

**Common Cytokine Receptor γ Chain Deficiency
in Dendritic Cells:
Implications for Immunity**

**BY
CHIARA BEILIN**

**INSTITUTE OF CHILD HEALTH
UNIVERSITY COLLEGE LONDON**

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Declaration

I, Chiara Beilin, declare that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

X-linked Severe Combined Immunodeficiency (X-SCID) caused by mutations in the common cytokine receptor γ chain (γ_c) is a fatal disease in which the immune system is severely compromised as T and NK cells are absent or profoundly diminished. Although dendritic cells (DCs) are key antigen presenting cells and are crucial for the induction of normal immune responses, little is known about their function in this disease. This is of specific importance as the myeloid compartment, including DCs, frequently remains of host origin, and therefore γ_c -deficient, after bone marrow transplant or gene therapy for this form of SCID. Curiously, even after curative treatment, patients with X-SCID continue to have a long-term susceptibility to human papilloma virus infection. We hypothesised that this could be due to persistent myeloid defects.

Using a murine model of X-SCID, we demonstrated that, in the absence of functional γ_c , DCs are less efficient at priming antigen-specific responses in γ_c -expressing CD4⁺ T cells. Despite the widely held view that IL-15R α is the chain responsible for IL-15 transpresentation to NK and CD8⁺ T cells, here we have shown that expression of the γ_c by DCs is necessary for effective transpresentation to CD4⁺ T cells. In a novel system of supported planar bilayers mimicking the T cell interface, we demonstrated that DC-expressed γ_c colocalises with MHC II clusters and mediates IL-15R α recruitment to the immunological synapse (IS). This depended on simultaneous TCR engagement with MHC-bound antigen, suggesting that IL-15 transpresentation may have important costimulatory function for T cell activation. Altogether, these data indicate that γ_c -deficiency impairs DC functions and reveal a novel mechanism for recruitment or stabilization of DC IL-15/IL-15R α complexes at the IS leading to localized transpresentation of IL-15. The contribution of myeloid γ_c -deficiency to persistent immunodeficiency in transplanted X-SCID patients warrants further investigation.

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Abbreviations

γ c	common cytokine receptor γ chain
ADA	adenosine deaminase
ANOVA	analysis of variance
APC	antigen presenting cell
BM	bone marrow
BM-DCs	murine bone marrow derived DCs
CCR	chemokine receptor
CDP	common DC-restricted progenitors
CHR	cytokine-binding homology region
CLEC	c-type lectin receptor 1
CNTF	ciliary neurotrophic factor
cSMAC	central supramolecular activation cluster
CT-1	cardiotrophin 1
CTL	cytotoxic T cell
CTLA-4	cytotoxic T-lymphocyte antigen-4
D1	N-terminal fibronectin domain
D2	C-terminal fibronectin domain
DAMP	damage-associated molecular pattern molecules
DAPI	diamidino-2-phenylindole
DC	dendritic cell
DIC	differential interference contrast
dSMAC	distal supramolecular activation cluster
DUSP	dual specificity phosphatases
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
Flt-3	FMS-like tyrosine kinase 3 ligand
F-Actin	filamentous actin
FRET	förster resonance energy transfer
GM-CSF	granulocyte/monocyte colony-stimulating factor
GPI	glycosylphosphatidylinositols
hGF	hepatocyte growth factor
HLA	human leukocyte antigen
HMGB1	high-mobility group box 1 proteins
HPV	human papilloma virus
HSCT	haematopoietic stem cell transplantation
HSP	heat-shock proteins
ICAM1	intercellular adhesion molecule 1
IFN	interferon
Ig	immunoglobulin
ILT	immunoglobulin-like transcript
iNOS	inducible nitric-oxide synthase
IS	immunological synapse
ITAMS	immunoreceptor tyrosine-based activation motifs
JAK	janus-activated kinase
LAT	linker for activation of T cells
LC	langerhans cells
LIF	leukaemia-inhibitory factor
LFA-1	lymphocyte function-associated antigen-1

LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MC	microclusters
MDP	monocyte-DC progenitors
MFI	mean fluorescence intensities
MHC	major histocompatibility complex
MICA	MHC-class-I-polypeptide-related sequence A
MIIC	MHC class II-rich compartments
MP	myeloid progenitors
MR	mannose receptor
MTOC	microtubule organizing center
MVB	multivesicular bodies
NFAT	nuclear factor of activated T cells
NF- κ B	nuclear factor- κ B
Ni-NTA	nickel-nitrilotriacetic acid
NLR	nucleotide oligomerization domain (NOD)-like receptors
NK	natural killer
NKG2D	natural-killer group 2 member D
NKIS	NK cell immunologic synapse
OSM	oncostatin M
OVA	ovalbumin
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered solution
PDL-1	programmed cell death ligand-1
PKC	protein kinase C
PI3k	phosphoinositide 3-kinase
PLC	phospholipase
PFA	paraformaldehyde
PPR	pattern recognition receptors
pSMAC	peripheral supramolecular activation cluster
RAG	recombination activating gene
RSV	respiratory syncytial virus
SCID	severe combined immunodeficiency diseases
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	SRC homology 2 domain
SHC	SH2 domain-containing adapter protein
SOCS	suppressors of cytokine signalling
SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
STAT	signal transducer and activator of transcription
TCR	T cell receptor
Th	T helper
TIRFM	total internal reflection microscopy
TLR	toll-like receptors
TNF	tumor-necrosis factor
TSLP	thymic stromal-derived lymphopoietin
WASp	wiskott-aldrich syndrome protein
WB	western blotting
WSXWS	Trp-Ser-X-Trp-Ser motif
X-SCID	X-linked severe combined immunodeficiency diseases
ZAP-70	zeta-chain-associated protein kinase 70

Chapter One- Introduction

1.1 γ c-family Cytokines and Related Immunodeficiencies

1.1.1 γ c-family Cytokine Receptors

1.1.1.1 Cytokines and Cytokine Receptor Families

Cytokines are broadly defined as group of soluble molecules secreted by one cell and acting on others¹. The term cytokine is quite general but includes many interleukins and interferons, as well as haematopoietic and growth factors. By binding to specific surface receptors, cytokines can initiate important cellular functions including differentiation, survival and apoptosis.

According to their structure, cytokines have been categorized in different structural groups including four-helical cytokines, β -trefoil growth factors, trimeric tumor necrosis factor (TNF) family and cysteine knot growth factors². Cytokines have also been classified according to the receptor they bind to. Six major families of cytokine receptors have been identified; class I cytokine receptors, class II cytokine receptors, TNF receptors, IL-1 receptors, tyrosine kinase receptors and chemokine receptors. Class I cytokine receptors form the largest group and bind to class I cytokines, defined by a four-helix bundle motif.

1.1.1.2 Shared Receptors of the Class I Cytokine Receptor Family

A distinguishing feature of class I cytokine receptors is the use of a common, shared receptor subunit. Receptors belonging to this family are classified on the basis of their ability to associate with one of three different types of shared signaling components; gp130, β chain (β c) or γ chain (γ c)². gp130 is the shared receptor for “gp130 long-chain cytokines” such as IL-6, IL-11, oncostatin M (OSM), cardiotrophin 1 (CT-1), CNTF (ciliary neurotrophic factor), and LIF (leukaemia-inhibitory factor). The β c is the common signaling component for cytokines such as IL-3, IL-5 and GM-CSF whereas the γ c is the shared cytokine receptor for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21.

Class I cytokine receptors share similar structural properties (Figure 1.1). A significant feature of most members of this receptor family is a conserved extracellular domain of approximately 200 amino acids which contains a cytokine-binding homology region (CHR) consisting of two fibronectin type III domains (D) interconnected by a linker. In contrast, the intracellular domains of the class I cytokine family members are not as well conserved as the extracellular ones, although they display some sequence similarities³.

While γ c consists only of the basic extracellular structure comprising a single CHR region, the gp130 and β c shared receptors possess additional domains which are required for cytokine binding and signal transduction². For example, the extracellular portion of gp130 contains six β -sandwich domains coated with a single amino-terminal immunoglobulin (Ig)-like domain, followed by one CHR domain and by three fibronectin type III domains. The β c extracellular portion instead consists of two consecutive CHR domains in an antiparallel orientation.

With the exception of certain class I cytokine receptors such as the hepatocyte growth factor (hGF) and erythropoietin receptors, receptor homodimerization is normally insufficient for signal transduction. Shared receptors of the class I cytokine receptor family typically display very low cytokine binding affinity on their own and therefore need to associate with hetero-subunits to bind and transduce cytokine signals. Unlike many other cytokine receptors, class I cytokine receptors lack

intrinsic enzymatic activity but signal mainly through the Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway³.

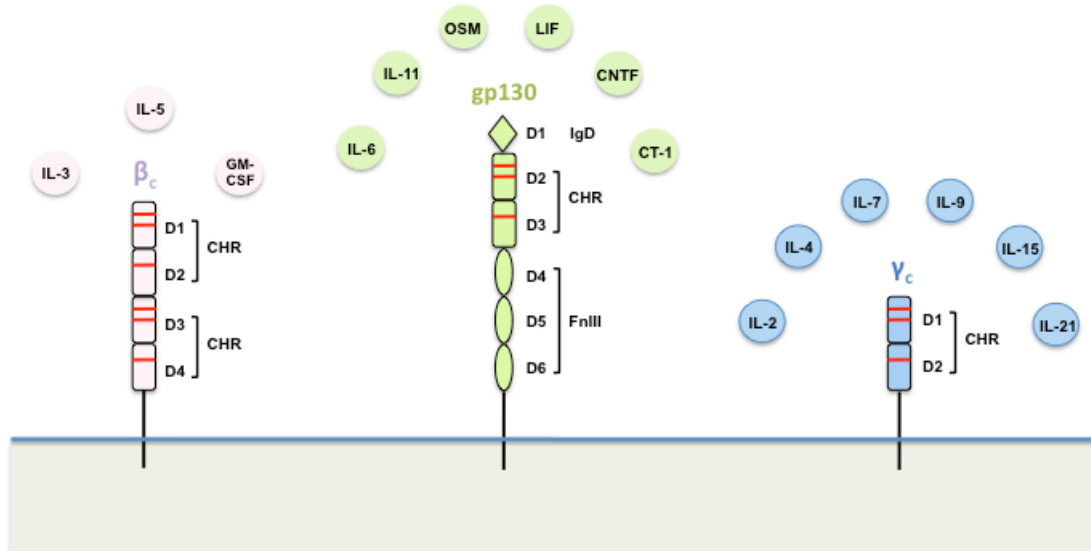


Figure 1.1 Schematic representation of the conserved structure of β_c , gp130 and γ_c shared class I cytokine receptors and of their respective interacting cytokines. Classically, within the CHR of class I cytokine receptors, the N-terminal fibronectin domain (D) contains 4 conserved cysteine residues whereas the more proximal domain has a conserved Trp-Ser-X-Trp-Ser (WSXWS) motif, all of which are important for tertiary structure. Adapted from “Structural Biology of Shared Cytokine Receptors”, Wang *et al.*, 2009².

1.1.1.3 γ c-family Cytokine Receptors

In general, γ c-family cytokine receptors are heterodimers composed of the common γ c and of cytokine-specific α chains (IL-4R, IL-7R, IL-9R and IL-21R)⁴. The situation is slightly different for the IL-2R and IL-15R which instead heterotrimerise the common γ c with cytokine-specific α chains and shared IL-2/15R β chains (Figure 1.2). Another interesting feature of γ c-family cytokine receptors is the ability of the α chains to associate with different subunits to form alternative receptors. The IL-4R α chain, for example, can also bind to the IL-13R α 1 to form type II IL-4R, which is the functional receptor of both IL-4 and IL-13⁵. Similarly, IL-7R α can associate with TSLPR (thymic stromal-derived lymphopoietin receptor) to recognise TSLP⁶.

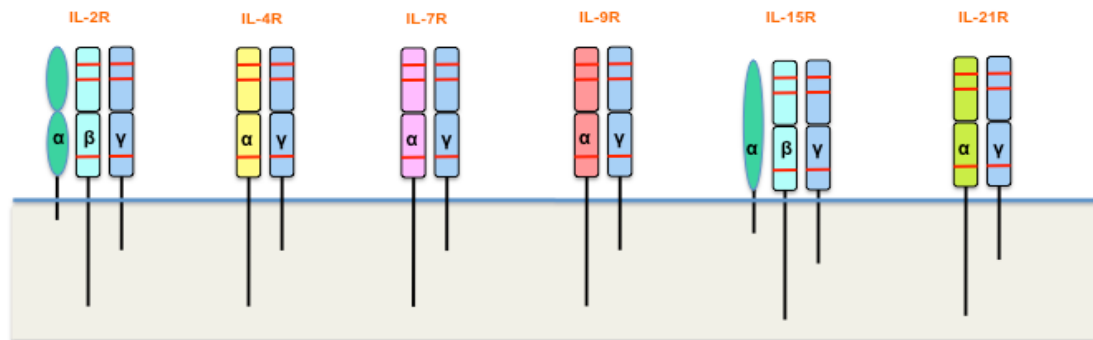


Figure 1.2 Schematic representation of the γ c-family cytokine receptors. Shown are the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Each receptor is composed of the common γ c and of a unique α chain. IL-2 and IL-15R also have an additional subunit; the shared IL-2/15R β chain. Adapted from “New insights into the regulation of T cell by γ c family cytokines”, Rochman *et al.*, 2009⁴.

1.1.1.4 Structure of the Trimeric γ c-family Cytokine Receptors

The γ c was first identified as a component of the IL-2R⁷. Subsequent studies showed that IL-2R has a very similar architecture to the one of the other trimeric γ c-family receptor, IL-15R⁸. Both receptors consist of unique cytokine specific α chains, shared IL-2/15R β and common γ c which together form the high-affinity receptor. In both cases, association of IL-2/15R β and γ c gives rise to the intermediate-affinity receptor, which is expressed on T and NK cells. Isolated IL-2R α has low binding affinity for IL-2 and has been termed low-affinity receptor. In contrast, IL-15R α binds IL-15 with high affinity even in the absence of the IL-2/15R β and γ c receptor subunits⁹ (Figure 1.3). Unlike other subunits of the class I cytokine receptor family which mediate cytokine binding through the classic CHR domains, both IL-2R α and IL-15R α bind their respective cytokines through unique sushi domains¹⁰⁻¹². Notably, IL-2R α 's extracellular domain has two sushi domains whilst IL-15R α only has one.

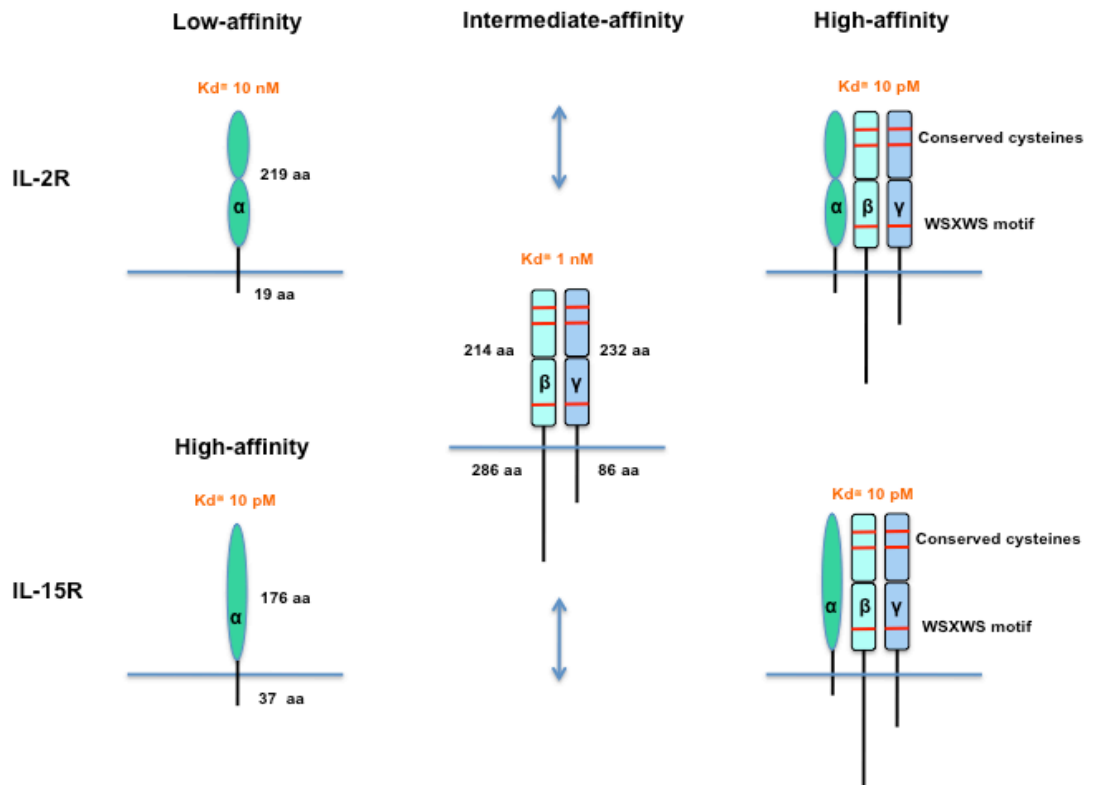


Figure 1.3 Schematic representation of the three types of IL-2 and IL-15Rs. Low-affinity IL-2R consists of a unique α chain only and has a dissociation constant (K_d) of 10nM^{13} . In contrast, IL-15R α binds IL-15 with high affinity (10 pM)⁹. Intermediate-affinity receptors contain the shared IL-2/15R β and common γ c ($K_d = 1\text{nM}$); high-affinity IL-2R and IL-15R contain unique α , shared IL-2/15R β and common γ c ($K_d = 10\text{pM}$).

1.1.1.5 Assembly of the Trimeric γ c-family Cytokine Receptors

The structural paradigms for γ c-family cytokine receptor assembly are derived from the prototypical IL-2R system. Studies of IL-2 ligand/receptor interactions were initiated in the early 1980s but surprisingly, we still do not have a full understanding of the mode of assembly of this receptor. The assembly of the quaternary IL-2- α - β - γ receptor complex is in fact discussed very controversially in the literature and various alternative assembly pathways have been suggested¹⁴.

The “stepwise binding model” proposes that IL-2R α and IL-2/15R β exist unassociated and that IL-2 initially binds to IL-2R α , due to its faster association rate, followed by stepwise binding of the IL-2/15R β and γ c to form the quaternary complex. In contrast, the “ α - β chain preassembly model” suggests that IL-2R α and IL-2/15R β exist in a preformed “pseudo-high” affinity state that serves as the initial interaction complex for IL-2 and then as a docking site for final γ c recruitment to form the quaternary complex. More recently, a third model, the “ β - γ chain preassembly model”, has been proposed and suggests that IL-2/15R β and γ c preassemble and that IL-2 binding induces the translocation of this preassembled complex in lipid rafts¹⁵. Although IL-2R α has been observed to be mainly localised in lipid-enriched membrane microdomains¹⁶, this model postulates that the few free IL-2R α would bind at last to the ternary IL-2/ IL-2R β / γ c complex locking IL-2 in a membrane-bound state.

Given that the architecture of the quaternary IL-15R complex is thought to be similar to that of the IL-2R, similar mechanistic assembly pathways remain possible¹⁷.

1.1.1.6 Signaling Downstream of γ c-family Cytokine Receptors

As noted in section 1.1.1.2, γ c-family class I cytokine receptors signal through the JAK/STAT pathway. There are a total of four JAK (JAK1, JAK2, JAK3 and TYK2) and seven STAT (STAT1–4, STAT5A, STAT5B and STAT6) proteins^{3,18}. JAKs are large cytoplasmic proteins containing a conserved kinase domain and an inactive pseudo-kinase domain. JAK1, JAK2 and TYK2 are ubiquitously expressed whereas JAK3 expression is restricted to hematopoietic cells and is inducible. STAT proteins are cytoplasmic transcription factors which are recruited to cytokine receptors through their conserved SRC homology 2 (SH2) domain. In addition, STAT proteins also comprise a transactivation domain and a homologous DNA-binding domain.

JAKs constitutively associate to Box 1 and 2 motifs in the intracellular domains of γ c-family class I cytokine receptors. The more cytokine specific IL-2R β , IL-4R α , IL-7R α , IL-9R and IL-21Rs associate with JAK1 whereas the γ c associates with JAK3³. Cytokine binding induces the activation of JAK1/JAK3, resulting in JAK-assisted phosphorylation of specific tyrosines in the intracellular domains of γ c-cytokine receptors, which then serve as docking sites for the SH2 domains of STAT proteins¹⁹⁻²². Receptor associated STAT proteins are in turn phosphorylated resulting in homodimerization (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) or heterodimerization (STAT1 and STAT2, STAT5A and STAT5B, STAT1 and STAT3). Dimerised STAT proteins translocate to the nucleus where they bind to DNA and act as nuclear transcription factors (Figure 1.4). Whereas STAT3 and STAT5A/B are activated by most γ c-family cytokines, STAT6 is specifically activated by IL-4 (Table 1.1).

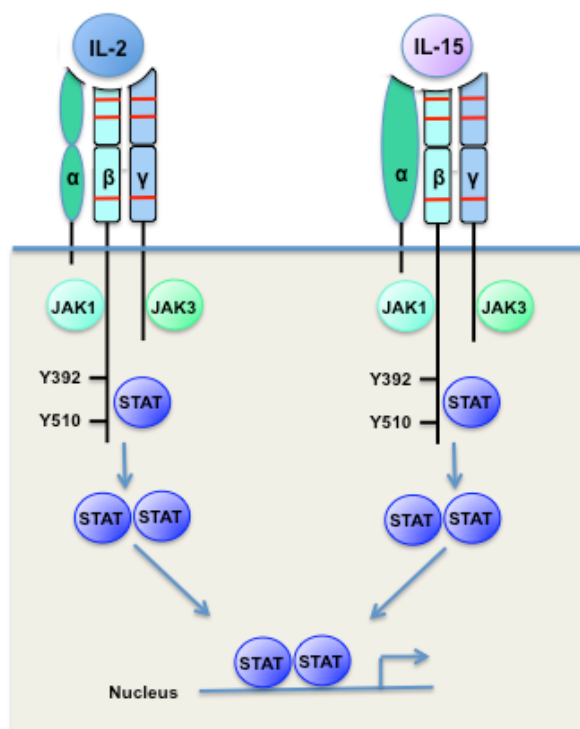


Figure 1.4 Schematic representation of signaling through the JAK/STAT pathway by typical class I cytokines such as IL-2 and IL-15. For IL-2R β , the location of tyrosines (Y392 and Y510) required for the docking and activation of STAT proteins are indicated²³.

<u>Ligands</u>	<u>JAKs</u>	<u>STATs</u>
IL-2	JAK1, JAK3	STAT5, STAT3
IL-4	JAK1, JAK3	STAT6
IL-7	JAK1, JAK3	STAT5, STAT3
IL-9	JAK1, JAK3	STAT5, STAT3
IL-15	JAK1, JAK3	STAT5, STAT3
IL-21	JAK1, JAK3	STAT3, STAT5, STAT1

Table 1.1 Cytokine-specific JAK and STAT activation. γ c-family cytokines transduce signals through unique sets of JAK/STAT proteins. STAT5 refers to both STAT5A and STAT5B. Adapted from “Cytokines and immunodeficiency diseases”, Kovanen *et al.*, 2004²².

Genes regulated by γ c-family cytokines regulate a vast array of biological functions, mainly related to cellular differentiation and proliferation. Consistent with the notion that cytokine signals sustain and amplify antigen-induced stimulation, γ c-family cytokines tend to activate gene patterns which are highly overlapping with those induced by TCR-like signals²⁴. In addition, another major set of genes regulated by γ c-family cytokines are those implicated in the negative regulation of JAK/STAT signaling. Amongst these are members of the SOCS (suppressors of cytokine signaling) and DUSP (dual specificity phosphatases) gene family^{25,26}. SOCS inhibit cytokine signaling either by direct inhibition of JAK catalytic activity (SOCS1 and SOCS3) or by binding to STATs thereby preventing their association with cytokine receptors (SOCS2) whereas DUSP hydrolyse phosphorylated residues.

In addition to the JAK/STAT pathway, the IL-2, IL-4, IL-7 and IL-15 γ c-family cytokines can also activate other signaling pathways such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI-3K)/Akt pathways. Here, coupling of cytokine receptors to these signaling pathways is thought to be mediated by the SH2 domain-containing (SHC) adapter protein²².

1.1.1.7 Unique Functions of Non-classical γ c-family Cytokine Receptors

As discussed in section 1.1.4, owing to replacement of the typical extracellular CHR domains with sushi domains, both IL-2R α and IL-15R α are not regarded as classical type I cytokine receptors. For a long time, both were considered non-signaling receptors mainly because of the lack of signaling motifs or docking sites in their intracellular tails. It has more recently been discovered that IL-15R α has functions other than cytokine binding, such as receptor recycling²⁷. IL-15R α can also signal independently of the β and γ chains, as shown in several hematopoietic and non-hematopoietic cells line²⁸⁻³⁰. Little is known about the downstream signaling pathways but in fibroblasts, IL-15 mediates anti-apoptotic activities and these are achieved through the constitutive association of IL-15R α with the tyrosine kinase, Ax1, which induces PI3K/Akt activation and upregulation of Bcl-2 and Bcl-x1³¹.

Another distinguishing feature of the non-classical IL-15R is its ability to present IL-15 by two distinct mechanisms. The IL-15R α ectodomain consists of a single sushi domain which is interconnected by a linker to a membrane-proximal proline-threonine-rich (PT) region¹⁰. This unique architecture confers IL-15R α with a particularly flexible structure which allows it to present IL-15 both *in cis* and *in trans*³². Antigen-presenting cells (APCs) are especially important for IL-15R α -mediated transpresentation. In these cells, IL-15/ IL-15R α complexes are assembled intracellularly, prior to delivery to the surface^{33,34}, then presented *in trans* to β/γ c expressing Natural Killer (NK) and CD8+ T cells upon cell-cell interaction. Transpresentation of IL-15 is now known to be critical for both NK and CD8+ T cell homeostasis and priming, as demonstrated both *in vitro* and *in vivo*³⁵⁻⁴⁰. IL-2R α has also been shown to transpresent IL-2 but surprisingly, the biological importance of this process remains unexplored⁴¹.

1.1.2 X-linked Severe Combined Immunodeficiency

1.1.2.1 The common γ c is mutated in X-SCID

Severe combined immunodeficiency diseases (SCID) represent the most severe forms of primary immunodeficiency disorders⁴². They are characterised by defects in the T and B cell immune compartments and by variable abnormalities of NK cells. The majority of SCIDs are caused by mutations in genes affecting cytokine signaling (γ c, JAK3, IL-7R), antigen receptor recombination (RAG1, RAG2, ARTEMIS) or metabolism (ADA)²² (Table 1.2).

<u>Gene defect</u>	<u>Mechanism</u>	<u>Phenotype</u>	
		<u>Human</u>	<u>Mouse</u>
γ c	Cytokine signaling	T-B+NK-	T-B-NK-
JAK3	Cytokine signaling	T-B+NK-	T-B-NK-
IL7R	Cytokine signaling	T-B+NK+	T-B-NK+
RAG1	Ag receptor recombination	T-B-NK+	T-B-NK+
RAG2	Ag receptor recombination	T-B-NK+	T-B-NK+
ARTEMIS	Ag receptor recombination	T-B-NK+	T-B-NK+
ADA	Metabolim	T-B-NK-	T-B-NK-

Table 1.2 Severe Combined Immunodeficiencies. Adapted from “Cytokines and Immunodeficiency diseases”, Kovanen *et al.*, 2004²².

X-SCID is the most common form of SCID with an incidence of 1 in 50,000 to 100,000 live births⁴². The finding that the genetic locus of the γ c mapped to the same region as the X-SCID locus (Xq13⁴³) led to the discovery that mutations in the γ c are the cause of X-SCID⁴⁴. In this study, all of the genotyped X-SCID patients had different nonsense mutations in the γ c, each resulting in premature stop codons or in truncations of the cytoplasmic/signalling domain, underlining the importance of this region for γ c-cytokine-mediated functions. More than 100 γ c-defects causing X-SCID are now known⁴⁵. The majority of mutations lead to a complete loss of function and to a severe clinical phenotype. These span along the whole length of the protein and comprise nonsense and missense point mutations as well as insertion/deletion frameshift mutations (Figure 1.5). Mutations in the extracellular domain, for example, result in loss of cytokine binding whereas those in the

intracellular domain inhibit binding of signalling partners, therefore interfering with signal transduction^{45,46}. A few cases of hypomorphic mutations have also been described leading instead to milder disease⁴⁷.

An identical clinical phenotype to severe X-SCID is seen with loss of function mutations in the γ c-binding partner JAK3⁴⁸, highlighting the importance of γ c-cytokine signaling for development of this disease.

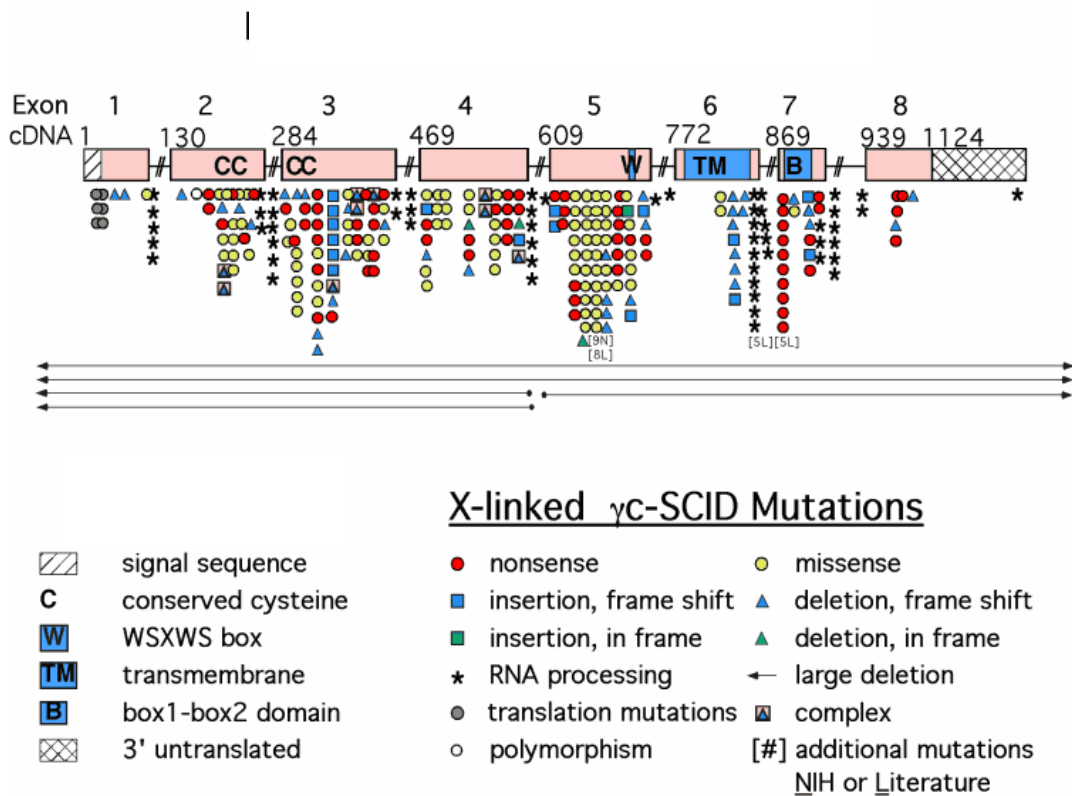


Figure 1.5 Schematic representation of the mutations found in X-SCID patients, obtained from the IL2RBbase of the Human Genome Research Institute, National Institutes of Health.

1.1.2.2 Critical Role of γ c-family Cytokines for the Immune System

The γ c was first identified as a component of the IL-2R⁴. The finding that both humans and mice lacking IL-2 had normal lymphoid development^{49,50} led to the logical conclusion that the γ c must be a shared component of other cytokine receptors. As previously described in section 1.1.1.3, the γ c is now known to be a functional component of the IL-4, IL-7, IL-9, IL-15 and IL-21R.

γ c deficiency results in a T-B+NK- form of SCID in which human T and NK cells are absent or profoundly diminished and B cells are present but poorly functional²². These profound immunological impairments are due to the wide range of biological functions exerted by γ c-family cytokines, especially IL-2, IL-7 and IL-15. IL-2, for example, has multiple effects. It is mainly known as T-cell growth factor, as was first established *in vitro*⁵¹, but also promotes NK cytolytic activity and regulates Ig production by B cells^{49,50}. IL-7 is probably the most important of the γ c-family cytokines for lymphocyte development. As seen with γ c-deficient mice⁵², in mice deficient in IL-7 or IL-7R α both T and B cell development are abrogated^{53,54}. In contrast, IL-7R α -deficient patients lack T cells but have normal B-cell numbers⁵⁵, as do γ c-deficient patients, highlighting the important differences in IL-7 function between humans and mice. In humans, intrinsic B-cell defects (defective B-cell activation and abnormal class switching) are instead thought to be the result of a combination of lack of T cell help and of absent IL-4 and IL-21 signaling²². These cytokines work together to control B-cell function by regulating Ig production and IgG and IgE class switching. IL-15 is also important for immunity and owing to the similar structure of the IL-2 and IL-15 receptors, IL-15 exerts similar functions to IL-2. *In vitro*, both cytokines can stimulate T and NK cell proliferation and NK cytolytic activity²². However, *in vivo*, these cytokines differ in their effects; as noted above, whereas mice deficient in IL-2 have normal lymphoid development, those deficient in IL-15 or IL-15R α instead lack NK cells, thus pointing to a critical role for IL-15 in NK cell development^{56,57}. In addition, IL-15 and IL-15R α -deficient mice also lack naïve and memory CD8+ T cells, therefore highlighting the importance of IL-15 as regulator of CD8+ T cell homeostasis. IL-15R α knock-out mice also have reduced numbers of CD4+ T cells. Although the defect is less dramatic than that

observed with CD8+ T cells, it still suggests a possible involvement for IL-15 in CD4+ T cell homeostasis.

Taken together, these findings point to a complex interaction of cytokine dysfunction in γc deficiency resulting in a severe combined immunodeficiency.

1.1.2.3 Treatment of X-SCID

Haematopoietic stem cell transplantation (HSCT) is the treatment of choice for the cure of X-SCID. Allogeneic stem cell transplantation can be carried out between HLA-identical donors with high survival rates (>90%)⁵⁸. Due to the lack of T cells in X-SCID patients, successful transplantation does not require myelosuppressive conditioning. Where HLA-matched donors are not available, haploidentical transplants remain one of the few alternatives. However, even after the introduction of T-cell depleted transplants which efficiently prevent graft-versus-host disease, the success rates of HLA-mismatched transplants remain lower (66%).

An alternative therapeutic approach to X-SCID is gene therapy. This treatment involves *ex vivo* retroviral-mediated gene transfer into hematopoietic progenitor cells. Various clinical trials have established the efficacy of this treatment for immune reconstitution⁵⁹. The development of leukemia as a result of insertional mutagenesis in one of the clinical trials has however raised issues on the safety/applicability of this approach^{60,61}.

1.1.2.4 Persistent Immunodeficiency in Patients with γc /JAK3 Forms of SCID

Although SCID patients cured with HSCT generally have a good prognosis, they can suffer from long-term complications. Two independent studies have reported a high incidence of severe cutaneous Human Papilloma Virus (HPV) disease, late after treatment. In the first case, 9/41 SCID patients who were alive 10 years or longer after HSCT developed severe cutaneous HPV disease⁶², whereas in the second, 9/76 immunodeficiency patients who successfully received HSCT developed chronic HPV infection⁶³. Interestingly, the only risk factor associated with the development of HPV disease was molecular diagnosis as all of the affected patients had either γc or JAK3 forms of SCID. On the contrary, susceptibility to chronic HPV was not

predicted by transplantation conditions or subsequent immune reconstitution, therefore suggesting a residual defect related to the original genotype of this group.

Of note is the fact that, as many SCID patients receive transplants without any chemotherapy conditioning, in the majority of cases, the B-lymphocyte and myeloid lineages, including dermal dendritic cells (DCs) and their epidermal counterpart, Langerhans cells (LC), remain of recipient origin⁶⁴ and therefore γ c-deficient. Furthermore, both of these cells are known to be maintained by local populations of proliferating precursors under steady state conditions^{65,66}, thus making it is even more likely that defective host DC/LC may persist post-transplant. As HPV is mainly confined to epithelial layers, a major role for these cells, which in fact are the antigen presenting cells (APCs) of the skin⁶⁷, is predicted in the induction of anti-HPV immunity. This supports the possibility that residual DC/LC defects may account for persistent immunodeficiency and HPV disease in γ c- and JAK3-deficient SCID patients.

Interestingly, keratinocytes, have also been suggested to contribute to residual immunodeficiency in transplanted SCID patients⁶². Apart from being the point of entry to HPV, keratinocytes also represent one of the first lines of defence to pathogens in general. Together with resident immune cells of the skin, keratinocytes orchestrate innate immune surveillance. In response to stimulation, keratinocytes produce antimicrobial peptides and immunoregulatory cytokines⁶⁷. The latter induce the recruitment of other immune cells from the blood. Keratinocytes sense the environment through pattern recognition receptors such as Toll-Like Receptors (TLRs)⁶⁸. Signaling cascades downstream of the activation of TLRs regulate the expression of proinflammatory cytokines that act on the endothelia to further facilitate the extravasation of leukocytes into the skin. Notably, keratinocyte biology also appears to be regulated by γ c-dependent signaling. Keratinocytes have been shown to express and signal through γ c-containing receptors⁶⁹⁻⁷². Ligation of keratinocyte IL-4 and IL-15R, for example, results in proinflammatory cytokine release and enhances expression of several chemokines leading to leukocyte recruitment and local T cell expansion^{70,73,74}. Thus, these findings raise the possibility

that defective γ c signaling in keratinocytes may also contribute to the susceptibility to HPV in γ c and JAK3 deficient patients.

1.2 Origin and Development of Dendritic Cells (DCs)

1.2.1 DCs; Biology and Subsets

1.2.1.1 Introduction to DCs

DCs are widely distributed cells of hematopoietic origin critical for the initiation of non-specific (innate) and specific (adaptive) immune responses and for the maintenance of tolerance to self-peptides⁷⁵. DCs were first discovered in preparations of adherent cells from murine lymphoid organs⁷⁶ and were distinguished from other splenic mononuclear cells for their unique morphology. DCs in fact derive their name from thin and long processes, termed dendrites, which extend and retract in a non-polarised fashion from the cell body. Owing to their unique shape and motility, DCs are specialised APCs⁷⁷. In the immature state, they act as sentinels of the immune system patrolling peripheral tissues for the presence of antigen. Encounter with a microbial product or with a danger signal, triggers their activation and enhanced migration to the draining lymph nodes where they present processed antigen, in the form of a surface peptide-major histocompatibility complex (MHC) to antigen-specific T cells. In addition, DCs express costimulatory molecules which are essential for the induction of immunity.

1.2.1.2 Categories of DCs

DCs are very heterogeneous cells. Since their original discovery, various DC subsets have been identified, both in mice and in humans, each with a distinct location and/or function in the immune system. DCs have been categorised according to their developmental stage, location, origin and function. Although these criteria broadly apply to both mouse and human DCs, there are differences in DC subsets between the species⁷⁸. This chapter will focus on murine DC subsets and development.

One initial distinction that can be made in mouse DC populations is between conventional DCs (cDCs), ie cells at a mature developmental stage, and pre-DCs, meaning cells that need further differentiation⁷⁹. Whilst cDCs occur at the steady-state, pre-DCs typically need an inflammatory signal to complete their development. The classical example of pre-DCs are type I interferon-producing plasmacytoid DCs (pDCs). pDCs can be isolated from the spleen, lymph nodes and thymus and are poor inducers of T cell proliferation⁸⁰. Upon activation, pDCs develop into mature DCs

with enhanced levels of MHC II and T cell stimulatory capacity. On the other hand, cDCs can be already found in a mature state in the circulation and in both lymphoid/non-lymphoid tissues. cDCs can be further subdivided into migratory DCs (classical text book DCs which collect antigen from the periphery and migrate to the lymph nodes) and lymphoid-tissue resident DCs, whose function and origin are restricted to the lymphoid organ itself. In addition to cDCs and pre-DCs, a third group is represented by inflammatory DCs, ie DCs that are generated from monocytes in response to an inflammatory signal. These are termed TipDCs as they produce tumor-necrosis factor (TNF) and inducible nitric-oxide synthase (iNOS)⁸¹.

1.2.1.3 cDC subtypes

cDCs are mature DCs that express CD11c (integrin α -chain) and moderate to high levels of MHC II, according to their level of activation⁷⁸. cDCs also display “T-cell markers” such as CD4 and the CD8 α dimer, which should not be confused with the TCR-associated CD8 $\alpha\beta$ isoform. Other markers used to classify cDCs are CD11b (integrin β -chain) and CD205 (multilectin domain molecule DEC205). These markers are especially useful in the segregation of lymphoid-resident DCs in distinct functional subsets. Spleen DCs, for example, can be divided into 3 major subsets, according to the differential expression of CD4 and CD8⁸² (Table 1.3). The first is represented by CD8+CD205+ cells which are mainly located in the T-cell area and whose main function is to present antigens from dying cells and bacteria on MHC I molecules. The second comprises CD8- CD11b+ cells which can be instead found in the red pulp and present antigens on MHC II molecules. Finally, CD8-CD4+ cells represent the third category and are the dominant population of DCs in the spleen. The lymph nodes also contain CD8+CD205+ DCs and CD8-CD11b+ DCs⁸³. In addition to these populations, the lymph nodes also contain migratory DCs which typically express Langerin, a characteristic marker of LCs, and carry antigen from the periphery to then transfer it to CD8+ resident DCs.

Migratory DCs, which originate from a multitude of non-lymphoid tissues such as the skin, intestine, lung, liver and kidney, are typically classified according to the expression of the epithelial cell-specific alpha E integrin, CD103⁸³. The skin contains LCs in the epidermis and either CD103+ CD11b^{lo} Langerin+ DCs or CD103- CD11b^{hi} Langerin- DCs in the dermis. Whilst CD103+ CD11b^{lo} Langerin+ DCs have

been shown to be involved in antiviral responses⁸⁴, the functions of the other two are still unclear. DC populations in the intestine are also varied. CD103+ CD11b^{lo} DCs are found in Peyer's patches whilst CD103- CD11b^{hi} and CD103+ CD11b+ cells populate the lamina propria and have been implicated in the transport of intestinal bacteria to the mesenteric lymph nodes. Together with CD103- CD11b^h, CD103+ CD11b- DCs also constitute one of the two main DC subsets in the lung, liver and kidney and have been recently been shown to be developmentally related to CD8+ CD205+ DCs present in the spleen.

Organ	DC subset	Phenotype
Spleen	CD8+ CD8- CD8-	CD8+CD4-CD205+CD11b- CD8-CD4-33D1+CD11b+ CD8-CD4+CD205-CD11b+
Lymph node	CD8+ CD8- Migratory DC	CD8+ CD205+ CD8-CD11b+ Langerin+CD40 ^{hi} CCR7+
Skin	Epidermal LC Dermal CD103+ Dermal CD103-	Langerin+CD1a+ CD103+CD11b ^{hi} Langerin+ CD103-CD11b ^{lo} Langerin-
Intestine	CD103+ CD103+ CD103-	CD103+CD11b- CD103+CD11b+ CD103-CD11b+
Lung, liver, kidney	CD103+ CD103-	CD103+CD11b- CD103-CD11b+

Table 1.3 cDCs in Lymphoid and Non-Lymphoid Tissues. Adapted from “Origin and Development of Dendritic Cells”, Liu *et al.*, 2010⁸³.

1.2.2 Development of DCs

1.2.2.1 Bone Marrow Origin of DCs

Initially, it was thought that monocytes might be the direct precursors of DCs. It is now accepted that steady state DCs originate from BM-derived hematopoietic stem cells (HSCs) rather than from monocytes⁸³. The current view of steady-state DC development has relied on the identification of common monocyte-DC progenitor (MDP) in the BM⁸⁵. MDPs express specific markers, including c-kit/CD117, flt-3/CD135 and c-fms/CD115, and give rise either to monocytes or to DC-restricted progenitors (CDP). CDPs generate both pDCs and pre-cDCs; the latter then migrate from the BM to the periphery where they undergo further differentiation into mature cDCs (Figure 1.6).

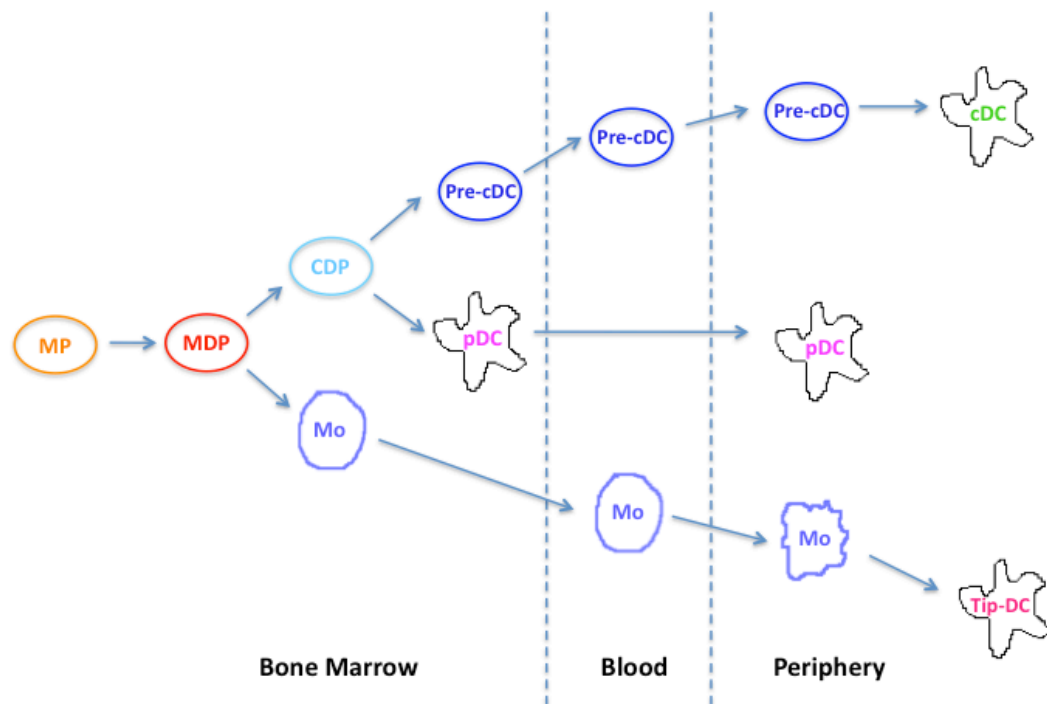


Figure 1.6 BM origin of DCs. DC precursors in the BM are myeloid progenitors (MP), common monocyte-DC progenitors (MDP) and the DC-restricted progenitors (CDP). cDCs are thought to derive from CDP-derived precursors that migrate from the BM through the blood to the lymphoid organs. pDC are also generated in the BM from CDPs. Monocytes derive from the MDPs and, upon activation, give rise to inflammatory Tip-DCs in the periphery. Adapted from “Origin and Development of Dendritic Cells”, Liu *et al.*, 2010⁸³.

1.2.2.2 *In Vitro* Models of DC Development

DC development has been extensively studied through the use of *in vitro* cytokine-dependent culture systems. The most classical example of *in vitro* generated DCs are those derived from human monocytes and early progenitor cells cultured with granulocyte/monocyte colony-stimulating factor (GM-CSF)⁸⁶. Similarly, treatment of mouse BM cells with GM-CSF also leads to the generation of antigen-presenting DCs displaying high levels of CD11c and MHC II⁸⁷. As GM-CSF levels are normally low in the steady state, it has been postulated that GM-CSF derived DCs might model an inflammatory rather than homeostatic type of DC⁷⁸.

DCs can also be generated from mouse BM by culture with FMS-like tyrosine kinase 3 ligand (flt-3)⁸⁸. Differently from GM-CSF, this cytokine is critical for homeostatic DC development and accordingly, flt-3-derived DCs mimic steady-state pDCs and cDCs⁷⁹. Although these *in vitro* models have greatly assisted the understanding of DC development, it is important to underline that they however fail to faithfully recapitulate the more relevant *in vivo* DC phenotypes observed with whole animal studies.

1.3 Immunoregulation by DCs

Historically, the main function of DCs is to initiate T-cell dependent immune responses^{77,89}. The classical model of DC-mediated regulation of T cell responses includes a role for DCs both in steady-state and inflammatory conditions. In the first scenario, a constant low-level entry of DCs into the lymphoid tissues maintains peripheral tolerance to self-antigens. In the second case, sensing of a pathogen-associated danger signal induces the maturation of DCs from antigen-capturing to antigen-presenting cells and enhances their migration to the lymphoid organs where they can then prime antigen-specific immune responses. In addition, DCs have also been shown to be important for humoral immunity, both through direct and indirect activation of B cells. Furthermore, recent accumulating evidence has demonstrated that, in addition to regulating adaptive immunity (T and B cells), DCs also play a critical role in orchestrating the first lines of defense against infection; ie, innate immunity. This chapter will focus on the mechanisms by which DCs regulate T and NK cells.

1.3.1 Immunogenic Properties and Functions of DCs

1.3.1.1 Antigen Uptake by Immature DCs

The main function of immature DCs is to search peripheral tissues for the presence of pathogens, which they sense either by recognizing pathogen-associated components or by receiving signals from the activated innate immune system. Immature DCs have distinctive features that make them ideal antigen-capturing cells. First of all, chemokine responsiveness and chemokine receptor expression allow immature DC recruitment to areas of inflammation. Notably, immature DCs express the chemokine receptors CCR1, CCR2, CCR5, CCR6 and CXCR1 and are attracted to inflammation sites by MIP-1/3 α , MIP5 and RANTES⁸⁹. Secondly, functions such as phagocytosis, macropinocytosis and receptor-mediated uptake render immature DCs fully competent for antigen capture. DC membrane invagination and ruffling enable the uptake of solid and liquid material through phagocytosis⁹⁰ and macropinocytosis⁹¹, respectively. Endocytosis-receptors capture and direct antigen to specialized DC antigen-processing compartments. The major representatives of this group are members of the C-type lectin family. These include both type I

C-lectins, such as DEC-205⁹², and type II C-lectins, including DC immunoreceptor (DCIR)⁹³, DC-associated C-type lectin-2 (dectin-2)⁹⁴ and C-type lectin receptor 1 (CLEC-1)⁹⁵. Additional receptors for antigen uptake include the macrophage mannose receptor (MR)⁸⁶ and Fc γ and Fc ϵ receptors for immunoglobulins⁸⁶. Antigen-capturing functions are downregulated upon activation further confirming their specific roles in immature DC biology⁷⁷.

1.3.1.2 Activation of DCs by Danger Signals

Once DCs have captured antigen, they undergo a series of changes that allow their maturation into antigen-presenting cells^{77,89}. These comprise i) changes in morphology including loss of adhesive structures and cytoskeletal changes which confer DCs with higher motility ii) decreased uptake capacities due to downregulation of receptors for endocytosis iii) increased secretion of chemokines such as IL-8, MIP-1 α and β iv) upregulation of adhesion (LFA-3, ICAM-1) and costimulatory molecules (B7-1, B7-2, CD40, CD58) v) translocation of MHC II compartments to the cell membrane vi) secretion of cytokines that recruit effector cells.

In addition to antigen, DCs also require a second “danger signal”, such as microbial components, to elicit powerful immune responses. Pattern recognition receptors (PRRs) on DCs recognize conserved molecular patterns, referred to as pathogen-associated molecular patterns (PAMPs), on a broad group of microbial species including Gram+/- bacteria, viruses, mycoplasma and yeast. Examples of PAMPs are unmethylated CpG motifs in bacterial DNA, single and double-stranded viral RNA, lipopolysaccharide (LPS) of Gram- bacteria and bacterial flagellin. Examples of PRRs expressed on DCs are Toll-Like Receptors (TLRs), cell surface C-type lectin receptors (CLRs) and intracytoplasmic nucleotide oligomerization domain (NOD)-like receptors (NLRs).

TLRS are the best-characterized PRRs. They were first identified as important developmental regulators in *Drosophila* embryos⁹⁶. Their mammalian homologue (now termed TLR4) was then discovered in humans where it was shown to regulate inflammatory responses⁹⁷. The TLR family now consists of 13 members (TLR1–TLR13) of which TLRs 1–9 are conserved in humans and mice, TLR10 is expressed

only in humans, TLR11 is functional in mice and TLRs 12-13 have still not yet been fully characterized⁹⁸. TLRs can be categorized according to their location and DC-subtype expression; TLR1, 2, 4, 5 and 6, for example, are expressed on the surface and are specialized mainly in the recognition of bacterial products whereas TLR3, 7, 8 and 9 localise in intracellular compartments and typically recognise nucleic acids⁹⁹. Whereas in humans there are clear distinctions in TLR expression amongst DC classes, in mice there is less specificity. cDCs, for example, express all TLRs, with the exception of splenic CD8+ T cells which lack TLR7. Similarly, pDCs express mRNAs for all TLRs but display preferential expression for TLR7 and TLR9¹⁰⁰ (Table 1.4).

	<u>CD4+</u>	<u>CD8+</u>	<u>pDCs</u>
TLR1	++	++	++
TLR2	++	++	++
TLR3	+	+++	+/-
TLR4	+	+	+
TLR5	+++	+	++
TLR6	+++	++	++
TLR7	++	-	+++
TLR8	++	++	++
TLR9	++	++	+++

Table 1.4 TLR expression by murine splenic DC subsets. The expression of the TLRs was confirmed by mRNA expression. Adapted from “Toll-like receptors and dendritic cells: for whom the bug tolls”, *Reis e Sousa*, 2004¹⁰¹.

Signaling through TLRs, via MyD88 or TRIF pathways, regulates a vast array of crucial DC functions such as migration, expression of cell-surface molecules and secretion of cytokines and chemokines¹⁰¹. Remarkably, TLRs differ in their ability to induce cytokine secretion. For example, signaling through MyD88-dependent TLRs induces the secretion of cytokines such as IL-12, IL-6, TNF whereas MyD88-independent TLRs lead to type I IFN (IFN- α/β) secretion. IFN- β release has also been implicated in signaling via the MyD88-dependent pathway¹⁰² and all together, type I IFN release occurs predominantly in pDCs and to a much lesser extent in cDCs. Another interesting aspect of TLR signaling is that cytokine release can be

differentially regulated by the binding of different ligands to the same TLR. This is the case of IL-12p70, the bioactive form of IL-12, which is triggered by TLR2 ligands contained in mycobacteria but not from atypical LPS from *P. gingivalis*, also ligand for TLR2¹⁰¹.

DCs can also undergo indirect microbial activation through capture of necrotic products of cells dying in response to infection. All together, these necrotic products are referred to as damage-associated molecular pattern molecules (DAMPs) and include heat-shock proteins (HSPs), high-mobility group box 1 proteins (HMGB1), β -defensins and uric acid, which can all enhance the immunostimulatory capacity of DCs¹⁰³.

Indirect activation of DCs by microbes can also be achieved by cells of the innate immune system which are activated by pathogen invasion⁸⁹. Through the release of soluble factors, innate cells can modulate both the type and function of DCs. This has been observed for neutrophils and macrophages (IL-1, IL-6 and TNF), mast cells (GM-CSF, IL-4 and TNF), pDCs (type I IFN), keratinocytes (IL-15, GM-CSF, TSLP) and NK cells (TNF, IFN- γ , GM-CSF and HMGB1).

1.3.1.3 Antigen Processing and Presentation

Mature DCs are uniquely equipped for the processing and presentation of peptide fragments on surface MHC molecules and are therefore very efficient at processing a vast array of both foreign and self-peptides, even when at low concentrations. Typically, DCs present exogenous antigens on MHC II and endogenous antigens on MHC I. It is generally accepted that peptides of exogenous origin are loaded on MHC II in late endocytic compartments whereas antigens present in the cytoplasm are loaded on MHC I in the endoplasmic reticulum (ER)¹⁰⁴.

For class II presentation, internalized proteins are enzymatically degraded in endosomes and lysosomes by proteases which function at the low pH of these organelles. MHC II molecules are synthesized in the ER and are then transported to endosomal vesicles in association with the invariant chain (Ii), which occupies the peptide-binding pocket of MHC II. These vesicles have shared lysosomal/endosomal features, are rich in HLA-DM (H-2M in mice) and hydrolytic enzymes and are

specifically termed MHC class II-rich compartments (MIIC)¹⁰⁵. Here, Ii is proteolytically cleaved and the remaining CLIP fragment is removed by HLA-DM molecules so that processed peptides can be loaded on MHC II, before being transported to the surface. Historically, lysosomes were considered very inefficient sites for antigen recycling to the membrane but it is now evident that they are critical regulators of MHCII-peptide assembly and delivery to the surface. Exactly how these lysosomal immunogenic complexes reach the membrane is still unclear. One possibility is that MHCII-peptide complexes are delivered from lysosomes to the plasma membrane via vesicular structures which become progressively devoid of lysosomal components as they reach the cell surface¹⁰⁶. Another view is that MHC II transport occurs through tubulation of MHC II-containing lysosomal compartments which then fuse with the plasma membrane¹⁰⁷.

As discussed earlier, antigen presentation is regulated by maturation status. Mature DCs are very efficient in the formation/accumulation of MHC class II-peptide complexes within lysosomal compartments and in their delivery to the membrane, whereas this process is instead very inefficient in immature DCs¹⁰⁸. In addition, DC-LAMP, a lysosomal-associated glycoprotein specifically expressed in MIICs, is upregulated in mature DCs and is thought to promote the transport of antigen-loaded MHC II complexes to the cell surface¹⁰⁹. DC maturation also influences MHC II turnover as demonstrated by less ubiquitination and greater stability at the surface¹¹⁰.

For the induction of anti-viral and anti-tumoral cytotoxic T cell (CTL)-mediated immunity, DCs instead need to present antigens in association with MHC I. This is quite straightforward when a virally-infected cell uses its own machinery to synthesize viral proteins and then transports them to the surface via the classical MHC class I presentation pathway; ie, proteasome-digested products are translocated from the cytosol to the ER where they are loaded on newly synthesized MHC I molecules and then shuffled through the Golgi apparatus to the surface. Targeting of MHC I on endocytosed proteins which have no access to the cytosol requires a more complicated intracellular mechanism, commonly referred to as “cross-presentation”¹¹¹. Cross-presentation of extracellular antigens on MHC I has been shown to occur in stable early or recycling endosomes which are distinct from the classical ER MHC class I-loading pathway. A variety of cell surface receptors have

been implicated in uptake of antigen for cross-presentation including Fc receptors and members of the C-type lectin family and similarly to class II presentation of soluble antigens, class I cross-presentation is also enhanced by DC maturation¹¹².

1.3.1.4 DC Migration *In Vivo*

As noted in section 1.3.1.1, immature DCs are attracted to sites of inflammation by cytokines that bind to CCR1, CCR2, CCR5, CCR6 and CXCR1. The maturation of DCs is instead associated with their migration from the peripheral tissue to the draining lymphoid organ. Systemic administration of LPS or cytokines such as TNF- α and IL-1 α induces the mobilization of DCs from the heart, kidney and skin¹¹³. Mobilization to the lymphoid tissues depends on the upregulation of the lymph node homing receptor CCR7 which confers responsiveness to CCL19 and CCL21 chemokine ligands expressed on lymphatic vessels and secondary lymphoid organs¹¹⁴. Classically, CCR7 was thought to be an essential mediator of dermal and epidermal DC migration. More recently, CCR7 has also been shown to influence the migration of a population of CD40^{lo} CD80^{lo} CD86^{lo} DCs therefore indicating that it is a regulator of skin DC trafficking not only in inflammatory but also in steady-state conditions¹¹⁵.

1.3.2 DCs and the Control of Immune Responses

1.3.2.1 DC-T cell Cross-talk in the Context of Immune Responses

DCs are the key APCs capable of priming naïve T cells and are very important both for the induction and regulation of T-cell immune responses⁷⁷. *In vivo*, activated DCs migrate to the lymphoid organs where they can interact with antigen-specific naïve CD4⁺ and CD8⁺ T cells. To initiate a productive T cell response, DCs deliver at least two signals to naïve T cells: presentation of peptide-MHC complexes to the T cell receptor (TCR) provides the antigen-specific “signal 1” whereas ligation of B7 molecules by CD28 on the T cell provides the costimulatory “signal 2”¹¹⁶. A third cytokine-related signal also contributes to the activation of naïve (CD8⁺) T cells and is provided by IL-12 and type I IFN¹¹⁷. Recognition of these DC-mediated signals by naïve T cells induces cytokine secretion, clonal expansion (as a result of IL-2 induced autocrine proliferation) and differentiation into either effector or memory cells. In the effector phase of the response, naïve CD4⁺ cells undergo differentiation into different T helper (Th) effector cells (Th1, Th2 or Th17), whilst CD8⁺ T cells differentiate into CTLs with the ability to kill infected cells, either through the release of cytotoxic granule proteins, such as granzymes and perforin, or by Fas-ligand mediated pathways¹¹⁸. Alternatively, naïve T cells develop into memory CD4⁺ or CD8⁺ memory T cells, responsible for accelerated immune responses upon re-exposure to the same pathogen¹¹⁹.

Similar to DC-mediated regulation of T cell functions, T cells can also regulate the function of DCs. An important mechanism is achieved through ligation of DC CD40 and TNF with TNF family proteins expressed on activated and memory T cells (ie. CD40L). In the case of CD40/CD40L interaction, this leads to DC upregulation of B7-1 and B7-2, secretion of IL-12 and expression of other TNF family members such as CD70, 4-1BBL and OX40L⁸⁹. This has feedback effects on T cell function as CD70 and 4-1BBL are both important for the priming and differentiation of naïve CD8⁺ T cells^{120,121} and OX40L induces Th2 differentiation¹²². Another way that T cells can alter DC functions is through CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4), which both bind to CD80 and CD86 and deliver activating/inhibitory signals, respectively⁸⁹.

1.3.2.2 DCs Direct T Helper Cell Responses

DCs have the capacity to direct the T cell response by activating distinct CD4+ T cell subsets which provide different “helper” functions to the immune system (Figure 1.7). Upon TLR triggering, DCs secrete IL-12 and induce the differentiation of naïve CD4+ T cells into IFN- γ producing Th1 cells, which in turn promote the differentiation of CD8+ T cells in CTLs and cellular immunity to intracellular pathogens and viruses. In contrast, with IL-4 secretion, DCs regulate the differentiation of CD4+ T cells into Th2 cells, which principally secrete IL-4 and IL-5, most notable for their involvement in humoral immunity mainly to extracellular pathogens¹²³.

The situation is likely to be more complex than this as different TLR signals can result in different DC responses. Activation of DCs with LPS from *E. Coli* induces IL-12 production and Th1 responses whereas exposure to LPS from *P. gingivalis* skews T cell response towards the Th2 type¹²⁴. In addition, the polarization of the Th cell response can also be influenced by which DC subtype is activated. This is the case for splenic DCs as demonstrated by the fact that CD8 α - DCs induce a Th2-type response, whereas CD8 α + DCs lead to Th1 differentiation and cytokine secretion¹²⁵.

Advances in the field of T-cell immunity have added a third developmental branch to the dual Th1/Th2 differentiation model. Secretion by DCs of IL-23, another IL-12 family molecule, has recently implicated in the development of another Th subtype; the IL-17-producing effector CD4+ T cell (Th-17). These cells differentiate along a developmental program that is distinct from that of Th1 and Th2 lineages and which is inhibited by IFN- γ and IL-4¹²⁶. Th17 cells trigger potent proinflammatory responses and have been implicated in the pathogenesis of autoimmune and inflammatory disease such as rheumatoid arthritis, lupus, asthma and allograft rejection¹²⁷. In contrast, DC-derived IL-27, another member of the IL-12 cytokine family, has been shown to inhibit the differentiation of Th17 cells and to act as an anti-inflammatory agent *in vivo*¹²⁸.

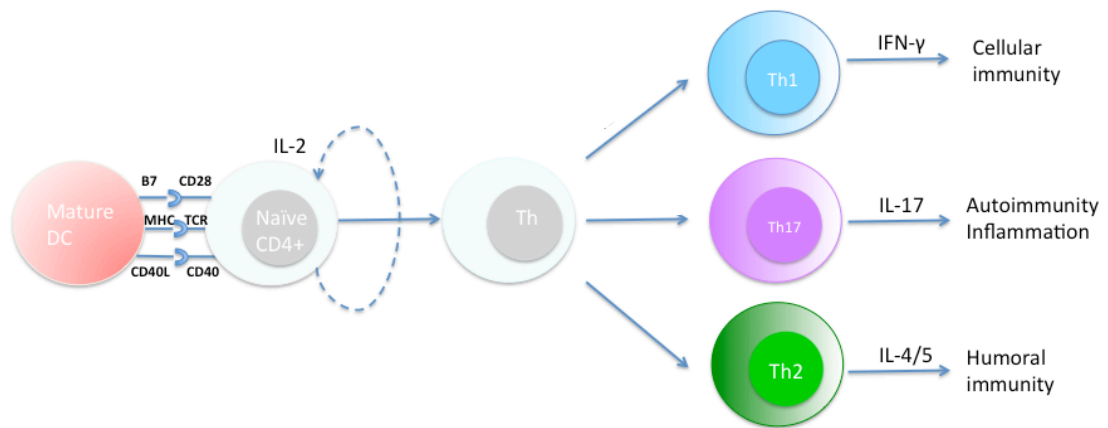


Figure 1.7 DCs direct the T cell response. CD4⁺ T helper (TH) cells are important regulators of T cell-mediated immune responses and inflammatory diseases. Mature DCs present MHCp in association with costimulatory signals to naïve CD4⁺ Th cells and control their differentiation into TH1, TH2 and TH17 effector cells by secreting IL-12, IL-4 and IL-23, respectively. TH1 cells produce IFN- γ and mediate cellular immunity, TH2 cells produce IL-4/5 and mediate humoral immunity and allergic responses, TH17 cells secrete IL-23 and are involved in autoimmune and inflammatory diseases.

1.3.2.3 DCs and the Control of T Cell Tolerance

Besides being critical initiators of T cell immunity, DCs also play an important role in tolerizing T cells to self-derived antigens through the induction and maintenance of central and peripheral tolerance.

Central tolerance is mainly achieved by thymic DCs, which together with the thymic medullary epithelium, induce the deletion (negative selection) of thymocytes bearing high-affinity TCRs for self pMHC complexes¹²⁹.

Peripheral tolerance is instead required to ensure the elimination of self-reactive T cells that escape deletion in the thymus, possibly because of their lower affinity for self-antigens. DCs play a critical role in the induction of this process. One mechanism by which this is achieved is through the maintenance of an immature phenotype. In the absence of inflammation, immature DCs uptake, process and present tissue antigens leading to antigen-specific T-cell unresponsiveness (also known as clonal anergy) or deletion¹³⁰. This mechanism appears to be especially important for tolerization to tissue-specific self-antigens which have no access to the thymus¹³¹. Mature DCs can also participate in the maintenance of peripheral

tolerance by facilitating the clonal expansion of the naturally occurring thymus-derived Foxp3+CD4+CD25+ regulatory T cells¹³².

Recent advances in this field of study now also support a role for specialized tolerogenic DC subsets in the maintenance of peripheral tolerance through the induction of T cells with suppressive properties¹³³. Differentiation into tolerogenic DCs can be specifically regulated by pathogenic agents. Infection with Respiratory Syncytial Virus (RSV) induces DC expression of inhibitory Ig-like transcripts (ILT) and of programmed cell death ligand-1 (PD-L1)¹³⁴, molecules implicated in the delivery of T cell inhibitory/anergic signals, whilst *Bordetella Pertussis* stimulates DC IL-10 secretion leading to the induction of IL-10 releasing regulatory T cells¹³⁵.

1.3.2.4 DCs and the Control of NK Cell Activation

In addition to initiating and regulating adaptive immunity, DCs are also important for the innate arm of immunity, in particular for NK cells. NK cells are specialised in the elimination of pathogen-infected and tumor cells, either by direct lytic mechanisms (granule exocytosis and death-receptor engagement) or by cytokine secretion (for example, IFN- γ)¹³⁶.

One way DCs can regulate the functions of NK cells is through cytokines. By secreting IL-2, IL-12, IL-15 or IL-18, DCs have been shown to participate in NK cell activation¹³⁷⁻¹³⁹. Notably, soluble DC-derived IL-15 is known to synergise with IL-12 in inducing NK cell IFN- γ production whereas membrane-bound IL-15 on DCs is essential for NK cell survival and function, as already discussed in section 1.1.17.

Cell-contact also plays an important role in DC-mediated activation of NK cells. Of note is the fact that NK activation is regulated by a balance between signals delivered through activating and inhibiting receptors and that DCs can regulate the expression of ligands for these receptors¹⁴⁰. For example, immature DCs constitutively express ligands for the activating receptors 2B4 and CD27 (CD48 and CD70, respectively) whilst mature DCs upregulate ligands with analogous activating functions under inflammatory conditions. This is the case of NKG2D (natural-killer group 2, member D) ligands which are specifically expressed in response to type I IFNs¹⁴¹. On the other hand, mature DCs can also downregulate the expression of ligands for

inhibitory receptors such as NKR-P1B (NK-cell receptor protein 1B) and NKR-P1D66¹⁴². Furthermore, DC adhesion molecules also play a role in NK cell contact dependent activation; for example, LFA-1 (lymphocyte function-associated antigen 1) interacts with ICAM-1 (intercellular adhesion molecule 1) in the context of the DC-NK immunological synapse^{143,144}. This will further discussed in chapter 1.4.

1.3.2.5 Orchestration of Immune Responses by DC Chemokine Expression

DCs are thus critical orchestrators of both the innate and adaptive arms of the immune system. One way DCs can coordinate all of these processes to finally produce an effective immune response is through regulated chemokine expression. This has recently been demonstrated for human blood DCs in response to viral infection and has been shown to occur in 3 sequential waves of chemokine secretion¹⁴⁵. Here, the first cytokines were produced within 2 to 4 hours from pathogen exposure and attracted innate effectors such as neutrophils (CXCL8) and NK cells (CXCL1-3), thereby limiting the initial spread of infection. The second wave was instead produced within 8 to 12 hours and recruited effector memory T cells to sites of infection (CXCL9-11 and CCL3-5). Finally, the third wave occurred at a later stage (after 24 hours), once DCs had reached the secondary lymphoid organs, and attracted B and follicular T helper cells for the induction of a humoral response (CXCL13), naïve cells to amplify the immune response (CCL19, CCL22) and finally regulatory T cells to control it (CCL22).

1.4 Structure and Functions of Immunological Synapses (IS)

1.4.1 Molecular Definition of the IS

The study of the molecular organization of the T-APC interface, which we now refer to as the immunological synapse (IS), began with *in vitro* imaging of fixed cellular conjugates. The first molecular reorganization to be observed was the polarization of the microtubule organizing centre (MTOC) to the interface between CD8+ T cells and their target cells¹⁴⁶. This initial finding was followed by a series of studies which demonstrated a marked redistribution of the TCR, its co-receptor CD4, the integrin LFA-1 and the integrin-associated signaling protein talin at the T-APC contact zone¹⁴⁷⁻¹⁴⁹. Although very valuable, these findings failed to provide information on the spatial organization of these molecules. Springer *et al.* were the first to address this issue by proposing a size-dependent segregation of molecular complexes in distinct domains at the interface between T cell and APCs¹⁵⁰. Here, they introduced the idea that contact zones enriched in TCR/MHC and CD2/CD58 interactions would be divided from those containing LFA-1/ICAM-1 interactions or large CD45 phosphatases according to ectodomain size. This model was later confirmed by Kupfer in a series of experiments which employed image deconvolution of a collected stack of images¹⁵¹. This technique allowed detailed 3-dimensional views of the structure of the interfaces and revealed the formation of a bull's eye arrangement with a central cluster of TCR/MHCp, referred to as cSMAC (central supramolecular activation cluster), surrounded by a peripheral ring of LFA-1/ICAM-1, the peripheral SMAC (pSMAC). Subsequent studies later revealed the existence of a third zone, the distal SMAC (dSMAC), containing CD45 phosphatase¹⁵² (Figure 1.8).

Deeper insights into the molecular organization of contact interfaces were achieved thanks to the supported planar bilayer technology, established by McConnell^{153,154} and discussed in more detail in 1.4.2. The first dynamic view of SMAC formation was derived from studies with supported planar bilayers containing adhesion molecules¹⁵⁵. Here, CD58 and ICAM-1 were anchored to the bilayers via glycosylphosphatidylinositols (GPIs). Fluorescent tagging of these molecules then allowed direct visualization of the interaction with their binding partners through total internal reflection microscopy (TIRFM). These studies demonstrated similar phenomena to the ones described above ie, segregation of CD2-CD58 interactions

from LFA-1/ICAM-1 interactions, and led to the initial definition of the IS as a specialized junction defined by adhesion, polarization and stability. The following year, supported planar bilayers containing fluorescent MHCp complexes and LFA-1 and ICAM-1 provided further insight into the dynamics of the IS and showed that nascent TCR microclusters (MCs) formed in the dSMAC are transported to the cSMAC, within a few minutes from their formation, in an F-actin dependent manner¹⁵⁶. These findings recapitulated the patterns observed by Kupfer¹⁵¹ but also provided an additional twist to the story in that they showed that IS formation correlates with full T cell activation and is important for sustained signaling.

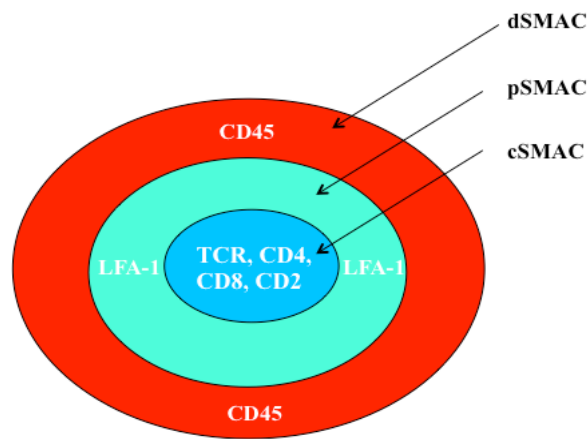


Figure 1.8 Structure of the IS. En face view of the “organized IS” with a bull’s eye pattern of supramolecular activation complex (SMACs). In the center is the cSMAC, which contains TCR, CD4, CD8 and CD2 receptors. More recently, the cSMAC has been resolved in an inner compartment containing the TCR and in an outer one containing CD28 and PKC- θ (not shown). Surrounding the cSMAC is the pSMAC which is populated by LFA-1 and outside of the pSMAC is an even more peripheral domain known as the dSMAC. Besides segregating CD45 phosphatases from TCR, it is becoming increasingly clear that the dSMAC is also a site of active membrane movement and signaling. Adapted from “Understanding the Structure and Function of the Immunological Synapse”, Dustin *et al.*, 2010¹⁵⁷.

1.4.2 Supported bilayers for the study of IS

Supported planar bilayers are an important technological tool for the study of molecular interactions at contact interfaces. Glass-supported bilayers were initially developed in the early 1980s¹⁵⁸ and were subsequently applied to study of the IS¹⁵³. Glass-supported bilayers are generated by the deposition of a phospholipid bilayer on a glass surface. Bilayer deposition can be achieved either by fusion of preformed liposomes or by monolayer transfer from an air-water interface¹⁵⁹. In the first case, liposomes absorb and fuse on the surface generating a continuous phospholipid bilayer whilst in the second, the phospholipid bilayer is deposited one leaflet at a time. Nowadays, in addition to glass, other commonly used forms of substrates are quartz, mica and silica. Irrespective of the method of assembly or type of substrate, the common underlying principle of supported planar bilayers is the creation of a balance of attractive and repulsive forces between the bilayer and the substrate to ensure trapping of the bilayer next to the surface.

A distinguishing feature of lipid bilayers is their mobility. In fact, all lipids and lipid-associated proteins exhibit remarkable lateral mobility within the bilayer. This includes streptavidin-labeled molecules attached to biotinylated lipids and His-tagged molecules linked to Ni²⁺ chelating lipids, such as nickel-nitrilotriacetic acid (Ni-NTA)-modified lipids.

With respect to the cell-based system, the advantages of supported lipid bilayers are controlled composition, superior resolution and simplicity of interpretation of bilayer-derived images. Furthermore, by virtue of their planar geometry and close distance to the substrate, supported lipid bilayers can be imaged via a multitude of microscopy techniques including conventional epifluorescence, confocal imaging, TIRFM, reflection and fluorescence interference and Förster resonance energy transfer (FRET). Wide field epifluorescence imaging is normally used to test the mobility of the bilayers; reflection and fluorescence interference are instead particularly useful to resolve the topography of structures whilst FRET provides important information on intermolecular distances. A very valuable advance in this field has been the application of TIRFM to planar bilayers and was first achieved by McConnell allowing successful visualization of MHC/TCR interactions¹⁵⁴. TIRFM

relies on illuminating an interface with a laser at such an angle that the incident light is totally internally reflected and creates an evanescent wave which decays exponentially thereby penetrating only a short distance from the interface. This short evanescent wave ($\approx 200\text{nm}$) reduces background fluorescence and increases contrast on planar events at the bilayer interface offering unique imaging advantages in terms of resolution and image-derived information.

1.4.3 TCR signaling and Activation in the Context of the IS

T cell activation is a very well characterized process which consists of a defined series of molecular events¹⁶⁰. Ligation of the TCR by peptide-MHC complexes is an essential stimulus for T cell activation. Upon peptide recognition, the TCR relocates within specialized glycolipid-enriched membrane microdomains, known as lipid rafts, where active signaling molecules reside¹⁶¹. The Src protein kinase Lck is located within these rafts and mediates the phosphorylation of tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) of the invariant CD3 and ζ -chains of the TCR complex. Phosphorylated ITAMs then act as docking sites for the zeta-chain-associated protein kinase 70 (ZAP-70) tyrosine kinase which is in turn activated and phosphorylates multiple adaptor proteins including Linker for activation of T cells (LAT) and SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). LAT also resides within lipid rafts and is critical for the recruitment/activation of a number of signaling proteins such as PLC- γ 1, PI-3K and Ras¹⁶². Active PLC- γ 1 leads to Ca^{2+} release, calcineurin activation and Protein Kinase C (PKC) activation; PI3K is responsible for the generation of inositol phospholipids and the Ras-dependent signaling pathway mediates activation of MAP kinases. SLP-76, the other adaptor protein, is also required for TCR activation and by interacting with Grb2 and PLC- γ 1 has been reported to provide a functional link with the Ras and Ca^{2+} signaling pathways¹⁶³. Altogether, these signaling pathways participate in the activation of transcription factors, such as nuclear factor of activated T cells (NFAT), AP-1 and nuclear factor-kB (NF-kB), which control cell proliferation, death and differentiation. All of these proximal intracellular events occur within 5 min from T cell activation, which is the time frame in which the IS forms¹⁶⁰.

In recent years, efforts have focused on understanding the importance of IS formation for TCR signaling and T cell activation. Initially, the cSMAC was thought to be important to sustain T cell signaling. This was suggested by early studies in which signaling molecules, such as PKC- θ and Lck, were observed within cSMACs formed by T-APC cells¹⁵¹. However, experiments with planar bilayers demonstrated that Ca²⁺ signaling is initiated before cSMAC formation and that phosphorylated Lck localizes in the pSMAC rather than in the cSMAC¹⁵⁶. These findings led to the current model of T cell activation whereby TCR MCs represent the signaling units of T cells. TCR MCs have been implicated in TCR signaling by the way of elegant experiments in which treatment with anti-MHC, which blocks new MC formation, eliminates Ca²⁺ signaling after 2 min, the time frame in which MCs assemble in the dSMAC and reach the cSMAC¹⁶⁴. Rather than a site of signal initiation, the cSMAC is now viewed as one of signal termination and TCR degradation¹⁶⁵. Altogether, these findings suggested that signaling is sustained solely during the lifetime of these MCs and that prolonged signaling requires continuous MC formation and translocation. MC involvement in TCR signaling was further confirmed by parallel studies demonstrating their colocalization with signaling molecules, including ZAP-70, LAT and SLP-76, in fixed cells^{166,167}. Altogether, these studies demonstrate that IS formation is not required for the initiation of TCR signaling but that it is instead critical for sustained signaling and full T cell activation.

1.4.4 Adhesion and Costimulation in IS formation and T cell activation

In addition to TCR/MHC-peptide interactions, IS formation and T cell activation also require the engagement of costimulatory receptor pairs. The most important costimulatory receptors on T cells and their ligand on APCs are LFA-1– ICAM-1 and CD28-B7.

The LFA-1–ICAM-1 adhesion receptor pair plays an important role in both IS formation and T cell activation. By binding to ICAM-1, its DC counterpart, LFA-1 facilitates IS formation¹⁶⁸. In addition to promoting adhesion of T cells to APCs thereby facilitating TCR/MHCp interactions, LFA-1 can also enhance TCR activation by lowering the amounts of antigen necessary for T cell activation¹⁶⁹.

Similar to LFA-1, CD28 is an important costimulatory molecule which promotes full activation of T cells, in this case by way of direct effects on gene activation, cell cycle and survival¹⁷⁰. CD28 has been observed to enhance both the earliest processes associated with TCR signaling pathways, such as increase in Ca^{2+} levels^{171,172}, activation of NFAT¹⁷¹ and NF-KB¹⁷³, as well as later processes such as IL-2 release¹⁷⁴. At present, the molecular mechanism by which CD28 amplifies TCR-mediated T cell activation is still unclear but its localization in the IS suggests it may manipulate TCR signaling through spatial coordination. In fact, in contrast to LFA-1, CD28 resides in the cSMAC. Within the cSMAC, CD28 has been observed to colocalise with PKC θ and to be segregated from inactive TCRs¹⁷⁵. This has led to a new functional concept of the cSMAC, defined by a separate inner compartment of inactive TCR and an outer compartment containing CD28 and the associated PKC θ component. Furthermore, CD28 has been shown to facilitate the transport of peptide-MHC complexes at the IS suggesting that increased MHC-TCR receptor densities at the membrane might account for its costimulatory effect¹⁷⁶.

The CD2 is a transmembrane protein that also participates in T cell activation and functions both as an adhesion and costimulatory receptor. CD2-CD58 interactions have been shown to promote APC-T cell adhesion and to lower the threshold required for T cell activation¹⁷⁷. In addition to its role in adhesion, CD2 engagement also promotes signaling through unique phosphorylation of PLC γ 1 via Fyk kinase¹⁷⁸. A recent study has shown that CD2 signals in a manner which is reminiscent of that described for the TCR in that it also induces the clustering of TCR- ζ , Lck, and LAT into microdomains¹⁷⁹. Due to the similar interface distance created between CD2/CD58 and TCR/MHC receptor pairs¹⁵⁵, CD2 was initially thought to reside exclusively within the cSMAC and to facilitate T cell signal transduction through exclusion of CD45 phosphatases. This notion has been challenged by the finding that active CD2 localises in the periphery and that it is spatially segregated from the TCR-enriched cSMAC zone¹⁷⁹.

1.4.5 The Actin Cytoskeleton in T Cell Activation and in IS Formation

The actin cytoskeleton plays an important role in T cell activation. One of the ways this is achieved is through changes in T cell polarity and shape, including formation of the IS. Importantly, the T cell cytoskeleton participates in the assembly of both the

classical IS, a stable T-APC junction, and the immunological kinapse, a moving cell junction¹⁸⁰.

Prior to antigen encounter, chemokine induced T cell polarization takes place at the leading edge of the T cell leading to loss of a rounded shape. This results in the establishment of an asymmetric synapse (kinapse) and to fast cell migration and scanning. Migrating T cells are characterized by an F-actin rich leading edge (lamellipodium) trailed by a myosin II-rich uropod¹⁶⁰. These two structures mediate actin polymerization and retraction respectively, resulting in oscillatory contractions. Although the kinapse has not been extensively studied, lamellipodia have been associated to dSMACs which push the actin meshwork (lamellas) to one side (pSMAC) leading to asymmetry and migration. The uropod is instead thought to correspond to the cSMAC, in that both structures are relatively devoid of F-actin (Figure 1.9)¹⁸¹. Altogether, these changes at the leading edge of polarized lymphocytes are also important for increased sensitivity to antigen as demonstrated by studies in the which lamellipodia were found to be at least 10 times more sensitive than the uropod to antigen¹⁸². Polarization is also required for effective adhesion as shown by enhanced integrin engagement at the leading edge and by disruption of adhesive interactions at the uropod¹⁸³.

Upon TCR engagement, there is a reversal in the polarity of actin movement resulting in a symmetric IS; actin starts moving centripetally assisting the inward transport of distinct SMACs, as has been demonstrated for TCR MCs^{176,184}. The current view of actin-assisted IS formation envisages that upon their formation in the dSMAC, both TCR and LFA-1 MCs are shuffled to the pSMAC by an actin flow which then dissipates in the pSMAC allowing the diffusion of LFA-1 in this region. In contrast, TCR MCs are incorporated into the cSMAC and this has recently been demonstrated to be mediated by Tsg101, a protein that recognizes ubiquitinated substrates and mediates their transport into multivesicular bodies (MVBs)¹⁸⁵.

Another intriguing feature of the cytoskeleton is that it can mediate the interconversion between the stable IS and the migratory kinapse¹⁸⁰. In the first case, a centripetal actin flow maintains an axis of radial symmetry and allows cells to slow or stop their motility upon encounter of a strong TCR signal. In the second,

retrograde actin flow breaks the symmetry of the synapse and restores motility leading to net migration and scanning of APC surfaces. In this model, PKC- θ is thought to promote symmetry breaking (kinapses) through myosin II contraction in the pSMAC whereas Wiskott Aldrich Protein (WASp) is believed to suppress myosin II contraction thereby promoting symmetry of the contact area (stable IS)¹⁸⁶.

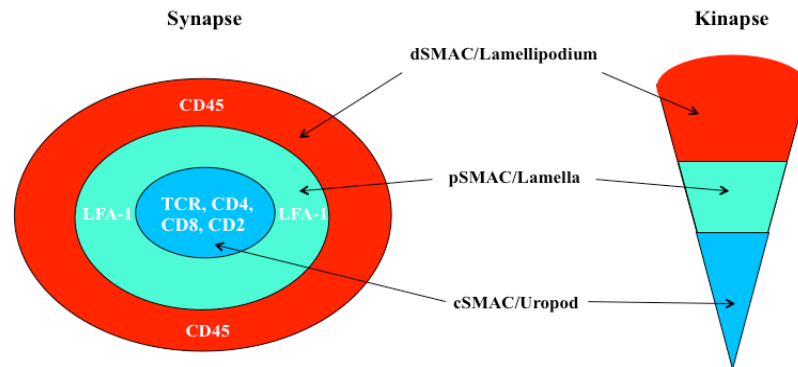


Figure 1.9 Pairing the Structures of the Immunological synapse/kinapse. The kinapse is a motile/asymmetric T-APC junction which consists of an F-actin rich leading edge/lamellipodium (corresponding to the dSMAC) and of an F-actin depleted trailing uropod (corresponding to the cSMAC). Retrograde actin flow leads to condensation of lamella on one side (pSMAC) and resultant asymmetry/migration. Adapted from the book “Multichain Immune Recognition Receptor Signaling: From Spatiotemporal Organization to Human Disease”.

Besides their participation in IS formation and stability, another important function of actin filaments is the formation of a scaffold for signaling components. PKC- θ , for example, is recruited to TCR clusters within the mature IS and this seems to depend on Vav-mediated actin polymerization¹⁸⁷. Furthermore, the actin scaffold has also been implicated in the recruitment and stabilization of glycolipid membrane domains enriched in signaling molecules, thereby influencing TCR activation^{188,189}. An emerging concept is that T cell activation and the actin cytoskeleton reciprocally regulate each others functions, as suggested by the fact that actin polymerization itself can be influenced by TCR signaling¹⁹⁰. For example, upon TCR ligation, activated SLP-76 recruits Vav1, the guanine exchange factor for Rho-family G-proteins Cdc42 and Rac. Activated Cdc42 interacts with WASp to induce actin polymerization and consequent enhancement of T cell activation via clustering of

glycolipid-rich membrane domains and concentration of signaling components, as noted above.

Further evidence of actin involvement in TCR signaling is given by the fact that upon reaching the F-actin depleted c-SMAC, the signaling components dissociate from TCR MCs leading to signal termination and TCR degradation¹⁸³. The actin cytoskeleton therefore confers the IS with an organized structure based on symmetry and on an actin-depleted central zone, important for vesicle trafficking to and from the plasma membrane.

1.4.6 Functions of the IS

Due to the many definitions of the IS, its purpose has for long been a very controversial issue. It is now generally agreed that the IS can have various functions, which depend on its architecture and on the cell type involved. This section will therefore focus on those functions of the IS that depart from those discussed above (signal amplification, termination and costimulation) but instead relate to contact stability, directed secretion and cell fate determination.

One role of the IS is to act as a brake on T –cell motility allowing T cells to dwell on APCs for long periods of time. Full T cell activation is known to require stable contacts of up to several hours^{191,192}. The high durability of the IS, which can last up to approximately 15 hours¹⁹³, reinforces the idea that one the functions of the IS might be to allow stable interactions necessary for full T cell activation.

In addition to a role in contact formation, the organized structure of the IS has been suggested to act as a conduit for directed secretion of components, such as lytic granules and cytokines. This has been observed for CTLs where directed IS-mediated secretion seems to play a major role in cytolytic killing. Here, the F-actin, depleted cSMAC is thought to represent a site of polarized secretion of cytokines and cytolytic agents whereas the pSMAC might function as a tight seal to prevent the escape of these molecules from the cSMAC¹⁵⁷. The polarization of the MTOC at the IS has been implicated in the directed secretion of cytokines and cytolytic granules towards the target cell contact site¹⁹⁴ and direct evidence of the involvement of the IS in directed secretion was first provided by a study clearly demonstrating that the

pSMAC plays a role in reorienting the MTOC¹⁹⁵. Accordingly, LFA-1-ICAM-1 interactions in the pSMAC were later shown to be essential for directed cytolytic granule release at the contact site with APCs¹⁹⁶.

Targeted secretion of cytokines is not unique to CD8+ T cells but can also be mediated by other cells of the immune system. Synaptic delivery of IL-12 by DCs¹⁹⁷ and of IL-2 and IFN- γ by CD4+ T-cells^{194,198} suggests that a specific cytokine micro-environment at the IS is likely to be important for DC or T-cell effector functions. Though not in soluble form, IL-15 might also play a role in specific cell-cell interactions at the IS through transpresentation, as suggested by localization of IL-15R α at DC-NK cell synapses¹⁹⁹.

Another interesting feature of the IS is that it appears to play a role in establishing effector CD4+ T cell fates. For example, the receptor for IFN- γ (classic Th1 cytokine) has been shown to be asymmetrically distributed at the IS and to be asymmetrically partitioned during asymmetric cell division. This has been suggested to promote differentiation into Th1 rather than Th2/Th17 cellular fates²⁰⁰. Furthermore, Th1 and Th2 cells have been observed to be predisposed to by hyperstable or unstable synapses, respectively¹⁸⁶, thereby suggesting that to retain potential for both Th1 and Th2 phenotypes, naïve CD4+ cells must maintain a balance between stable and symmetry breaking immune synapses/kinapses.

1.4.7 The Natural Killer IS

Although the IS was initially described as a specialized junction of adhesion, polarization and stability between a T cell and an APC, these definitions also apply to the NK-cell IS. Studies on the NK-cell IS paralleled those on the T-cell IS highlighting some interesting comparisons between these two synapses. However, owing to NK specialization in cytolytic killing, certain functions of the IS, such as directed secretion of lytic granules, seem to be prevalent in NK synapses²⁰¹.

NK cell effector functions are regulated by a balance between activating and inhibitory surface receptors. NK cells can form activating or inhibitory IS depending on the molecules recruited. Inhibitory IS are rich in inhibitory receptors that recognize self-determinants and prevent the lysis of healthy cells. In contrast,

activating NK cell immunologic synapses (NKIS) mediate cytotoxic killing¹⁴⁴. In humans, NK activating receptors, such as NKG2D²⁰², bind to stress-inducible polymorphic MHC I-like molecules (MICA) and associate with immunoreceptor tyrosine-based activatory (ITAM-bearing signal transduction chains). Activating NK receptors signal in a way which is comparable to the TCR in that they initiate a signaling cascade involving Syk, ZAP-70 and Lck²⁰³. Similarly to T cell IS, the activating NKIS also recruits signaling molecules to a central cluster. In addition, the NKIS cSMAC distinctively accumulates perforin and the pSMAC contains CD2, CD11a, CD11b and F-actin²⁰⁴. Notably, actin reorganization at the NKIS leads to receptor clustering and lipid raft aggregation whilst the redistribution of lytic granules to the contact area with the target cell depends on MTOC polarization¹⁴⁴.

1.4.8 The other side of the IS; the DC

The IS is a bipartite structure composed of a T/NK cell and a DC. Most studies on the IS have focused on the T/NK side of the IS whereas the DC IS has received much less attention. This was partly due to the initial belief that DCs played a passive role in IS formation as well as to the lack of specific tools, such as the well defined lipid bilayer system which has instead been fundamental for imaging and describing the T cell IS. Experiments with cultured or primary DCs have shown that the structure of the DC IS departs from the canonical bull's eye arrangement observed with planar bilayers in that DCs preferentially forms multifocal patterns of MHC-TCR interactions rather than a central one²⁰⁵. The main distinction between the T-DC and the T-bilayer system is the active contribution of the DC cytoskeleton to IS formation, as demonstrated by the finding that paralysation of the DC cytoskeleton impairs T cell activation²⁰⁶. It is now well accepted that DCs participate in IS formation through cytoskeletal rearrangement, as confirmed by various reports²⁰⁷⁻²¹⁰.

In support of its important role during IS formation, the DC cytoskeleton actin has also been observed to be critical for MHC recruitment to the DC IS. DC cytoskeletal reorganization has been shown to be required for both the delivery of MHC/peptide complexes to the surface¹⁰⁶ and for their clustering at the contact interface with T cells²¹¹. This is in contrast to studies with other APCs such as B cells which have instead shown that disruption of the actin cytoskeleton has no effect on MHC II accumulation at the synapse²¹² and that MHC II aggregation occurs only through

TCR-mediated trapping of raft-associated MHC II-peptide complexes²¹³.

In addition to its function in the formation of IS, the DC cytoskeleton is also thought to play an active role in the multifocal patterning of the IS. In fact, by interacting with MHCp clusters, the DC cytoskeleton has been proposed to limit the free diffusion of TCR MCs to the cSMAC²⁰⁹. Altogether, these findings highlight the importance of the DC cytoskeleton for both IS function and patterning.

Chapter Two Materials and Methods

2.1 Materials

Unless otherwise stated, all tissue culture reagents were supplied by Gibco (Invitrogen) and all general chemicals by Sigma-Aldrich.

2.1.1 Buffers and Solutions

Blocking buffer	5% milk solution in 0.1% Tween/ PBS
DNA loading buffer	10mM Tris pH 7.5, 50mM EDTA, 10% Ficoll 400, 0.4% Orange G
FACS buffer	1% FCS/PBS
Loading buffer *	4× NuPage sample buffer, 1× NuPage sample reducing agent (Invitrogen)
Loading buffer **	1.5× NuPage loading buffer, 1× NuPage sample reducing agent (Invitrogen)
Luria-Bertani (LB) Broth	2g LB in 1L H ₂ O
Lysis buffer	1% NP40 (AppliChem), 50 mM HEPES pH 7.4, 150 mM NaCl, 1mM EDTA, 1/40 protease inhibitor cocktail
Running Buffer	50ml NuPAGE 20X MOPS SDS running buffer (Invitrogen), 950ml H ₂ O
TAE buffer (50×)	242g Trizma base, 100ml 0.5M EDTA pH8.0, 57.2ml glacial acetic acid in final volume of 1L
Transfer Buffer	30ml methanol, 30ml NuPAGE Transfer Buffer (Invitrogen), 240 ml H ₂ O
Wash buffer	0.1% Tween/ PBS

* WB (Western Blotting)

** IP (Immunoprecipitation)

2.1.2 Primary antibodies (Table 2.1)

Antibody	Clone	Manufacturer
rat anti-mouse B220-FITC	RAF-6B2	BD Pharmingen
mouse anti-mouse CD4-PE-Cy7	SK3	BD Pharmingen
rat anti-mouse CD4-APC	RM4-5	BD Pharmingen
rat anti-mouse CD8 α -PercP	53-6.7	BD Pharmingen
rat anti-mouse CD11b-PE	M1/70	BD Pharmingen
hamster anti-mouse CD11c-APC	HL3	BD Pharmingen
rat anti-mouse CD16/CD32	2.4G2	BD Pharmingen
hamster anti-mouse CD69-PE-Cy5	H1.2F3	BD Pharmingen
rat anti-mouse CD86-FITC	GL1	BD Pharmingen
rat anti-mouse CD132	4G3	BD Pharmingen
mouse anti-GADPH	6C5	Santa Cruz Biotechnology
rat anti-mouse I-A/I-E	M5/114.15.2	BD Pharmingen
rat anti-mouse I-A/I-E-PE	2G9	BD Pharmingen
rat anti-mouse IL-2R α -FITC	PC61.5	BD Pharmingen
rat anti-mouse IL2R β -FITC	TM-b1	BD Pharmingen
rabbit anti-IL-2R γ	M-20	Santa Cruz Biotechnology
biotin rat anti-mouse IL-12p40/p70	C17.8	BioLegend
goat anti-mouse IL-15R α -FITC	FAB551F	R&D Systems
goat anti-mouse IL-15R α	AF551	R&D Systems
goat anti-IL-15R α	N-19	Santa Cruz Biotechnology
rabbit anti-IL-15R α	H-107	Santa Cruz Biotechnology
rat anti-mouse IL-21R-PE	eBio4A9	BD Pharmingen
rabbit anti-JAK3	C-21	Santa Cruz Biotechnology
mouse anti-mouse NK1.1-APC	PK136	BD Pharmingen
rabbit anti-phospho-AKT (Ser473)	D9E	Cell Signaling Technology
rabbit anti-phospho-JAK3(Tyr785)	ab61102	Abcam
anti-phospho-PLC γ 1 (Ser1248)	4G10	Cell Signaling Technology
rabbit anti phospho-STAT5(Tyr694)	C71E5	Cell Signaling Technology
rabbit anti phospho-STAT5(Tyr694)	D47E7	Cell Signaling Technology
mouse anti-pSTAT5-PE (Tyr694)	47	BD Pharmingen
rabbit anti-STAT5	3H7	Cell Signaling Technology
anti-TCR ζ Alexa 647		Dustin Lab

2.1.3 Cell Lines

293T	Human embryonic kidney cell line
B017.4	Kindly provided by Dr. Derek Minney, Department of Medicine, Dalhousie University, Halifax, Canada. This cell line is a T cell hybridoma with a TCR specific for OVA ₃₂₃₋₃₃₉ peptide in the context of I-A ^b .
ED7R	Kindly provided by Prof. Ian Alexander at the Children's Hospital, Westmead, Sydney, Australia. This cell line is derived from a γ c-deficient human leukaemia T cell line ED40515 ²¹⁴ .

2.1.4 Mice

All experiments were approved by and performed according to UK Home Office Animal Welfare Legislation. Mice were C57BL/6 wild-type mice and OTII transgenic mice (OVA₃₂₃₋₃₃₉ peptide-specific CD4) and were obtained from Charles River (Kent, UK). γ c/Rag 2^{-/-} mice were backcrossed at least 10 times with C57BL/6 and were kindly provided by Dr F. Colucci (Babraham Institute, Cambridge, UK) and bred at Institute of Child Health facilities.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Culture of Adherent Cell Lines

Adherent cell lines used in this study were 293T cells. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing GlutaMAX supplemented with 10% foetal calf serum (FCS) and 1% Penicillin/Streptomycin (complete DMEM). Cells were grown in 25 cm², 80 cm² or 175cm² tissue culture flasks at 37°C with 5% CO₂. Cells were passaged when 80% confluent: the monolayers were first washed with phosphate buffered solution (PBS), then incubated for 5 minutes at 37°C with trypsin/EDTA. Cells were split 1:5 in complete DMEM and transferred to a new flask.

2.2.1.2 Culture of Non-adherent Cell Lines

Non-adherent cell lines used in this study were B017.4 and ED7R cells. Cells were maintained in RPMI medium 1640 supplemented with 10% FCS and 1% Penicillin/Streptomycin (complete RPMI). 1% HEPES was also added to the medium for culture of B017.4 cells. Cells were grown in 25 cm², 80 cm² or 175cm² tissue culture flasks (standing upright) at 37°C with 5% CO₂. Cells were passaged every 3-4 days; cells were split 1:10 in complete RPMI and transferred to a new flask.

2.2.1.3 Generation of Murine Dendritic Cells (DCs)

Using a syringe, BM cells were flushed from the femurs and tibia of C57BL/6 wild-type mice or γ c/Rag 2^{-/-} mice with culture medium (complete RPMI) on a 70 μ m cell strainer. Cells were mashed and passed through the cell strainer with a plunger then centrifuged at 1400rpm. Cell pellets were resuspended in 5mls Red Cell Lysis Buffer for 5 minutes at 37°C. Cells were then washed with culture medium and pelleted by centrifugation. 4 \times 10⁶ cells were plated in 10 cm² plates in 10ml of culture medium containing 20ng/ml GM-CSF (BioSource). BM cells were left to differentiate into DCs over a period of 7 days. 5ml of culture medium containing 20ng/ml GM-CSF were also added on day 3 and 5 of culture.

2.2.1.4 Culture of Murine DCs

On day 7 of culture, DCs were scraped from the tissue culture plates, washed and pelleted by centrifugation. 2×10^6 DCs were then plated in 6-well tissue culture plates and matured by overnight stimulation with 100ng/ml LPS. For experiments with CD4⁺ T cells, DCs were also pulsed with 100 μ g/mL ovalbumin (OVA).

2.2.1.5 Isolation of CD4⁺ T cells

CD4⁺ T cells were obtained from the spleens of OTII transgenic mice. Single cell suspensions were generated from spleens by mashing and passing whole cell material through a 70 μ m cell strainer using a plunger. Cells were pelleted by centrifugation at 1400rpm and resuspended in 5ml Red Cell Lysis Buffer for 5 minutes at 37°C. Cells were then washed with culture medium. CD4⁺ T cells were subsequently isolated by negative selection using a CD4⁺ T Cell Isolation Kit and magnetic bead separation according to manufacturer's instructions (Miltenyi Biotech).

2.2.1.6 Isolation of NK cells

NK cells were obtained from the spleens of C57BL/6 mice. Spleens were treated as described in 2.2.1.5. NK cells were subsequently isolated by positive selection using CD49b (DX5) MicroBeads according to manufacturer's instructions (Miltenyi Biotech).

2.2.2 Molecular Cloning

2.2.2.1 Polymerase Chain Reaction (PCR)

The PCR reaction was performed to create a γ c^{mut}-GFP protein lacking 77 amino acids at the carboxy terminus of the γ c. Primers for the PCR reaction were designed so as to introduce an AgeI site at amino acid 289 of γ c^{WT} cDNA and were ordered from MWG Eurofins. The following primers were designed:

Forward primer: GAAGACACCGACTCTAGAGCCACCATGTTG

Reverse primer: CAACCGGTGGGCATCGTCCGTTCCAG

Each primer was made to 100pmol/ μ l in distilled H₂O. 100ng of DNA template were incubated with 5 μ l 10 \times reaction buffer, 100pmol forward primer, 100pmol reverse primer, 1 μ l dNTP mix (all from Promega) to a final volume of 50 μ l. 1 μ l of Pfa DNA Polymerase (Promega) was added to the sample and the reaction was then cycled at the following conditions:

Cycle 1	95°C	2 minutes	initial denaturation
Cycle 2-30	95°C	45 seconds	denaturation
	58°C	45 seconds	annealing
	72°C	1 minute	extension
Cycle 31	72°C	5 minutes	final extension

The PCR product was then purified using the QIAGEN PCR Purification Kit and finally resuspended in 35 μ l of distilled H₂O.

2.2.2.2 Plasmid DNA Digestion

The PCR product and 2 μ g of γ c^{WT}-GFP cDNA were both digested for 2 hours with 2 μ l of Age1 and 1 μ l of Xba1 enzymes. Digests were performed in a final volume of 30-50 μ l in the buffers and at the temperature recommended by the manufacturers (Promega and New England Biolabs).

2.2.2.3 Agarose Gel Electrophoresis

DNA fragments were resolved by electrophoresis. 2 μ l of DNA samples were resuspended in loading buffer and loaded in 1-2% agarose gels. A 1 Kb plus ladder was loaded alongside the samples. Gels were stained with 1 μ l Ethidium Bromide and run in 1 \times TAE buffer using a voltage of 50-100V (up to 150 mA) for 1-2 hours depending on the size of the fragment to be resolved. The separated DNA fragments were visualised by exposure to UV light using an UVIdoc gel documentation system.

2.2.2.4 Gel Purification of DNA

Following electrophoresis, DNA fragments were excised from agarose gels and purified using the Gel Extraction Kit according to manufacturer's instructions

(QIAGEN). DNA concentration was measured by reading the absorbance at OD 260nm using a Genequant Calcular (Pharmacia).

2.2.2.5 Ligation

Ligation reactions were performed using 1:5 vector to insert ratio (100ng vector DNA) in a final volume of 12 μ l containing 10 \times T4 ligase buffer and 1 μ l T4 DNA ligase (Promega). Reactions were incubated for 4 hours at room temperature.

2.2.2.6 Transformation of Competent Bacteria

5 μ l of the ligated DNA were incubated on ice with 100 μ l Stbl3 competent *E.Coli* (Invitrogen) for 30 minutes. Bacteria were heat-shocked at 42°C for 45 seconds then held on ice for 1 minute. 1ml of LB was added to the bacteria and incubated at 37°C for 1 hour at 225rpm on a shaking incubator. 25-100 μ l from each transformation were then spread on pre-warmed Agar ampicillin plates and incubated overnight at 37°C.

2.2.2.7 Plasmid DNA Purification

Selected colonies were incubated overnight in 5ml LB containing ampicillin (50 μ g/ml) in a shaking incubator at 37°C. Purification of plasmid DNA was performed using the Mini-prep kit according to manufacturers' instructions (QIAGEN). The DNA was digested to confirm the presence of the correct plasmid DNA construct within the bacteria.

2.2.2.8 Large-scale Plasmid DNA Production

500ml LB-ampicillin were inoculated with 500 μ l of a fresh bacterial culture and incubated overnight in a shaking incubator at 37°C. For lentiviral production, plasmid DNA was prepared using Maxi-prep kit according to manufacturers' instructions (QIAGEN). For transfections, DNA was prepared using the Endo-Free Plasmid Maxi Kit according to manufacturers' instructions (QIAGEN).

2.2.3 Lentiviral Preparation, Transductions and Transfections

2.2.3.1 Lentiviral Production

Recombinant lentiviruses were produced by transient transfection of subconfluent 293T cells. 30µg of the packaging plasmid pCMV-dR8.7, 10µg of the envelope plasmid pMDG2 (VSV-G) and 50 µg of one the transfer vectors encoding LifeAct-Ruby, γ^{WT} -GFP or γ^{Mut} -GFP DNA were added to 5ml OPTIMEM and filtered through a 0.22 µm filter then combined with 5ml filtered OPTIMEM supplemented with 1µl polyethylenimine (10mM). The transfection reaction was incubated at room temperature for 20 minutes, during which time 293T cells were washed with OPTIMEM. 10 ml of the transfection mixture were then added to each flask and cells were incubated for 4 hours at 37°C with 5% CO₂, after which time the media was replaced with fresh complete DMEM. After 48 and 72 hours, the viral supernatant was harvested, filtered through a 0.22 µm filter then concentrated by ultracentrifugation at 100,000g for 2 hours using a Beckman Centrifuge. Lentiviral pellets were resuspended in OPTIMEM and stored at -80°C.

2.2.3.2 Titration of Lentiviral Supernatants

Viral titre was determined by infection of 293T cells. 5 x 10⁵ cells (in 200µl) were seeded in 24-well plates and left to adhere overnight. 100µl of medium containing serially diluted lentiviral supernatant were then added to the cells. After 72 hours, the cells were harvested and analysed by flow cytometry to determine the percentage of transgene positive cells. The titre was calculated by dividing the number of infected cells by the amount of virus used (ml) and was expressed as infectious units/ml.

2.2.3.3 Lentiviral Transduction of Cell Lines

Lentiviral infection was achieved by seeding cells at a density of 0.3 x 10⁶ cells/ml in complete medium. Concentrated lentiviral supernatant was directly added to the cells in culture at the required multiplicity of infection (MOI).

2.2.3.4 Lentiviral Transduction of DCs

DCs were grown as described in section 2.2.1.3. On day 6, cells were scraped off and resuspended at 1.5× 10⁵ cells/ml. Lentiviral infection was performed by adding viral supernatant (MOI 25) and protamine sulphate (8ug/ml) to 1.5× 10⁶ cells in a 15ml

Falcon tube. Cells were spun with virus at 1200g for 2 hrs at 25°C then plated in conditioned medium in a 6-well plate. Experiments with transduced cells were performed 48 hours after infection.

2.2.3.5 Lentiviral Transfection of DCs

DCs were grown as described in section 2.2.1.3. On day 6, cells were scraped off and resuspended at 2.5×10^5 cells/ml. Lentiviral transfection was achieved by nucleoporating 2.5×10^5 DCs per transfection with 5ug lentiviral DNA or 2ug pmaxGFP control vector using the Amaxa Mouse Dendritic Cell Nucleofector Kit (Lonza), according to manufacturers instructions. Cells were then seeded in 48-well plates and cultured at 37°C with 5% CO₂. Experiments with transfected cells were performed 24 hours after nucleoporation.

2.2.4 Protein Isolation, Immunoblotting and Immunoprecipitation

2.2.4.1 Western Blotting (WB)

1×10^6 cells were lysed for 15 minutes on ice in 20µl lysis buffer and cleared by centrifugation in a table-top centrifuge (13 000 rpm for 10 min). 5µl of loading buffer were added to the cell lysates and the samples were heated for 5 minutes at 100°C. The proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 1 hour at 120V in running buffer and transferred on a PVDF membrane (Millipore) using a semi-dry transfer method for 30 min at 18V in transfer buffer. Membranes were blocked in blocking buffer for 1 hour at room temperature, then incubated in blocking buffer containing a 1:100 dilution of primary antibody (anti-IL-15Rα N-19 and anti-IL-2Rγ M-20). Membranes were then washed 5 times with wash buffer before incubation for 45 minutes in blocking buffer containing a 1:1000 dilution of secondary horseradish peroxidase-conjugated antibody. The blots were washed 5 times. Protein bands were visualised by incubating the membrane with enhanced chemiluminescence (ECL) solution for 1 minute and digitally captured using a gel documentation system (UVITEC) and software (UVISOFT). When necessary, membranes were stripped by washing with 0.2M glycine, 0.5M NaCl for 5 minutes before rinsing with 0.1M NaOH for 10 minutes. Membranes were then re-probed for GADPH (1:500 dilution) as a loading control.

2.2.4.2 Immunoprecipitation (IP)

Prior to immunoprecipitation, 50µl of Protein G Dynabeads (Invitrogen) were transferred to an appendorf, placed on a magnet and washed in PBS. 4µg of antibody (anti-IL-15R α H-107) diluted in 500µl PBS were then added to the beads and incubated with rotation for 1 hour at room temperature. The bead-antibody complex was then resuspended in 200µl lysis buffer and added to the cell lysates. Cell lysates were derived from 5×10^6 cells lysed for 15 minutes on ice in 500µl lysis buffer and cleared by centrifugation (13 000 rpm for 10 min). Samples were incubated with rotation at 4°C overnight then washed 5 times with PBS. Beads were finally resuspended in 30µl loading buffer, boiled for 5 minutes at 100°C and placed on a magnet. The supernatants were then separated by SDS-PAGE and immunoblotted with anti-I-A/I-E (M5/114.15.2) as described in 2.2.4.1.

2.2.5 Flow cytometry

Cells were analysed using a Cyan flow cytometer (Dako) and either Summit software (DakoCytomation) or FlowJo software.

2.2.5.1 Surface Staining of Primary Cells

Prior to staining, 2×10^5 cells were resuspended in 100µl PBS containing a 1:50 dilution of rat anti-mouse Fc Receptor Block (CD16/CD32) and incubated for 30 minutes on ice. Cells were then pelleted by centrifugation at 1200 rpm for 5 minutes, resuspended in 100µl FACS buffer containing a 1:25 (unconjugated), 1:25 (FITC-conjugated) or 1:50 (PE-, APC-, PE-Cy5- and PercP-conjugated) dilution of the appropriate antibody and incubated on ice for 30 minutes in the dark. For analysis of DC surface expression, cells were stained with the following antibodies: CD132, B220-FITC, CD86-FITC, IL-2R α -FITC, IL-15R α -FITC, IL2R β -FITC, CD11b-PE, MHC II-PE, IL-21R-PE, CD11c-APC and CD8 α -PercP. For analysis of T cell surface expression, cells were stained with the following antibodies: CD4-APC, CD69-PE-Cy5. For analysis of NK cell surface expression, cells were stained with the following antibodies: NK1.1-APC. Where an unconjugated antibody was used, cells were washed once with 1ml FACS buffer, pelleted by centrifugation and resuspended in 100µl FACS buffer containing a 1:50 dilution of secondary antibody for 30 minutes on ice in the dark. Following staining, cells were washed once with

1ml FACS buffer, pelleted by centrifugation, resuspended in 300µl FACS buffer and analysed by flow cytometry. If cells were not analysed immediately after staining, they were resuspended in 300µl 1% paraformaldehyde (PFA)/PBS and stored in the dark at 4°C.

2.2.5.2 Intracellular Staining of Primary Cells

Prior to staining, 2×10^5 cells were fixed in 100µl 2% PFA/PBS for 30 minutes at room temperature. Cells were then washed with PBS, pelleted by centrifugation and permeabilised in 100µl 0.1% saponin/PBS containing a 1:50 dilution of FcR block for 30 minutes on ice. Staining was then performed as described in 2.2.5.1. Antibodies used were IL-15R α -FITC.

2.2.5.3 Intracellular Cytokine Staining

2×10^5 DCs were seeded in 96-well plates and stimulated for 18 hours in 200µl complete RPMI containing 100ng/ml LPS alone or in combination with 100ng/ml murine IL-21 (R&D Systems). The following day, cells were treated for 4 hours with 10 µg/ml Brefeldin A. Cells were washed 3 times with PBS and subsequently stained for surface CD11c (1:50 dilution in PBS) for 30 minutes on ice. Cells were washed once in PBS then resuspended in 200µl Cytofix/Cytoperm solution (BD Pharmingen) for 30 minutes on ice. Cells were washed once with Perm/Wash solution (BD Pharmingen) then stained with biotinylated anti-IL-12 p40/p70 or isotype control (1:50 dilution in Perm/Wash) for 30 minutes on ice. Cells were washed once with Perm/Wash solution then stained with Streptavidin PE (BD Pharmingen) (1:100 dilution in Perm/Wash) for 30 minutes in the dark on ice.

2.2.5.4 Intracellular Phalloidin Staining

5×10^5 DCs were fixed in 100µl 4% PFA for 30 minutes at room temperature then washed in PBS. Cells were resuspended in 100µl Perm/Wash solution, incubated for 5 minutes at room temperature then washed. Subsequently cells were resuspended in 100µl Perm/Wash solution containing 2µL Rhodamine Phalloidin (Molecular Probes, Invitrogen) and incubated for 20 minutes on ice in the dark. Cells were washed and analysed.

2.2.5.5 Analysis of Endocytic Activity

2×10^5 DCs were incubated in 100 μ l complete RPMI with or without 50ng/ml LPS for 30 minutes at 37°C in polystyrene round-bottom tubes. Cells were washed in PBS then cultured with 2 mg/ml FITC dextran on ice for 30 min. Samples were incubated at 37°C for the indicated timepoints, then washed twice with PBS and analysed at the flow cytometer.

2.2.6 Analysis of Cytokine Release

2.2.6.1 Analysis of Cytokine Release by Luminex

DCs were CD11c selected using magnetic bead separation according to manufacturer's instructions (Miltenyi Biotech). 1×10^5 CD11c selected DCs were cultured overnight in 100 μ l RPMI 1% FCS with one of the following TLR ligands at the indicated final concentrations: 0.5 μ g/ml Pam3CSK4 (TLR1/2), 10^8 cells/ml HKLM (TLR2), 25 μ g/ml Poly I:C (TLR3), 100ng/ml LPS *E.Coli* (TLR4), 1 μ g/ml Flagellin (TLR5), 0.5 μ g/ml FSL-1 (TLR6/2), 1 μ g/ml ssRNA40 (TLR8), 5 μ M CpG (TLR9). Supernatants were assayed using the Luminex Beadlyte® system or on mouse IFN- α (PBL Biomedical Labs), TNF- α and IL-6 (eBioscience) ELISAs. Optimal TLR ligand concentrations for stimulation were pre-determined by assessing the release of the surrogate marker IL-12 by ELISA (eBioscience).

2.2.6.1 Enzyme-linked Immunosorbent Assay (ELISA)

ELISAs were performed according to manufacturers' instructions and absorbances were measured at 450 nm using a FLUOstar OPTIMA (BMG LABTECH).

2.2.7 Analysis of JAK/STAT Signaling

2.2.7.1 Detection of JAK/STAT Signaling by Immunofluorescence.

Prior to cytokine stimulation, 1×10^5 DCs were starved for 30 minutes on ice in RPMI medium then plated in RPMI 1% FCS on polylysine coated slides and left to adhere for 30 min. Cells were stimulated for 10 min with 20ng/ml murine IL-2 (Peprotech) or murine IL-21 (R&D Systems) at 37°C then fixed in ice-cold 1% PFA for 5 minutes. Cells were permeabilised with 0.5% Triton X100 for 5 min, washed and blocked with 2% normal mouse serum for 30 minutes. Cells were stained with rabbit

anti-pJAK3 (10 μ g/ml) or rabbit anti-pSTAT5 (C71E5) (1:100) for 1 hour, washed then stained with anti-rabbit Alexa Fluor 488 (10 μ g/ml) and rhodamine phalloidin for 45 min (1:100) (all from Molecular Probes, Invitrogen). Washes and antibody stains were performed in PBS. Coverslips were mounted on slides with DAPI-containing mounting medium (Vector Laboratories) and examined using a laser scanning confocal microscope (TCS SP2 Leica).

2.2.7.2 Detection of JAK/STAT Signaling by Flow Cytometry

For intracellular detection of pSTAT5 in DCs and ED7R cells, cells were starved for 60 minutes in RPMI medium. Subsequently, 2×10^5 cells were cultured for 10 minutes in 100 μ l medium with or without murine/human IL-2 (20ng/ml and 100ng/ml, respectively) at 37°C then fixed with FACS lyse/fix buffer (BD Pharmingen) for 10 minutes at 37°C. Cells were pelleted by centrifugation and permeabilised with cold Perm Buffer III (BD Pharmingen) for 30 min on ice. Cells were washed with FACS buffer then stained with anti-pSTAT5-PE (47) and analysed at the flow cytometer.

For intracellular detection of pSTAT5 in CD4+ T cells, cells were starved for 3 hours in RPMI medium. 1×10^5 T cells (in 100 μ l) were then mixed in polystyrene round-bottom tubes at a 1:1 ratio with DCs, which had been pre-treated for 30 minutes with 20 μ g/ml anti-IL-15R α (AF551) or with isotype matched control at 37°C. Cells were centrifuged for 2 minutes at 600 rpm to encourage conjugate formation, incubated for 10 minutes at 37°C then fixed with FACS lyse/fix buffer. For intracellular detection of pSTAT5, cells were treated as described above. Conjugates were also stained with anti-CD4-PE-Cy7 and anti-CD11c-APC (to discriminate between T cells and DCs) then analysed at the flow cytometer.

2.2.7.3 Detection of JAK/STAT Signaling by Western Blotting

1×10^6 DCs were cultured with or without 100ng/ml IL-21 and analysed by immunoblotting as previously described in in 2.2.4.1. Immunoblotting was performed with rabbit anti p-STAT5 (C71E5) (1:1000 dilution) and anti-rabbit IgG horseradish peroxidase. Membranes were stripped and reprobed with rabbit anti-STAT5 (1:1000 dilution).

2.2.8 Analysis of Uptake and Processing of OVA

2.2.8.1 Analysis of DQ-OVA Uptake and Processing

0.25×10^6 LPS-matured DCs were seeded in 48-well plates and incubated in 300 μ l complete RPMI medium (without Phenol Red) containing 5 μ g/ml DQ-OVA (Molecular Probes, Invitrogen). The fluorescence was then measured at the FLUOstar OPTIMA (BMG LABTECH) over a period of 16 hours.

2.2.8.2 Analysis of OVA Presentation

1×10^5 DCs were incubated for 2 hours at 37°C in 100 μ l complete RPMI medium containing OVA₃₂₃₋₃₃₉ peptide (AnaSpec) (0.1 μ M, 1 μ M or 10 μ M final concentration). B017.4 T cells were then cultured with OVA-pulsed DCs at a 10:1 ratio for 18 hours. Supernatants were assayed for IL-2 using an anti-mouse IL-2 ELISA kit (R&D Systems).

2.2.9 DC: T cell *In Vitro* Experiments

2.2.9.1 Analysis of DC-mediated T cell priming

1×10^6 CD4⁺ T cells were resuspended in 1ml RPMI, labeled with 5 μ M CFSE (Molecular Probes, Invitrogen) for 20 min at 37°C then washed twice in complete media. Cells were then seeded in 96-well plates (1×10^5 cells/well in 100 μ l) and cultured at 1:1, 1:5 and 1:10 DC:T cell ratios with LPS-matured, OVA-pulsed DCs in a 37°C/5%CO₂ incubator. For IL-15R α blocking experiments, DCs were pre-treated for 30 minutes 37°C with anti-IL-15R α (AF551) or with isotype matched control at concentrations ranging from 1.25 μ g/ml to 20 μ g/ml, prior to coculture with T cells. After 24 hours, cocultures were washed, pelleted by centrifugation, stained with anti-CD4 and anti-CD69 and analysed at the flow cytometer. Alternatively, after 72 hours, cocultures were washed and pelleted by centrifugation; this time, the cell pellets were stained with anti-CD4 and CFSE dilution was measured at the flow cytometer whilst the supernatants were assayed for IL-2 production using a mouse IL-2 ELISA kit.

2.2.9.2 Analysis of DC-T cell Adhesion by Flow Cytometry

2×10^6 DCs (LPS-matured, pulsed with or without OVA) were resuspended in 5ml RPMI and labeled with $0.2 \mu\text{M}$ CMFDA (Molecular Probes, Invitrogen). 5×10^6 CD4⁺ T cells were resuspended in 5ml RPMI and labeled in $1 \mu\text{g/ml}$ DDAO (Molecular Probes, Invitrogen). Both cell types were cultured for 30 min at 37°C , then washed and resuspended in complete RPMI for 30 min at 37°C . 1×10^6 DCs were blocked with Fc Receptor block (1:50) for 30 min on ice then washed and resuspended in 1ml complete RPMI containing $20 \mu\text{g/ml}$ anti-ICAM-1 or isotype control Ab for 30 min at 37°C . CD4⁺ T cells were also resuspended in 1ml complete RPMI then mixed 1:1 with DCs in polystyrene round-bottom tubes and centrifuged for 10 min at 600 rpm to encourage conjugate formation. Cells were incubated for 1 hour at 37°C then analysed at the flow cytometer.

2.2.9.3 Analysis of DC-T Cell Conjugates by Confocal Microscopy

0.5×10^6 CD4⁺ T cells and 0.5×10^6 DCs (LPS-matured, pulsed with or without OVA) were mixed 1:1 in polystyrene round-bottom tubes (1ml total volume) and were centrifuged for 10 min at 600 rpm to encourage conjugate formation. After incubation at 37°C for 1 hour, cells were fixed with 2% PFA and allowed to adhere briefly on polylysine-coated coverslips. Cells were permeabilised with 0.1% saponin, quenched with 50mM glycine and blocked with blocking buffer (10% FCS, 2% Fc block in PBS). Cells were stained with anti-TCR ζ Alexa 647 (1:50) and rat anti-LFA-1 ($15 \mu\text{g/ml}$) for 1 hour, washed then stained with anti rat Alexa 488 ($10 \mu\text{g/ml}$) (Molecular Probes) for 45 minutes. Washes were performed in wash buffer (2% FCS in PBS) and antibody stains in staining buffer (0.01% saponin in wash buffer). After staining, coverslips were mounted on glass slides with ProLong Gold Antifade mounting medium (Invitrogen) and examined using Zeiss LSM 510 confocal microscope (63x, 1.4 NA, oil immersion objective).

2.2.9.4 Analysis of Contact Stability and T Cell Ca^{2+} Signaling by Live Cell Imaging

1×10^5 DCs (LPS-matured, pulsed with or without OVA) were resuspended in 0.5ml complete RPMI, plated in 35mm FluoroDishes (World Precision Instruments) and

left to adhere for 30 minutes at 37°C. In the mean time, 1×10^6 CD4+ T cells were resuspended in 1ml RPMI, labeled with $2 \mu\text{M}$ Fluo4-AM (Molecular Probes, Invitrogen) for 20 min at 37°C, then washed twice in complete media. 5×10^5 T cells were resuspended in 0.5ml complete RPMI and co-cultured with the adhered DCs at 37°C/5%CO₂ in the environmental chamber of a Zeiss LSM 710 confocal microscope (25x, 0.8 NA, oil immersion objective). Cellular interactions and Ca²⁺ fluxes were monitored simultaneously by differential interference contrast (DIC) and fluorescence over 30 minutes at 10-second intervals using Zeiss software.

2.2.10 DC: NK cell *In Vitro* Experiments

2.2.10.1 Analysis of DC-mediated NK cell Survival and Activation

DCs were matured overnight with 1μg/ml LPS. The following day, cells were washed, seeded in 96-well plates (2×10^5 cells/well in 100ul) and cultured at a 2:1 ratio with DX-5 selected NK cells at 37°C. After 24 hours, DC:NK cocultures were washed and pelleted by centrifugation. Cell pellets were stained with anti-NK1.1 and analysed for cell viability using the Annexin V-PE Apoptosis Detection Kit according to manufacturer's instructions (BD Pharmingen). The supernatants were instead assayed for IFN-γ using a mouse IFN-γ ELISA kit (Invitrogen).

2.2.10.2 Analysis of DC-mediated NK cell Proliferation.

0.5×10^6 NK cells were resuspended in 1ml RPMI, labeled with $2.5 \mu\text{M}$ CFSE for 20 min at 37°C then washed twice in complete media. Cells were seeded in 96-well plates (1×10^5 cells/well in 100μl) and cultured at 1:1 and 1:5 ratios with LPS-matured DCs for 48 hours at 37°C. Cell pellets were then stained with anti-NK1.1 and CFSE dilution was measured at the flow cytometer.

2.2.11 Lipid Bilayer Experiments

Experiments involving the lipid bilayer/TIRFM platform are part of a collaboration with Prof. Michael Dustin at the Skirball Institute of Biomolecular Medicine, New York University. For experiments with CD4+ T cells, the lipid composition of the bilayers was the following: 0.4mM DOPC, 12.5 mol% Ni-NTA (all from Avanti Polar Lipids). I-Ab/OVA, IL-15/ IL15Rα and ICAM-1 are His6-tagged and were

linked to the bilayer via Ni-NTA. For experiments with DCs, the lipid composition of the bilayers was the following: 0.4mM DOPC, 0.05 mol% Cap-bio, 12.5 mol% Ni-NTA (all from Avanti Polar Lipids). The α MHCII Fab-bt1 fragment was attached to the bilayer using streptavidin as a bridge. The LFA-1-His6-domain was attached to the bilayer via Ni-NTA. Preparation of lipid bilayers and image acquisition (Nikon Ti TIRFM, x100 Apo TIRF objective, NA 1.49) are the work of Dr. Kaushik Choudhuri. For these experiments, I have performed cell culture, transfection, cell preparation, immunofluorescence staining and data analysis, as detailed below.

2.2.11.1 Experiments with CD4+ T cells

CD4+ T cells were incubated at 37°C on glass-supported planar bilayers containing 500 molec/ μm^2 ICAM-1, 300 molec/ μm^2 CD80, molec/ μm^2 I-A^b pOVA and 2 $\mu\text{g}/\text{ml}$ IL-15/ IL15R α complex (eBioscience) in the presence of fluorescent anti-TCR Fab. After 30 minutes, cells interacting with lipid bilayers were fixed by injecting 2% PFA into the flow chamber. Cells were then permeabilised with 0.1% saponin/PBS for 5 minutes, quenched with 50mM glycine for 30 minutes and blocked with 5% BSA/PBS for 1 hour. Washes were performed in 2% BSA in PBS and stains in 2% BSA, 0.1% saponin/PBS. Primary antibodies were incubated for 1 hour and were rabbit anti-phospho-STAT5 (D47E7), anti-phospho-PLC γ 1 and anti-phospho-AKT (all at 1:50 dilution). Secondary antibodies were incubated for 45 minutes and were anti-rabbit AlexaFluor488 (10 $\mu\text{g}/\text{ml}$) (Molecular Probes, Invitrogen).

2.2.11.2 Experiments with DCs

LPS-matured, OVA-pulsed DCs were incubated at 37°C on glass-supported planar bilayers containing LFA-1 and Alexa-568-I-A^b Fab. Cells interacting with lipid bilayers were fixed at the indicated timepoints by injecting 2% PFA into the flow chamber. Cells were then permeabilised and stained as described in section 2.2.11.1. Primary antibodies were rabbit anti-IL-15R α (H-107) (10 $\mu\text{g}/\text{ml}$). Secondary antibodies were anti-rabbit AlexaFluor488 (10 $\mu\text{g}/\text{ml}$) (Molecular Probes, Invitrogen). Cells were also stained with Alexa 488-phalloidin (1:50) (Molecular Probes, Invitrogen).

2.2.12 Image Processing and Data Analysis

For live cell imaging, images were analysed using *Volocity Version 5.3.0* software. Only cells that could be observed for at least 30 seconds after the formation of a tight T cell-DC contact were analysed. Contact times are presented either as cumulative or individual contact times of T cells interacting with DCs. The total Fluo-4 intensity per T cell was quantified as F/F_0 , where F is the integrated fluorescence intensity during contact with a DC and F_0 corresponds to the mean integrated fluorescence intensity of non contacting T cells. Ca^{2+} oscillations are presented as the variance of Fluo-4 intensities of contacting T cells. For presentation, images were corrected using the “gamma” option of *ImageJ* software.

For analysis of cell-conjugates, confocal images were first background subtracted. The enrichment of molecules at the interface was then determined using the “ROI” option of the *Metamorph v6.1* software and is expressed as ratio of fluorescence intensity at contact interface with DC versus non contact regions of the plasma membrane. For *en face* synapse reconstructions, z-stacks of appropriately oriented cell conjugates were collected at 150nm steps to a distance of $\sim 15\mu m$. Z-stacks were deconvolved using the Iterative Deconvolve 3D algorithm in *ImageJ* to produce XZ reconstructions of the contact interface.

For analysis of TIRFM images, images were first background subtracted. T cell signaling was measured by identifying regions of cell contact using a threshold on TCR intensity. Total (integrated) and average fluorescence of pSTAT5, pAKT and pPLC γ 1 was measured within these regions. For analysis of MHC II accumulation at DC interfaces, total and average fluorescence, representing accumulation of bound MHC II at the contact interface, was measured for each DC in a 60-minute timeseries acquired at 4 frames/minute. A similar method was used for GFP accumulation. Colocalisation in fixed and live cells was measured using Pearson’s correlation coefficient (Rr). This was calculated by intensity correlation analysis using the “colocalization” option in *ImageJ*. Image analysis was performed using a combination of *Metamorph v6.1* and *ImageJ*.

2.2.13 Statistical Analysis

Prism Version 5 (GraphPad Software) was used for statistical analysis. Two-tailed student's t-test with 95% confidence bounds was applied when comparing the means of two normally-distributed samples; linear regression analysis was used to analyse the relationship between two variables x and y; one-way analysis of variance (ANOVA) (Bonferroni's multiple comparison test) was performed to test for significant differences amongst two or more independent groups. Mean values are shown and error bars represent s.e.m. (standard error of the mean). P-values greater than 0.05 are indicated as n.s. (nonsignificant).

*Chapter Three- Analysis of the Role of the Common Cytokine
Receptor γ c in Dendritic Cell Biology and Functions*

3.0 Aims

- Assess γ c expression and signaling in DCs.
- Assess the importance of the γ c for DC functions.

3.1 Introduction

Mutations in the γ c cause X-SCID. X-SCID patients lack T and NK cells thereby indicating that γ c-family receptors play a critical role in the development of these cells. A clinically indistinguishable phenotype is seen with loss of function mutations in the γ c signaling mediator JAK3^{48,215}, highlighting the importance of γ c-related signaling for the homeostasis of these lymphocyte subsets.

DCs are still present in X-SCID patients. Although little is known about DCs in this disease, in the literature there is an increasing body of evidence suggesting that γ c expression and signaling are important for DC functions. Murine DCs express various γ c-family receptors such as IL-2R, IL-4R, IL-15R and IL-21R^{37,216-220} and the related cytokines have been reported to play a role in murine DC biology. IL-2, for example, augments IFN- γ production by DCs²¹⁷ and enhances antigen uptake via IL-2R mediated endocytosis²¹⁸. IL-4 is instead a survival factor and in combination with GM-CSF skews monocyte differentiation into DCs, thus pointing to a role for γ c signaling in DC development²²¹. Unlike IL-4, IL-21 is not required for DC differentiation but can alter DC functions by keeping DCs in an immature, low immunogenic state²²⁰. When stimulated with IL-21, DCs in fact develop a phenotype characterised by reduced MHC II and co-stimulatory molecule expression, high antigen uptake and reduced release of pro-inflammatory cytokines. Similarly, IL-7 also downregulates MHC II expression and thereby maintains DC in an immature state²²². In contrast, IL-15 has an activating effect on DC functions. Notably, stimulation of DCs with IL-15 results in the increased survival of mature DCs, in the upregulation of co-stimulatory molecules and increased antigen presentation. IL-15 also acts during DC development and been reported to skew human monocyte differentiation into DCs with LC phenotype *in vitro*²²³ and into mature DCs *ex vivo*²²⁴.

Taken together, these reports provided strong evidence of a critical role of γ c-family cytokines in regulating DC physiology and pointed to the possibility that DC function might be impaired in X-SCID. To address this hypothesis, the functional importance of the γ c and of its associated receptors was assessed in murine bone-marrow derived DCs (BM-DCs).

3.2 γ c-family Cytokine Receptor Expression and Signaling in Dendritic Cells

3.2.1 DCs Express the γ c and Signal through the JAK-STAT Pathway

Whereas murine BM-DCs have previously been reported to express γ c RNA²¹⁹, protein expression of the γ c has not been examined. To confirm γ c protein expression, DCs were generated from the BM of WT C57BL/6 mice cultured over 7 days with GM-CSF then stained with an antibody against γ c. γ c protein expression was confirmed by flow cytometry in CD11c+ DCs (Figure 3.1).

The γ c is a component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21⁴. All of these cytokines signal through the JAK/STAT pathway. Cytokine binding results in JAK1/JAK3 tyrosine phosphorylation and in downstream activation and phosphorylation of STAT molecules which then localize to the nucleus and act as transcription factors¹⁹⁻²². To determine whether JAK/STAT signaling occurs downstream of γ c in DCs, cells were stimulated with IL-21 and stained with antibodies specific for phosphorylated forms of JAK3 (Y785) and STAT5 (Y694). By confocal microscopy we observed that IL-21 stimulation results in an increase in the phosphorylation of both JAK3 and STAT5 proteins compared to unstimulated controls, thereby indicating that γ c signaling through the JAK/STAT pathway occurs in DCs. As expected, phosphorylated JAK3 was mostly membrane associated whereas phosphorylated STAT5 predominantly accumulated in the nucleus (Figure 3.2A,B).

A

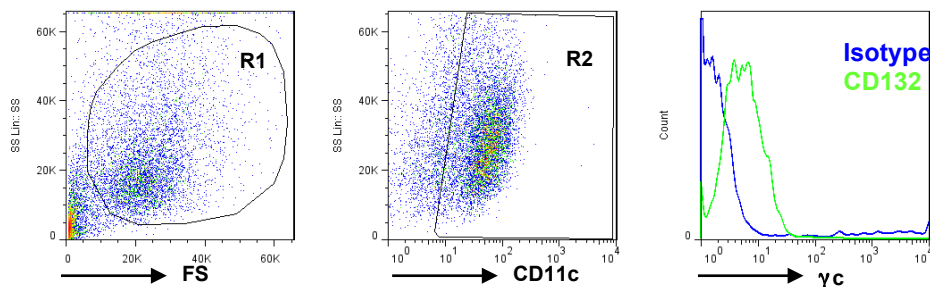
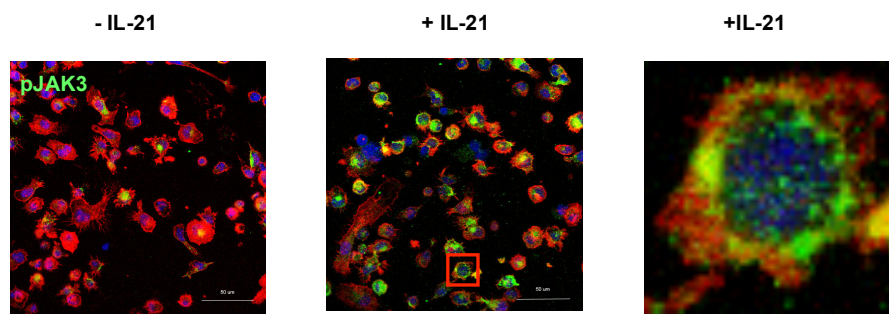


Figure 3.1 DCs express the γ c. DCs derived from the BM of WT C57BL/6 mice were stained with anti- γ c (CD132) or with an isotype control antibody. Right panel indicates surface expression of the γ c in DCs gated according to size (R1) and CD11c expression (R2) (left and middle panel, respectively).

A



B

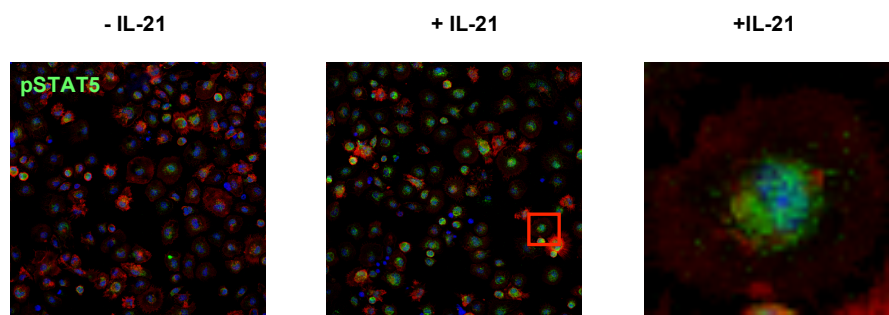


Figure 3.2. DCs express the γc and signal through the JAK/STAT pathway. DCs were cultured for 10 min with or without IL-21 (20ng/ml). Representative images showing expression of pJAK3 (green) (A) and pSTAT5 (green) (B) (left and middle panel) and localization of pJAK3 (A) and pSTAT5 (B) (right panel). The right panel is a high order magnification of cells shown in red box. Nuclei and F-actin were stained with DAPI and with rhodamine phalloidin, respectively.

3.2.2 Generation of DCs from Bone Marrow of γ c-deficient Mice (γ c^{-/-} DCs).

To assess the functional importance of γ c expression and signaling in DCs, BM-DCs were generated from a murine model of γ c-deficiency: the γ c/RAG-2^{-/-} mouse. These mice harbour a deletion spanning from exon 2-6 of γ c corresponding to the extracellular and transmembrane domains of the protein, which results in aberrant protein expression. These mice also lack lymphoid restricted recombinase activating gene 2 (RAG-2), to eliminate low levels of persisting T cells seen in γ c single knock out strains²²⁵, whereas RAG-2 deficiency is instead predicted not to influence myeloid lineages. By flow cytometry analysis, we observed that BM-DCs derived from these γ c-deficient mice (γ c^{-/-} DCs) expressed similar levels of CD11c compared to WT (Figure 3.3A). As expected, γ c^{-/-} DCs completely lacked γ c expression, as demonstrated by WB analysis (Figure 3.3B).

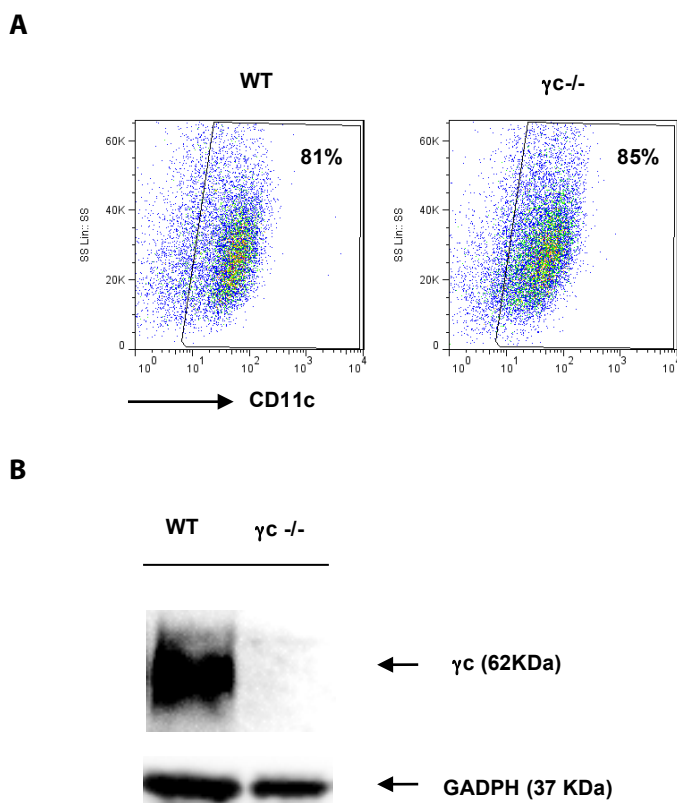
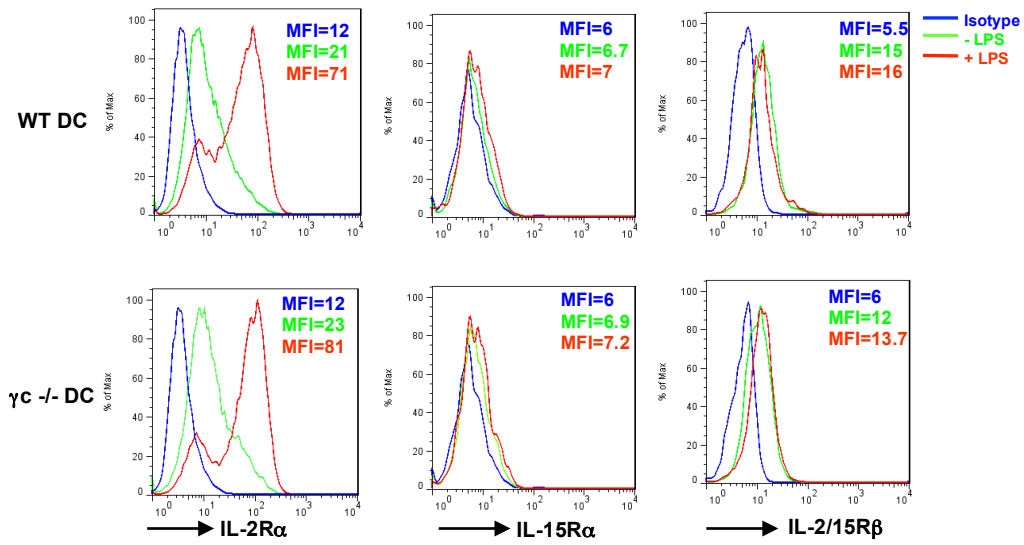


Figure 3.3 DCs from BM of γ c/RAG-2^{-/-} mice. DCs were generated from the BM of WT and γ c/RAG-2^{-/-} C57BL/6 mice cultured for 7 days with GM-CSF. (A) Surface expression of CD11c (B) Immunoblot analysis of γ c expression. Cell lysates were separated by SDS-page and immunoblotted with anti- γ c. The blot was stripped and re-probed with anti-GADPH (lower lane) to demonstrate protein loading in each lane.

3.2.3 γ c^{-/-} DCs Express Normal Levels of the other Subunits of the IL2/IL-15R.

The γ c was first discovered as a component of the receptor for IL-2⁷. The IL-2 receptor complex (IL-2R) is closely related to the IL-15 receptor complex (IL-15R) in that they share common β and γ chains². In addition each receptor has its own private α subunit which together with the IL-2/15R β and γ c heterodimer forms the complete high affinity IL-2 or IL-15R⁴. To verify whether the absence of the γ c might affect surface expression of the other components of the IL-2/15R complex, DCs were stained with antibodies against IL-2R α , IL-15R α or IL-2/15R β . Analysis at the flow cytometer confirmed that γ c^{-/-} DCs express these receptor chains at comparable levels to WT (Figure 3.4A). IL-2R α expression is known to be induced by a variety of stimuli, including antigens, pathogens and cytokines²⁶. In keeping with the literature, our data also shows that IL-2R α is upregulated upon LPS stimulation and that this occurs similarly in WT and γ c^{-/-} DCs (\approx 3-fold increase in mean fluorescence intensity) (Figure 3.4A,B). Expression levels of IL-15R α and IL-2/15R β were also comparable in LPS-treated WT and γ c^{-/-} DCs but in contrast to IL-2R α , these subunits failed to be upregulated upon stimulation. Taken together, these results indicate that γ c deficiency does not affect surface expression of the other IL-2 and IL-15R subunits on the surface of DCs.

A



B

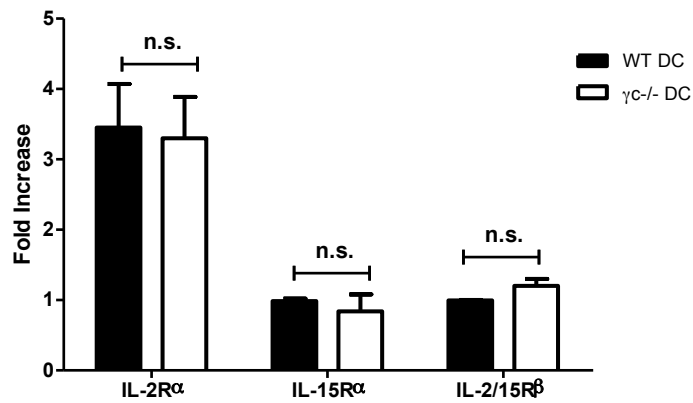


Figure 3.4 $\gamma c^{-/-}$ DCs express the other components of IL-2/IL-15R. DCs were cultured overnight with or without LPS (A) Representative plots showing surface expression of IL-2R α , IL-15R α and IL2/15R β in CD11c+ gated cells (MFI=mean fluorescence intensities) (B) Fold increase (MFI values) of the indicated receptors upon LPS stimulation relative to unstimulated cells. Data are from 3 independent experiments (mean \pm s.e.m). P-values, two-tailed students t-test.

3.2.4 γc $-/-$ DCs Fail to Transduce IL-2 Signals through the JAK-STAT Pathway

The IL-2R is the prototype for the family of γc -receptors and the best characterised. As a result of combining the various IL-2R subunits, several forms of receptor complexes with different cytokine affinities exist on the cell surface. Isolated IL-2R α , ie low-affinity receptor, is not be involved in signal transduction whereas the IL-2R $\beta/\gamma c$ heterodimer, ie intermediate-affinity receptor, is necessary and sufficient to transduce IL-2 signals¹⁹. We were therefore interested to confirm that the absence of DC γc would abrogate signaling downstream of the IL-2R, despite normal expression of the other receptor subunits. To this end, DCs were stimulated with IL-2 and analysed for STAT5 activation by flow cytometry and confocal microscopy. This showed that, in contrast to WT DCs, γc - $-$ DCs mice fail to signal through the γc in response to stimulation with IL-2, as demonstrated by absent pSTAT5 staining (Figure 3.5A,B).

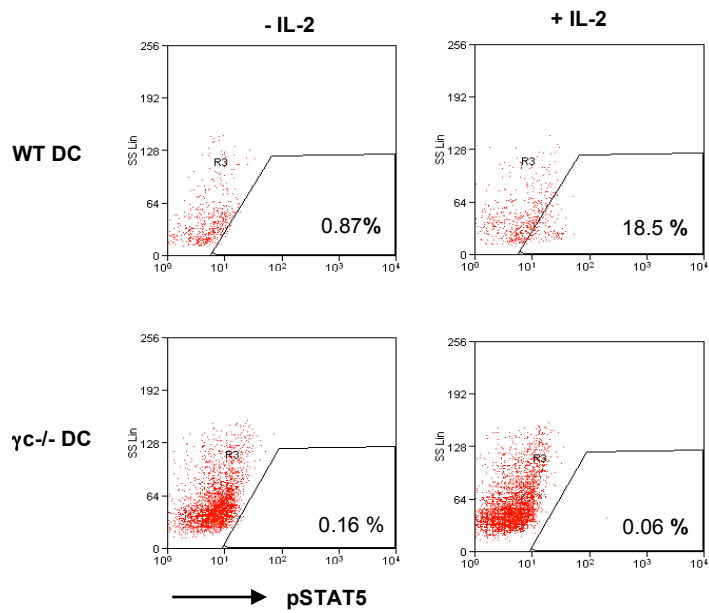
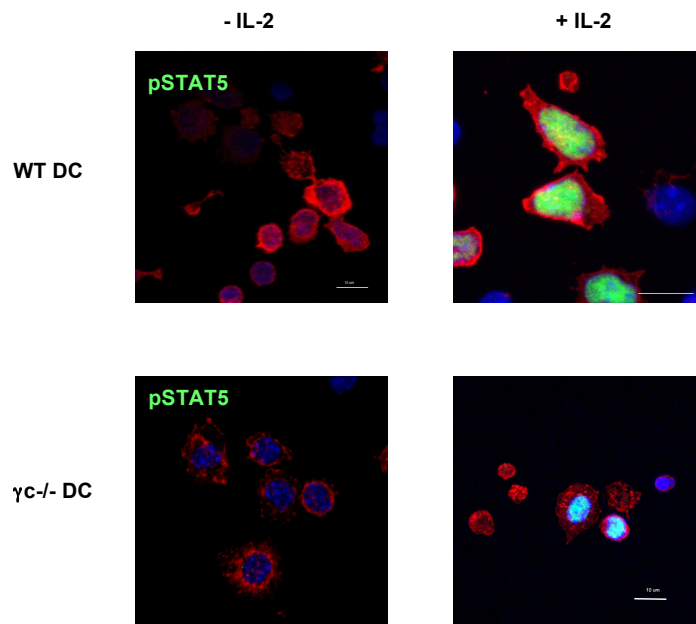
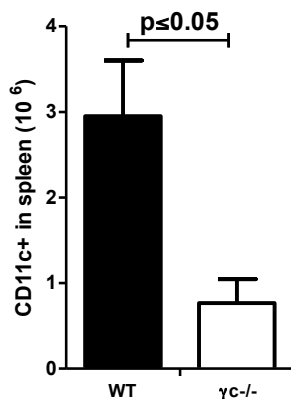
A**B**

Figure 3.5 γ C^{-/-} DCs fail to transduce IL-2 signals. DCs were cultured for 10 min with or without IL-2 (20 ng/ml). Cells were then fixed and stained with an anti pSTAT5. (A) Flow cytometry analysis of pSTAT5 expression on CD11c⁺ gated cells. R3 is set relative to unstained control (B) Confocal microscopy analysis of pSTAT5 expression (green). Nuclei and F-actin were stained with DAPI and with rhodamine phalloidin, respectively. Data presented is from 2 independent experiments.

3.2.5 γ c/RAG-2^{-/-} Mice Have Fewer Splenic DCs

In order to assess the influence of γ c receptors on DC development *in vivo*, DC numbers and subtype composition were analysed in γ c/RAG-2^{-/-} mice. This revealed that there are significantly fewer CD11c⁺ DCs in the spleens of these mice when compared to WT controls ($p \leq 0.05$); γ c/RAG-2^{-/-} mice typically had 30% of the DC numbers found in WT mice (Figure 3.6A). However, within the CD11c-enriched fraction, WT and γ c/RAG-2^{-/-} mice had similar frequencies of CD11c⁺ CD11b⁺ and CD11c⁺ CD8 α ⁺ conventional DCs. CD11c⁺ B220⁺ plasmacytoid DC were also present in comparable numbers in the spleens of WT and γ c/RAG-2^{-/-} mice (Figure 3.6B). Thus, these results suggest that, *in vivo*, whereas γ c is probably dispensable for the DC differentiation, it is instead required for DC development. Further investigation is required to establish whether this is due to a direct effect of γ c-family cytokines on DCs or to an indirect effect determined by the lack of T cells.

A



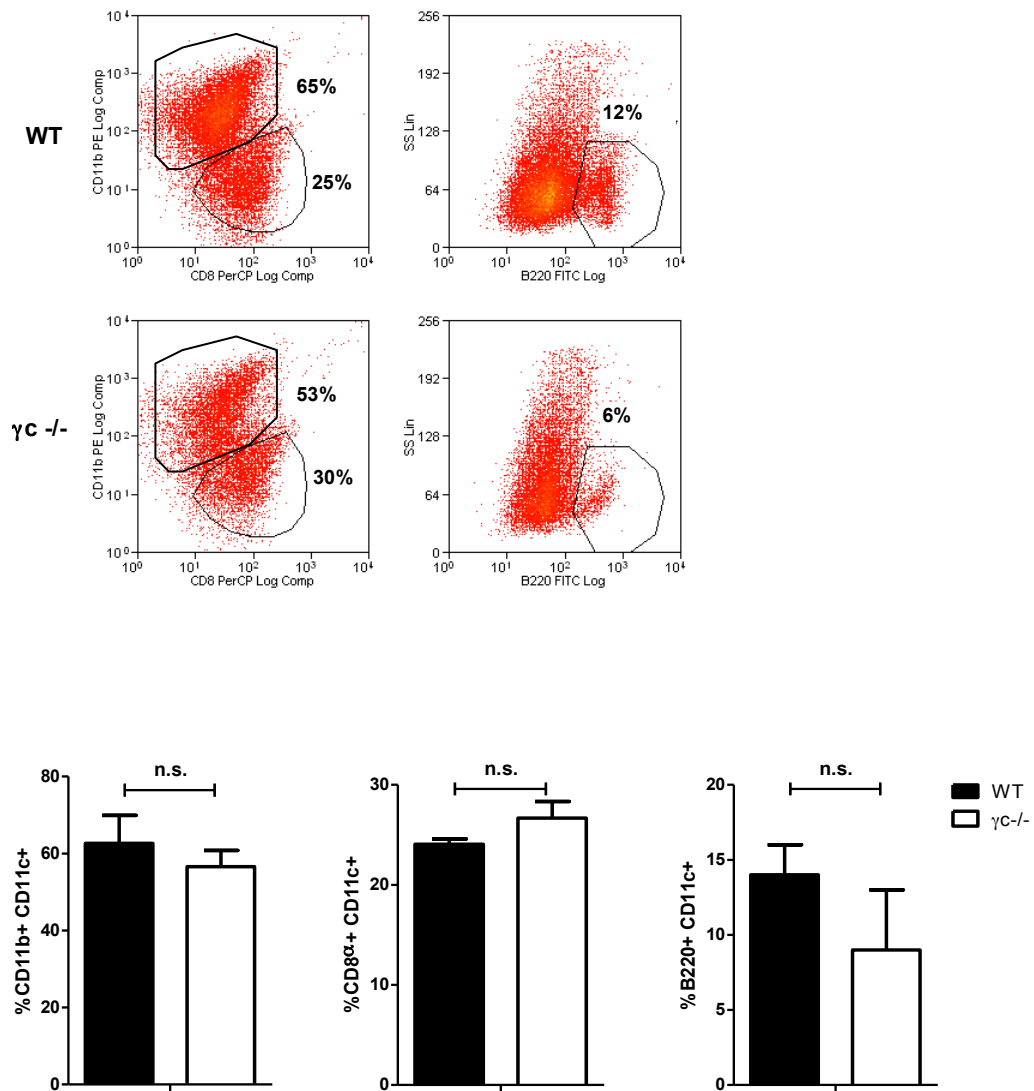
B

Figure 3.6 DC numbers and subtype composition in $\gamma c/RAG-2^{-/-}$ mice. Single cell suspensions were obtained from the spleens of WT and $\gamma c/RAG-2^{-/-}$ C57BL/6 mice and enriched in CD11c+ DCs by magnetic bead selection. (A) CD11c+ DC frequency per spleen (B) Density plot of DCs (gated on CD11c+ cells) stained with anti-CD11b, CD8 α and B220 to identify CD11c+ CD11b+ CD8 α -, CD11c+ CD8 α + CD11b- and CD11c+ B220+ cells. Mean percentage of CD11c+ CD11b+, CD11c+ CD8 α + and CD11c+ B220+ out of total CD11c+ cells in spleens of 3 WT and $\gamma c/RAG-2^{-/-}$. P values, two-tailed students t-test.

3.3 Absence of the γc in Dendritic Cells Does Not Affect Antigen Uptake or Maturation in Response to Activation Stimuli

3.3.1 $\gamma c^{-/-}$ DCs Exhibit Normal Endocytic Activity

Classically, immature DCs are antigen-capturing cells. To verify whether this function is affected by γc deficiency, we compared the endocytic activity of immature WT and $\gamma c^{-/-}$ DCs using a fluorescent antigen. Briefly, DCs were incubated with FITC-dextran for 10 minutes and uptake was measured at early timepoints at the flow-cytometer. Antigen uptake is expressed as percentage of maximum endocytosis (ie, fluorescence intensity at a given timepoint relative to maximal fluorescence throughout the experiment) and was shown to be comparable between WT and $\gamma c^{-/-}$ DCs over a period of 1 hour (Figure 3.7).

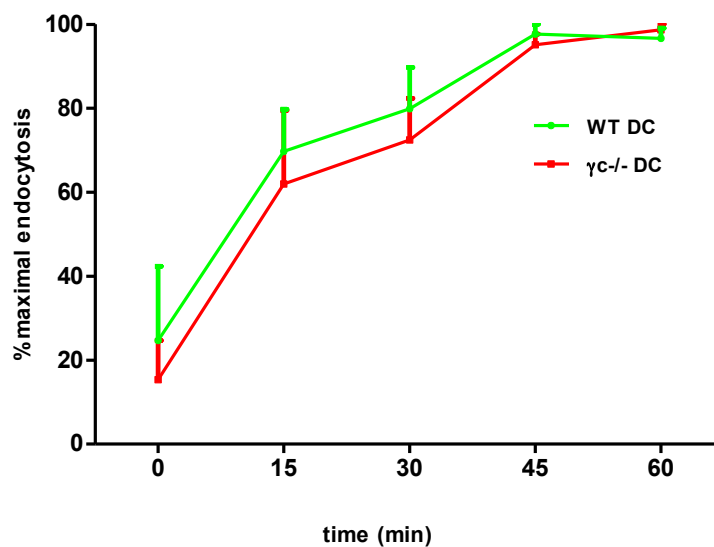


Figure 3.7 $\gamma c^{-/-}$ DCs exhibit normal endocytic activity. DCs were cultured with FITC dextran for 20 min on ice followed by incubation at 37 °C for the indicated timepoints. Cells were then washed and internalised FITC-dextran was measured by flow cytometry after gating on CD11c⁺ gated cells. % of maximal endocytosis is expressed in mean fluorescence intensity (MFI). Data is from 3 independent experiments (mean \pm s.e.m). P values, two-tailed students t-test.

Although an accepted hallmark of DC maturation is eventual downregulation of antigen uptake, transient enhancements of endocytic activity have been observed immediately after maturation, peaking 45 minutes after LPS stimulation²²⁷. We were therefore interested to test whether γ c deficiency might alter the acute response to bacterial stimulation in terms of antigen uptake. To this end, DCs were activated with LPS and analysed for endocytic activity of FITC-dextran. To our surprise, we failed to detect an increase in FITC-dextran uptake upon LPS stimulation, as has been previously reported (Figure 3.8). A similar uptake of FITC-dextran was however observed in LPS-stimulated γ c^{-/-} DCs compared to the LPS-stimulated WT counterpart (Figure 3.8). Taken together, these results suggest that γ c deficiency does not alter the endocytic activity of DCs, regardless of their activation status.

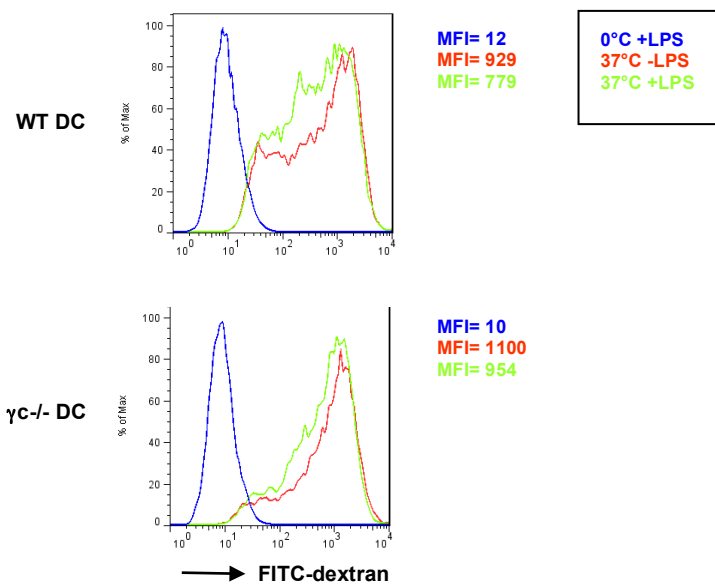


Figure 3.8 DC endocytic activity is not affected by LPS stimulation. DCs, with or without prior exposure to 50ng/ml LPS (for 10 min at 37 °C), were cultured with FITC dextran for 20 min on ice, followed by incubation on ice or at 37 °C for 45 min. Mean fluorescence intensity (MFI). One representative experiment of 3 is shown.

3.3.2 $\gamma c^{-/-}$ DCs Express Normal Levels of CD86 and MHCII Molecules

Upon encounter of bacterial products such as LPS, DCs acquire a mature phenotype and upregulate MHC II and co-stimulatory molecules such as CD80 and CD86 for presentation and costimulation to T cells⁷⁷. To assess whether γc signaling affects the maturation of DCs, cells were cultured with or without LPS and assessed for the expression of CD86 and MHCII. This showed that, at steady state, $\gamma c^{-/-}$ DCs express similar levels of CD86 and MHC II molecules to WT DCs (Figure 3.9A). Upon LPS stimulation, $\gamma c^{-/-}$ DCs were observed to upregulate both CD86 and MHC II (≈ 2 and 3-fold increase in mean fluorescence intensity, respectively) and this was also comparable to WT (Figure 3.9A,B).

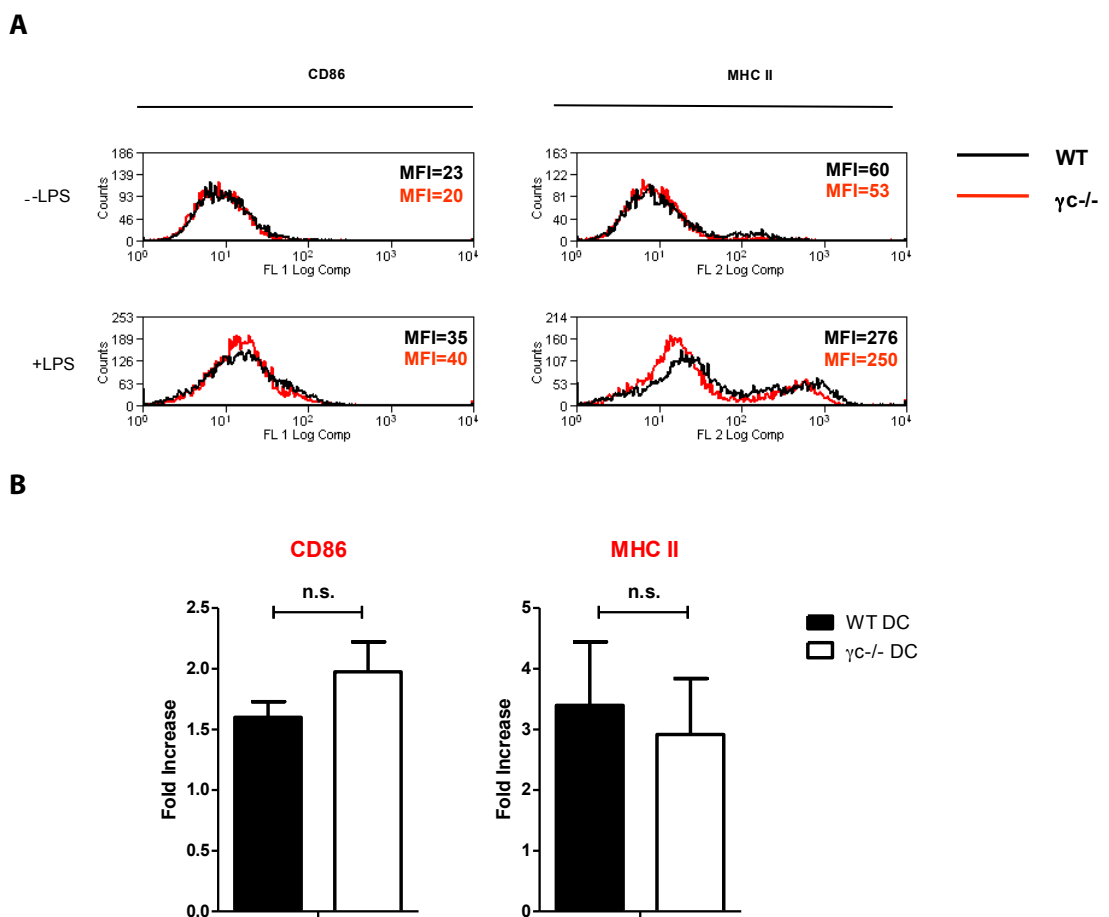


Figure 3.9 $\gamma c^{-/-}$ DCs express normal levels of CD86 and MHCII molecules. DCs were cultured overnight with or without LPS. (A) Representative plots showing surface expression of CD86 and MHCII molecules on CD11c⁺ gated cells (MFI=mean fluorescence intensities) (B) Fold increase (MFI values) upon LPS stimulation for CD86 and MHCII staining relative to unstimulated DCs. Data presented are from 3 independent experiments (mean \pm s.e.m). P-values, two-tailed students t-test.

3.3.3 $\gamma c^{-/-}$ DCs Display Normal Cytokine Secretion Profiles

A hallmark of DC activation by bacterial products is the release of proinflammatory cytokines. To assess whether this is affected by γc signaling, DCs were exposed to ligands for TLRs 1-9 and cytokine release profiles were tested using the Luminex cytokine bead array (Beadlyte®) system and by ELISA. Prior to ligand exposure cells were selected for CD11c expression through magnetic bead selection, thus ensuring high levels of DC purity (approximately 90%) (Figure 3.10). Optimal TLR ligand concentrations for stimulation were pre-determined by assessing the release of the surrogate marker IL-12 by WT DCs. Collectively, the data shown in 3.11, highlights normal levels of proinflammatory (IL-1 β , IL-2, IL-6, IL-12, IFN- α , TNF- α) and anti-inflammatory (IL-10) cytokine secretion by $\gamma c^{-/-}$ DCs upon stimulation with ligands to TLR2 (HKLM), TLR3 (Poly I:C), TLR4 (LPS), TLR6/2 (FSL-1) and TLR9 (CpG). Interestingly, as already reported for JAK3 $^{-/-}$ DCs²²⁸, $\gamma c^{-/-}$ DCs were observed to release significantly greater amounts of IL-12 upon LPS stimulation ($p \leq 0.05$). No cytokine release was detected, even in WT DCs, by stimulation with ligands for TLR1 (Pam3CSK4), TLR5 (Flagellin *S.typhimurium*) and TLR8 (ssRNA) and therefore these results are not shown.

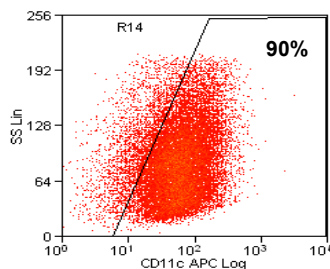


Figure 3.10 DC purity after CD11c selection. CD11c⁺ DCs isolated through magnetic bead selection from BM cultures were stained with anti-CD11c to confirm purity.

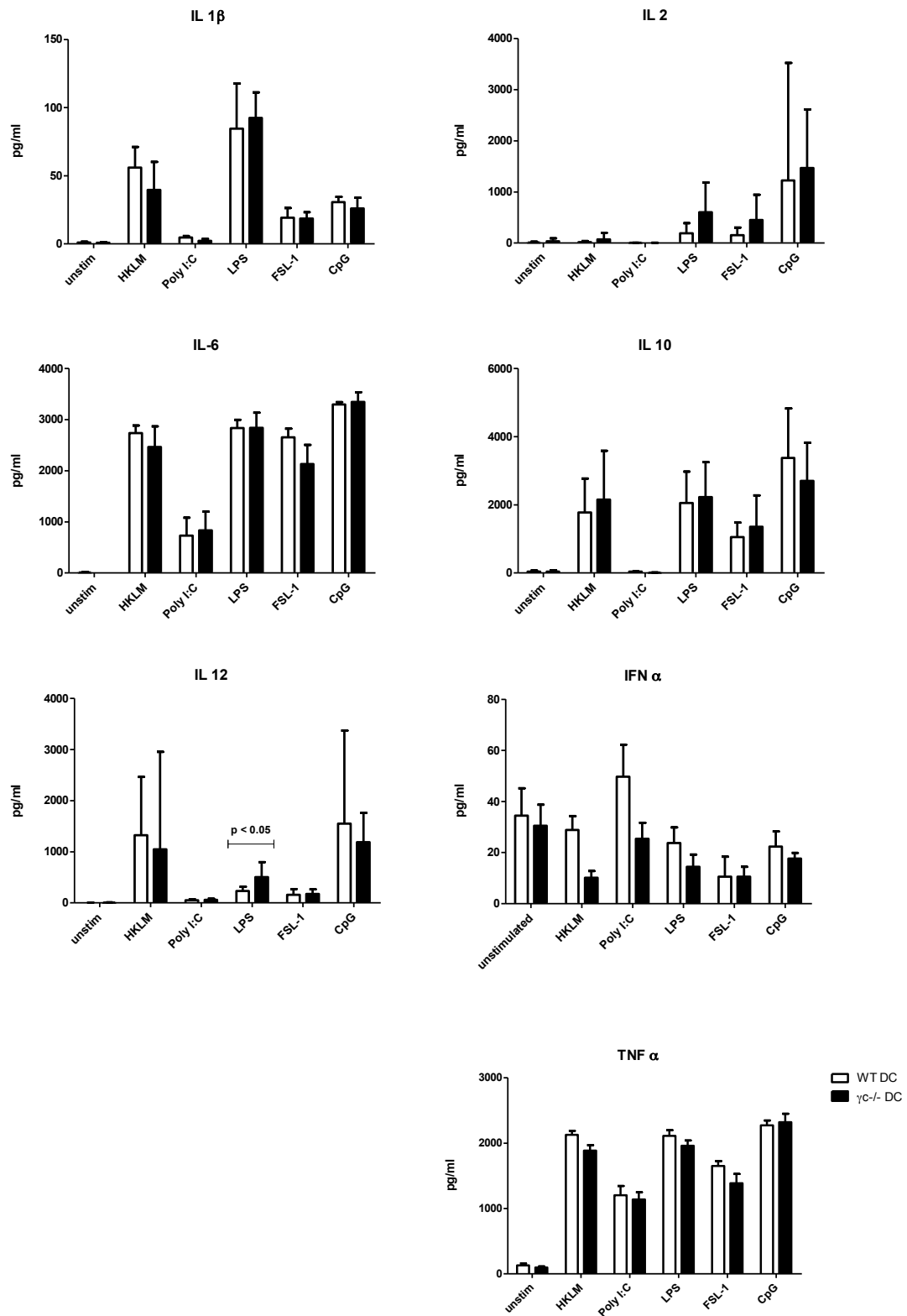


Figure 3.11 Cytokine secretion profile of DCs in response to stimulation with TLR ligands. CD11c selected DCs were cultured overnight with a panel of TLR ligands at optimal concentrations. The supernatants were assayed using the Beadlyte® system (IL-1 β , IL-2, IL-10, IL-12) and on ELISA (IL-6, IFN- α and TNF- α). Data are from 8 independent experiments (mean \pm s.e.m). P values, two-tailed students t-test.

3.4 Absence of the Common Cytokine Receptor γc Impairs Dendritic Cell Responses to IL-21-mediated Inhibitory Effects

3.4.1 $\gamma c^{-/-}$ DCs Express but Fail to Signal through the IL-21R

Like IL-2, IL-21 has its own private high-affinity receptor chain and shares the common γc cytokine receptor. To analyse whether γc deficiency affects surface IL-21R expression, DCs were stained with an antibody against IL-21R. This showed that $\gamma c^{-/-}$ DCs express IL-21R at comparable levels to WT (Figure 3.12A). Furthermore, we observed that surface IL-21R expression is not affected by LPS stimulation, both in WT and $\gamma c^{-/-}$ DCs (Figure 3.12B).

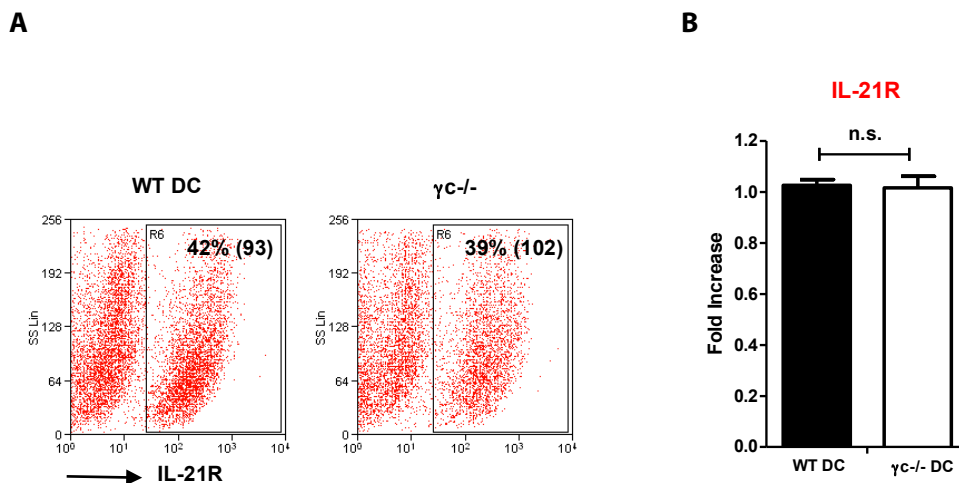


Figure 3.12 DCs express the IL-21R. DCs were cultured overnight with or without LPS.

(A) Representative plots showing surface expression of IL-21R on unstimulated DCs (gated on CD11c⁺ cells). The percentages of IL-21R-expressing DCs and MFI (in parenthesis) are shown

(B) Fold increase (MFI values) of the IL-21R upon LPS stimulation relative to unstimulated cells.

Data presented are from 3 independent experiments (mean \pm s.e.m). P-values, two-tailed students t-test.

Next, to confirm that γc deficiency abrogates signaling downstream of the IL-21R, DCs were stimulated for 10 minutes with IL-21, then analysed for STAT5 activation by WB analysis. This showed that $\gamma c^{-/-}$ DCs fail to phosphorylate STAT5 protein upon IL-21 stimulation, regardless of normal levels of total STAT5 (Figure 3.13).

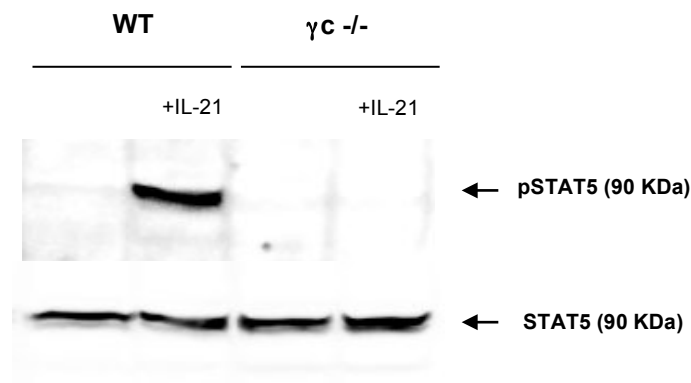
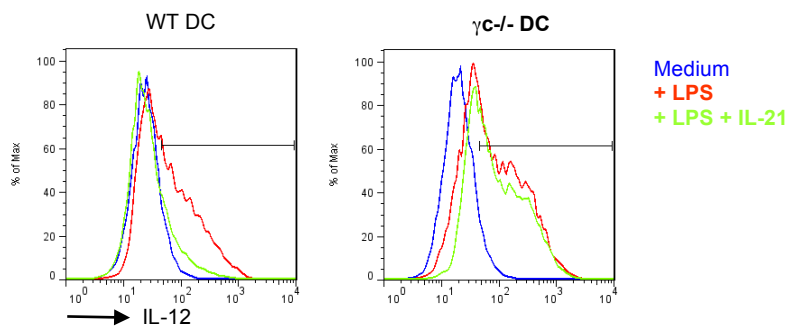


Figure 3.13 $\gamma c^{-/-}$ DCs fail to signal through the IL-21R. DCs were cultured for 10 min with or without IL-21 (100ng/ml) prior to lysis. DC lysates were then separated by SDS-page and immunoblotted with anti-pSTAT5. The blot was stripped and re-probed with anti-STAT5 (lower lane) to demonstrate levels of total STAT5 in each lane.

3.4.2 γc $-/-$ DCs Fail to Downregulate IL-12 Production in Response to IL-21 Stimulation

It is well known that IL-21 prevents functional aspects of LPS-induced DC maturation, including pro-inflammatory cytokine release²¹⁹. To test whether γc deficiency makes DCs unresponsive to IL-21 immunosuppressive effects, DCs were stimulated overnight with LPS alone or in combination with IL-21 and assessed for intracellular expression of the IL-12 inflammatory cytokine by flow cytometry. This showed that, unlike WT DCs, γc -/- DCs did not downregulate LPS-induced production of IL-12 upon IL-21 stimulation (Figure 3.14A,B). Taken together, these results suggest that IL-21 stimulation fails to inhibit the functional maturation of γc -deficient DCs, in terms of IL-12 cytokine secretion.

A



B

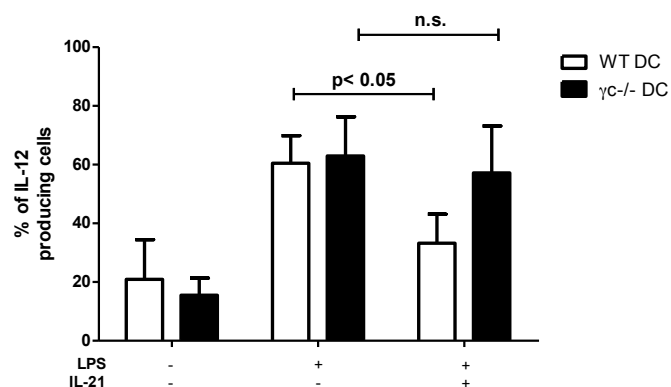


Figure 3.14 IL-21 fails to inhibit LPS-induced IL-12 production in γc -/- DCs. DCs were either left unstimulated or stimulated overnight with LPS alone or in combination with IL-21(100ng/ml). Prior to analysis, cells were treated with Brefeldin A for 4 hours, fixed and stained with anti-IL-12. (A) Representative plots showing intracellular expression of IL-12 in CD11c+ gated cells. Bars represent IL-12-expressing cells and are set according to isotype control (B) Bar graph indicating IL-12 expressing cells (A). Data are from 3 independent experiments (mean \pm s.e.m). P values, two-tailed students t-test.

3.5 Conclusions

In this chapter, the importance of γ c signaling for basic DC functions was assessed. Although, this has already been suggested by others through functional responses to γ c-containing cytokines^{37,216-220,222-224}, here we provided first direct evidence of γ c protein expression and downstream signalling in WT BM-DCs. In addition, to directly address the role of γ c in DC functions, our study relied on a murine model of γ c-deficiency (γ c/RAG-2^{-/-} mouse) and as expected, DCs generated from the BM of these mice (γ c^{-/-} DCs) lacked γ c expression and downstream signaling, despite expressing normal levels of other γ c-containing receptor subunits. Otherwise, γ c^{-/-} DCs were indistinguishable from their WT counterpart in terms of expression of the CD11c phenotypic marker, MHC II and the CD86 co-stimulatory molecule.

γ c signaling was dispensable for several specific functions of DCs examined, such as antigen uptake and maturation in response to activation stimuli. When stimulated by LPS (ligand for TLR4), γ c^{-/-} DCs underwent normal upregulation of maturation markers (CD86 and MHC II). Cytokine release, as measured by Luminex, was also normal in response to stimulation by LPS as well as by ligands for TLR2, 3, 6 and 9. We did not find reduced levels of IL-2 secretion in response to LPS as suggested by others²²⁹. We did however observe enhanced IL-12 release by LPS-stimulated γ c^{-/-} DCs, in agreement with similar findings in JAK3-deficient DCs²²⁸. This difference was detected by Luminex and not by intracellular cytokine staining, possibly because the latter is a less sensitive method for detection and quantification of cytokines. Notably, low levels of IFN- α secretion were observed for all ligands shown and this could be due to the fact that type I IFNs are mainly secreted by plasmacytoid DCs as opposed to more conventional type DCs, such as BM-DCs. Furthermore, negligible levels of cytokine release were detected upon stimulation with ligands for TLR1, 5 and 8. A possible explanation for this is that ligand concentrations were titrated according to the release of IL-12 and this may not have been an ideal readout for all TLR ligands as it is preferentially induced by stimulation through the TLR4 and TLR9 pathways. Regardless of these technical limitations, the results presented here suggest that altogether, TLR-mediated cytokine release is not generally affected by the γ c-JAK/STAT signaling pathway.

Although several general functions of DCs were unaffected by γ c-deficiency, specific functional responses to γ c-family cytokines were observed. This is in keeping with reports indicating that signaling in response to γ c-family cytokines is important for DC functions such as differentiation, survival and induction of both immunogenic and non-immunogenic phenotypes⁴. In this chapter, functional deficits were specifically demonstrated for the IL-21 immunosuppressive cytokine, which failed to inhibit LPS-induced IL-12 secretion in γ c^{-/-} DCs. In the light of these findings, it therefore seems plausible to hypothesise that *in vivo*, upon exposure to a rich inflammatory cytokine milieu, γ c-deficiency may affect many DC functions. Failure of γ c-family cytokine signaling also appeared to affect DC homeostasis, as demonstrated by reduced numbers of DCs in the spleens of γ c/RAG-2^{-/-} mice (70% reduction). This is consistent with a previous report in which RAG-2^{-/-} and γ c^{-/-} mice (both T cell deficient) were also shown to have fewer epidermal and lymph node DCs (75% reduction)²³⁰. Here, defects in DC numbers were corrected by the infusion of normal T cells suggesting that T cell-derived signals, including γ c-family cytokines, affect the survival of DCs. Further investigation is required to determine the direct contribution of γ c-family cytokines on DC homeostasis and/or development *in vivo*.

3.6 Final Conclusions

- Murine BM-DCs express and signal through the γ c.
- Despite normal expression of other γ c-containing receptor subunits, BM-DCs derived from γ c/RAG-2-deficient mice (γ c^{-/-} DCs) fail to signal through the γ c-JAK/STAT pathway.
- γ c^{-/-} DCs undergo normal antigen uptake and maturation.
- γ c^{-/-} DCs fail to respond to IL-21-mediated inhibitory effects.

***Chapter Four- Analysis of the Role of the Common Cytokine Receptor
 γ c in Dendritic Cells for Antigen-Specific CD4⁺ T Cell Activation***

4.0 Aims

- Assess whether DC-expressed γ c affects antigen-specific activation of γ c-expressing CD4+ T cells.
- Establish the specific role of DC-expressed IL-15R in promoting CD4+ T cell activation

4.1 Introduction

To explain the mechanisms underlying DC-mediated T cell activation, a multi-signal hypothesis has been constructed. This model envisages that, in order to be fully activated, T cells must be presented with peptide-MHC complexes (signal 1) and must receive co-stimulatory signals (signal 2) from interacting DCs. The integration of these signals involves the reorganization of specific adhesion and signaling molecules into an immunological synapse (IS). The mature IS is a site of continuous TCR signaling and the maintenance of a stable IS is required for full T cell activation i.e, T cell proliferation and cytokine release^{156,231-233}. Cytokines are also implicated in the optimal generation of T cell responses (signal 3) and interestingly, some cytokine signals have been shown to be delivered specifically at the IS^{194,197,198}.

Of particular interest to this study is the fact γ c-family cytokines can especially influence T cell activation. γ c-family cytokine signals can be delivered to T cells either directly, as indicated by IL-15 mediated regulation of T cell proliferation²³⁴⁻²³⁸, or indirectly, by acting on other γ c-family cytokine receptor expressing immune cells, such as DCs, which can in turn affect T cell activation. IL-7, for example, maintains DCs in an immature state and thereby decreases the homeostatic proliferation of CD4+ T cells²²². IL-21-stimulated DCs also retain an immature phenotype and inhibit antigen-specific T cell proliferation *in vitro* and contact hypersensitivity *in vivo*^{219,220}. A particularly interesting case is the one of IL-15. Owing to the particularly flexible structure of its receptor³², this cytokine can promote T cell activation by DCs, by acting either in *cis* or in *trans*. In the first scenario, DC-derived IL-15 functions in an autocrine manner to increase cell survival, upregulation of co-stimulatory molecules and presentation of antigen to CD4+ and CD8+ T cells⁴. Alternatively, IL-15 is assembled intracellularly with IL-15R α in DCs before transport to the surface for transpresentation to neighboring

cells expressing membrane $\gamma\text{c}/\text{IL-2}/\text{15R}\beta$ complexes, such as NK and CD8+ T cells^{27,33,239}. This is thought to be the main mechanism for IL-15 signaling as demonstrated by a study in CD8+ T cells where transpresented IL-15 was found to be more effective than soluble IL-15 in augmenting memory CD8+ T cell proliferation in response to TCR stimulation²⁴⁰. Remarkably, IL-15R α -IL-15 complexes have been shown to enhance proliferation of both naïve and memory antigen-specific CD8+ cells *in vivo* and to participate in tumor clearance²⁴¹⁻²⁴³ therefore confirming the importance of IL-15 transpresentation for CD8+ T cell priming.

Taken together, these reports provided strong evidence of a critical role of γc -family cytokines in regulating DC-mediated T cell activation and pointed to the possibility that residual myeloid γc -deficiency in bone marrow transplanted SCID patients might contribute to persistent immunodeficiency through defective T cell activation. To address this hypothesis, we investigated the role of the γc in DC-induced priming of a naïve CD4+ T cell response.

4.2 Absence of the Common Cytokine Receptor γ_c in Dendritic Cells Impairs Antigen-Specific CD4+ T cell Activation

4.2.1 γ_c -/- DCs Prime Antigen-specific CD4+ T cells Less Efficiently

To investigate the role the γ_c plays in DC-induced priming of a naive antigen-specific CD4+ T cell response, DCs from wild type and γ_c /Rag 2^{-/-} C57BL/6 mice were combined *in vitro* with T cells from OTII transgenic mice. The OTII mice harbour a TCR which specifically recognizes the ovalbumin peptide₃₂₃₋₃₃₉ presented in the context of I-A^b. In our experimental system, LPS-matured DCs were pulsed with ovalbumin (OVA) and co-cultured with CFSE-stained CD4+ OTII cells. CD69 upregulation was used as a marker of early CD4+ T cell activation and assessed after 24 hours whereas T cell proliferation and IL-2 release were taken as late activation readouts and analysed 72 hours from initial T-DC coculture. Flow cytometry analysis showed that T cells induced to express CD69 were significantly diminished ($p \leq 0.05$) when cultured with antigen-pulsed γ_c -/- DCs as compared to WT ($\approx 30\%$ reduction) (Figure 4.1 A,B). γ_c -/- DC-induced proliferation of CD4+ antigen-specific T cells, as determined by CFSE dilution, was also significantly lower ($p \leq 0.05$) than that induced by WT DCs ($\approx 30\%$ decrease) (Figure 4.1 C, D). In addition, OVA-pulsed γ_c -/- DCs supported significantly lower IL-2 secretion in T cell co-cultures ($p \leq 0.05$, Figure 4.1E). As previously described²⁴⁴, DC IL-2 release under these conditions was very low (≈ 40 pg/ml, data not shown) hence, T cells were considered the main source of IL-2 release. Taken together, these data suggest that γ_c -/- DCs are defective in inducing antigen-specific CD4+ T cell activation.

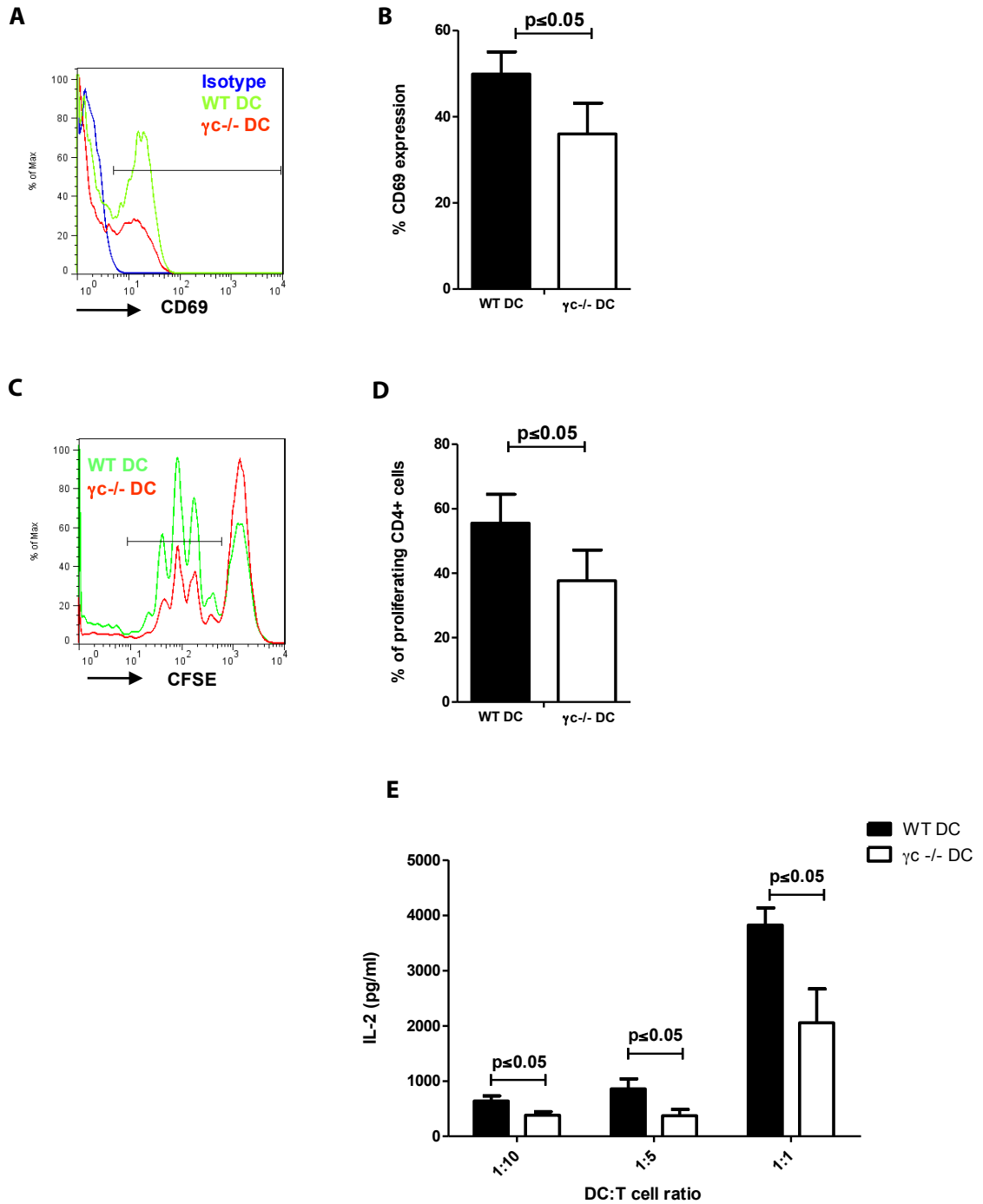


Figure 4.1 $\gamma C^{-/-}$ DCs fail to induce full CD4⁺ T cell activation. DCs were stimulated overnight with LPS and OVA. The following day, cells were washed and co-cultured with CFSE-labelled CD4⁺ OTII cells. Representative plots of CD69 expression (A) and CFSE dilution (C) in CD4⁺ gated T cells after co-culture with DCs at 1:1 DC:T cell ratios for 24 hours and at 1:10 DC:T cell ratios for 72 hours, respectively. Bars represent CD69 expressing (A) and CFSE diluting (C) CD4⁺ T cells. Quantification of percentages of CD69 expression (B) and proliferation (D). (E) IL-2 secretion from supernatants of CD4⁺ T cells cultured with DCs for 72 hours at the indicated ratios. Data are from 4 independent experiments (mean \pm s.e.m). P values, two-tailed students t-test.

4.2.2 Antigen Processing and Presentation are not Affected by γ c-deficiency in DCs

The initiation of a vigorous antigen-specific CD4⁺ T cell response requires correct antigen processing and presentation by DCs. To exclude the possibility that the reduced ability of γ c^{-/-} DCs to prime an antigen-specific response might be due to defective antigen uptake and processing, LPS-matured DCs were incubated with DQ-OVA; self-quenched conjugate of OVA that emits fluorescence upon uptake and degradation. Measurement of fluorescence derived from DQ-OVA pulsed DCs showed that γ c^{-/-} DCs uptake and process antigen as efficiently as WT DCs over a period of 16 hours (Figure 4.2A). Furthermore, to test whether γ c deficiency influences DC capacity to present antigen/MHC II complexes to T cells, the B017.4 hybridoma was cultured with DCs pulsed with OVA₃₂₃₋₃₃₉ peptide and assessed for IL-2 production. B017.4 T cells have a TCR specific for OVA₃₂₃₋₃₃₉ peptide presented by MHC II and secrete IL-2 independently of costimulation and proportionally to the amount of antigen they are presented with. Our data shows that B017.4 T cells cultured with either WT or γ c^{-/-} DCs secrete comparable amounts of IL-2 thereby suggesting that γ c deficiency in DCs does not impair antigen presentation (Figure 4.2B). Taken together, these results indicate that defective antigen-specific CD4⁺ T cell priming by γ c^{-/-} DCs is not due to ineffective processing and presentation of OVA.

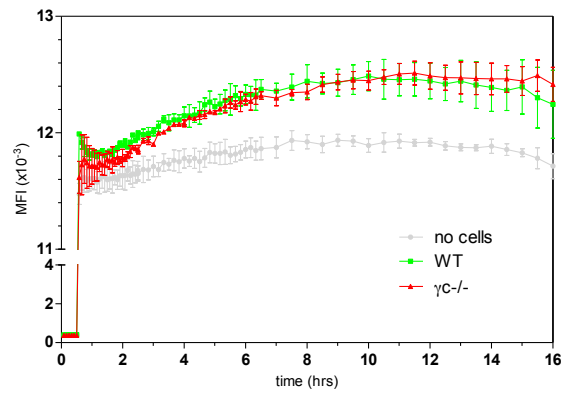
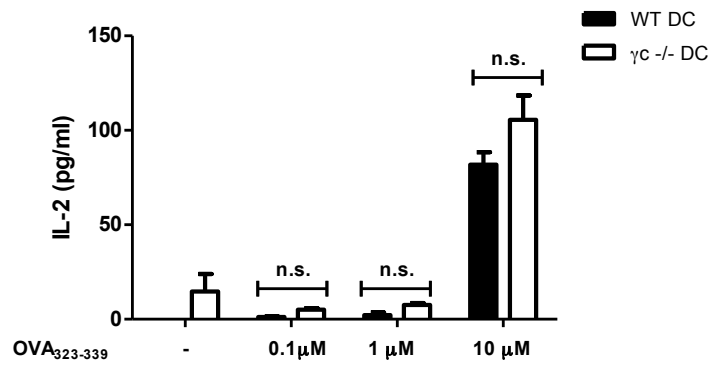
A**B**

Figure 4.2 $\gamma C^{-/-}$ DCs process and present antigen normally (A) LPS-matured DCs were cultured for 16 hrs with DQ-OVA. Fluorescence was then measured at 515 nm (B) LPS-matured DCs were pulsed with OVA₃₂₃₋₃₃₉ for 2 hrs at the indicated concentrations then cultured with B017.4 T cells at a 1:10 ratio. Supernatants were assayed for IL-2 production by ELISA after a 24hr co-culture. Error bars represent s.e.m. of triplicate measurements. P values, two-tailed students t-test.

4.2.3 Conjugate Formation is not Affected by γc -deficiency in DCs

Normal cytokine secretion profiles by LPS-matured γc -/- DCs, as described in 3.2, strongly suggested that soluble cytokine factors might not be critical contributors to the observed defects in antigen-specific CD4+ T cell activation. Hence, we next investigated whether cognate cell contacts might be affected. The most obvious interaction to study was LFA-1-ICAM-1 integrin-mediated adhesion as this is well described to be the most prominent adhesion interaction at the IS. To determine whether inefficient CD4+ T cell activation by γc -/- DCs could be due to defective adhesion between these two cell-types, conjugate-formation was assessed by flow-cytometry as described by others²⁴⁵. CFMDA-labelled CD4+ OTII cells were co-cultured for 1 hour with DDAO-labelled LPS-matured DCs, which had been pulsed with or without OVA. Prior to co-culture, DCs were pre-treated with a blocking anti-ICAM-1 antibody or with an isotype-matched control antibody and the proportion of events positive for both CFMDA and DDAO were then used as a marker of conjugate-formation (adhesion) between CD4+ T cells and DCs, as shown in Figure 4.3A. In keeping with previous reports describing the formation of antigen-independent synapses^{246,247}, conjugate formation was observed also in the absence of antigen, both for WT and γc -/- DCs (3.4% and 4.2%, respectively) (Figure 4.3B). The presence of antigen led to an increase in the number of conjugates and this occurred to a similar extent in both WT and γc -/- DCs (≈ 1.4 and ≈ 1.3 fold increase, respectively). Remarkably, ICAM-1 blockade inhibited both antigen-independent and dependent adhesion and this occurred similarly in WT and γc -/- DCs (3.4% to 2.5% and 4.9% to 2.2%, respectively, in WT DCs compared with 4.2% to 2.2% and 5.5% to 2.2% in γc -/- DCs.). Taken together, these results suggest that a primary (ICAM-1 mediated) adhesion defect is not responsible for defective T cell activation observed with γc -/- DCs. To be noted however is the fact that the percentages of conjugates in our system were lower than those previously described in systems using B cell as APCs ($\approx 20\%$)²⁴⁵. We hypothesise that this may be due to the adhesion of DC-containing conjugates to the culture plates in our assay, which may not have occurred with less adhesive APCs, such as B cells.

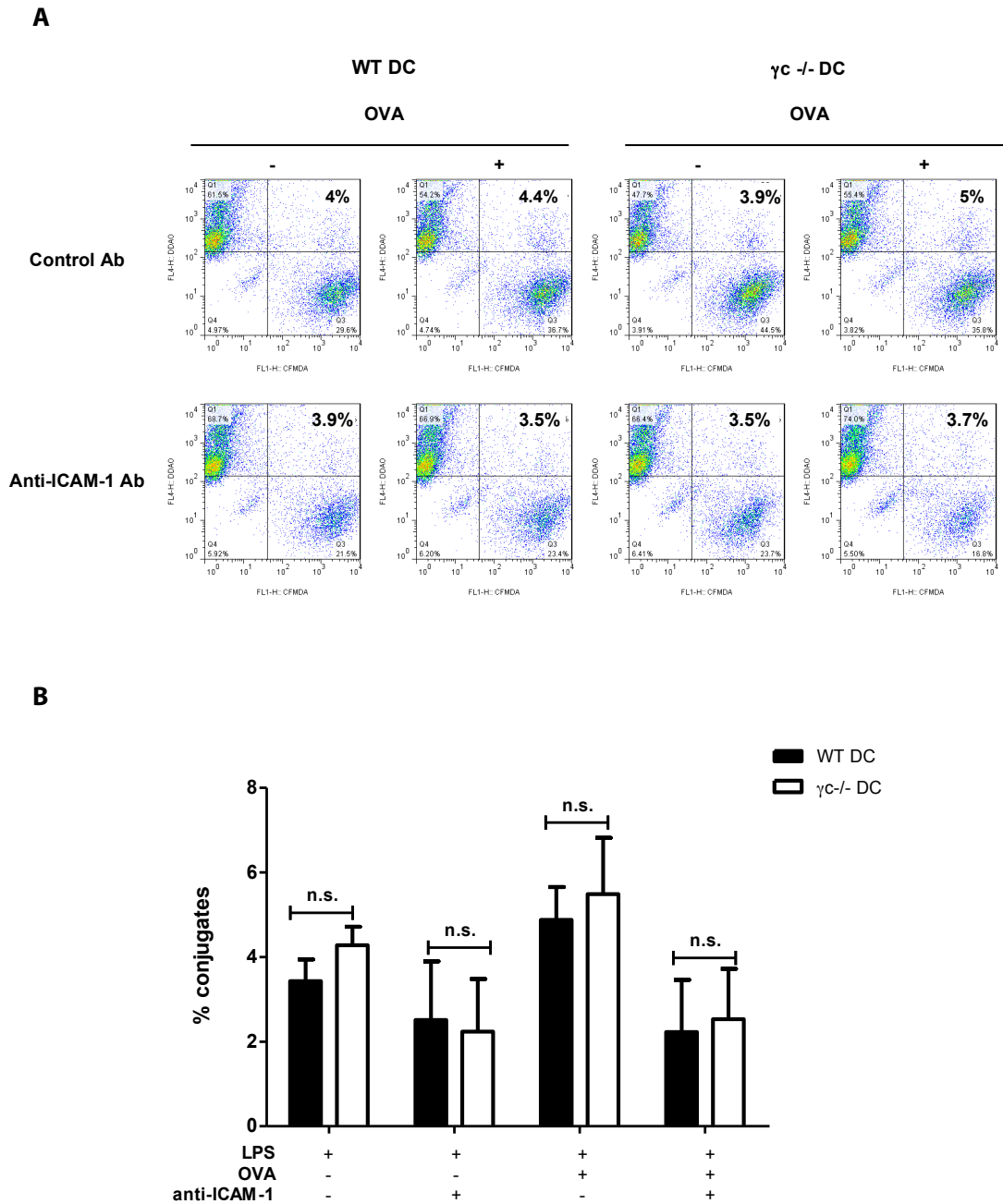


Figure 4.3 Conjugate formation is not affected by the absence of the γc in DCs. DCs were cultured overnight with LPS alone or in combination with OVA. The following day, DCs were labeled briefly with CFMDA then treated with a blocking anti-ICAM-1 antibody or with an isotype-matched control antibody for 1 hr at 37°C. DCs were then mixed at a 1:1 ratio with DDAO-labelled CD4⁺ T cells and brought into contact by centrifugation for 10 min at 600 rpm. After 1 hr incubation at 37°C, cells were analysed by flow cytometry. (A) Density plot representation of mixed cell populations. Indicated are percentages of double-positive cells (DC-CD4⁺ T cell conjugates) (B) Quantification of conjugate formation under the indicated conditions. Data are from 3 independent experiments (mean \pm s.e.m). P values, two-tailed students t-test.

4.2.4 Conjugate Duration and Stability are not Affected by γ c-deficiency in DCs

Long-lived DC-T cell contacts are important for achieving full T cell activation²³³. To exclude the possibility that the inefficiency of γ c^{-/-} DCs to prime a CD4⁺ antigen-specific T cell response might be due to altered contact stability between these two cell types, the duration of contacts was probed *in vitro*. To this end, LPS-matured DCs were pulsed with or without OVA then co-cultured for 30 minutes with CD4⁺ T cells on an environmental chamber on an inverted microscope stage. The contacts were then followed by live cell imaging and their duration was quantified. Interestingly, this showed that the total contact time established by either WT or γ c^{-/-} DCs with T cells was not affected by the presence or absence of antigen (Figure 4.4A). The situation was however different when the dynamics of individual T-DC interactions were analysed. In fact, whereas in the absence of antigen, both WT and γ c^{-/-} DCs established multiple short contacts (1.8 ± 0.16 and 1.7 ± 0.16 minutes, respectively) which never lasted over 5 minutes (Figure 4.4B), in the presence of antigen DCs behaved quite differently, actively embracing T cells by membrane protrusion and establishing stable contacts. Contacts with T cells were in fact significantly longer ($p \leq 0.001$) for both WT (3.7 ± 0.5 minutes) and γ c^{-/-} (4.2 ± 0.48 minutes) OVA-pulsed DCs and the proportion of long/stable contacts (ie, >5minutes) increased to 16% for WT DCs and to 23% for γ c^{-/-} DCs. Taken together, these data indicate that there are no significant differences in the duration of total nor individual contacts formed between T cells and WT/ γ c^{-/-} DCs, neither in antigen dependent nor independent contacts (Figure 4.4B), thereby suggesting that the duration and stability of DC-T cell conjugates are not affected by DC γ c-deficiency.

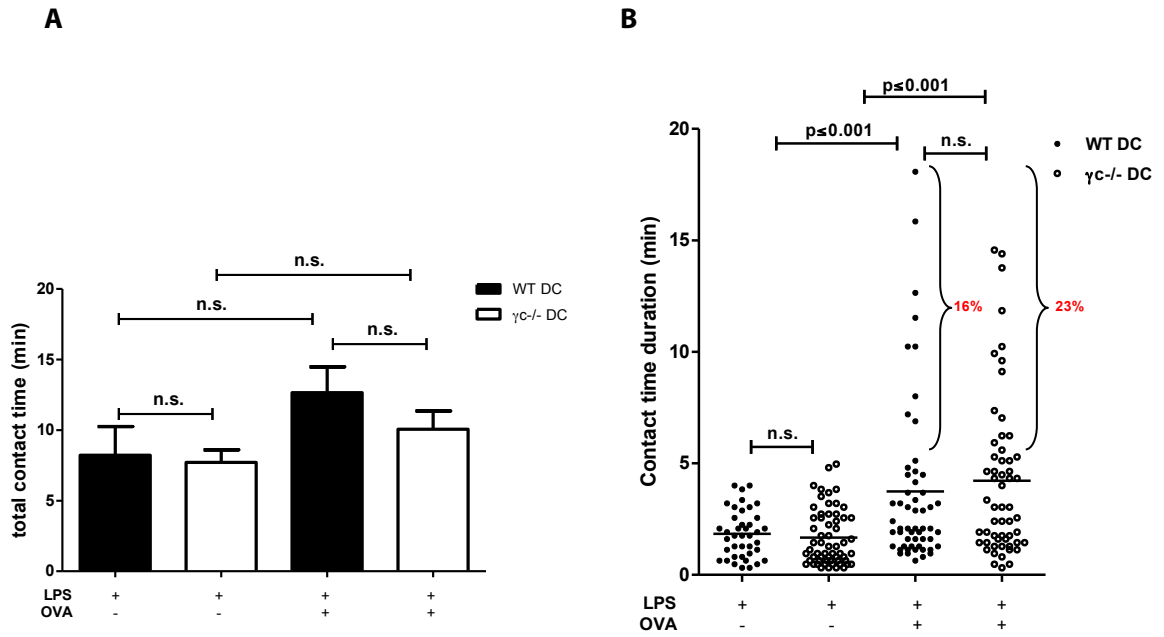
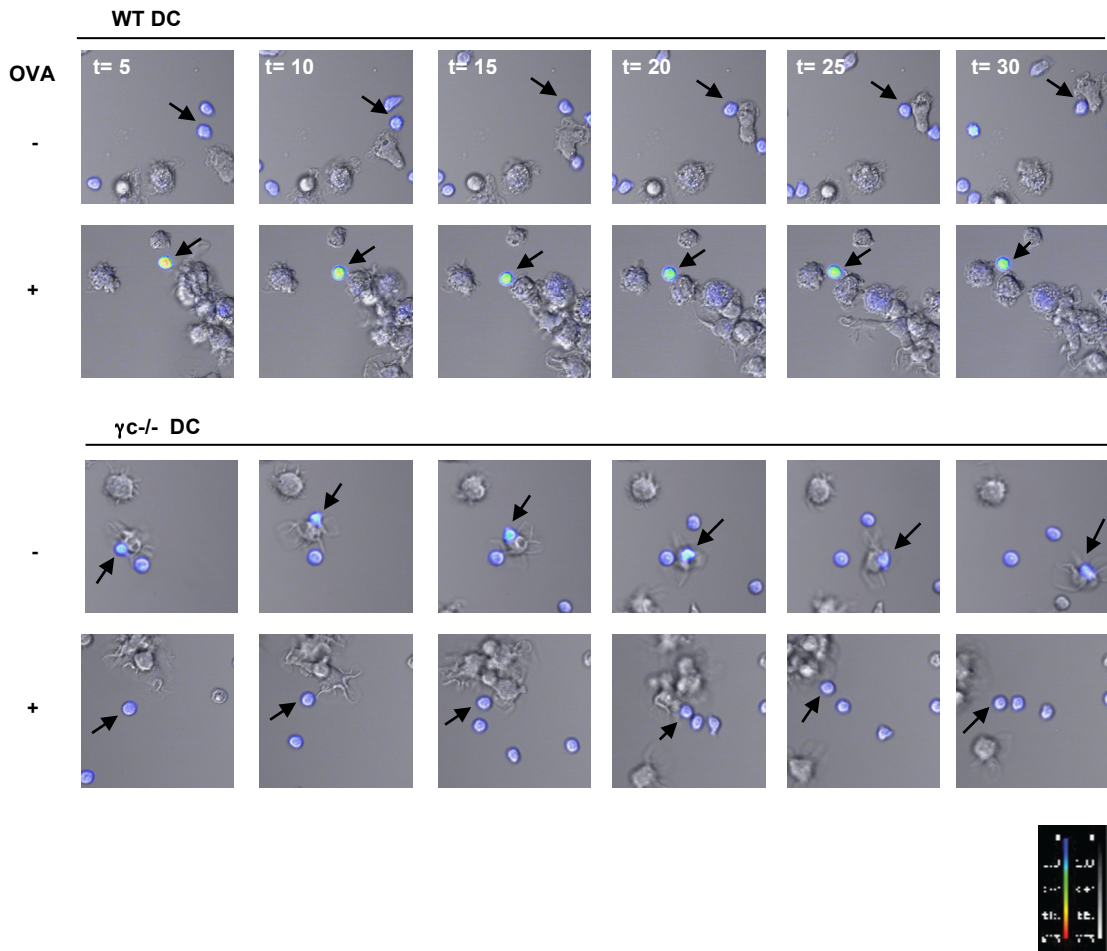


Figure 4.4 Conjugate duration is not affected by the absence of the γc in DCs. DCs were stimulated overnight with LPS alone or in combination with OVA. The following day, DCs were co-cultured at a 1:5 ratio with CD4⁺ T cells for 30 min in an environmental chamber on an inverted microscope stage. (A) Total contact time of CD4⁺ T cells interacting with DCs under the indicated conditions (B) Duration of individual contacts formed by CD4⁺ T cells with DCs. Data are from 2 independent experiments (mean \pm s.e.m). For each condition, 40-60 cells were evaluated. P values, one-way ANOVA (Bonferroni's multiple comparison test).

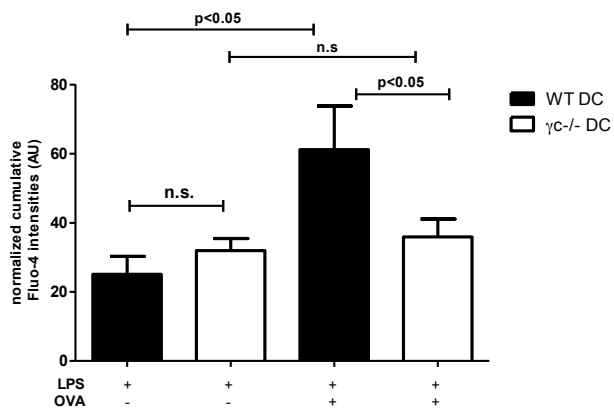
4.2.5 $\gamma c^{-/-}$ DCs induce TCR-evoked Ca^{2+} signaling in CD4⁺ T cells Less Efficiently

Sustained TCR signalling culminates in elevation of intracellular Ca^{2+} concentration and is required for effective T cell activation²⁴⁸. Having demonstrated defective CD4⁺ T cell activation by $\gamma c^{-/-}$ DCs, we were then interested to assess whether γc deficiency would alter DC capacity to induce T cell Ca^{2+} responses. To this end, LPS-matured DCs were stimulated with or without OVA and co-cultured with CD4⁺ T cells loaded with the Ca^{2+} sensitive Fluo-4 dye. Ca^{2+} fluxes were then monitored over 30 minutes at 10-second intervals through live cell imaging. Representative images of Ca^{2+} signals are shown for T cells contacting DCs in the presence or absence of antigen; emission intensities are displayed on a pseudocolor scale from red (low Ca^{2+}) to green (high Ca^{2+}) (Figure 4.5A). T cell activation was analysed by measuring total intracellular Ca^{2+} levels; ie, a integrated Fluo-4 intensities for a T cell when in contact with one or multiple DCs. In keeping with the literature²⁴⁷, antigen-independent contacts were characterised by low level Ca^{2+} fluxes whereas interaction with antigen-pulsed (WT) DCs led to a significant rise ($p \leq 0.05$) in total levels of T cell intracellular Ca^{2+} (≈ 2.5 fold increase) (Figure 4.5A,B). In contrast, antigen-pulsed $\gamma c^{-/-}$ DCs failed to induce an enhanced Ca^{2+} response and the total levels of T cell Ca^{2+} were significantly lower ($p \leq 0.05$) compared to those induced by WT antigen-pulsed DCs ($\approx 40\%$ reduction), regardless of similar total contact times (Figure 4.4A). Ca^{2+} oscillations were also used as a marker of T cell activation as they have been shown to play an important role in regulating lymphocyte effector functions^{182,249,250}, and are presented as the variance of Fluo-4 intensities of contacting T cells. Representative Ca^{2+} traces are shown as a function of time for T cells under each condition (Figure 4.5C). In keeping with the literature²⁵¹, Ca^{2+} responses were also observed in the absence of antigen and were characterized by rare and small transients. The most obvious difference in single-cell Ca^{2+} signals was a markedly greater frequency of Ca^{2+} spiking in T cells interacting with WT DCs in the presence of antigen, as demonstrated by an increase in Fluo-4 signal variance (Figure 4.5C,D). Ca^{2+} traces of T cells interacting with $\gamma c^{-/-}$ OVA-pulsed DCs showed much lower amplitudes, as confirmed by a significant decrease ($p \leq 0.0001$) in the variance of Fluo-4 intensities compared to WT OVA-pulsed DCs ($\approx 70\%$ reduction). Taken together, these data suggest weaker TCR activation by $\gamma c^{-/-}$ DCs.

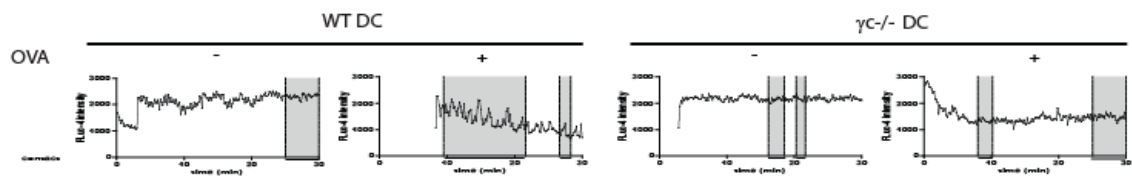
A



B



C



D

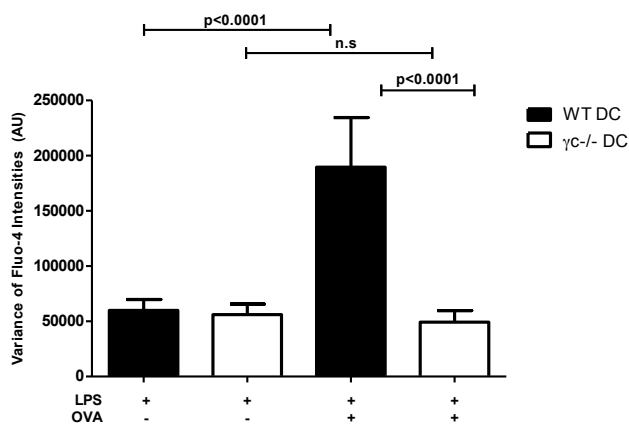


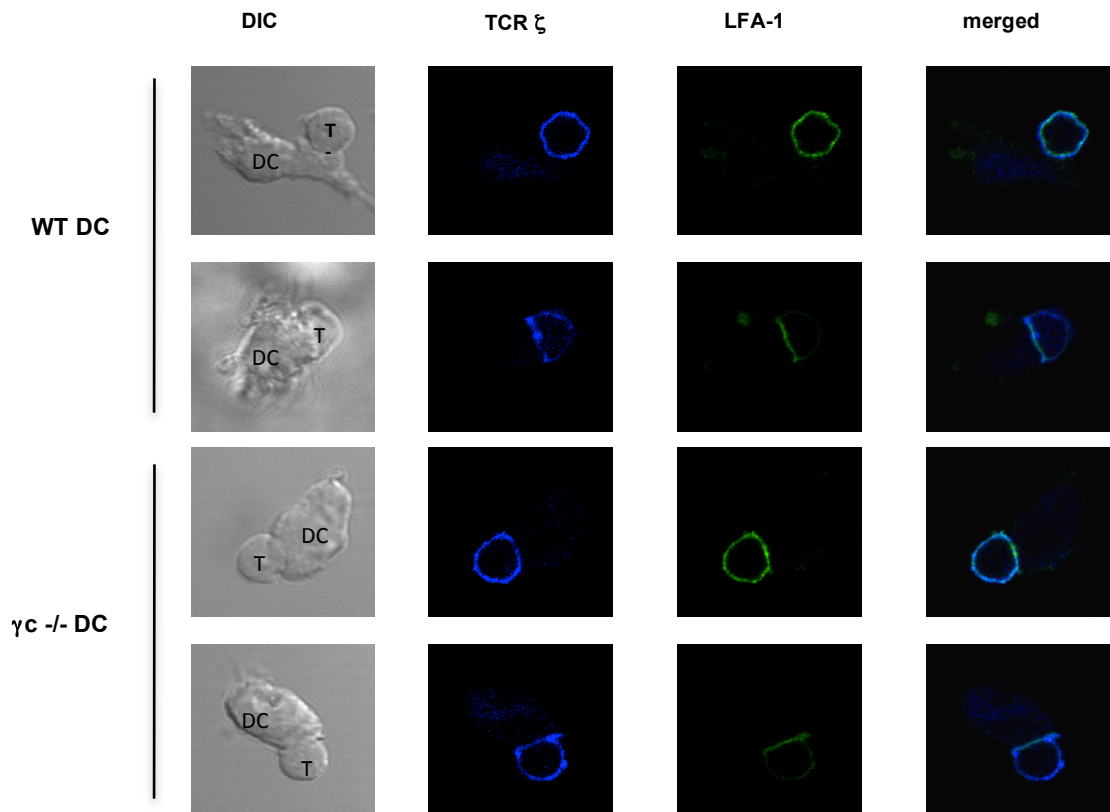
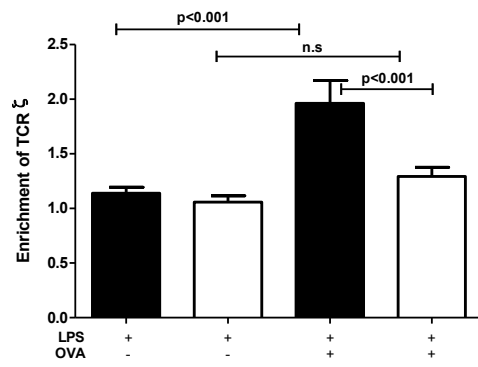
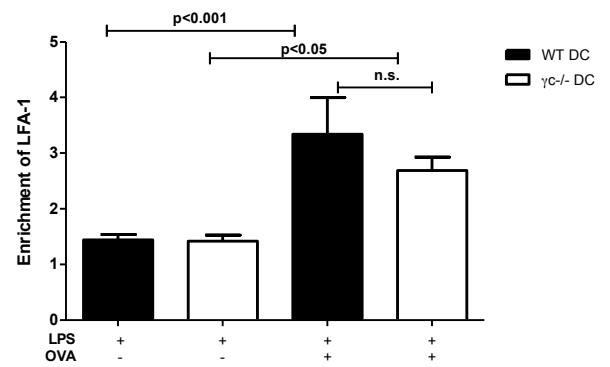
Figure 4.5 Decreased TCR-driven Ca²⁺ signaling in CD4⁺ T cells stimulated by γC^{-/-} DCs.

DCs were stimulated overnight with LPS alone or in combination with OVA. The following day, cells were washed and co-cultured with Fluo-4 labelled CD4⁺ T cells for 30 min in an environmental chamber on an inverted microscope stage. (A) Time-lapse confocal microscopy pseudocolor representative images of Fluo-4 labelled CD4⁺ T cells interacting with DCs under each of the tested conditions (images were corrected with a gamma coefficient of 0.8). The time scale is indicated (min). Black arrows indicate CD4⁺ T cells interacting with DC (B) Integrated Fluo-4 intensities in arbitrary units (AU) of individual T cells during interactions with DCs normalized over basal fluorescence (C) Representative Ca²⁺ traces showing the oscillation of Fluo-4 intensities over time for T cells under each of the tested conditions. The grey shaded areas indicate periods of contact (D) Quantification of variance of Fluo-4 intensities in AU of individual cells contacting DCs. For each condition, 30-45 cells were evaluated. Data are from 2 independent experiments (mean ± s.e.m). P values, one-way ANOVA (Bonferroni's multiple comparison test).

4.2.5 $\gamma c^{-/-}$ DCs Fail to Induce TCR ζ Recruitment at the Contact Area Regardless of Normal LFA-1 Recruitment

TCR-mediated signaling leads to accumulation of TCR and LFA-1 at the IS¹⁵¹. To assess whether γc deficiency alters the capacity to induce redistribution of such molecules at the DC-T cell interface, CD4⁺ T cells were conjugated with LPS-matured DCs that had been stimulated with or without OVA and conjugates were stained for TCR ζ and LFA-1. Figure 4.6A shows the labelling of TCR ζ and LFA-1 in T cell-DC conjugates. Remarkably, under antigen stimulation, unlike WT DCs, $\gamma c^{-/-}$ DCs failed to induce redistribution of total surface TCR ζ to the T cell contact area (Figure 4.6A,B). Significant differences ($p \leq 0.001$) were in fact detected between the level of TCR ζ enrichment at the interfaces formed between T cells and antigen-pulsed WT/ $\gamma c^{-/-}$ DCs (≈ 1.9 for WT versus ≈ 1.2 -fold enrichment for $\gamma c^{-/-}$ DCs). En face reconstructions of the DC-T cell contact area showed multifocal TCR polarizations rather than the classical cSMAC configuration, as has been previously suggested by other studies of naïve T cell-DC interactions (Figure 4.6D)²⁰⁵. Overall, similar patterns of TCR ζ clustering were observed at T cell interfaces with either WT or $\gamma c^{-/-}$ DCs.

Analysis of LFA-1 accumulation at the interface instead showed similar enrichment of this molecule at the contact area formed between T cells and either WT or $\gamma c^{-/-}$ OVA-pulsed DCs (≈ 3.3 . and ≈ 2.7 -fold enrichment, respectively) (Figure 4.6A,B,D) confirming our findings that integrin-mediated adhesion is not affected by the absence of the γc in DCs.

A**B****C**

D

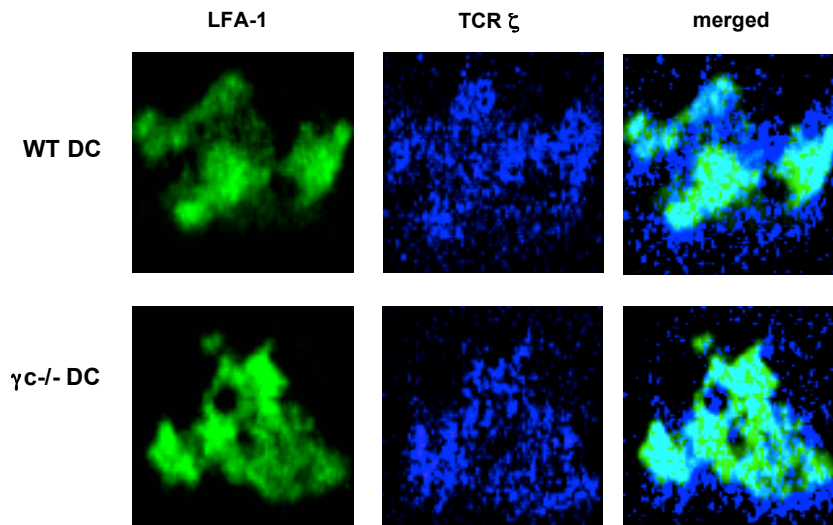


Figure 4.6 Decreased recruitment of TCR ζ in T- γ c $^{-/-}$ DC conjugates. DCs were stimulated overnight with LPS alone or in combination with OVA. The following day, DCs were mixed at a 1:1 ratio with CD4 $^{+}$ T cells and brought in contact by centrifugation for 10 min at 600 rpm. After 1 hour incubation at 37°C, cells were fixed and stained for TCR ζ and LFA-1. (A) Differential interference contrast (DIC) acquisitions and representative deconvolved images showing localization of TCR ζ (blue), LFA-1 (green) and their corresponding overlays in DC-T cell conjugates (B) Quantitative analysis of the enrichment of TCR ζ (B) and LFA-1 (C) at the T-DC contact area (D) En face XZ reconstructions of deconvolved interfaces showing the distribution at the interface of TCR ζ (blue), LFA-1 (green) and their corresponding overlays. Data are from 2 independent experiments (mean \pm s.e.m). For each condition, 35–48 conjugates were evaluated. P values, one-way ANOVA (Bonferroni's multiple comparison test).

4.3 Absence of the Common Cytokine Receptor γc in Dendritic Cells Impairs IL-15 transpresentation

4.3.1 $\gamma c^{-/-}$ DCs Fail to Induce IL-15 Transpresentation-dependent STAT5 Activation in CD4+ T cells

Normal cytokine secretion profiles by $\gamma c^{-/-}$ DCs (chapter 3.3.2) suggested that inefficient CD4+ T cell activation might be explained by defects in cognate cell contacts rather by defects in release of soluble factors. Having ruled out the contribution of adhesion to the observed defects, IL-15 transpresentation therefore seemed the other prominent cognate interaction to explore, especially because it involves the γc .

IL-15 transpresented by IL-15R α has been shown to activate the JAK-STAT pathway *in trans* through the β/γ chain complex expressed on NK and CD8+ T cells²⁴¹. Although the γc is described to play predominantly a signaling role in IL-15R biology, we wondered whether the absence of DC γc might affect transpresentation to CD4+ T cells. To test this, STAT5 phosphorylation was examined in CD4+ T cells following 10-minute incubation with DCs that had been pretreated with LPS alone or in combination with OVA. Since surface IL-15/IL-15R α complexes are induced in DCs upon TLR stimulation³³, we hypothesized that γc -expressing LPS-matured DCs would activate JAK/STAT signaling through the CD4+ T cell β/γ chain. Surprisingly, γc -expressing DCs stimulated with LPS alone did not induce STAT5 activation in CD4+ T cells whereas stimulation with LPS in combination with OVA resulted in a significant enhancement in STAT5 phosphorylation ($p \leq 0.05$), suggesting that DC-mediated CD4+ T cell STAT5 activation depends on a cognate TCR:MHC signal (Figure 4.7 A,C). Interestingly, T cell STAT5 activation also appeared to depend on DC γc as indicated by the failure of $\gamma c^{-/-}$ DCs to induce STAT5 phosphorylation in CD4+ T cells, under any of the conditions tested (Figure 4.67A,C). To confirm that the induction of JAK/STAT activation observed in CD4+ T cells stimulated with antigen-pulsed γc -expressing DCs was dependent on IL-15 transpresentation, DCs were treated with anti-IL-15R α prior to co-culture with T cells. IL-15R α -blockade in antigen-pulsed γc -expressing DCs abrogated CD4+ T cell STAT5 phosphorylation ($p \leq 0.05$), therefore suggesting that the T cell activation observed occurred through IL-15 transpresentation (Figure

4.7B,C). $\gamma c^{-/-}$ DCs were unaffected by treatment with anti-IL-15R α . Taken together, these data suggest that the γc in DCs is involved in mediating antigen-dependent IL-15 transpresentation to CD4 $^{+}$ T cells.

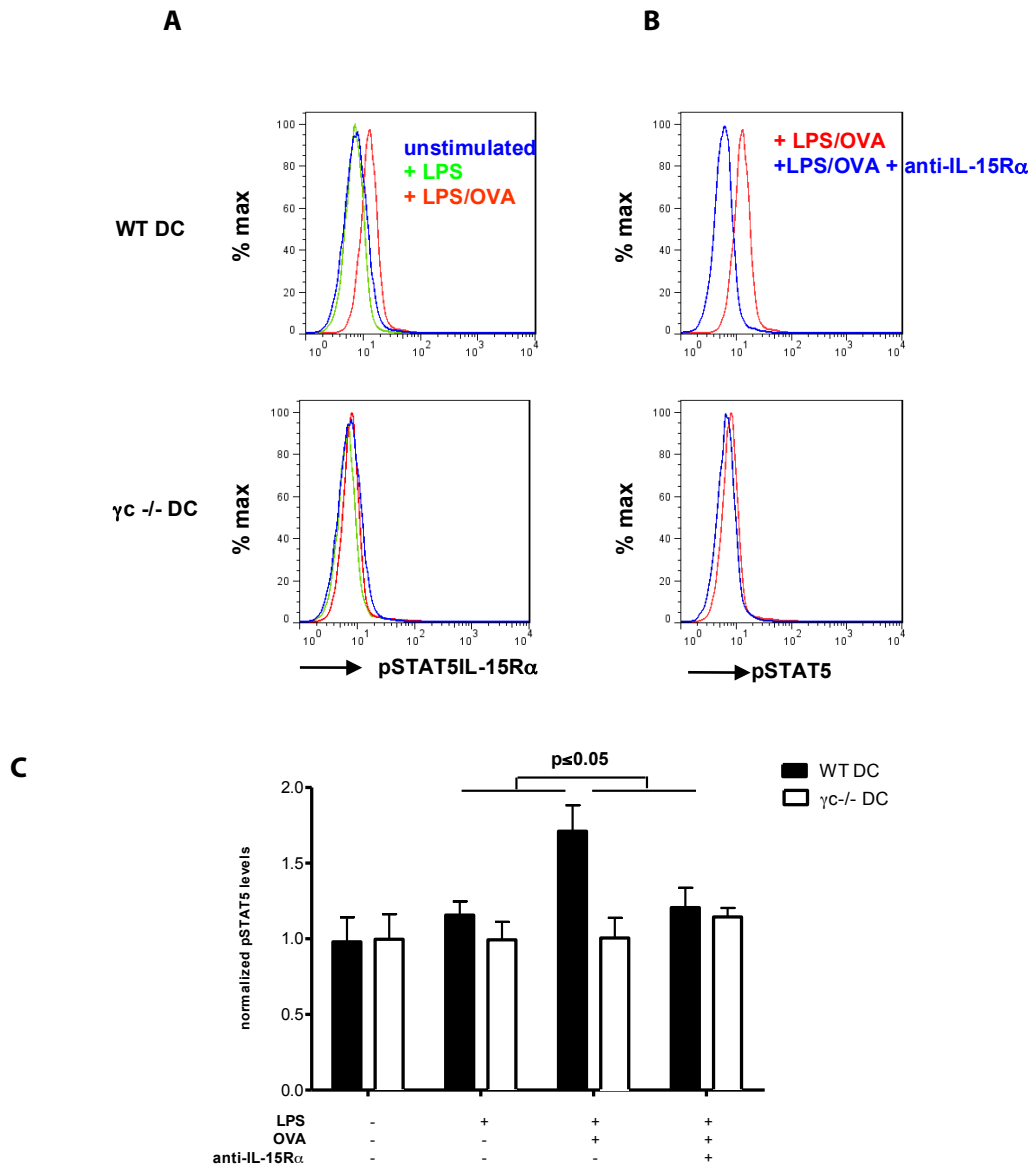


Figure 4.7 $\gamma c^{-/-}$ DCs fail to induce STAT5 activation in CD4 $^{+}$ T cells. DCs were either left untreated or stimulated overnight with LPS alone or in combination with OVA. The following day, cells were washed, treated for 30 min with 20 μ g/ml anti-IL15R α or isotype control, then cultured at a 1:1 ratio for 10 min with CD4 $^{+}$ T cells, which had been serum-starved for 3 hours. Cells were then fixed and stained with anti-pSTAT5. (A,B) pSTAT5 expression on CD4 $^{+}$ CD11c $^{-}$ gated cells stimulated with DCs under the indicated conditions (C) Normalized pSTAT5 fluorescence intensities relative to unstimulated control. Data presented are from 3 independent experiments (mean \pm s.e.m). P values, one-way ANOVA (Bonferroni's multiple comparison test).

4.3.2 $\gamma c^{-/-}$ DCs Express Normal Levels of Intracellular IL-15R α

The results above indicated that DC γc is required for IL-15R α -mediated transpresentation of IL-15 to CD4 $^{+}$ T cells. IL-15/IL-15R α complexes are mainly assembled intracellularly prior to delivery to the surface and having previously established normal surface expression of IL-15R α (Figure 3.4), we were then interested to verify the levels of intracellular IL-15R α in $\gamma c^{-/-}$ DCs. Western blot and flow cytometry analysis showed that $\gamma c^{-/-}$ DCs express intracellular IL-15R α similarly to WT (Figure 4.8A,B). Furthermore, we observed normal upregulation of IL-15R α upon LPS stimulation (1.3 and 1.4 fold increase for WT and $\gamma c^{-/-}$ DCs, respectively) (Figure 4.8B). In keeping with the literature¹⁹⁹, analysis of mean fluorescence intensities of IL-15R α staining showed that intracellular IL-15R α (Figure 4.8B) is relatively more abundant than surface IL-15R α (Figure 3.4) in both immature and mature DCs (≈ 2 and 2.5-fold increase, respectively). Again, this was also unaffected by the absence of DC γc .

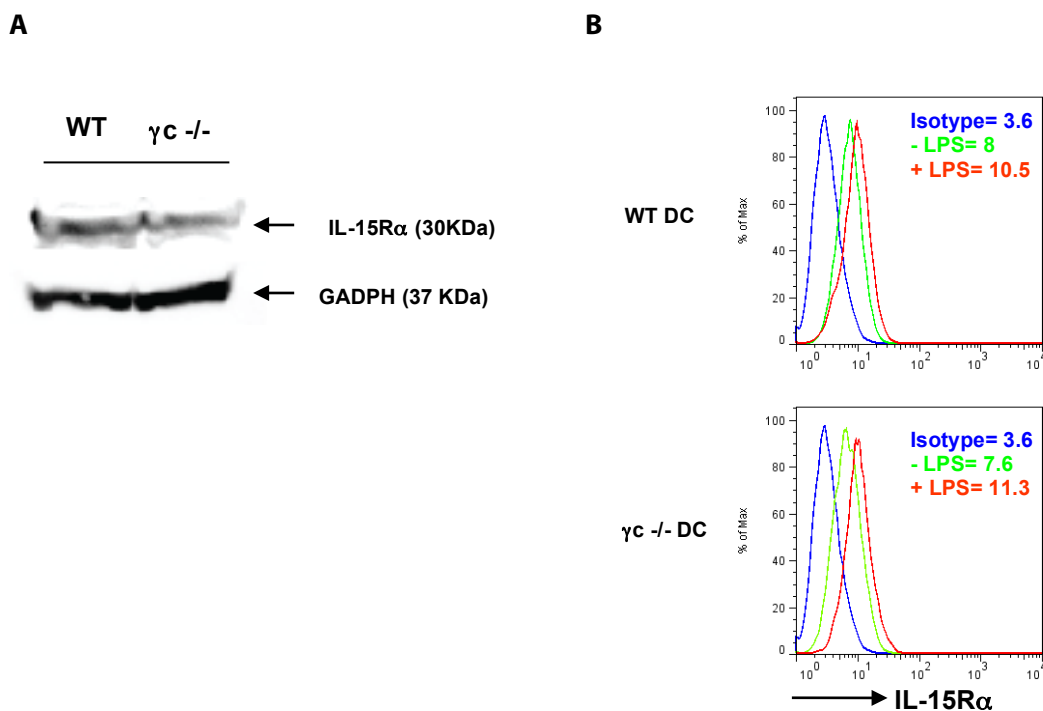


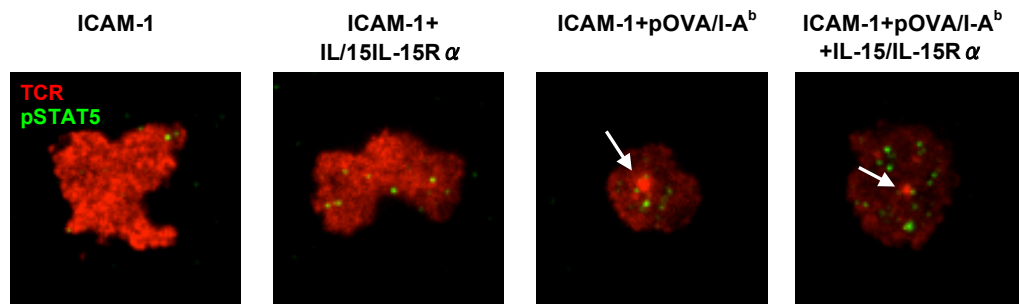
Figure 4.8 $\gamma c^{-/-}$ DCs express and upregulate intracellular IL-15R α normally. DCs were cultured overnight with or without LPS. (A) Immunoblot analysis of total IL-15R α expression in unstimulated DCs. GADPH is used as a loading control (B) Representative plots showing intracellular expression of IL-15R α in CD11c $^{+}$ gated cells under the indicated conditions (MFI=mean fluorescence intensities).

4.3.3 TCR Engagement is Required for Efficient IL-15 Trans-signaling in CD4+ T cells

To fully characterize antigen-dependent IL-15 transpresentation to CD4+ T cells, we studied the response of CD4+ T cells to stimulation with IL-15/IL-15R α and peptide/MHC complexes by total internal reflection microscopy (TIRFM). To this end, CD4+ T cells were incubated for 30 minutes on lipid bilayers presenting ICAM-1, CD80, I-A^b/pOVA and IL-15/IL-15R α as indicated in Figure 4.9A and fluorescently-labelled for pSTAT5 and TCR. This showed that, upon interaction with bilayers presenting I-A^b/pOVA, naïve CD4+ T cells developed TCR microclusters, as previously observed by others²⁰⁵ (Figure 4.9A arrows). Interestingly, upon stimulation with IL-15/IL-15R α complex in combination with I-A^b/pOVA, CD4+ T cells accumulated significantly more pSTAT5 microclusters at the cell-bilayer interfaces compared to either stimulus alone (\approx 1.5-fold difference) ($p < 0.001$) (Figure 4.9A,B). In addition, we also observed that pSTAT5 microclusters were not recruited within TCR-antigen microclusters but were homogenously distributed at the contact interface, possibly suggesting spatial segregation of the IL-15R and TCR signaling pathways.

Consistent with the flow cytometry results obtained with DC-T cell conjugates (Figure 4.7), these data reiterate the idea that IL-15/IL-15R α -mediated CD4+ T cell STAT5 activation depends on simultaneous TCR engagement.

A



B

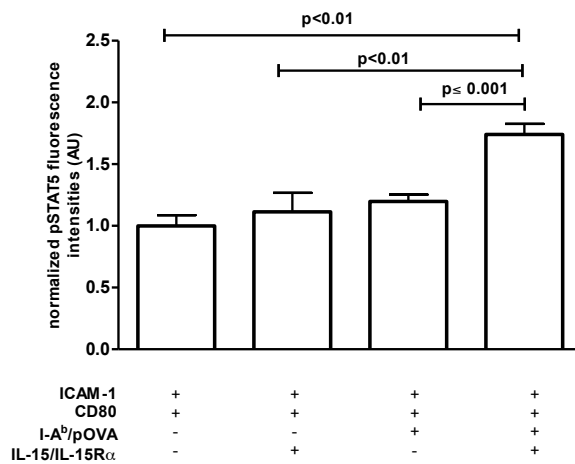


Figure 4.9 IL-15/IL-15R α mediated STAT5 activation in CD4⁺T cells requires TCR engagement. CD4⁺ T cells were incubated on glass-supported planar bilayers containing ICAM-1, CD80, I-A^bpOVA and IL-15/ IL15R α as indicated in the presence of fluorescent anti-TCR Fab. After 30 min incubation at 37°C, cells were fixed and stained for pSTAT5. (A) Representative TIRFM images showing pSTAT5 (green) and TCR (red) accumulation at the T cell contact interface (B) Quantification in arbitrary units (AU) of integrated pSTAT5 fluorescence intensities (mean \pm s.e.m) at contact interfaces shown in A normalized relative to ICAM-1 control. For each condition, 32-57 cells were evaluated. P values, one-way ANOVA (Bonferroni's multiple comparison test).

4.3.4 IL-15 Transpresentation Does Not Modulate TCR Signaling

The findings that IL-15 trans-signaling requires simultaneous TCR engagement pointed to the possibility that IL-15 transpresentation might act as a costimulatory signal to facilitate TCR signal transduction. To study whether IL-15 transpresentation enhances TCR mediated early signalling events, CD4+ T cells were incubated on lipid bilayers described in 4.3.3 and stained for markers of early TCR activation, such as phosphorylated PLC- γ 1. Unlike the classical CD28 costimulatory signal¹⁷¹, the signal supplied by IL-15/IL-15R α did not lead to increased PLC- γ 1 phosphorylation compared to stimulation with I-A^b/pOVA alone (Figure 4.10 A,B), suggesting that IL-15 trans-signaling does not have an effect on proximal TCR signaling events.

PI3K-dependent AKT activation is considered another marker of early T cell activation. Given that PI3K is also activated by a number of different receptors alongside the TCR, including co-stimulatory receptors and cytokine receptors²⁵², we hypothesized that IL-15 transpresentation might enhance TCR induced PI3K-dependent AKT activation. Phosphorylation of AKT in response to TCR ligation with antigen was very moderately enhanced by simultaneous stimulation with IL-15/IL-15R α complex (\approx 1.1-fold increase) (Figure 4.10 C,D). Though statistically significant ($p \leq 0.05$), the magnitude of this effect was small and therefore unlikely to be physiologically relevant. Taken together, these data suggest that IL-15 transpresentation does not amplify TCR-mediated early signalling events, though this might still relate to signal saturation due to the strong TCR stimulation provided in this experimental system.

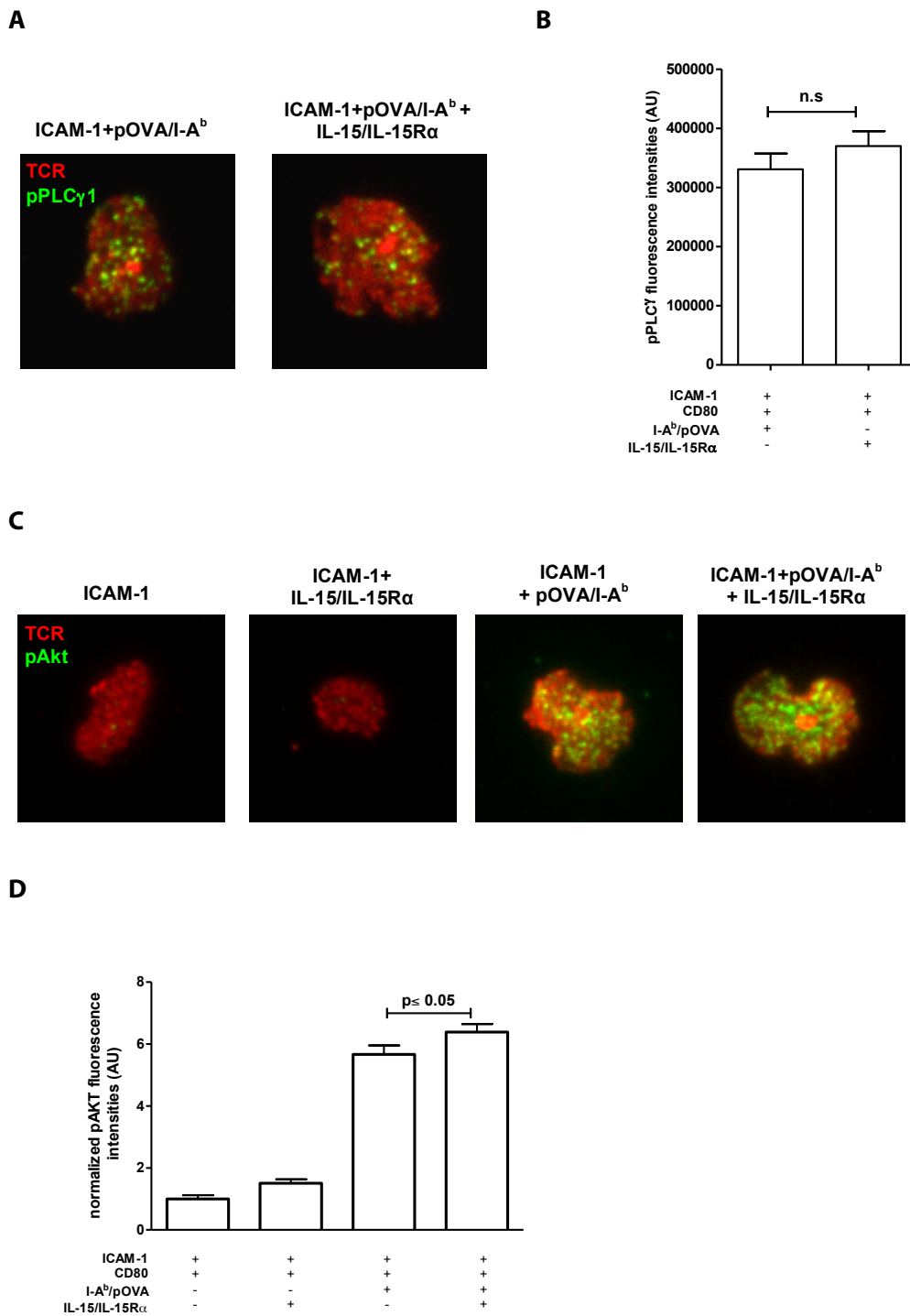


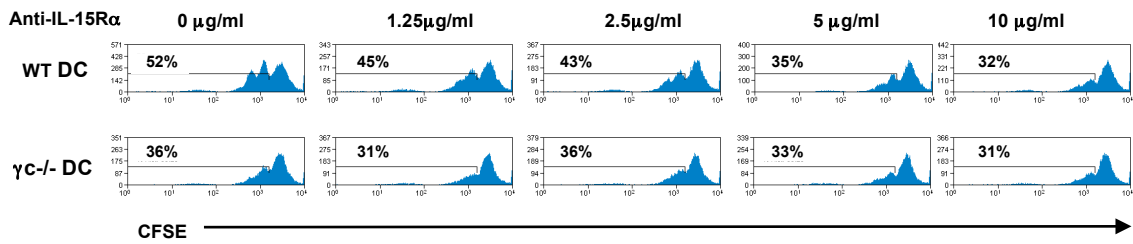
Figure 4.10 TCR-mediated signals are not modulated by stimulation with IL-15/IL-15Rα.

CD4⁺ T cells were incubated on glass-supported planar bilayers containing ICAM-1, CD80, I-A^bpOVA and IL-15/IL15Rα as indicated in the presence of fluorescent anti-TCR Fab (red). After 30 min incubation at 37°C, cells were fixed and stained for (A) pPLCγ1 (green) and (C) pAKT (green). Bar graphs indicate quantification in arbitrary units (AU) of integrated pPLCγ1 (B) and pAKT (D) fluorescence intensities (mean ± s.e.m) normalized relative to ICAM-1 control at contact interfaces. For each condition, 35–65 cells were evaluated. P values, one-way ANOVA.

4.3.5 IL-15R α Blockade in γ c-expressing DCs Inhibits Antigen-specific CD4+ T Cell Activation

Activation of STAT5 is critical for IL-15 mediated NK and CD8+ T cell survival, proliferation and function^{253,254}. It therefore seemed plausible to hypothesise that IL-15 mediated STAT5 activation might also play a role in the induction of CD4+ T cell functions. To address whether IL-15 transpresentation influences antigen-specific CD4+ T-cell activation, antigen-pulsed DCs were treated with anti-IL-15R α prior to co-culture with CD4+ T cells. Interestingly, IL-15R α blockade significantly reduced T cell proliferation induced by WT DCs ($p \leq 0.002$) and in keeping with defective IL-15 transpresentation by γ c $^{-/-}$ DCs, this did not occur in γ c $^{-/-}$ DCs (Figure 4.11A,B). Interestingly, treatment of WT DCs with the higher range of blocking antibody concentrations (5-10 μ g/ml) reproduced the γ c-deficient phenotype (Figure 4.11A). Taken together, these data suggest that inefficient IL-15R α -mediated IL-15 transpresentation by γ c $^{-/-}$ DCs accounts for the original defects observed in DC-mediated T cell activation (Figure 4.1).

A



B

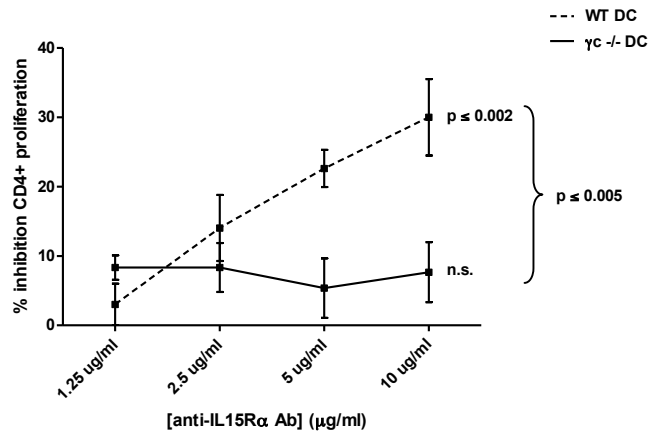


Figure 4.11 IL-15R α blockade in γ c-expressing DCs inhibits antigen-specific CD4⁺ T cell activation. DCs were stimulated overnight with LPS in combination with OVA. The following day, cells were washed and pre-treated for 30 minutes with IL-15R α blocking Ab or isotype-matched control at the indicated concentrations. Cells were then cocultured with CFSE-labelled CD4⁺ OTII cells. (A) Representative plots of CFSE dilution in CD4⁺ gated T cells after co-culture with OVA-pulsed DCs at 1:5 DC:T cell ratios for 72 hours respectively. Bars represent CFSE diluting CD4⁺ T cells (B) Quantification of % inhibition (relative to isotype-matched control Ab) of proliferation of CD4⁺ T cells shown in B. Data are representative of 3 independent experiments (mean \pm s.e.m). P values, linear regression analysis.

4.4 Conclusions

Productive T cell activation by DCs depends on coordinated delivery of signals from antigen (signal 1), adhesion and co-stimulatory molecules (signal 2) and soluble factors (signal 3). In the present chapter, we showed that, despite normal uptake, processing and presentation of antigen (signal 1), $\gamma c^{-/-}$ DCs were less efficient at inducing full CD4⁺ T cell activation as indicated by decreased proliferation, expression of the CD69 activation marker and IL-2 release. We excluded that this defect could be due aberrant DC maturation as, in response to LPS stimulation, $\gamma c^{-/-}$ DCs underwent normal upregulation of maturation markers (CD86 and MHC II) and secretion of pro-inflammatory cytokines, including IL-2 and IL-12. Interestingly, both of these cytokines have been shown to be important for T cell effector functions. DC-derived IL-12, for example, is known to be critical for the initiation of Th1 responses by promoting the differentiation of naive CD4⁺ cells into IFN- γ -producing cells²⁵⁵. Similarly, IL-2 has been proposed to enhance DC-mediated T cell activation as suggested by defective CD4⁺ and CD8⁺ T cell priming by IL-2-deficient DCs^{244,256}. Normal IL-2 and IL-12 secretion profiles therefore indicated that cytokine release (signal 3) may not be a critical factor in the priming of antigen-specific naïve CD4⁺ T cell responses by $\gamma c^{-/-}$ DCs.

Long-lived intercellular contacts are established through adhesive interactions and are required to elicit full T cell activation^{192,233}. To rule out the contribution of integrin-mediated adhesion to the defects observed, we next studied the LFA-1-ICAM-1 interaction. Notably, LFA-1 polarization was preserved at the contact interface of T cells with antigen-pulsed $\gamma c^{-/-}$ DCs and, as expected, this led to normal LFA-1 dependent adhesion. In agreement with these findings, the duration and stability of DC-T cell contacts were also unaffected by the absence of DC-expressed γc in live cell assays. Taken together, these findings suggested that LFA-1-mediated adhesion and related signaling (signal 2) might not account for the observed defects.

Having partly excluded the contribution of signal 1, 2 and 3 to the defects in antigen-specific CD4⁺ T cell activation, we next investigated whether other pathways and cognate interactions might be affected. The most obvious cognate interaction to study involving the γc was IL-15 transpresentation due to its well established role in

both NK and CD8+ T cell homeostasis and priming^{33,36,257}. Despite the classical view that DC-mediated IL-15 transpresentation is important for the activation and homeostatic regulation of these cells, in this chapter we showed that IL-15 is also transpresented to CD4+ T cells. Intriguingly, our co-culture experiments demonstrated that DC-mediated transpresentation of IL-15 results in STAT5 activation in CD4+ T cells and that this depends upon cognate TCR interactions, as has been recently described for CD8+ T cells²⁴². This was confirmed in a bilayer system in which CD4+ T cell STAT5 activation was achieved only upon simultaneous stimulation with IL-15/IL-15R α and peptide/MHC complexes. Altogether, our results pointed to the possibility that IL-15 transpresentation might have an important costimulatory role in CD4+ T cell effector functions and according to our expectations, blockade of IL-15R α in γ c-expressing DCs resulted in reduced antigen-specific T cell proliferation.

Having established defective induction of antigen-specific T cell proliferation by γ c^{-/-} DCs, we investigated the requirement of the γ c for IL-15 transpresentation to CD4+ T cells. Interestingly, our co-culture experiments showed that, regardless of normal levels of IL-15R α , γ c^{-/-} DCs failed to induce IL-15 trans-signaling in CD4+ T cells, as demonstrated by lack of STAT5 activation. Similarly, IL-15R α blockade did not result in a marked reduction in CD4+ T cell proliferation as observed for γ c-expressing DCs. In contrast with the widely held view that IL-15R α is the critical subunit for IL-15 transpresentation^{36,38} while the γ c plays predominantly a signaling role downstream of β/γ c subunit ligation, here we showed that the γ c is also required for effective IL-15 transpresentation to CD4+ T cells. This suggests that effective IL-15 transpresentation may require the *cis* interaction of IL-15R α with the β/γ c complex and is in keeping with a previous study in which blocking IL-2R β resulted in an inhibition of IL-15R α dependent NK activation³⁷.

In addition to defective induction of IL-15 trans-signaling in CD4+ T cells, the data presented in this chapter also suggested inefficient triggering of TCR-mediated Ca²⁺ signaling by γ c^{-/-} DCs, as demonstrated by reduced T cell total Ca²⁺ levels and Ca²⁺ oscillations. Cross-communication between diverse signaling networks represents a way of fine-tuning cellular reactions hence we were interested to verify whether

IL-15 transpresentation and trans-signaling might regulate TCR proximal events. Since both PLC- γ 1 and AKT signaling pathways are involved in IP₃ generation and in intracellular Ca²⁺ responses, we set out to assess whether IL-15 transpresentation might act as a canonical CD28-like costimulatory signal and enhance their activation. In contrast to our expectations, phosphorylation of neither PLC- γ 1 nor AKT in response to TCR ligation was substantially enhanced following stimulation with IL-15/IL-15R α complexes in bilayer experiments. It therefore remains unclear how IL-15 transpresentation might regulate early TCR signaling events. Furthermore, normal conjugate formation also led us to exclude the possibility that the interaction established through the IL-15R might facilitate membrane apposition and lead to enhanced PLC- γ activation and Ca²⁺ response, as has instead been proposed for the CD28-B7 interaction¹⁷¹. In the light of these findings, the TCR Ca²⁺ signaling defects are thus problematic to explain in the context of IL-15 transpresentation. Alternatively, it is possible that these results could represent a technical artifact due to the type of analysis adopted in this study (ie single-parameter as opposed to ratiometric). Our analysis may have in fact been hampered by artifactual differences in fluorescence signal due to variations in dye loading or cell size. In contrast, ratiometric measurements of Ca²⁺ signals are considered a more accurate way to analyse Ca²⁺ responses as they involve the measurement of two different fluorescence signals from within the same cell. The ratio of emissions at two different wavelengths is generally consistent from cell to cell and therefore the above described variables tend to be cancelled out resulting in more reliable interpretations. Hence, it is possible that, due to analysis with Fluo-4 alone, we may have overestimated the differences in the Ca²⁺ responses elicited by WT/ γ c^{-/-} DCs. Qualification of the data is required to clarify whether the observed differences are real.

Through confocal imaging of DC-T cell conjugates, here we also provided evidence of defective TCR ζ recruitment at the contact interface with γ c^{-/-} DCs. Stabilization of TCR ζ clusters at the centre of the interface is regulated by signaling- and cytoskeleton-dependent processes^{151,258}. Despite the unlikely contribution of IL-15-dependant signals to TCR signal transduction, it still remains possible that IL-15 transpresentation might promote TCR translocation. This is suggested by the fact that

other costimulatory receptor pairs, such as CD28/B7, have been proposed to enhance the aggregation of TCR-containing membrane microdomains at the contact interface by mediating cytoskeleton-mediated membrane raft movement^{161,176,189}. Intriguingly, both TCR ζ and γc have been observed to associate with the cytoskeleton in T cells^{259,260} therefore raising the intriguing possibility that the IL-15R receptor pair might affect T cell actin remodeling and TCR translocation at the contact interface.

Taken together, these data suggested that IL-15 transpresentation could represent a mechanism by which DCs deliver cytokine-related costimulatory signals to CD4+ T cells to enhance their activation. In addition, we have provided evidence that TCR ζ recruitment at the contact interface may also be influenced by IL-15 transpresentation though this requires further investigation.

4.5 Final Conclusions

- IL-15 transpresentation depends on simultaneous TCR engagement with MHC-bound antigen and functions as a costimulatory signal for CD4+ T cell activation.
- DC-expressed γc is involved in regulating IL-15 transpresentation to CD4+ T cells.
- $\gamma c^{-/-}$ DCs are hence defective in inducing full antigen-specific CD4+ T cell activation

*Chapter Five- Analysis of the Role of the Common Cytokine
Receptor γc in Dendritic Cells for the Organisation of Molecules at
the Immunological Synapse*

5.0 Aims

- Study immunological synapse (IS) formation by $\gamma c^{-/-}$ DCs through TIRFM of DC-lipid bilayer interfaces
- Characterise the role of the γc in controlling the localization of its related receptors at the DC interface

5.1 Introduction

The IS is a specialised junction between the T cell and the APC characterised by an organised clustering of specific signaling and adhesion molecules^{151,156}. Besides being a site of delivery of membrane bound receptors and cytosolic signaling proteins, the IS is also a site of polarized cytokine secretion. Cytoskeletal rearrangement plays a critical role in the targeted secretion of cytokines at the IS. This is achieved through reorientation of the MTOC and consequent alignment of intracellular organelles involved in cytokine secretion towards the contact site. This process has been well defined in T cells, where it regulates delivery of cytokines, such as IL-2, IL-4, IL-5 and IFN- γ and sustains TCR signaling^{194,198,261}. Similarly, polarization of IL-12/IL-18 and IL-2 has been observed in DCs at the contact sites with NK and CD8+ T cells, respectively^{137,244,262}. Recently, MTOC polarization has been specifically implicated in regulating the targeted delivery of cytokines at the DC IS with CD8+ T cells, as has been shown for IL-12¹⁹⁷.

Whereas the formation of the T cells IS has been extensively characterised, much less is known about the molecular events underlying DC IS formation. As already discussed in section 1.4.8, there is now growing evidence suggesting that DCs also play an active role in IS formation, through cytoskeletal rearrangement.

We therefore hypothesised that inefficient T cell activation by $\gamma c^{-/-}$ DCs could be due to defective IS formation. To address this, we specifically investigated the role of the γc in regulating molecular events on the DC side of the IS.

5.2 Immunological Synapse Formation by γc -/- DCs

5.2.1 Establishment of TIRFM/lipid Bilayer Platform for High Molecular Resolution Imaging of the DC Synaptic Membrane

Given the central role of IS formation for T cell priming²⁶³, we hypothesised that DC γc might affect the correct assembly of the IS. To study the organization of DC synaptic interfaces, a planar lipid bilayer platform was developed in collaboration with Prof. Dustin's laboratory at the Skirball Institute of Biomolecular Medicine in New York, and imaged using TIRFM. Bilayers were loaded with LFA-1 and Fab fragments specific for I-A^b for ligation of DC ICAM-1 and MHC II respectively, thereby modelling the T cell side of the IS. Briefly, LFA-1 was tagged to a His6 tail and linked to Nickel-NTA modified lipids whilst monobiotinylated anti-I-A^b Fabs were fluorescently labelled and attached to biotinylated lipids using streptavidin as a bridge (Figure 5.1). TIRFM imaging allowed us to follow the spatial-temporal dynamics of ligated MHC II molecules at high molecular resolution, ie within 200 nm of the interface between the bilayer and the DC plasma membrane.

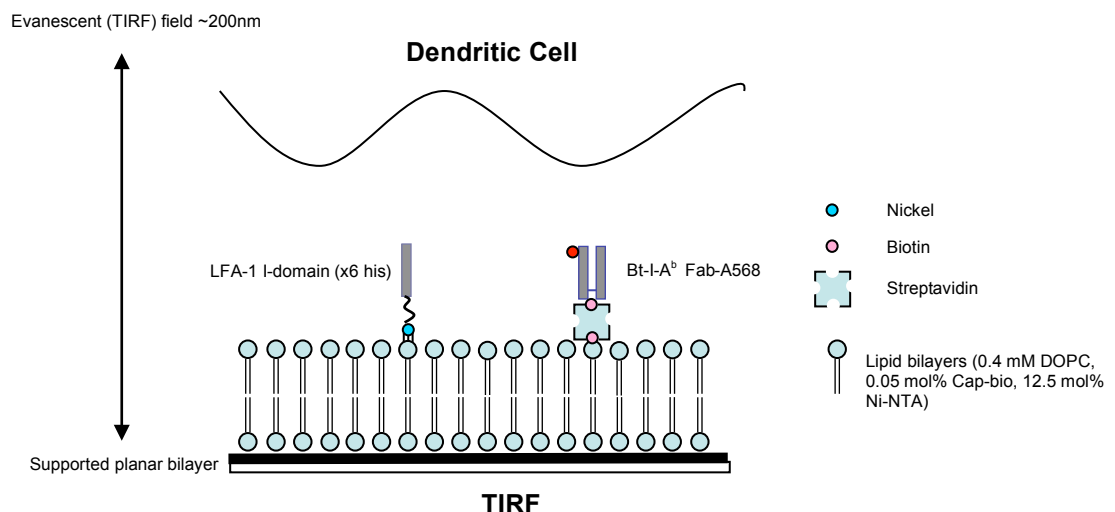


Figure 5.1 Schematic diagram of a planar lipid bilayer containing unlabelled LFA-1 domain and Alexa-568 anti-I-A^b Fabs.

5.2.2 Organisation of MHC II Microclusters at $\gamma c^{-/-}$ DC Interfaces

To study the organisation of MHCII-TCR interactions, LPS-matured OVA-pulsed DCs were settled on bilayers described in 5.2.1 and followed through live cell imaging using TIRFM. Upon interaction with the bilayers, WT γc -expressing DCs spread over the surface and started aggregating MHC II molecules in microclusters. These microclusters were highly dynamic and, once the cells had fully adhered to the membrane, moved towards the centre of the contact where they underwent progressive accumulation. $\gamma c^{-/-}$ DCs were also observed to cluster MHC II at the contact interfaces (Figure 5.2A). Quantitative analysis of fluorescence intensities collected by TIRFM at the interfaces revealed similar levels of total MHC II accumulation in WT and $\gamma c^{-/-}$ DCs over time ($p=0.64$, $t=20$ min; $p=0.07$, $t=60$ min) (Figure 5.2A,B), thereby suggesting that DC γc does not affect MHC II recruitment at the IS.

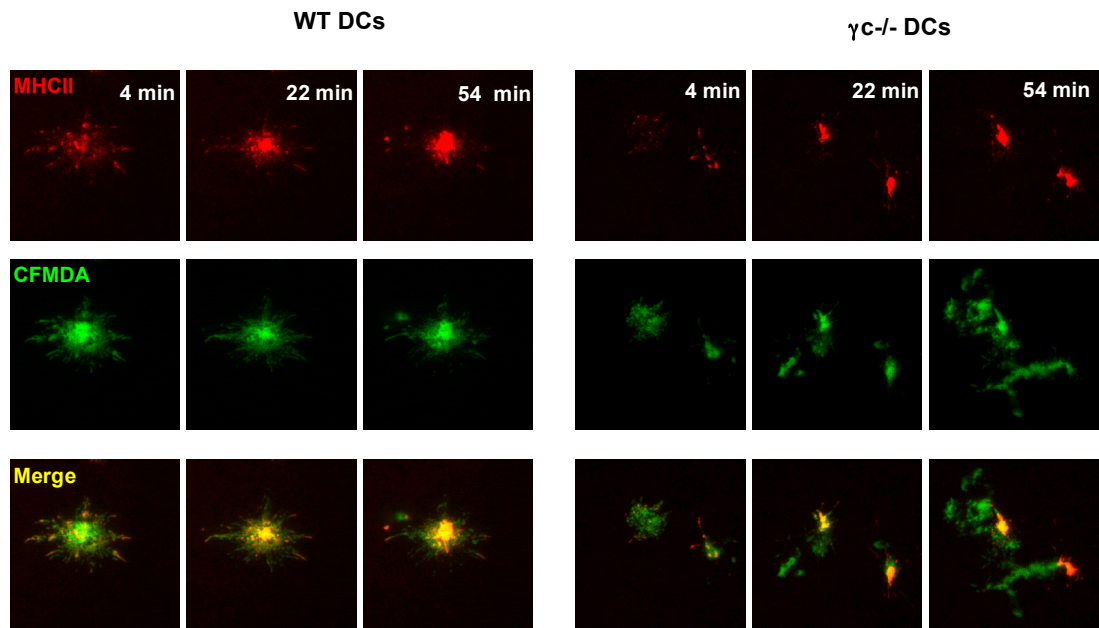
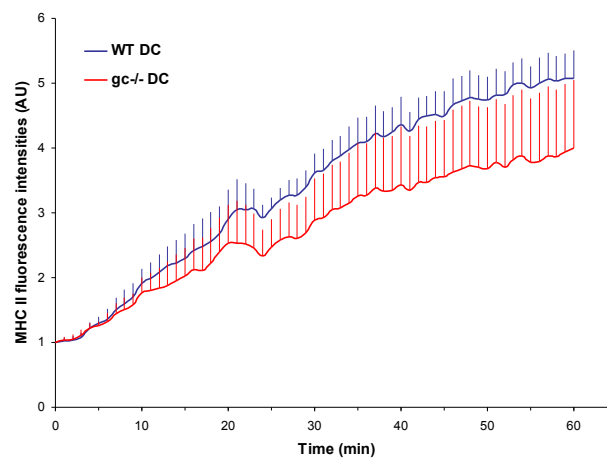
A**B**

Figure 5.2 WT and $\gamma c^{-/-}$ DCs recruit MHC II microclusters at the contact interface.

LPS-matured OVA-pulsed DCs were incubated on glass-supported planar bilayers containing LFA-1 and Alexa-568-I-A^b Fab and imaged by TIRFM at 37°C for 1 hr. (A) Representative TIRFM images of MHC II (red) clusters at WT and $\gamma c^{-/-}$ DC interfaces. Counterstaining of cytoplasm with CFMDA (green) (B) Time course analysis of MHC II integrated fluorescence intensities in AU at the contact interface normalized with respect to fluorescence at t=0 (mean \pm s.e.m, n=4). P values, two-tailed students t-test.

5.2.3 F-actin is Recruited to γc -/- DC Interfaces

The DC actin cytoskeleton has been reported to play an active role during IS formation²⁶⁴ and MHC clustering²¹¹. We were therefore interested to compare actin recruitment at WT and γc -/- DC contact interfaces. To this end, DCs were transduced with a lentivector containing a marker of filamentous actin (F-actin), Life-Act-Ruby (kindly provided by Dr. Bouma at Molecular Immunology Unit, Institute of Child Health). 48 hours post infection, LPS-matured OVA-pulsed DCs were settled on lipid bilayers and followed by live cell TIRFM imaging. Consistently with previous reports demonstrating actin polarization at the contact site with T cells²⁰⁶, we observed that upon interaction with the bilayers, actin was dynamically recruited to the contact interfaces of both WT and γc -/- DCs. Interestingly, actin distribution almost perfectly correlated with MHC II and this was also observed to be similar in the presence or absence of DC γc (Figure 5.3).

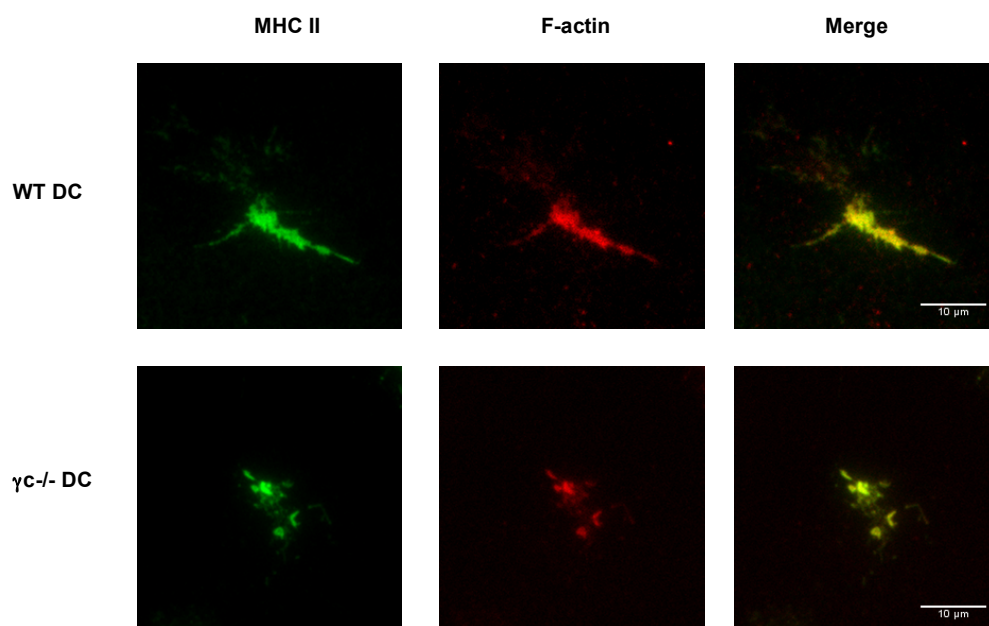


Figure 5.3 WT and γc -/- DCs recruit F-actin at the contact interface. DCs were transduced with lentiviral vectors encoding Life-Act Ruby (see Appendix 1 for vector details). 24 hours post infection, cells were LPS-matured and pulsed with OVA overnight. Cells were then incubated on glass-supported planar bilayers containing LFA-1 and Alexa-488-I-A^b Fab and imaged by TIRFM at 37°C for 30 min. Shown are representative TIRFM images demonstrating MHC II (green), F-actin (red) and corresponding overlays.

5.2.4 $\gamma c^{-/-}$ DC Polarize Less F-actin at the Interface

Having observed a normal pattern of F-actin recruitment to $\gamma c^{-/-}$ DC interfaces, we then set out to investigate whether the levels of polymerized actin at DC interfaces might be affected by the absence of the γc . For this purpose, DCs cells were left to interact with bilayers for 30 minutes, then fixed and stained with fluorescently-labelled phalloidin (high-affinity marker of F-actin). Interestingly, F-actin accumulation was significantly reduced ($p \leq 0.05$) at $\gamma c^{-/-}$ DC interfaces compared to WT (Figure 5.4A,B), despite similar levels of total F-actin (Figure 5.4C). This was confirmed by flow cytometry after cell permeabilization and staining with fluorescently-labelled phalloidin. Taken together, these results suggest abnormal F-actin rearrangement at $\gamma c^{-/-}$ DC contact interfaces.

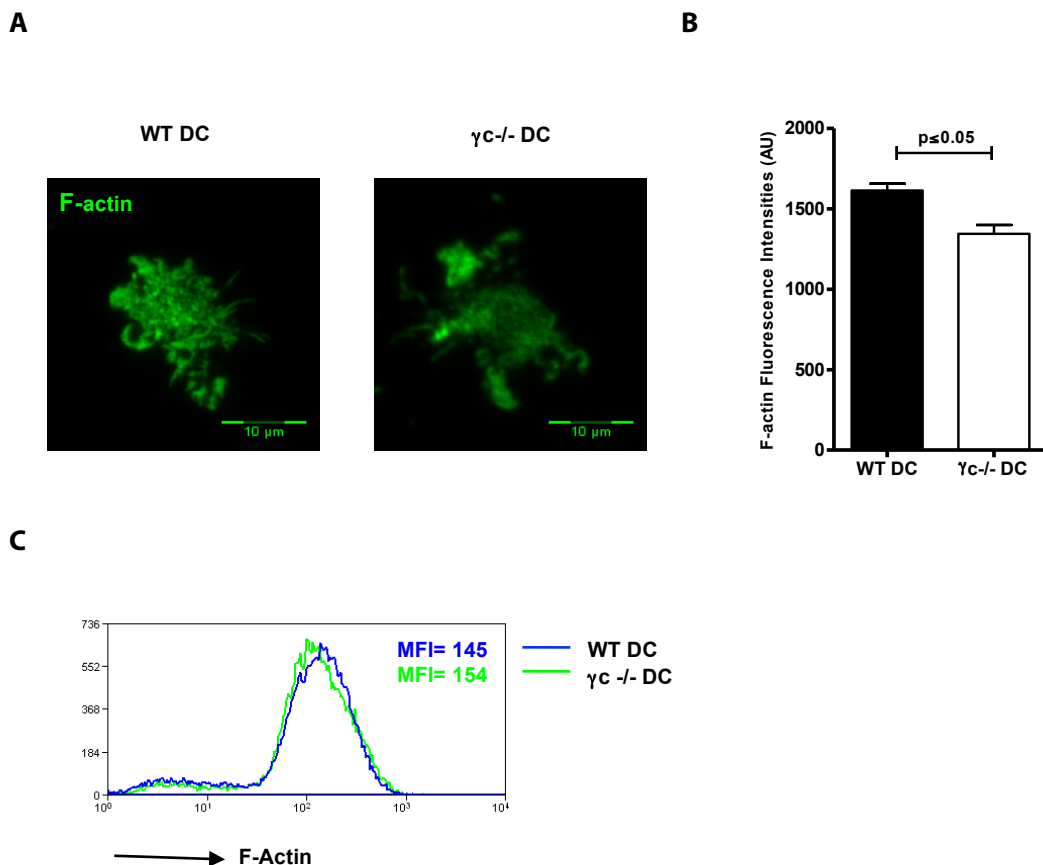


Figure 5.4 $\gamma c^{-/-}$ DCs inefficiently polarize F-actin at the contact interface. (A-B) LPS-matured OVA-pulsed DCs were incubated on glass-supported planar bilayers containing LFA-1 and I-A^b Fab for 30 min. Cells were then fixed and stained for F-actin with Alexa-488-phalloidin. (A) Representative TIRFM images showing F-actin (green) at contact interfaces (B) Quantification in

arbitrary units (AU) of average F-actin fluorescence intensities (mean \pm s.e.m) at contact interfaces shown in A. For each condition, 20 cells were evaluated. P values, two-tailed student's t-test

(C) LPS-matured OVA-pulsed DCs were permeabilised and stained for F-actin with rhodamine-phalloidin. Representative plots showing intracellular expression of F-actin in CD11c⁺ gated cells (MFI= mean fluorescence intensity).

5.3 γ c is Recruited to the DC Interface and Controls Localization of Related Receptors

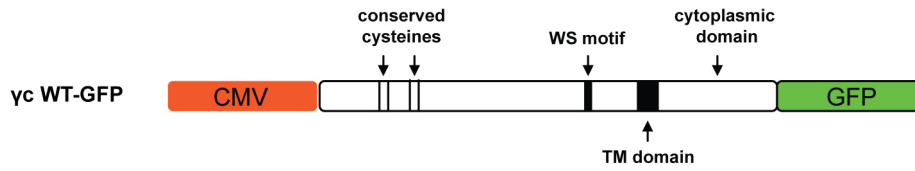
5.3.1 Generation of γ c-GFP Fusion Protein Variants for Analysis of γ c Recruitment to the DC IS

The results presented in chapter 3 indicated that a TCR/MHC signal is needed for DC-mediated IL-15 transpresentation to CD4⁺ T cells. This suggested that the TCR and IL-15R might be colocalised at the T cell interface and raised the intriguing possibility that one way this could happen was if the γ c and its associated receptors were recruited to and associated with MHC II at the DC contact interface.

To visualise γ c localization at the DC IS, we made use of a lentiviral construct encoding a GFP-tagged full-length γ c (γ c^{WT}-GFP) (Figure 5.5.). The γ c^{WT}-GFP construct was kindly provided by Toni Cathomen's laboratory, Hannover Medical School, and was particularly useful not only because of the lack of specific anti- γ c antibodies but also because it allowed us to follow the dynamics of γ c recruitment at the interface. Furthermore, to investigate the requirement of the γ c cytoplasmic/signaling domain for its recruitment to the DC IS, the γ c^{WT}-GFP fusion protein described above was mutated so as to create a truncated a signaling-deficient version (γ c^{mut}-GFP). This mutant was designed to retain the γ c ecto- and transmembrane domains but to lack 77 amino acids at the carboxy-terminus thus resembling a stop mutation (R289X) that has been described in an X-SCID patient²⁶⁵ (Figure 5.5B).

γ c^{WT} and γ c^{mut} expression and signaling were assessed in the γ c-null human leukemia ED7R T cell line. Flow cytometry analysis of γ c-null human ED7R T cells transduced with either γ c^{WT}- or γ c^{mut}-GFP revealed similar levels of γ c surface expression therefore confirming that the truncation of the cytoplasmic domain does not affect the expression of the γ c extracellular domain (Figure 5.6A). However, in contrast to cells transduced with γ c^{WT}-GFP, cells expressing γ c^{mut}-GFP failed to phosphorylate STAT5 following stimulation with IL-2 (Figure 5.6B), regardless of similar levels of transduction (45% versus 40% GFP⁺ cells following infection with γ c^{WT}-GFP and γ c^{mut}-GFP, respectively). These results therefore confirm that the truncation of the carboxy-terminus of the γ c abolishes its signaling functions.

A



B

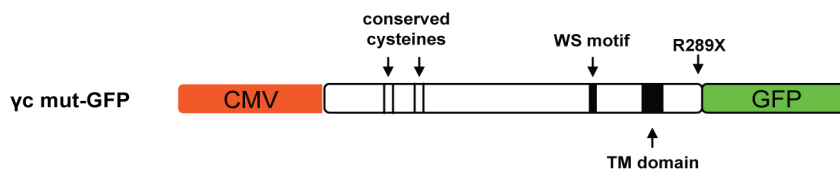


Figure 5.5 Schematic diagrams of CMV-driven γc^{WT} - and γc^{mut} -GFP encoding constructs. Schematic representations of full length (γc^{WT}) (A) and truncated (γc^{Mut}) (B) γc proteins attached to GFP via an 8 amino acid linker. A truncated γc was created by introduction by PCR of an AgeI site at amino acid 289 of the γc . The PCR product was then religated into the original vector to create a γc -GFP fusion lacking 77 amino acids at the C terminus (γc^{Mut} -GFP). See Appendices 2 and 3 for vector details.

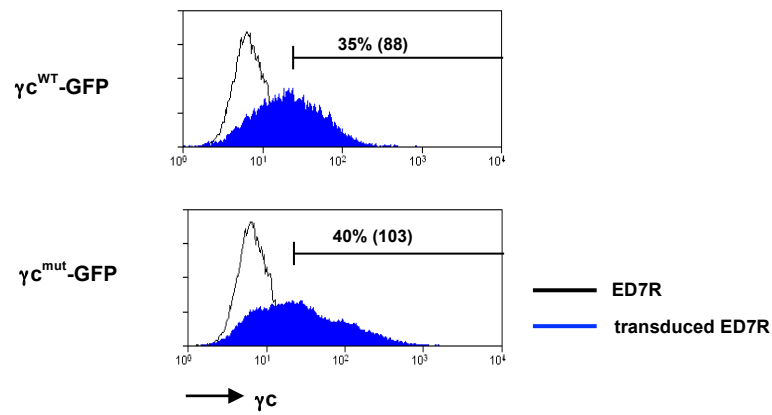
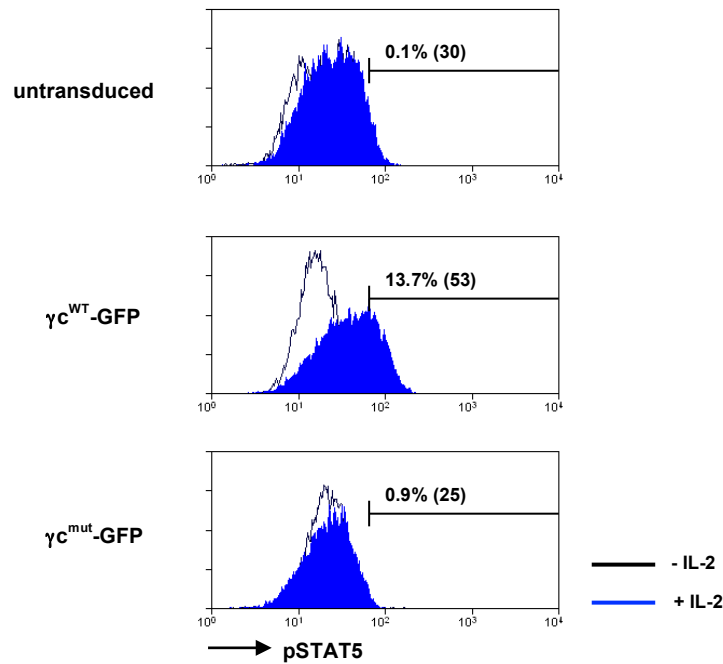
A**B**

Figure 5.6 γC expression and signaling in ED7R cells infected with γC^{WT} - and γC^{mut} -GFP encoding lentivectors. ED7R cells were transduced with γC^{WT} - and γC^{mut} -GFP encoding lentivectors at MOIs of 25. Cells were analysed 72 hours post-infection. (A) Surface expression of the γC in GFP+ cells. The percentages of γC -expressing cells and MFI (in parenthesis) are shown (B) Cells were cultured with or without IL-2 (100 ng/ml). pSTAT5 expression in GFP+ cells. Untransduced cells (GFP-) are a negative control. The percentages of pSTAT5-expressing cells and MFI (in parenthesis) are shown.

5.3.2 γ c is Recruited to the DC Contact Interface and this Does Not Depend on its Signaling Function

To investigate whether γ c localizes at the DC IS, γ c^{-/-} DCs were transfected with γ c^{WT}-GFP, γ c^{Mut}-GFP or with GFP-control vector. Similar transduction efficiencies were achieved as demonstrated by GFP expression (Figure 5.7A). After overnight stimulation with LPS and OVA, transduced DCs were settled on bilayers and followed by live cell imaging. Figure 5.7B shows representative images of GFP-expressing DCs interacting with bilayers over time. Notably, γ c^{WT}-GFP was recruited to the DC interface and accumulated within MHC II clusters. Furthermore, γ c^{WT}-GFP progressively colocalised with MHC II, as demonstrated by an increase in the coefficient of colocalisation (Rr) from 0.6 to 0.9 over 25 minute (Figure 5.7C). In contrast, no increase in Rr was seen in GFP control-transfected cells, thereby suggesting specific γ c transport to and association with MHC II at the DC IS. Interestingly, γ c recruitment to the DC contact interface did not seem to depend on its signaling functions as γ c^{mut}-GFP accumulated to the interface to a similar degree and with similar kinetics as γ c^{WT}-GFP (Figure 5.7D). Final GFP mean fluorescence intensities were significantly higher ($p = 0.002$) in cells expressing either γ c^{WT/mut}-GFP compared to those expressing the GFP control vector (≈ 4 -fold increase), thus indicating specific γ c enrichment at the DC interface (Figure 5.7D). Taken together, these data indicate γ c recruitment within MHC II clusters at the DC IS and suggest that this does not depend on its signaling function.

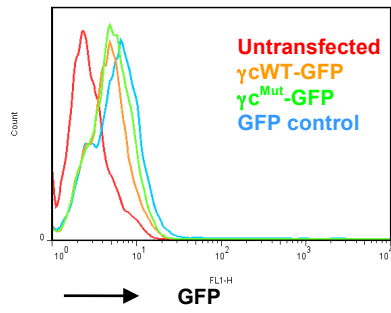
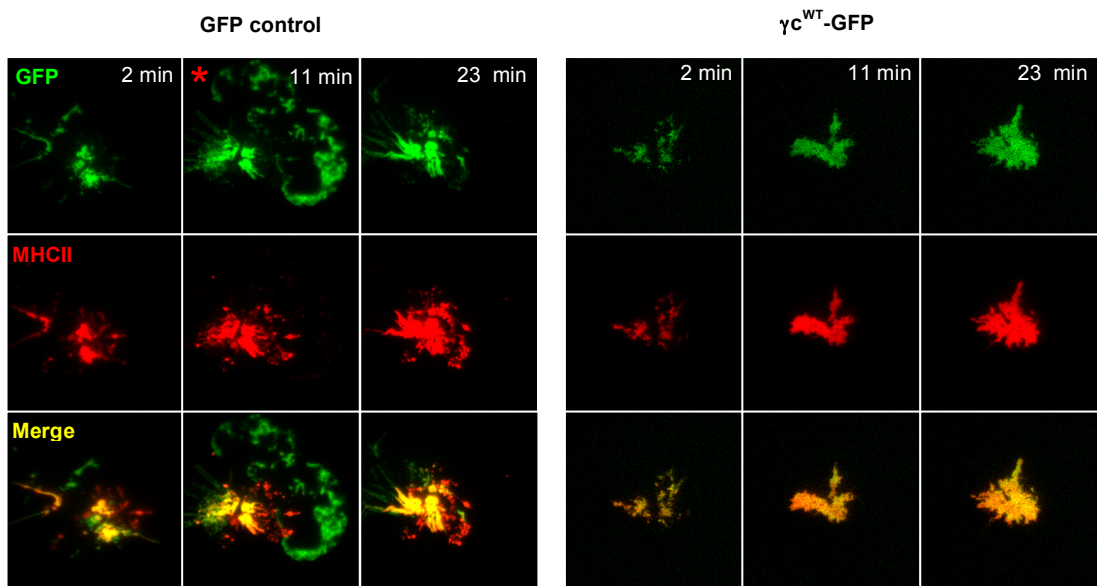
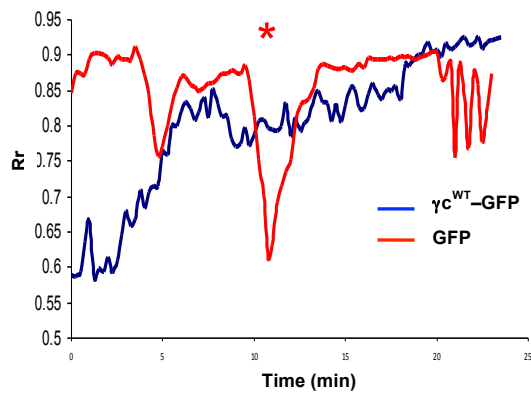
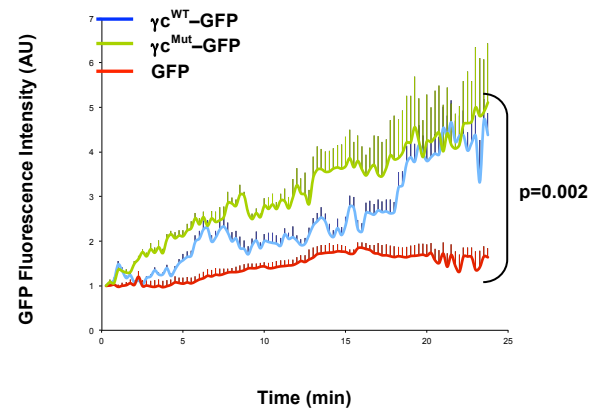
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Figure 5.7 γ_c is recruited to the DC interface and colocalises with MHC II. $\gamma_c^{-/-}$ DCs were transfected either with lentiviral vectors encoding γ_c^{WT} -GFP, γ_c^{Mut} -GFP or with pmaxGFP® Control Vector. 4 hours post transfection, cells were LPS matured and pulsed with OVA. 24 hours post transfection, cells were (A) assayed for GFP surface expression and (B-D) incubated on glass-supported planar bilayers and imaged by TIRFM at 37°C for 25 min. (B) Representative TIRFM images showing GFP (green) and MHC II (red) accumulation at the contact interface of $\gamma_c^{-/-}$ DCs expressing GFP control (left) or γ_c^{WT} -GFP (right) vectors (C) Time course analysis of GFP and MHC II colocalisation (R_r =coefficient of colocalisation). Asterix (*) corresponds to lamellipodial extension indicated in B and explains drop in R_r (D) Time course analysis of GFP average fluorescence intensities in arbitrary units (AU) at the contact interface normalized with respect to fluorescence at $t=0$ (mean \pm s.e.m, n=4). P values, two-tailed students t-test.

5.3.3 IL-15R α Associates with MHC II in DCs

We next examined whether the γ c heterodimer, IL-15R α , associates with MHC II in DCs, as has been reported for lipid rafts of human T lymphoma cells²⁶⁶. To this end, DC lysates were immunoprecipitated with anti-MHC II and immunoblotted with anti-IL-15R α . Interestingly, immunoprecipitation of MHC II yielded considerable amounts of co-precipitated IL-15R α and this occurred comparably in both WT and γ c^{-/-} DCs (Figure 5.8) thereby suggesting that the γ c does not influence the cellular association of IL-15R α and MHCII.

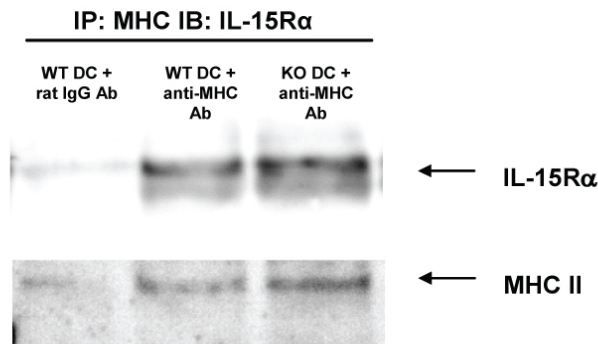


Figure 5.8 Cellular association of DC IL-15R α and MHCII occurs independently of the γ c. Cell lysates of LPS-matured OVA-pulsed WT and γ c^{-/-} DCs were immunoprecipitated with anti-MHC II, separated by SDS-page and immunoblotted with anti-IL-15R α . The blot was re-probed with anti-MHC II (lower lane) to demonstrate protein loading in each lane.

5.3.4 $\gamma c^{-/-}$ DCs Inefficiently Recruit and Associate IL-15R α with MHC II at the DC Interface

Having demonstrated that DC-expressed γc is required for IL-15 transpresentation and that it is recruited to the IS, we hypothesised that that the γc might play a role in recruiting or stabilising IL-15R α at the DC IS. To test this, LPS-matured OVA-pulsed DCs were incubated on lipid bilayers coated with or without fluorescently labelled I-A^b then stained for IL-15R α . This showed that in WT DCs IL-15R α accumulated at the DC interface and colocalised with MHC II (Rr=0.7) (Figure 5.9A,D). Interestingly, IL-15R α accumulation at the WT DC contact interface was significantly enhanced upon interaction with bilayers coated with I-A^b compared to those coated without ($p \leq 0.001$, Figure 5.9B). Despite similar levels of MHC recruitment to the interface (Figure 5.9C), IL-15R α recruitment upon MHC II ligation was significantly reduced in $\gamma c^{-/-}$ DCs compared to WT ($p \leq 0.001$, Figure 5.9B). Colocalization of MHC II and IL-15R α was also significantly reduced in $\gamma c^{-/-}$ DCs ($p \leq 0.005$) (Figure 5.9A,D). Taken together, these data suggest IL-15R α polarization at the IS during MHC II ligation and implicate the γc in regulating this process.

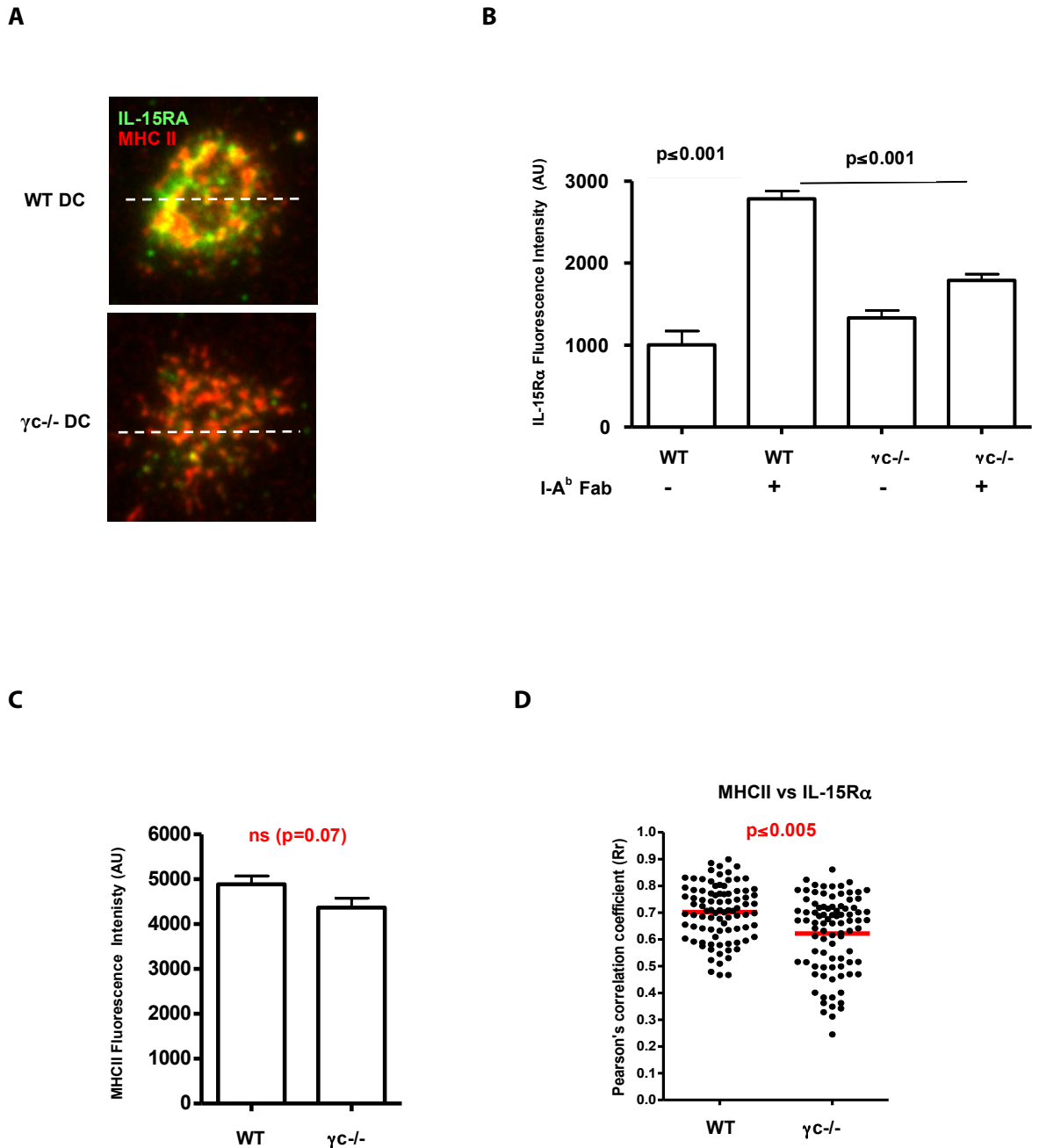


Figure 5.9 $\gamma c^{-/-}$ DCs inefficiently recruit and associate IL-15R α with MHC II at the DC interface. DCs were LPS-matured and pulsed with OVA. The following day, cells were incubated on glass-supported planar bilayers containing LFA-1+/- Alexa-568-I-A^b Fab. After 15 min incubation at 37°C, cells were fixed and stained for IL-15R α . (A) Representative TIRFM images showing IL-15R α (green) and MHC II (red) accumulation at the DC contact interface (B) Bar graphs indicate quantification in arbitrary units (AU) of average IL-15R α (B) and MHC II (C) fluorescence intensities (mean \pm s.e.m) at contact interfaces (D) Quantification of level of colocalization (Rr) of MHC II and IL-15R α at DC interfaces. P values, one-way ANOVA (Bonferroni's multiple comparison test).

5.4 γc Restores IL-15R α Recruitment and Transpresenting Functions at the DC Interface

5.4.1 γc is Required for IL-15R α Recruitment to the DC Interface

To confirm the requirement of the γc for IL-15R α recruitment to the DC interface, γc^{WT} - and γc^{mut} -GFP were reintroduced in $\gamma c^{-/-}$ DCs. After overnight stimulation with LPS and OVA, transduced DCs were settled on bilayers coated with unlabelled LFA-1 and I-A^b then stained for IL-15R α . As expected, reintroduction of both γc -GFP constructs resulted in the restoration of IL-15R α accumulation at the interface (Figure 5.10A,B). A significant increase in the levels of IL-15R α was observed in cells transfected with γc^{WT} -GFP compared to the control GFP vector (≈ 1.9 -fold increase) ($p \leq 0.05$). An increase in the level of IL-15R α recruitment was also seen with γc^{mut} -GFP (≈ 1.7 -fold increase), although this did not achieve significance (Figure 5.9A,B). A correlation of 0.53 and 0.73 (R^2) was observed between IL-15R α and γc^{WT} - or γc^{mut} -GFP, respectively, and in both cases this was significantly higher ($p \leq 0.001$) compared to GFP control transfected cells (Figure 5.10C). Taken together, these data confirm that the defects observed in IL-15R α recruitment at the DC interface can be specifically attributed to the lack of γc expression.

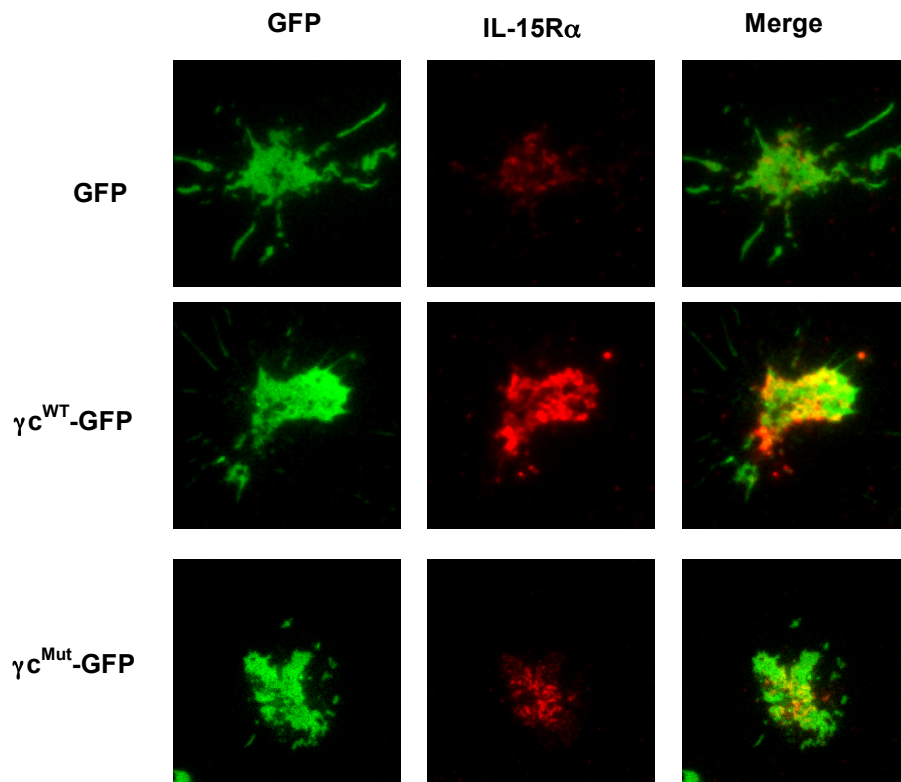
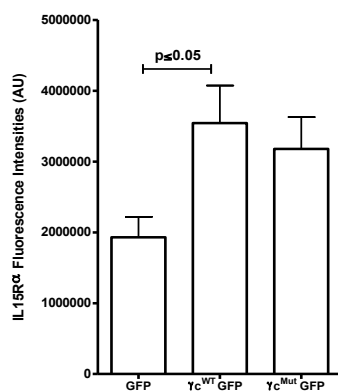
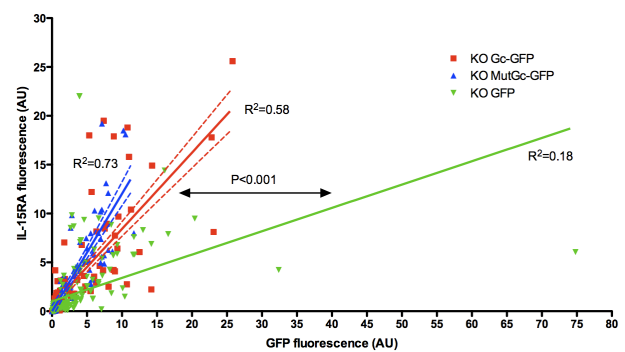
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Figure 5.10 γ_C is required for IL-15R α recruitment to the DC interface. $\gamma_C^{-/-}$ DCs were transfected either with lentiviral vectors encoding $\gamma_C^{WT/mut}$ -GFP or with pmaxGFP® Control Vector. 4 hours post transfection, cells were LPS-matured and pulsed with OVA. 24 hrs post transfection, cells were incubated on glass-supported planar bilayers containing LFA-1 and I-A^b Fab for 15 mins. Cells were then fixed and stained for IL-15R α . (A) Representative TIRFM images showing GFP (green)

and IL-15R α (red) accumulation at the DC contact interface (B) Quantification in arbitrary units (AU) of integrated IL-15R α fluorescence intensities (mean \pm s.e.m) at contact interfaces for DCs transfected with each construct (C) Linear regression analysis of GFP versus IL-15R α fluorescence intensities (expressed in AU $\times 10^6$). For each condition, 38-43 cells were evaluated. P values, one-way ANOVA (Bonferroni's multiple comparison test).

5.4.2 γ c is Required for IL-15R α Transpresenting Functions at the DC Interface

To confirm that the γ c is required for IL-15R α -mediated IL-15 transpresentation to CD4+ T cells, STAT5 activation was analysed in CD4+ T cells after coculture with γ c^{-/-} DCs transfected with γ c^{WT}-, γ c^{mut}- or control GFP vectors. WT DCs transduced with GFP control vector were used as a positive control. Despite similar levels of transduction compared to control transfected cells (Figure 5.11A), γ c^{-/-} antigen-pulsed DCs expressing either of the γ c-GFP constructs induced higher levels of STAT5 phosphorylation in CD4+ T cells compared to γ c^{-/-} antigen-pulsed GFP control-transfected cells ($p \leq 0.05$) (Figure 5.11B,C). Similar restoration of STAT5 activation was observed in CD4+ T cells by re-expression of γ c^{WT} and γ c^{mut}-GFP in γ c^{-/-} DCs (Figure 5.11B,C), suggesting that γ c expression as opposed to signaling is required for DC-mediated IL-15 transpresentation. Taken together, these data strongly implicate that, by controlling the recruitment of IL-15R α at the DC IS, the γ c is required for optimal IL-15 transpresentation to CD4+ T cells.

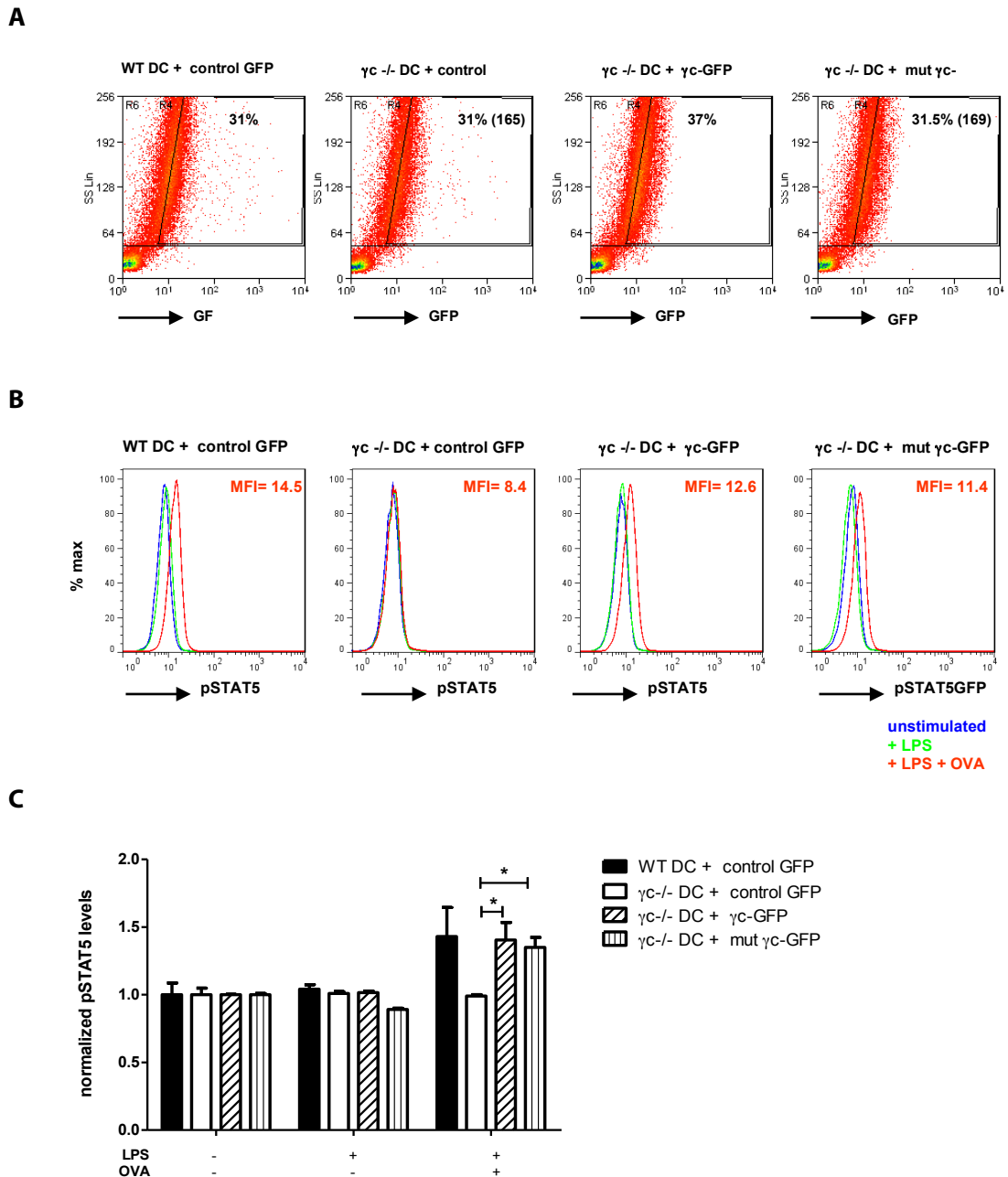


Figure 5.11 γc in DCs is required for STAT5 activation in CD4⁺ T cells. DCs were transfected either with lentiviral vectors encoding $\gamma c^{WT/mut}$ -GFP or with pmaxGFP® Control Vector. 4 hours post transfection, cells were either left untreated or stimulated overnight with LPS alone or in combination with OVA. The following days, cells were co-cultured with CD4⁺ T cells, which had been serum-starved for 3 hours, at a 1:1 ratio for 10 min. Cells were then fixed and stained with anti-pSTAT5. (A) Transduction efficiencies measured as GFP expression in DCs transfected with the indicated constructs. DCs are gated according to side scatter (SS). The percentages of GFP-expressing DCs and MFI (in parenthesis) are shown (B) pSTAT5 expression in CD4⁺ GFP- gated cells. Shown are MFIs for CD4⁺ T cells cultured with OVA-pulsed DCs under the indicated conditions (C) Normalized pSTAT5 fluorescence intensities relative to unstimulated control. Data presented are from 2 independent experiments. * $p \leq 0.05$, two-tailed student's t-test.

5.5 Conclusions

To date, imaging of the DC IS has only been approached through 3-dimensional reconstructions of DC-T cell interface. Because of the limitations of fluorescence imaging, the molecular events underlying the formation of the DC IS have been poorly defined. The TIRFM/lipid bilayer platform developed in collaboration with Prof. Dustin's laboratory thus provided a novel approach to study the DC synaptic membrane at high molecular resolution. The data presented in this chapter is, to our knowledge, the first detailed view of DC synapses as they occur *in vitro*. The fundamental findings are twofold. First, DC synapses are very dynamic, as demonstrated by active MHC II and actin recruitment and accumulation at contact interfaces. Secondly, the DC IS accumulates cytokine receptors which are important for effective T cell activation. Collectively, our data highlights the importance of events on the DC side for IS assembly and organisation and implicates the γ_c in regulating cytokine receptor recruitment at the DC IS.

Based on early video imaging studies, it has been inferred that DC-T cell synapses are very motile structures^{251,267}; however, the spatio-temporal dynamics of these events has never been studied. Through live cell TIRFM imaging of DCs interacting with our novel lipid bilayer system containing LFA-1 and Fab fragments specific for I-A^b, we observed dynamic clustering of MHC II at the interface. MHC II was shown to progressively accumulate at the DC IS and interestingly this was not affected by the absence of DC γ_c . Furthermore, in contrast to T cells where TCR engagement delivers a stop signal that strongly reduces T-cell mobility²⁶⁸, DC mobility at the contact interface never completely stopped, even after MHC II ligation. We observed continuous crawling and spreading of the DC along the bilayer, and this probably explains sustained T-cell membrane ruffling at synaptic interfaces, as has been observed by others²⁶⁹. We hypothesize that continuous DC mobility, which most likely relates to the observed dynamic actin rearrangement at the interface, also probably influences the patterning of the synapse, and may explain why bull-eye structures in antigen-dependent T cell-DC synapses are much less frequent than multifocal ones, as has been previously described²⁰⁵.

Synaptic delivery of IL-12 by DC¹⁹⁷ and of IL-2, IL-4, IL-5 and IFN- γ by CD4+ T cells^{194,198} have previously been reported and suggest that cytokine enrichment at the IS might be critical for T cell effector functions. Having established that IL-15 transpresentation depends on both DC γ c expression and on MHC II ligation, we hypothesised that the γ c might be recruited to the DC IS and influence the synaptic localization of IL-15R α . Using lipid bilayers, we showed that DC γ c is recruited to the DC interface and progressively colocalises with MHC II. Interestingly, this did not depend on its signaling functions as expression of a truncated version of the γ c lacking the cytoplasmic signaling domain (γ c^{mut}-GFP) resulted in similar localization to the DC contact interface. Furthermore, we observed that the γ c heterodimer, IL-15R α , is also recruited to the DC IS and colocalises with MHC II, as been previously been shown on the surface of T cells²⁶⁶. In addition, we demonstrated cellular association of IL-15R α with MHC II and, although we have no evidence for this, it might be possible to hypothesise that these two molecules might also traffic to the membrane as a complex, as has been proposed for MHC II and the costimulatory B7 molecule¹⁰⁶.

Of particular note was our finding that IL-15R α recruitment to the DC IS occurs only upon MHC II ligation. This suggested to us MHC-driven lateral surface redistribution of IL-15R α to the synaptic interface and may explain why IL-15 transpresentation and STAT5 signaling are in seen in T cells only after TCR engagement. IL-15R α polarization to the IS has been previously observed at DC-NK cell synapses although here, IL-15R α was specifically shown to accumulate on the NK cell whereas no selective aggregation was observed on the DC¹⁹⁹. The discrepancy with our findings may relate to the failure to detect specific enrichment through confocal microscopy imaging or to inherent differences in the DC:NK synapses. However, we think that IL-15R α must be present at the DC IS to transpresent to NK cells so technical explanations remain more likely.

In this chapter we also showed that, despite normal levels of intracellular IL-15R α and MHC II recruitment to the DC IS, γ c^{-/-} DCs inefficiently accumulated and colocalised IL-15R α with MHC II at the contact interface. This was also independent

of γ_c signaling functions as re-expression of either γ_c^{WT} - or γ_c^{mut} -GFP restored IL-15R α localization and transpresenting functions at the DC interface. Taken together these observations raise the exciting possibility that the γ_c is required for lateral membrane redistribution or stabilization of IL-15R α at the DC interface. We favour the prediction that this might occur through direct interaction of the γ_c and of IL-15R α given that these two receptors have been previously shown to be associated and compartmentalized within the same membrane microdomains²⁶⁶ and that γ_c signaling function was shown to be dispensable. Alternatively, we hypothesise that this might occur through cytoskeletal-mediated processes. This was initially suggested to us by the reported association of IL-2/15R β and γ_c with both tubulin and actin and by the fact that inhibition of polymerization of either cytoskeletal components resulted in attenuated γ_c -associated signaling^{15,260}. Interestingly, we observed that in the absence of the γ_c , DCs polymerize less filamentous actin at the interface and we therefore hypothesise that defects in IL-15R α accumulation might be due to defective cytoskeletal confinement at the IS. This would be consistent with recent reports showing that IL-2R receptor confinement in membrane nanodomains is achieved through interaction with the cytoplasmic meshwork¹⁵ and similarly, that the DC cytoskeleton plays an important role in restricting cytokine delivery at the IS¹⁹⁷. Further investigation is required to determine which of these plausible mechanisms contributes to γ_c regulation and molecular organization of the DC IS.

5.6 Final Conclusions

- the DC IS is a dynamic structure characterised by active recruitment of MHC II and actin.
- selective synaptic recruitment of DC IL-15R α upon MHC II ligation suggests that IL-15 transpresentation may represent a mechanism by which DCs deliver cytokine signals specifically at the interface with activated T cells.
- DC-expressed γ c is recruited to the IS where it colocalises with MHC II and mediates recruitment of IL-15R α , independent of its signaling functions.

*Chapter Six- Analysis of the Role of the Common Cytokine
Receptor γ c in Dendritic Cells for Regulating NK Cell Functions*

6.0 Aims

- Assess whether DC-expressed γ_c affects activation of γ_c -expressing NK cells.

6.1 Introduction

NK cells are innate immune cells that play a key role in protecting the host against viral pathogens or transformed cells through recognition of foreign or altered MHC molecules. The function of NK cells results from a balance between inhibitory and activating signals delivered through a vast array of surface receptors belonging to the immunoglobulin-like receptor and C-type-lectin receptor families²⁷⁰ and is also affected by pro-inflammatory cytokines¹⁴⁰. In addition to their direct effect on the activation and proliferation of specific NK cell subsets, cytokines also can modify the signals delivered by receptor-mediated interactions by influencing the expression of NK-specific receptors/ligands¹⁴¹.

Although their principal function is the activation of naïve T cells, DCs have also been shown to participate in the activation NK cells through their secretion of IL-2, IL-12, IL-18 and IFN- α ^{140,141,271-273}. The γ_c -family IL-15 cytokine has been found to synergise with IL-12 in inducing NK cell IFN- γ production¹³⁹ and it has been more recently recognized that IL-15 exerts its actions on NK cells mainly through DC-mediated IL-15 transpresentation. This mechanism has been shown to promote NK cell activation and proliferation, *in vitro* and *in vivo*^{33,37,243,274}. IL-15 also plays an important role in NK cell physiology as evidenced by the fact that IL-15^{-/-} and IL-15R α ^{-/-} mice lack NK cells^{56,57}. In keeping with this, IL-15 transpresentation has been also shown to be important for the maintenance/survival of NK cells in peripheral tissues^{36,275}.

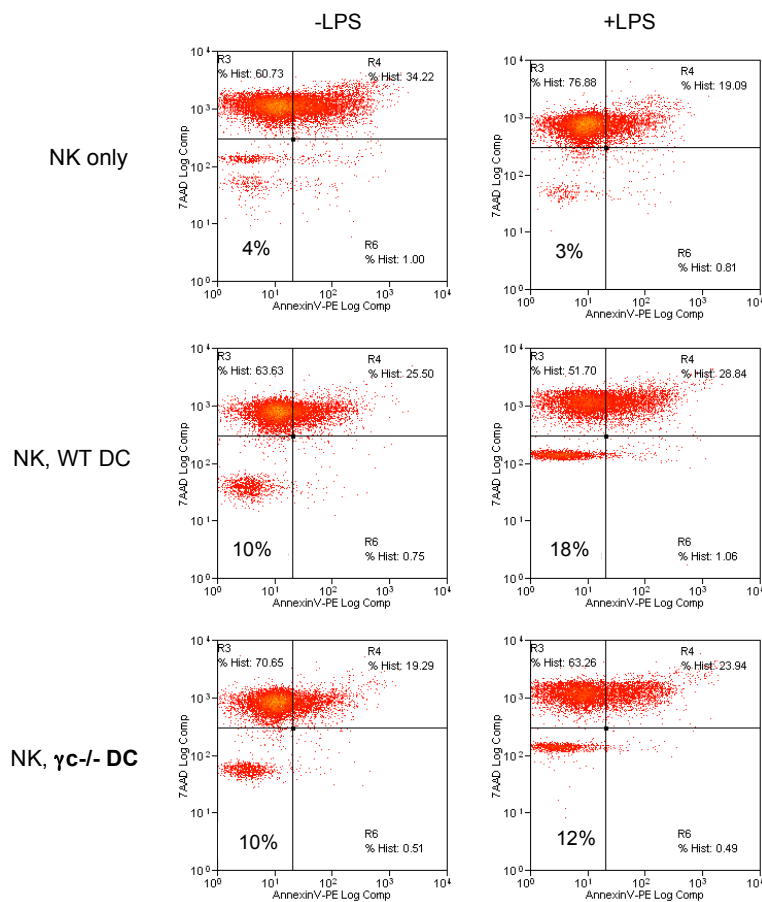
In the light of the findings described in Chapter 4 and 5, there was the possibility that DC-expressed γ_c might play a critical role in regulating NK cell functions, possibly through IL-15-related processes as observed for CD4⁺ T cells.

6.2 Absence of the Common Cytokine Receptor γ_c in Dendritic Cells Impairs NK Homeostasis and Activation

6.2.1 γ_c $-/-$ DCs Support NK Cell Survival Less Efficiently

LPS-stimulated DCs have previously been shown to provide essential survival signals to NK cells through upregulation of surface IL-15/IL-15R α complex expression³⁷. Having established defective IL-15 transpresentation to CD4+ T cells by γ_c $-/-$ DCs, it therefore seemed plausible to hypothesise that γ_c deficiency might alter the capacity of DCs to provide survival signals to NK cells. To test this, NK cells were cultured with DCs, which had been cultured with or without LPS, and quantified for NK cell viability after 24 hours of coculture. In keeping with the literature, LPS-stimulation of WT DCs induced a significant increase ($p \leq 0.05$) in NK cell survival compared to untreated DCs (≈ 1.8 -fold increase). Interestingly, this did not occur in γ_c $-/-$ DCs. Overall, LPS-stimulated γ_c $-/-$ DCs supported NK cell survival less efficiently compared to WT DCs ($14 \pm 1.5\%$ versus $19 \pm 0.6\%$ viable cells), although this did not achieve significance ($p = 0.06$) (Figure 6.1A,B).

A



B

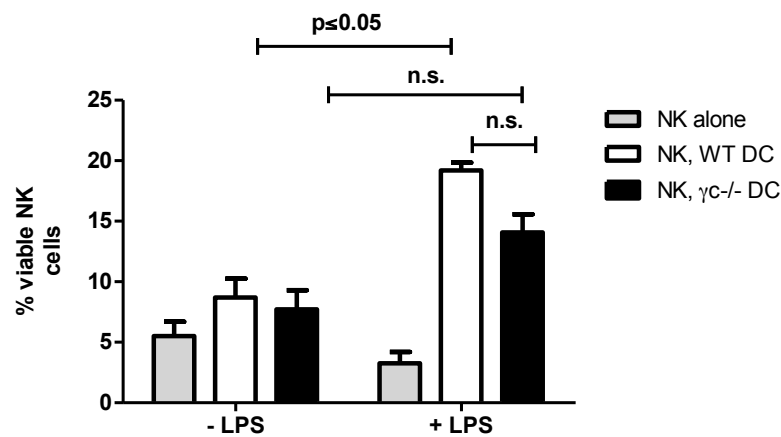


Figure 6.1 $\gamma c^{-/-}$ DCs are less efficient at inducing NK survival. DCs were cultured overnight in the presence or absence of LPS. The following day, cells were washed and co-cultured for 24h hrs with DX5 selected NK cells derived from spleen of C57BL/6 WT mice. Viable cells are Annexin V-PE and 7-AAD negative cells. (A) Representative plots showing Annexin V-PE and 7-AAD staining on NK1.1 gated cells (B) Bar graphs of data shown in A. Data are from 3 independent experiments (mean \pm s.e.m). P-values, one-way ANOVA (Bonferroni's multiple comparison test).

6.2.2 $\gamma c^{-/-}$ DCs Fail to Induce NK Cell Activation.

To determine whether DC γc is required for NK cell activation as well as for NK cell survival, the supernatants from the cocultures described in 6.2.1 were tested for IFN- γ release. Stimulation of WT DCs with LPS induced the production of IFN- γ by NK cells (≈ 2.3 -fold increase). Remarkably, LPS-matured $\gamma c^{-/-}$ DCs stimulated significantly less ($p \leq 0.001$) NK cell IFN- γ production compared to WT DCs (0.5 ± 0.4 ng/ml versus 2.8 ± 0.6 ng/ml) (Figure 6.2).

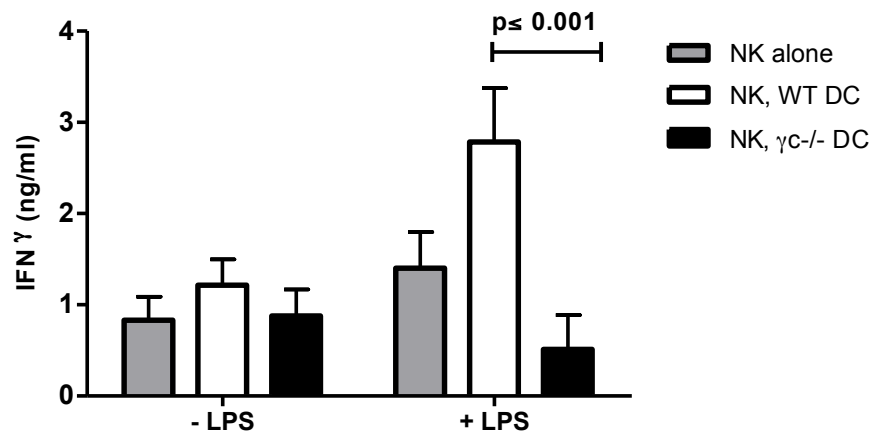


Figure 6.2 $\gamma c^{-/-}$ DCs are unable to activate NK cells. Supernatants from the experiment described in section 6.2.1 were assayed for IFN- γ production by ELISA. Data are from 3 independent experiments (mean \pm s.e.m). P values, one-way ANOVA (Bonferroni's multiple comparison test).

6.2.3 $\gamma c^{-/-}$ DCs Induce Normal Proliferation of NK Cells.

IL-15/IL-15R α complexes have been shown to induce NK cell proliferation *in vivo*²⁴³. To analyse whether γc deficiency alters the capacity of LPS-stimulated DCs to induce NK cell proliferation, CFSE labelled NK cells were cultured *in vitro* with DCs and analysed for CFSE dilution after 48 hours of co-culture. Although, minimal levels of proliferation were observed, $\gamma c^{-/-}$ DCs induced NK cell proliferation as efficiently as WT, at all of the tested ratios (Figure 6.3A,B).

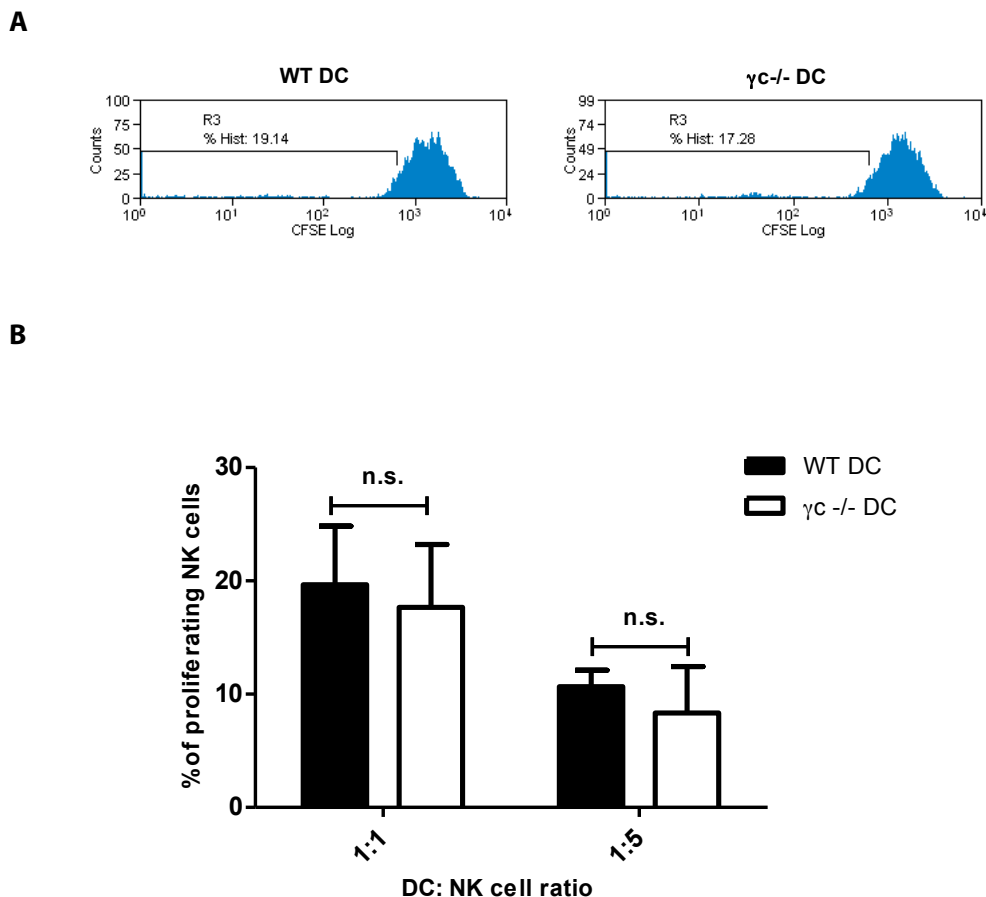


Figure 6.3 $\gamma c^{-/-}$ DCs induce normal NK cell proliferation. DCs were cultured overnight with LPS. The following day, cells were washed and cocultured for 48 hrs with CFSE-labelled DX5 selected NK cells derived from spleen of C57BL/6 WT mice at 1:1 and 1:5 DC:NK cell ratios (A) Representative plots of CFSE dilution in NK1.1 gated cells after co-coculture with DCs at 1:1 DC:NK cell ratios. Gates are set on NK cells cultured alone (B) Bar graphs represent quantification of NK proliferation from 3 independent experiments (mean \pm s.e.m). P values, one-way ANOVA (Bonferroni's multiple comparison test).

6.3 Conclusions

Although there remains much controversy about where the DC/NK cell encounter takes place *in vivo*, it is clear that the outcome of this cellular interaction critically depends on the activation state of the DC. Indeed, TLR-stimulated DCs have been found to regulate NK cell functions and one way this is thought to happen is through secreted cytokines, such as IL-2, IL-12, IL-18 and IFN- α ¹⁴⁰. In this chapter, we have shown that LPS-stimulated $\gamma c^{-/-}$ DCs are inefficient in mediating NK survival and activation, despite normal IL-2, IL-12 and IFN- α release (Figure 3.11). This suggested that soluble cytokines might not account for the observed defects but that these might rather be explained by cell-contact dependant factors. Notably, DC expressed IL-15/IL-15R α complexes have been shown to be critical for NK homeostasis and priming^{33,37,243,274} and in the light of the data presented in Chapters 4 and 5, we hypothesise that DC-expressed γc might also be critical for efficient IL-15 trans-signaling and presentation to NK cells. In contrast, NK proliferation appeared not to be affected by the absence of DC-expressed γc . However, these results may not be very reliable as the levels of proliferation detected were minimal and much lower compared to those previously observed²⁴³. This may be due to the fact that in those systems, NK cells were directly stimulated with IL-15/IL-15R α complexes, which may have provided a stronger stimulus.

Time did not allow further investigation on defective NK cell activation by $\gamma c^{-/-}$ DCs but future work could focus on more experiments examining NK cell functions and on confirming whether the defects observed here are also due to failure to transpresent IL-15, as observed for CD4+ T cells. Of note, polarization of DC-derived IL-12 at the synaptic interface has been shown to be important for NK functions¹³⁷ and IL-15R α has been observed to localise at the DC:NK IS, although not specifically on the DC side of the synapse, suggesting that IS-restricted cytokine signaling might be critical for NK functions. It would therefore be very interesting to study the localisation of DC IL-15R α at the DC-NK cell IS. Furthermore, in the light of our findings that IL-15R α accumulates at the DC/bilayer interface upon MHC II engagement, we hypothesise that IL-15 transpresentation to NK cells might also be regulated by selective engagement of certain NK-DC receptor pairs. Notably, murine DCs have been shown to express various ligands for NK-cell activating

receptors, such as MICA and/or MICB¹⁴¹. Future work could address whether IL-15R α recruitment to the DC:NK IS depends on the specific engagement of activating receptors.

Altogether, the data presented in this chapter represent a preliminary insight on how DC-expressed γ c may regulate NK functions and hold promise for exciting future investigations.

6.4 Final Conclusions

- γ c^{-/-}DCs inefficiently regulate NK functions, such as survival and activation.
- Further work is required to clarify whether this is due to defective IL-15 transpresentation.

Chapter Seven-Discussion

The γ_c is best known for its importance in T and NK cell homeostasis and function^{4,276}. This is demonstrated by the human disease X-SCID where the lack of γ_c expression and function results in failure of T and NK cell development. Although there is little data regarding DCs in X-SCID, an increasing body of evidence suggests that γ_c /JAK3 expression and/or signaling are important for their normal function^{37,216-220}. In agreement with these observations, here we specifically demonstrate that DC-expressed γ_c is important for CD4+ T cell activation during cognate interaction. IL-15R α is the heterodimer of the γ_c and is critical for DC-mediated transpresentation of IL-15 to β/γ_c expressing cells^{27,36,257}, hence we reasoned that the lack of γ_c might affect IL-15 transpresentation with potential influences on CD4+ T cell activation. It is already known that IL-15 transpresentation is important for NK and CD8+ T cell homeostasis and function^{33,36,257} but our data intriguingly showed that DCs also transpresent IL-15 to CD4+ T cells. Interestingly, this appeared to depend on a TCR:MHC signal and is consistent with previous findings in CD8+ T cells where simultaneous administration of IL-15 complex together with a TCR signal resulted in enhanced proliferation²⁴². Our findings are particularly novel as, although IL-15 has previously been reported to enhance the proliferation of CD4+ T cells in response to mitogens and TCR stimulation^{29,277-280}, a specific role for IL-15 transpresentation to CD4+ T cells has never been documented. Most importantly, here we demonstrate that the γ_c is required for effective IL-15 transpresentation. Our findings therefore challenge the widely held view that IL-15R α is the chain responsible for cytokine transpresentation as has been inferred through *in vivo* studies with IL-15R α -competent, IL-2/15R β -deficient cells^{36,38}.

Having demonstrated that IL-15 transpresentation depends both on TCR engagement and on DC γ_c expression, we reasoned that the lack of the γ_c might lead to defective CD4+ T cell activation by affecting the specific localisation of IL-15R α to the IS. This was suggested to us by two factors. Firstly, γ_c -containing receptors have been shown to be present in supramolecular complexes of MHC and adhesion molecules on the surface of T cells²⁶⁶ and secondly, IL-15R α has been reported to localise at the IS with NK cells¹⁹⁹. Using a novel lipid bilayer system mimicking the T cell side of the IS, we visualised the DC synaptic membrane at high molecular resolution and observed that IL-15R α is specifically recruited to the DC interface and colocalises

with MHC II. Remarkably, we also showed that IL-15R α recruitment to the IS occurs only upon MHC II ligation and this is consistent with the idea that IL-15 trans-signaling depends on cognate TCR interactions, as proposed above. We therefore envisage a scenario in which TCR engagement induces MHC-driven lateral surface redistribution of IL-15R α at the IS thereby allowing correct positioning for effective transpresentation to T cells. Most importantly, we also demonstrated that the γ_c is recruited within MHC II microclusters at the DC interface and mediates recruitment of IL-15R α . Interestingly, γ_c -/- DCs exhibited impaired accumulation of IL-15R α at contact interfaces as well as diminished colocalisation with MHC II thereby suggesting that the γ_c could act as a bridge between MHC II and IL-15R α . DC-mediated IL-15 transpresentation seemed to be independent of γ_c signaling function as a γ_c -GFP fusion protein lacking the signaling domain (γ_c^{mut} -GFP) was as efficient as full-length γ_c (γ_c^{WT} -GFP) in restoring IL-15R α localisation and transpresenting functions at the IS in γ_c -deficient cells.

Having excluded the contribution of its signaling function, we hypothesized that the γ_c might instead influence DC-mediated IL-15 transpresentation by stabilising the assembly of IL-15R subunits at the IS. Although the precise mechanisms of γ_c -family cytokine receptor assembly are incompletely understood, the γ_c has been observed to associate with the IL-15R α and β chains at the cell surface even in the absence of IL-15²⁶⁶, suggesting preassembly of IL-15R. Furthermore, blocking IL-15R α association with IL-2/15R β has been shown to inhibit IL-15 transpresentation³⁷ therefore suggesting that stable interaction between receptor subunits might be required for effective transpresentation. Having observed normal surface expression of both the α and β subunits of the IL-15R, our data raises the exciting possibility that the γ_c may modulate IL-15 signalling *in trans* by directing the assembly and/or spatial positioning of associated cytokine receptors. Altogether, our findings using an *in vitro* system highlight the importance of DC γ_c for the regulation of molecular events on the DC side of the IS. Further investigation is required to dissect possible mechanisms and importantly, to address the relative significance of these phenomena for DC function *in vivo*. Whether the localisation of

IL-2R β at the IS is influenced by DC γ c will also be an interesting area of future study.

In addition, it is also possible to speculate that the γ c might regulate IL-15 transpresentation by assisting the binding of IL-15 to IL-15R α . However, given that the intrinsic binding affinity of IL-15 to the γ c and IL-2/15R β heterodimer ($K_d \cong 1$ nM) is much lower compared to IL-15R α alone ($K_d \cong 10$ pM)⁸, we think that this is unlikely. IL-15 binding to IL-15R α has been difficult to assess as most of the commercially available anti-IL-15 antibodies bind to epitopes that are masked when IL-15 is complexed with IL-15R α . Free IL-15 was undetectable in culture supernatants of both WT and γ c -/- DCs (data not shown) and given that assembled IL-15/IL-15R α complexes can either stay cell bound or be proteolytically released^{33,34}, our results suggest normal binding of IL-15 to IL-15R α thus masking detection. Definite conclusions to this question will be an interesting area for further study and may require competitive binding assays with radiolabelled IL-15.

Taken together, our data supports the idea that IL-15 transpresentation acts as costimulatory signal for antigen-mediated T cell activation (Figure 7.1). We favour a model in which TCR engagement induces MHC-driven lateral surface redistribution of DC IL-15R α to the synaptic interface. In this scenario, the γ c appears to mediate the recruitment and/or stabilisation of IL-15R α at the DC IS and to be necessary for IL-15 dependant STAT5 activation. STAT5 activation is known to be important for a variety of functions of T cells, including homeostatic proliferation²⁵³. We have shown that IL-15R α blockade in γ c-expressing DCs results in reduced antigen-mediated proliferation and thereby suggest that IL-15 transpresentation and downstream STAT5 signaling could function as a costimulatory signal to augment TCR function. This would be consistent with studies in STAT5A knockout mice where defective T cell proliferation was observed in response to TCR stimulation²⁸¹.

Furthermore, we predict that costimulation provided to T cells through IL-15 transpresentation specifically occurs through localised cytokine signaling at the IS. In contrast to previous reports demonstrating that STAT5 activation can be induced by a TCR antibody²⁸², our data indicates that this requires both an IL-15R and TCR

signal. This suggests that the ability of IL-15R α to trans-present IL-15 might depend on the appropriate positioning of IL-15R next to sites of TCR engagement. Previous reports have also suggested that spatial synergism between IL-15R and TCR is critical for enhanced CD8⁺ T cell responses²⁴⁰. Here, CD8⁺ T cell responses were amplified by co-presentation of IL-15 and anti-CD3 on the same surface. Similarly, STAT5 has been shown to assemble with the TCR and interestingly, this appeared to be mediated by the Lck kinase through the generation of tyrosine-phosphorylated docking sites within the associated TCR ζ chains²⁸². However, given that to our knowledge, the JAK-STAT and TCR signalling pathways are independent from each other, it still remains unclear whether or how cytokine-mediated STAT activation can be achieved upon simultaneous stimulation through the TCR. One possibility for this could be the creation of a shared signaling environment. A “kinase promiscuity model” has already been proposed to explain the synergism between the TCR and CD28 signaling pathways whereby TCR-induced activation of downstream signaling mediators would be amplified by the activate kinases provided by CD28 costimulation^{189,283}. Similarly, we envisage that TCR engagement could recruit kinases in proximity of the IL-15R and promote associated JAK/STAT signal transduction. Alternatively, removal of inhibitory phosphatase activity near engaged TCR, ie. “kinetic segregation model”, might contribute to enhancing JAK/STAT signaling. CD45 is a candidate molecule as it is excluded from the cSMAC and it is a suppressor of JAK kinases therefore a negative regulator of cytokine receptor signaling²⁸⁴. Altogether, our data raises the exciting possibility that IL-15 transpresentation to T cells could require both the TCR and cytokine receptors and that spatial coordination between these two signaling pathways might constitute a way to regulate cytokine receptor signaling and associated cellular functions. Nonetheless, it is still interesting to speculate about why we failed to detect colocalisation of TCR and pSTAT5 microclusters in our bilayer experiments. We believe that this could be explained by technical reasons. Naïve T cells have been recently shown to internalise the TCR very rapidly sometimes even bypassing cSMAC formation²⁸⁵ and it is possible that we may have failed to detect colocalisation at the chosen timepoint (ie. 30 minutes). Alternatively, as pSTAT5 leaves the cytokine receptor sites and translocate to the nucleus, we may not have detected association of pSTAT5 and TCR within the TIRFM field (\approx 200nm from the plasma membrane).

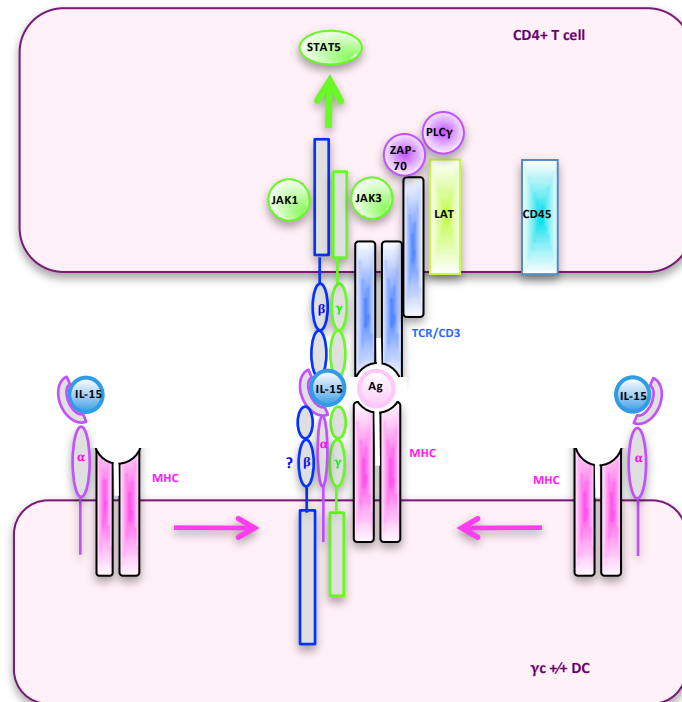


Figure 7.1 Spatial coordination model for IL-15 transpresentation. MHC II ligation induces lateral surface redistribution of DC IL-15R α to the synaptic interface. Effective IL-15 trans-signaling may require consequent IL-15R positioning next to sites of TCR engagement. This may be due to shared signaling environment/kinetic segregation of phosphatases.

Moreover, our findings have important clinical implications as they suggest that X-SCID patients lacking γc expression may also have defects in DC functions. Of course this may not be so relevant in a disease which is characterised by lack of T and NK cell populations but may instead be more important in patients where immune reconstitution is achieved through bone marrow transplant or gene therapy but were absence of chemotherapeutic conditioning results in retention of γc -deficient myeloid lineages. Interestingly, some of these transplanted SCID patients have been observed to suffer from long-term complications that are independent of lymphoid immune reconstitution, including a high incidence of severe cutaneous HPV infection^{62,63}, thereby suggesting defects of myeloid immunity. To investigate increased susceptibility to HPV infection, we chose to study the functional outcomes of DC γc -deficiency using a transgenic CD4+ T cell system. This was mainly because of the central role played by CD4+ T cells in the induction of anti-viral²⁸⁶

and more specifically in anti-HPV immunity, as suggested by the fact low CD4+ T cell counts associated with HIV infection are amongst the strongest risk factors for HPV persistence and related malignancies²⁸⁷. Of note, NK cells are also important for HPV immunity²⁸⁸ and we have some preliminary data suggesting defective NK activation by γ c-deficient DCs. Further studies will be required to dissect the importance of DC-expressed γ c for the induction of both innate and adaptive immune responses to HPV, both *in vitro* and *in vivo*. This could be approached through the use of HPV virus-like particles (VLPs), composed of the immunogenic wild-type L1 major capsid protein, or of long peptides spanning the HPV E6/E7 sequences. Both of these approaches have been extensively employed to examine specific anti-HPV responses *in vitro*^{289,290} and would be very useful to examine the role of DC-expressed γ c in HPV-specific immunity.

Finally, it is important to underline that keratinocytes, the HPV host cells, have been specifically shown to express and signal through γ c-containing receptors⁶⁹⁻⁷². It will therefore be interesting to examine whether defective γ c signaling in host keratinocytes might contribute to the increased susceptibility to HPV observed in bone-marrow transplanted SCID patients. One way to address this would be grow skin rafts from γ c-deficient keratinocytes and assess whether γ c-deficiency alters HPV life cycle and progression to disease.

Collectively, this study provides new insight into the molecular events that regulate cytokine transpresentation in DCs and demonstrates a novel role for IL-15 transpresentation in the induction of a productive CD4+ T cell response. Clearly, further investigations are required to shed light on the relevance of these events for anti-HPV immunity.

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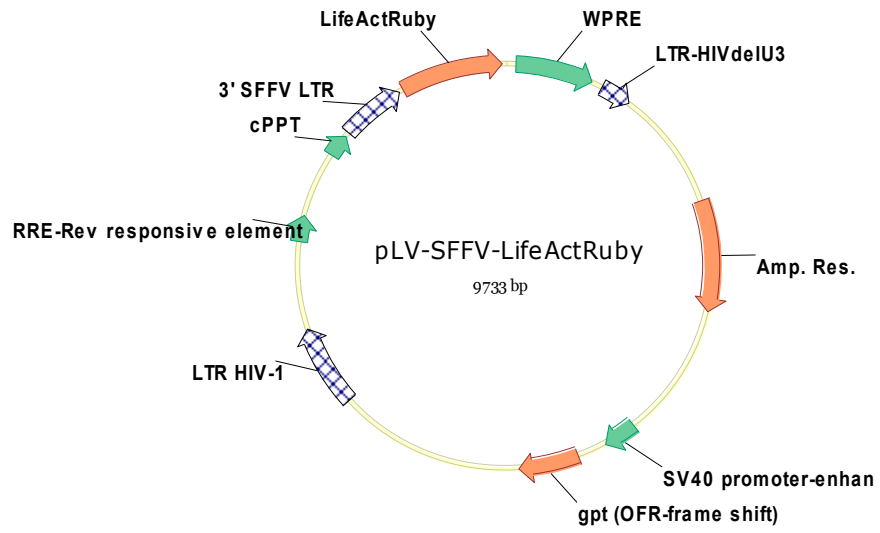
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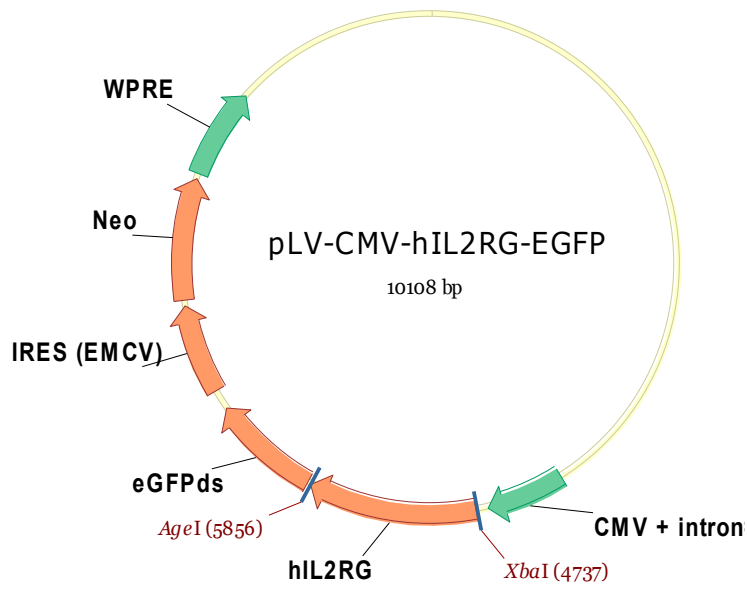
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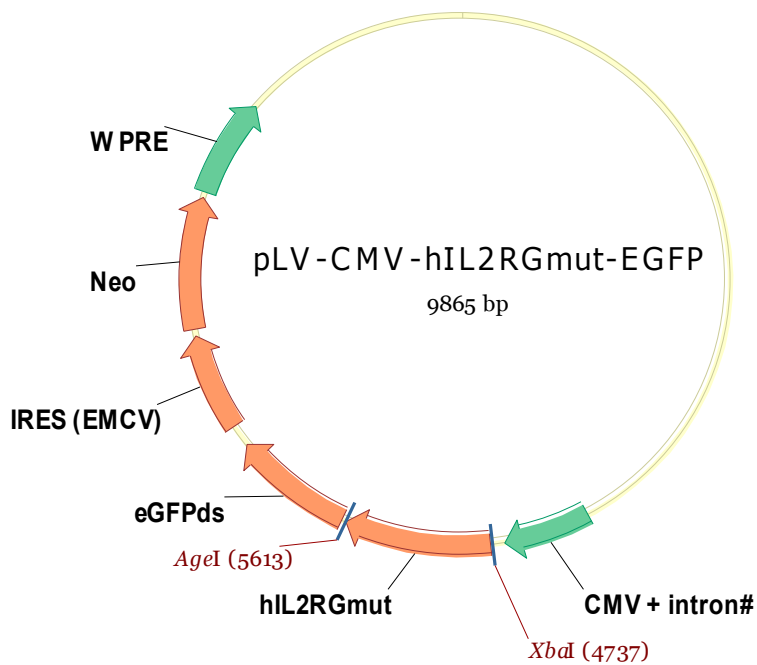
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Appendix 1 LiFeAct-Ruby Lentiviral Vector Map



Appendix 2 γc^{WT} -GFP Lentiviral Vector Map



Appendix 3 γc^{Mut} -GFP Lentiviral Vector Map

