974). Other molecules include lactoferrin and transferrin that are responsible for chelating iron, an essential metal in bacterial nutrition and growth (Singh et al., 2002).

The third facet of innate immunity is cellular agents. These immune cells offer a potent, rapid and broad first line of defence. Some are resident in tissues and organs such as macrophages and dendritic cells. Other are recruited to the site of inflammation such as neutrophils, eosinophils, monocytes and natural killer (NK) cells (Figure 1.1).

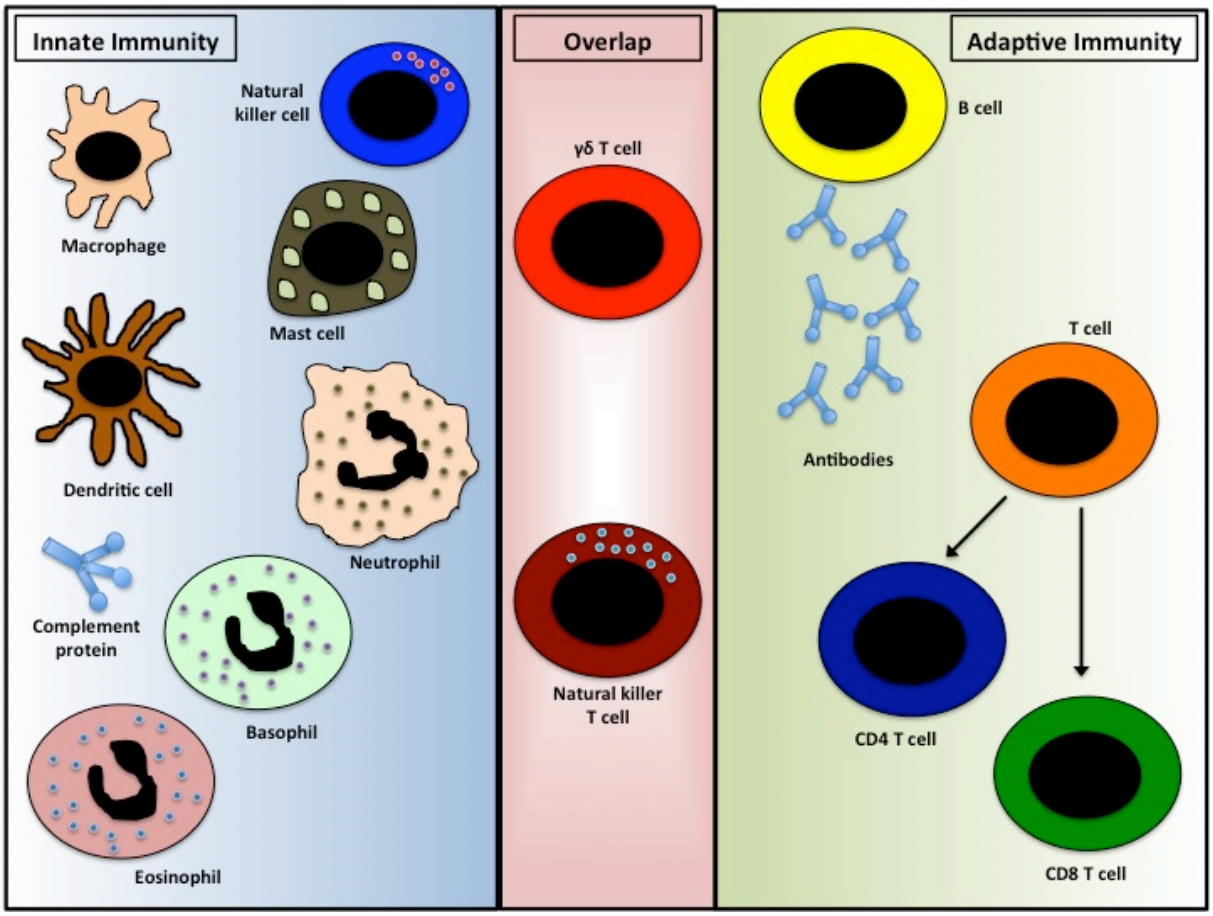

Figure 1.1. Cells and mediators of the innate and adaptive immune system.

The innate immune response functions as the first line of defense against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including basophils, eosinophils, neutrophils, mast cells, macrophages, dendritic cells and natural killer cells. Natural killer T cells and γδ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity.

Neutrophils and eosinophils are polymorphonuclear cells both of which possess granules containing highly reactive compounds that kill bacteria (Faurschou and Borregaard, 2003, Borregaard et al., 1993) and parasites (Molina et al., 1988, Hamann et al., 1987, Gleich et al., 1979) respectively but in addition cause collateral tissue damage (Weissmann et al., 1978, Goldyne, 1975). Neutrophils also phagocytose foreign organisms and kill them intracellularly (Dale et al., 2008). Resident macrophages and dendritic cells, and migratory inflammatory monocytes that differentiate primarily into dendritic

cells but also macrophages at sites of inflammation (Banchereau et al., 2000), function to phagocytose and kill microorganisms intracellularly (Nagl et al., 2002, Banchereau et al., 2000). Furthermore, macrophages contribute to inflammation resolution and tissue repair (Serhan et al., 2006, Serhan and Savill, 2005) and dendritic cells, and to a much lesser extent macrophages, act as antigen-presenting cells (APC) that are crucial for the initiation of the adaptive immune responses (Banchereau and Steinman, 1998, Steinman and Hemmi, 2006). NK cells, although not usually involved in the classical inflammatory response, are important innate immune cells required for the detection and eradication of virally infected cells and tumour cells (Cerwenka and Lanier, 2001, Lodoen and Lanier, 2005).

1.1.2. Adaptive Immunity

Antigen presenting cells (APC), like dendritic cells (Inaba et al., 1990), are involved in innate immunity but also are a paramount link to the adaptive immune system by participating in the activation of both T and B lymphocytes (Jego et al., 2005, Ni and O'Neill, 1997). A characteristic feature of APC is the expression of cell surface molecules, class I and class II major histocompatibility complex (MHC), which forms a complex with antigens (components from foreign microbes processed when endocytosed) and is presented so that the appropriate lymphocytes can be activated. B cells also express class II MHC molecules and also function as APC. B cells are very effective in presenting antigen to memory T cells, especially when the antigen concentration is low because surface immunoglobulin on the B cells binds antigen with a high affinity (Rodríguez-Pinto, 2005). After exposure to antigen, naïve B and T cells differentiate into effector lymphocytes. B cells differentiate into effector B cells called plasma cells whose primary function is the production of antibodies. Similarly, T cells can differentiate into either a single subset of T cytotoxic (Tc) cells that primarily operate to destroy tumour cells and virally infected cells or a single subset of T helper (Th) cells that largely provide help to cytotoxic T-lymphocytes and B-lymphocytes by augmenting the roles of these

cells.

Antigen specificity of T and B cells is accomplished through recognition by the T cell receptor (TCR) and the B cell receptor (BCR), respectively. The TCR and BCR are similar in that each receptor is specific for one antigenic determinant but they differ in that BCR are divalent and have their antigen receptors cross-linked by antigen while TCR are monovalent and do not require cross-linking (Weiss and Littman, 1994, Alberola-Ila et al., 1997). Each B and T cell has a receptor that is unique for a particular antigenic determinant and there is a vast array of different antigen receptors on both B and T cells (Janeway, 2005, Honjo et al., 1989). B cells utilise cell surface-bound immunoglobulin as antigen receptor and the specificity of that receptor is the same as the immunoglobulin that it is able to secrete after activation. B cells recognize the following antigens in soluble form: proteins, nucleic acids, polysaccharides, lipids and haptens (Elgert, 2009). In contrast, the overwhelming majority of antigens for T cells are proteins, and these must be fragmented and recognized in association with MHC products expressed on the surface of APC, not in a soluble form. T cells are grouped functionally according to the class of MHC molecules that associate with the peptide fragments of the protein; helper T cells recognize only those peptides associated with class II MHC molecules, and cytotoxic T cells recognize only those peptides associated with class I MHC molecules. This constrained detection of peptides by T cells is established during T

cell development in the thymus and is termed self-MHC restriction. For T cells to recognize and respond to a foreign protein antigen, it must recognize the MHC on the APC as self-MHC. Helper T cells recognize antigen in context of class II self-MHC and cytotoxic T cells recognize antigen in context of class I self-MHC (Doyle and Mamula, 2001, Koch and Stockinger, 1991).

After Th cells recognize specific antigen presented by an APC, they initiate several key immune processes that include: 1) selection of appropriate effector mechanisms; 2) induction of proliferation of appropriate effector cells; and 3) enhancement of the functional activities of other leukocytes. Classically, Th effector subsets were categorised into two groups. But over the last decade, further insight into patho-physiological conditions has led to the discovery of new Th effector subsets, namely Th17 (Annunziato and Romagnani, 2009, Liew, 2002, Ma et al., 2010, Stockinger et al., 2007). When naïve T cells encounter antigen in secondary lymphoid tissues, they become activated; these activated, non-committed Th0 cells and are capable of differentiating into Th1 cells, Th2 cells, Th17 cells, which are distinguished by the cytokines they produce. Whether a Th0 cells becomes a Th1, a Th2 or a T17 cell depends upon the cytokine microenvironment, which is influenced by antigen-bearing APC (Feili-Hariri et al., 2005, Gutcher and Becher, 2007, Steinman and Hemmi, 2006). Some antigens stimulate IL-4 production that favours the generation of Th2 cells while other antigens stimulate IL-12

production, which favours the generation of Th1 cells. Equally important, each subset can exert inhibitory influences on the other; Th1-derived IFN-γ inhibits proliferation of Th2 cells and differentiation of Th17 cells, and Th2-derived IL-10 inhibits the production of IFN-γ by Th1 cells. In addition, IL-4 inhibits formation of Th1 cells and differentiation of Th17 cells (Farrar et al., 2002, Flavell et al., 1999, Male et al., 2006). Subsequently, the differentiating T cells initiate transcriptional pathways as well as activate production of cytokines in order to promote and propagate lineage commitment of the T helper cell subset (Figure 1.1.2.1). Thus, the immune response is adapted and directed to deal with the pathogen encountered – cell-mediated responses for intracellular pathogens or antibody responses for extracellular pathogens.

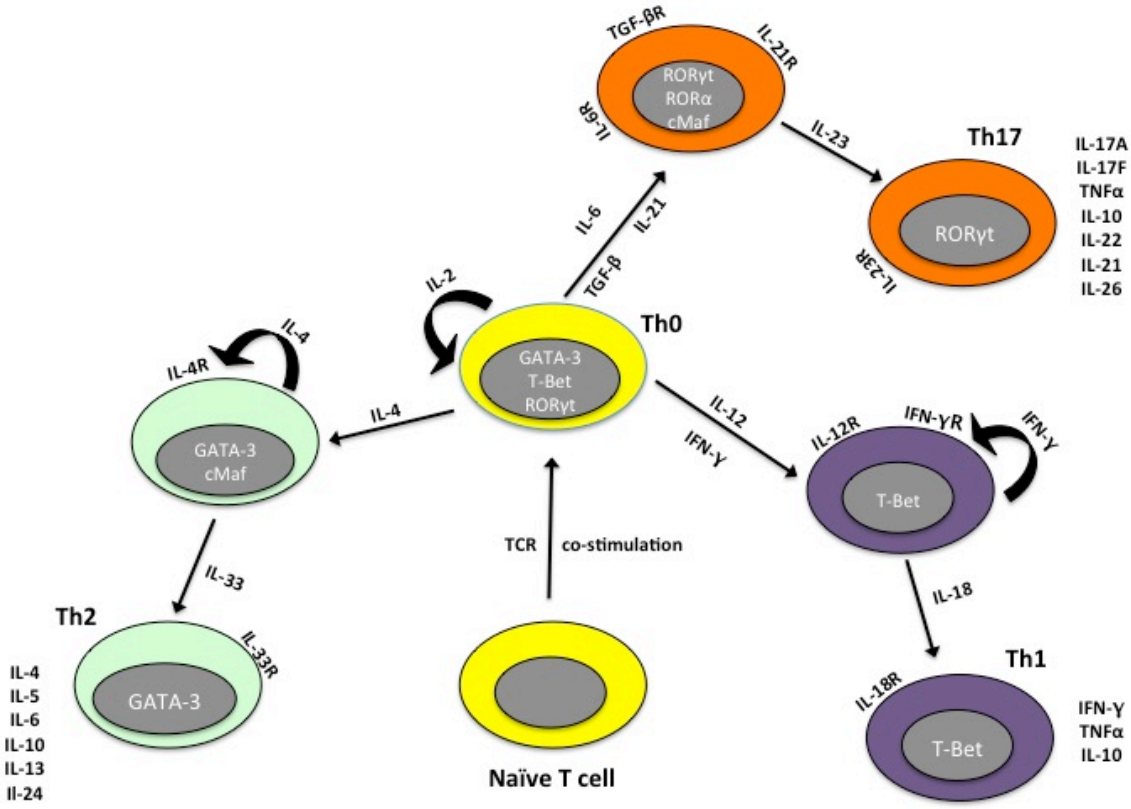

Figure 1.1.2.1. Intrinsic commitment and microenvironment signalling resulting in T helper cell subset differentiation.

Naïve T cells become activated by APC and in the presence of a select APC-derived and T-cell-derived cytokine milieu initiate distinct transcription factors to drive the development of diverse T helper cell subsets.

Th1, Th2 and Th17 cells affect different cells and influence the type of immune response; Th1 cytokines activate macrophages and participate in the generation of cytotoxic lymphocytes (CTL), resulting in a cell-mediated immune response. In contrast Th2 cytokines help to activate B cells, resulting in antibody production; Th2 cytokines also activate eosinophils, mast cells and basophils, which are central to immune responses to extracellular parasites such as helminths. The relatively recently identified Th17 cells constitute a group of T cells collectively termed neutrophil-regulatory T (Tn) cells that also

include γδ T cells and NKT cells (Ley et al., 2006). All these cell types secrete IL-17A, which stimulate epithelial cells, endothelial cells and fibroblasts to secrete IL-8 (Fossiez et al., 1996), a potent neutrophil chemokine, which activates and recruits neutrophil to inflammatory sites. Th17 cells also produce IL-23, which has been reported to play a prominent role in the regulation of granulopoiesis (Figure 1.1.2.2) (Smith et al., 2007, Stark et al., 2005).

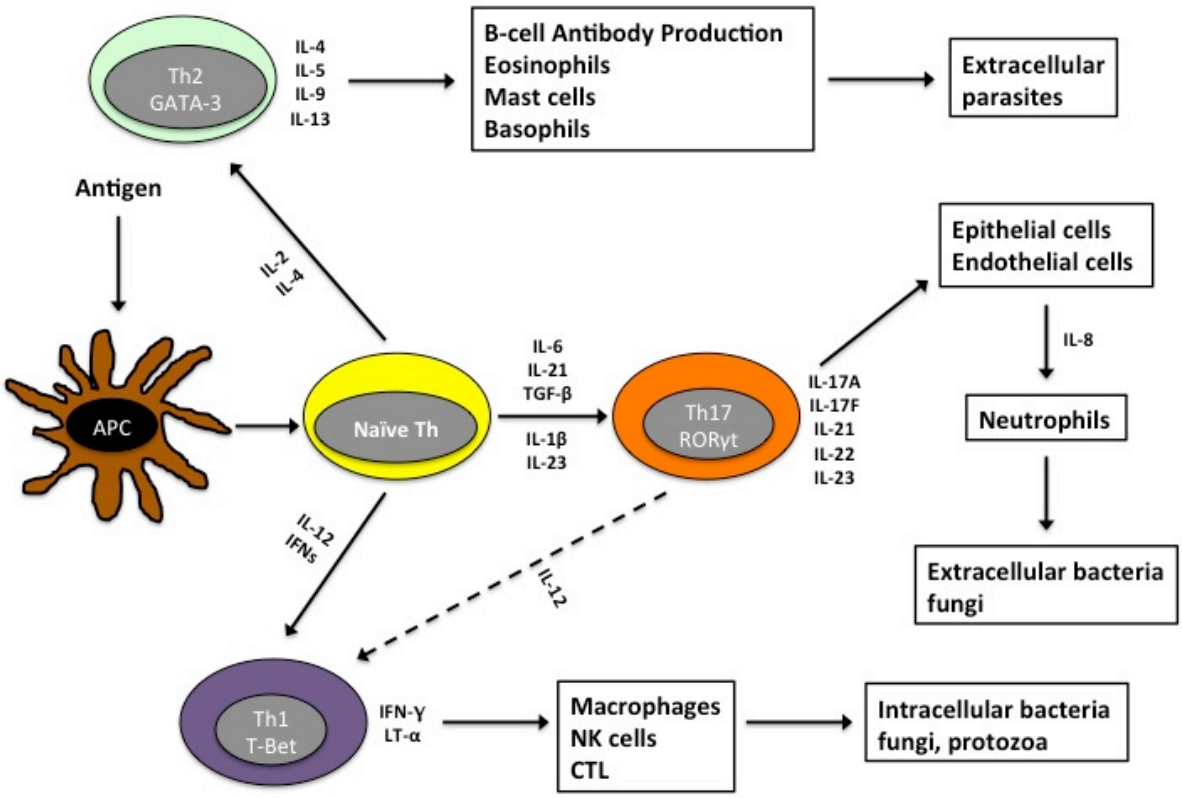

Figure 1.1.2.2. Main populations of CD4$^+$ T helper cells and their effector functions.

Effector T helper cell subsets and the biological significance of their effector functions in driving immunity against an array of pathogenic infections. Also their patho-physiological contributions to human diseases and different disease models.

1.2. DENDRITIC CELLS

Dendritic cells (DC), a heterogeneous population of leukocytes, are unique antigen-presenting cells with the ability to induce primary immune responses. DC capture and transfer information from the external environment to the cells of the adaptive immune system (Steinman, 1991, Steinman et al., 1980). DC are not only critical for the induction of primary immune responses, thus permitting establishment of immunological memory; but may also be important for the induction of immunological tolerance, as well as for the regulation of the type of T cell–mediated immune response (Steinman et al., 2000).

The identification of dendritic cells by Steinman and Cohn (Steinman and Cohn, 1973) and the elucidation of their crucial role as antigen-presenting cells (Steinman, 1991) was a major advancement in immunology. DC are the central policing leukocytes of the immune system, which initiate innate as well as adaptive immune responses; they begin in an immature form as sentinels constantly sampling antigens at peripheral sites. On encountering microbial products or danger signals they migrate to lymphoid organs and mature into an antigen-presenting entity now capable of activating T cells.

To detect pathogens or other forms of danger, DC are equipped with a variety of pattern recognitions receptors (PRR). Amongst the PRR, the Toll-like receptors (TLR) are a family of highly conserved, germline-encoded transmembrane receptors (Medzhitov and Janeway, 2000, Mogensen, 2009). Structurally, TLR are typical type I receptors with an extracellular domain consisting of leucine-rich repeats (LRR), one short transmembrane region and an intracellular domain. The latter is highly homologous with the respective counterparts of the IL-1 receptor and Toll and thus named the Toll-IL-1R (TIR) domain (Akira, 2004). The LRR domains of the TLR directly bind invariant "pathogen associated molecular patterns" (PAMPs) that are conserved products of microbial metabolism expressed by virtually all classes of microorganisms, including bacteria, viruses fungi and parasites. One characteristic common to all PAMPS is that they are produced by microorganisms but not by host cells (Bianchi, 2007).

At present, **thirteen mammalian TLR** motifs have been identified, ten in human and twelve in mice, as well as their co-receptors, accessory molecules and each individual signal transduction. TLR2 in combination (heterodimerisation) with TLR1 or TLR6 recognises a variety of lipoproteins, lipopeptides or polysaccharides of bacterial, viral or fungal origin (Akira and Hemmi, 2003, Into et al., 2004, Takeda and Akira, 2004, van der Aar et al., 2007, Zeuthen et al., 2008). TLR3 seems to be highly specific for double-stranded (ds)RNA,

which might be either genomic viral RNA or dsRNA intermediates produced during viral replication. TLR4, the first mammalian TLR identified, is the main receptor for bacterial derived lipopolysaccharides and in addition senses some viral envelope proteins. TLR4 may also act as a receptor for stress-induced molecules such as heat shock proteins (HSP) (Vabulas et al., 2002). TLR5 recognises flagellin. TLR7 and TLR8 represents receptors for single stranded RNA (ssRNA) from RNA viruses like influenza or HIV (Diebold et al., 2004, Heil et al., 2004). Synthetic ligands for TLR7 have also been identified, which include guanosine analogs like loxoribrine, imiquimod and resiquimod (also acts through human TLR8). TLR9 originally was described as a receptor for CpG motif containing unmethylated bacterial or synthetic DNA but recently the spectrum was broadened to include viral DNA from viruses of the herpes family including HSV-1, HSV-2 and MCMV-5 (Lund et al., 2003, Krug et al., 2004, Hochrein et al., 2004). Hemozoin, a digestion product of haemoglobin by the malaria parasite has been recently added to the list of TLR9 ligands. Hemozoin is until now the only non-nucleic acid ligand for TLR9 (Coban et al., 2005).

TLRs can be classified using several categories: cellular localisation, amino acid homology, nature of their specific ligands or use of adaptor molecules. TLR7, -8, -9 are grouped together into the TLR9 subfamily based on their high amino acid sequence similarity, localisation in endosomes, the requirement for endosomal acidification for ligand binding and their structurally related ligands:

nucleic acids (Wagner, 2004). At least in DC, location and ligand recognition of nucleic acids are also attributes of TLR3 however sequence homology to the TLR9 subfamily is comparatively poor. TLR2 family members (TLR1, -2, -6), TLR4 and TLR5 are localised to the extracellular membrane and thus recognise extracellular PAMPs and TLR ligands.

1.3. DENDRITIC CELLS AS ANTIGEN-PRESENTING CELLS

Upon pathogenic encounter or tissue insult, DC initiate a maturation process into highly specialised antigen-presenting cells (APC), thereby activating cellular mechanisms in order to decrease antigen (Ag)-processing capacities, to enhance expression of MHC Class I or II and co-stimulatory molecules and migrate to secondary lymph nodes, where they trigger naïve T cells. The fate of naive T cells is determined by three stimulatory signals that are provided by pathogen-primed mature DC (Ni and O'Neill, 1997). **Signal 1** results from the ligation of the T-cell receptor (TCR) to pathogen-derived peptides, presented on MHC class I or II molecules on the cell surface of DC, and determines the antigen-specificity of the response (Nel and Slaughter, 2002). However, the initiation of protective immunity also requires T-cell co-stimulation. **Signal 2** is the co-stimulatory signal, which involves a balanced combination of signals to T-cell via the binding of the DC co-stimulatory surface molecules particularly butyrophilin family members B7-1 (cluster of differentiation (CD) 80) and B7-2 (CD86) to their T-cell cell surface counterparts CD28 or cytotoxic T lymphocyte antigen 4 (CTLA-4/CD152) on naïve T cells as well as reciprocal signalling from the T-cell to the DC via CD40 on DC ligating to CD40L on activated T cells (Chambers and Allison, 1997).

The affinities of the B7 molecules and their interactions with CD28 or CTLA-4 differ substantially. B7-1 binds to CTLA-4 with highest affinity (K_d = 0.2μM); B7-1-CD28 and B7-2-CTLA-4 bind with intermediate affinities (K_d = 4μM and 2.6μM respectively); and B7-2 binds to CD28 with lowest affinity (K_d = 20μM) (Bhatia et al., 2006). In addition, these molecules have differential expression and regulation profiles on APC. B7-2 is constitutively expressed and rapidly up-regulated upon stimulation; in contrast, B7-1 is generally absent on immature cells and is induced upon activation with slower expression kinetics (Coyle and Gutierrez-Ramos, 2001). These observations have led to the hypothesis that B7-2 interactions are required for initial T cell co-stimulation, whereas B7-1-CD28 interactions are more involved in the sustained T cell activation; however this model remains to be corroborated.

Recently, intense research in the field of co-stimulation was to dissect whether B7-1 and B7-2 have overlapping and redundant roles in determining polarisation of Th effector cells. Ligation of CD28 and CTLA-4 may directly regulate non-polarised T helper (Th) differentiation because CD28 activation facilitates Th2 responses, whereas CTLA-4 inhibits Th2 responses. Furthermore, these findings suggest a model that the engagement of either CD28 or CTLA-4 is more critical in the differentiation fate of non-polarised Th cells rather than the B7 molecules themselves (Khattri et al., 1999, Oosterwegel

et al., 1999b). Although, emerging evidence strongly support the importance of B7 molecules in signalling to regulatory T cells. Mice lacking B7-1 and B7-2 have a profound decrease in the $CD4^+$ $CD25^+$ regulatory T cell subset population (Salomon et al., 2000). In the absence of signal 2, Th cells become anergic (Gimmi et al., 1993); this is important when DC present self-antigens to naïve Th cells.

TCR stimulation and co-stimulation allow naive Th cells to develop into effector Th cells, undoubtedly accompanied by high-level expression of selective sets of cytokines (Curtsinger et al., 1999). The balance of these cytokines and the resulting class of immune response strongly depend on the conditions under which DC are primed for the expression of the T-cell-polarizing molecules, which constitute **signal 3**. Indeed, bioactive pathogen-derived compounds that polarize DC for their expression of these Th-polarizing cytokines largely tune the adaptive immune response to pathogens. These DC-derived cytokines with Th-cell-polarizing capacity have been well documented and are integral to T-cell polarisation (Figure 1.3.1). Several Th1-cell-polarizing cytokines are interleukin (IL)-12 and its family members IL-23 and IL-27, and Type 1 IFN; whereas Th2-cell-polarizing cytokines are monocytic chemotactic protein 1 (MCP-1)/CCL2; and the regulatory T-cell-polarizing factors IL-10 and transforming-growth factor-β (TGF-β) (de Jong et al., 2005, Kapsenberg, 2003). DC activation also triggers production of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 (Blanco et al.,

2008), which are suspect in the initial inflammatory scenario.

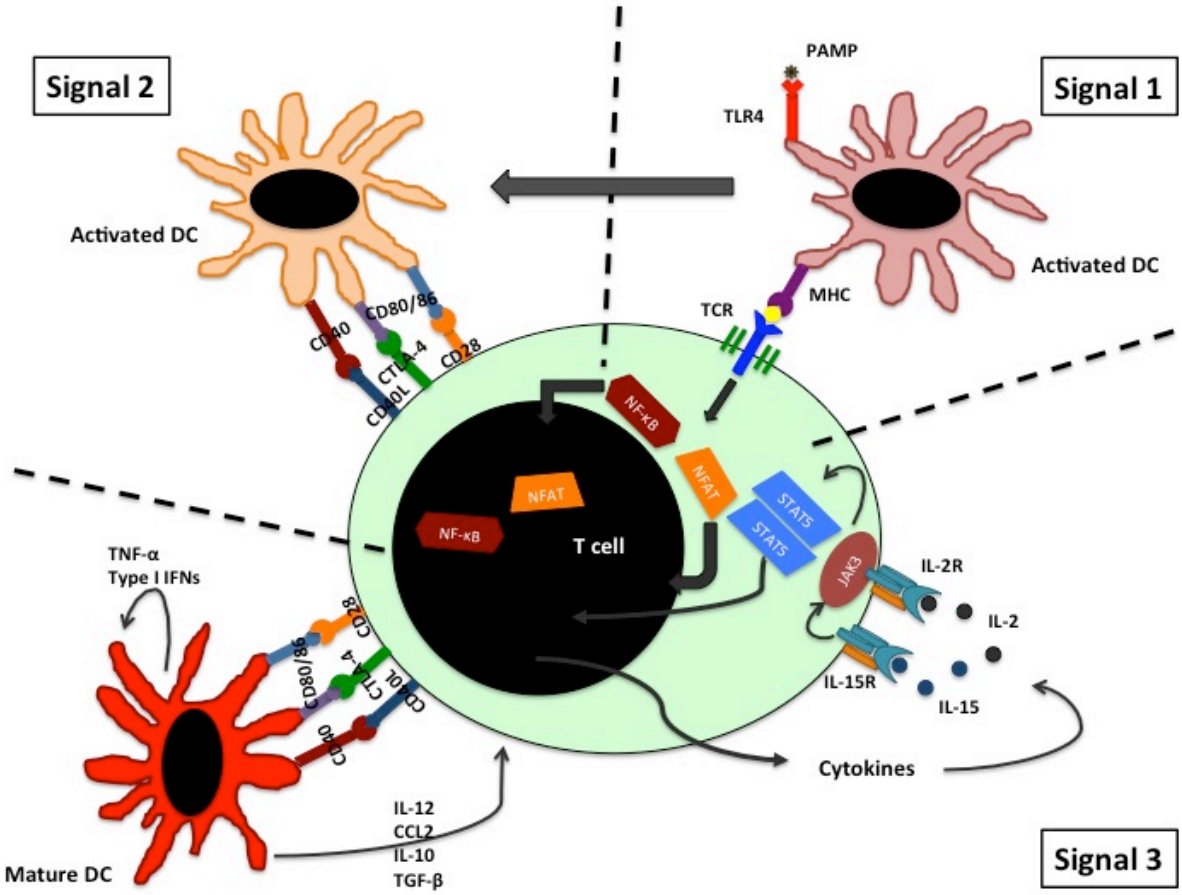

Figure 1.3.1. The three signals required for activating naïve T cells.

The three signalling events necessary for T cell activation: 1. T-cell receptor engagement and the activation events signalling through the TCR and the co-receptor, CD3. 2. Co-stimulatory molecule ligation and the signalling cascades through their counterparts. 3. Cytokine receptor signalling acting either in an autocrine manner, such as DC-derived TNF-α inducing itself and IL-1β, or in a paracrine manner, for instance the polarising cytokines IL-12 and CCL2.

1.4. DENDRITIC CELL SUBSETS

The development of culture systems for the production of DC from monocytes or from myeloid precursors under the influence of granulocyte-macrophage colony stimulating factor (GM-CSF) has provided a readily available model for detailed study (Caux et al., 1996, Inaba et al., 1992). This unifying theory of an experimentally practical culture model has also lead to the discovery of DC with distinctive phenotypes within lymphoid tissue.

All DC subsets possess the fundamental functionalities: capture foreign and host entities and then process them into antigens for MHC presentation to T cells, which, in turn, become activated. But there are additional features of DC functions of immunological significance as well as many specialisations that provide biological rationale to justify the existence of multiple DC subtypes (Shortman, 2000).

The logic for the existence of different DC subsets is that DC are localised in various tissues and organs and consequentially these tissue-specific DC display differences in chemokine receptors, adhesion molecules and addressins to maintain their residency in their respective tissue sites. These distinct DC types also share

variation in their expression recognition and phagocytic-endocytic receptors, such as TLR, lectin receptors, allowing for recognition and response to different yet diverse ranges of insult and potential pathogens and also dictate functional differences. This possibly suggests that the actions of discrete DC subtypes streamline the initiation of the appropriate adaptive responses by influencing T cell activation and differentiation into unique effector T cell subsets to target the stimuli encountered (Manickasingham et al., 2003). DC are involved not only in inducing immunity, but also in regulating immune tolerance. Although these two central functions can be correlated to various developmental points of a single DC subset, it is plausible that further specialisation is required for inducing tolerogenic DC functional potential.

The presence of microbial products or tissue damage in the sampling environment of the DC initiates a transition from antigen-capturing cells to antigen-presenting cells that correlate with decreased antigen uptake, increased half-life of surface MHC-peptide complexes, upregulation of co-stimulatory molecules, alteration in chemokine receptor expression and production of cytokine that are central to effector T cell differentiation. Despite the fact that the transition results in little phenotypic resemblance to its immature counterpart, the lifecycle of DC during an immune response is a very well defined process; but not all DC life cycles share this common fate (Wu and Dakic, 2004).

A DC lineage-specific marker has not yet been identified and the subsets of DC in humans and mice are therefore currently defined by lineage$^-$ (Lin$^-$) MHC class II$^+$ (MHC-II$^+$) cells in combination with various cell surface markers. Although the development of murine DC from early haematopoietic precursors is currently a rapidly expanding field with intense intellectual dissection, there are five major DC subsets that have been identified, to date, in lymphoid tissues of uninfected mice. Phenotypically, all mouse DC are defined by a co-expression of CD11c and MHC-II; high expression levels of these markers is characteristic of all mature DC in mice (Metlay et al., 1990). Further characterisation is based on the expression four other surface markers that distinguishes the DC subsets; and these are CD4, CD8$\alpha\alpha$-homodimer, CD11b and DEC-205 (CD205). These murine DC subsets have loosely identifiable human DC equivalents (Figure 1.4.1).

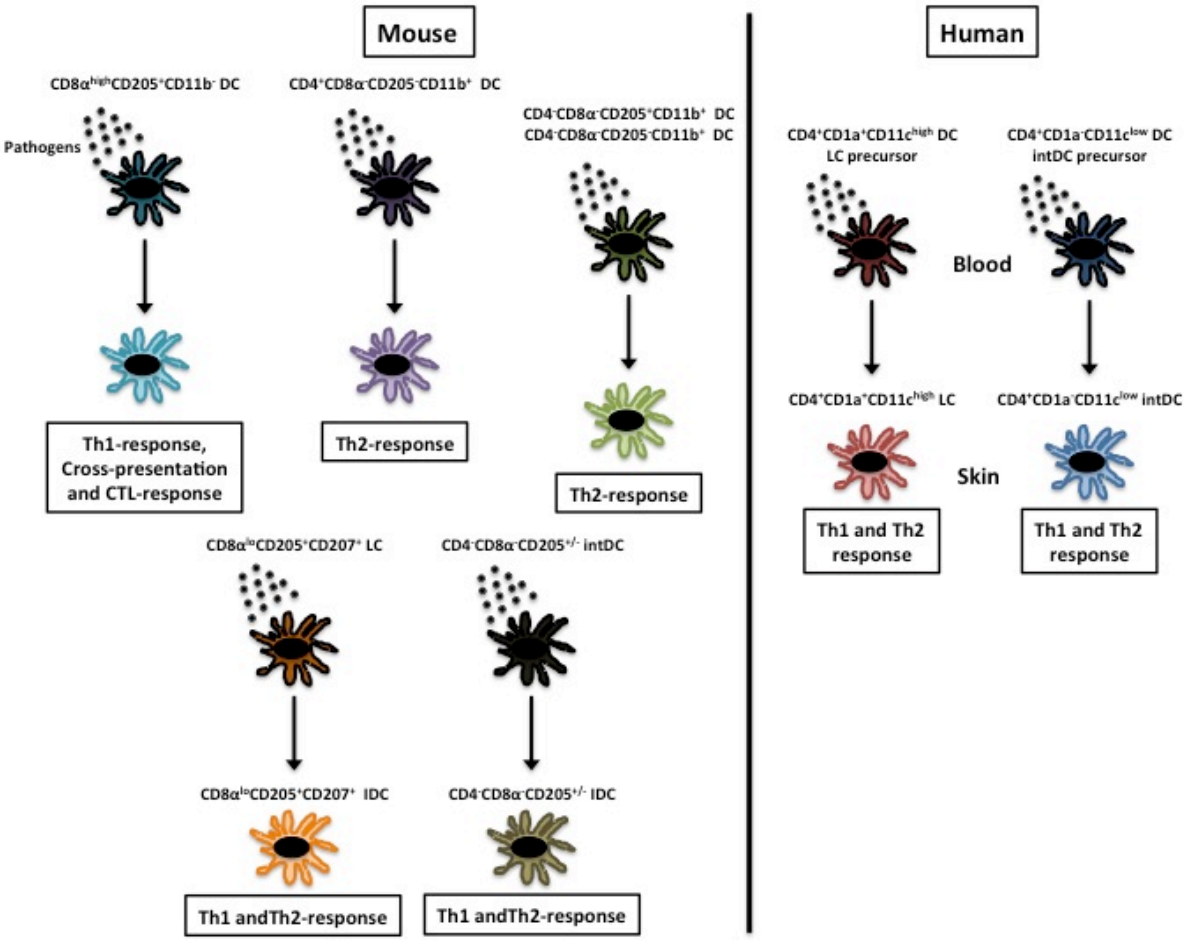

Figure 1.4.1. Dendritic cell subsets and their major function *in vivo*.

The different dendritic cell subtypes in human and mouse and their consequent influence on distinctive T cell responses.

Anatomically, the thymus, spleen and lymph nodes (mesenteric and skin-draining) are DC-rich sites of the lymphoid environments. The subset populations that constitute thymic DC include mainly $CD8\alpha^{+}CD4^{-}CD205^{hi}CD11b^{-}$ cells (~70%) and to a minor extent $CD8\alpha^{lo/-}CD4^{-}CD205^{+}CD11b^{-}$ cells (~30%) (Crowley et al., 1989, Henri et al., 2001, Vremec et al., 2000). However in the spleen and lymph nodes, only about 20% of DC are $CD8\alpha^{+}$ phenotype. The mouse $CD8\alpha^{+}$ DC are non-migrating resident DC derived from a bone marrow precursor, distinct from monocytes that continuously propagate the subset populous in lymphoid organs. These cells vary

in a number of central functions from their CD8α^- counterparts. In a homeostatic state, CD8α^+ DC are in an immature state with high surface levels of MHC class II, low but detectable levels of co-stimulatory molecules (Wilson et al., 2003) and produce TGF-β (Yamazaki et al., 2008). The CD8α^+CD205$^+$ DC are mainly found in T-cell rich areas of the peripheral lymphoid organs such as the periarteriolar lymphatic sheaths (PALS) of the spleen (Pulendran et al., 1997) and paracortical regions of the lymph nodes (Inaba et al., 1997). Upon activation or during intracellular pathogenic infection, they are chief producers of IL-12 and become the main presenters of pathogen antigens including efficiently cross-presenting exogenous cell-bound and soluble antigens on MHC class I (den Haan et al., 2000, Pooley et al., 2001). Therefore, promoting CD8α^+ CTL and antibody responses.

CD8α^+ DC also have immunoregulatory properties and help maintain tolerance to self-tissues. In the thymus, CD8α^+ DC are proposed to play a principal role in the purging of self-reactive T cells from the developing repertoire. Also, CD8α^+ DC are believed to have a unique regulatory role in preventing self-reactive responses (Fazekas de St Groth, 1998). *In vitro* studies revealed that when immature splenic CD8α^+ DC, isolated from mice, are co-cultured with primary CD4$^+$ or CD8$^+$ T cells, or secondary CD4$^+$ T cells they produced reduced proliferative responses in all cases compared to CD8α^- DC counterparts (Kronin et al., 2001, Winkel et al., 1994). Further

studies have shown that despite equivalent activation states between CD8α^+ and CD8α^- DC as well as similar initial T-cell activation events, CD8α^+ DC induced a higher incidence of apoptotic T-cell death per division cycle (Rizzitelli et al., 2006). These early signals from CD8α^+ DC to T cells control the extent of T-cell proliferation; the nature of the signals is unclear but it is apparent that it is not Fas-ligand dependent (Suss and Shortman, 1996, Winkel et al., 1997).

All DC subsets express some members of the TLR family, for instance TLR9, but CD8α^+ DC exclusively express TLR3 that equip these cells with the ability to mount potent immune responses against viral infections (Belz et al., 2004). In addition to being the instigators of robust CTL responses, in the periphery, CD8α^+ DC are restricted to lymphoid organs therefore, these cells are highly capable of acquiring antigens transferred from migrating DC that enter lymph nodes (Carbone et al., 2004, Vermaelen et al., 2001) as well as cross-presentation (den Haan et al., 2000). Consequently, CD8α^+ DC are noteworthy in directing CTL immunity, recalling virus-specific memory CD8$^+$ T cells (Belz et al., 2007) and priming T cells against intracellular infections such as *Listeria monocytogenes* (Belz et al., 2005), *Salmonella typhimurium* (Kirby et al., 2002), *Toxoplasma gondii* (Yarovinsky et al., 2006, Yarovinsky et al., 2005) and malarial *Plasmodium* spp (Lundie et al., 2008, Sponaas et al., 2006).

Although most characteristic DC surface markers, like CD4, are also

expressed on human DC, CD8α is not. But several recent reports described BDCA3$^+$ (blood dendritic cell antigen 3 or thrombomodulin; CD141$^+$) DC as the human DC equivalent to murine CD8α DC. Villadangos and Shortman reviewed this human equivalent to the CD8α$^+$ DC and highlighted the similarities between the mouse and human counterparts (Villadangos and Shortman, 2010). BDCA3$^+$ DC are a subset member of conventional human blood DC but also are well known to possess CD8α$^+$ DC-like properties such as DNGR-1 (ClecA9) expression. Similar to CD8α$^+$ DC, BDCA3$^+$ DC effectively phagocytose apoptotic and dead cells and are also efficient at cross-presentation of antigens. Both BDCA3$^+$ and CD8α$^+$ DC subtypes exclusively express TLR3 that is crucial in viral recognition and are potent producers of IL-12, in concert, resulting in rigorous CTL responses. The DC subsets both require the transcription factors Baft3 and IRF-8 to drive their developmental pathways, but not IRF-4 (Villadangos and Shortman, 2010). The ethical and logistical implications to purifying lymphoid-resident human DC limit such probable identification and characterisation of human CD8α$^+$ DC. Although, one very compelling piece of evidence equating the myeloid-derived blood (BDCA3$^+$) DC and the lymphoid-resident CD8α$^+$ DC is the restricted expression of the chemokine receptor XCR1. Mouse CD8α$^+$ DC are the only murine cells known to express this molecule. XCR1 is responsive to its ligand XCL1, also known as lymphotactin, an exceedingly specific and potent chemokine for CD8α$^+$ DC subset. XCL1 is abundantly secreted chemoattractant by

activated $CD8^+$ T cells allowing recruitment these DC subsets to CD8 T cells after antigen recognition - increasing the pool of antigen-specific CD8 T cells and their capacity to produce IFN-γ (Dorner et al., 2009).

The $CD8\alpha^-$ DC subset can be delineated into three additional subtypes based on further characterisation. These are the $CD4^+CD8\alpha^-CD205^-CD11b^+$ population, which is predominately present in the spleen (50-70% of splenic DC), and the $CD4^-CD8\alpha^-CD205^+CD11b^+$ ('double-negative') and the $CD4^-CD8\alpha^-CD205^-CD11b^+$ ('triple-negative') populations, majority of which are found in the lymph nodes. In the mesenteric lymph nodes, the $CD4^-CD8\alpha^-CD205^-CD11b^+$ subset comprise about 40% of the total LN DC whilst skin-draining LN DC constitute similar proportions (20-30%) of the $CD4^-CD8\alpha^-CD205^+$ and the $CD4^-CD8\alpha^-CD205^-$ DC, and about 10-20% of the $CD8\alpha^+CD205^+$ DC subsets (Shortman and Liu, 2002). Interestingly, also in skin-draining lymph nodes of healthy mice, about 30% of DC are $CD8\alpha^{lo}CD205^+$ that express high levels of MHC class II and co-stimulatory molecules as well as langerin (CD207). It is suggestive that these cells are post-migrating Langerhans cells (Pulendran et al., 1997).

The $CD4^+CD8\alpha^-CD205^-CD11b^+$ DC, $CD4^-CD8\alpha^-CD205^+CD11b^+$ DC and $CD4^-CD8\alpha^-CD205^-CD11b^+$ DC from the spleen and lymph nodes all resemble cells of a myeloid lineage; furthermore, $CD4^+CD8\alpha^-CD205^-$

$CD11b^+$ DC and $CD4^-CD8\alpha^-CD205^-CD11b^+$ DC even express myeloid markers like 33D1 and F4/80 (Vremec et al., 2000). They are localised to the marginal zone and migrate to T cell areas upon stimulation; both subsets are effective stimulators of $CD4^+$ and $CD8^+$ T cells and demonstrate efficient MHC class II presentation to antigen-specific $CD4^+$ T cells (Pooley et al., 2001). $CD4^+CD8\alpha^-$ DC and $CD4^-CD8\alpha^-$ DC do differ phenotypically and biologically. The former has a greater adhesion capacity, higher levels of F4/80 expression but lower cytokine production post-stimulation compared to the latter (Hochrein et al., 2001, Pulendran et al., 1997, Vremec et al., 2000).

The relationship between these subsets in the spleen and LN was addressed using continuous BrdU labelling of DC precursors *in vivo*, which illustrated that all five DC subsets arise from independent, dividing, self-renewing precursors, i.e. one subset does not seem to mature into another subset. These populations are short lived in lymphoid tissues (3-5 days) (Kamath et al., 2002). It had been proposed that the different DC populations originated from lymphoid or myeloid cell lineages principally based on cell surface marker expression and the common development of $CD8^+$ DC and lymphocytes from $CD4^{lo}$ early thymic precursors; further studies have either supported or contested this concept (Ardavin, 2003, Shortman and Liu, 2002). However, it is now apparent that the major classes of DC can be derived from either a common myeloid or common

lymphoid haematopoietic precursor, as well from a specific blood derived DC precursor (del Hoyo et al., 2002); in addition, $CD8\alpha^+$ and $CD8\alpha^-$ DC may arise from peripheral blood monocytes upon extravasation into tissues (Leon et al., 2004), similar to DC differentiation from monocytes upon migration into the lymph in humans (Randolph et al., 1998, Randolph et al., 1999, Randolph et al., 2002).

Research on murine DC has been vastly extensive in contrast to the relatively few studies on human DC due to the ease of accessibility of freshly isolated DC. Blood is the only readily available source of human DC with rare cases of lymphoid tissues such as tonsils, spleen and thymus as a source. As aforementioned, human DC are defined by Lin^- $MHC\text{-}II^+$ cells and, like mouse DC, consist of several subset populations of which, all express CD4. These subset differences might be a reflection of maturation status rather than distinct DC lineages (Sato and Fujita, 2007).

Human DC are divided into two subsets of conventional $CD11c^+$ DC of a myeloid origin and a $CD11c^-$ plasmacytoid DC of a lymphoid lineage. The two conventional myeloid DC are present in peripheral blood and express the blood dendritic cell antigens (BDCA). These are the $CD4^+CD11c^{high}CD1c^+$/BDCA-1^+CD1a^+ DC comprising of 0.6% of PBMC and the $CD4^+CD11c^{low}CD141^+$/BDCA-3^+CD1a^- DC, which are found only in trace numbers in blood (<0.05% of PBMC) (Narbutt et

al., 2004, Dzionek et al., 2000). Under appropriate culture conditions, BDCA-1^+ DC give rise to cells with characteristic LC features of E-cadherin and Langerin expression with typical Birbeck granules but not BDCA-3^+ DC; therefore, BDCA-1^+ DC and BDCA-3^+ DC are probable direct precursors of LC and interstitial DC (intDC) in peripheral blood respectively (Ito et al., 1999). Both myeloid DC subsets are capable of stimulating T cells and their T-cell stimulatory capacity is enhanced with GM-CSF stimulation. These DC can also elicit Th1 or Th2 responses depending on the inflammatory environments (Eksioglu et al., 2007, Liu et al., 2001, Liu, 2001, Liu et al., 2007, Wang et al., 2006).

1.5. DENDRITIC CELL BIOLOGY

1.5.1. Dendritic Cell Activation: Immature versus Mature DC

In most tissues, DC are present in an immature state and are generally incapable of stimulating T cells. Although these DC lack the co-stimulatory molecules required for full T cell activation, they are very well equipped to capture antigen in the periphery. Once immature DC have encountered foreign antigens (soluble or processed from a pathogen), they initiate maturation and migrate to the T cell areas of secondary lymphoid organs where the mature DC activate the adaptive immune response.

Immature DC have several means of antigen capture. Firstly, they can engulf particles and microbes via phagocytosis (Caux et al., 1992, Svensson et al., 1997). Secondly, immature DC can perform a process called macropinocytosis whereby large pinocytic vesicles are formed that endocytose extracellular fluid and soluble particulates (Sallusto et al., 1995). The vesicular contents are then sampled by the DC for foreign antigens. The third method is *via* receptors that mediate adsorptive endocytosis, which include the carbohydrate receptors – C-type lectin receptors, mannose receptors and DEC-205,

as well as the immunoglobulin receptors Fcγ and Fcε receptors (Jiang et al., 1995, Sallusto and Lanzavecchia, 1994).

In immature DC, macropinocytosis and receptor-mediated antigen uptake are exceedingly proficient processes, requiring picomolar and nanomolar concentrations of antigen, considerably lower levels typically required by other APC. However, once a non-domestic entity is encountered or a non-self antigen is captured, signals are transduced to mature DC and their ability for antigen sampling is rapidly lessened.

The captured pathogen or antigens enter the endocytic pathway and are directed to specialised lysosomes containing large amounts of class II MHC molecules. These MHCII-rich compartments (MIIC), which are abundant in immature DC, make it possible for DC to display numerous cell surface class II MHC-antigen complexes (Nijman et al., 1995, Pierre et al., 1997, Winzler et al., 1997). During DC maturation, MIIC convert to non-lysosomal vesicles and release their MHCII-antigen complexes onto the cell surface (Cella et al., 1997a, Pierre et al., 1997).

Once activated, DC migrate to lymph nodes to present antigen on MHC complex to naïve lymphocytes, which become activated and differentiate into effector T and B cells and memory T and B cells. DC maturation is crucial for the initiation of antigen-specific immunity and this process is characterised by reduced antigen-capture

capacity, and increased surface MHCII expression and surface expression of co-stimulatory molecules. Full DC maturation is only achieved upon interaction with appropriate T cells, i.e. T cells with the TCR that recognise the antigen being presented. Expression of the co-stimulatory molecules on mature DC that interact with appropriate receptors on T cells to enhance adhesion and TCR signalling include CD54, CD58, CD40, CD80 and CD86. Dendritic cells are a major source of many cytokines, namely, IFN-α, IL-1, IL-6, IL-7, IL-12, IL-15 and MIP-1γ, all of which are important in eliciting of a primary immune response (Cella et al., 1996, Grouard et al., 1996, Macatonia et al., 1995, Mohamadzadeh et al., 1996, Strobl et al., 1996, Zhou and Tedder, 1995).

1.5.2. TLR4 and other TLR Signalling Pathways

Signalling via TLR expressed by DC results in an array of activation events including phenotypic and morphological alterations, cytokine production and migration. TLR4 signalling pathway is the best described of all TLR signalling pathways and selectively binds LPS but does not solely recognise LPS. LPS binding to TLR4 is aided by its co-receptor, CD14, which forms as a complex with a membrane-bound molecule, myeloid differentiation protein 2 (MD-2 also know as lymphocyte antigen 96, LY96). LPS binds initially to CD14 and then to TLR4-MD-2 receptor complex. TLR4 undergoes a conformational change in the cytoplasmic domain in response to LPS binding. The intracellular signalling domain of each TLR is known as the Toll/Interleukin-1 (IL-1) receptor (TIR) domain, which confers the conformational change and signals the recruitment of the cytoplasmic protein, myeloid differentiation factor 88 (MyD88), which possesses a TIR binding motif, and binds to the TIR and acts as an adaptor for signal transduction. A central adaptor molecule required for TLR signalling, MyD88 becomes recruited by homophile TIR interactions to the corresponding TLRs (Medzhitov et al., 1998). Consequently, IL-1R-assocated kinase (IRAK) 4 becomes recruited, which binds to IRAK1 and forms a complex with the tumour necrosis factor (TNF) associated factor (TRAF) 6. IRAK1 and TRAF6 dissociate from the TLR complex and TRAF6 then forms a complex with the transforming

growth factor (TGF)-β activated kinase 1 (TAK1) and the TAK1 binding molecules TAB1 and TAB2. This leads to the activation of TAK1 which in turn results in activation of two signal transduction pathways the nuclear factor (NF)-κB pathway and the mitogen-activated protein (MAP) kinase pathway which includes p38, c-Jun N-terminal kinase (JNK) 1/2 and extracellular signal-regulated kinase (ERK) 1/2 (Akira and Takeda, 2004, Wagner, 2001). NF-κB and activator protein 1 (AP-1), a gene product of the Jun oncogene, transcriptionally activate several pro-inflammatory genes, particularly the cytokines, chemokines and other inflammatory mediators.

TLR2 and TLR9 subfamilies as well as TLR5 are wholly dependent on MyD88 for signalling; during MyD88-dependent TLR2 and TLR4 signalling, MyD88 heterodimerizes with the adaptor molecule TIR domain-containing adaptor protein (TIRAP also named Mal) (Horng et al., 2002). Knockout studies have confirmed that many, but not all, TLR signals are abolished by targeted deletion of MyD88 gene; thus defining alternative signalling pathways to LPS/TLR4 responses (Yamamoto and Akira, 2005).

TLR3 and TLR4 exploit this additional signalling pathway, which is MyD88 independent. These signals utilize the adaptor molecule TIR domain-containing adaptor inducing interferon (IFN)-β (TRIF also named TICAM). With respect to TRIF-dependent TLR4 signalling, an auxiliary adaptor protein called TIR domain-containing adaptor (TRAM

also known as TICAM-2) is involved (Fitzgerald et al., 2003b, Yamamoto et al., 2003b). This pathway induces delayed activation of NF-κB and IFN regulatory factor (IRF)-3 that results in MyD88-independent DC maturation and induction of IFN-β respectively.

Collectively, these converging signalling cascades acting via distinct Toll-like receptors are graphically represented in the simple schematic below (Figure 1.5.2.1).

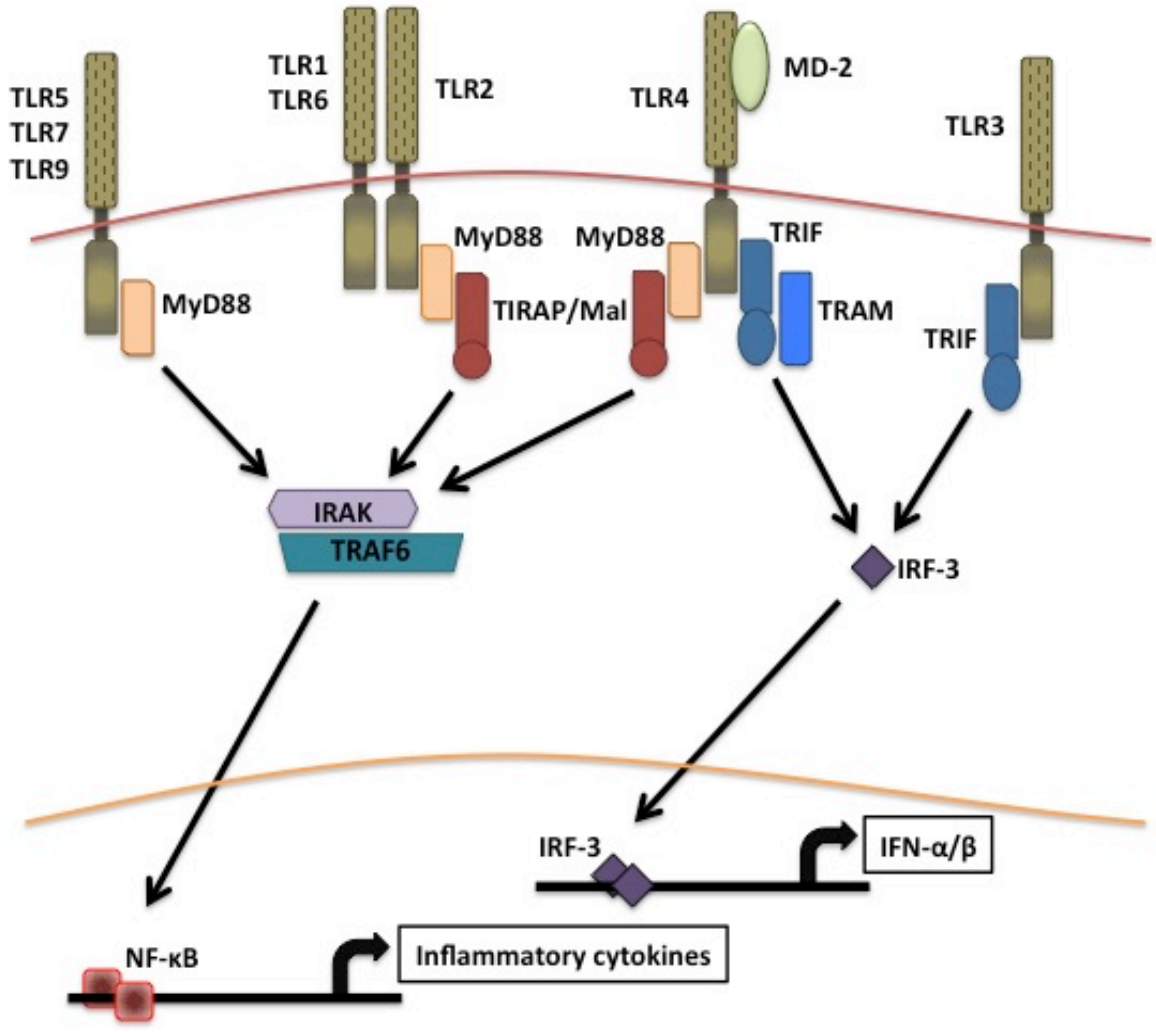

Figure 1.5.2.1. Schematic representation of the TLR post-receptor early signalling events.

Illustration showing the adaptor proteins that directly associate with the Toll/IL-1 receptor (TIR) domains and the early signalling molecules that lead to activation of individual transcription factors.

The activation of NF-κB is initiated by either activation of TRAF6 or receptor interacting protein (RIP) 1 upon binding to TRIF (Meylan et al., 2004, Sato et al., 2003). The cascade resulting in the activation of IRF-3 depends on the non-canonical IκB kinase homologs, IκB kinase-epsilon (IKKε also known as IKK*i*) and TANK-binding kinase-1 (TBK1) (Fitzgerald et al., 2003a, Sharma et al., 2003). Following an

initial NF-κB and IRF-3 activation, transcription factor-dependent induction of IFN-β and IFN-α4 occurs; these cytokines acting through their IFN-I receptors activate the Janus kinases (JAK) that then phosphorylate signal transducer and activator of transcription (STAT) family of transcription factor (TF) proteins. The STAT proteins, in turn, induce IRF-7 thus initiating transcription of multiple IFN-α genes such as the IFN-inducible protein (IP-10) gene and the inducible nitric oxide (NO) synthase (iNOS) gene (Levy et al., 2002, Takeda and Akira, 2005).

1.6. NUCLEAR FACTOR (NF)-κB: THE PLURIPOTENT TRANSCRIPTION FACTOR

In nearly all nucleated cells, NF-κB serves as a comprehensive transcription factor that regulates numerous genes, which play significant roles in inter- and intra-cellular signalling, stress responses, cellular growth, survival and apoptosis (Baichwal and Baeuerle, 1997, Baldwin, 2001, Guttridge et al., 1999, Karin and Lin, 2002). NF-κB is activated by a broad range of agents including cytokines, viruses, lipopolysaccharide, T cell mitogens and okadaic acid. It is surprising how all these assorted agents, which elicit different intracellular reactions, can cause the same event, that is, the release of IκB from NF-κB (Barnes, 1997, Schmidt et al., 1995a, Schmidt et al., 1995b, Traenckner et al., 1995, Thanos and Maniatis, 1995). Control of NF-κB-responsive genes, in a selective and temporal manner, is of biological importance particularly in physiological and patho-physiological inflammatory scenarios (Hoffmann et al., 2006, Hoffmann et al., 2002).

Five subunits partake in NF-κB functions: RelA/p65, cRel, RelB, p50 and p52 that form various dimers with each other to produce biologically active species and virtually in all cells, the p65:p50 heterodimer is the most prevalent complex (Hoffmann and Baltimore,

2006, Siebenlist et al., 1994). Genetic studies of NF-κB binding sites have shown that its binding to regulatory sequences (promoter and/or enhancer regions) is responsible for transcriptional activation of genes in response to stimulation of cells by IL-1, TNF-α, bacterial LPS, double-stranded RNA and active phorbol esters. Published works have shown that NF-κB is present in the cytosol in a sequestered form, of resting cells, by a family of inhibitors called IκB (inhibitor of κB), which when activated is released from its inhibitory proteins. This inhibitor of DNA binding selectively and reversibly inactivates NF-κB (Baeuerle and Baltimore, 1989, Baeuerle and Baltimore, 1988b, Baeuerle and Baltimore, 1988a). Stimulated cells induce signalling cascades to activate IκB kinase (IKK) complex, which phosphorylates all three IκB isoforms (IκB-α, -β and -ε) followed by ubiquitination. The ubiquitinated IκB proteins enter the proteolytic pathway and are degraded allowing NF-κB to localise to the nucleus and bind DNA (Ghosh et al., 1998).

Previous literature has highlighted that overexpression of the p65 subunit, by transfection or retroviral infection, markedly increases endogenous IκB-α mRNA and protein but overproduction of p50 is unable to induce a transcriptional response of the IκB-α gene. Free IκB protein is unstable therefore at basal conditions IκB levels must continuously equate or exceed those of the transcription factor. However, interaction between the IκB and p65 stabilises the inhibitory protein allowing protein levels to increase rapidly. In

addition to protein stabilisation, p65 also enhances the induction of the IκB-α gene thus rapidly increasing mRNA and protein levels (Scott et al., 1993, Brown et al., 1993). Hence, activation of many cells types by specific stimuli such as LPS, phorbol 12-myristate 13-acetate (PMA), or TNF-α would lead to dissociation of p65:p50 heterodimer from IκB and IκB degradation. The free NF-κB binds to consensus sequences of DNA and helps promote the initiation of transcription of many genes, in particular the p65 subunit supports the transcription of IκB gene. The inhibitor protein levels, specifically IκB-α, accumulate and bind NF-κB, which becomes deactivated. To surmise, IκB-α synthesis is controlled by an exceedingly NF-κB-responsive promoter causing autoregulation of NF-κB signalling (Figure 1.6.1) (Scott et al., 1993).

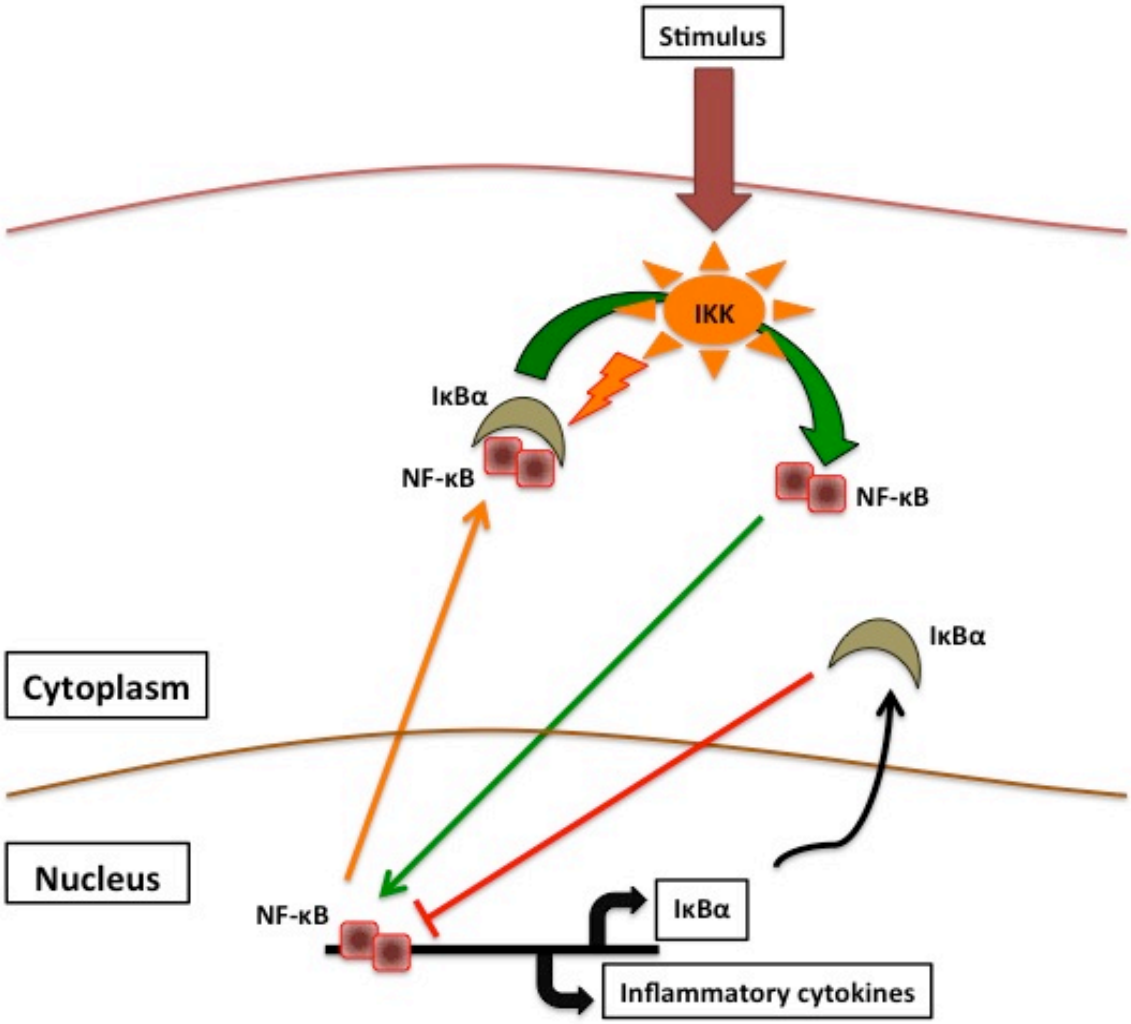

Figure 1.6.1. Negative feedback loop of the IκB-α–NF-κB signalling cascade.

NF-κB is held as an inactive complex in the cytoplasm by the IκB protein isoform complex. Cells exposed to stimuli leads to IκB kinase (IKK) activation, which then phosphorylate the three IκB isoforms of the protein complex and results in their degradation; allowing for NF-κB nuclear translocation. Translocated NF-κB binds to responsive elements of promoter regions and transcribes inflammatory genes as well as the genes of IκB isoforms: IκB-α. IκB-β (not shown) and IκB-ε (not shown). IκB-α induction is the most predominant of the inhibitor proteins and is responsible for cytosolic sequestering of NF-κB and arresting NF-κB activity.

Genetic analyses have further dissected the precise role of each IκB isoforms in regulating NF-κB. Deletional studies reveal that expression of only IκB-α results in an extremely oscillatory NF-κB response indicating that after NF-κB activation, there is a rapid and potent inhibition of NF-κB by newly synthesized IκB-α. Further stimulus signal results in a swift IκB-α degradation, releasing and activating NF-κB and thus creating a cycle of sinusoidal activation patterns of NF-κB. In contrast, solitary expression of the IκB-β or the IκB-ε isoforms lead to an uniform increase in nuclear NF-κB that eventually plateau without further suppression (Hoffmann et al., 2002). Unlike IκB-α, neither IκB-β nor IκB-ε can completely inhibit NF-κB activity and also highlights redundancy of inhibitory activity between these two isoforms.

Both IκB-α and IκB-β are inactivated via protein kinase A and C signalling pathways, but in contrast to IκB-α, IκB-β loses its inhibiting activity through a dephosphorylation process (Link et al., 1992). Therefore, IκB-β is only partially degraded in response to most extracellular signals. Unphosphorylated, resynthesized IκB-β forms a stable complex with NF-κB in the cytosol; however, this binding fails to mask the nuclear localization signal and DNA binding domain on NF-κB, and the IκB-β–NF-κB complex enters the nucleus allowing transcription of NF-κB-inducible genes (Chen et al., 2004, Suyang et al., 1996).

IκB-β exists in at least two different forms: one that is bound to the NF-κB dimer and the other bound to both NF-κB and κB-Ras, a Ras-like small G protein. Removal of cellular κB-Ras augments induced IκB-β degradation whereas excess κB-Ras impedes induced IκB-β degradation and astonishingly, κB-Ras functions in both GDP- and GTP-bound states. While signal-induced degradation of IκB-α is responsible for rapid, oscillatory pattern of NF-κB activation; prolonged activation of NF-κB, which is fundamental for certain biological functions such as T-cell activation, requires IκB-β degradation (Chen et al., 2003, Chen et al., 2004).

1.7. IMMUNE TOLERANCE

Immunological tolerance is the failure to mount an immune response to an antigen; it is not simply a failure to recognise an antigen but an active process of detection of a particular epitope with equal specificity as an immunogenic response. There are two main modes of tolerance: natural or self-tolerance and induced tolerance (Khurana, 2006, Miller et al., 2009, Stockinger, 1999). Self-tolerance is the recognition of self-antigens without eliciting an immune response against them. This mechanism is paramount to lymphocyte development especially T cells. The second type of tolerance is a deliberate manipulation of adaptive immunity to usually nontoxic environmental antigens. A break in this induced tolerance typically results in atypical allergic reactions to food, insect sting and plant pollen or abnormal host rejection reactions such as miscarriage of pregnancy (Nossal, 1993, Weiner et al., 2011, Munoz-Suano et al., 2011, du Pré and Samsom, 2011, Guerin et al., 2009, Saito et al., 2007, Mahnke et al., 2003) Both T and B cells can be tolerised but a greater emphasis is aimed towards T cell tolerance. Overall B cell tolerance is not a rigidly regulated system as T cell tolerance but tolerising B cells is still a noteworthy procedure to general immunoregulation. B cells require the aid of T cells to make antibodies to most antigens hence tolerising T cells in general

prevents B cell differentiation into antibody-secreting plasma cells (Janeway, 2005). T and B cell tolerance is subdivided into central tolerance and peripheral tolerance.

Central tolerance of T cells is critical for their normal development in the thymus and usually occurs in tandem with the MHC restriction process. During sampling of self-peptides to determine class I or class II MHC recognition, developing T cells also must surpass the checkpoints that monitor the affinity and strength of TCR binding to these self-antigen/MHC complexes. T cells that recognise self-antigens too strongly are prevented to proceed through development and receive signals to induce apoptosis. This mechanism is termed negative selection (McCaughtry and Hogquist, 2008). The APC of the thymus, thymic DC and thymic medullary epithelial cells (TEC), present peptide fragments derived from many housekeeping genes found in all cells but some proteins are only expressed in differentiated cells, such as insulin and the thyroxine pro-protein, thyroglobulin. Thus preventing maturation of reactive T cells against such proteins, thymic APC largely thymic DC express the transcription factor, autoimmune regulator (AIRE), which can initiate the transcription of numerous tissue-specific gene products that are presented during negative selection (Heino et al., 1999, Ramsey et al., 2006). This checkpoint affords the necessary signals to T cells to continue developing appropriately therefore, mature naïve T cells that emerge from the thymus are sufficiently equipped to mount immune

responses against non-self peptides and not self peptides (Palmer, 2003, Prud'homme, 2004).

Central tolerance of B cells occurs in a similar fashion as T cells that is, checkpoint controls during development into immature B lymphocytes. B cells are generated and differentiate in the bone marrow and a vast proportion of developing B cells construct a polyreactive BCR that are capable of binding self-antigens. Like T cells, B cells with a self reactive BCR that binds robustly to self-antigens receive signals that halt their development. Instead these B cells suffer one of two fates: either induce cell death via BCR signalling upon self antigen ligation or instigate **receptor editing** program of the BCR, which induces rearrangement of the gene segments that encode the heavy and light chains of the BCR in order to construct a new BCR that is not self reactive. Additionally, B cells that bind antigens with lower avidity become anergic and induce developmental arrest due to lack of receptiveness for survival signals (Ferry et al., 2006, Goodnow, 1992).

Mature naïve T and immature B cells that immigrate into the periphery are reasonably safe but despite central tolerance, some self-reactive lymphocytes do escape this strict system and enter the periphery. Activation of these lymphocytes may result in autoimmune or immunopathological diseases and thus they do need to be made tolerant in the periphery.

Peripheral tolerance, like central tolerance is heavily geared towards regulating T cell responses as apposed to B cell responses. Peripheral T cell tolerance is achieved via several mechanisms: these are characterised as **immunological ignorance**, **deletion**, **regulation** and **suppression**. Immunologic ignorance occurs when autoreactive T cells fail to encounter their self-antigen due to anatomical barriers preventing access to the particular tissue sites. Under physiological conditions, these immune privileged sites, such as the eye, testes and brain, restrict T cell entry. Therefore the resident DC in these tissues cannot present self antigens to possibly autoreactive T cells, thereby maintaining self tolerance (Butcher and Picker, 1996).

Self-peptides presented to T cells by dendritic cells usually occur in the absence of co-stimulation and thereby fail to prime. These T cells commit to either of two fates; one of which is CD95- or Fas-mediated cell death. Engagement of the Fas receptor with its ligand (FasL) induces apoptosis in Fas-positive cells. The importance of this mechanism is illustrated by constitutive expression of FasL by DC and other cell types of immune privileged sites; T cells granted entry into such organs, for instance the eye, that are CD95-positive will undergo apoptosis without compromising tissue homeostasis (Griffith and Ferguson, 1997, Griffith et al., 1995).

Regulation of T cell responses is another process of maintaining self-

tolerance in the periphery and it broadly occurs by two means: **anergy** or **inhibition**. Anergy is the alternative outcome to deletion when presentation of antigens occurs in the absence of co-stimulation. Instead of T cell apoptosis in a Fas-dependent manner, insufficiently primed T cells fail to produce IL-2, the quintessential cytokine required for complete T cell activation, and thus regulating the action of T cells. The other approach to T cell regulation is inhibition; T cells that do encounter antigen in the presence of co-stimulation must engage the co-stimulatory B7 ligands on DC with the co-stimulatory receptor CD28 instead of the co-inhibitory receptor CD152 (or CTLA-4) on T cells for full activation. In order to prevent aberrant T cell activation, CD152 preferentially binds the B7 ligands on DC and B cells inhibiting T cell activation or responses of activated T cells. Whilst this signalling path is not the only co-inhibitory system (another is PD-1 with PD-L1 and PD-L2 (Carter et al., 2002, Freeman et al., 2000)), the B7-CTLA-4 cascade is the most robust co-inhibitory signalling pathway (Brunner et al., 1999, Oosterwegel et al., 1999a, Rudd, 2009).

Dysregulation or breach of either central or peripheral tolerance has been associated with the development of autoimmunity, and though adaptive immunity appears to be a central component of autoimmune pathogenesis, clearly a variety of cell types contribute to autoimmune diseases (Benson et al., 2010). It has recently been proposed that the progression to autoimmune pathologies is a four-step process,

which does not require antigen-specificity. These four hypothesized steps are:

(1) T cell activation regardless of antigen specificity;

(2) Local events inducing tissue-specific accumulation of activated T cells;

(3) Enhanced sensitivity to T cell-derived cytokines in populations of cells in affected tissue;

(4) Activation of a cytokine-dependent IL-6 amplification loop**,** triggered by CD4+ T cell-derived cytokines such as IL-17A (Ogura et al., 2008).

In general, mechanistic understanding of human autoimmune diseases results from aberrant responses that were not centrally tolerised or are peripherally breached. Unlike many other conditions, animal models of autoimmune diseases have tended to focus on the later stages of disease pathogenesis. However, recent clinical studies tracking biomarkers associated with disease development revealed that the most critical checkpoint, breach of self-tolerance, occurs months or years prior to clinical presentation Therefore, little is known about the immunological processes that lead to the spontaneous loss of tolerance that is characteristic of human disease. This represents a bottleneck to the clinical development of prevention and early intervention strategies. (Benson et al., 2011, Rantapää-Dahlqvist et al., 2003, Majka et al., 2008, Nielen et al., 2005)

Immunosuppression is the fourth form of T cell tolerance. Unlike the other means of T cell tolerance that involve the functionality of APC predominantly DC but on occasion B cells, this method is commonly employ a unique effector T cell – regulatory T cell (Treg), to immobilize activation of naïve and effector memory T cells. Originally called suppressor T cells (Ts cells), the significance of T-cell mediated immunosuppression had not been fully appreciated until recently and hindrance in the development or function of Tregs is a major cause of autoimmune and inflammatory diseases (Sakaguchi et al., 2008). Extensive animal studies of normal mice involving thymectomy at different life stages resulted in tissue-specific autoantibody production and autoimmune damage. These findings provide surprising evidence that normal mice harbour potentially pathogenic autoreactive T cells but also dominantly functional Tregs that suppress autoimmunity (Sakaguchi et al., 1982). Tregs are represented by three major cell populations: the naturally occurring Treg, Tr1 cells, Th3 cells, $CD8^+CD28^-$ T cells and Qa-1 restricted T cells.

The naturally occurring $CD4^+CD25^+$ Tregs was first described in mice as a population of cells consisting of about 5-10% of the T helper subset that demonstrated regulatory properties both *in vivo* and *in vitro* (Sakaguchi et al., 2001, Sakaguchi et al., 1995). Using a colitis model in severe combined immunodeficient mice, injection of $CD4^+CD25^-$ T cells induced bowel disease while co-injection of these T

cells with $CD4^+CD25^+$ T cells prevented inflammation of the colon. Characteristically, naturally occurring Tregs are identified by their inability to proliferate upon TCR activation or stimulation via mitogenic antibodies.

These Tregs require exogenous sources of IL-2, albeit autocrine or paracrine, for suppressive function that uniquely involves the inhibition of transcriptional induction of IL-2 in $CD25^-$ T cells (Shevach, 2002, Thornton et al., 2004). Treg suppressor effect has been shown, at least with *in vitro* culture models, to be dependent mainly on cell-cell contact and partially on granzyme B (Gondek et al., 2005).

Animal investigations have highlighted that $CD4^+CD25^+$ Treg activation is antigen-specific and a requirement of their inhibitory function but these activated Tregs suppress T cell responses in a antigen-nonspecific manner; suggesting a broad spectrum regulatory role of these Tregs towards a variety of pathogen-specific effector T cells (Sakaguchi, 2003).

The $CD4^+CD25^+$ T-cell population is heterogeneous and only a proportion of this population exerts regulatory functions, namely the $CD4^+CD25^{high}$ T cells. Also, it is apparent that not all $CD25^+$ T cells exercise a suppressive activity. Therefore, Tregs are typical characterised by expression of intracellular CTLA-4, neuropilin-1

(Npr-1), glucocorticoid-induced tumour necrosis factor family-related receptor, CCR4, CCR8, CD62L (L-selectin), lymphocyte activation gene-3 (LAG-3) and CD103 (integrin $\alpha_\varepsilon\beta_7$). Naturally occurring Tregs specifically express the transcription factor forkhead box P3 (Foxp3), a master regulator of Treg development and function and a member of forkhead/winged-helix family of transcription factors. Foxp3 expression in T cells is quite restricted; only peripheral $CD4^+CD25^+$ T cells and $CD4^+CD25^+CD8^-$ thymocytes express the gene. Studies employing ectopic retroviral transduction of the *Foxp3* gene have shown that transduced $CD4^+CD25^-$ T cells are converted to $CD4^+CD25^+$ Treg-like cells with suppressive activity towards other proliferating T cells. Moreover, *Foxp3* transduction in naïve T cells upregulates the expression of CD25, CTLA-4 and the glucocorticoid-induced TNF receptor family-related gene (GITR), whereas it represses the production of IL-2, IL-4 and IFN-γ (Hori et al., 2003, Hori and Sakaguchi, 2004). Naturally occurring Tregs, in mice, also regulate T cell responses by modulation tryptophan metabolism, an essential amino acid required for proliferation of effector T cells. $CD4^+$ $CD25^+$ cells constitutively express CTLA-4; this surface antigen binds the B7 molecules on DC and triggers a B7–dependent induction of indoleamine 2,3-dioxygenase (IDO) activation in DC. IDO catalyses the initial tryptophan degradation reaction; this is the rate-limiting step for the degradation process and thus results in tryptophan deficiency in the microenvironment. Consequently, in a tryptophan-deprived environment, effector T cells undergo apoptosis.

1.8. TOLEROGENIC DENDRITIC CELLS

Each DC subset has exceptional functional plasticity and thus it was proposed that a specific DC lineage determines the outcome of the T cell effector subset, that is, tolerance or immunity. Several studies suggest that tolerogenic DC represent a particular subtype. Kronin *et al*. demonstrated that splenic $CD8\alpha^+$ lymphoid DC induce a lesser proliferative response in $CD4^+$ T cells than its $CD8\alpha^-$ lymphoid counterparts (Kronin et al., 1997b). Furthermore, these DC preferentially induce Fas-dependent apoptosis of $CD4^+$ T cells *in vitro* implicating their tolerogenic properties (Kronin et al., 1997b, Suss and Shortman, 1996, Kronin et al., 1997a). Conversely, numerous reports have counter argued this hypothesis by exhibiting that $CD8\alpha^+$ DC are also high producers of IL-12 that can stimulate cytotoxic $CD8^+$ T cells during antiviral immunity (Dalod et al., 2002). In humans and mice, plasmacytoid DC (pDC) are the most potent producers of type I interferon and are the key effector cells in early antiviral innate immunity (Barchet et al., 2005, Kadowaki et al., 2001). pDC-derived type I IFN might have a significant role in driving myeloid DC maturation with an immunogenic nature (Kadowaki and Liu, 2002).

During homeostasis, both myeloid and plasmacytoid immature DC efficiently capture apoptotic cells and non-pathogenic bodies from the environment by phagocytosis, macropinocytosis or endocytosis, but

these DC are unable to present antigens from these sources effectively and thus induce T cell anergy or a regulatory T cell phenotype (Dhodapkar and Steinman, 2002, Hawiger et al., 2001). This is due to low-level expression of MHC class II and undetectable levels of co-stimulatory molecules by immature DC – the surface receptors required for T cell activation. On the other hand, under steady-state conditions, DC that patrol periphery must undergo some spontaneous maturation indicative of CCR7 up-regulation (Ip and Lau, 2004), which is crucial for migration to lymphoid organs, thus supporting the hypothesis that maturing DC, not immature DC, are involved in triggering T cell tolerance (Huang et al., 2000, Scheinecker et al., 2002).

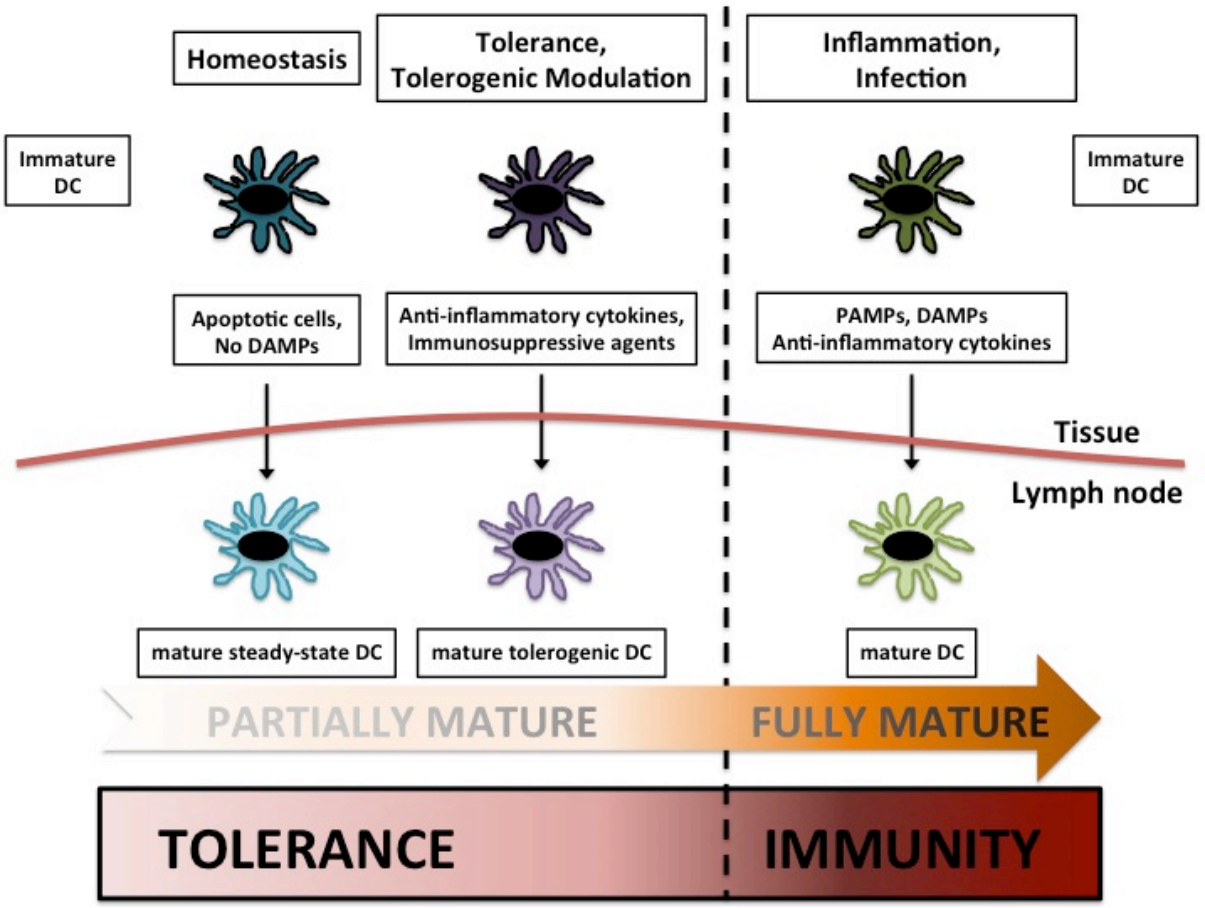

Figure 1.8.1. Dendritic cells are the homeostatic mediators in the balance between immunity and tolerance.

Schematic illustrating signals mediated to the adaptive immune arm by DC based on external stimuli and DC microenvironment that leads to either immunity or tolerance.

The phenotypical and morphological changes that occur when DC encounter tissue antigens and/or non-infectious material is a maturation process, which steady-state migrating DC evidently undergo (Figure 1.8.1). These steady-state migrating DC do not terminally differentiate into fully mature immunogenic DC but appear to arrest in a semi-mature state of maturation (Voigtlander et al., 2006, Lutz and Schuler, 2002). Therefore, the degree of maturation causes them to lose their ability to capture foreign matter efficiently, to up-regulate class I and II MHC and co-stimulatory molecules, to some extent or in a few cases not at all dependent on the stimuli.

These DC do proficiently process and present the antigens from non-immunogenic origin and accordingly possess antigen-presenting properties capable of engaging T cells. Typically, mature DC are also characterised by IL-12 production and this is apparently absent in these semi-mature DC; in addition, these DC may or may not secrete IL-10 as their major cytokine. All of the qualities of these semi-mature DC are noticeable in tolerogenic DC (Lutz and Schuler, 2002). For these reasons, the activation or maturation status of dendritic cells determines the immunogenicity or tolerogenicity of dendritic cells. Therefore, tolerogenicity of DC is not a characteristic feature of a specific subset but rather an adapted consequence on DC due to environmental pressures. A more encompassing model is that discrete DC developmental and activation phases are responsible for the induction of either immunity or tolerance.

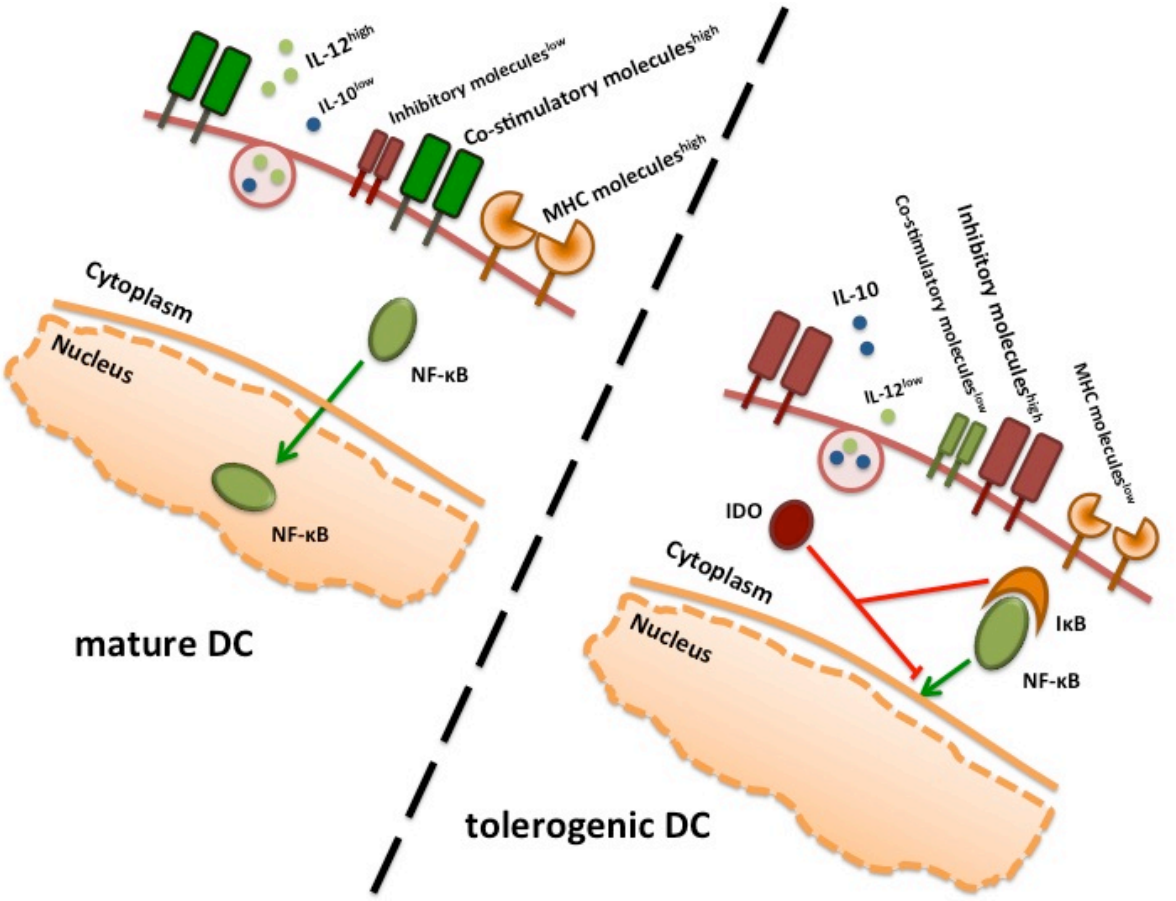

Figure 1.8.2. Simplified scheme summarising the contrasting features in phenotype and function between immunogenic (mature) and tolerogenic DC.

A basic illustrative drawing highlighting the phenotypic differences between immunogenic and tolerogenic DC.

Despite corresponding to a functional phenotype, tolerogenic DC do loosely display particular characteristics; the expression of inhibitory molecules such as signalling lymphocyte activation molecule (SLAM), programmed cell death ligand (PDL)-1 and -2 (PDL-1 and PDL-2), DEC-205 (CD205) and immunoglobulin-like transcript (ILT) family (ILT3/ILT4) are highly associated with these DC. Also, tolerogenic DC may express high levels of IL-10 and IDO (Figure 1.8.2) (Smits et al., 2005). The myriad of co-signalling to augment or inhibit TCR-mediated T cell activation is pictured below (Figure 1.8.3). It is noteworthy to emphasize that the co-inhibitory signals supersede co-

stimulatory signals thus stressing the importance of stringently regulating T cell activation.

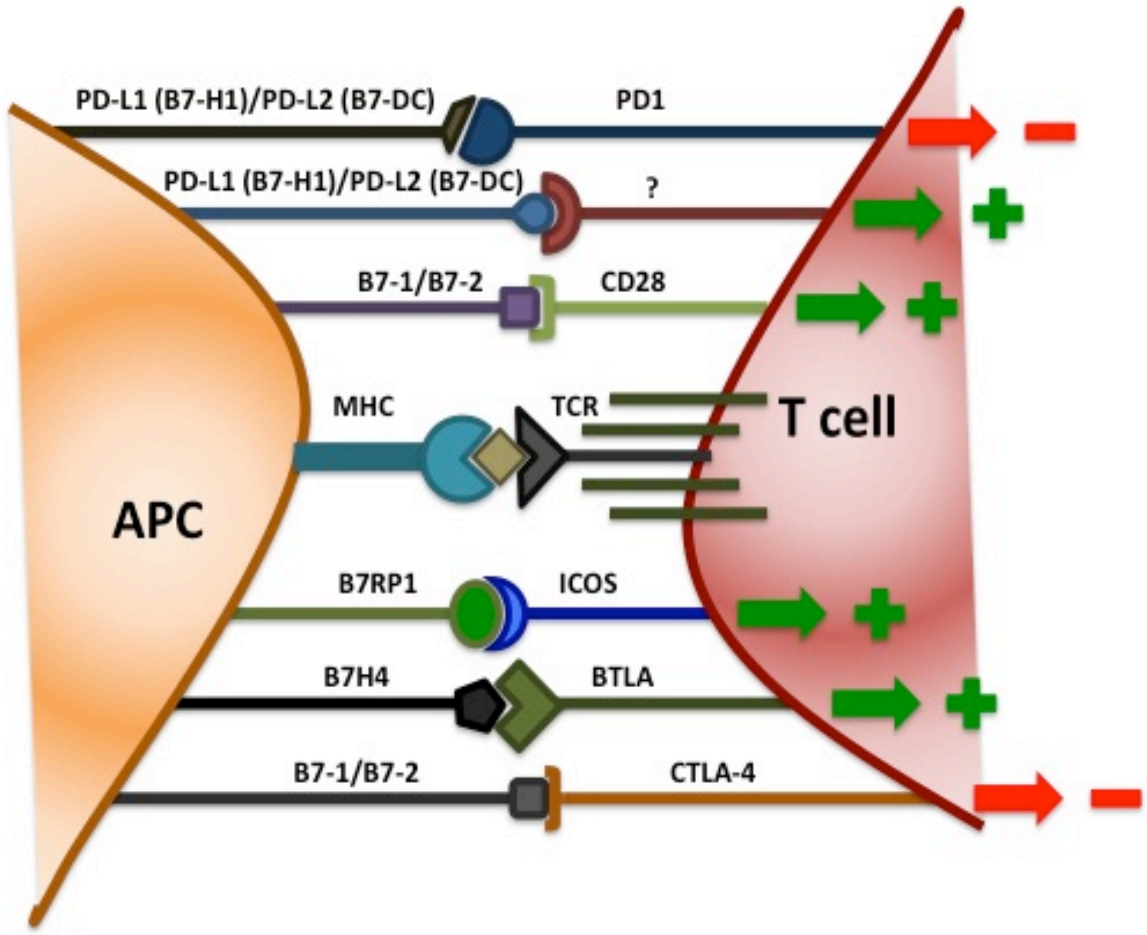

Figure 1.8.3. Schematic representation of co-stimulatory molecules expressed on T cells and DC, with a focus on inhibitory pathways.

Green arrows represent positive co-stimulatory signals and the *red arrows* represent negative or inhibitory signals transduced to T cells.

Dendritic cells can be tolerised by a range of cytokines and endogenous mediators as well as some invading pathogens, primarily parasites, greatly exploit these negative regulatory mechanisms to benefit their own survival. Conventionally, IL-10 and/or TGF-β have been heavily implicated in the *in vitro* differentiation of tolerogenic DC. More recently, new and uncategorised functions of single cytokines and hormones, such as vasoactive intestinal peptide (VIP), have been highlighted in the development of tolerogenic DC (Table 1.8.1) (Rutella et al., 2006). *Plasmodium*-infected erythrocytes bind CD36 on human monocyte-derived DC (moDC) and inhibit the LPS-induced maturation of moDC and subsequent T cell stimulation. *Plasmodium* utilises this method of immune evasion by inducing immature regulatory DC (Urban et al., 1999, Pain et al., 2001). Alternatively, in a TLR2-dependent manner, *Schistosoma*-derived lysophosphatidylserine (lyso-PS), primes DC maturation that initiate high IL-10 production and low IL-12 production and also drive the generation of IL-10-producing regulatory T cells (Rutella et al., 2006, Smits et al., 2005). *Plasmodium falciparum* in the asexual, erythrocytic stages of infection impair the ability of human DC to mature *in vitro* (Urban et al., 1999). *P. falciparum*-infected children showed reduced levels of the MHC molecule, HLA-DR, on peripheral blood DC compared with DC counterparts of uninfected children, suggesting a reduced state of activation (Urban et al., 2001). Subsequent to presentation of heterologous antigen by parasitized erythrocytes (pRBC)-exposed DC, activation of DC is drastically

altered partly because of deposition of the malarial pigment hemozoin (HZ) within these cells. These DC trigger an abrogated expansion of $CD4^+$ T-helper cells and thus defective B-cell expansion and differentiation and a failure of the antibody response. Thus, the ability of malarial parasites to inhibit DC maturation could be involved not only in parasite-specific immunosuppression but also in the suppression of responses to heterologous antigens and unrelated pathogens (Millington et al., 2007, Millington et al., 2006)

Cytokine, experimental model	Additional stimuli applied during in vitro differentiation
TNF-α	
Suppression of EAT	Supernatant of a GM-CSF–transfected cell line
Suppression of EAE	Supernatant of a GM-CSF–transfected cell line or GM-CSF
GM-CSF	
Suppression of EAT after in vivo provision of GM-CSF	NA
IL-10/TGF-β	
Protection from lethal GVHD	GM-CSF
G-CSF	
Differentiation of human monocyte-derived DCs	Post–G-CSF serum containing high levels of IL-10/IFN-α
Provision of G-CSF in vivo to bone marrow donor mice	NA
Provision of G-CSF in vivo to mice with EAE, diabetes, and lupus nephritis	NA
IFN-λ	
Differentiation of human monocyte-derived DCs	GM-CSF/IL-4
VIP	
Differentiation of mouse bone marrow–derived DCs for subsequent use in EAE and rheumatoid arthritis	GM-CSF
Differentiation of mouse bone marrow–derived DCs for subsequent use in posttransplantation GVHD	GM-CSF
M-CSF	
Differentiation of human monocyte-derived DCs	IL-4
TGF-β	
Treatment of EAE mice with thioglycolate-elicited, mouse peritoneal exudate cells	Antigen (MBP)
HGF	
Differentiation of human monocyte-derived DCs	None or GM-CSF
IL-16/TPO	
Differentiation of human CD34-derived DCs	GM-CSF/IL-4/FltSL/TNF-α/SCF
IL-21	
Differentiation of mouse bone marrow–derived DCs	GM-CSF
IL-10	
Differentiation of human monocyte–derived DCs	GM-CSF/IL-13
Differentiation of mouse bone marrow–derived DCs; isolation of their natural in vivo counterpart	GM-CSF/TNF-α
TSLP	
Differentiation of human $CD4^+$ $CD25^+$ $FoxP3^+$ thymocytes by TSLP-treated $CD11c^+$ thymic DCs	None

EAT, indicates experimental autoimmune thyroiditis; EAE, experimental autoimmune encephalomyelitis; NA, not applicable; TSLP, thymic stromal lymphopoietin; and MBP, myelin basic protein.

Table 1.8.1 Endogenous mediators implicated in the *in vitro* or *in vivo* differentiation of tolerogenic DC.

Table showing the cytokines and mediators involved in tolerogenic DC development. Excerpt taken from (Rutella et al., 2006).

A number of studies describing tolerogenic DC, particularly in models of infection, have either focussed on the regulatory effects on T cell responses or the modulating effects on DC without addressing the eventual outcome for effector T cell responses. In this respect, it is important to note the production of anti-inflammatory mediators by immature or mature tolerogenic DC that in turn induce Treg responses. This is crucial for the resolution process (Smits et al., 2005). Some actions of tolerogenic DC are tabulated below (Table 1.8.2).

Regulatory DCs	Characteristics	Function	Site of action
'Steady-state' immature mDCs or pDCs	Co-stimulatory molecules: low; CCR7: low or high depending on conditions; IL-12 low, IL-10 high	Tolerance to autoantigens and ubiquitous antigens during homeostasis	Peripheral tissues; lymph nodes (resident population?)
'Semi-mature' DCs	Co-stimulatory molecules: high; CCR7: high; cytokines: low	Tolerance to autoantigens and ubiquitous antigens during homeostasis	Peripheral tissues (?); lymph nodes
'Steady-state' mature DCs	Co-stimulatory molecules: high; CCR7: high; cytokines?	Tolerance to harmless antigens during homeostasis	Lymph nodes
'Exposed' tissue-dwelling DCs [gut, lung or anterior chamber (AC) of eye]	Co-stimulatory molecules: high; CCR7: high; CD40? or low (AC of eye); IL-12 low, IL-10 high; extra inhibitory membrane molecules?	Tolerance to continuous exposed harmless antigens	Lymph nodes
Pathogen-driven regulatory immature DCs	Co-stimulatory molecules: low; CCR7?; IL-12 low, IL-10 high; extra inhibitory membrane molecules?	Protection of host; immune evasion of pathogen	Peripheral tissues; lymph nodes (?)
Pathogen-driven regulatory mature DCs	Co-stimulatory molecules: high; CCR7: high; IL-12 low, IL-10 moderate to high; extra inhibitory membrane molecules (?)	Protection of host; immune evasion of pathogen	Peripheral tissues (?); lymph nodes

Table 1.8.2. Different regulatory DC subsets categorized by their phenotypic characteristics, putative function and site of action.

Phenotype and functionality of the individual tolerogenic DC subtypes in tolerance and/or homeostasis. Excerpt taken from (Smits et al., 2005).

1.9. ANNEXINS

The **annexins** are a superfamily of proteins consisting of 13 members and are composed of an evolutionarily conserved carboxyl terminal protein core of four (Annexin-A1) or eight (Annexin-A6) repeats of five α-helices of 70 amino acids and a distinctive N-terminal domain for each family member. The annexins are a family of phospholipid binding proteins characterised by the ability of their conserved core to bind negatively charged membrane phospholipids in a Ca^{2+}-regulated manner. The unique N-terminal segment of annexins accounts for the diversified roles of each family member; these include controlled organisation of membrane domains, membrane-cytoskeleton scaffolding, certain endocytic and exocytic transport, regulation of ion fluxes, and growth control (Gerke et al., 2005, Gerke and Moss, 2002).

Annexins binding to phospholipid matrices is reversible process such that removal of Ca^{2+} ions results in the release of these membrane-bound annexins. As a result, annexins are involved in exocytosis, endocytosis, macropinocytosis, phagocytosis and vesicle trafficking (Raynal and Pollard, 1994). Annexins can also mediate clustering of phospholipids and the stabilisation of the plasmalemma and organelle membrane microdomains and lipid rafts (Hu et al., 2008, Menke et

al., 2005).

In addition to lipids and lipid structures, annexins form complexes with a number of other molecules that include proteins like EF-hand family of Ca^{2+}-binding proteins such as the S100 subfamily (Mailliard et al., 1996), cytoskeletal proteins such as F-actin and profilin (Alvarez-Martinez et al., 1996, Alvarez-Martinez et al., 1997, Filipenko and Waisman, 2001, Hayes et al., 2004); and macromolecules such as large polymer molecules like glycosaminogylcans (GAG) (Ishitsuka et al., 1998) and ribonucleic acid (RNA) (Hirata and Hirata, 1999).

Surprisingly, annexins do not possess a secretory consensus sequence (Christmas et al., 1991, Haigler and Christmas, 1990), considering most annexins are closely associated with the machinery involved with vesicle trafficking, vesicle fusion, regulated exocytosis and the classical (canonical) secretory pathway (Donnelly and Moss, 1997). Despite the absence of secretory signal sequence, annexins are sequestered to the plasma membrane and/or secreted into the extracellular compartment where they elicit biological effects in both an autocrine and/or paracrine manner for example anti-inflammatory actions of Annexin-A1 (Hayhoe et al., 2006, Perretti and Flower, 1995, Perretti and Flower, 2004, Perretti and Gavins, 2003) and the anti-thrombogenic actions of Annexin-A2 (Hajjar et al., 1994, Ling et al., 2004).

1.9.1. Glucocorticoids

Glucocorticoids (GC) are a class of steroid hormones that derives their name from their role in the regulation of the metabolism of glucose and their synthesis in the adrenal cortex. As the name implies, glucocorticoids have long been well known for the stimulatory effects on gluconeogenesis in the liver and for the catabolic effects on such peripheral tissues as the skeletal muscle and thymus. For these actions, GC exert crucial homeostatic actions and may perhaps participate in regulation of carbohydrate, protein and fat metabolism of the body. In large doses, GC are known also to act as potent anti-inflammatory and immunosuppressive agents. For a family of compounds that can wield such profound effects on the immune system and that are so widely used therapeutically (Figure 1.9.1.1), the mechanisms by which the glucocorticosteroids exert their immunosuppressive and anti-inflammatory effects are poorly understood (Yeager et al., 2004, Guyre et al., 1984, Guyre et al., 1981, Goulding, 2004, Wilckens and De Rijk, 1997). Early studies concentrated on the inhibitory effects of these hormones on cell trafficking patterns and cell functions, such as plasma cell Ig production, T-cell activities, and most functions of monocytes and macrophages (Rose et al., 2010, Newton, 2000).

GC cause their effects by binding to the glucocorticoid receptor (GR), which is a type of nuclear receptor found in practically all cells. The activated GR complex directly binds distinct segments of DNA called glucocorticoid response elements (GRE) and up-regulates the expression of anti-inflammatory and immunosuppressive agents (a process known as transactivation) and represses the expression of pro-inflammatory proteins in the cytosol by preventing nuclear translocation of transcription factors, principally NF-κB and AP-1, in the cytosol (transrepression) as well as down-regulates the transcriptional and post-transcriptional events of cytokine genes including IL-1, IL-6, TNF-α, MIP-1α and IL-10 (Franchimont et al., 1999, John et al., 1998). Generally, endogenous GC play a prominent part of the feedback mechanism in the immune system by dampening down the inflammatory response but GC actions on the immune system are in fact complex by both suppressing and inducing several pro- and anti-inflammatory mediators (Goulding, 2004, Rhen and Cidlowski, 2005). GC depress the inflammatory cascade by enhancing certain events of the resolution phase (Heasman et al., 2003).

Glucocorticoids also modulate the formation and the action of several growth factors and other mediators. At physiological concentrations GC favour the synthesis and release of these growth factors. GC also favours their effect by increasing the expression of specific receptors. All these actions contribute to the hormonal effect of GC (Wilckens, 1995). At higher concentrations, natural and synthetic GC inhibit the

synthesis of cytokines and other factors and this is of major importance for bringing about their effect on the immune system. Several of the anti-inflammatory actions exerted by potent glucocorticoids are mediated by the endogenous protein, Annexin-A1 (Goulding and Guyre, 1992, D'Acquisto et al., 2008b, Perretti and D'Acquisto, 2009).

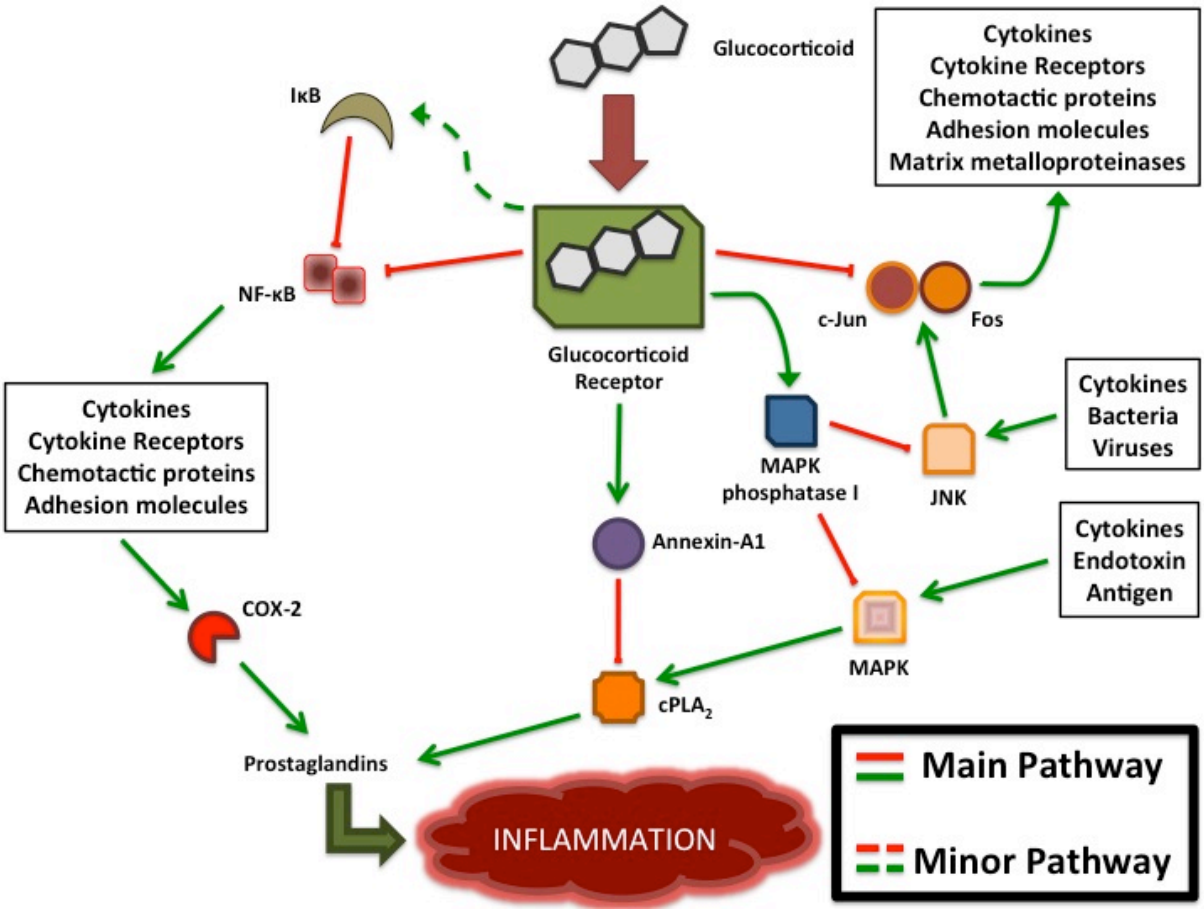

Figure 1.9.1.1. Partial Signalling Network of the Glucocorticoid-Induced Antagonism of Inflammation.

Interplay of positive pathways of inflammation (*green arrows*) and anti-inflammation (*red blocked arrows*) are highlighted above. The glucocorticoid receptor act at multiple points by directly inhibiting inflammatory mediators by inducing anti-inflammatory proteins.

1.9.2. Annexin-A1

Formerly termed lipocortin 1, Annexin-A1 (Anx-A1 or AnxA1) was originally identified as a glucocorticoid-inducible 37-kDa protein, which inhibits phospholipase A_2 (PLA_2) activity and thus prostaglandin production (Di Rosa et al., 1984, Flower, 1988). The human Annexin A1 (ANXA1) gene is located on the q arm of chromosome 9 at locus q12-21.2 and transcribes three protein-encoding gene transcripts; and murine annexin 1 gene (Anxa1), found on chromosome 19q19, transcribes two gene transcripts that encode proteins. The conserved core of Anx-A1 contains four 70-amino acid repeating domains, which include the Ca^{2+} binding sites (Figure 1.9.2.1) and phospholipid binding sites (Rosengarth and Luecke, 2003).

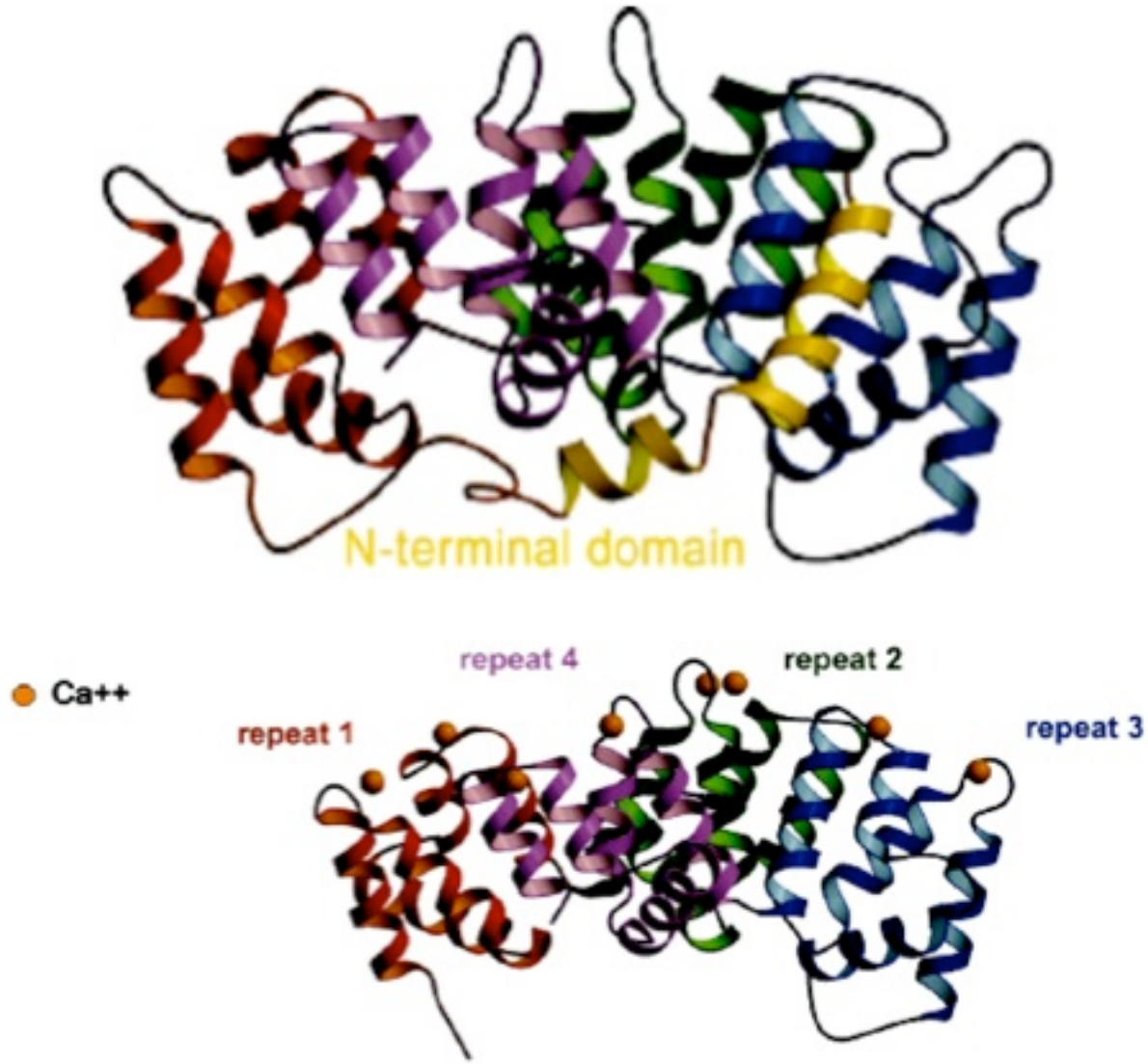

Figure 1.9.2.1. Tertiary structure of Annexin-A1 monomer.

The ribbon diagrams of one monomer of full-length annexin-A1 in comparison with full-length annexin-A1 in the absence (top cartoon) and presence (bottom cartoon) of calcium. Image excerpted from (Rosengarth and Luecke, 2003)

The 49-amino acid N-terminal domain of the protein is buried in groove of the α-helices of the 3rd repeat of the protein core. In the presence of Ca^{2+} ($\geq$ 1mM), a conformational change occurs within the protein and exposes N-terminal tail (Figure 1.9.2.2) (Rosengarth and Luecke, 2003), which is responsible for the biological activity of Annexin-A1 (Cirino et al., 1993). The N-terminus contains phosphorylation and proteolysis sites whereby the revealed N-

terminus undergoes proteolytic cleavage, which inactivates the anti-inflammatory actions of the full-length protein (Vong et al., 2007).

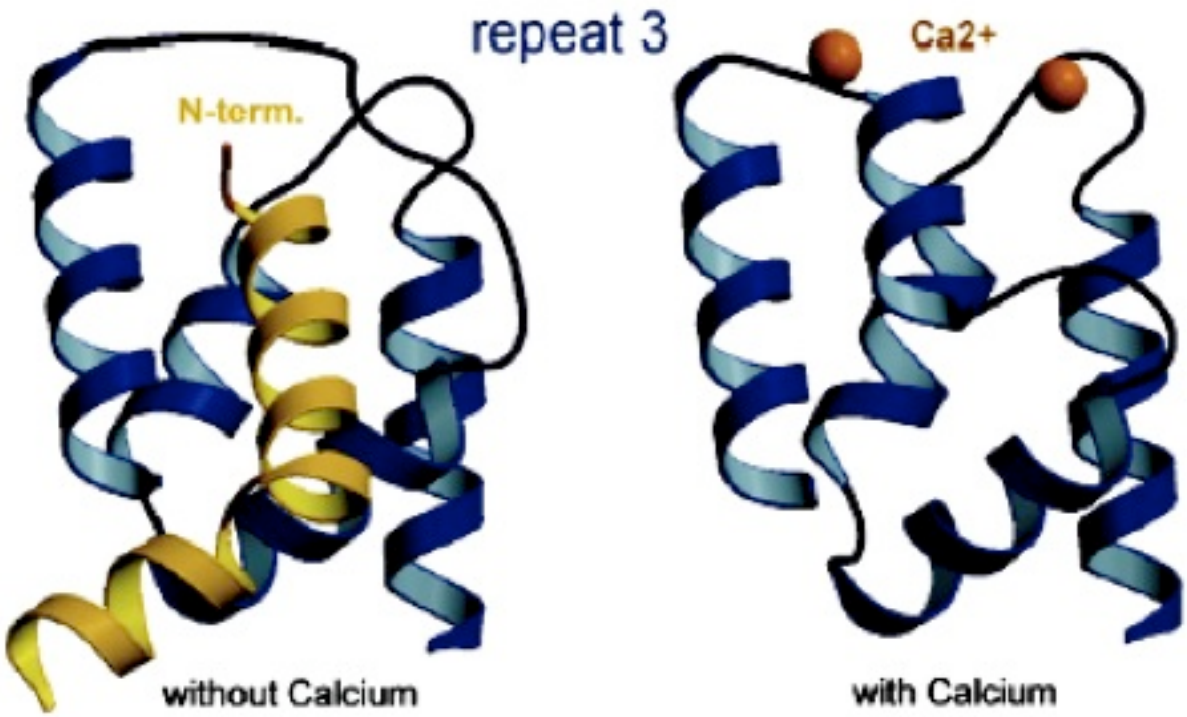

Figure 1.9.2.2. Tertiary structure of the N-terminus in the pocket of third α-helical loop of Annexin-A1.

The diagrams of conformational change in the third α-helical loop full-length annexin-A1 in the presence (right image) of calcium from the native annexin-A1 in the absence (left image) of calcium. Image excerpted from (Rosengarth and Luecke, 2003)

Annexin-A1 is present in many cell types and tissues and is detectable intracellularly in all leukocyte populations (Morand et al., 1995, Spurr et al., 2011). In neutrophils, Anx-A1 is compartmentalised in gelatinase granules (Perretti et al., 2000) and generally the Anx-A1 protein expression tends to be greater in differentiated cells compared to precursor cells, for instance, higher levels of Anx-A1 expression in macrophages compared to their monocyte precursors (Ambrose et al., 1992). Neutrophils, monocytes

and NK cells express higher levels of Anx-A1 and compared to T or B cells. Furthermore, specific T cell subsets revealed higher Anx-A1 levels in activated $CD25^+$ and memory $CD45RO^+$ CD4 T cells compared to resting $CD25^-$ or naïve $CD45RA^+$ CD4 T cells. (Spurr et al., 2011)

After treatment with GC, cytosolic Anx-A1 is mobilised to the plasmalemma, where by a mechanism not fully elucidated, the protein is secreted (Perretti et al., 1996) and it has been reported that after exogenous administration of corticosteroids resulted in changes in Anx-A1 distribution as well as concentration in human peripheral blood leukocytes (Goulding et al., 1990c, Goulding et al., 1990b, Goulding et al., 1990a). There is even evidence of a paracrine role of Anx-A1 in the hypothalamo-hypophysial system and a further endocrine involvement in the hypothalamo-pituitary-adrenocortical (HPA) axis (Traverso et al., 1999, Buckingham and Flower, 1997) as well as a relationship with serum cortisol levels (Mulla et al., 2005).

T cells treated with glucocorticoids, particularly dexamethasone, decreased Anx-A1 mRNA and protein levels in a time-dependent fashion and almost abolished its expression after 12h of incubation. TCR-mediated activation of T cells, which were pre-incubated with dexamethasone for 12h, led to significant reduction of IL-2 production. Moreover, addition of human recombinant Anx-A1 to dexamethasone-treated T cells reversed the inhibitory effects of the

steroids on the TCR-induced IL-2 production. Therefore, GC suppress Anx-A1 expression in T cells *in vitro* and also *in vivo* – the steroid treatment of rheumatic patients decreased Anx-A1 expression in T cells (D'Acquisto et al., 2008a).

D'Acquisto *et al* proposed a novel pathway for regulating the adaptive immune response *via* steroids and suggest that Anx-A1 may represent a target for the treatment of autoimmune diseases (D'Acquisto, 2009, Paschalidis et al., 2009).

1.9.3. Annexin-A1 Receptor

Human Anx-A1 is the endogenous ligand of the human formyl peptide receptor 2 (FPR2), also termed the ALX receptor because it is also the receptor for Lipoxin A_4 (LXA_4), formerly named formyl peptide receptor-like-1 (FPRL1). FPR2 is a member of a family of G-protein coupled seven transmembrane receptors constituting of two other family members: FPR1, previously known as FPR, and FPR3, formerly designated FPRL2 (Gavins et al., 2005, Ye et al., 2009, Perretti, 2003).

The FPR family classically recognise formyl peptides derived from bacterial and mitochondrial proteins and is involved in host defence against pathogens as well as sensing internal molecules that constitute signals of cellular dysfunction. In addition, peripheral blood mononuclear cells (PBMC) treated with different glucocorticoids *in vitro* demonstrated an up-regulation of the FPR1/ALX receptor (Sawmynaden and Perretti, 2006). Peptides derived from the N-terminus of Anx-A1 are agonists for all three receptors of the human FPR family *in vitro* (Dahlgren et al., 2000, Gavins et al., 2005, Perretti et al., 2002).

The FPR family vary markedly in their gene expression between

different mammalian species; the mouse FPR gene family has at least eight known members including related sequences (Fpr1, Fpr-rs1 – Fpr-rs8) clustered on chromosome 17 whilst only three human genes cluster on chromosome 19. Fpr1 gene product is the mouse orthologue of human FPR1 and Fpr-rs1 and Fpr-rs2 are both structurally related to human FPR2/ALX gene product with mouse Fpr-rs2 gene product being a low affinity receptor fMLF. There are no known mouse orthologues for human FPR3/FPRL2 (Ernst et al., 2004, Migeotte et al., 2006).

A recent study performed by Spurr *et al* addressed the differential expression of FPR2 and Anx-A1 in leukocyte populations (Spurr et al., 2011). Both PMN and PBMC express surface FPR2 with the latter showing a bifurcation within the population: a $FPR2^+$ subset (~12%) and a $FPR2^-$ subset (~78%). However, these differences were masked when examining total FPR2 expression (surface and cytosolic FPR2) suggesting that FPR2 is localised intracellularly in the majority of PBMC while a small PBMC subpopulation readily expressed FPR2 on the cell membrane. Overall, the FPR2 expression pattern appeared to mirror AnxA1 expression, with PMN expressing about 3 times more FPR2 compared to PBMC (Spurr et al., 2011).

In analysing $CD3^+$ T cell subsets: $CD4^+$ and $CD8^+$ T cells virtually do not display membrane FPR expression however, intracellular expression of FPR was found to express slightly higher levels (~25–

30% more) in $CD4^+$ T cells compared to $CD8^+$ T cells. Previous reports have shown that the AnxA1/FPR2 system is engaged upon TCR-mediated activation of naïve T cells (D'Acquisto et al., 2007a, D'Acquisto et al., 2007b). Analysis on activated, naïve or memory T cells revealed surface FPR2/ALX was about 30% higher in $CD4^+CD25^+$ T cells compared to $CD4^+CD25^-$ T cells, while there were no differences in the intracellular levels of FPR2. Comparison between naïve and memory T cells highlighted that $CD4^+CD45RA^+$ naïve T cells expressed slightly higher levels of surface FPR2/ALX compared to $CD4^+CD45RO^+$ memory T cells. Meanwhile, intracellular FPR2/ALX was equally expressed in these subsets. These findings are summarised in the table below (Table 1.9.3.1) (Spurr et al., 2011).

Cell type	External AnxA1	Total AnxA1	External FPR2/ALX	Total FPR2/alx
PMN	2.9±0.9	514±38.2	41.8±3.8	984.4±36.4
PBMC	4.6±1.2	160.4±19.3	20.8±2.3	376.2±27.0
$CD14^+$	16.6±2.0	784.9±47.8	149.5±14.9	275.9±20.4
$CD19^+$	4.9±1.1	110.7±8.7	12.8±1.0	92.9±2.8
$CD335^+$	4.3±1.0	24.2±2.4	77.7±7.6	145.1±14.5
$CD3^+$	3.6±0.9	25.8±3.6	9.2±1.4	143.7±14.6
$CD4^+$	1.7±0.4	48.3±4.4	8.7±1.1	334.1±60.9
$CD8^+$	3.1±0.9	36.3±3.2	27.7±4.0	232.3±22.3
$CD4^+CD45RA^+$	1.3±0.4	107.2±9.1	18.6±2.3	152.6±15.0
$CD4^+CD45RO$	12.9±1.7	394.4±6.0	13.2±1.2	397.4±14.8

Table 1.9.3.1. Analysis of the AnxA1/FPR2 system in human leukocyte subsets.

Table indicate expression of Anx-A1 and FPR2 in human leukocyte populations. Numbers indicate the Mean Fluorescence Intensity (MFI)±S.E.M. obtained from n=5 different donors (Spurr et al., 2011).

Human DC express functional FPR and FPRL2 in an immature state and lose expression of FPR upon maturation whilst FPRL2 expression is maintained; FPRL1 is not expressed in human DC (Yang et al., 2002). Therefore it is believed that the action of Anx-A1 on DC is mediated by binding these two receptors in man.

After binding of their agonists, like Anx-A1, the FPR family of receptors activate the receptor-coupled heterotrimeric G_i protein resulting in dissociation of the respective α and βγ subunits, activating signalling molecules and pathways shared with most chemoattractant receptors. The α and $\beta\gamma$ subunits initiate a series of signal transduction events *via* phosphoinositide 3-kinase γ (PI3Kγ) and phospholipase Cβ (PLCβ). PLCβ hydrolyses phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP_3) and

diacylgylcerol (DAG), intermediatory second messengers that releases endoplasmic calcium stores and activates protein kinase C (PKC) isoforms respectively. PKC activation induces heterologous desensitization of unrelated chemotactic receptors; PI3Kγ induces the mitogen-activated protein kinases (MAPK) ERK1/2, JNK and p38. Other signal mediators include phospholipase A_2 and D, and tyrosine kinases such as lyn and $p125^{FAK}$. ERK1/2 play a role in FPR-mediated phospholipase D activity (Gripentrog and Miettinen, 2008, Gripentrog and Miettinen, 2005, Lopez-Ilasaca et al., 1997, Migeotte et al., 2006).

1.9.4. Annexin-A1 in Innate Immunity

Anx-A1 is expressed highly and predominately in differentiated cells and cells of the haematopoietic lineage such as tissue-specific macrophages, monocytes, mast cells and particularly polymorphonuclear leukocytes, mainly in neutrophils. This expression has been widely documented to increase upon GC treatment *in vitro* (Ambrose et al., 1992) and *in vivo* (De Caterina et al., 1993). Localisation of Anx-A1 is varied amongst the immune cell types; for instance, neutrophils compartmentalise the protein in gelatinase granules (Perretti et al., 2000), likewise in or on α-granules within mast cells (Oliani et al., 2000). Whilst in most other cells including macrophages, Anx-A1 is localised chiefly in the cytosol as well as being associated with the phospholipid layers (plasmalemma and nuclear). Several studies with Anx-A1 and its related N-terminal peptide, Ac2-26 have shown pharmacological actions of inhibition of PMN adhesion to vascular beds and transmigration via induction of L-selectin shedding, inhibition of trafficking of monocytes and phagocytic activity of macrophages in the zymosan-peritonitis mouse model (Getting et al., 1997, Harris et al., 1995). Anx-A1 has been revealed to mediate neutrophil apoptosis at inflammatory sites through dephosphorylation of the pro-apoptotic intermediary BAD (Solito et al., 2003).

Exploiting transgenic tools in the generation of the Annexin-1-null mouse has uncovered valuable information of the role of endogenous Anx-A1 *in vivo* as well as confirming prior evidence of the anti-inflammatory role of this protein (Hannon et al., 2003).

1.9.5. Annexin-A1 in Adaptive Immunity

The role of Anx-A1 in cells of the adaptive immune system is an emerging field and is not as well characterised as in innate immune responses. Both human and murine T lymphocytes express moderate levels of Anx-A1 (Morand et al., 1995). Expression profiles of Anx-A1 in human and mouse leukocytes have shown that the protein is expressed at higher levels in PMN, monocyte/macrophages and other innate immune cells as compared with T and B lymphocytes (Kamal et al., 2005, Perretti and Flower, 2004, Perretti and Flower, 1996). In addition, extravasated lymphocytes all express Anx-A1 protein in contrast to peripheral blood T lymphocytes, which are about 50% positive for the protein (Perretti et al., 1999).

Human recombinant (hr) Anx-A1 sensitises murine T lymphocytes to CD3/28 stimulation in a dose-dependent manner as manifested by increased cell proliferation and IL-2 production. Dissecting the T cell activation profile highlighted that high-levels of Anx-A1 (300nM, 600nM) promote T cell activation with sub-optimal CD3/28 stimulation (1.25 μg/ml) suggesting that Anx-A1 amplifies the TCR signalling. This finding was compounded by increased number of cells positive for the activation markers CD69 and CD25 in hrAnx-A1 treated groups. Naive T cells stimulated with hrAnx-A1 in Th1 (IL-2,

IFN-γ and α-IL-4) conditions or Th2 (IL-2, IL-4 and α-IFN-γ) conditions and later restimulated with αCD3 produced higher levels of IL-2 and IFN-γ and lower levels of IL-4. Furthermore, analysis of the two major transcriptional switches in Th1 or Th2 differentiation, T-bet and GATA-3 (Glimcher and Murphy, 2000), showed that cells differentiated in the presence of hrAnx-A1 expressed a higher level of T-bet and a lower level of GATA-3 (D'Acquisto et al., 2007a).

Conversely, in Annxein-A1$^{-/-}$ T cells stimulated with different stimuli such as αCD3 or αCD3/CD28 or PMA and ionomycin showed reduced IL-2 mRNA expression and protein production as well as reduced number of cells positive for the activation markers CD69 and CD25 when stimulated with αCD3/CD28. Proliferation experiments were consistent with cytokine and surface activation molecules expression and exhibited significantly attenuated proliferation in Anx-A1$^{-/-}$ T cells. This lack of proliferation was not due to defective IL-2 signalling in these T cells since administration of recombinant IL-2 partially rescued the proliferative ability (D'Acquisto et al., 2007b).

THESIS AIM

Published reports from our Centre have dogmatically shown the anti-inflammatory role and the mechanism of action of Annexin-A1 in several immune cells indicating this protein is an effector of a pivotal pathway for the action of glucocorticoids in innate immunity. But more recently, annexin-A1 has been revealed as a positive immunomodulatory protein in the adaptive immune system, at least, in T lymphocytes.

Is Annexin-A1 a positive or negative modulator of dendritic cell biology including their ability to instruct the adaptive immune response?

The aims of my thesis are:

To investigate the role of endogenous Anx-A1 in DC biology by characterising the phenotype of Anx-A1$^{-/-}$ DC and the biological response to TLR agonist, lipopolysaccharide (LPS);

To examine the molecular determinants responsible for DC maturation;

To study the consequential impact of AnxA1 deficiency in DC on their polarizing effect on T cells;

To dissect the role of endogenous AnxA1 in governing DC responses

to CD4 or CD8 T cells.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. Antibodies for Flow Cytometry

Expression of DC surface antigens was quantified by flow cytometry using the following FITC-, PE- or biotin-conjugated, affinity-purified, rat or hamster IgG anti-mouse monoclonal antibodies: I-$A^{b,d,q}$/I-$E^{d,k}$ (clone M5/114.15.2), CD16/32 (clone 93) CD80/B7-1 (clone 1G10), CD86/B7-2 (clone Gl-1), and CD40 (clone 1C10), CD197/CCR7 (clone 4B12), CD54/ICAM-1 (clone YN1/1.7.4), CD34 (clone RAM34) (eBioscience, Middlesex, UK) and CD11c (clone HL3; BD Pharmingen, Oxford UK).

2.1.2. Antibodies for Western Blotting

The mouse monoclonal antibody used to detect phosphorylated ERK1/2 (E-4) was purchased from Santa Cruz Biotechnology and recognises the short amino acid sequence containing phosphorylated tyrosine 204 of ERK1 and the correspondingly phosphorylated ERK2. The rabbit polyclonal antibodies used for recognition of the C-terminus of total ERK1 (C-16) and total ERK2 (C-14) were also bought from Santa Cruz Biotechnology. Whereas the rabbit monoclonal antibodies against phosphorylated Akt recognise either phosphorylated serine residue 473 (clone 193H12) or phosphorylated threonine 308 (clone 244F9) were purchased from Cell Signaling Technology. In addition, detection of total Akt1/2/3 levels were performed using a rabbit polyclonal antibody also acquired from Cell Signaling Technology.

2.1.3. Mice

BABL/c and C57BL/6 male mice were obtained from the B&K Universal Ltd (Hull, UK). Anx-A1 null mice on C57BL/6 background were generated in our lab and bred in pathogen free conditions in our animal facilities. OT-I (specific TCR for H-2K^{b}/OVA$_{257-264}$) and OT-II (specific TCR for I-A^{b}/OVA$_{323-339}$) transgenic mice on Rag-1-deficient background were kindly provided by Professor Hans Strauss (Royal Free Hospital, UCL, London, UK) and were also maintained in pathogen free conditions in our animal facilities. All mice were 6-8 weeks old and had a body weight of 24-28g. Food and water were available *ad libitum*. Animals were kept under standard conditions and maintained in a 12-hr light/dark cycle at 22±1 ^{0}C. Wild type animals were used 7 days after arrival according to guidelines laid down by the Ethical Committee for the use of Animals, Barts and The London School of Medicine. Animal work was performed according to Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986). All mice used in these studies were aged between 8 and 12 weeks.

2.1.4. X63 Supernatant: Active Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

The mouse myeloma cell line P3-X63-Ag8 does not express immunoglobulin heavy or light chains. This clone X63-Ag8.653 can be used for efficient fusion with antibody-forming cells to obtain hybrid cell lines producing pure monoclonal antibodies (Kearney et al., 1979). Culture supernatant from X63.Ag865 cell line stably transfected with the cDNA of murine granulocyte-macrophage colony stimulating factor (X63-GM-CSF) (kindly provided by Dr B. Stockinger, Molecular Immunology, National Institute for Medical Research, London, UK) was used as a biologically active source of GM-CSF (Volkmann et al., 1997, Volkmann et al., 1996). This supernatant was used to supplement complete Iscove's Modified Dulbecco's Media (IMDM) into a GM-CSF-rich media.

2.2. METHODOLOGY

2.2.1. X63-GM-CSF Cell Line

The murine GM-CSF-transfected X63.Ag8653 cell line is cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated Foetal Calf Serum (FCS), 50mg/ml gentamicin, 2mM L-glutamine and 1mg/ml geneticin on non-adherent plastic T175 culture flasks. The P3-X63-Ag8.653-mGM-CSF gene construct contains a geneticin-resistance gene insert but the DNA vector is episomally expressed in the myeloma cells and therefore, it is essential that the cell line be maintained under antibiotic selection.

The X63-GM-CSF cells, brought up from frozen stock, were washed free of Dimethyl Sulfoxide (DMSO) and then first cultured in the specific IMDM medium in non-adherent plastic T25 flasks until confluent. Cells were washed in the culture medium, counted, passaged by a dilution method with culture medium and then split into several T175 flasks at a 1:10 ratio. Maintaining episomal expression of the transfected cells, the cell cultures are split twice a week.

For preparation of supernatant, the cells are grown to a cell density of

60 x 10^6 cells/ml. The cells are then resuspended at a cell density of 60 x 10^6 cells/200ml in antibiotic-free IMDM medium in non-adherent plastic T175 culture flasks for 48hr. The supernatant is harvested and frozen at –20^{o}C. It is important to note that the secreted GM-CSF protein forms aggregates in solution and filtering the supernatant will result in loss of most of the cytokine activity.

2.2.2. Cell Culture

Bone marrow-derived DC were generated as previously described (Lutz et al., 1999). Bone marrow cells from tibias and femurs were flushed and cultured in IMDM (Sigma-Aldrich, Dorset, UK) supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 100U/ml penicillin, 100mg/ml streptomycin: complete IMDM (all from Sigma-Aldrich) and 10% culture supernatant from X63-GM-CSF. Bone marrow-derived DC were generated by culturing $2x10^7$ bone marrow cells in 10ml of medium in 100-mm Petri (Sterilin) dishes at 37°C under humidified 5% CO_2.

Cell density was calculated using a cell counting protocol and a haemocytometer. 50μl of freshly isolated bone marrow cells were transferred to a 1.5ml Eppendorf tube containing 200μl of Phosphate buffered saline (PBS) and 250μl of 0.4% Trypan Blue solution and the cell suspension is mixed thoroughly (dilution factor of 10). With a cover-slip placed onto the haemocytometer chamber, 10-20μl of diluted cell suspension is slowly and carefully pipetted at the edge of the cover-slip allowing the suspension to fill the chamber by capillary action (Figure 2.2.2.1).

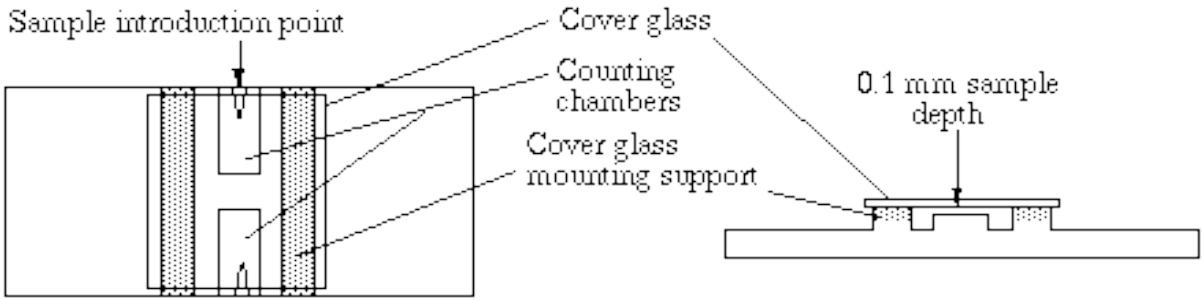

Figure 2.2.2.1. Schematic diagram of the haemocytometer slide. (Image source: *http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html*.)

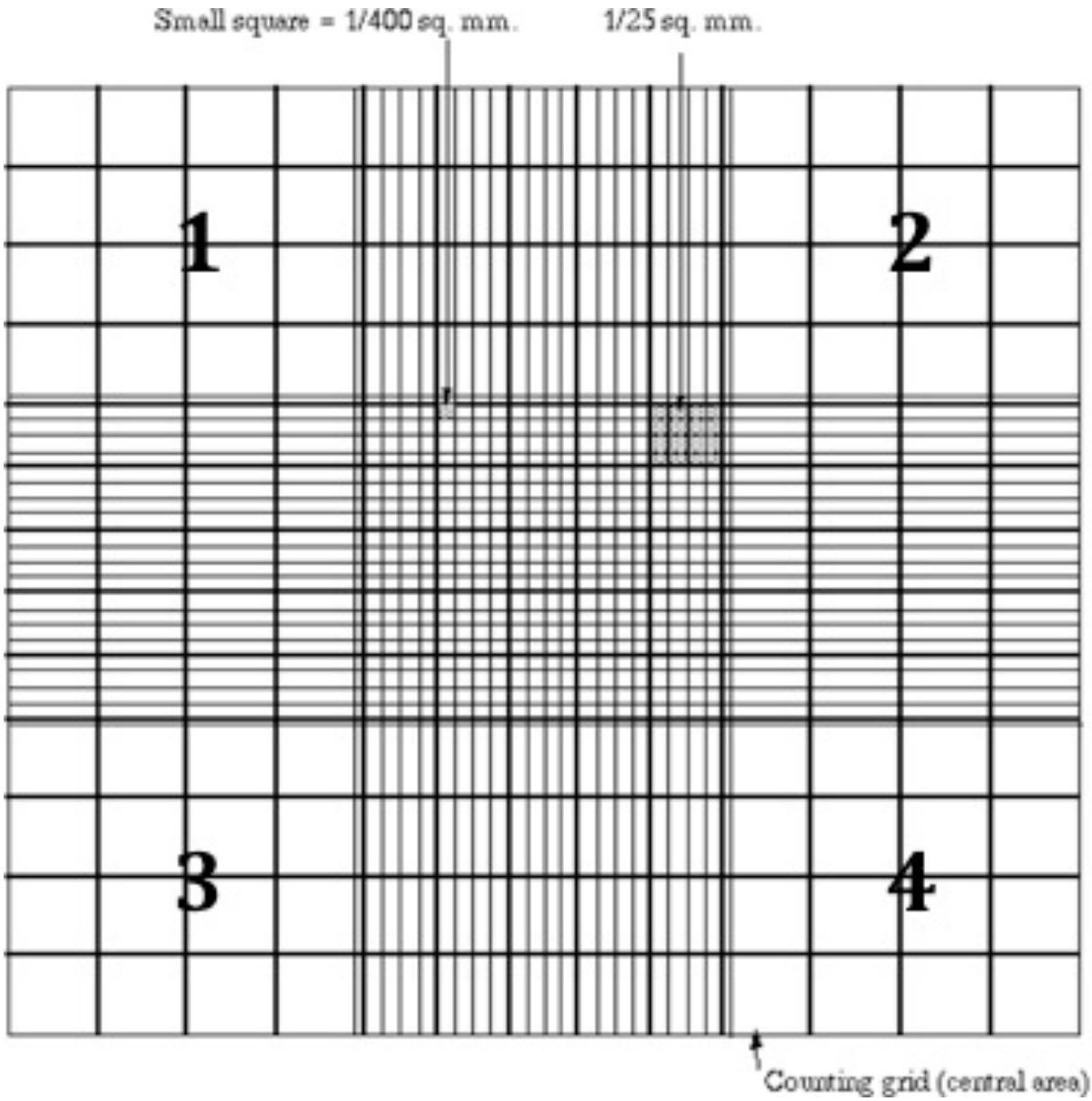

Figure 2.2.2.2. Schematic diagram of the counting grids of the haemocytometer chamber. (Image source: *http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html*.)

All the viable cells are counted (non-viable cells take up the dye and stain blue, viable cells remain opaque) in the four corner squares.

Each square of the haemocytometer (with the cover-slip in place) represents a total volume of $0.1mm^3$ or $10^{-4}cm^3$ (Figure 2.2.2.2). Since $1cm^3$ is equivalent to 1ml, the subsequent concentration per ml, and the total number of cells, is determined using the following

formulae:

Cells per ml = the average count per 4 squares x the dilution factor x 10^4

Total cell number = cells per ml x the original volume of fluid from which cell sample was taken

On day 2, an additional 10ml of medium was added. Starting on day 4, 10ml of medium was replaced every 2 days for a total differentiation period of 6 days. On day 7 of culture, cells were harvested as immature dendritic cells (Figure 2.2.2.3) and phenotypic analysis was performed via flow cytometry to verify features of dendritic cells.

Figure 2.2.2.3. Diagram showing the differentiation protocol of bone marrow progenitor cells into dendritic cells and maturation with LPS (lipopolysaccharide).

The changes in phenotypical surface expression of the antigen-presenting receptor and co-stimulatory ligands on wild-type bone marrow-derived dendritic cells were tracked to show the archetypal alterations during DC differentiation (Figure 2.2.2.4).

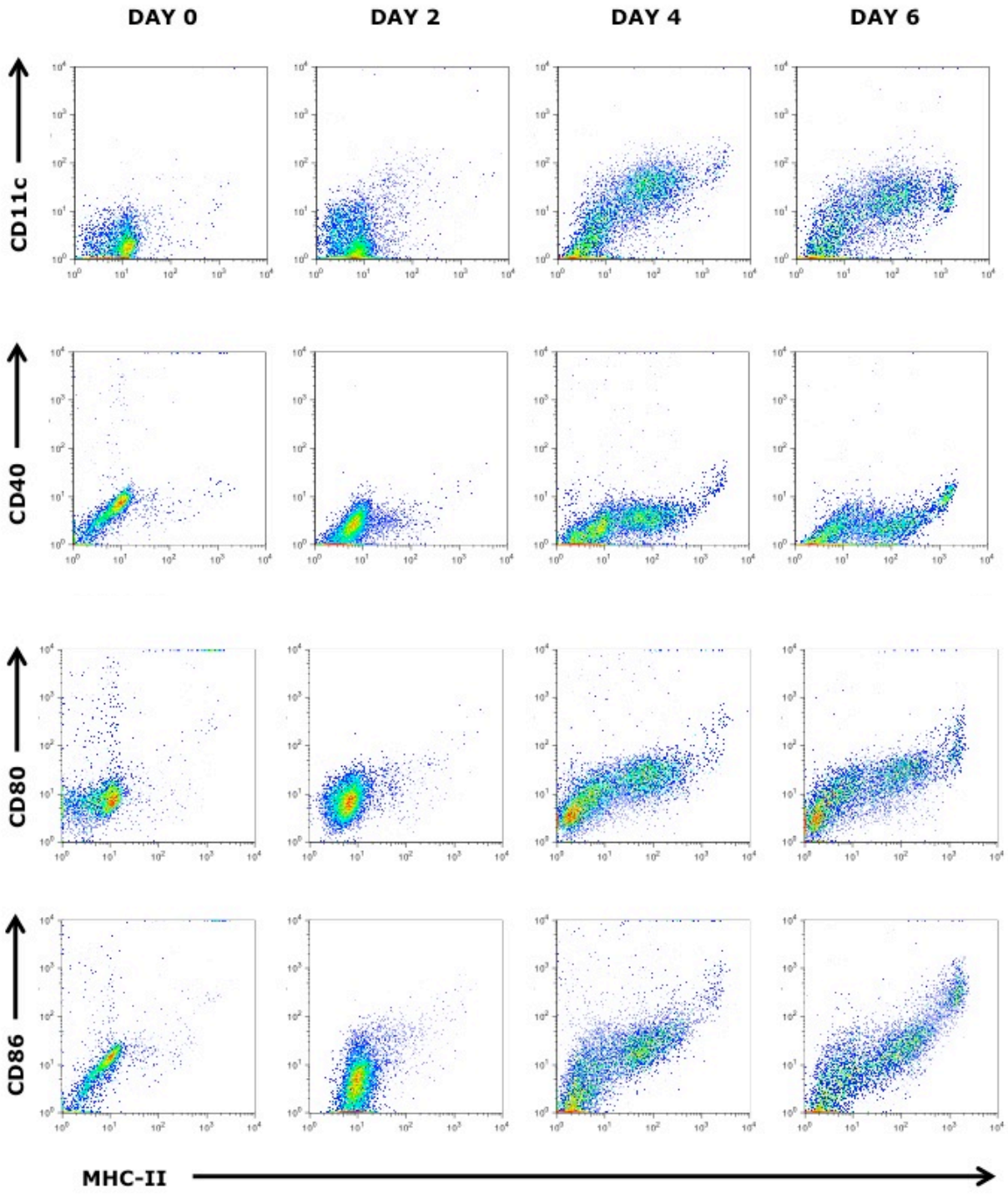

Figure 2.2.2.4. Dendritic Cell-Surface Receptor profiles of differentiating bone marrow progenitors into dendritic cells using a GM-CSF-rich medium.

2.2.3. LPS Stimulation of BMDC

Immature AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC were collected from the bacteriological plates by scraping and aspirating adherent, semi-adherent, and non-adherent cells. The cell cultures were washed free of dead and apoptotic cell with GM-CSF-free IMDM medium, counted and plated at a density of 1x10^6/well in triplicates in polystyrene-treated (adherent) plastic 24-well plates. Thereafter, the cells were incubated with medium alone or with 100ng/ml lipopolysaccharide (LPS, *S. minnesota*; Alexis Biochemicals, Nottingham, UK) at 37^{o}C under humidified 5% CO_2 for various time-points before the cells and supernatants were used in assay analyses.

In some cases, after incubation, cells and supernatants were harvested and frozen at –20^{o}C prior to analysis by Western Blotting, immunoprecipitation, enzyme-linked immunosorbent assay (ELISA) and electrophoretic mobility shift assay (EMSA). Also, cells were frozen at –80^{o}C prior to analysis by RNA extraction, cDNA synthesis and gene expression profiles (real-time PCR).

2.2.4. Flow Cytometry

AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC were treated as described in subheading 2.2.3. The BMDC, in the 24-well culture plates, were centrifuged at 2000rpm for 5min and subsequently cell-free supernatants were collected and stored at -20^{0}C; then the BMDC were first preincubated in FACS buffer (PBS containing 5% FCS and 0.02% NaN_3) containing anti-CD16/32 monoclonal antibody (1:1000 dilution) for 30min at 4^{o}C, to avoid nonspecific binding of the reported monoclonal antibodies to the FcγII/IIIR. Thereafter, cells were washed and incubated with the appropriate concentration of the reported monoclonal antibodies (1:500 dilution for PE-conjugated antibodies and 1:250 dilution for FITC-conjugated antibodies) in FACS buffer for 30min at 4^{o}C. Cells were washed, resuspended in FACS buffer and subsequently analysed on the flow cytometer (FACSCalibur®; Becton & Dickinson, Oxford, UK).

Forward and side scatters were calibrated to exclude erythrocytes and dead cells, and at least 2x10^4 DC were analysed per sample. Determination of positive and negative populations was performed based on the control staining with an irrelevant IgG isotype labelled with PE or FITC.

2.2.5. Endocytosis Assay

Quantitative analysis of DC endocytic activity was performed as previously described (Sallusto et al., 1995) with minor modifications. AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC previously treated as in subheading 2.2.3 were washed free of GM-CSF-rich medium and diluted to a cell density of 0.5x10^6 cells/ml in GM-CSF-free IMDM medium. 2x10^6 AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC were incubated in complete medium with FITC-dextran (1mg/ml, MW 145000) on non-adherent plastic Falcon tubes at either 37^{0}C (water bath) or 4^{0}C (on ice) for 60 minutes. Following the incubation, the cells were washed three times with cold FACS solution (PBS containing 5% FCS and 0.02% NaN_3).

The cells were stained with PE-conjugated CD11c as described in subheading 2.2.3 as a marker to identify DC and then analysed by flow cytometry. Forward and side scatters were calibrated to exclude erythrocytes and dead cells, and at least 5x10^4 DC were analysed per sample. BMDC treated with FITC-dextran at 4^{0}C was used as a background reference and MFI values for corresponding samples were always subtracted.

2.2.6. *In Vivo* Migration

Following AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC treatment described in subheading 2.2.3, the cells were washed twice in warm PBS (37^{0}C) and resuspended at cell density of 25-50x10^6 cells/ml in warm PBS solution containing free carrier protein (PBS/0.1%BSA). AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC were resuspended in 1-5ml warm CFSE solution (PBS/1μM CFSE/0.1%BSA) at cell density of 25-50x10^6 cells/ml in non-adherent plastic Falcon tubes and incubated for 15 minutes in order for CFSE labelling to occur. Five times-volumes of cold PBS solution (PBS/1%BSA) were added to the cell suspension to cease the labelling; the cells were then washed at least three times in this cold PBS solution. The BMDC were resuspened in serum-free medium (IMDM medium, 50mg/ml gentamicin) at a concentration of 20x10^6 cells/ml.

100μl (2x10^6 DC) of CFSE-labelled cells were injected subcutaneously in the hind paws of C57BL/6 mice and 48h post-injection the draining inguinal and popliteal lymph nodes were harvested. Lymph nodes were isolated and processed into a single-cell suspension by crushing and homogenising the lymph nodes through a 70-μm cell strainer. Single-cell suspensions were washed and resuspended in FACS solution and then stained with PE-labelled CD11c as previously stated

in subheading 2.2.3 followed by analysis on the FACSCalibur™.

2.2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC culture supernatants post-incubation were collected and analysed for TNF-α, IL-1β and IL-12 content and the mixed leukocyte reaction (MLR) co-culture supernatants were also collected and tested for IL-2 and IFN-γ levels according to the manufacturer's instructions (eBioscience). 96-well EIA/RIA high-binding plates were coated with cytokine capture antibody and incubated overnight at 4^{o}C. Protein cytokine of known concentration was diluted a 2-fold serial dilutions to make the standard curve. 100μl/well protein cytokine and sample was added to the coated wells and incubated for 2 hours at room temperature or 37^{o}C in order to allow the protein present in the cell free supernatants to bind to the immobilised antibody already bound to the plate. After the incubation, the plates were washed extensively with the wash buffer (1x PBS/0.05% Tween-20 (pH 7.4)) in order to remove any unbound sample. The bound protein to the antibody was detected by the addition of 100 μl of a detection antibody specific for the desired cytokine following incubation of 1-hour. Thereafter, the plates were incubated for 30min with the enzyme Avidin-Horseradish Peroxidase (HRP), and after an extensive wash to remove unbound antibody-enzyme reagent, 100 μl of substrate solution (tetramethylbenzidine TMB) was added to produce colour. The reaction was terminated by

the addition of 50 μl of 1M H_3PO_4 turning the samples from blue to yellow and the absorbance was read at a wavelength of 450nm using a spectrophotometer (Labsystems Multiskan Bichromatic). The concentration was calculated from the standard curve using the software Graph Pad Prism™.

2.2.8. Western Blotting (Immunoblotting)

AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC were lysed in ice-cold lysis buffer (1% Triton-X, 20mM Tris pH7.5, 150mM NaCl, 1mM DTT - 1, 4-Dithio-DL-threitol) containing the protease and phosphatase inhibitors: 1mM $MgCl_2$ (magnesium chloride), 1mM EGTA (ethylene glycol tetraacetic acid), 0.5mM PMSF (phenylmethylsulfonyl fluoride), 1mM Aprotinin, 1mM Leupeptin, 1mM Pepstatin, 50mM NaF, 10mM $Na_4P_2O_7$ (tetrasodium phosphonatophosphate), and 1mM $NaVO_4$ (sodium orthovanadate), 1mM β-glycerophosphate. The lysates were centrifuged at 14,000 rpm for 5min at 4°C and the supernatants collected and stored at -20°C. To immunoprecipitate extracellular released Anx-A1, 5μl of anti-Anx-A1 antibody (purified polyclonal rabbit IgG; Zymed, California, USA) and 35μl of protein A/G sepharose beads were added to 500μl of culture supernatants obtained from 1x10^6 DC. Samples were incubated overnight at 4°C under continuous rotation and thereafter, washed with PBS. Lysates and immunoprecipitates were denatured with hot 6x sample buffer and subjected to electrophoresis on SDS-12% polyacrylamide gel. After subsequent transfer onto nitrocellulose membranes, these were incubated overnight with antibodies diluted in Tris-buffered saline solution containing Tween-20 (TTBS: 130mM NaCl; 2.68mM KCl; 19mM Tris-HCl; 0.001% v/v Tween-20; pH 7.4) with 5% non-fat dry

milk at 4ºC. For the experiments with anti-pERK1/2 and anti-pAkt, the antibodies were diluted in 5% milk/TTBS buffer supplemented with 50mM NaF and membrane washes post incubation with TTBS was adjusted to 500mM NaCl. For each condition, extract equivalents obtained from the same number of cells were used. Immunoblotting and visualization of proteins by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) were performed according to manufacturer's instructions.

2.2.9. Nuclear and Cytoplasmic Extraction

Nuclear extracts were harvested from $3x10^6$ cells according to a previously described protocol with modifications (Jorritsma et al., 2003). 24h post-LPS stimulation of $AnxA1^{+/+}$ and $AnxA1^{-/-}$ BMDC, viable cells were recovered and washed in ice-cold PBS and resuspended in 200μl of cold Buffer A (10mM HEPES pH 7.9, 2mM $MgCl_2$, 10mM KCl, 10mM NaF, 0.1mM EDTA, 10mM $Na_4P_2O_7$, 0.5mM DTT plus a mixture of protease/phosphatase inhibitors) then incubated on ice. After 10min on ice, cells were lysed by addition of Nonidet P-40 (NP-40) in a final concentration of 1% and allowed to incubate at room temperature for 4min. Lysates were centrifuged at 5000rpm for 2min. Supernatants of cytoplasmic contents were aspirated, collected and stored at -20^0C. Pellets were washed with 200μl of Buffer A and then 50μl of Buffer B (20mM HEPES pH 7.9, 50mM $MgCl_2$, 0.4M NaCl, 0.2mM EDTA, 0.5mM DTT plus protease/phosphatase inhibitors) was added without resuspension. The samples were then continuously vortexed for 45-60min then centrifuged for 5 min at 14,000 rpm under 4^0C conditions. Supernatants containing the nuclear fractions were stored at -80°C. Protein/nucleic acid concentrations of the nuclear extracts were determined by Bradford assay using the Bio-Rad Protein Dye Reagent (dye, H_2SO_4 and CH_3OH) diluted 1:5 with H2O.

2.2.10. Electrophoretic Mobility Shift Assay (EMSA)

NF-κB DNA binding was analysed by EMSA using 5μg of nuclear extracts were incubated with 2μg of poly(dI:dC) in 20μl of NF-κB binding buffer with ^{32}P end-labelled, double–stranded NF-κB oligonucleotide probes ($5x10^5$ c.p.m). Following binding, the DNA-protein complexes were fractionated from free probe by electrophoresis in a non-denaturing 4%-polyacrylamide gel (29:1 cross-linking ratio) in 0.5% Tris-base/Borate/EDTA (TBE) buffer for 3h at 150 volts. Gels were dried and exposed to autoradiography.

The NF-κB binding buffer (10X) consists of 100mM Tris-HCl pH 7.5, 500mM NaCl, 10mM EDTA, 10mg/ml BSA, 30mMGTP and 10mM DTT. The non-denaturing gel 4%-polyacrylamide gel is made up of 13.5ml 30% Acrylamide:Bisacrylamide (29:1) solution, 2.5ml 10x TBE, 500μl 10% Ammonium Persulphate (APS), 85μl TEMED (N,N,N',N'-tetramethylenediamine) and water up to a total volume of 50ml.

2.2.11. RNA Extraction

Total RNA was extracted from immature or LPS-matured AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC using RNeasy mini-spin columns kit (Qiagen). Briefly, 5x10^{6} cells were pelleted and lysed using buffer RLT, a lysis buffer that contains guanidine isothiocyanate and supplemented with 1% β-mercaptoethanol (both responsible for the inactivation of RNAases). The samples were homogenized by passing the lysates at least 5 times through a 20-gauge needle and 70% (v/v) ethanol was added. The samples were loaded onto RNAeasy mini columns, and centrifuged to allow adsorption of RNA onto the column membrane. Following several washes with wash buffers RW1 (low-concentration guanidine isothiocyanate/ethanol buffer) and RPE (high salt/ethanol buffer) to remove contaminants, the RNA was eluted in TE buffer (Tris EDTA; 10 mM Tris, 1 mM EDTA, pH 7.5).

2.2.12. cDNA Synthesis

cDNA synthesis was carried out using the AMV reverse transcriptase kit (Promega). Briefly, 5-10 μg of total RNA were mixed with 0.5 μg oligo $(dT)_{15}$ and diethyl pyrocarbonate (DEPC)-treated H_2O and incubated at 70°C for 10 min. Thereafter, the mix was transferred on ice and supplemented with 10 units AMV reverse transcriptase, 40units RNase inhibitor, and 1.25mM each dNTPs and incubated at 42°C for 45min, then at 70 C for 10 min. Thereafter, samples were stored at -20°C. DNA concentrations were determined by spectrophotometry using NanoDrop spectrophotometer

2.2.13. Real-time PCR

Real-time PCR was carried out by using 5μl cDNA, ABsolute™ QPCR ROX Mix and the fluorescent QuantiTect primers for the following murine genes: Fpr1 (QT01165899), Fpr-rs1 (QT01063216), Fpr-rs2 (QT00171514), Anx-A1 (QT00145915), TLR4 (QT00259042), IL-10 (QT00106169), IDO (QT00103936), IDOL1 (QT01066345) and TGF-β1 (QT00145250).

Cycling conditions were set according to manufacturer's instructions. Sequence specific fluorescent signal was detected by 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, Cheshire, UK). mRNA data was normalized relative to GAPDH or 18S RNA and then used to calculate expression levels. We used the comparative Ct method to measure the gene transcription in samples (Pfaffl, 2001). The results are expressed as relative units based on calculation of $2^{-\Delta\Delta Ct}$, which gives the relative amount of gene normalized to endogenous control (GAPDH) and to the sample with the lowest expression set as one.

2.2.14. Mixed Leukocyte Reaction (MLR)

Immature or LPS-matured AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDC (C57BL/6 background) were treated with 50 ng/ml Mitomycin C in complete IMDM for 1h at 37°C. Thereafter, cells were washed with complete IMDM and incubated with 1x10^5 allogeneic naïve T cells from BALB/c mice at two ratios (1:1, 1:5) in 96-well U-bottom culture plates at 37^{o}C under humidified 5% CO_2. T cells were obtained as previously described (Shankar et al., 2004). Briefly, the axillary, inguinal and mesenteric lymph nodes of BALB/c mice were isolated and processed into a single cell suspension; then naïve T cells were purified on a MACS column using a CD4$^+$ CD62L$^+$ T cell isolation kit (Miltenyi Biotec). After 3 days, T cell proliferation was measured by adding 1mCi [^{3}H]-thymidine in the final 12h of co-culture incubation thereafter, cells were harvested using a semi-automatic cell harvester (Skatron 7022). The incorporated radioactivity was measured by automated scintillation counter (Tri-Carb 1900TR, Packard, Berkshire, UK).

2.2.15. Antigen-specific Leukocyte Reactions of OT-I/RAG-$1^{-/-}$ and OT-II/RAG-$1^{-/-}$ Transgenic TCR T cells

LPS-matured AnxA$1^{+/+}$ and AnxA$1^{-/-}$ BMDC were treated as aforementioned under subheading 2.2.3 with the addition of the chicken ovalbumin peptides $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) and $OVA_{257-264}$ (SIINFEKL) in 96-well U-bottom culture plates for 24h. The cervical, axillary, inguinal and mesenteric lymph nodes of OT-I/RAG-$1^{-/-}$ and OT-II/RAG-$1^{-/-}$ mice were harvested, isolated and processed into a single cell suspension. Thereafter, $2x10^5$ transgenic TCR T cells were appropriately added to the culture plates of OVA-pulsed, LPS-matured AnxA$1^{+/+}$ and AnxA$1^{-/-}$ BMDC at 37^{o}C under humidified 5% CO_2 for 5 and/or 7 days. After 4 days, 1mCi [^{3}H]-thymidine in the final 18h of the 5-day co-culture incubations; this irradiation step was repeated on day 6 on the 7-day co-cultures. Thereafter, cells were harvested using a semi-automatic cell harvester (Skatron 7022). The incorporated radioactivity was measured by automated scintillation counter (Tri-Carb 1900TR, Packard, Berkshire, UK).

2.3. GRAPHICAL SOFTWARE AND STATISTICAL ANALYSIS

All statistical analysis was performed with Prism software (GraphPad software). All values are expressed as mean ± S.E.M. Statistical analysis was assessed either by Student's t test or one-way ANOVA where appropriate. A probability of $P < 0.05$ was considered significant.

3. RESULTS

3.1. Anx-A1 Mobilisation and Modulation during DC Maturation

Previous studies from our and other research groups have shown that activation of different cell types with inflammatory stimuli causes increased expression and release of endogenous Anx-A1 (Damazo et al., 2005, Rescher et al., 2006). To investigate if this phenomenon occurred also in DC, Anx-A1 expression was measured in immature and mature DC by Western blotting and real-time PCR. Immature DC express high levels of Anx-A1 that is localized mainly in the cytosol and constitutively released to a lower levels in the culture supernatant (Figure 3.1.1, Time 0, lower and upper panels, respectively). Stimulation with LPS over 24h induced a gradual and time-dependent release of Anx-A1 in the extracellular medium. The peak of the release occurs between 1 and 3h (Figure 3.1.1, top panel) and is paralleled by a depletion of the intracellular store followed by the resynthesis at later time points, between 12-24h (Figure 3.1.1, lower panel).

This pattern of the protein expression was mirrored at the mRNA

level since a marked downregulation at 3 and 6h followed by resynthesis at 24h was observed (Figure 3.1.2).

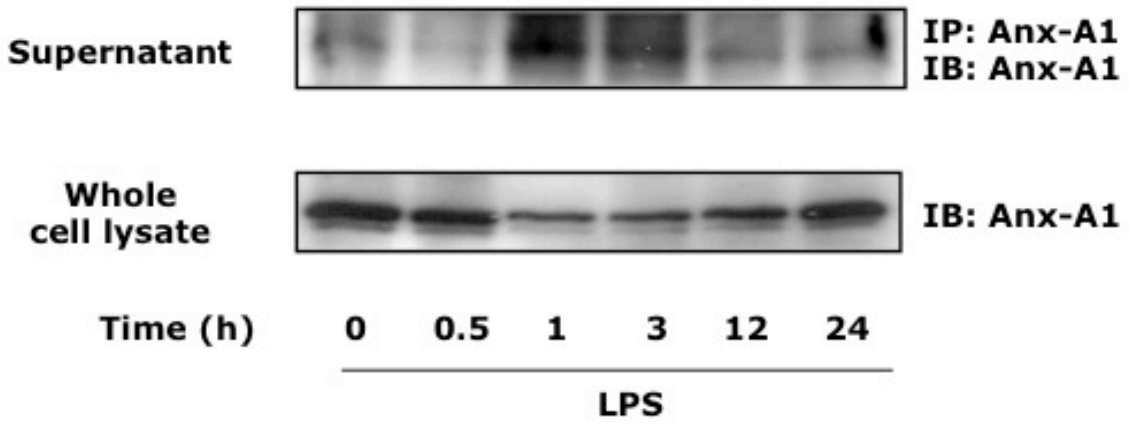

Figure 3.1.1. Anx-A1 protein modulation between immature and mature dendritic cells.

Western blotting analysis of Anx-A1 protein expression in culture supernatants (upper panel) and whole cell lysates (lower panel) of bone marrow-derived DC stimulated with LPS (100ng/ml) for the indicated times. To measure Anx-A1 levels in the culture supernatants samples were immunoprecipitated as described in Materials and Methods. Data shown is from a single experiment of n=3 mice and is representative of n=5 experiments.

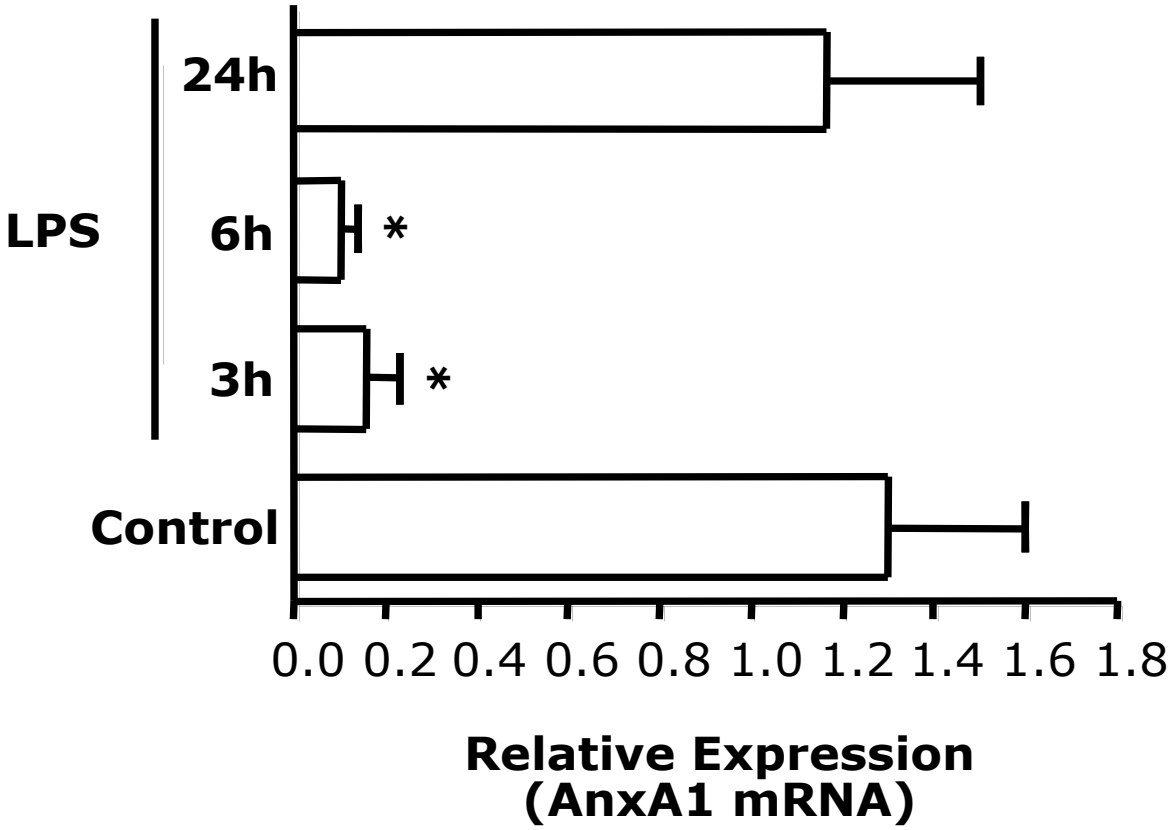

Figure 3.1.2. Anx-A1 mRNA expression in immature and mature dendritic cells.

Cumulative Real-time PCR analysis of Anx-A1 mRNA expression in Anx-A1$^{+/+}$ bone marrow-derived DC incubated with LPS (100ng/ml) over the indicated time course. Data shown is the mean $\Delta\Delta$CT values ± S.E.M. of n=3 mice per experiment from 4 separate experiments. P values were calculated using one-way ANOVA analysis and were determined from comparison of Control group *vs.* LPS-treated groups. *P<0.05.

3.2. Partial Maturation Status of Immature Anx-A1$^{-/-}$ DC

To investigate the role of endogenous Anx-A1 in DC maturation we compared the phenotype of bone marrow derived Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC. The first difference we observed was a significant increase in the number of CD11c-high positive cells in Anx-A1$^{-/-}$ DC (98.3±5.6%; $p< 0.001$) compared to Anx-A1$^{+/+}$ DC (56.2±4.6%; $p< 0.001$) (Figure 3.2.1). Further phenotypic characterization of these CD11c^{+} populations showed a similar expression of CD86 and MHC II and an increased expression of CD80 in Anx-A1$^{-/-}$ DC compared to the Anx-A1$^{+/+}$ (Figure 3.2.1).

One key feature of immature DC is to endocytose foreign antigens before presenting on MHC II. This function is reduced upon maturation and hence it is used to distinguish the immature from mature DC (Steinman and Hemmi, 2006). Accordingly, the capability of immature and mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC to endocytose FITC-dextran was compared as measure of antigen uptake. Consistent with the analysis shown in Figure 3.2.1, uptake of FITC-dextran by immature Anx-A1$^{-/-}$ DC was significantly lower than immature Anx-A1$^{+/+}$ DC while no differences were observed between mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$DC (Figure 3.2.2 and 3.2.3).

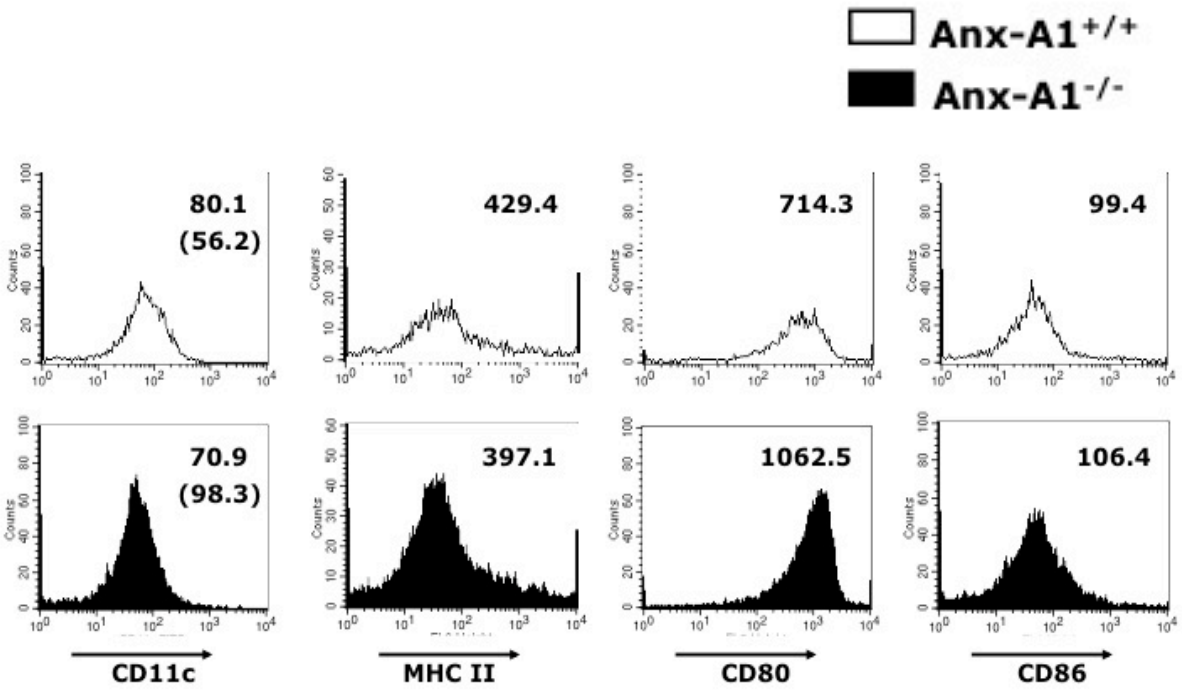

Figure 3.2.1. Heightened maturation phenotype of Anx-A1$^{-/-}$ DC.

Bone marrow-derived cells were differentiated *in vitro* with complete medium containing GMCSF for 6 days as described in Materials and Methods. Flow cytometric analysis of Anx-A1$^{+/+}$ (white histograms) and Anx-A1$^{-/-}$ DC (black histograms) showing mean fluorescence intensity (MFI) for CD11c, CD80, CD86 and MHC class II in immature Anx-A1$^{-/-}$ and Anx-A1$^{+/+}$ DC. Histograms and MFI values shown for CD80, CD86, MHC class II are gated on CD11c^{+} DC and percentages of CD11c^{+} cells are shown in brackets. Data and values shown are from a single experiment of n=3 mice and representative of 4 separate experiments.

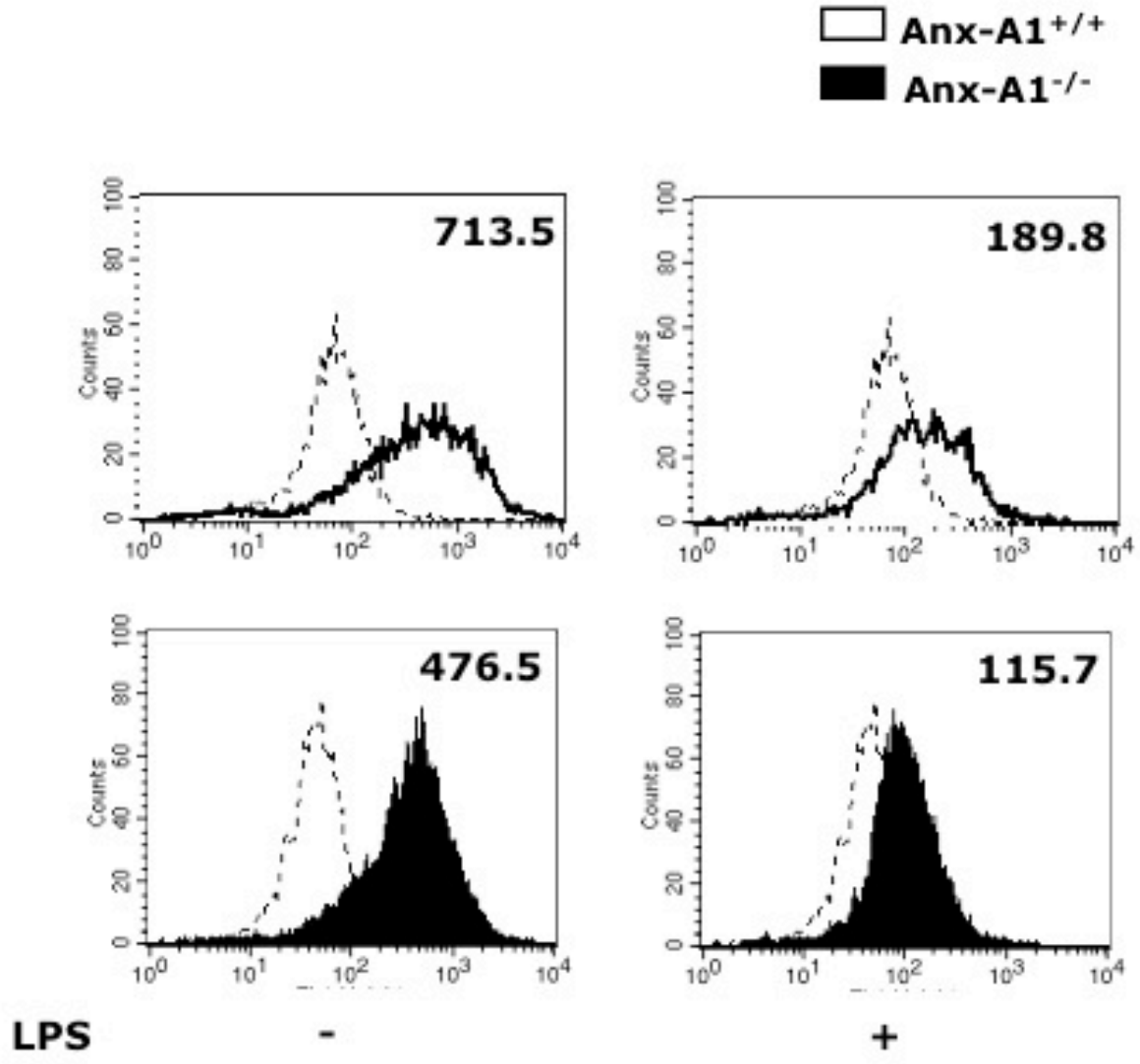

Figure 3.2.2. Comparison of endocytosis in Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC.

Analysis of FITC-dextran uptake by immature (left histograms) or LPS-mature (right histograms) Anx-A1$^{+/+}$ (white histograms) and Anx-A1$^{-/-}$ (black histograms) DC. Cells were incubated with FITC-dextran uptake at 37ºC (bold lines) as described under "Materials and Methods" and analysed by flow cytometry. FITC-dextran uptake at 4ºC served as negative control (dotted lines) Data shown is from a single experiment of n=3 mice and is representative of 3 experiments.

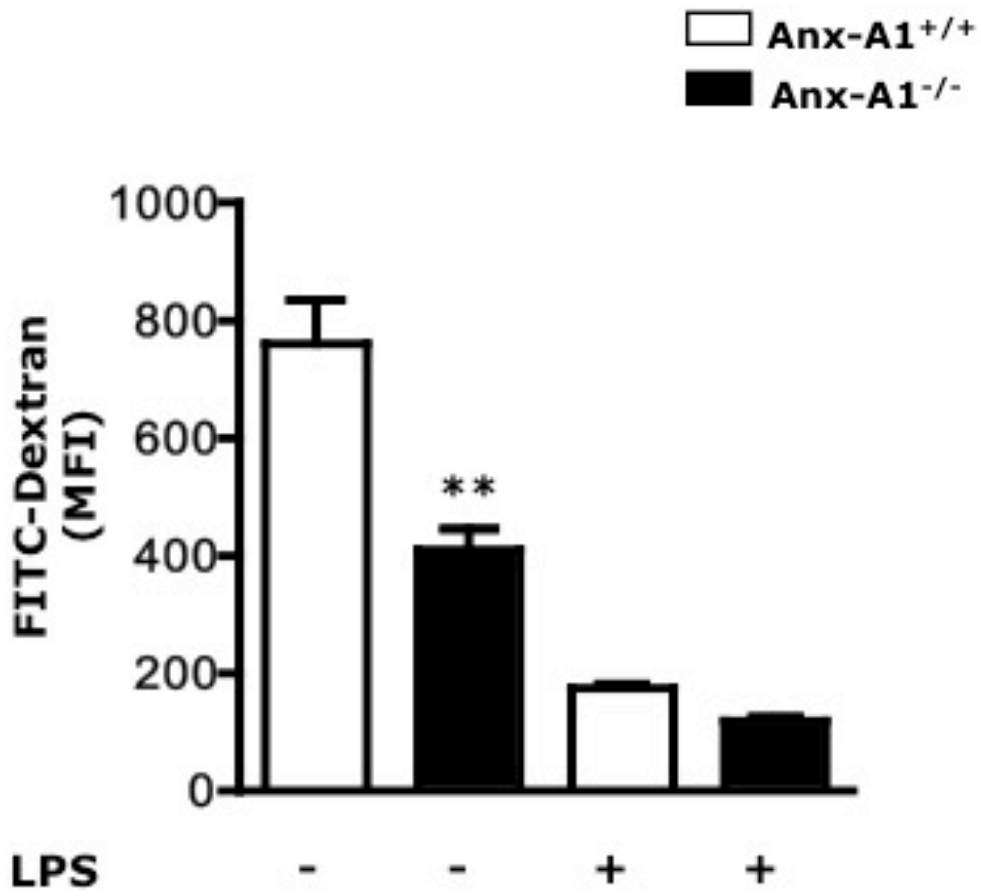

Figure 3.2.3. Endocytic analysis between Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC.

Cumulative results of FITC-dextran uptake in immature and LPS-mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC. Values represent the mean MFI ± S.E.M. of CD11c^{+}/FSC high-gated populations obtained from DC cultures of n=3 mice. Data shown are cumulative from 3 experiments and P values are calculated by Student's T-test analysis and are determined from comparison between the Anx-A1$^{+\backslash+}$ data sets and their respective Anx-A1$^{-\backslash-}$ data sets. **P<0.01.

3.3. Impaired Up-Regulation of Maturation Molecules on mature Anx-A1$^{-/-}$ DC

Upregulation in cell surface levels of MHC class I and II and co-stimulatory molecules characterise the transition of DC from the immature to the mature state. Escalated levels of these surface receptors in immature DC prompted us to further analyze the expression of phenotypical markers of DC maturation (Cella et al., 1997b). Thus, immature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC were stimulated with LPS for 24h and thereafter we stained the cells with antibodies against CD40, CD54, CD80 and CD86, CCR7 and MHC II. Immature Anx-A1$^{-/-}$ DC expressed similar levels of CD86 and MHC II, higher levels of CD40, CD54 and CD80 and decreased levels of CCR7 compared with Anx-A1$^{+/+}$ DC. Stimulation with LPS caused an expected increase in expression of the maturation markers in Anx-A1$^{+/+}$ however, surprisingly, failed to remotely induce an increase in Anx-A1$^{-/-}$ DC (Figure 3.3.1). This pattern was conserved over several experiments and the cumulative results, expressed as percentage increase over basal mean fluorescence intensity (MFI), are summarised (Figure 3.3.2).

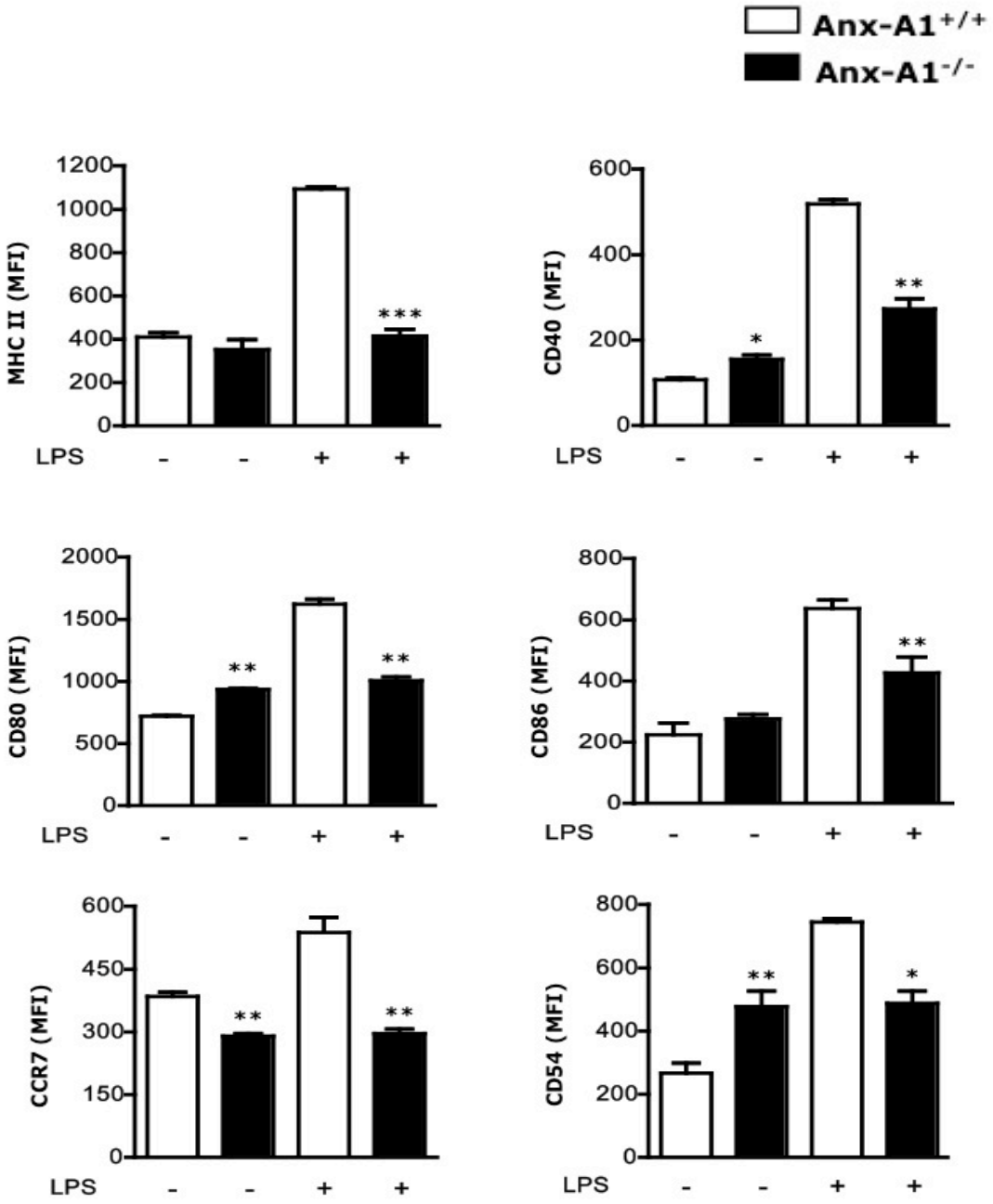

Figure 3.3.1. Expression of MHC class II and co-stimulatory molecules in Anx-A1$^{-/-}$ DC.

Bone marrow-derived Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC were incubated with culture medium or LPS for 24h and then analysed by FACS for the expression of MHC class II, CD40, CD80, CD86, CCR7 and CD54. Values in graphs are mean MFI of n=3 mice from a single experiment. Data shown are representative of 5 separate experiments and P values were calculated using one-way ANOVA and were determined from the comparison of the Anx-A1$^{+\backslash+}$ sample groups with their respective Anx-A1$^{-\backslash-}$ sample group counterparts. *P<0.05, **P<0.01, ***P<0.001.

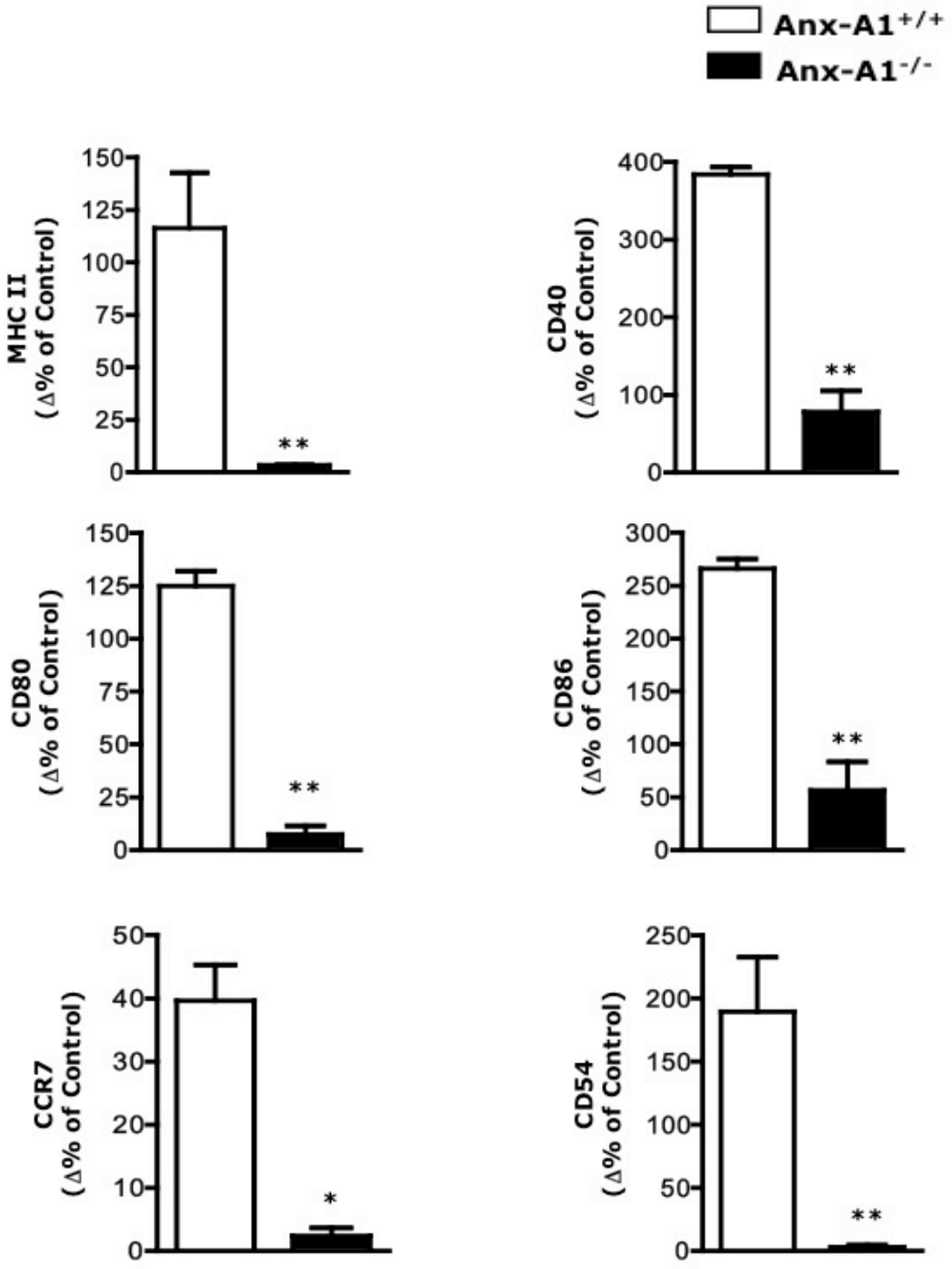

Figure 3.3.2 Defective up-regulation of maturation markers in Anx-A1$^{-/-}$ DC.

Cumulative analysis of LPS-induced MHC class II, CD40, CD80, CD86, CCR7 and CD54 up-regulation in Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC. Values are expressed as mean percentages of increase of MFI ± S.E.M. of mature *vs*. immature DC. Data shown are cumulative results from n=3 mice per experiment and a summation of 5 different experiments. P values were calculated using one-way ANOVA and were determined from the comparison of the Anx-A1$^{+\backslash+}$ data sets with their respective Anx-A1$^{-\backslash-}$ data sets. *P<0.05, **P<0.01.

3.4. Abated Cytokine Production by Anx-A1$^{-/-}$ DC

Stimulation of DC with LPS induced the production of pro-inflammatory and T helper cell skewing cytokines. The fact that Anx-A1$^{-/-}$ DC showed an altered phenotype even in the absence of LPS and a mediocre increase in the surface expression of maturation markers upon stimulation encouraged us to investigate whether this phenotype was also accompanied by an impaired cytokine production.

To this aim, immature DC were stimulated with LPS over several time points and measured for the production of IL-1β, TNF-α and IL-12 in the culture supernatants. As shown in Figure 3.4.1, stimulation of Anx-A1$^{+/+}$ DC with LPS induced a time dependent increase in IL-1β, TNF-α and IL-12 production that became significant after 3 or 6h of stimulation. Activation of Anx-A1$^{-/-}$ DC in the same conditions showed a decreased production of all cytokines compared to Anx-A1$^{+/+}$ DC (Figure 3.4.1).

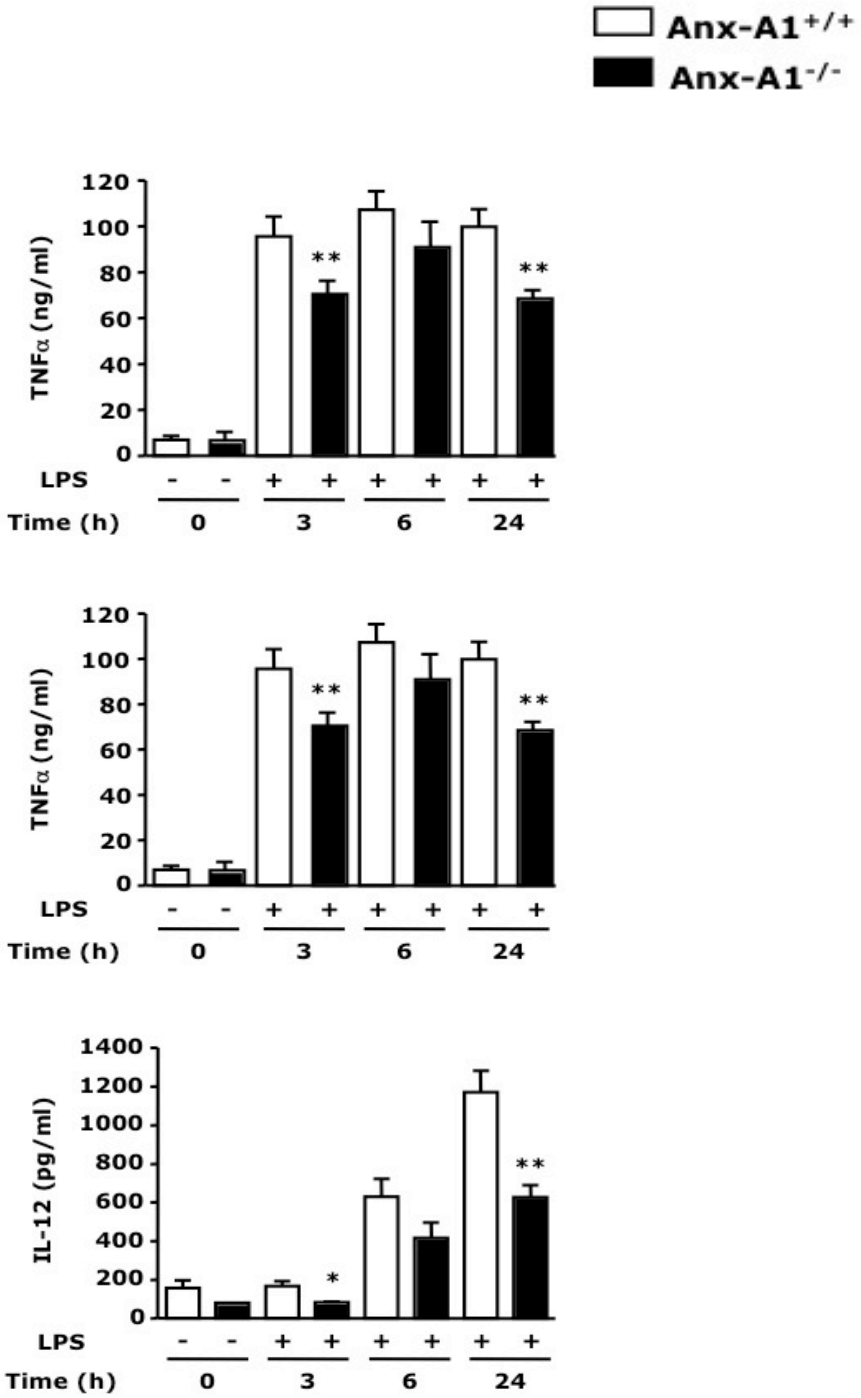

Figure 3.4.1. Impaired production of TNF-α, IL-1β and IL-12 in Anx-A1$^{-/-}$ DC.

Bone marrow-derived Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC were incubated with LPS (100ng/ml) for the indicated times and the supernatants collected and analysed for their content of IL-1β, TNF-α and IL-12. Values are mean ± S.E.M. of n=3 mice from a single experiment and are representative of 6 different experiments. P values were calculated using Student's T-test and statistical analysis was determined from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data within the same sample group. *P<0.05, **P<0.01.

3.5. Elevated IDO expression in Anx-A1$^{-/-}$ DC

Immature Anx-A1$^{-/-}$ DC have an increased surface expression of the maturation markers: CD40 and CD80, and inhibited production of pro-inflammatory cytokines: IL-1β and TNF-α as well as T-cell skewing cytokine, IL-12 when stimulated. This phenotype, to some extent, resembles semi-mature, tolerogenic DC. These DC are usually characterized by production of TGF-β, IL-10 and/or indoleamine 2,3-dioxygenase (IDO). Indoleamine 2,3-dioxygenase is a rate-limiting enzyme for tryptophan metabolism; tryptophan is an indispensable amino acid involved in T cell activation and proliferation. Hence, the expression of TGF-β, IL-10, indoleamine 2,3-dioxygenase and indoleamine 2,3-dioxygenase 2 (indoleamine 2,3-dioxygenase-like-1, IDOL1) mRNA was analyzed by real-time PCR in immature Anx-A1$^{+/+}$ DC and Anx-A1$^{-/-}$ DC stimulated with LPS. TGF-β mRNA and protein levels were not detectable by real-time PCR and ELISA respectively.

In Figure 3.5.1, Anx-A1$^{-/-}$ DC stimulated with LPS did not induce significant levels above basal expression of IL-10 mRNA whilst IL-10 mRNA expression was downregulated upon LPS stimulation in Anx-A1$^{+/+}$ DC that became significant at 6h and 24h of stimulation. Alternatively, both IDO and IDOL1 mRNA expression were upregulated in Anx-A1$^{-/-}$ DC when stimulated with LPS within the first

6h followed by a homeostatic downregulation of IDOL1 mRNA to basal level but a sustained elevation IDO mRNA levels at 24h post-LPS stimulation (Figure 3.5.2).

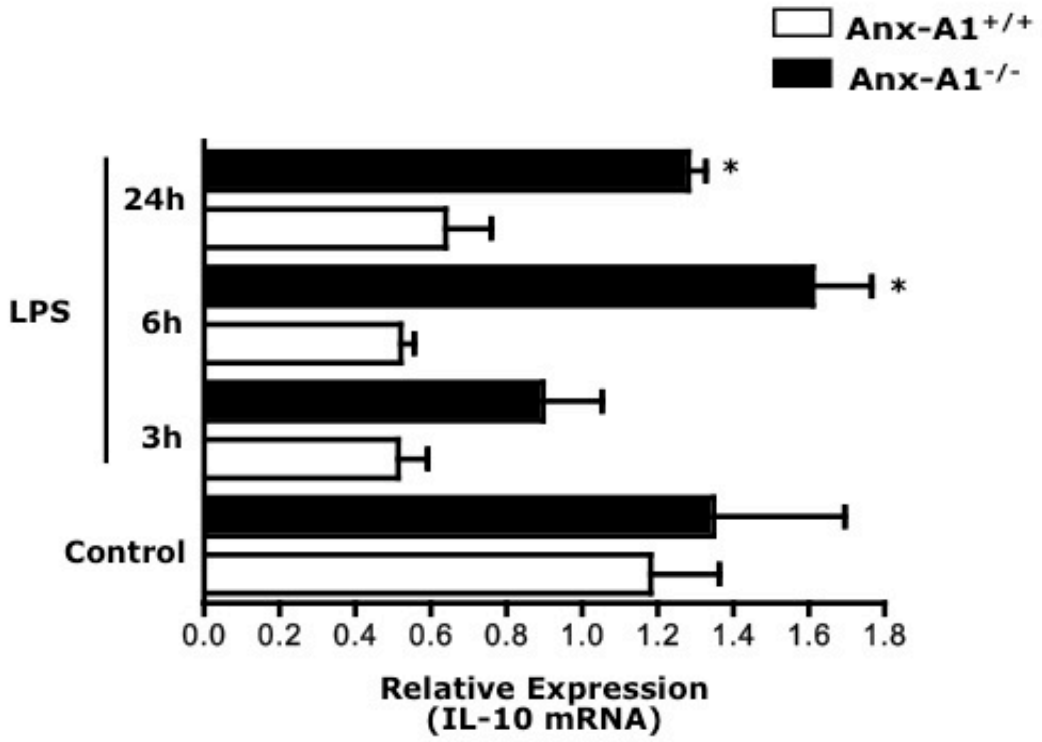

Figure 3.5.1. IL-10 mRNA Expression between Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC.

Real Time PCR analysis of IL-10 expression in Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC incubated with culture medium (Control) or LPS (LPS; 100ng/ml) for the indicated times. Values are mean ΔΔCT ± S.E.M. of n= 3 mice. Data shown are from a single experiment and P values are determined by Student's T-test and calculated from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data within the same sample group. *P<0.05.

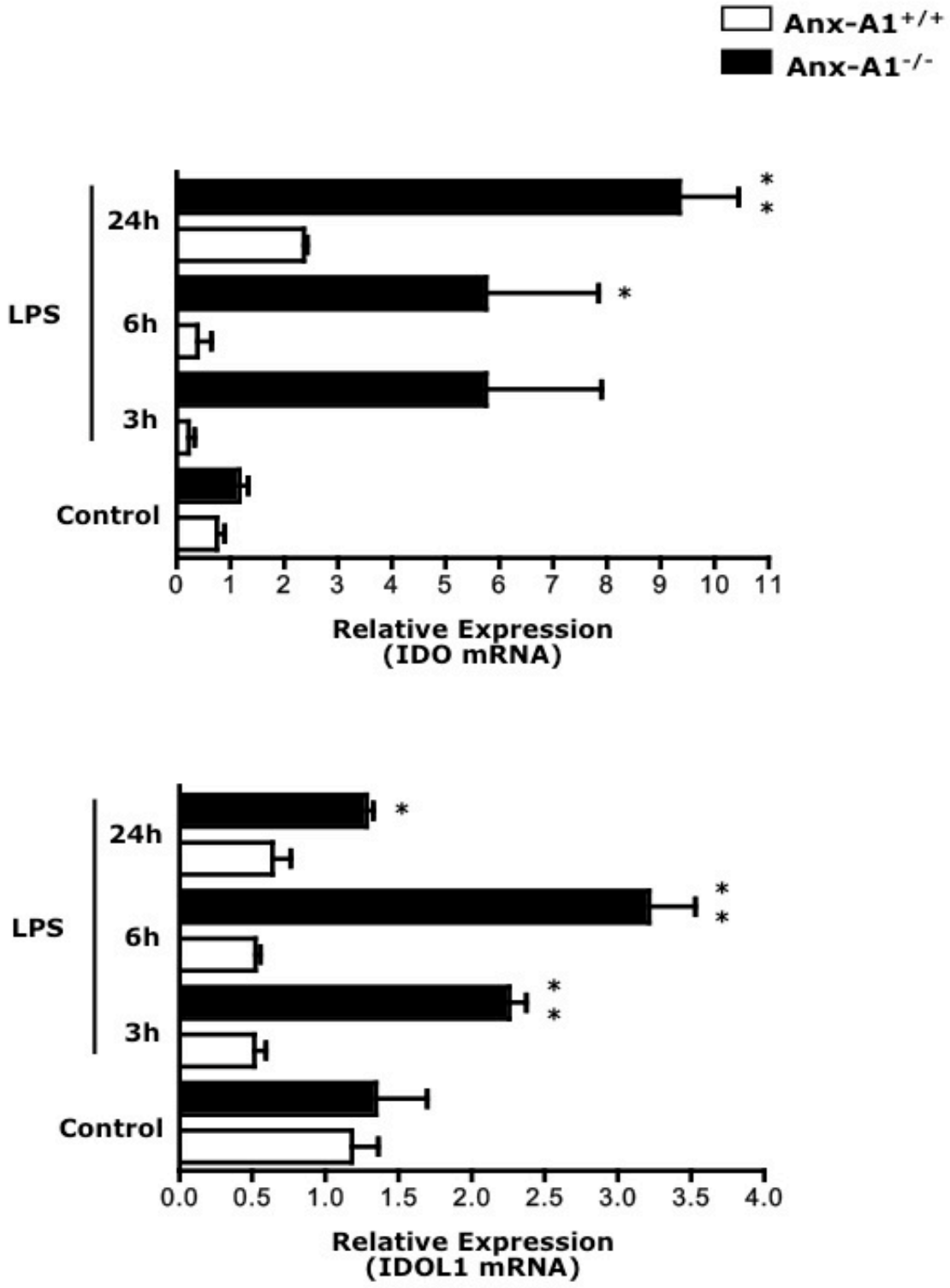

Figure 3.5.2. IDO and IDOL1 mRNA Expression between Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC.

Real Time PCR analysis of IDO and IDOL1 expression in Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC incubated with culture medium (Control) or LPS (LPS; 100ng/ml) for the indicated times. Values are mean ΔΔCT ± S.E.M. of n=3 mice. Data shown are from a single experiment and P values were determined by Student's T-test and calculated from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data within the same sample group. *P<0.05, **P<0.01

3.6. TLR4 Expression is Comparable in Anx-A1$^{-/-}$ DC

LPS is recognised by the TLR4 member of the PRR subfamily of receptors and upon LPS binding the receptor, signalling cascades are initiated. Pharmacologically, the frequency of a particular receptor expression effects cellular response to a specific stimulus; case in point, the co-stimulatory receptors expression and cytokine production. The expression of TLR4 on Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC was analysed by real-time PCR to eliminate the level of TLR4 expression as a factor accounting for poor activation status of Anx-A1$^{-/-}$ DC. Surprisingly, as shown in Figure 3.6.1, Anx-A1$^{-/-}$ DC stimulated with LPS in a time-dependent manner portrayed a slightly greater but not significant expression of TLR4 in contrast to Anx-A1$^{+/+}$ DC. However, one may assume that TLR4 mRNA expression in BMDC was generally unmodulated by LPS stimulation.

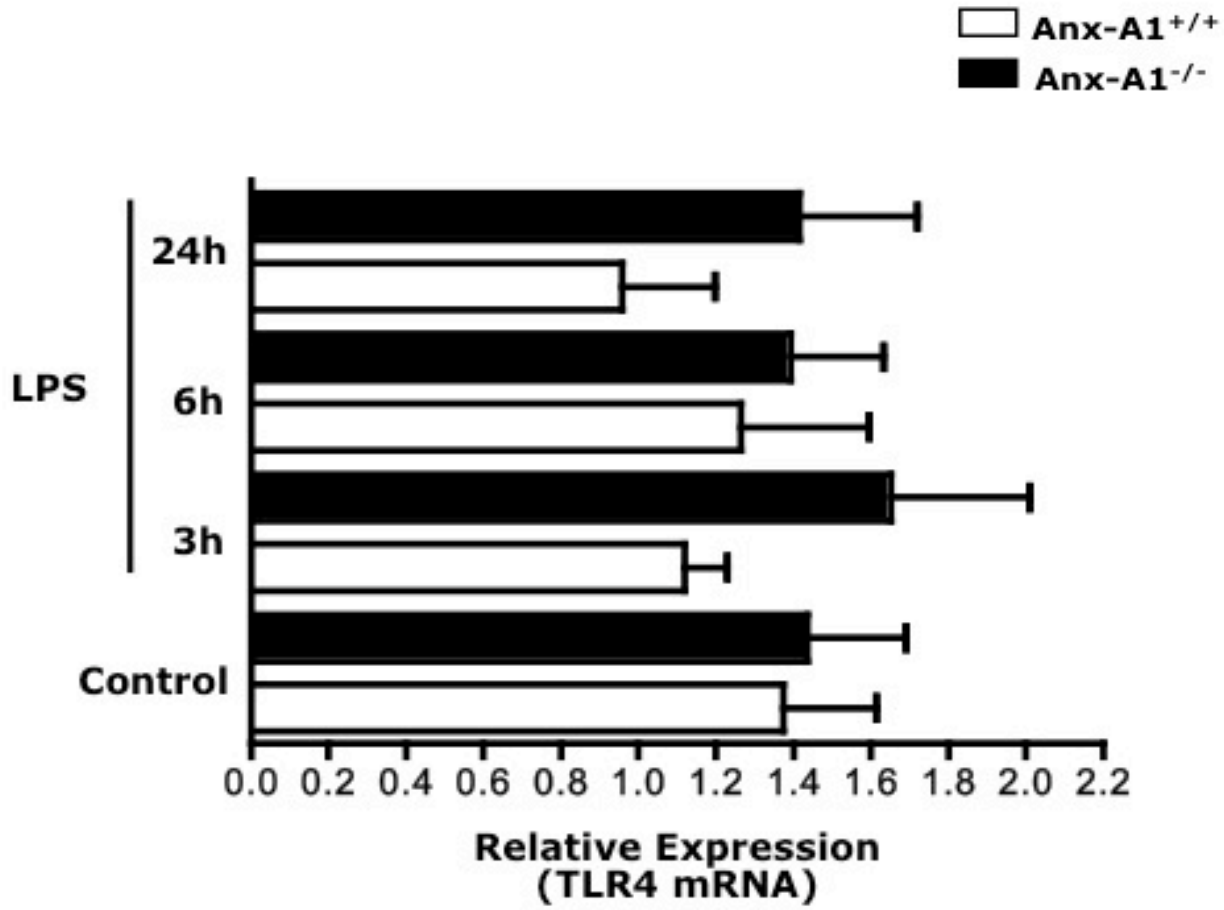

Figure 3.6.1. Paralleled TLR4 mRNA Expression between Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC.

Real Time PCR analysis of TLR4 expression in Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC incubated with culture medium (Control) or LPS (LPS; 100ng/ml) for the indicated times. Values are mean ΔΔCT ± S.E.M. of n=3 mice. Data shown are from a single experiment and P values were determined by Student's T-test and calculated from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data within the same sample group.

3.7. Diminished NF-κB activation in Anx-A1$^{-/-}$ DC

LPS activates TLR4 and induces a cascade of signalling events culminating in the activation of NF-κB and thus, the induction of numerous pro-inflammatory genes including the cytokines IL-1β and TNF-α. Given the attenuated production of these cytokines in Anx-A1$^{-/-}$ DC, we investigated whether these differences were due to an impaired activation of this transcription factor. DC from Anx-A1$^{+/+}$ or Anx-A1$^{-/-}$ mice were stimulated with LPS for various times and then analyzed for their NF-κB/DNA-binding activity by EMSA. Activation of Anx-A1$^{+/+}$ DC with LPS induced a time-dependent increase and oscillatory pattern of NF-κB/DNA-binding activity. When we performed a similar analysis in Anx-A1$^{-/-}$ DC we observed a delayed onset of oscillatory pattern and a marked reduction in NF-κB/DNA-binding activity (Figure 3.7.1).

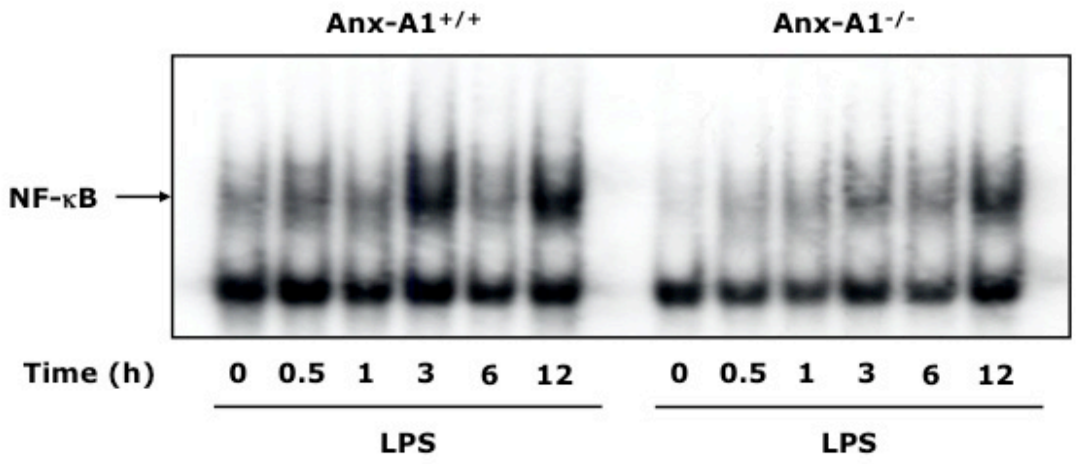

Figure 3.7.1. Delayed and defective NF-κB activation in Anx-A1$^{-/-}$ DC.

Electrophoretic mobility shift assay showing NF-κB/DNA-binding activity in nuclear extracts of Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC stimulated with LPS (100ng/ml) for the indicated times. Autoradiogram shown is representative of n=5 experiments.

3.8. Waned Re-synthesis of IκBα in Anx-A1$^{-/-}$ DC

Activation of NF-κB occurs via the phosphorylation, ubiquitination and degradation of its inhibitory subunit IκBα. This process is followed by the resynthesis of IκBα promoted by activated NF-κB itself as a negative feedback mechanism to prevent prolonged NF-κB activation (Hoffmann et al., 2002). Analysis of LPS-induced IκBα degradation in Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC showed a similar pattern with an almost complete degradation of the protein between 15 and 30min. However, at 60min post-stimulation (time when IκBα resynthesizes) Anx-A1$^{-/-}$ DC showed a virtually complete inability to regenerate protein as compared to Anx-A1$^{+/+}$ cells (Figure 3.8.1). These findings are consistent with EMSA results (Figure 3.7.1), which suggest that the reduced activation of this transcription factor might be in turn responsible of the limited resynthesis of IκBα.

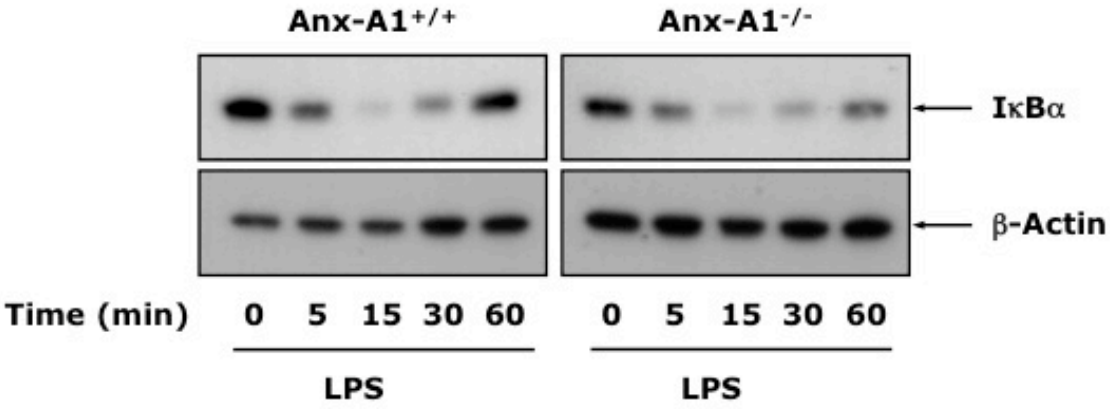

Figure 3.8.1. Impaired IκBα regeneration in Anx-A1$^{-/-}$ DC.

Western blotting analysis of IκBα degradation profile in LPS-treated Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC. Autoradiograms and blots shown are representative of n=5 experiments.

3.9. Impaired FPR Signalling in Anx-A1$^{-/-}$ DC

The binding of Anx-A1 to its receptor FPR induces a cascade of signalling events that contribute to cell activation. To investigate whether the exhausted phenotype of mature Anx-A1$^{-/-}$ DC was due to impaired FPR activation, we compared ERK1/2 and Akt phosphorylation in LPS-stimulated Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC. Figure 3.9.1 highlights that stimulation of Anx-A1$^{+/+}$ DC with LPS caused a rapid phosphorylation of both ERK1/2 and Akt whilst in Anx-A1$^{-/-}$ DC there was minimal phosphorylation of all kinases; confirming the previous observation that the lack of endogenous Anx-A1 result in diminished FPR signalling (Alldridge et al., 1999).

Physiologically, the absence of ligand may lead to modulation of its receptor expression, which can in turn be responsible for different extent of downstream signalling activation. To confirm the defect in ERK1/2 and Akt phosphorylation observed in the Anx-A1$^{-/-}$ DC was due to the absence of Anx-A1 and not to a differential expression of its receptor, Fpr1, FPR-rs1 and FPR-rs2 mRNA expression in mature and immature DC were analysed. Fpr1, FPR-rs1 and FPR-rs2 were expressed at similar levels in immature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC. Stimulation with LPS for 24h induced an upregulation of FPR and FPR-rs2 but not FPR-rs1 with no significant differences being observed in

Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC (Figure 3.9.2).

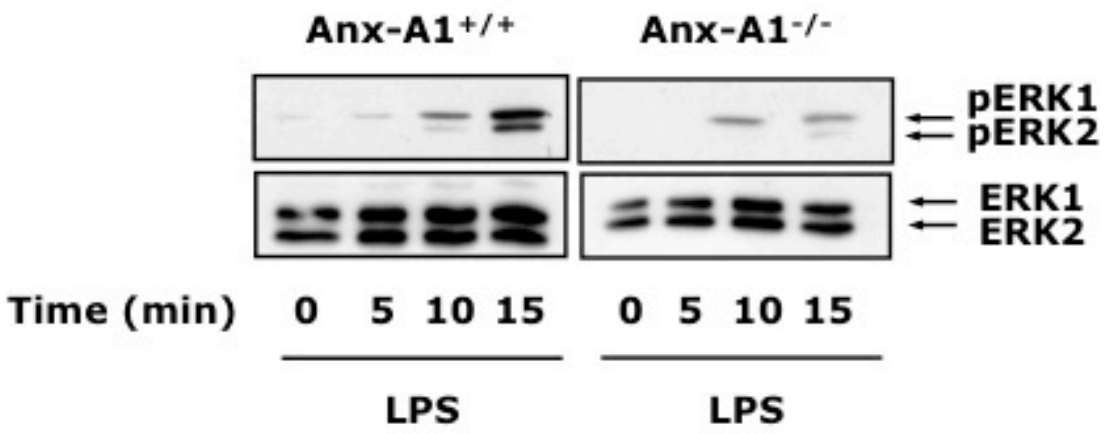

Figure 3.9.1. Impaired FPR signalling in Anx-A1$^{-/-}$ DC.

Western blot analysis of phosphorylated and total ERK1/2 and Akt of cell lysates of Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC stimulated with LPS (100ng/ml) for the indicated times. Autoradiograms and blots shown are representative of n=5 experiments.

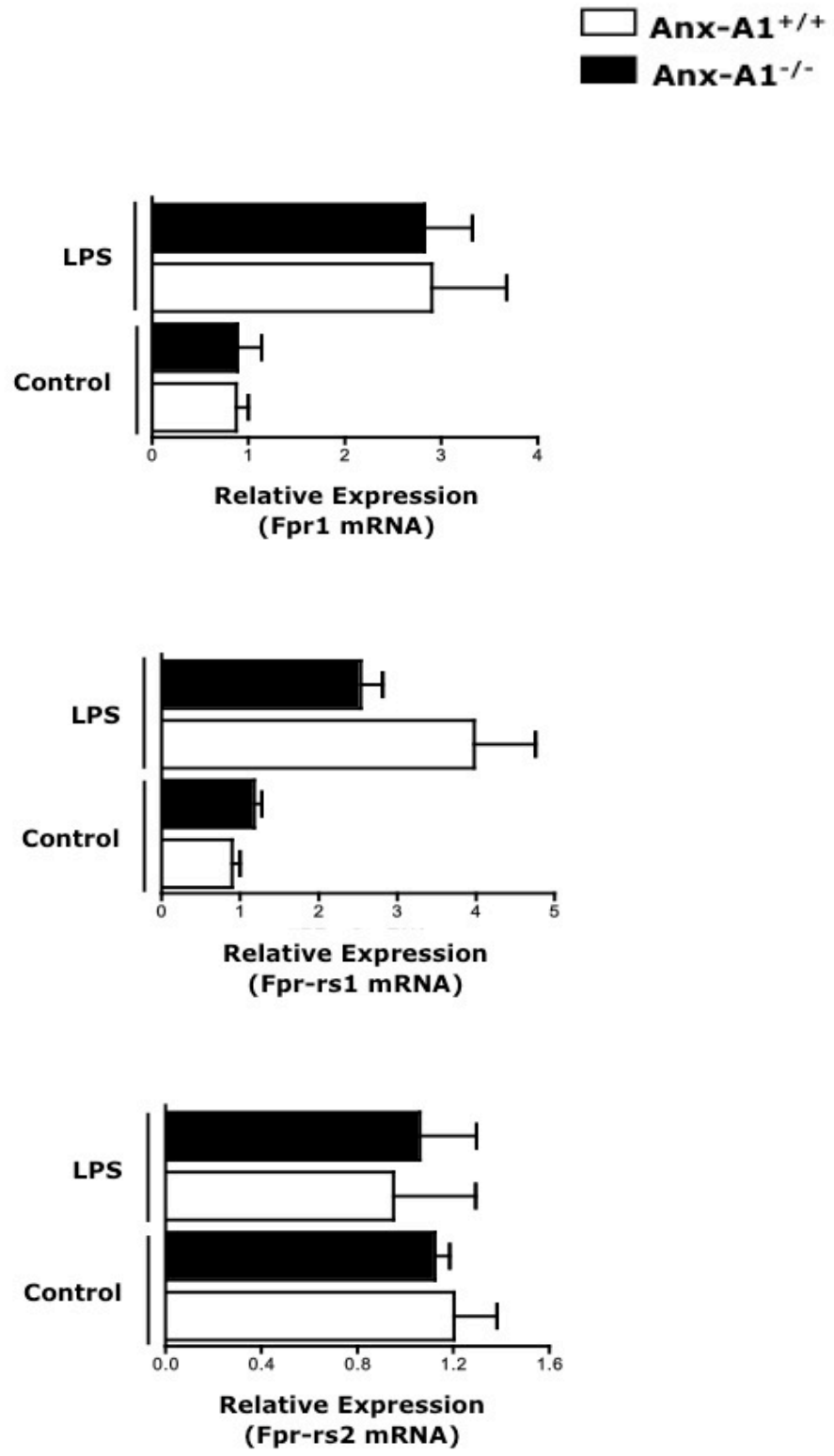

Figure 3.9.2. Analysis of FPR expression in Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC.

Real Time PCR analysis of Fpr1, Fpr-rs1 and Fpr-rs2 expression in Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC incubated with culture medium (Control) or LPS (LPS; 100ng/ml) for 24h. Values and data are mean ΔΔCT ± S.E.M. of n=3 mice per experiment and are cumulative of 5 separate experiments. P values were determined by one-way ANOVA and calculated from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data within the same sample group.

3.10. Impaired Migratory Capacity of Anx-A1$^{-/-}$ DC In Vivo

The maturation of DC at the site of infection is followed by their migration to the local draining lymph node where DC present the antigen to T cells. This process is mainly driven by the expression of the chemokine receptor CCR7 in mature DC and the high levels of its ligands CCL19 and CCL21 in the lymph nodes (Forster et al., 2008, Sanchez-Sanchez et al., 2006). Given the impaired ability of mature Anx-A1$^{-/-}$ DC to up-regulate CCR7 (as shown in Figure 3.3.1), the functional consequences of this impaired phenotype was investigated by evaluating their ability to migrate to the draining lymph nodes of host recipient mice. Therefore, we subcutaneously injected CSFE-labelled mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC into control C57BL/6 mice as previously reported (de Noronha et al., 2005, Lammermann and Sixt, 2008, Sixt et al., 2005). FACS analysis of lymph node cells collected from mice injected with wild-type DC contained about 0.3 % of CD11c^{+} CFSE^{+} cells while about 40 % less cells could be recovered from mice injected with Anx-A1$^{-/-}$ DC (Figure 3.10.1).

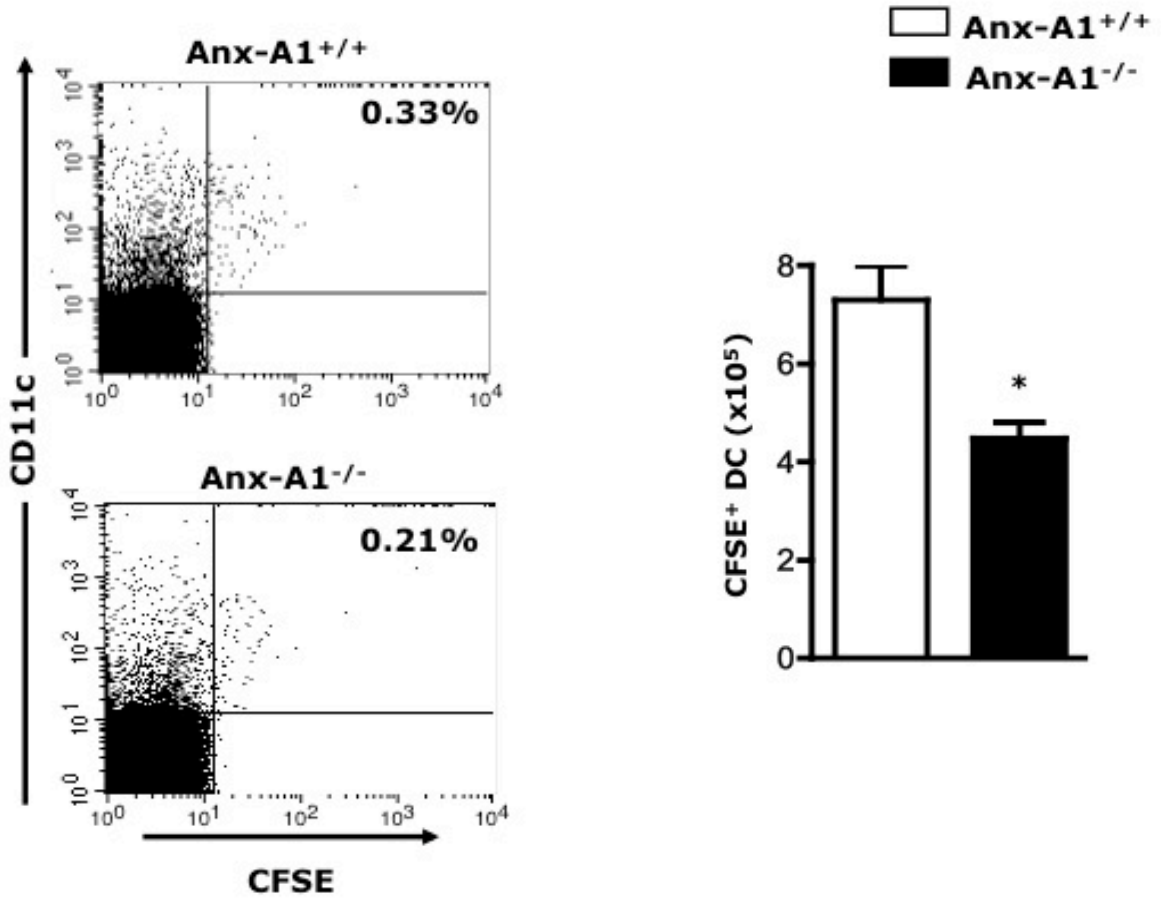

Figure 3.10.1. Impaired migratory capacity *in vivo* of Anx-A1$^{-/-}$ DC.

LPS-matured Anx-A1$^{-/-}$ and Anx-A1$^{+/+}$ DC were labelled with CFSE and injected subcutaneously into the hind paws of C57BL/6 mice. DC migration to the draining lymph nodes was assessed as described in Materials and Methods 48 hours after the injection. The numbers in the dot plots indicate the percentage of CD11c^{+}/CFSE^{+} DC recovered from a single mouse and are representative of 3 different experiments with similar results. The bar graph shows the mean number ± S.E.M. of CD11c^{+}/CFSE^{+} DC recovered from n=3 mice and is representative of 3 different experiments with similar results. P values were determined by one-way ANOVA and calculated from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data. *P<0.05.

3.11. Decreased T-cell Stimulatory Capacity of Anx-A1$^{-/-}$ DC

The impaired capacity of Anx-A1$^{-/-}$ DC to fully mature prompted us to assess their ability to initiate T cell-driven responses. As a result, immature and mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC from C57BL/6 mice were co-cultured with allogeneic naïve T cells form BALB/c mice and T cell proliferation, IL-2 and IFN-γ production were recorded as a measure of T-cell stimulatory ability. Consistent with the findings showed thus far, mature Anx-A1$^{+/+}$ DC induced a vigorous proliferative response and elevated IL-2 and IFN-γ production whereas Anx-A1$^{-/-}$ DC showed a significantly reduced ability to induce either cell proliferation or IL-2 and IFN-γ production. Surprisingly, incubation of naïve T cells with immature Anx-A1$^{-/-}$ DC showed increased but not significant levels of ^{3}H-thymidine incorporation, IL-2 and IFN-γ production compared to Anx-A1$^{+/+}$ counterparts (Figure 3.11.1). Together these results further demonstrate that, in absence of Anx-A1, DC lose their capacity to stimulate a robust T cell mediated effector response.

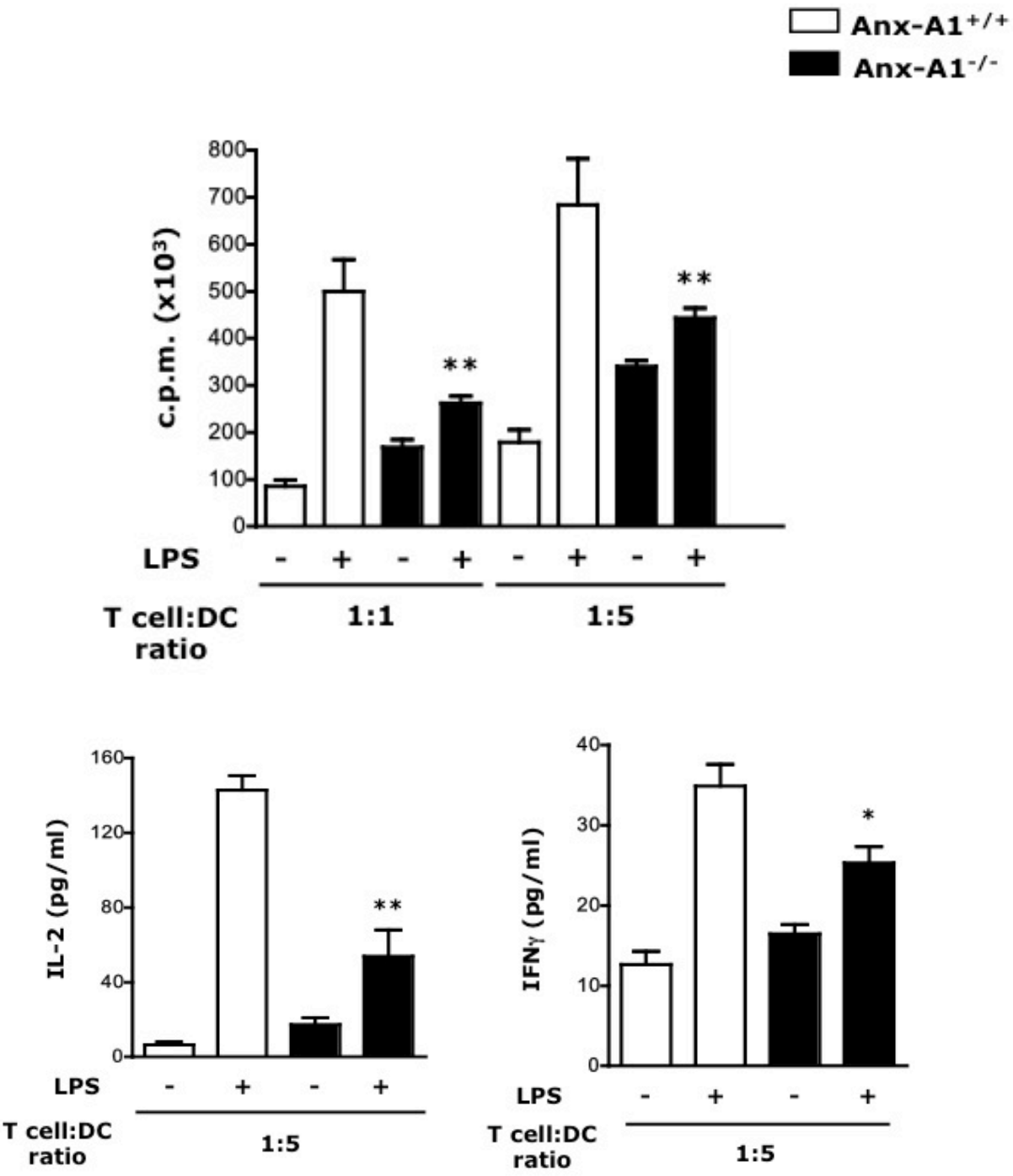

Figure 3.11.1. Abated stimulatory activity and Th1 skewing capacity of LPS-matured Anx-A1$^{-/-}$ DC.

^{3}H-thymidine incorporation, IL-2 and IFN-γ production from a mixed leukocyte reaction of naïve CD4^{+} T cells from BALB/c mice cultured with allogeneic immature or mature Anx-A1$^{+/+}$ or Anx-A1$^{-/-}$ DC from C57BL/6 mice at two ratios (1:1 or 1:5) for 72h. Values are mean ± S.E.M. of cytokine concentration from n=3 mice and is representative of 4 different experiments. P values were determined by Student's T-test and calculated from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ comparison of the respective data sets. *P<0.05, **P<0.01.

3.12. Diminished Activation of Antigen-specific T cells by Anx-A1$^{-/-}$ DC

Confirmation of the impaired ability of Anx-A1$^{-/-}$ DC to initiate T cell-driven responses prompted further dissection of this poor APC function of Anx-A1$^{-/-}$ DC. Therefore, the use of transgenic TCR mouse models was employed to emphasize if the effect of Anx-A1 is more substantial in certain types of antigen-specific T-cell reactions; to perform this, the OT-I/RAG-1$^{-/-}$ and OT-II/RAG-1$^{-/-}$ transgenic mice were exploited to this end. OT-I/RAG-1$^{-/-}$ and OT-II/RAG-1$^{-/-}$ mice generate mature naïve T lymphocytes with a single functional TCR epitope that recognise the peptide sequences SIINFEKL ($OVA_{257-264}$) and ISQAVHAAHAEINEAGR ($OVA_{323-339}$) from chicken ovalbumin respectively.

Hence, LPS-matured Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC were pulsed with corresponding chicken ovalbumin peptides ($OVA_{257-264}$ or $OVA_{323-339}$) then co-cultured with transgenic TCR T cells form OT-I/RAG-1$^{-/-}$ (OT-I CD8) and OT-II/RAG-1$^{-/-}$ (OT-II CD4) mice. T cell proliferation was noted as a measure of DC stimulatory potential within antigen-specific T-cell responses. The findings showed that peptide-pulsed, mature Anx-A1$^{+/+}$ DC induced a strong, concentration-independent proliferative response in OT-I CD8 T cells and a strong, concentration-dependent proliferation of OT-II CD4 T cells during

both 5-day co-culture (Figure 3.12.1) and 7-day co-culture (Figure 3.12.2) incubations. Whereas Anx-A1$^{-/-}$ DC demonstrated a significantly reduced ability to induce T cell proliferation in both OT-I CD8 and OT-II CD4 T cells during 5-day co-culture (Figure 3.12.1). Though the lack of proliferation was sustained in co-cultures with OT-I CD8 T cells and Anx-A1$^{-/-}$ DC for 7-day co-cultures, this proliferative insufficiency, interestingly, was markedly abolished in similar incubations between Anx-A1$^{-/-}$ DC and OT-II CD4 T cells as seen in Figure 3.12.2.

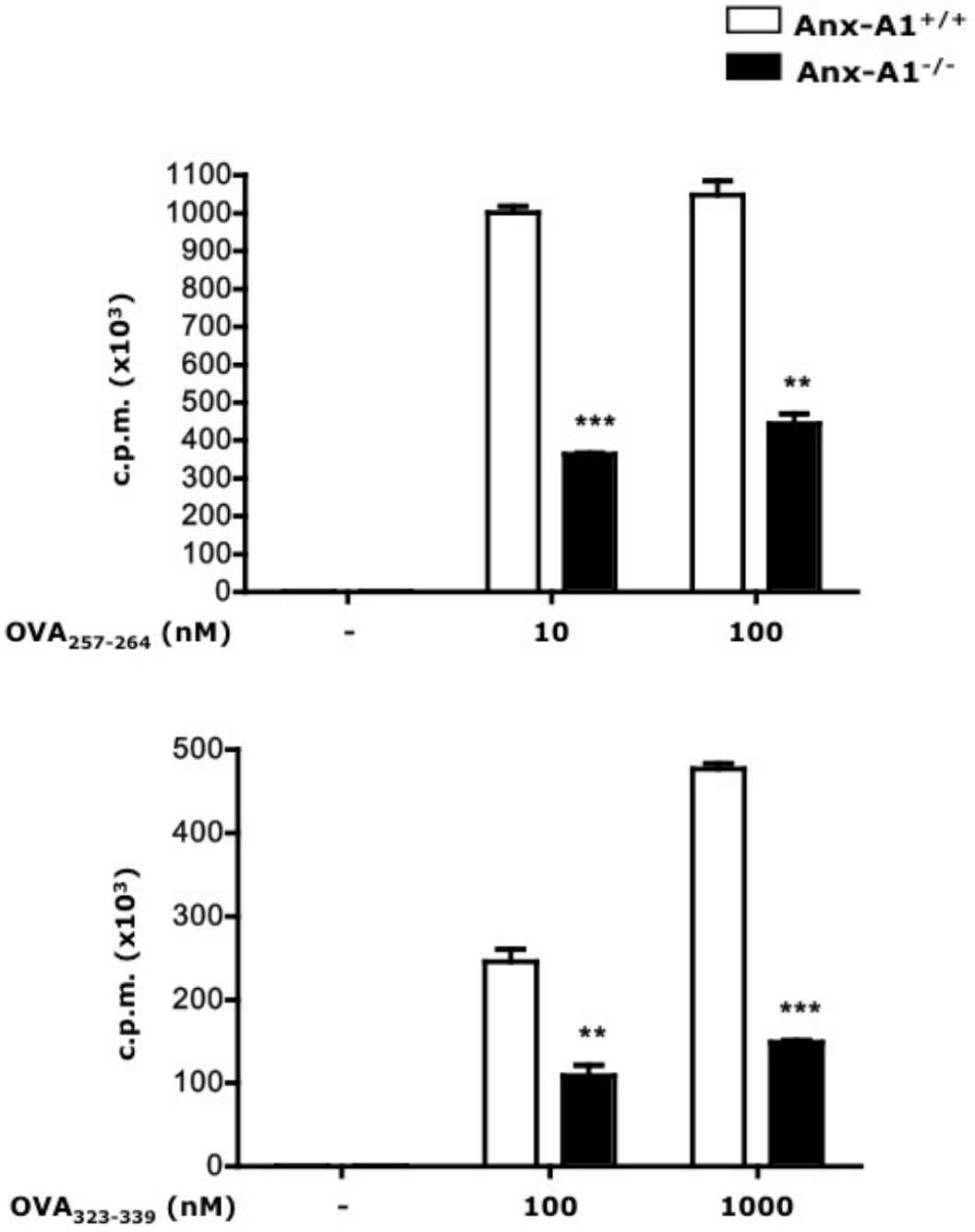

Figure 3.12.1. Weakened stimulatory activity of peptide-pulsed, LPS-matured Anx-A1$^{-/-}$ DC in 5-day co-cultures.

^{3}H-thymidine incorporation from mixed leukocyte reactions of OT-I CD8 T cells (upper panel) and OT-II CD4 T cells (lower panel) cultured with OVA-loaded, mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC for 5 days. Values are mean radioactivity ± S.E.M. of 4 separate experiments with similar results. P values were determined by Student's T-test and calculated from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ comparison of the respective data sets. **P<0.01, ***P<0.001.

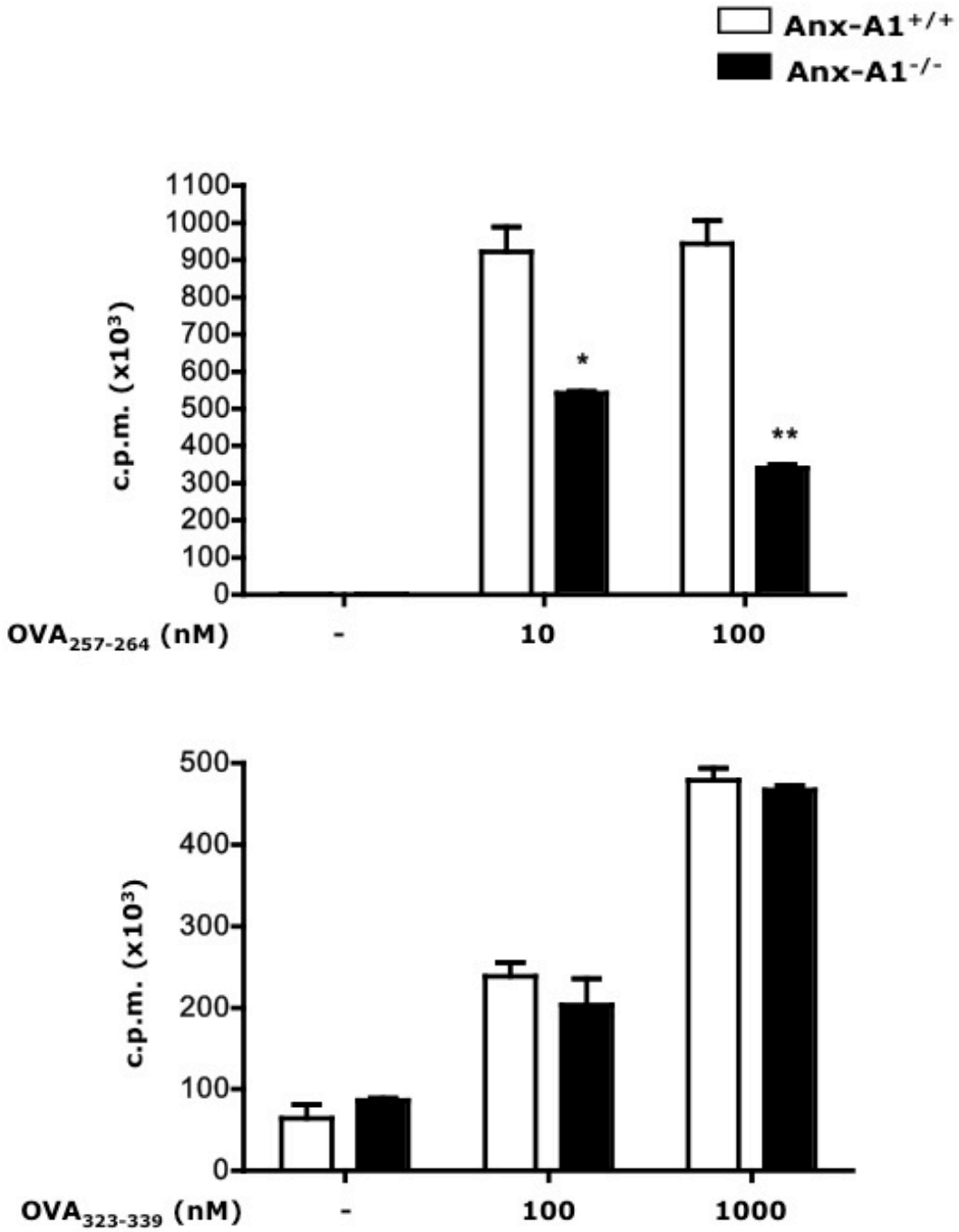

Figure 3.12.2. Lessened Stimulatory effect in 7-day co-cultures of peptide-pulsed, LPS-matured Anx-A1$^{-/-}$ DC with OT-I CD8 T cells.

^{3}H-thymidine incorporation from mixed leukocyte reactions of OT-I CD8 T cells (upper panel) and OT-II CD4 T cells (lower panel) cultured with OVA-loaded, mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC for 7 days. Values are mean radioactivity ± S.E.M. of 4 separate experiments with similar results. P values were determined by Student's T-test and calculated from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ comparison of the respective data sets. *P<0.05, **P<0.01.

3.13. Reduced IL-2 Production in OT-I CD8 T cell co-cultures with Anx-A1$^{-/-}$ DC

Observing the impaired ability of Anx-A1$^{-/-}$ DC to initiate OT-I CD8 T cell proliferation over 5-day and 7-day co-cultures and OT-II CD4 T cell expansion in 5-day cultures encouraged further scrutiny of T cell-driven responses. Therefore, the supernatants of OVA-pulsed, LPS-matured Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC co-cultured with OT-I/RAG-1$^{-/-}$ (OT-I CD8) and OT-II/RAG-1$^{-/-}$ (OT-II CD4) transgenic TCR T cells were collected for cytokine analysis. The T-cell-specific mitogenic cytokine, IL-2, was tested as a measure of T-cell growth.

OVA-pulsed, mature Anx-A1$^{+/+}$ DC provoked a potent, concentration-dependent increase in IL-2 co-culture levels containing both OT-I CD8 T cells and OT-II CD4 T cells during 5-day co-culture as well as 7-day co-culture incubations (Figure 3.13.1 and Figure 3.13.2 respectively). Anx-A1$^{-/-}$ DC stimulated an abridged IL-2 production in both OT-I CD8 and OT-II CD4 T cell co-cultures, which were significant at certain concentrations, during 5-day co-culture incubations (Figure 3.13.1). Although this suppression in IL-2 levels persisted in co-cultures with Anx-A1$^{-/-}$ DC and OT-I CD8 T cells for 7-day co-cultures, this IL-2 deficiency was completely rescued in 7-day incubations between Anx-A1$^{-/-}$ DC and OT-II CD4 T cells (Figure 3.13.2).

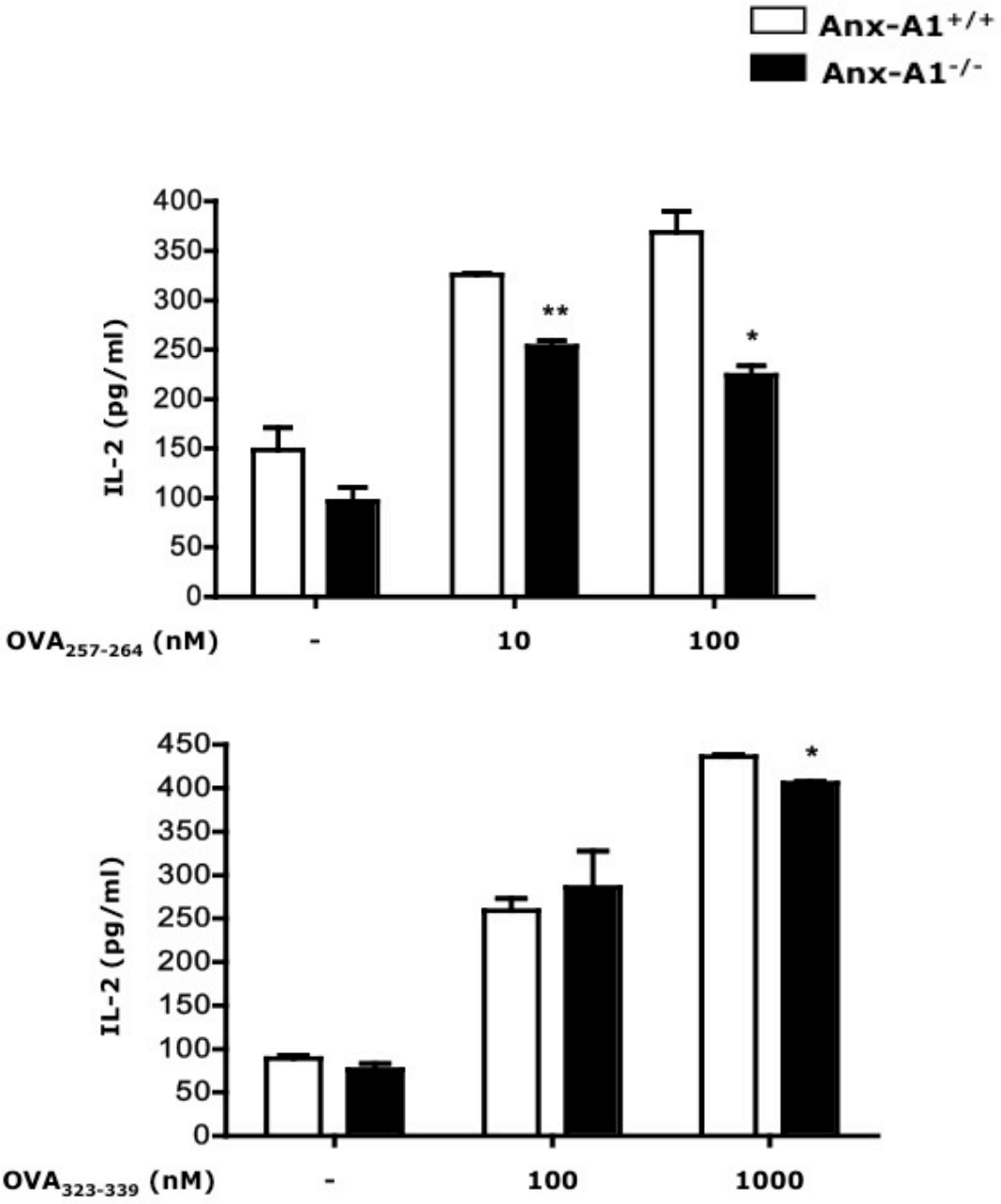

Figure 3.13.1. Attenuated IL-2 content in 5-day co-cultures with OVA-pulsed, LPS-matured Anx-A1$^{-/-}$ DC.

Specific IL-2 ELISA analysis from mixed leukocyte reactions of OT-I CD8 T cells (upper panel) and OT-II CD4 T cells (lower panel) cultured with OVA-loaded, mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC for 5 days. Values are mean concentration ± S.E.M. of 4 separate experiments with similar results. P values were calculated using Student's T-test and statistical analysis was determined from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data within the same sample group. *P<0.05, **P<0.01.

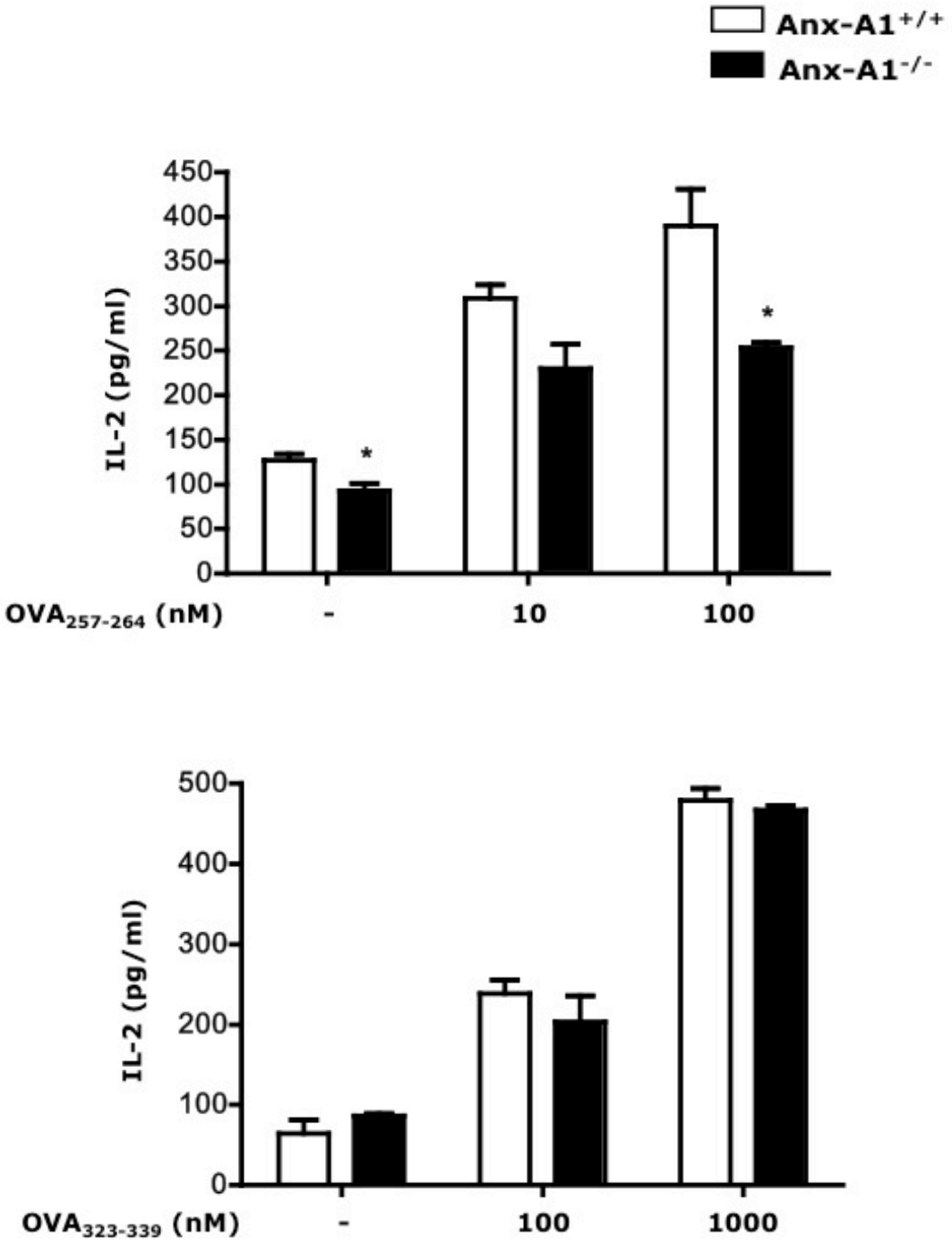

Figure 3.13.2. Suppressed IL-2 levels in 7-day co-cultures of OVA-pulsed, LPS-matured Anx-A1$^{-/-}$ DC with OT-I CD8 T cells.

Specific IL-2 ELISA analysis from mixed leukocyte reactions of OT-I CD8 T cells (upper panel) and OT-II CD4 T cells (lower panel) cultured with OVA-loaded, mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC for 7 days. Values are mean concentration ± S.E.M. of 4 separate experiments with similar results. P values were calculated using Student's T-test and statistical analysis was determined from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data within the same sample group. *P<0.05, **P<0.01.

3.14. Impaired IFN-γ Production in OT-I and OT-II T cell co-cultures with Anx-A1$^{-/-}$ DC

Surveying the T cell proliferation and IL-2 production of OT-I and OT-II T cells when incubated with Anx-A1$^{+/+}$ DC and Anx-A1$^{-/-}$ DC during 5-day and 7-day co-cultures revealed intricate data that suggest a varying extent of the role of DC-derived Anx-A1 in CD8 and CD4 T-cell responses. Hence, additional exploration of this effect on a differentiating T-helper-like phenotype was pursued. Therefore, the supernatants of OVA-pulsed, LPS-matured Anx-A1$^{+/+}$ DC and Anx-A1$^{-/-}$ DC co-cultured with OT-I/RAG-1$^{-/-}$ (OT-I CD8) and OT-II/RAG-1$^{-/-}$ (OT-II CD4) transgenic TCR T cells used for the IL-2 investigations were also examined for their IFN-γ, IL-4, IL-17 and IL-10 content.

Co-culture supernatants of OVA-pulsed, mature Anx-A1$^{+/+}$ DC and Anx-A1$^{-/-}$ DC and both OT-I CD8 T cells and OT-II CD4 T cells during 5-day as well as 7-day incubations did not trigger measurable levels of IL-4 and the IL-17 and IL-10 cytokine profiles were not reproducible and thus inconclusive. However, OVA-pulsed, mature Anx-A1$^{+/+}$ DC elicited a profound IFN-γ release in co-cultures with OT-I CD8 T cells, in a concentration-independent manner, and OT-II CD4 T cells, in a concentration-dependent manner, during both 5-day co-culture and 7-day co-culture incubations (Figure 3.14.1 and 3.14.2 respectively). Meanwhile, Anx-A1$^{-/-}$ DC stimulated a significant deficit

in IFN-γ production in both OT-I CD8 and OT-II CD4 T cell co-cultures during both 5-day co-cultures (Figure 3.14.1) and 7-day co-cultures (Figure 3.14.2).

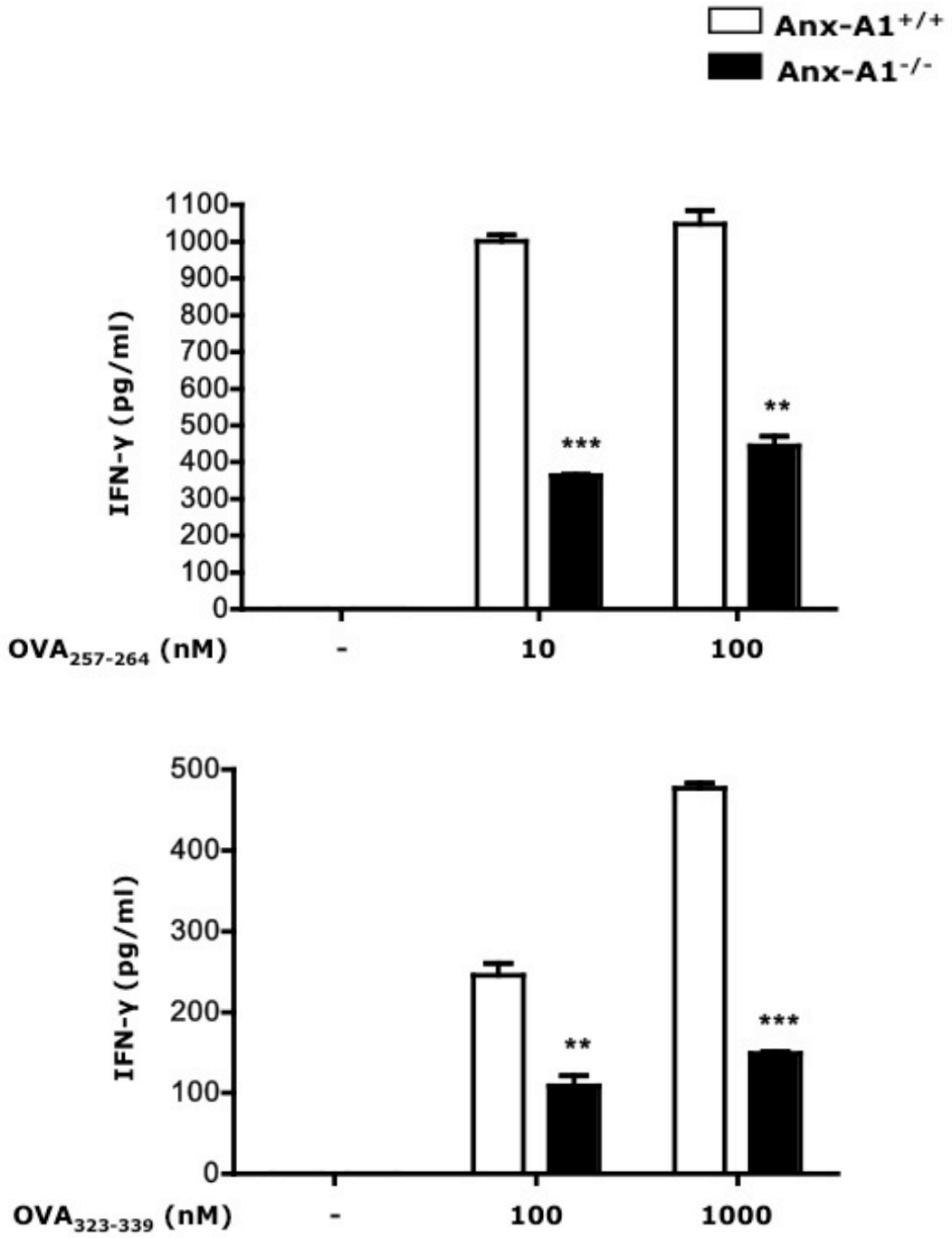

Figure 3.14.1. Diminutive IFN-γ concentrations in 5-day co-cultures of OVA-pulsed, LPS-matured Anx-A1$^{-/-}$ DC.

IFN-γ production in co-cultures of OT-I CD8 T cells (upper panel) and OT-II CD4 T cells (lower panel) and OVA-loaded, mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC for 5 days. Values are mean concentration ± S.E.M. of 4 separate experiments with similar results. P values were calculated using Student's T-test and statistical analysis was determined from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data within the same sample group. **P<0.01, ***P<0.001.

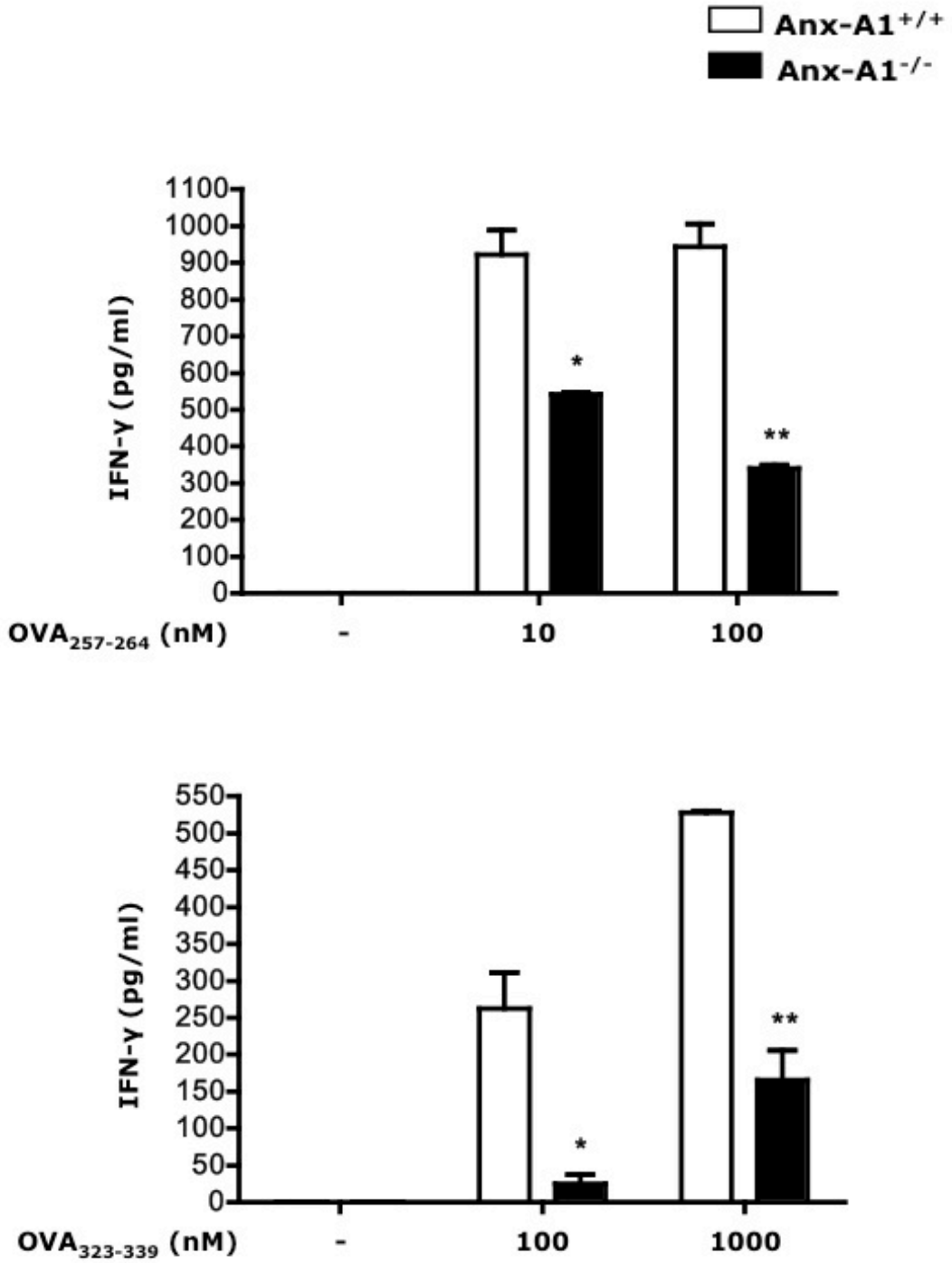

Figure 3.14.2. Decreased IFN-γ production in 7-day co-cultures of OVA-pulsed, LPS-matured Anx-A1$^{-/-}$ DC and OT-I CD8 T cells.

IFN-γ content in co-cultures of OT-I CD8 T cells (upper panel) and OT-II CD4 T cells (lower panel) and OVA-loaded, mature Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC for 7 days. Values are mean concentration ± S.E.M. of 4 separate experiments with similar results. P values were calculated using Student's T-test and statistical analysis was determined from Anx-A1$^{+\backslash+}$ *vs.* Anx-A1$^{-\backslash-}$ data within the same sample group. *P<0.05, **P<0.01.

4. DISCUSSION

Recent focus on the role of Anx-A1 in the adaptive immune response has revealed that this protein modulates T cell activation by fine-tuning the strength of TCR signalling (D'Acquisto et al., 2008a, D'Acquisto et al., 2007a, D'Acquisto et al., 2007b). In this study, I sought to determine the role of endogenous Anx-A1 in DC biology examining events preceding T cell activation, but with a clear overspill on the consequential ability to activate T cells. To this aim, we generated bone marrow DC from Anx-A1$^{-/-}$ mice and compared them to their wild-type littermates.

4.1. Anx-A1 Expression and Modulation in BMDC

Prior to published data based on this thesis work, Anx-A1 had not been shown to be expressed in human and murine dendritic cells *ex vivo* nor *in vitro*. Extensive work had been performed, on very closely related cells, by focussing on the actions, the expression and distribution and the modulation of Anx-A1, in monocytes and more particularly, macrophages (Ambrose et al., 1992, Perretti et al.,

1993b, Getting et al., 1997). Therefore, firstly, I examined if Anx-A1 is present in bone marrow-derived dendritic cells and whether the protein was modulated during DC activation via TLR, and DC maturation. I found that Anx-A1 protein was preformed in the cytosolic compartment of terminally differentiated immature bone marrow-derived dendritic cells with the full-length protein at predominant levels compared to the N-terminal cleaved species, which was at minimal levels. In addition, the activation of TLR4 by LPS resulted in the secretion of the protein into the extracellular space with no detectable increase in protein cleavage. For the recorded time-points, the secretion peaked at 3h post-LPS stimulation and the protein was being resynthesized by 12h post-stimulation and was completely restored to basal levels at 24h after activation. I also scrutinised the profile of messenger transcript levels during maturation process and the data complemented the protein modulation.

4.2. Semi-mature Profile of Immature Anx-A1$^{-/-}$ DC

The first difference that I observed when I examined the classical phenotypical characteristics of immature BMDC was that immature Anx-A1$^{-/-}$ DC displayed an increased number of CD11c^{+} cells and, in addition, expressing high levels of CD40, CD54, CD80, reduced levels of CCR7 but similar levels of MHC II and CD86. One of the primary

functional attributes of immature DC is their ability to patrol and to endocytose foreign matter as potential danger-associated molecular patterns (DAMP). If DC stimulation does not occur in the periphery, DC remain immature and tissue-resident, expressing low levels of MHC class II (MHC-II) and co-stimulatory molecules. Therefore, the immature Anx-A1$^{-/-}$ DC exhibited the phenotypic features of DC that underwent a partial- or semi-maturation process.

These semi-mature DC develop typically under steady-state conditions from immature, tissue-resident DC that encounter self-antigens and commence a maturation process driven by TNF-α, which is expressed in considerable quantities in murine immature DC. Generation of semi-mature DC from either murine precursors or human peripheral blood monocytes possess a tolerogenic profile and induce either T cell anergy or, in most cases, a regulatory T cell pool (McGuirk et al., 2002, Akbari et al., 2001, Menges et al., 2002, Jonuleit et al., 2000). The characteristics of these DC are high expression of MHC-II and co-stimulatory molecules, but low or absent production of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and in particular, IL-12p70 and some of cases production of anti-inflammatory cytokines, such as IL-10 (Christensen et al., 2002).

4.3. Weakened Up-Regulation of Maturation Molecules on mature Anx-A1$^{-/-}$ DC

To further compare the characteristics of semi-mature tolerogenic DC, I analyzed the surface receptors in LPS-matured Anx-A1$^{-/-}$ DC to observe if there would be a switch from tolerogenic to immunogenic functional profile (Granucci et al., 2001). Surprisingly, my observations uncovered an impaired capacity to up-regulate the expression of most of the receptors investigated and a virtual inability of MHC-II, CD80, CD54 and CCR7 up-regulation compared to Anx-A1$^{+/+}$ DC.

This loss of plasticity in immature Anx-A1$^{-/-}$ DC to resume maturation from its arrested, semi-mature state to a fully mature, immunogenic DC when exposed to stimuli such as LPS was not expected. Immature Anx-A1$^{-/-}$ DC appeared to emerge from the DC differentiation process in a peculiar state of terminally matured DC and application of LPS only provoked a minor increase of up-regulation in a couple of receptors examined. Specifically, the most significant defect was the down-regulated expression of CCR7 and the failure of its upregulation upon exposure of Anx-A1$^{-/-}$ DC to LPS. I proceeded to question prominent and distinctive qualities of both immature DC, such as endocytosis, and mature DC, such as cytokine production and migration.

4.4 Reduced Endocytic Ability of Immature Anx-A1$^{-/-}$ DC

One of the main functional characteristics of immature DC is endocytosis (Blander and Medzhitov, 2006, Silverstein et al., 1977). Considering that immature Anx-A1$^{-/-}$ DC display a semi-mature phenotypic profile and the intimate involvement of the annexins with actin and actin-dependent process such as phagocytosis (Gerke and Moss, 1997, Hayes et al., 2004), I, therefore, addressed the endocytic capacity of immature Anx-A1$^{-/-}$ DC. Immature Anx-A1$^{-/-}$ DC demonstrated an inhibition of endocytosis - a 34% reduction of mean fluorescence intensity of endocytosed FITC-dextran by immature Anx-A1$^{-/-}$ DC compared to immature Anx-A1$^{+/+}$ DC. DC maturation triggers a transformation process that converts immature, antigen-processing DC into mature, antigen-presenting DC. As expected, endocytosis mechanisms are down-regulated in mature DC, upon LPS stimulation both mature Anx-A1$^{+/+}$ DC and Anx-A1$^{-/-}$ DC endocytosed considerably less FITC-dextran compared to their immature counterparts.

4.5. Impaired Mature DC Functions displayed by Anx-A1$^{-/-}$ DC

Stimulation of DC with microbial products - such as LPS - instigate a complex maturation process in immature DC characterized by their migration from tissues to secondary lymphoid organs (Reis e Sousa et al., 2003) and up-regulation of MHC as well as co-stimulatory molecules that are pivotal in T cell priming and the secretion of pro-inflammatory cytokines including TNF-α and IL-1β as well as IL-12, a cytokine that is essential in driving Th1 cells polarization (Macagno et al., 2007).

My investigations revealed that LPS-matured Anx-A1$^{-/-}$ DC failed to effectively migrate to the draining afferent lymph nodes in an adoptive transfer experimental protocol. This was conclusive by the lower percentage of migrated, CFSE-positive Anx-A1$^{-/-}$ DC present in the draining lymph nodes compared to the percentage of migrated, CFSE-positive Anx-A1$^{+/+}$ DC present in similar tissues.

The down-regulated expression of CCR7 in immature Anx-A1$^{-/-}$ DC, which ceased to increase its expression upon activation with LPS was a key indicator of a probable migratory deficiency. Inflammatory DC, as well as steady-state migratory DC, absolutely require the up-regulation of surface CCR7 in order to initiate migration along

CCL19/CCL21 chemokine gradient to proximal lymph nodes for DC-T-cell interactions (Randolph, 2001, Sallusto et al., 1999, Sallusto and Lanzavecchia, 2000, Ohl et al., 2004)

Examining cytokine production, in my experiments, the strongest impairment was observed at the early time points (range from 0.5h to 6h) suggesting that the absence of Anx-A1 exerts a stronger influence on the first phase of LPS-induced DC activation and that this might be responsible for the ablated production of IL-1β, TNF-α and IL-12.

Several studies have shown that cytokine production by DC is subjected to tight control (Reis e Sousa, 2004b, Reis e Sousa, 2004a). In particular, it has been shown that the production of TNF-α and IL-1β occur in the first few hours post-stimulation and the production of IL-12 peak at later time points. These and other studies demonstrated that in response to LPS, the kinetics of DC activation is extremely important in regulating their capacity to prime T cells (Langenkamp et al., 2002, Langenkamp et al., 2000).

4.6. Defective oscillatory NF-κB activation in AnxA1-deficient DC

To understand the mechanism responsible for decreased TNF-α, IL-1β and IL-12 production in Anx-A1$^{-/-}$ DC, I investigated early and late LPS signalling in these cells.

LPS activates TLR4, a member of a family of innate receptors that sense microbial products and trigger DC maturation and cytokine production. Activation of TLR4 leads to the recruitment of adaptor proteins, which in turn lead to a cascade of events culminating in the activation of NF-κB (Reis e Sousa, 2004b). LPS signals through TLR4 and activates two downstream pathways, each of which is thought to directly activate NF-κB (Kawasaki et al., 2003, Barton and Medzhitov, 2003). The MyD88-dependent pathway recruits IRAK1 and IRAK4 kinases, which phosphorylate TRAF6, and in turn leads to the activation of the IKK complex. The MyD88-independent cascade that results in NF-κB activation is yet to be fully elucidated. The pathway is dependent on the TRIF, TRAM, RIP1 and RIP3 have been identified as important factors in the pathway (Meylan et al., 2004, Yamamoto et al., 2003a, Yamamoto et al., 2003b). However, the end result of these pathways is the same as the end point of the TNFα-activated pathway: degradation of IκB, which is followed by activation of IκBα gene transcription (Ashall et al., 2009, Sun et al., 1993). NF-κB

activation through the MyD88-dependent pathway occurs earlier than activation by the MyD88-independent pathway (Yamamoto et al., 2003a). This suggested that the non-oscillatory behaviour of NF-κB activation through TLR4 could be due to the interaction of the two pathways.

I, first, investigated if the phenotype of Anx-A1$^{-/-}$ DC might be due to an impaired expression of this receptor and we did not find any difference. Thereafter I analyzed NF-κB activation over several time points. Stimulation of Anx-A1$^{+/+}$ DC with LPS induced a strong sinusoidal pattern of NF-κB/DNA-binding activity, which is atypical of LPS-induced NF-κB activation pattern, even when compared with that of LPS-activated macrophages (Covert et al., 2005). In contrast, Anx-A1$^{-/-}$ DC showed a delayed kinetics and marked decrease in NF-κB activation during the first 6h of stimulation and then a partial recovery at 12h. This impaired NF-κB activation coincided with the period where we observed a significantly decreased production of IL-1β and TNF-α.

The lag in NF-κB kinetics resultant from stimulation by LPS compared to that of TNFα could transpire through two modes:
the MyD88-independent pathway could initiate much slower kinetics than that of the MyD88-dependent pathway;
the MyD88-dependent and MyD88-independent pathways could display similar kinetics, in which case the initiation of the MyD88-

independent pathway signalling must be delayed.

The activation of NF-κB *via* the TRIF-dependent pathway occurs through a secondary response mediated by IRF-3 and NF-κB-independent TNFα, establishing an autocrine feedback for a delayed NF-κB activation. The combination of two asynchronous oscillatory-based responses appears to allow for the stable and consistent early NF-κB response to LPS. The temporally decreased production of TNF-α in Anx-A1$^{-/-}$ DC probably accounts for the partial recovery of NF-κB activation. Unfortunately, I did not examine either IRF-3 or TNF-α mRNA, or intracellular TNF-α protein in Anx-A1$^{-/-}$ DC to confirm this hypothesis.

4.7. Anx-A1/FPR cascade: synergism with TLR signalling?

In order to elucidate why Anx-A1$^{-/-}$ DC showed this defect in NF-κB activation, I investigated classical 'Anx-A1/FPR' signalling events. Anx-A1 and its derived peptide Ac.2-26 are ligands for the FPRs, which are a family of seven transmembrane G protein coupled receptors with three members identified in humans to date: FPR/FPR1, FPR-like-1 (FPRL-1)/FPR2 and FPR-like-2 (FPRL-2)/FPR3 (Wang and Ye, 2002, Ye et al., 2009, Walther et al., 2000). Upon binding of the ligands, heterotrimeric Gi proteins coupled to FPR

rapidly dissociate into α and $\beta\gamma$ subunits, activating signalling molecules and pathways shared with most other leukocyte chemotactic receptors. The $\beta\gamma$ subunit initiate a series of signal transduction events via phosphoinositide 3-kinase g (PI3Kγ) and phospholipase Cγ (PLCγ) where the former results in activation of Ras/Raf proteins and in turn, the mitogen-activated protein kinase (MAPK) pathway, particularly ERK1/2 and the latter causes Ca^{2+} mobilization and activation of protein kinase C (PKC) (Lopez-Ilasaca et al., 1997, Gripentrog and Miettinen, 2005, Le et al., 2002a, Le et al., 2002b).

Previous studies have shown that immature monocyte-derived human DC express constitutive high basal levels of FPR1 and FPRL-2 but not FPRL-1. Upon maturation, DC lose FPR expression and maintain only FPRL-2 which is constitutively expressed on their plasmalemma (Yang et al., 2002). This study using murine bone marrow derived DC has shown that immature DC express message for Fpr1 and Fpr-rs2. Moreover, stimulation with LPS induced the upregulation of both Fpr1 and Fpr-rs2, with little effect on Fpr-rs1 (Ye et al., 2009). It is highly likely that differences between the human and mouse cells exist with respect to receptor expression and, possibly also, in the mechanisms controlling their expression. Most interestingly, I did not observe significant differences in the pattern of expression of these receptors in Anx-A1$^{+/+}$ and Anx-A1$^{-/-}$ DC but I did find an impaired activation of classical FPR-initiated signalling pathways such as ERK1/2 and Akt.

These results allow two important considerations.

First, the signalling cascade induced by LPS in immature DC includes the FPR/Anx-A1 system whose function is to integrate and contribute to the differentiation of mature DC. In fact, here I have shown that, in analogy with other cell types, activation of DC with LPS induced a time dependent release of Anx-A1 in the culture supernatant mirrored by a depletion of the intracellular stores. Most interestingly, I observed that the release of Anx-A1 peaked between 1-3h after LPS stimulation and then decline over the next 12-16h. Consistently, the intracellular levels decreased after 3h of stimulation and were subsequently restored. Cumulatively, these results suggest that the protein is promptly released soon after the activation of DC and that this might influence the signalling cascades activated by LPS.

Second, considering the parallel up-regulation of both Fpr1 and Fpr-rs2 upon LPS stimulation (the latter being the mouse ortholog of human FPRL-1/FPR2) it is not possible to fully associate the reduced activation of ERK1/2 and Akt to the lack of binding of Anx-A1 to only one of these two receptors. However, our and other research groups have demonstrated that most of the biological effects of Anx-A1 occurs via the activation of its receptor FPRL-1 (Gavins et al., 2003, Hayhoe et al., 2006, Maderna et al., 2010, Maderna et al., 2005, Perretti et al., 2001, Renshaw et al., 2010, Chatterjee et al., 2005, Damazo et al., 2005, Gavins et al., 2005, Hannon et al., 2003,

Perretti et al., 1993a, Perretti et al., 2002, Babbin et al., 2006), therefore it is possible to hypothesize that lack of Fpr-rs2 signalling might be the main cause of the impaired phenotype of Anx-A1$^{-/-}$ DC. Further experiments adding other agonists to this receptor, e.g. serum amyloid protein A or lipoxin A_4 or synthetic analogues such as Amgen compound 43 (Burli et al., 2006), would determine the validity of this hypothesis.

4.8. Synergism at the level of Transcriptional Factors: A possible co-operation of TLR-induced NF-κB and FPR-activated ERK

The transcription factor NF-κB is paramount in activating the inflammatory genes involved in the innate immune response. Recent studies have shown that NF-κB typically demonstrates a sinusoidal pattern of activation because NF-κB transcribes not only pro-inflammatory genes but also transcribes the genes to its inhibitors, thus producing a regulatory feedback on its own activation (Ghosh and Karin, 2002, Thompson et al., 1995). Surprisingly, the pattern of NF-κB activation in Anx-A1$^{-/-}$ DC lacks this sinusoidal modulation. I was not able to determine the molecular mechanisms underlying this effects but it is tempting to speculate that the reduced degree of ERK activation, due to the lack of "Anx-A1/FPRL-1 signal", might at least contribute to the impaired temporal control of NF-κB activation. To this regard, it would be also interesting to investigate whether FPR-

deficient DC display the same phenotype or which member of the FPR family is responsible for these effects.

4.9. T-cell stimulatory ability of Anx-A1$^{-/-}$ DC

The up-regulated expression of co-stimulatory molecules together with MHC during maturation synergistically enhances the T cell stimulatory capacity of DC. In fact, the increased expression of MHC ensures the presentation of more epitopes of the antigens while the other co-stimulatory molecules engage with their respective co-receptors on T cells and ensure an effective amplification of the signalling to T cells (Yoshimura et al., 2001, Cella et al., 1997a, Inaba et al., 2000, Kassiotis et al., 2003). When I analysed the allogeneic T cell stimulatory capacity of immature Anx-A1$^{-/-}$ DC, I observed an increase in T cell proliferation and IL-2 production compared to Anx-A1$^{+/+}$ DC. However, I noticed that with LPS-matured Anx-A1$^{-/-}$ DC I obtained the opposite results suggesting that stimulation of Anx-A1$^{-/-}$ DC induces a tolerogenic phenotype manifested as a reduced ability of these cells to elicit a robust T-cell response.

Activated DC act as IL-12–producing APC for peripheral tissue-homing NK cells and memory T cells at the site of infection. Beyond the role of a pro-inflammatory cytokine, IL-12 is integral in dictating

adaptive immune responses. IL-12 possesses T helper polarising properties thus maturing naïve T cells into effector Th1 cells. Engagement of CD28 by CD80 or CD86 is required for T cell priming by DC and the IL-12 produced by DC is a potent and obligatory inducer of differentiation of IFN-γ–producing T cells (Cella et al., 1996, Schulz et al., 2000). Consistent with the IL-12 data, LPS-matured Anx-A1$^{-/-}$ DC showed a reduced capability to induce Th1 cytokine production compared to the mature Anx-A1$^{+/+}$ DC. We can enticingly speculate from these findings that Anx-A1 plays a definitive role in DC differentiation, whereby its lack would favour differentiation to a 'semi-mature' DC status with a potential tolerogenic phenotype (Lutz and Schuler, 2002, van Duivenvoorde et al., 2006).

Recent literature in the field of peripheral tolerance postulates that DC are paramount in deciphering the myriad of stimuli (self-antigens, harmless environmental agents or pathogens) encountered simultaneously in order to trigger the appropriate maturation status. The stage of DC maturation heavily influences the type of T cell response (Adler and Steinbrink, 2007, Steinman et al., 2003).

Therefore, I pursued a specific system to monitor the influence of DC-derived Anx-A1, or lack thereof, on particular T cell subset responses. I utilized two single-TCR transgenic mouse models – the OT-I/RAG-1$^{-/-}$ mouse model and the OT-II/RAG-1$^{-/-}$ mouse model whereby only

CD8 T cells expressing a single TCR epitope and only CD4 T cells also expressing a single TCR epitope are generated respectfully. OT-I/RAG-$1^{-/-}$ transgenic mice express a single T-cell receptor specific for chicken ovalbumin peptide 257-264 ($OVA_{257-264}$; SIINFEKL) presented by the MHC class I molecule, H-$2K^{b}$. This is possible because of a MHC-I restriction of budding T cells in the thymus of these mice and mature naïve $CD8^{+}$ T cells expressing only this specific receptor enter the periphery. OT-II/RAG-$1^{-/-}$ transgenic mice express a T-cell receptor specific for chicken ovalbumin peptide 323-339 ($OVA_{323-339}$; ISQAVHAAHAEINEAGR) presented by the MHC class II molecule I-A^{b}. This is achieved by a MHC-II restriction of developing T cells in the thymus of these mice and mature naïve $CD4^{+}$ T cells expressing only one receptor specific for this peptide emerge from the thymus.

Hence, I incubated $OVA_{257-264}$-pulsed Anx-$A1^{+/+}$ DC and Anx-$A1^{-/-}$ DC with OT-I/RAG-$1^{-/-}$ T cells or $OVA_{323-339}$-pulsed Anx-$A1^{+/+}$ DC and Anx-$A1^{-/-}$ DC with OT-II/RAG-$1^{-/-}$ T cells in co-culture systems and assessed T-cell proliferation and cytokine production. Immature Anx-$A1^{-/-}$ DC results were not fully reproducible and thus inconclusive. About one-third of experiments showed an expected, elevated cell proliferation level of both OT-I CD8 T cells and OT-II CD4 T cells co-cultured with peptide-pulsed, immature Anx-$A1^{-/-}$ DC whilst, one-half of investigations with peptide-pulsed, immature Anx-$A1^{-/-}$ DC unpredictably illustrated opposing data, and the remaining results were questionable. On the other hand, peptide-pulsed, mature Anx-

$A1^{-/-}$ DC consistently demonstrated that these cells are drastically defective in stimulating both OT-I CD8 T-cell and OT-II CD4 T-cell proliferation in 5-day cultures as well as OT-I CD8 T-cell proliferation but not OT-II CD4 T-cell proliferation in 7-day cultures when compared to T-cell proliferation in co-cultures with peptide-pulsed, mature Anx-$A1^{+/+}$ DC.

IL-2, the T-cell mitogenic cytokine, was also measured as an additional marker of T-cell activation. These results complemented the T-cell proliferation patterns whereby both OT-I CD8 T-cell and OT-II CD4 T-cell 5-day co-cultures as well as 7-day co-cultures with mature Anx-$A1^{+/+}$ DC induced potent levels of IL-2. Whereas there was an abated IL-2 production in OT-I CD8 T-cell and OT-II CD4 T-cell 5-day co-cultures as well as OT-I CD8 T-cell 7-day co-cultures with mature Anx-$A1^{-/-}$ DC but IL-2 levels were restored in OT-II CD4 T-cell 7-day co-cultures

These data compounded the aforementioned hypothesis that Anx-A1 may have a prominent role in early events involved in fully maturing DC to an immunogenic state. This appears to have a knock-on effect in activating T cells and initiating T-cell proliferation during relatively early DC-T-cell interactions although while this impact seems to be maintained during DC interactions with certain T cell subsets, my observations suggest that this influence is less significant or probably even insignificant between DC-T-cell interactions of other T cell

subsets.

Deciphering which were the likely effector T cells that would possibly result from these co-culture combinations, I scanned IFN-γ production within the co-cultures as an implication that the extent of IFN-γ-producing cells may dictate effector subset classification. Fascinatingly, IFN-γ production was severely encumbered in all the OT-I CD8 T-cell and OT-II CD4 T-cell co-cultures with mature Anx-A1$^{-/-}$ DC during both 5-day and 7-day incubation periods when compared to IFN-γ levels in co-cultures with mature Anx-A1$^{+/+}$ DC.

4.10. Synopsis: Proposed Role of Annexin-A1 in Dendritic Cell Function.

Annexin-A1 appears to have a clear influence on dendritic cell biology and this effect is noticeable from the point of the terminally differentiated immature dendritic cells; that is, the up-regulated expression of the maturation markers CD40 and CD80. This phenomenon may have even taken effect prior to the complete differentiation process.

Immature Anx-A1$^{-\backslash-}$ DC emerge from their developmental process in an advanced state of maturation; therefore, further maturation does not modify the phenotype and biology of these cells to any great extent. Once exposed to maturation stimuli, these Anx-A1$^{-\backslash-}$ DC do

not function as expected of typical mature DC because when these cells become activated, they fail to undergo the ubiquitous DC maturation process:

1. Anx-A1$^{-\backslash-}$ DC fail to up-regulate MHC class II and co-stimulatory molecules proficiently.
2. Anx-A1$^{-\backslash-}$ DC fail to produce large amounts of inflammatory cytokines.
3. Anx-A1$^{-\backslash-}$ DC fail to effectively initiate and propagate T-cell activation and proliferation; and in the proportion of T-cell priming that occurs, a Treg phenotype may be induced due to the microenvironment created by these DC.

A summary of these interpretations is illustrated in Figure 4.10.2 below.

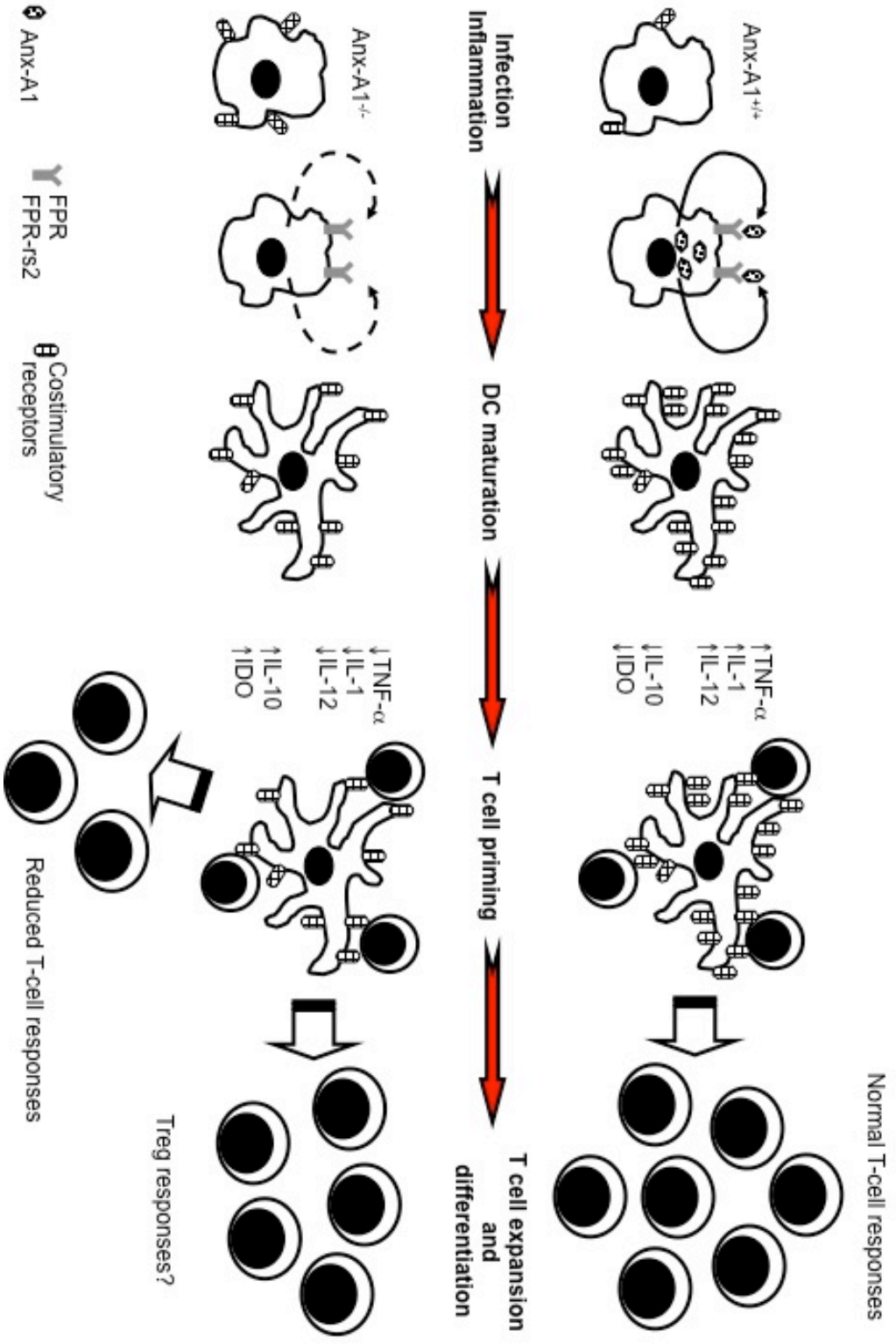

Figure 4.10.1. Overview of the effect of the absence of Annexin-A1 on dendritic cell biology.

CONCLUDING REMARKS

In conclusion, this study provides the first evidence for an unexplored role of endogenous Anx-A1 in DC biology. It can be concluded that the role of Anx-A1 is strongly indicative of a positive modulator for DC maturation with immunogenic features. These findings complement our on-going research on the role of Anx-A1 in the adaptive immune response and further suggest that therapies targeting Anx-A1 function in DC might represent a novel way to limit uncontrolled adaptive immune response typical of autoimmune diseases.

5. FUTURE DIRECTIONS

The first question I would pose is to investigate to what extent the role of DC-derived Annexin-A1 is involved in or influences CD4 T-cell and CD8 T-cell responses. And would the end product be similar if Annexin-A1-deficient, single-TCR transgenic T cells are co-cultured with 'wild-type' DC; that is, is the source of Anx-A1 vital to the immunological outcome?

Another question to address would be to illuminate the exact murine FPR that mediates the effects of Annexin-A1 in mature DC and how this would reconcile with the role of Anx-A1/FPR signalling in human DC.

To address these questions, I would pre-treat immature Anx-A1$^{+\backslash+}$ DC with selective FPR antagonists (cyclosporine H blocks FPR1 and WRW4 peptide blocks FPR2) followed by LPS stimulation. I would then again pre-treat the mature Anx-A1$^{+\backslash+}$ DC with selective FPR antagonists prior to establishing the co-cultures with the OT-I/RAG-1$^{-\backslash-}$ and OT-II/RAG-1$^{-\backslash-}$ T cells in order to prevent modulation of DC effector functions by Annexin-A1 during T-cell-DC interactions. This would be a 2-fold approach examining the Anx-A1/FPR1 and Anx-

A1/FPR2 signalling in DC and the effect of these two signalling cascades in DC biology – DC maturation and APC-stimulatory capacity.

In addition, is this relationship between Annexin-A1 and dendritic cell activation and maturation unique to LPS/TLR4 signalling? Does Annexin-A1 acts in a similar manner in dendritic cells during other TLR signalling cascades?

One final inquiry to tackle is the full dissection of the possible synergism between TLR signalling and Anx-A1/FPR signalling.

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