Role of Syk-coupled C-type lectin receptors in T cell immunity to fungal stimuli

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Declaration

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

Abstract

Innate signals are fundamental to determine the class of adaptive response against infection. The translation of microbial signatures into adaptive immunity is mediated by pattern recognition receptors (PRRs), which are expressed in specialized leukocytes called dendritic cells (DCs) and results in responses matched to the nature of the offending microbe. Here, I provide evidence that two Syk-coupled C-type lectin receptors (CLRs), Dectin-1 and Dectin-2, act in the coordination of adaptive immune responses to fungal stimuli and fungal pathogens.

DC activated via Dectin-1 are strong elicitors of IL-17 production by $CD4^+$ T cells. Results presented in this thesis demonstrate that Dectin-1 signalling in DCs results in the generation and $Foxp3^+$-IL-17^+ T cells, a cell type that defies either regulatory or Th17 classification. This process is dependent on IL-23, which is produced by DCs upon Dectin-1 ligation.

In addition, this work identified an additional CLR responsible for the induction of Th17 responses during the course of fungal infections. This thesis demonstrates that Dectin-2 is a second Syk-coupled PRR involved in DC activation by fungi. In a model of *Candida albicans* systemic infection, Dectin-2 is essential for the induction of Th17 responses to the organism.

Finally, I have generated tools to study responses of T cells specific for a fungal-associated antigen. Furthermore, I provide preliminary evidence regarding the activation, proliferation and trafficking of antigen-specific $CD4^+$ and $CD8^+$ T cells during systemic fungal infections.

Statement of Collaboration

Data presented in this Thesis is my own. Nonetheless, collaboration with people within the lab and elsewhere made this work possible. Specific collaborations are as follows:

Salomé LeibundGut-Landmann (previous member of the Immunobiology Lab) inspired the work of Dectin-1 signalling in DC activation and initiation of Th17 responses. We worked closely to elucidate the mechanisms that drive the initiation of Th17 responses downstream of Dectin-1. Matthew Robinson (previous member of the Immunobiology Lab) worked in parallel with me revealing the role of Dectin-2 in DC activation, cytokine production and coordination of adaptive immunity to fungal stimuli.

Colitis experiments designed to address the role of curdlan in the generation of $Foxp3^{+}$-$IL\text{-}17^{+}$ T cells were carried out at the laboratory of Dr Fiona Powrie in Oxford and were performed by myself together with Dr Philip Ahern. Lentiviral particles containing a shRNA to Dectin-2 were produced in the laboratory of Dr Luis F. Moita (Instituto de Medicina Molecular, Universidade de Lisboa, Lisboa, Portugal).

Dr Philip Taylor (Cardiff University, Cardiff, UK) provided helpful advice in the Dectin-2 project, and also provided the anti-Dectin-2 antibody. In addition, Matthew Robinson and myself performed the systemic infections as part of the Dectin-2 project in Dr Taylor's laboratory. *Candida albicans* strains and vectors were obtained in collaboration with Dr Alistair Brown (University of Aberdeen, Aberdeen, UK).

Syk deficient foetal liver was provided by Dr Victor Tybulevicz (NIMR, Mill Hill, UK) and Dectin-1 deficient bone marrow was provided by Dr Gordon Brown University of Aberdeen, Aberdeen, UK).

Acknowledgement

I am very grateful to Caetano for giving me the chance to study towards my PhD in his laboratory. He has been a great mentor and a supportive supervisor. Having the chance to receive such a great scientific education as a member of his lab has deeply influenced my life. I want also to thank past and present members of the Immunobiology Lab for support and advice. In particular, I would like to mention Salomé and Matthew who worked closely with me and provided scientific advice and guidance.

Above everybody else, I want to thank José for being such a wonderful man who is always there to support me and give me the strength to keep going throughout difficult times.

I want to thank David, Olivier, Delphine, Diego and Santiago for making the lab a cheerful place.

Without my close friends Andrea, Dan, Viduth and Diego, probably my PhD would not have been possible. They were the people who made me feel “at home” and I will never forget our endless discussions in “the Ship” or in “the George”.

And finally, I want to thank my family for always supporting me throughout this challenge and for teaching me that the distance that separates people cannot be measured in kilometres.

This thesis is dedicated to José and to my beautiful nieces Javiera and Sofía.

Publications resulting from this thesis

For convenience, publications containing work presented in this thesis are reproduced at the end of the reference list.

- Robinson MJ*, Osorio F*, Rosas M, Freitas RP, Schweighoffer E, Gross O, Verbeek JS, Ruland J, Tybulewicz V, Brown GD, Moita LF, Taylor PR, Reis e Sousa C. 2009. **Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection**. *Journal of Experimental Medicine* 206(9): 2037-2051.
  *These authors contributed equally to this work.

- Osorio F, LeibundGut-Landmann S, Lochner M, Lahl K, Sparwasser T, Eberl G and Reis e Sousa C. 2008. **DC activated via dectin-1 convert Treg into IL-17 producers**. *European Journal of Immunology* 38(12): 3274-3281.

In addition, the publication of LeibundGut-Landmann et al (containing work described in figures 3.1 and 3.2 of this thesis) is reproduced at the end of the reference list.

- LeibundGut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, Schweighoffer E, Tybulewicz V, Brown GD, Ruland J, Reis e Sousa C. 2007. **Syk-and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17**. *Nature Immunology* 8(6): 630-638.

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Abbreviations

APC	Antigen Presenting cell
BMDC	Bone-marrow derived dendritic cell (GM-CSF-derived unless otherwise stated)
CMC	Chronic mucocutaneous candidiasis
DC	Dendritic cell
FACS	Fluorescence Activated Cell Sorting
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
IFN	Interferon
IL	Interleukin
IRF4	Interferon Regulatory Factor 4
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
MAP	Mitogen-Activated Protein
MHC	Major Histocompatibility complex
ROS	Reactive Oxygen Species
TGF-β	Tumour Growth Factor β
Th1	Type 1 helper cell
Th2	Type 2 helper cell
Th17	Type 17 helper cell
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell

Chapter 1. Introduction

Our immune system has evolved specialized mechanisms to coordinate an appropriate anti-pathogen response while maintaining host homeostasis. The immune signalling pathways involved in pathogen recognition and clearance are controlled by microbial sensors that detect features present in different organisms. The best-characterized pathogen sensors of the immune system are the so-called pattern recognition receptors (PRRs), first defined by Charles Janeway Jr. in his seminal essay in 1989 (Janeway, 1989). He defined PRRs as germ-line encoded receptors that are non-clonally distributed and are involved in the recognition of invariant pathogen-associated molecular patterns (PAMPs) not found in the host (Janeway, 1989). Janeway's theory postulated that innate recognition by PRRs preceded the generation of adaptive immunity by regulating the induction of costimulatory signals required for lymphocyte activation (Janeway, 1989).

Five years after the publication of Janeway's theory, an essay written by Polly Matzinger provided new perspectives to the immunological field (Matzinger, 1994). She challenged Janeway's opinion by stating that the immune system is not designed to discriminate between "self" and "not self" but it has evolved to detect any form of danger signals, even in absence of pathogen presence (Matzinger, 1994). Danger signals are intrinsically immunogenic and correspond to endogenous molecules that become exposed in cells upon stress, injury and death. In later years, these molecules have been referred to as damaged-associated molecular patterns (DAMPs) and there are numerous examples including uric acid, calreticulin, ribonucleoproteins and ATP among others. Notably, the detection of DAMP presence is also mediated by PRRs, which orchestrate the initiation of the immune response in response to the appropriate insult (Rock and Kono, 2008).

In recent years, it has become apparent that both Janeway and Matzinger were correct. PAMPs and DAMPs are elicitors of the immune system in conditions of infection and/or sterile injury. Whether the immune system distinguishes these two types of signals to generate qualitatively different types of immune responses is at present unclear.

1.1 The discovery of pattern recognition receptors

According to Janeway's theory, a PRR should fulfil the three following criteria: first, it must localize to the cell surface; second, it must be expressed by APCs such as macrophages and DCs; and third, it must be capable of triggering signalling pathways that result in the upregulation of costimulatory molecules (Medzhitov, 2009).

At that time, it was known that stimulation of cells with various innate agents led to the activation of the transcription factor NF-κB (Kopp and Ghosh, 1995). NF-κB activation was also noted in response to IL-1 receptor triggering (Kopp and Ghosh, 1995). Furthermore, the identification of a particular cytosolic signalling domain present in the human IL-1R that shared similarity to the Drosophila Toll Protein and to the Tobacco Resistance protein N, known as Toll, IL-1R and Resistance domain or TIR (Gay and Keith, 1991, Whitham et al., 1994), received much attention. Indeed, the first receptor capable of triggering an anti-microbial response directly was the Toll protein from *Drosophila melanogaster* (Lemaitre et al., 1996). In this report, Jules Hoffmann's group identified the Spätzle/Toll/Cactus signalling pathway as an essential axis of antifungal resistance in Drosophila (Lemaitre et al., 1996). It was discovered that activation of Spätzle by proteolytic cleavage allow it to bind to Toll receptor, and transmit signals to Cactus (an IκB-related protein) to control the expression of the antifungal peptide drosomycin (Lemaitre et al., 1996).

The groundbreaking discovery of an innate signalling pathway associated with host defense in Drosophila reinforced the idea that signalling PRRs must exist in higher eukaryotes. In 1997, Medzhitov and Janeway first reported the identification of a human toll homolog that signalled for cell activation (Medzhitov et al., 1997). The human Toll protein shared many similarities with Drosophila Toll such as the presence of leucine rich repeats (LRRs) and a TIR domain, and the ability to signal through the NF-κB pathway (Medzhitov et al., 1997). Furthermore, activation of human Toll resulted in cytokine production and upregulation of costimulatory molecules suggesting a role for this protein in the coordination of adaptive immunity ((Medzhitov et al., 1997) and reviewed in (Medzhitov and Janeway, 1997)).

The identification of the first toll-like receptor provided experimental evidence for Charles Janeway's theory and opened an immense field of research dedicated to the identification of additional mammalian PRRs and their contribution in immunity.

At present, four major classes of PRRs have been identified and include members of Toll-like receptor, RIG-I-like receptor, NOD-like receptor and C-type lectin receptor families.

1.2 Pattern recognition receptors

1.2.1 Toll-like receptors (TLRs)

Following the identification of the first TLR, the discovery of the TLR family made rapid progress. Twelve members were identified in mammals. TLRs together with IL-1 receptors constitute a superfamily based on the homology of the cytoplasmic region. Within the TIR domain, the similarities map at the level of three conserved boxes that are essential for signalling (Akira and Takeda, 2004).

In addition to the intracellular signalling domain, TLRs possess an extracellular region containing 19-25 tandem copies of LRRs. This region is not present in IL-1R and forms a surface suited for PAMP recognition (Akira and Takeda, 2004).

TLRs reside either in the plasma membrane or in intracellular compartments (Figure 1.1). TLRs expressed at the cell surface (TLR 1, 2, 4, 5 and 6) recognize a broad range of microbial signatures. TLR1, TLR2 and TLR6 recognize lipids including peptidoglycan, lipoarabinomannan and phospholipomannan (TLR2), lipoteichoic acids, Gram-positive bacteria and bacterial proteins (TLR1/TLR2) (TLR2/TLR6) (Akira et al., 2006, Iwasaki and Medzhitov, 2010). TLR4 recognizes LPS from Gram-negative bacteria whereas TLR5 recognizes flagellin (Iwasaki and Medzhitov, 2010). TLRs that are found in endosomal/lysosomal compartments recognize microbial nucleic acids including double-stranded RNA (TLR3), single-stranded RNA (TLR7) and double-stranded DNA (TLR9) (Iwasaki and Medzhitov, 2010).

Upon ligand binding, TLRs initiate downstream signalling responses by recruiting a TIR domain-containing adaptor such as Myeloid Differentiation primary-response

protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP) and TIR-domain-containing adaptor protein inducing IFN-β (TRIF) (Akira and Takeda, 2004). TLR-adaptor interaction favours the formation of a complex involving IL-1R-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6). This ultimately results in activation of NF-κB and MAPKs for induction of proinflammatory cytokines (reviewed in (Akira and Takeda, 2004) and (Akira et al., 2006)).

Although most TLRs signal in a MyD88-dependent manner, TLR3 utilizes the adaptor TRIF for NF-κB activation and production of type I IFNs (Akira et al., 2006). Thus, MyD88 and TRIF are responsible for the activation of signalling pathways downstream of TLRs leading to the induction of proinflammatory cytokines and type I IFNs (Akira et al., 2006).

1.2.2 Cytosolic PRRs

1.2.2.1 RIG-I-like receptors (RLRs)

The RIG-I-like receptor family of cytosolic RNA helicases is involved in the detection of nucleic acids following viral infection (Figure 1.1). This family comprises three members: retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and Laboratory of genetics and physiology 2 (LGP2). Detection of viral RNA by RIG-I and MDA5 results in activation of the adaptor MAVS (mitochondrial antiviral signalling), which initiates a signalling cascade involving interferon response factor 3 (IRF3), IRF7 and NF-κB. This results in the production of type I IFNs and other proinflammatory cytokines (Pichlmair and Reis e Sousa, 2007). RIG-I and MDA5 recognize different ligands, are activated upon infection with different set of viruses and play a non-redundant role in the coordination of antiviral defense (Rehwinkel and Reis e Sousa, 2010).

1.2.2.2 NOD-like receptors (NLRs)

NLRs constitute a large family of cytosolic sensors involved in the recognition of pathogens and danger signals (Figure 1.1). NLR members have three characteristic domains: an LRR region, a nucleotide oligomerization domain (NOD or also called NACHT) and an effector motif such as CARD, PYRIN or BIR domain (Martinon et al., 2009).

Some members of the NLR family are involved in the recognition of bacterial signatures. Two different components present in the bacterial peptidoglycan: γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyldipeptide (MDP) are the ligands for NOD1 and NOD2, respectively (Akira et al., 2006). Upon ligand binding, NOD 1-2 signal to NF-κB via activation of the kinase RIP2/RICK (Akira et al., 2006).

Additional members of the NLR family are involved in the activation of a protein complex responsible for processing of the cytokines IL-1β/IL-18/IL-33 named inflammasome. Is important to note that in the original sense of the terms described above, these NLR members do not fulfill the criterion of PRRs; as activation of the inflammasome does not culminate in gene regulation. On the other hand, inflammasome assembly leads to the activation of caspase-1, which is responsible for the processing and secretion of proinflammatory cytokines.

The NLRs NALP1, NALP3 and IPAF are prototype activators of the inflammasome in response to factors derived from bacteria and viruses or to endogenous danger signals such as uric acid, potassium efflux, ROS and membrane disruption (reviewed in (Martinon et al., 2009)). Inflammasome activation has emerged as a crucial sensor of harmful microbial presence and metabolic stress.

1.2.3 C-type lectin receptors (CLRs)

The term "C-type lectin" was first used to differentiate a group of Ca^{+2} dependent carbohydrate-binding (lectin) proteins from the rest of animal lectins (Zelensky and Gready, 2005). At that time, the analysis of Ca^{+2} dependent lectins identified a particular carbohydrate recognition domain (CRD) that was called "C-type CRD" or "C-type lectin domain" (Zelensky and Gready, 2005). The identification of many more proteins containing a C-type CRD revealed that not all members of this group bind to carbohydrates or even calcium. This contradiction was later solved by introducing a more general term; "C-type lectin-like domain" (CTLD) to name proteins containing a homologous C-type CRD (Zelensky and Gready, 2005).

The definition of "C-type lectin" corresponds to any protein possessing one or more CTLDs (Kerrigan and Brown, 2010). According to that definition, CLRs encompass a

large family of proteins with diverse functions including cell adhesion, pathogen recognition, regulation of natural killer function, complement activation, tissue remodelling, platelet activation, endocytosis and phagocytosis among others (Zelensky and Gready, 2005, Kerrigan and Brown, 2010, Robinson et al., 2006). Notably, there is a subset of CLRs that function as transmembrane innate PRRs that are mainly expressed on myeloid cells (Figure 1.1).

CLRs that act as PRRs recognize microbial signatures including mannose, fucose and glucan moieties. Glucans are main constituents of the cell wall of fungi, plants and mycobacteria species; mannose and high mannose structures are highly expressed by some viruses, fungi and mycobacteria. Fucose structures are found on the surface of helminths and some bacterial species (Geijtenbeek and Gringhuis, 2009).

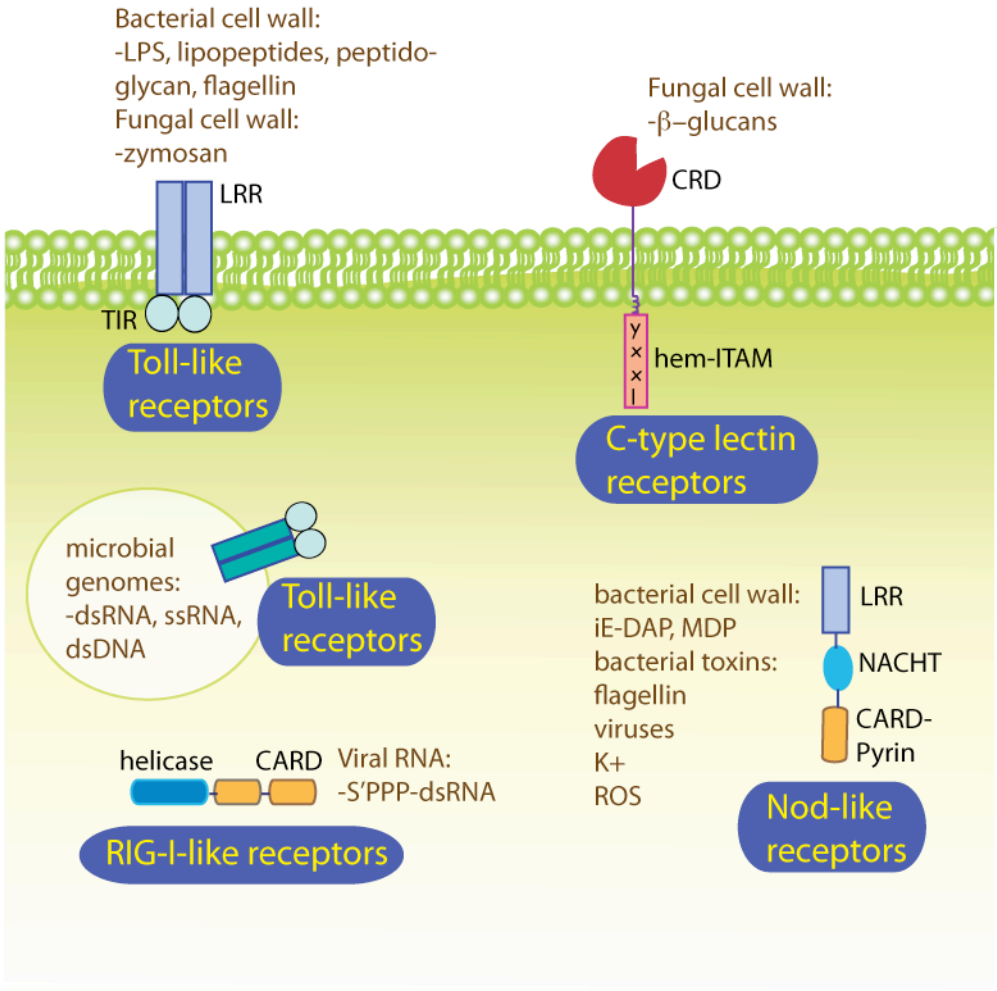


Figure 1-1: Major classes of cell-associated PRRs in mammals
Upon PAMP recognition, transmembrane or intracellular PRRs trigger signalling pathways involved in the activation of innate and adaptive immunity. (Drawn with reference to (Medzhitov, 2009)).

1.3 Myeloid CLRs in innate immunity

According to the nature of their CTLD, myeloid CLRs can be divided into two groups: group I contains CLRs belonging to the mannose receptor family and group II contain CLRs that belong to the asialoglycoprotein receptor family, which can be further divided into Dectin-1 or DCIR subfamilies (Geijtenbeek and Gringhuis, 2009).

An additional criterion to classify myeloid CLRs is based on their ability to trigger an intracellular signalling event. A group of CLRs has been shown to induce signalling pathways by associating with signalling adaptors containing an immunoreceptor tyrosine-based activation motif or ITAM (YXXL/I $X_{6,8}$ YXXL/I (Ivashkiv, 2009)).

Other CLRs possess an ITAM-like motif (named "hemITAM"; for detailed description see section 1.3.2.1) within their intracellular tail, which allows them to activate protein cascades directly. A third group of CLRs possesses an immunoreceptor tyrosine-based inhibitory motif or ITIM (S/I/V/LxYxxI/V/L (Barrow and Trowsdale, 2006)) that is thought to inhibit myeloid cell activation. These types of CLRs are illustrated in figure 1.2.

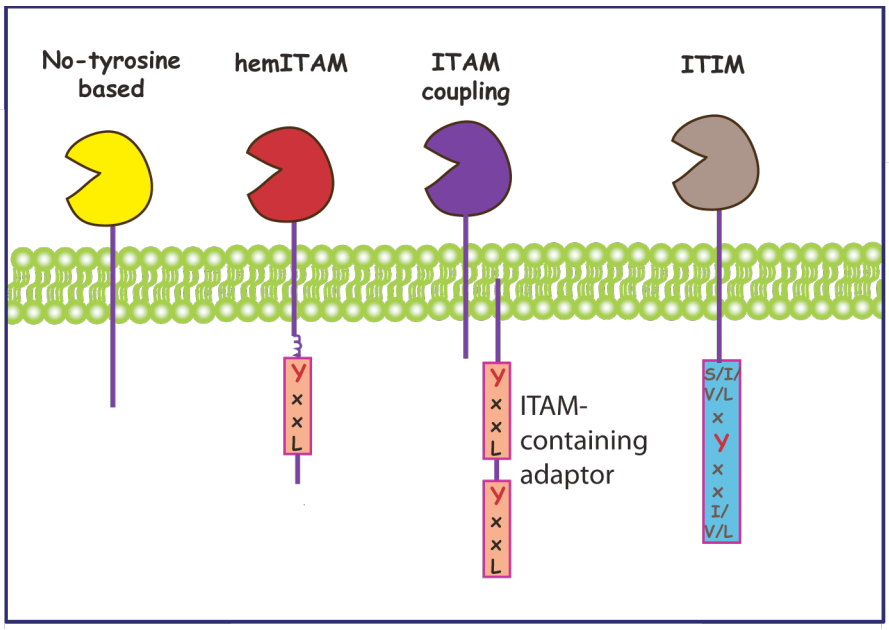


Figure 1-2: Upon ligand binding, myeloid CLRs induce protein cascades by using distinct proximal signalling mechanisms.
ITAM, hemITAM and ITIM signalling starts with the phosphorylation of the tyrosines located within the tyrosine based signalling motif. In the case of ITAM and hemITAM-coupled CLRs, tyrosine phosphorylation leads to activation of the kinase Syk and initiation of signalling cascades. In contrast, ITIM-containing CLRs can couple to SHP-1 or SHP-2 phosphatases, which may inhibit cell activation. Tyrosines contained within the signalling motifs are depicted in red and 'x' corresponds to any amino acid. Drawn with reference to (Robinson et al., 2006).

For simplicity purposes, CLRs have been associated based on the presence of tyrosine-based motifs. Myeloid CLRs falling into these four categories are shown in table 1.

Table 1: Myeloid CLRs associated by their signalling motifs.
With reference to (Robinson et al., 2006) and (Geijtenbeek and Gringhuis, 2009).

CLR	Tyrosine-based motif	Ligand
Mannose Receptor	None	High mannose, fucose
DEC-205	None	ND
DC-SIGN	None	High mannose and fucose
MGL	None	Terminal GalNAc
CLEC5a	ITAM-based (adaptor DAP10/DAP12)	ND (Dengue virus)
Dectin-2 (CLEC4n)	ITAM-based (adaptor FcRγ chain)	High mannose, α-mannans
Mincle (CLE4E)	ITAM-based (adaptor FcRγ chain)	α-mannose, glycolipids
BDCA-2	ITAM-based (adaptor FcRγ chain)	ND
Dectin-1 (CLEC7a)	hemITAM-based	β-1,3 glucans
CLEC-2	hemITAM-based	ND
DNGR-1 (CLEC9a)	hemITAM-based	ND
DCIR (CLEC4a)	ITIM-based	ND
CLEC12B	ITIM-based	ND
MICL (CLEC12a)	ITIM-based	ND

1.3.1 Endocytic CLRs lacking classical signalling motifs

DC-SIGN, DEC-205 and mannose receptor (MR) are CLRs that have endocytic activity and mediate internalization of their ligands into intracellular compartments (Geijtenbeek and Gringhuis, 2009). Furthermore, these CLRs can direct the cargo into the appropriate route for antigen processing and presentation. Despite their involvement in antigen trafficking, these CLRs do not contain classical signalling motifs within their cytoplasmic tails. Thus, there is limited evidence indicating that they behave as signalling PRRs for gene transcription and cytokine production in myeloid cells. It is also unclear whether the endocytic properties of these CLRs require signalling via

protein cascades. On the other hand, CLRs with endocytic activity expressed in myeloid DCs such as DEC-205 have become interesting candidates for vaccine studies. In fact, targeting of antigens to DEC-205 enhances antigen presentation and leads to T cell immunity (reviewed in (Steinman et al., 2003)).

1.3.2 CLRs that signal via the spleen tyrosine kinase Syk

1.3.2.1 Dectin-1, a 'hemITAM' containing CLR

Although not all CLRs are competent to trigger an intracellular signalling event, some CLRs have shown to directly couple PAMP recognition to gene induction. The classical example of a CLR that induces intracellular signalling cascades is Dectin-1 ("DC-associated C-type lectin 1"). Despite its name, Dectin-1 is not restricted to DC and it is expressed in many other cell types including monocytes, macrophages, neutrophils, a subset of T cells, and CCR6-expressing γδ T cells (Taylor et al., 2002, Martin et al., 2009).

Dectin-1 is a receptor with specificity for β-linked glucans that are present in the cell wall of fungi, but also some bacteria and plants (Brown, 2006). Studies using oligosaccharide microarray technology have shown that Dectin-1 specifically recognizes β-(1,3)-linked glucans (Palma et al., 2006). Although it was first described as a receptor for the crude yeast cell wall preparation zymosan (Brown and Gordon, 2001), Dectin-1 also binds to fungal pathogens including *Candida* spp*., Aspergillus* spp*., Coccidiodes* spp*., Pneumocystis* spp. and bacterial pathogens such as mycobacteria (Brown, 2006, Geijtenbeek and Gringhuis, 2009, Rothfuchs et al., 2007). In addition to the ligands derived from pathogens, this receptor possesses an unidentified endogenous ligand expressed in T cells (Ariizumi et al., 2000b).

Dectin-1 is a type II transmembrane protein that contains a single extracellular CTLD and an ITAM-like motif on its intracellular tail (Ariizumi et al., 2000b). Classical ITAM signalling starts with the phosphorylation of two tyrosines located within the ITAM motif by members of Src family kinases. This process provides appropriate docking sites for dual interaction with the tandem SH2 domains of Syk family kinases (Syk and ZAP-70), leading to the initiation of downstream signal transduction events (Ivashkiv, 2009).

In contrast to the conventional ITAM sequence, the amino terminal tyrosine (Tyr3) of Dectin-1 is not located within an appropriate YXXL context and has a highly charged triacidic motif (DED) located proximal to distal tyrosine 15 (Ariizumi et al., 2000b, Underhill et al., 2005). Despite this unusual sequence, the ITAM-like motif of Dectin-1 is able to directly recruit and activate Syk upon zymosan binding (Rogers et al., 2005, Underhill et al., 2005). Work from the Immunobiology Laboratory has first demonstrated that a single phosphorylation on the membrane-tyrosine 15 in the tail of Dectin-1 is necessary and sufficient to mediate signalling via Syk (Rogers et al., 2005). The single YXXL motif responsible for this unusual mode of Syk activation has been termed "hemITAM" ((Rogers et al., 2005) and reviewed in (Robinson et al., 2006)).

The relevance of Syk signalling in fungal recognition is underscored by the inability of Syk deficient DCs to produce IL-2 and IL-10 upon zymosan stimulation, reinforcing the notion that Syk is non redundant for innate responses to fungal stimuli (Rogers et al., 2005). Surprisingly, unlike Syk deficient DCs, DCs lacking Dectin-1 are not impaired in their ability to produce IL-2 and IL-10 upon zymosan stimulation indicating that additional Syk-coupled PRRs are likely to be involved in fungal recognition (LeibundGut-Landmann et al., 2007, Robinson et al., 2009).

On average, glucans account for 54.7% of yeast zymosan (Di Carlo and Fiore, 1958). The remaining fraction contains a large proportion of mannans (18.8%), proteins (14.5%), fat (6.6%), inorganic materials (3.2%) and chitin (0.8%) (Di Carlo and Fiore, 1958). Not surprisingly, this crude preparation has been shown to contain ligands for various PRRs including Dectin-1, TLR2/6, mannose receptor and complement receptor among others (Brown and Gordon, 2001, Ezekowitz et al., 1984, Sung et al., 1983, Gantner et al., 2003). Upon zymosan stimulation, PRRs such as Dectin-1 and TLR2 are engaged simultaneously and have shown to collaborate to enhance the production of proinflammatory cytokines (Gantner et al., 2003).

Despite its ability to interact with TLRs for modulating of inflammatory responses to complex stimuli, Dectin-1 can also function as a signalling PRR in isolation. Dectin-1 signalling directs NF-κB activation by recruiting the adaptor protein CARD9 (Gross et al., 2006, Goodridge et al., 2009). This adaptor signals via Bcl10 and Malt-1 for NF-κB

activation and is essential for antifungal immunity to *Candida albicans in vivo* (Gross et al., 2006). In addition to the activation of the canonical NF-κB pathway in response to Syk/CARD9 signalling, Dectin-1 ligation also results in activation of the non-canonical NF-κB pathway. Activation of both NF-κB pathways downstream of Dectin-1 involves signalling via Syk and also via the serine/threonine kinase RAF-1 (Gringhuis et al., 2009).

Notably, CARD9 signalling in response to Dectin-1 activation seems to be cell-type dependent. Whereas GM-CSF-derived BMDC, GM-CSF/IFN-γ primed macrophages and peritoneal cells activate NF-κB and produce robust amounts of TNF in response to Dectin-1 ligation, FLT3-L-derived BMDCs and M-CSF-derived macrophages are unresponsive to a Dectin-1 agonist (Goodridge et al., 2009). The heterogeneity in Dectin-1 signalling in different subsets of APC may be of relevance in the study of antifungal immunity.

The intracellular consequences of Dectin-1/Syk activation extend beyond the activation of NF-κB. Activation of Dectin-1/Syk by fungal stimuli results in production of reactive oxygen species (ROS) and activation of ERK MAP kinase (Underhill et al., 2005, Slack et al., 2007). Moreover, ligation of Dectin-1 but not TLR2 results in NFAT activation (Goodridge et al., 2007). In DCs stimulated with fungal particles, NFAT controls the production of IL-2, IL-10 and IL-12p70 (Goodridge et al., 2007).

These studies elegantly dissected the role of Dectin-1 in DC responses to complex fungal stimuli such as zymosan or *C. albicans*. Nonetheless, the contribution of Dectin-1 to the initiation of adaptive immunity was unknown due to the lack of a purified agonist. In 2005, Yoshitomi et al. showed that a purified preparation of β-glucan known as curdlan was able to induce DC activation. Curdlan is a β-(1,3)-glucan polymer produced by culture fermentation from a non-pathogenic and non-toxico strain of the bacterium *Alcaligenes faecalis* (Spicer et al., 1999). BMDCs stimulated with curdlan produced TNF in a MyD88-independent manner (Yoshitomi et al., 2005). Remarkably, the addition of a blocking antibody to Dectin-1 prevented the induction of TNF by curdlan-activated BMDCs suggesting that Dectin-1 was a PRR for this β-glucan

preparation (Yoshitomi et al., 2005). At that time, it was unclear whether curdlan could act as a specific agonist for Dectin-1.

Dr Salomé LeibundGut-Landmann (former member of the Immunobiology Laboratory, Cancer Research UK) validated curdlan as a specific Dectin-1 agonist and established the outcome of the Dectin-1 signalling pathway in DCs. Specific triggering of Dectin-1 induced upregulation of the costimulatory molecules CD86, CD80 and CD40 and activation of the MAP kinases p38, Erk and Jnk together with NF-κB in a Syk dependent manner (LeibundGut-Landmann et al., 2007). In addition, curdlan-activated DCs produced a distinct pattern of cytokines with robust levels of IL-2, IL-10, TNF, IL-6 and IL-23. Importantly, that response was dependent on Dectin-1, Syk and CARD9 and independent of MyD88 (LeibundGut-Landmann et al., 2007). Altogether, this work demonstrated for the first time that selective activation of Dectin-1/Syk/CARD9 signalling pathway in isolation was sufficient to induce full DC activation (LeibundGut-Landmann et al., 2007). The contribution of Dectin-1 to the initiation of adaptive immunity is the main research topic of chapter 3 presented in this thesis.

Like Dectin-1, there are additional members of this family containing a hemITAM motif including CLEC-2 and DNGR-1 (also known as CLEC9A). CLEC-2 recognizes a ligand present in the snake venom toxin rhodocytin and in HIV-1. In addition, CLEC-2 possesses an endogenous ligand called podoplanin, which is involved in platelet aggregation, lymphatic vessel formation and tumor metastasis (Kerrigan and Brown, 2010). Importantly, CLEC-2 behaves as a Syk-coupled PRR in various cell types including DCs and platelets (Diego Mourão-Sá, unpublished observations and (Suzuki-Inoue et al., 2006). DNGR-1 recognizes an unidentified ligand exposed during cell necrosis and mediates crosspresentation of dead-cell material by DCs through a hemITAM-Syk interaction (Sancho et al., 2009).

1.3.2.2 CLRs signalling via ITAM-containing adaptors

In addition to the CLRs that recruit Syk directly via their hemITAM motif, there is a second group of receptors that do not contain intracellular signalling regions but trigger protein cascades through association with ITAM-containing adaptor molecules. Members of this category include Mincle (macrophage inducible C-type lectin, also

known as CLEC4E), Dectin-2 (also known as CLEC4n), BDCA2 (blood DC antigen 2 protein, also known as CLEC4C) and CLEC5A (also known as myeloid DAP12-associating lectin-1, MDL-1 (Bakker et al., 1999)) (Geijtenbeek and Gringhuis, 2009).

Mincle is expressed in macrophages and recognizes mannose residues present in fungi including *C. albicans* and *Malasezzia sp*, and glycolipids present in *M. tuberculosis* (Bugarcic et al., 2008, Wells et al., 2008, Yamasaki et al., 2009, Ishikawa et al., 2009). This receptor also recognizes the endogenous ribonucleoprotein SAP130, which is released upon cell death (Yamasaki et al., 2008). Upon ligand binding, Mincle associates with the ITAM-bearing molecule Fc receptor γ chain (FcRγ) and transduces an FcRγ chain-dependent signal that involves Syk and CARD9 (Yamasaki et al., 2008). Signalling via Mincle results in the production of TNF by macrophages (Yamasaki et al., 2008, Wells et al., 2008). In response to pathogen recognition, Mincle largely accounts for the MyD88 independent aspects of mycobacteria recognition and it is involved in host defense to *C. albicans* (Ishikawa et al., 2009) (Wells et al., 2008).

Dectin-2, like Dectin-1, was identified by subtractive cDNA cloning in DC lines and despite its name, is only 19.6% homologous to Dectin-1 (Ariizumi et al., 2000a). Dectin-2 is a type II transmembrane CLR involved in the recognition of zymosan and various pathogens including *C. albicans*, *Saccharomyces cereviseae, Mycobacterium tuberculosis*, *Paracoccoides brasiliensis*, *Histoplasma capsulatum, Microsporum audouinii, Trichophyton rubrum, Criptococcus neoformans* with specificity for the hyphae over the yeast form (McGreal et al., 2006, Sato et al., 2006). Dectin-2 is also involved in the recognition of house dust mite allergens (Barrett et al., 2009) and glycan microarray data revealed that Dectin-2 has specificity for high-mannose structures (McGreal et al., 2006). Recently, it was reported that Dectin-2 recognizes α-mannans present in *C. albicans* cell wall (Saijo et al., 2010). As previously noticed for other CLRs, Dectin-2 also possesses an unknown endogenous ligand, which is expressed in $CD4^+CD25^+$ T cells upon UV radiation (Aragane et al., 2003). Although it was originally described as a DC-restricted receptor, particularly expressed in Langerhans cells (Ariizumi et al., 2000a, Bonkobara et al., 2001), further studies have demonstrated that Dectin-2 is expressed in various cell types including tissue macrophages and peripheral blood monocytes (Taylor et al., 2005). Dectin-2 associates with FcRγ chain

(Sato et al., 2006, Barrett et al., 2009), and hyphal stimulation of Dectin-2-transfected macrophages results in protein phosphorylation, NF-κB activation and production of TNF and IL-1Ra (Sato et al., 2006). Whether this reflected direct signalling by Dectin-2 or was a consequence of enhanced fungal binding is unclear. Results presented in chapter 5 revealed that Dectin-2 is indeed a Syk-coupled CLR for fungal stimuli in DCs. Furthermore; data presented in chapter 5 indicates that Dectin-2 mediates the induction of Th17 responses to *C. albicans* infection (Robinson et al., 2009).

BDCA-2 (blood dendritic cell antigen 2) is mainly expressed in plasmacytoid DCs (Dzionek et al., 2000, Dzionek et al., 2001). BDCA2 forms a complex with the ITAM-containing transmembrane adaptor FcεRIγ, and leads to a signalling cascade similar to that activated downstream of the B cell receptor (BCR)(Cao et al., 2007). The BCR-like 'signalosome' recruited by BDCA-2 involves Syk, BLNK and PLCγ2 and interferes with the production of type-I IFN induced by TLR agonists (Cao et al., 2007, Rock et al., 2007). It is unclear whether BDCA-2 acts only to prevent TLR signalling or can trigger cytokine responses in isolation.

Finally, CLEC5A is a CLR expressed in monocytes, macrophages and osteoclasts (Bakker et al., 1999, Chen et al., 2008, Inui et al., 2009) and associates with the ITAM-bearing molecules DAP10 and DAP12 (Bakker et al., 1999, Inui et al., 2009). Recently, it has been shown that CLEC5A is a PRR for Dengue virus and regulates the production of TNF in response to viral challenge (Chen et al., 2008). Whether CLEC5A is a Syk-coupled receptor has yet to be determined.

It is important to note that members of this group possess CTLD of different nature and fall into different subfamilies of the group II CLRs (Geijtenbeek and Gringhuis, 2009) but have been associated here for functional purposes.

1.3.2.3 Consequences of Syk activation downstream of CLRs.

Syk activation has pleiotropic effects in cellular biology including differentiation, cytoskeletal rearrangements, proliferation, survival and cytokine production (Mocsai et al., 2010). The relevance of Syk signalling in innate immunity is starting to be elucidated. Although Dectin-1 has been broadly used as the model PRR to study Syk

activation, it is now clear that many other CLRs might activate Syk and it will be important to reveal the role of this kinase in different types of innate challenge.

As mentioned above, Syk activation downstream of Dectin-1 ligation results in the activation of MAPK including ERK and activation of NFAT (Slack et al., 2007, LeibundGut-Landmann et al., 2007, Goodridge et al., 2007). In addition, Syk phosphorylation results in the activation of NF-κB through CARD9, which is essential for antifungal immunity (Gross et al., 2006). As a consequence, Syk deficient DCs and macrophages are greatly impaired at producing of IL-2, IL-10 and ROS in response to fungal stimulation (Rogers et al., 2005, Underhill et al., 2005, Gross et al., 2009). Syk also has been involved in phagocytosis of fungal particles. Nonetheless, this effect seems to be cell-type dependent as phagocytosis of zymosan is a Syk-independent process in macrophages but it depends on Syk in DCs (Underhill et al., 2005, Rogers et al., 2005, Gross et al., 2006).

Another aspect of Syk activation involves the regulation IL-1β production during fungal infection (Gross et al., 2009). IL-1β is a cytokine with a crucial role in antifungal immunity (Vonk et al., 2006) that is transcriptionally regulated by NF-κB and is post-translationally regulated by the NALP3-inflammasome (described above) (Martinon et al., 2009). Whereas the Syk/CARD9 pathway activates the synthesis of pro-IL-1β, Syk also activates the NALP3-inflammasome in a ROS-dependent manner, resulting in proteolytic processing of IL-1β (Gross et al., 2009). Accordingly, NALP3 deficient mice are highly susceptible to fungal infection ((Gross et al., 2009), reviewed in (Mocsai et al., 2010)).

Interestingly, the role of the Syk axis in immunity to pathogens extends beyond fungal recognition. The mechanism of adjuvanticity of the mycobacterial cord factor Trehalose-6-6-dimycolate (TDM) requires Syk and CARD9 signalling (Werninghaus et al., 2009). Mincle has been recently reported the Syk-coupled PRR for TDM (Ishikawa et al., 2009) (Schoenen et al., 2010).

In the context of sterile injury, ligation of Mincle and DNGR-1 by dead cells triggers Syk-dependent responses. Whereas Mincle drives the infiltration of neutrophils to the

damaged tissue, DNGR-1 promotes cross-presentation of dead-cell derived antigens for priming of cytotoxic T cells (Yamasaki et al., 2008, Sancho et al., 2009).

Notably, Syk functions downstream of CLRs might also encompass non-immune responses. Those aspects include osteoclastogenesis; platelet function and vascular development (reviewed in (Mocsai et al., 2010)) and CLRs including CLEC5A and CLEC2 are likely to be involved.

Is important to note that these findings might be just a small fraction of the Syk "signature" which will have to be addressed using genome-wide screens in various immune scenarios.

1.3.3 CLRs signalling via the serine/ threonine protein kinase RAF1

In addition to the signalling pathway controlled by Syk, some CLRs have been shown to induce a second protein cascade mediated by the serine/threonine protein RAF1. Those CLRs are DC-SIGN and Dectin-1 (Gringhuis et al., 2007, Gringhuis et al., 2009). DC-SIGN is a human PRR that recognizes mannose signatures present in *M. tuberculosis, M. leprae, C. albicans*, measles virus and HIV-1 (Geijtenbeek and Gringhuis, 2009). The initiation of the RAF1 signalling pathway starts with the activation of the small GTPase RAS, which binds to RAF1 for subsequent phosphorylation in defined tyrosine and serine residues ((Gringhuis et al., 2007) and reviewed in (Geijtenbeek and Gringhuis, 2009)). RAF1 activation downstream of DC-SIGN does not trigger responses in isolation but modulates TLR-mediated NF-κB activation. This modulation results in an increase in IL-10 production by unknown mechanisms (Gringhuis et al., 2007). Interestingly, the YXXL motif present in the intracellular tail of DC-SIGN is not required for activation of this signalling pathway (Gringhuis et al., 2007).

Dectin-1 also activates signalling cascades via RAF1. This pathway operates independently of Syk signalling but converges with the latter at the level of NF-κB (Gringhuis et al., 2009). Whereas Syk signalling results in activation of the canonical and non-canonical NF-κB pathways, RAF1 activation results in phosphorylation of the NF-κB subunit p65 (Gringhuis et al., 2009). Phosphorylated p65 can become acetylated

or it can generate RelB-p65 inactive dimers that do not bind to DNA (Gringhuis et al., 2009). Overall, RAF-1 activation enhances the expression of some Syk-dependent cytokines including IL-10, IL-12p35, IL-12p40, IL-6 and IL-1β and negatively regulates the production of IL-23 (Gringhuis et al., 2009). RAF-1 also regulates IL-12p70 production by human DCs and has been associated with the induction of Th1 responses downstream of Dectin-1 (Gringhuis et al., 2009).

The mechanisms underlying the activation of this innate signalling pathway upon DC-SIGN or Dectin-1 triggering are currently unknown.

1.3.4 CLRs signalling via ITIM motifs

In contrast to CLRs that trigger activatory signalling events, other members of this group including MICL (Myeloid inhibitory C-type lectin receptor) and DCIR (DC-inhibitory receptor) inhibit cell activation via an inhibitory ITIM motif (Robinson et al., 2006). ITIM phosphorylation in response to receptor ligation leads to the recruitment of tyrosine phosphatases such as SHP-1, SHP-2 or SHIP (Barrow and Trowsdale, 2006). Phosphatase recruitment results in reduced phosphorylation of the activatory protein cascade involving Syk, VAV, PLCγ, BLNK/SLP-76 and others (Barrow and Trowsdale, 2006).

In the context of innate recognition, ITIM signalling inhibits ITAM-initiated protein cascades and has been proposed to modulate Syk-coupled CLRs and TLR signalling (Robinson et al., 2006, Geijtenbeek and Gringhuis, 2009). In the case of DCIR, the receptor is expressed in monocytes, monocyte-derived DC, macrophages, B cells and DCs and inhibits TLR8-mediated induction of IL-12 and TNF by myeloid DCs and TLR9-induced IFN-α production in plasmacytoid DCs (Meyer-Wentrup et al., 2009, Meyer-Wentrup et al., 2008). On the other hand, MICL is expressed in human granulocytes, monocytes, macrophages and DCs and has shown to inhibit IL-12p40 and IL-12p70 to LPS and zymosan (Marshall et al., 2004, Chen et al., 2006). None of these receptors have proven to induce responses in absence of TLR signalling (reviewed in (Geijtenbeek and Gringhuis, 2009)). ITIM-signalling by CLRs illustrate a mechanism of regulating responses to other PRRs and probably modulating the initiation of adaptive immunity.

1.4 Linking innate into adaptive immunity

PAMP recognition by PRRs results in the mobilisation of anti-microbial mechanisms designed to contain infection. These aspects include the production of a broad variety of inflammatory mediators, which trigger cell recruitment to the infected sites for subsequent pathogen killing and tissue repair mechanisms. Furthermore, activation of a select group of PRRs may also result in the initiation of adaptive defense mechanisms. The translation of PAMP presence into priming of effector T cell responses is performed by DCs. Distinct properties confer to these leukocytes the ability to convey innate information to lymphocytes. First, DCs are able to sample antigens in the periphery and migrate into lymphoid organs to favour the contact of naïve T cells with foreign antigens (Banchereau and Steinman, 1998). Second, DCs sense the environmental milieu and generate an effector T cell response that is matched to the nature of the offending insult (Reis e Sousa, 2006). By expressing a broad range of PRRs, DCs can orchestrate the initiation of adaptive immunity.

When DCs encounter a pathogen in the periphery, they uptake the microbial cargo, become activated and migrate into draining lymphoid tissues for the presentation of antigenic peptides into MHC-I/II molecules. The transition from an "immature" DC to a "mature" DC that is competent to stimulate naïve T cells is the result of PRR ligation. The best-studied PRRs in the initiation of adaptive responses are the TLRs. Indeed, most TLR agonists induce upregulation of the costimulatory molecules CD80, CD86 and/or CD40 in DCs (Iwasaki and Medzhitov, 2004).

In addition to promoting DC "maturation", PRRs also activate DCs to decode pathogen information and instruct the differentiation of effector T cell fates. Effector $CD4^+$ T cells can be subdivided into Th1, Th2 and Th17 cells. The differentiation of effector T cell lineages is required for protection against infections but it must be strictly regulated in order to prevent autoimmunity. Aberrant Th17 and Th1 responses have been associated with the development of organ specific autoimmunity whereas uncontrolled Th2 responses contribute to the pathogenesis of allergic asthma (Korn et al., 2009, Zhou et al., 2009a). These types of excessive T cell responses can be kept under control by a specific subtype of T cells known as regulatory T cells (Tregs), which play an essential role in the maintenance of immune homeostasis.

1.4.1 Differentiation of effector T cell lineages

1.4.1.1 Th1 cells

Th1 cells produce the cytokine IFN-γ and are involved in activation of macrophages and other cell types for clearance of intracellular pathogens. Th1 cells also provide help to B cells to produce IgG2 type of antibodies (Mosmann and Coffman, 1989, Medzhitov, 2007).

The cytokines IFN-γ and IL-12 and the member of the T-box family of transcription factors T-bet, are crucial regulators of Th1 cell differentiation (Murphy and Reiner, 2002). Upon antigen recognition, the differentiation of Th1 cells starts with the induction of T-bet in response to IFN-γ signalling. In turn, T-bet triggers the production of IFN-γ and induces the expression of the β2 subunit of IL-12 receptor, conferring IL-12 responsiveness (Murphy and Reiner, 2002). This latter cytokine acts through signal transducer and activator of transcription 4 (STAT4) to amplify the production of IFN-γ and to fully establish the Th1 lineage. IL-12 also increases the expression of the receptor for IL-18, another STAT4 dependent inducer of IFN-γ (Murphy and Reiner, 2002). Terminally differentiated Th1 cells produce vast amounts of IFN-γ in response to TCR triggering.

During microbial infections, the initiation of Th1 responses is subjected to the activation of the innate immune system. It is proposed that in the context of antigen presentation, pathogen-activated cells such as NK cells might initially secrete IFN-γ to uncommitted Th1 cells to promote T-bet expression (Martin-Fontecha et al., 2004). The process of Th1 development continues with the supply of IL-12 and IL-18 by pathogen-activated macrophages and DCs, which results in the generation of mature IFN-γ-producing $CD4^+$ T cells (Murphy and Reiner, 2002) (Figure 1.3). Notably, various types of TLR ligands trigger the production of IL-12 by DCs and are involved in priming of Th1 cells (Medzhitov, 2007). Th1 cells emerge in response to IL-12 production induced by pathogens such as *Listeria monocytogenes* (Hsieh et al., 1993).

1.4.1.2 Th2 cells

Th2 cells play a role in immunity against extracellular parasites and produce cytokines IL-4, IL-5 and IL-13, which are involved in the activation of basophils/eosinophils and provide help for IgE production by B cells (Mosmann and Coffman, 1989, Medzhitov, 2007).

Th2 cells are instructed by the cytokine IL-4 and are characterized by the expression of the transcription factor GATA-3 (Murphy and Reiner, 2002). IL-4 signals via STAT6 to promote the expression of GATA-3, which leads to the remodelling of the IL-4 locus and subsequent transcription of the IL-4 gene by the transcription factors c-MAF, NFAT and AP1 (Murphy and Reiner, 2002). The mechanisms underlying the initiation of Th2 responses to pathogens or allergens are not fully understood. This is because the cellular sources of IL-4 in vivo have yet to be clearly defined. In particular, the initiation of Th2 responses to the helminth *Trichuris muris*, the allergen papain and immunoglobulin E complexes can be mediated by basophils (Sokol et al., 2007, Sokol et al., 2009, Perrigoue et al., 2009, Yoshimoto et al., 2009). Basophils are activated by the stimuli described above to produce the cytokines IL-4 and TSLP. It has been claimed that basophils can also act as antigen presenting cells for Th2 priming (Sokol et al., 2009, Perrigoue et al., 2009, Yoshimoto et al., 2009) (Figure 1.3). Nevertheless, this notion has been challenged by recent publications demonstrating that CD11c-expressing DCs are necessary and sufficient to prime Th2 responses to the parasite *Schistosoma mansoni*, to house dust mite allergens and to papain (Phythian-Adams et al., 2010, Hammad et al., 2010, Tang et al., 2010).

Additional subsets of innate leukocyte cells have been described as important sources of Th2 cytokines during helminth infections (Saenz et al., 2010, Neill et al., 2010, Moro et al., 2010). These subsets include ‘Nuocytes’, a multipotent progenitor known as MPP, and ‘natural helper’ cells (Saenz et al., 2010, Neill et al., 2010, Moro et al., 2010). Those cell types promote Th2 responses by producing large amounts of cytokines in response to helminth infection or by giving rise to cells involved in the initiation of Th2 responses such as basophils (Saenz et al., 2010, Neill et al., 2010, Moro et al., 2010).

The innate signalling pathways involved in controlling Th2 responses are not clearly defined. One reason accounting for these adversities might be that during innate recognition of allergens and parasites, the determinant is associated with the outcome of enzymatic activity rather than with the presence of a defined molecular signature. Therefore, is not surprising that the production of IgE in response to papain immunisation is a MyD88 independent process (Sokol et al., 2007).

As previously shown for other T cell lineages, the differentiation of Th2 cells is not a fixed process. It has been recently shown that TGF-β induces reprogramming of the Th2 cell lineage into an IL-9 producing T cell subset (named 'Th9' cells) (Dardalhon et al., 2008, Veldhoen et al., 2008b). Th9 cells are distinct from Th2 or any T cell effector lineage and their function is not fully elucidated as IL-9 can also be produced by other T cell subsets including Th17 cells (Nowak et al., 2009).

1.4.1.3 Th17 cells

Th17 is a recently described subset of $CD4^+$ cells that produces the cytokines IL-17A, IL-17F, IL-21 and IL-22 (Harrington et al., 2005, Langrish et al., 2005, Park et al., 2005, Nurieva et al., 2007, Korn et al., 2007, Liang et al., 2006). IL-17A and IL-17F belong to the same family of cytokines (Kolls and Linden, 2004) and IL-17 commonly denotes IL-17A. IL-17 acts on non-hematopoietic cells to upregulate chemokines for neutrophil recruitment to the site of infection and tissue repair (Weaver et al., 2007). Th17 cells play a role in host defense against extracellular bacteria and fungi (Korn et al., 2009).

Th17 cells were first described as a $CD4^+$ effector subset distinct from Th1 and Th2 in 2005 (Harrington et al., 2005, Park et al., 2005). However, the ability of $CD4^+$ T cells to produce IL-17 under some conditions of immune challenge was noted before (Happel et al., 2003, Langrish et al., 2005, Infante-Duarte et al., 2000). Over the last years, studies on Th17 function and differentiation have made substantial progress. This subset is currently characterized by the expression of the lineage-specific factors RORγt, RORα and the AP-1 protein BATF (Ivanov et al., 2006, Yang et al., 2008c, Schraml et al., 2009). Additional factors including IRF4 are also required for the generation of Th17

cells *in vitro* and *in vivo* (Brustle et al., 2007). IRF4 is not Th17-restricted and is also required for the development of Th2 cells (Brustle et al., 2007).

Upon antigen encounter, the Th17 differentiation process is initiated by the synchronized effect of IL-6 and TGF-β on naïve T cells (Mangan et al., 2006, Bettelli et al., 2006, Veldhoen et al., 2006a). In presence of TGF-β, IL-6 acts via STAT3 to direct lineage commitment and regulate the expression of RORγt, RORα and probably BATF (Yang et al., 2007, Mathur et al., 2007, Laurence et al., 2007, Yang et al., 2008c). The Th17 differentiation process results in the production of IL-17 and subsequent upregulation of the IL-23 receptor, which confers IL-23 responsiveness (Zhou et al., 2007, Nurieva et al., 2007). Although dispensable for the initial differentiation of Th17 cells, IL-23 is essential to sustain and expand committed Th17 cells. This cytokine promotes high levels of IL-17 production by activated $CD4^+$ T cells and is critical for the development of Th17 cells *in vivo* (Aggarwal et al., 2003, Langrish et al., 2005). Moreover, the acquisition of Th17 pathogenic function associated with autoimmunity in the central nervous system is dependent on IL-23 (Langrish et al., 2005, McGeachy et al., 2007). Along these lines, IL-23 deficient mice have reduced numbers of Th17 cells, which result in susceptibility to infection with the pathogen *Citrobacter rodentium* but also results in resistance to experimental autoimmune encephalomyelitis (EAE)(Cua et al., 2003, Mangan et al., 2006).

In addition to the differentiation programme induced by IL-6 and TGF-β, there is an alternative pathway of Th17 differentiation that involves the cytokine IL-21. IL-6 activated T cells produce large amounts of IL-21 in a RORγt-independent manner (Zhou et al., 2007). In turn, the combination of autocrine IL-21 with TGF-β can direct the Th17 differentiation program in absence of IL-6 (Nurieva et al., 2007, Korn et al., 2007, Zhou et al., 2007). Notably, the expression of IL-23R in response to IL-6 plus TGF-β requires IL-21 signalling (Nurieva et al., 2007, Korn et al., 2007, Zhou et al., 2007). Thus, the sequential engagement of IL-6, IL-21 and IL-23 pathways constitute a positive amplification loop that results in full Th17 differentiation. It is important to note that whereas IL-6, IL-21 and IL-23 act via STAT3 and induce mRNA transcripts encoding RORγt, none these factors are sufficient to drive Th17 differentiation on their

own. This indicates that additional signals provided by TGF-β are required for the production of IL-17 by T cells (Zhou et al., 2007).

The role of TGF-β in Th17 differentiation is well documented. Nonetheless, the mechanisms underlying TGF-β signalling are not fully understood. There is evidence indicating that the role of TGF-β in Th17 differentiation is dependent on the concentration of the cytokine. Low doses of TGF-β promote Th17 priming by synergizing with IL-6 and IL-21 whereas high doses of TGF-β represses RORγt expression and favours the generation of regulatory T cells (Zhou et al., 2008a). In addition, it has been argued that the role of TGF-β in Th17 priming is indirect, by inhibiting Th1 and Th2 differentiation. In favour of this notion, mice deficient in Th1 and Th2 cells ($Stat6^{-/-}$ $T\text{-}bet^{-/-}$ mice) generate Th17 responses in absence of TGF-β (Das et al., 2009). Similar results have been observed in $CD4^{+}$ cells isolated from $Stat6^{-/-}$ $T\text{-}bet^{-/-}$ mice bred with a mice transgenic for a dominant negative form of the TGF-β receptor ($Stat6^{-/-}$ $T\text{-}bet^{-/-}$ /TGF-βRIIDN mice) indicating that in absence of Th1 and Th2 signals, IL-6 is sufficient to drive Th17 differentiation (Das et al., 2009).

There are additional factors that have accessory roles in the initiation of Th17 responses. Notably, IL-1β and TNF increase the frequency of IL-17 producing cells *in vitro* (Veldhoen et al., 2006a). IL-1β synergizes with IL-6 and IL-23 for Th17 differentiation *in vitro* and is required for the generation of these responses *in vivo* (Sutton et al., 2006, Chung et al., 2009). Another modulator of Th17 cell function is the aryl hydrocarbon receptor (AhR)(Veldhoen et al., 2008a, Quintana et al., 2008). AhR is a cytosolic transcription factor that recognizes environmental toxins and becomes activated upon ligand binding. AhR ligands include some classes of aromatic hydrocarbons such as the toxin dioxin and the endogenous ligand FICZ (a tryptophan derived photoproduct) (Veldhoen et al., 2008a). AhR is selectively expressed by Th17 cells and controls IL-22 synthesis (Veldhoen et al., 2008a).

Like Th1 cells, the generation of Th17 cells is also controlled by activation of innate signalling pathways. Although DCs activated with TLR ligands are effective inducers of Th1 responses, addition of exogenous TGF-β to those same cultures deviates the differentiation to Th17 cells (Veldhoen et al., 2006a). TLR ligation results in the

production of IL-6, TNF and probably IL-1β by DCs, which synergize with TGF-β for the initiation of the Th17 differentiation program. *In vitro*, Treg-derived TGF-β is sufficient to support the differentiation of IL-17 producing cells in response to TLR triggering although is not clear whether Tregs perform similar functions *in vivo* (Veldhoen et al., 2006a).

Notably, Dectin-1 activation in DCs results in enhanced production of IL-6, TNF and IL-23 but little IL-12, suggesting that this type of innate activation is also suited for the generation of IL-17 producing cells (LeibundGut-Landmann et al., 2007). Working together with Salomé LeibundGut-Landmann, I found that activation of the Dectin-1/Syk/CARD9 axis leads to the initiation of Th17 responses (LeibundGut-Landmann et al., 2007). These results are presented in this thesis and effectively showed that Dectin-1 signalling in DCs leads to the initiation of adaptive immunity. Furthermore, results presented in this thesis together demonstrate that Dectin-1 activated DCs are competent to induce IL-17 production by T cells in an IL-23 dependent manner.

The cellular sources of Th17 polarizing cytokines *in vivo* have not been clearly elucidated. For Th17 cells generated during microbial infections, pathogen-activated innate immune cells like DCs might provide IL-6 and IL-23 necessary to sustain the Th17 response. In contrast, the cellular source of TGF-β has not been clearly defined but it might not correspond to member of the innate immune system. Although every cell in the body produces TGF-β, it has been reported that T-cell production of TGF-β is necessary for Th17 cell differentiation *in vivo* (Li et al., 2007). It is still unclear whether Treg-derived TGF-β accounts for this phenotype. Nonetheless, these results suggest that there must be a tight coordination between cells of the innate and adaptive immune system to enable the generation of a type 17 responses to immune challenge (Figure 1.3).

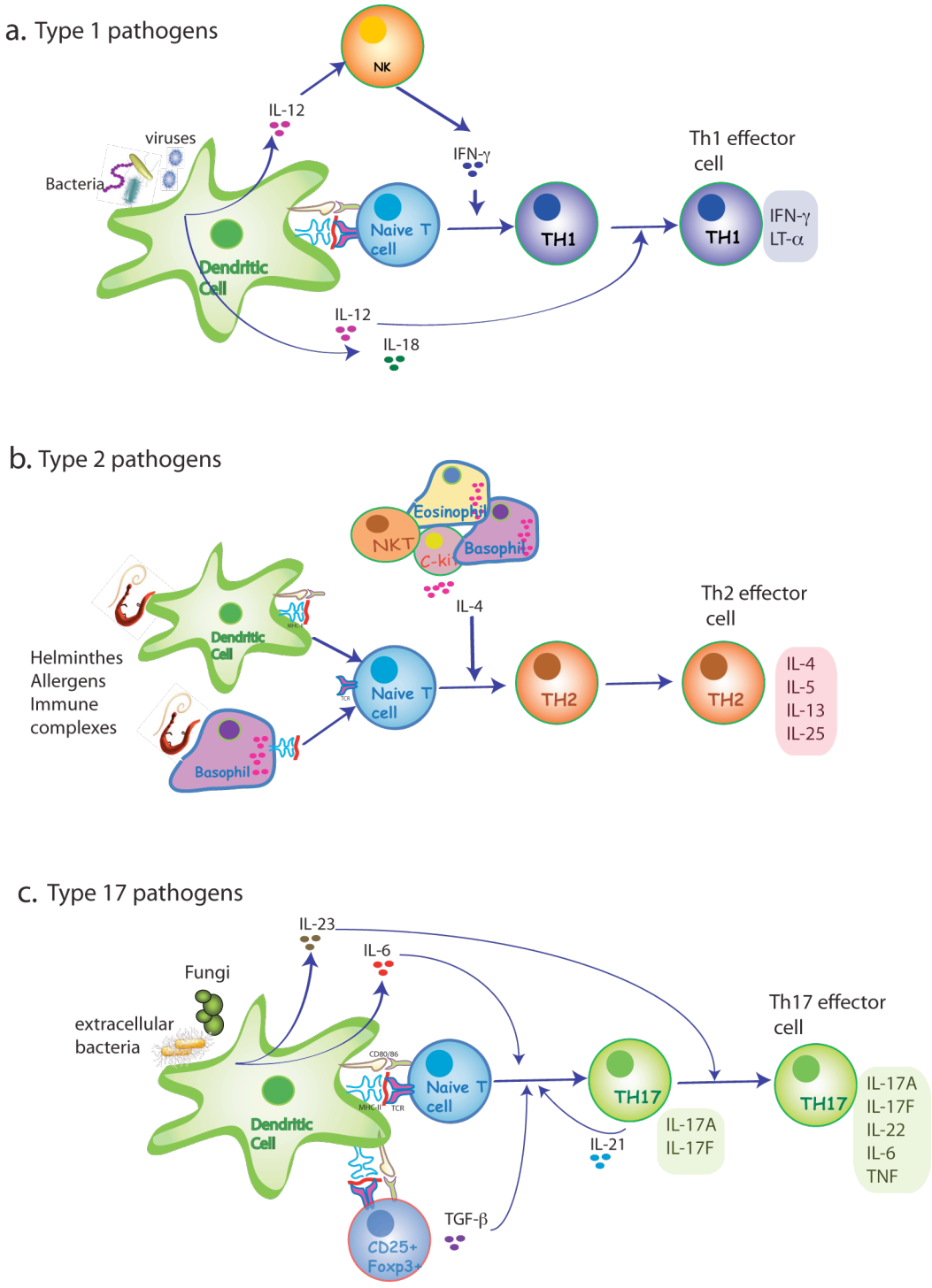


Figure 1-3: Effector T cell differentiation to microbes.
Models for (a) Th1, (b) Th2 and (c) Th17 differentiation in response to microbial challenge. Adapted from (Weaver et al., 2007, Korn et al., 2009).

1.4.1.4 Regulatory T cells (Tregs)

The specification lineage in Tregs is carried out by the transcription factor Foxp3 (Hori et al., 2003, Fontenot et al., 2003, Khattri et al., 2003). Foxp3 expressing cells can be subdivided in two groups: natural Tregs (nTregs) that are thymically derived and inducible (or adaptive) Tregs (iTregs), which differentiate in the periphery from naïve precursors under the influence of TGF-β (Weaver et al., 2006). The mechanisms of thymic differentiation of nTregs are not entirely defined but current models propose that nTreg development requires higher TCR affinity interactions than Foxp3 negative counterparts (Weaver et al., 2006). For peripherally induced iTregs it has been showed that activation of naïve T cells in presence of TGF-β results in upregulation of Foxp3 and acquisition of suppressive functions (Chen et al., 2003, Bettelli et al., 2006). This effect is dependent on the cytokine dose as Foxp3 expression is optimally induced by high concentrations of TGF-β (Zhou et al., 2008a). *In vivo*, TGF-β is required for the maintenance of Treg subsets outside the thymus and for the peripheral induction of iTregs (Weaver et al., 2006).

The relevance of Tregs *in vivo* is illustrated in studies in Scurfy mice (which have a frameshift mutation in the *Foxp3* gene), in Foxp3 deficient mice and in patients with Foxp3 mutations. Mice and humans develop a severe autoinflammatory lymphoproliferative disorder attributable to the absence of Tregs (Fontenot et al., 2003, Khattri et al., 2003).

The molecules that mediate Treg suppressive function are still being investigated. Treg-derived TGF-β and IL-10 contribute to maintain gut homeostasis (Maloy et al., 2003). In addition, Tregs have been recently shown to produce the cytokine IL-35, which is involved in the acquisition of suppressive function (Collison et al., 2007).

Some conditions of immune challenge overcome the dominant effect of Tregs in the control of T cell responses. Pasare and Medzhitov first described that T cell suppression by Tregs was reversed in cultures containing DCs stimulated with TLR agonists (Pasare and Medzhitov, 2003). TLR-activated DCs produce IL-6, which interferes with the suppressive abilities of Tregs by unknown mechanisms (Pasare and Medzhitov, 2003). Three years later, Veldhoen et al further demonstrated that in those cultures, the

combination of TLR-derived IL-6 plus Treg-derived TGF-β gave origin to Th17 cells, highlighting the role of Tregs in promoting appropriate T helper responses under some conditions of innate stimulation (Veldhoen et al., 2006a).

1.4.2 Relationship between Th17 and Tregs

As mentioned above, TGF-β is required for inducing both Th17 and iTreg cells. This highlights the close relationship between proinflammatory and regulatory T cell programs. Naïve T cells exposed to TGF-β upregulate both Foxp3 and RORγt (Zhou et al., 2008a, Ichiyama et al., 2008). This metastable population has the potential to become either Th17 or Treg cells depending on the cytokine milieu. Notably, the Foxp3-RORγt double positive T cell population has been found *in vivo*, particularly in the small lamina propria of mice and has shown to produce little or no IL-17 (Zhou et al., 2008a, Lochner et al., 2008). Moreover, it is proposed that $CD4^{+}Foxp3^{+}ROR\gamma t^{+}$ T cells behave as regulatory T cells and produce IL-10 (Lochner et al., 2008). The $Foxp3^{+}$-$ROR\gamma t^{+}$ T cell population has been recently identified in humans (Voo et al., 2009).

The observations showing that $Foxp3^{+}$-$ROR\gamma t^{+}$ T cells do not produce IL-17 in steady state suggests that Treg and Th17 cell programs are antagonistic. This notion is supported by the fact that Foxp3 can directly interact with RORγt to inhibit its effect. The interaction region of Foxp3 is located within the exon 2, which contains the forkhead (FKH) domain (Zhou et al., 2008a, Ichiyama et al., 2008). Similarly, Foxp3 can also inhibit RORα activity (Du et al., 2008, Yang et al., 2008b).

The Th17/Treg lineage commitment is strictly regulated by TGF-β. Low doses of TGF-β in combination with inflammatory cytokines favour Th17 differentiation by activating RORγt and inhibiting Foxp3 function. In contrast, high doses of TGF-β in absence of proinflammatory cytokines results in enhanced Foxp3 function and Treg development (Zhou et al., 2009a). Notably, TGF-β stimulation triggers binding of Foxp3 to active chromatin sites whereas TGF-β and IL-6 treatment results in decreased binding of Foxp3 to those same regions (Samanta et al., 2008). These observations provide a possible molecular explanation of the mechanisms controlling Treg or Th17 cell development.

The balance between Treg and Th17 cells is also influenced by additional factors particularly at mucosal sites. One example is retinoic acid (RA), which in combination with TGF-β favours Treg differentiation and inhibits Th17 development in the intestine (Mucida et al., 2007, Benson et al., 2007). A second example is IL-2, which is required for the generation of iTreg and survival of nTreg and the inhibition of Th17 differentiation via STAT5 (Laurence et al., 2007).

Notably, although Foxp3 inhibits IL-17 production, $Foxp3^+$ T cells from mice and humans are able to produce IL-17 upon stimulation with proinflammatory cytokines (Xu et al., 2007, Yang et al., 2008b, Zheng et al., 2008a, Koenen et al., 2008). This process is characterized by extinction of Foxp3 and subsequent upregulation of RORγt and IL-17 (Yang et al., 2008b). Genetic fate mapping studies have shown that at least one quarter of IL-17 producing T cells have expressed Foxp3 at some stage of their development (Zhou et al., 2009b). Results presented in this thesis demonstrate that DCs activated via Dectin-1 are competent to convert $Foxp3^+$ T cells into $Foxp3^+IL\text{-}17^+$ T cells, indicating that these cells may emerge under certain conditions of immune challenge (Osorio et al., 2008). The role of $Foxp3^+IL\text{-}17^+$ T cells in health and disease is yet to be elucidated.

1.4.3 Th17 effector cytokines in host defense

Intracellular bacteria and viruses are known elicitors of Th1 responses whereas parasite infection induces a Th2 cell response. Infection with pathogenic extracellular bacteria and fungi leads to the initiation of a type 17 response. Is important to note that Th17 cells are not the only source of IL-17 following immune stimulation. Various cell types including γδ T cells, lymphoid tissue inducer cells, NK cells, NKT cells, monocytes and neutrophils are non-$CD4^+$ T cell sources of IL-17 in response to microbial challenge (Martin et al., 2009, Duan et al., 2010, Takatori et al., 2009, Ferretti et al., 2003, Michel et al., 2007, Starnes et al., 2001). This indicates that the Th17 effector cytokines are produced sequentially by the innate and adaptive immune system for effective microbial clearance

The Th17-associated factors IL-17A, IL-17F and IL-22 regulate multiple aspects of an immune response. IL-17A modulates granulopoiesis by inducing the expression of G-

CSF in stromal cells (Fossiez et al., 1996). Activation of the IL-17A/G-CSF axis is controlled by the cytokine IL-23, which is produced by APCs upon pathogen recognition or during impaired clearance of apoptotic neutrophils (Stark et al., 2005). In addition, IL-17A and IL-17F mediate neutrophil recruitment to the infected site by inducing the expression of the chemokines CXCL1, CXCL2 and CXCL5 on epithelial and fibroblast cells (Ouyang et al., 2008, Kolls and Linden, 2004). IL-17 signalling also triggers an inflammatory response by inducing IL-6 and IL-8 production by keratinocytes (Ouyang et al., 2008).

IL-17F also has important functions in immune homeostasis. In addition to Th17 cells, innate cells and epithelial cells produce IL-17F (Ishigame et al., 2009). IL-17A and IL-17F are 55% homologous and they bind to the receptor IL-17RA/IL-17RC (Dubin and Kolls, 2009). Although these cytokines are functionally related and bind to the same receptor complexes, they have different immune functions. Unlike IL-17A, IL-17F is dispensable for the induction of autoimmune disease models including EAE (Yang et al., 2008a, Ishigame et al., 2009). IL-17F overexpression in lung epithelial cells *in vivo* leads to enhanced lung inflammation and mucus overproduction (Yang et al., 2008a). Together with IL-17A, IL-17F coordinates host defense to mucosal infection with extracellular pathogens. It is proposed that IL-17A has higher bioactivity than IL-17F, which provides an explanation for their different immune functions. In addition, it has been shown that IL-17A and IL-17F can form a heterodimer that possesses intermediate bioactivity (Dubin and Kolls, 2009).

IL-22 is usually coexpressed with IL-17 by Th17 cells and it has been associated with the maintenance of epithelial cell function. Nonetheless, various cell types including DCs and RORγt-expressing cells such as NK subsets and LTi cells are innate sources of IL-22 (Cella et al., 2009, Satoh-Takayama et al., 2008, Sanos et al., 2009, Takatori et al., 2009). Furthermore, IL-22 produced by innate cells is important in the protection to *C. rodentium* infection (Zheng et al., 2008b). Cells from innate and adaptive immune system can provide IL-22 to promote host defense, inflammatory responses, chemokines and antimicrobial peptides in epithelial cells (Ouyang et al., 2008). Notably, IL-17 and IL-22 cooperate to induce the expression of antimicrobial peptides

in the same target cells, highlighting the role of these cytokines in host defense (Liang et al., 2006).

1.4.3.1 Th17 effector cytokines during bacterial infections

Th17 effector cytokines mediate protection against various infections with extracellular bacteria. Blockade of IL-22 or IL-23, or deficiency in IL-17R and IL-17A results in enhanced susceptibility to *Klebsiella pneumoniae* infection (Ye et al., 2001, Happel et al., 2005, Aujla et al., 2008). In addition, IL-23, IL-22, IL-17A and IL-17F orchestrate host defense mechanisms against *C .rodentium* infection (Mangan et al., 2006, Zheng et al., 2008b, Ishigame et al., 2009). IL-17 derived from $CD4^+$ T cells mediates the formation of abscesses during *Bacteroides fragilis* infection *in vivo* (Chung et al., 2003). IL-17A and IL-17F are required for host protection to mucosal infection with *Staphylococcus aureus* and IL-23 is also involved in microbial clearance in a model of respiratory *Mycoplasma pneumoniae* infection (Ishigame et al., 2009, Wu et al., 2007).

Even though Th17 effector cytokines seem to be dispensable for controlling intracellular bacterial infections, they may have an indirect role in the initiation of Th1 responses. For example, although IFN-γ is critical in controlling *M. tuberculosis* infection; IL-17-producing T cells are required to attract protective Th1 cells to the site of infection (Khader et al., 2007). In addition, IL-17 has been shown to promote IFN-γ T cell responses and bacterial clearance during infection with the intracellular pathogen *Francisella tularensis* (Lin et al., 2009).

1.4.3.2 Th17 effector cytokines during fungal infections

Cytokines associated with the Th17 lineage confer protection in some models of fungal infection. IL-17 receptor knock out mice succumb to systemic candidiasis (Huang et al., 2004). Furthermore, the same cytokines are also important to confer protection to *C. albicans* in the oral cavity. IL-23, IL-17 and IL-22 deficient mice to a lesser extent, are susceptible to murine oropharyngeal candidiasis (OPC), a model that closely resembles features of human OPC (Conti et al., 2009). Notably, this model is dependent on adaptive immunity to the organism as illustrated by the enhanced susceptibility to OPC

in TCRβ deficient mice (Conti et al., 2009). Altogether, these results suggest that T cell-derived IL-17 might be crucial for the generation of an appropriate antifungal response in the oral cavity. Indeed, human T cells specific for *C. albicans* have a Th17 phenotype. In addition, patients with mutations in *STAT3* fail to generate Th17 cells and suffer from recurrent chronic mucocutaneous candidiasis (CMC) and *S. aureus* infections (Acosta-Rodriguez et al., 2007) (Ma et al., 2008, Milner et al., 2008, de Beaucoudrey et al., 2008). Notably, patients with deficiency in the autoimmune regulator (AIRE) gene suffer from recurrent CMC and generate autoantibodies against IL-17A, IL-17F and IL-22 (Puel et al., 2010, Kisand et al., 2010). These results indicate that IL-17 and Th17 cells mediate host protection to fungal infection. However, in a mouse model of gastric *C. albicans* infection, IL-23 and IL-17 exacerbate gut pathology (Zelante et al., 2007) indicating that the site of infection may influence the outcome of the Th17 response. The Th17 effector cytokines have been studied in other fungal infection models. In response to *P. carinii* infections, IL-23 deficient mice show higher fungal burden although they are competent to clear infection (Rudner et al., 2007). In addition, during *C. neoformans* infection, IL-23 deficiency is associated with reduced survival and delayed fungal clearance (Kleinschek et al., 2006), suggesting that the IL-17 axis of cytokines may contribute to antifungal defense.

1.4.4 Innate control of Th17 differentiation

Pathogens or PAMPs derived from *Propionibacterium acnes*, *B. burgdorferi*, *K. pneumoniae, B. fragilis*, *C. rodentium, C. albicans* and zymosan trigger the production of IL-17 by $CD4^+$ T cells (Perona-Wright et al., 2009, Infante-Duarte et al., 2000, Happel et al., 2005, Chung et al., 2003, Mangan et al., 2006, Acosta-Rodriguez et al., 2007, Veldhoen et al., 2006b). It is important to note that Th17 cells not only emerge during the course of infections but also are constantly induced by signals derived from the microbiota. Th17 accumulate preferentially in the intestine of mice and their frequency is drastically reduced under germ free conditions (Atarashi et al., 2008, Ivanov et al., 2008). Interestingly, monocolonization of germ free mice with a single commensal bacterium known as segmented filamentous bacterium (SFB) leads to the development of Th17 cells in the gut by unknown mechanisms (Ivanov et al., 2009). Microbiota-derived signals can also act as adjuvants for the initiation of Th17

responses. ATP derived from commensal bacteria activates a $CD70^{high}$ $CD11c^{low}$ subset of intestinal DCs to promote Th17 differentiation (Atarashi et al., 2008).

The role of innate signalling pathways in the initiation of Th17 responses to microbial challenge is yet to be elucidated. For *K. pneumoniae*, innate recognition by TLR4 controls the production of IL-23 and IL-17 (Happel et al., 2003). In response to the microbial flora, Th17 responses are preserved in MyD88-TRIF doubly deficient and RIP-2 deficient mice indicating that signals derived from TLRs and NLRs are either dispensable or redundant (Atarashi et al., 2008, Ivanov et al., 2008, Ivanov et al., 2009). The contribution of the CLR/Syk and CARD9 innate signalling pathway in the induction of intestinal Th17 cells has not been addressed.

Th17 cells also emerge in response to fungi and fungal PAMPs. In this context, fungal CLRs are likely to be crucial sensors of these responses. Zymosan contains ligands for various CLRs and is a strong elicitor of Th17 responses (Veldhoen et al., 2006b). Data presented in this thesis together with those from Salomé LeibundGut Landmann indicate that Dectin-1 ligation results in the generation of IL-17-producing $CD4^+$ T cells (LeibundGut-Landmann et al., 2007, Osorio et al., 2008). In addition to conventional $CD4^+$ T cells, γδ T cells are an important innate source of IL-17 to fungal challenge (Martin et al., 2009). Notably, γδ T cells express TLR2 and Dectin-1 on their surface and respond to curdlan, Pam_3CSK_4 and *C. albicans* by producing IL-17 (Martin et al., 2009). These data strongly suggests that innate production of IL-17 may be of vital importance for an appropriate coordination of antifungal immunity.

Signalling by CLRs directly contribute to the establishment of host defense to fungi. In addition to the enhanced susceptibility to *C. albicans* infection observed in CARD9 deficient animals, this strain of mice is unable to generate Th17 cells to the organism (LeibundGut-Landmann et al., 2007). It is important to note that Th17 but not Th1 cells are absent in CARD9 deficient mice infected with *C. albicans,* suggesting that TLR signalling can induce Th1 responses but cannot compensate for CLR signals in the establishment of a type 17 response to fungi (LeibundGut-Landmann et al., 2007). Notably, the Th17 deficiency observed in CARD9 deficient mice infected with *C. albicans* is not seen in mice lacking Dectin-1, indicating that additional Syk/CARD9

coupled receptors mediate Th17 responses to fungi (LeibundGut-Landmann et al., 2007). Results presented in Chapter 5 of this thesis demonstrate that Dectin-2 is the Syk/CARD9-coupled compensatory CLR responsible for the initiation of a type 17 response to *Candida* (Robinson et al., 2009).

1.5 Immunology of fungal infections

Whilst fungal infections are unusual in healthy individuals, fungal diseases occur in individuals with defects in the innate and/or adaptive arms of the immune system (Crameri and Blaser, 2002). Fungal organisms including *Histoplasma capsulatum, Paracoccidioides spp, C. neoformans, A. fumigatus, Pneumocystis jirovecii* are present in high frequencies in the environment (Romani, 2008). In addition, *C. albicans* is a long-lasting commensal that colonizes human skin, the gastrointestinal tract and the genitourinary tract (Kumamoto, 2008). *C. albicans* infection is the fourth most important cause of hospital acquired bloodstream infections and *A. fumigatus* and *A. terreus* infections are an important cause of death in patients undergoing transplantation (Romani, 2008). On the other hand, cutaneous fungal infections are characterized by colonization with the microorganisms *Microscporum spp, Trichophyton spp* and *Malassezia spp* (Romani, 2008).

The risks of fungal infections are clearly associated with various types of immunodeficiencies. Patients with primary (Hyper IgE syndrome; HIES, autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy; APECED, severe combined immunodeficiencies; SCID, and chronic granulomatosus disease; CMG among others) or secondary immunodeficiencies such as HIV infection are highly susceptible to fungal infections with *C. albicans* (Buckley, 2002, Kisand et al., 2010).

Candida infections vary from local colonization and superficial skin infections to lethal systemic invasion. The risk factors for systemic fungal infections are increased in patients that received organ transplantation, and in patients with leukemia leading to neutropenia and granulocytopenia (Crameri and Blaser, 2002). Indeed, neutropenia is a determinant risk factor for invasive *C. albicans* and *A. fumigatus* infections (Crameri and Blaser, 2002). Along these lines, patients with CMG have defective production of ROS by phagocytic cells and are highly susceptible to fungal infections (Crameri and

Blaser, 2002). The enhanced susceptibility to systemic fungal infections in patients receiving the immunosuppressant cyclosporine A has been recently linked to a defect in neutrophil-associated NFAT signalling in a murine model (Greenblatt et al., 2010). Altogether, these results indicate that fungal killing by phagocytic cells is an essential arm of the systemic antifungal response.

Antigen-specific T cells also play a role in controlling *Candida* infections. Notably, T-cell immunodeficiencies are not strongly linked to systemic mycoses but to infection at mucosal sites. Protection from CMC in nails, skin or mucosal surfaces is thought to be T cell mediated (Grubb et al., 2008, Crameri and Blaser, 2002). In mice models, T-cell deficiency does not render animals more susceptible to systemic *Candida* infection, but it contributes to resistance to mucosal candidiasis in the mouth and gastrointestinal tract (Jones-Carson et al., 2000, Giger et al., 1978, Cantorna and Balish, 1991, Conti et al., 2009). In humans, extensive OPC constitutes a clinical marker of disease in HIV-infected individuals (Greenblatt et al., 2010). Interestingly, these patients are not more susceptible to vaginal candididiasis suggesting that $CD4^+$ T cell protection may not be a determinant for all mucosal sites (Conti et al., 2009). Patients with gene mutations in Stat3 and AIRE are highly susceptible to CMC due to the lack of antifungal Th17 cells or the presence of autoantibodies against IL-17 and IL-22 (Ma et al., 2008, Milner et al., 2008, de Beaucoudrey et al., 2008, Puel et al., 2010, Kisand et al., 2010).

The contribution of antibodies to the resolution of fungal infections is not entirely understood. Even though any fungal organism can trigger an antibody response, it is unknown whether those responses contribute to antifungal immunity (Cutler et al., 2007). Furthermore, there is no clear association between antibody deficiency and susceptibility to fungal infections (Romani, 2004). Nonetheless, it has been shown that anti-β-glucan IgG antibodies elicited in response to β-glucan vaccination protect against systemic and vaginal infections with *C. albicans* (Torosantucci et al., 2005). In addition, glycopeptide vaccines made by conjugation of fungal β-mannan with specific *Candida*-derived antigens elicit antibody responses and induce host protection to systemic infections (Xin et al., 2008). These results indicate that antibody responses induced after vaccination may help resolve disseminated mycoses.

1.6 *C. albicans* recognition by the innate immune system

1.6.1 *C. albicans* cell wall composition

The fungal cell wall is a highly complex structure that preserves the integrity and viability of fungal organisms. The main structural components of *C. albicans* cell wall are β-(1,3)-glucans, β-(1,6)-glucans and chitin (a β-(1,4)-polymer of N-acetylglucosamine (GlcNAc)) (Netea et al., 2008). These molecules are covalently linked to form a dense net of fibrils located in the inner layer of the cell wall (Netea et al., 2008). The outer layer contains a large fraction of glycosylated proteins, including glycosylphosphatidylinositol (GPI)-anchor-dependent cell wall proteins, which are attached to the skeleton core. These glycosylated proteins are highly mannosylated with O-linked and N-linked mannans (Netea et al., 2008). O-linked mannans are short structures of α-(1,2)-mannans linked to Ser/Thr polypeptides. On the other hand, N-linked mannans are highly branched structures consisting of a long backbone of up to 150 α-(1,6) mannans with side chains of α-(1,2) and α-(1,3) mannans and phosphomannan attached to a core of $Man_8GlcNAc_2$ (Netea et al., 2008). According to this molecular organization, mannans are highly exposed PAMPs in *Candida* cell wall whereas β-glucans become accessible in bud scars regions (Netea et al., 2008, Gantner et al., 2005).

1.6.2 PRR recognition of *C. albicans*

Various PRR detect signatures present in *C. albicans*. N-linked mannans are preferentially recognized by mannose receptor, DC-SIGN and SIGNR1 in DCs and macrophages (Cambi et al., 2008, Taylor et al., 2004). It was recently shown that Dectin-2 recognizes α-mannans present in *C. albicans* cell wall and that TLR2 can detect phospholipomannan-containing molecules (Saijo et al., 2010, Jouault et al., 2003). Although Mincle has also been reported to be a PRR for *Candida* (Wells et al., 2008) another study has shown that this receptor recognizes the fungus *Malassezia* exclusively (Yamasaki et al., 2009). Additionally, sensing of O-linked mannans has been attributed to TLR4 (Netea et al., 2006).

C. albicans-derived β-glucans are ligands for Dectin-1, complement receptor 3 (CR3) and the scavenger receptors CD36 and SCARF1 (Gantner et al., 2005, Forsyth et al.,

1998, Means et al., 2009). Chitin is a potent inducer of Th2 responses (Reese et al., 2007) and is highly enriched in *C. albicans* cell wall. However no innate chitin receptors have been identified so far.

Although many PRRs are involved in innate recognition of *C. albicans*, they seem to coordinate different aspects of the antifungal response. Phagocytosis of *Candida* is likely to be mediated by opsonic and non-opsonic receptors, DC-SIGN, MR, and signalling CLRs. On the other hand, activation of the respiratory burst and cytokine production to fungi might be mediated by signalling CLRs and TLRs. PRRs involved in *C. albicans* recognition expressed at the cell membrane level of DCs and macrophages are depicted in figure 1.4.

The contribution of individual PRRs in the establishment of antifungal defense has been elucidated using *in vivo* models of fungal infection. These studies have shown that CR3 and FcγR receptors might have immunosuppressive effects during systemic candidiasis as enhanced resistance and lower fungal burden has been noted in CR3 and FcγR knock out mice infected with the organism (Romani et al., 2004). Along these lines, the contribution of individual TLRs has also been addressed *in vivo*. Two studies have shown that TLR2 deficient mice are more resistant to *C. albicans* infection suggesting an inhibitory role for this receptor during fungal recognition (Netea et al., 2004, Bellocchio et al., 2004). It is proposed that in response to *C. albicans*, TLR2 mediates the induction of IL-10-producing Tregs, which might be detrimental for antifungal defense (Netea et al., 2004). On the other hand, TLR4 or TLR9 deficiency does not impair survival to systemic candidiasis, suggesting that these receptors are redundant (Bellocchio et al., 2004). In contrast to single TLR deficiency, lack of the adaptor MyD88 renders mice highly susceptible to disseminated candidiasis (Bellocchio et al., 2004, Villamon et al., 2004). This effect might also encompass signalling via IL-1R, as IL-1R knock out mice are equally susceptible to systemic infection (Bellocchio et al., 2004). Interestingly, the negative regulator of TLR/IL-1R signalling TIR8 (Toll IL-1R8) is non-redundant in anti-*Candida* defense as TIR8 knock out mice succumbs to disseminated candidiasis (Bozza et al., 2008). These results indicate that a fine regulation of TLR/IL-1R signalling is necessary for effective clearance of fungal pathogens.

The contribution of CLR signalling to *C. albicans* infection is beginning to be elucidated. Mannose receptor is not involved in resistance to intraperitoneal *C. albicans* infection. Nonetheless, this model of infection is not an established model and does not resemble disseminated candidiasis (Lee et al., 2003). In addition, the role of Dectin-1 in systemic antifungal defense is controversial. Whereas one group reported high susceptibility of Dectin-1 deficient mice to *C. albicans* infection, another group reported no differences in infection with the same organism (Taylor et al., 2007, Saijo et al., 2007). The reason accounting for these differences are still unclear. Notably, patients with Dectin-1 deficiency suffer from vulvovaginal candidiasis and onychomycosis (fungal infection in the nail beds) but not from systemic fungal infections (Ferwerda et al., 2009). In murine settings, Dectin-1 and TLR2 control fungal dissemination and inflammasome activation in the model of OPC (Hise et al., 2009). These data indicate that those receptors mediate antifungal responses at least at mucosal sites. On the other hand, although Mincle was previously reported not to signal in response to *C. albicans* stimulation (Yamasaki et al., 2009), higher fungal burden is observed in Mincle deficient mice systemically infected with the organism (Wells et al., 2008).

Work presented in Chapter 5 helped to elucidate the role of Dectin-2 during systemic *Candida* infection. Although we did not observe a role for this receptor in innate resistance within the first week of infection, another group has recently reported the generation of the Dectin-2 deficient mice and has found enhanced susceptibility starting on day 10-post infection (Saijo et al., 2010). This data indicates that Dectin-2 is an additional CLR involved in antifungal defense.

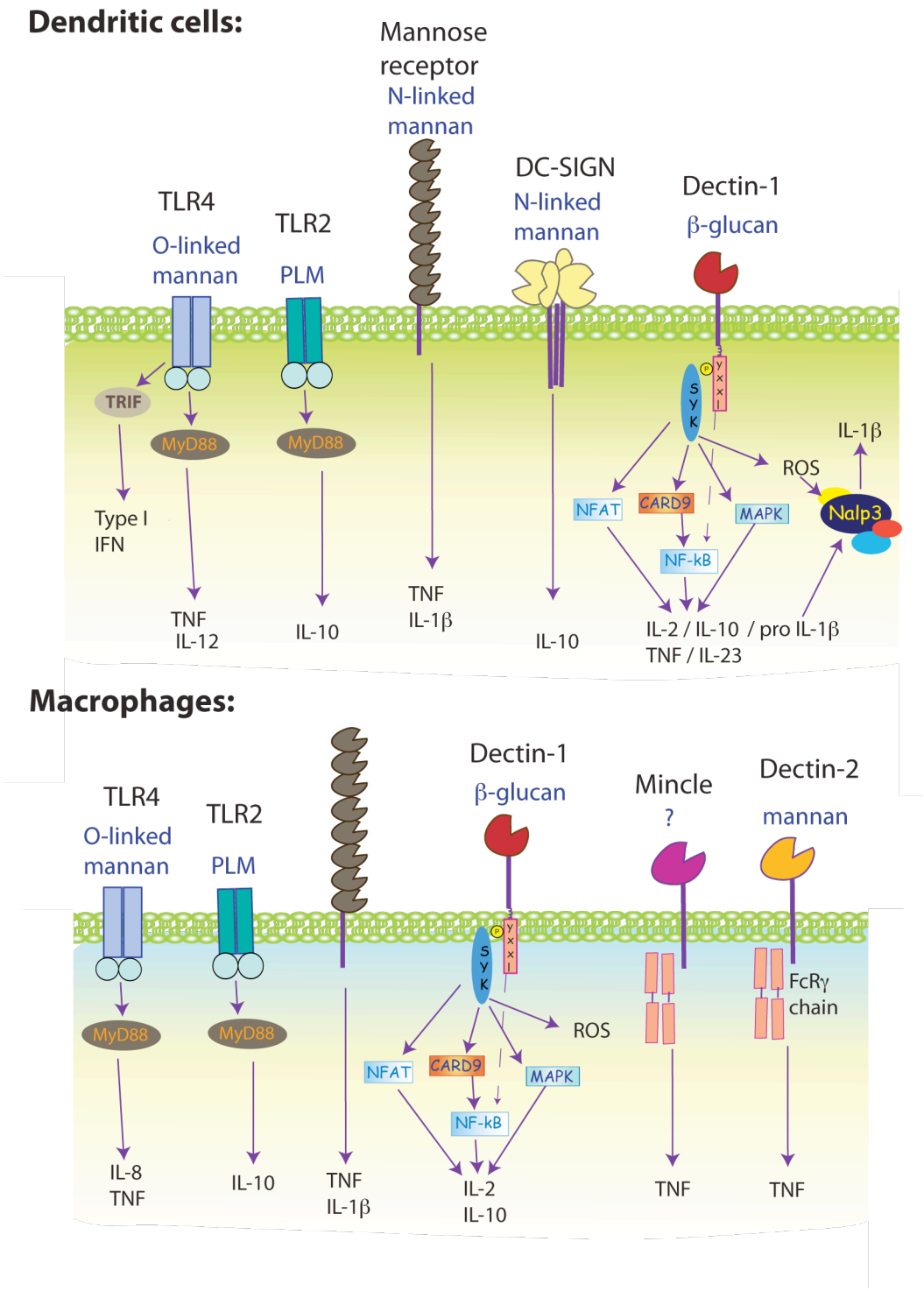


Figure 1-4: Recognition of *C. albicans* by PRRs expressed in DCs and macrophages. Adapted from (Netea et al., 2008, Gross et al., 2006, LeibundGut-Landmann et al., 2007, Wells et al., 2008).

1.7 Questions addressed in this Thesis

When this project was started, it had been recently demonstrated that Dectin-1/Syk/CARD9 constituted an innate signalling pathway that promoted DC activation in absence of TLR signals. Dectin-1 was the first member of the CLR family able to trigger intracellular signalling events resulting in the expression of innate response genes in DCs. Furthermore, Salomé LeibundGut Landmann had recently observed that DCs activated via Dectin-1 induced accumulation of IL-17 producing $CD4^+$ T cells, validating Dectin-1 as a bona fide PRR, according to Janeway's definition. These results opened many interesting avenues in the CLR field. An important question to address was how signalling via Dectin-1/Syk/CARD9 coupled to adaptive immunity. In addition, it was not known whether Dectin-1 was the only CLR competent to induce immunity via Syk and CARD9 or there were additional Syk-coupled CLRs that could account for the CARD9-dependent but Dectin-1-independent aspects of fungal recognition.

The aims of this project are to elucidate the contribution of fungal C-type lectin receptors to the initiation of effector T cell responses to fungal infection. Experiments in this thesis address the role of Dectin-1 and Dectin-2 in DC activation and Th17 cell induction. Questions addressed in each chapter can be summarised as:

1. What are the mechanisms underlying the generation of IL-17-producing $CD4^+$ T cells in response Dectin-1 signalling in DCs?

2. Is Dectin-2 a Syk-coupled PRR involved in Th17 responses to infection?

3. What is the phenotype of antigen-specific T cells generated in response to C. *albicans*-derived antigens during the course of infection? How the CLR signalling pathway contributes to this response?

Chapter 2. Materials and Methods

2.1 Reagents

2.1.1 Common Buffers, Solutions and Media

PBS-Dulbeccos: (GIBCO-BRL)

MACS-Buffer: PBS-Dulbecco containing 1% FCS (Autogen Bioclear, UK) and 2 mM EDTA (Sigma)

RPMI 1640 medium: (GIBCO-BRL)

R10: RPMI 1640 medium supplemented with 10% FCS (Autogen Bioclear), Penicillin 100U/ml, Streptomycin 100U/ml, L-Glutamine 0.3μg/ml (GIBCO-BRL), 50μm β-mercaptoethanol (GIBCO-BRL)

R10*: RPMI 1640 medium supplemented with 10% FCS (Autogen Bioclear), Penicillin 100U/ml, Streptomycin 100U/ml, L-Glutamine 0.3μg/ml (GIBCO-BRL), 50μM β-mercaptoethanol (GIBCO-BRL), 10mM HEPES (GIBCO-BRL), 1mM Sodium pyruvate (GIBCO-BRL), 0.1mM Non-essential Amino acids solution (GIBCO-BRL).

Red Blood Cell (RBC) lysis buffer: (Sigma)

ELISA block: PBS containing 2.5% FCS (Autogen Bioclear), 0.02% NaN3 (w/v) (Sigma)

ELISA coating buffer: 0.1M $NaHCO_3$ (Sigma) in dH_2O, pH 8.2

ELISA wash: 0.05% Tween-20 (Sigma) in PBS1X

LB medium: produced by Cancer Research UK.

Osmotic Stabilizing Buffer (OSB) mix: 0.2M Lithium Acetate solution (Sigma), pH 7.5, 40% Polyethylene glycol 8000 (Calbiohem), 0.1M Dithiothreitol (Invitrogen) and 250 μg/ml DNA MB grade from fish sperm (Roche Diagnostics).

Synthetic Defined (SD) media: ddH_20 containing 0.67% w/v Yeast Nitrogen base without aminoacids (Sigma), 2% glucose.

Synthetic Complete drop out (SC-URI) media: SD Agar media containing 2g/L drop out mix stock.

Drop out mix stock: amino acid composition is shown below (to prepare SC-URI, 2g Uridine must be omitted from the mix)

Drop out mix stock

Amino acids	**(g)**	**Amino acids**	**(g)**
L-Adenine	0.5 g	L-Lysine	2 g
L-Arginine	2 g	myo-Inositol	2 g
L-Asparagine	2 g	L-Methionine	2 g
L-Aspartic acid	2 g	*para*-Aminobenzidine	0.2 g
L-Cysteine	2 g	L-Phenylalanine	2 g
L-Glutamic acid	2 g	L-Proline	2 g
L-Glutamine	2 g	L-Serine	2 g
L-Glycine	2 g	L-Threonine	2 g
L-Histidine	2 g	L-Tryptophan	2 g
L-Isoleucine	2 g	L-Tyrosine	2 g
L-Leucine	10 g	L-Valine	2 g

(all from Sigma)

Yeast Peptone Dextrose (YPD) media: 500ml of ddH_20 containing 1% w/v Yeast Extract, 2% w/v Bactopeptone ± 2% w/v Bactoagar (all DIFCO), 2% glucose, 0.02% w/v adenine (Sigma).

YPD-Uri$^+$ media: YPD media containing 0.08g/L Uridine (Sigma).

2.1.2 Peptides, cytokines and neutralizing antibodies

Ovalbumin peptide (residues 323-339) and SIINFEKL peptide (residues 257-264) were synthesized and purified by high performance liquid chromatography at Cancer Research UK. Ovalbumin protein (OVA) was from Calbiochem.

Anti-CD3ε (clone 145-2C11) antibody was from BD pharmingen.

Recombinant cytokines used for in vitro assays were rmIL-23 (eBioscience), rhTGF-β1 (Sigma), rmIL-6 and rhIL-2 (RnD systems).

Neutralizing antibodies and isotype controls used in assays *in vitro* are described in the following table:

Antibody	**Clone**	**Company**
Anti-TGF-β	1D11	RnD systems
Anti-mouse IgG1	11711	RnD systems
Anti-p19	G23.8	eBioscience
Anti-rat IgG1	R3-34	BD Pharmingen
Anti-IFN-γ	XMG1.2	BD Pharmingen
Anti-IL-4	11B11	BD Pharmingen

2.1.3 Microbial Stimuli

Curdlan, purified from *Alcaligenes faecalis* was purchased from Wako (Osaka, Japan) and suspended in PBS at a concentration of 10mg/ml. CpG 1668 oligonucleotide (sequence TCCATGACGTTCCTGATGCT) was synthesized by Sigma.

Zymosan was purchased from InvivoGen (San Diego, CA). Lipopolysaccharide (LPS) from E. coli, Serotype R515 was purchased from Alexis Biochemicals (UK)

2.2 Cells and Mice

2.2.1 Mice

Wild-type C57BL/6 (H-2b) were obtained from Charles River or Cancer Research UK animal facility (Clare Hall, UK). Congenic B6.SJL.CD45.1 mice were from the Cancer Research UK animal facility (Clare Hall, UK).

C57BL/6 or congenic B6.SJL.CD45.1 OT-II mice bearing a transgenic TCR specific for $OVA_{323-339}$ presented on I-Ab were from the Cancer Research UK animal facility (Clare Hall, UK).

Congenic B6.SJL.CD45.1 OT-I mice bearing a transgenic TCR specific for $OVA_{257-264}$ (SIINFEKL) presented on H2-Kb were originally obtained from Dr Dimitris Kioussis (National Institute for Medical Research, Mill Hill, UK). OT-I transgenic mice on a *rag1*$^{-/-}$ background were originally obtained from Dr Fiona Powrie (Oxford University, UK). Both strains are bred at the Cancer Research UK animal facility (Clare Hall, UK).

MyD88 and TRIF deficient mice were a gift from Dr Shizuo Akira (Osaka, Japan) and MyD88-TRIF double knock out mice were generated by intercrossing the two strains. *Myd88*$^{-/-}$*trif*$^{/-}$ mice are bred at Cancer Research UK under specific pathogen free conditions.

DEREG mice (Lahl et al., 2007) are bred at Technische Universität München or at Cancer Research UK in specific pathogen-free conditions.

IL-23 p19-deficient mice (Ghilardi et al., 2004) were provided by A. MacDonald (Edinburgh, UK) with kind permission from N. Ghilardi, Genentech, and are bred at Cancer Research UK in specific pathogen-free conditions.

CD11cΔSyk mice used in this study is the intercross between the *CD11c cre*$^{+}$ mice strain (Kindly provided by Dr Boris Reizis, Columbia University medical center, New York and described in (Caton et al., 2007) and the *syk*$^{fl/fl}$ mice strain (Kindly provided by Dr Alexander Tarakhovsky, Rockefeller University, New York). CD11cΔSyk mice were genotyped by PCR for the expression of the Cre transgene and floxed alleles.

Rorc(γt)-Gfp^{TG} mice (Lochner et al., 2008) were bred at Institut Pasteur. Radiation chimeras in C57BL/6 hosts using bone marrow from DEREG or *Rorc(γt)-Gfp*^{TG} mice were generated at Cancer Research UK.

Bone marrow from *Clec7a*$^{-/-}$ mice (Taylor et al., 2007) was a kind gift from Dr G.D. Brown (University of Cape Town, South Africa) and fetal liver cells from *Syk*$^{-/-}$ embryos were a kind gift from Dr V.L. Tybulewicz (National Institute for Medical Research, Mill Hill, UK). Bone marrow from those mice was used to generate radiation chimeras in C57BL/6 hosts.

All animal experiments were performed in accordance with national and institutional guidelines for animal care.

2.2.2 Bone marrow chimeras

Host mice were kept on acid water (0.1 ml conc. HCL in 840 ml water, sterile filtered) for one week before irradiation and then for at least 4 weeks after bone marrow reconstitution. Mice received γ-irradiation using a ^{137}Cs source (IBL 637; CISbio International, Gif-sur-Yvette, France) with two doses of 5.5 Gy separated by 3 hrs. 20-24 hrs later, mice were reconstituted intravenously with >10^6 bone marrow or foetal liver cells in 200μl of sterile PBS. The health status of the mice was checked periodically. Six weeks after reconstitution blood samples were taken for FACS analysis of congenic marker expression on granulocytes and T cells. Full reconstitution was accepted when 95-100% of the granulocyte population were of donor origin.

2.2.3 Generation of GM-CSF BMDC

Bones from femurs and tibia from 8-12 weeks old mice were prepared under sterile conditions and the bone marrow was flushed with 2 ml of R10 medium into a 5 ml polypropylene tube using a 1ml syringe with a 23G needle. Cells were strained through a 70μm filter into 22.5 ml R10 containing GM-CSF (GM-CSF was made by Cancer Research protein purification service and was titrated for optimal growth conditions of BMDCs) and plated into a 6-well plate (Falcon). Cells were cultured at 37°C and 5% CO2. On day 2 of culture, 2.5ml of the culture medium was removed and was replaced with 3 ml of fresh R10 medium containing GM-CSF. On day 3 of culture, the wells were washed off extensively in order to remove all non-adherent and loosely adherent cells. The medium was replaced with 4 ml of fresh R10 medium containing GM-CSF. On day 5 of culture, cells were harvested using a sterile rubber policeman and collected in a 50ml tube (Falcon). CD11c-expressing cells were purified after enrichment with CD11c MACS beads (Miltenyi biotec) (30μl per 10^7 cells) using a LS MACS column according to manufacturer's instructions. CD11c$^+$ cells purified under this protocol are usually >95% pure and the yield is 10^7 cells approximately.

2.2.4 Isolation of conventional DC from spleen

Spleens were aseptically removed and were perfused with serum-free RPMI containing Liberase C1 (1.7 U/ml, Roche Diagnostics) and DNase I (0.2 mg/ml, Roche Diagnostics). Samples were incubated at 37°C for 30 min. Digested spleens were mashed through a 50μm cell strainer and washed with ice-cold MACS buffer. Single cell suspensions were suspended in 190μl of MACS buffer and 10μl of CD11c-MACS beads was added (clone N418, Mitenyi biotec, UK). Tubes were incubated for 10 min at 4°C and were washed with ice-cold MACS buffer. CD11c positive cells were positively selected using varioMACS and LS columns (Miltenyi Biotec) according to manufacturer's instructions. Alternatively, the CD11c enriched fraction was labelled with anti-CD8 FITC, anti-CD4 PerCP and anti-CD11c APC antibodies and $CD11c^+CD4^+$, $CD11c^+CD8^+$ and $CD11c^+CD8^-CD4^-$ populations were sorted by flow cytometry using a MoFlo (Dako cytomation) or a FACSAria (BD Biosciences).

2.2.5 $CD4^+$ T cell purification

Cell sorting of $CD4^+$ T cells was performed using a MoFlo (Dako cytomation) or a FACSAria (BD Biosciences).

For in vitro T cell assays containing T cells from OT-II mice (on C57BL/6 background), $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells were sorted from spleens by cell sorting. Alternatively, for studies of antigen presentation of *C. albicans*-associated antigens, $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ (naïve) cells from OT-II mice were sorted from spleens by cell sorting.

For in vitro T cell assays containing T cells from C57BL/6 mice, $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ (naïve) and $CD4^+CD25^+$ cells were sorted from spleens by cell sorting.

For in vitro T cell assays containing T cells from DEREG mice, $CD4^+GFP^+CD25^+$ and $CD4^+GFP^-CD25^-$ cells from DEREG mice or DEREG bone marrow chimeras were purified from spleen and lymph nodes by cell sorting.

For in vitro T cell assays containing T cells from *Rorc(γt)-Gfp*^{TG} bone marrow chimeras, $CD4^+GFP^+CD25^+FR4^+CD45.1^-$ and $CD4^+GFP^-CD25^+FR4^+CD45.1^-$ cells were purified from mesenteric lymph node by cell sorting.

For in vivo experiments involving naïve $CD4^+$ T cells from OT-II mice, naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ were sorted from spleens and lymph nodes by cell sorting.

2.2.6 $CD8^+$ T cell purification

$CD8^+$ T cells were isolated from spleen and lymph nodes from OT-I mice. Spleen was perfused in 5 ml of R10 and it was centrifuged. Red blood cells were lysed using 1 ml RBC buffer per spleen for 4 min and samples were washed with R10. Lymph nodes were mashed through a 50μm cell strainer to produce a cell suspension. Cells from spleen and lymph nodes were pooled and washed twice in ice-cold MACS-buffer. A lineage depletion cocktail of PE-conjugated antibodies was used for negative selection of $CD8^+$ T cells. All antibodies were used at 1:100 dilutions and were purchased from BD Pharmingen.

Antibody	**Antibody**
PE anti-CD4	PE anti CD16/CD32
PE anti-CD11b	PE anti-B220
PE anti-Ly6G/C	PE anti-CD11b

Antibody clone details are provided in the Flow cytometry section

Cells were incubated with PE-conjugated antibodies for 15 minutes at 4ºC and washed twice in ice-cold MACS buffer. The cell suspension was incubated with anti-PE multisort MACS beads (Miltenyi Biotec) for 10 minutes at 4ºC and then washed in ice-cold MACS buffer. $CD8^+$ T cells were negatively selected using MACS LS columns according to manufacturer's instructions.

2.2.7 CFSE labelling of cells

Cell suspensions were washed twice in PBS and suspended in 5 ml of PBS. 2μl of CFSE 5mM was added to the cells and tubes were incubated 12 min at 37ºC. Cells were

washed twice with PBS if used for *in vivo* experiments or with R10* if used for *in vitro* assays.

2.3 In vitro assays for DC activation

2.3.1 Stimulations of BMDC:

For analysis of cytokine production, 10^5 BMDC were cultured for 18-24 hrs in p96 U-bottom plates in 200μl of R10 supplemented with GM-CSF. Cells were incubated in the presence of the following stimuli: curdlan (50μg/ml), CpG (0.5μg/ml), LPS (100ng/ml), Zymosan (100μg/ml), heat-killed or live *C. albicans* yeast or hyphae (10^5–$5x10^5$). For cultures containing live *C. albicans*, 2.5μg/ml of fungizone (Invitrogen) or 50ng/ml caspofungin (Merck) was added to the cultures after 2 hrs of stimulation. Cytokines were detected in supernatants and quantified by sandwich ELISA.

2.3.2 Study of Dectin-2 function in BMDC:

10^5 BMDC were cultured as described above. Before the addition of stimuli, cells were pre-incubated for 2 hrs with 10μg/ml soluble anti-Dectin-2 antibody (clone 11E4, kindly provided by Dr Phillip Taylor, Cardiff university) or an isotype-matched control (Rat IgG2a). 10^5–$5x10^5$ heat-killed or live *C. albicans* were added to the cultures for a total time of 12-14 hrs. 2.5μg/ml of fungizone (Invitrogen) or 50ng/ml caspofungin (Merck) was added to the cultures 2 hrs after live *C. albicans* stimulation. Cytokine levels in the supernatants were evaluated by sandwich ELISA.

2.3.3 Dectin-1 and Dectin-2 staining by FACS

Splenic DCs were isolated as in section 2.2.4 and stained with anti-CD8 FITC, anti-CD4 PerCP and anti-CD11c-APC in ice-cold FACS buffer containing 1% mouse serum. Anti-Dectin-1 antibody (clone 2A11) or isotype matched control were used at a concentration of 10μg/ml. Alternatively, anti-Dectin-2 antibody (clone 11E4) or isotype matched control were used at a concentration of 10μg/ml. Cells were incubated for 30 min at 4ºC and washed in ice-cold FACS buffer. Anti-rat IgG-PE (1:200, Jackson ImmunoResearch laboratories) was used a secondary reagent for both Dectin-1 and

Dectin-2 antibodies. Samples were incubated for 30 min at 4ºC and washed in ice-cold FACS buffer prior to acquisition.

2.3.4 Lentiviral knockdown of Dectin-2 in BMDC:

The shRNA construct for mouse Dectin-2 in PLKO.1 lentiviral vector (TRCN0000066785, Open Biosystems) chosen for this study was based on a previous publication (Barrett et al., 2009). The hairpin construct consists of a 21-bp sense and antisense sequence and a 6-bp loop. The sequence for TRCN0000066785 is as follows:

CCGGGCGGTGTGTTTCAATAGTTTACTCGAGTAAACTATTGAAACACACCGC TTTTTG.

Color codes: sense, loop, antisense.

A scramble shRNA was used on these studies as a control. Lentiviral particles were generated in the laboratory of Dr Luis Ferreira Moita (Instituto de Medicina Molecular, Universidade de Lisboa, Portugal) after cotransfection of 293T cells with the PLKO.1 construct, the packaging vector Δ8.9 and the envelope vector VSV-G.

10^5 bone marrow precursors were cultured in R10 medium supplemented with GM-CSF in p96 U-bottom plates. At day 2, media was carefully removed and 10µl of the viral stocks was added to the cells. 40µl of media containing 8µg/ml hexadimethrine bromide was added immediately to the well and cells were centrifuged at 1,130 x g for 90 minutes, 37ºC. After centrifugation, the media-containing virus particles was carefully removed and replaced with 200µl R10 supplemented with GM-CSF. At day 4, 5µg/ml puromycin was added to the cultures. On day 6, cells were harvested and used for stimulations as described in section 1.3.1.

2.4 In vitro assays for T cell activation

$CD4^+$ T cells are isolated as described in section 2.2.5. Cells were cultured in p96 U bottom plates (Falcon) in a total volume of 200µl in R10*.

For assays containing T cells from OT-II mice, 5×10^4 $CD4^+CD25^-$ OT-II T cells were cultured in the presence or absence of 2.5×10^4 $CD4^+CD25^+$ OT-II T cells (2:1 mixture)

plus $1x10^4$ BMDCs and 2nM of $OVA_{323-339}$ peptide. Innate stimuli used were 50μg/ml curdlan and 0.5μg/ml CpG oligonucleotide 1668. Exogenous cytokines used were rhTGF-β1 (1, 10ng/ml) and neutralizing antibodies used were anti-TGF-β (10μg/ml) and anti-p19 (10μg/ml) or isotype matched antibodies.

For assays containing T cells from C57BL/6 mice, DEREG mice or Rorc(γt)-Gfp^{TG} chimeras, 5×10^4 sorted T cells were cocultured with $2x10^4$ BMDCs and 0.2μg/ml of soluble anti-CD3ε in the presence or absence of 50 μg/ml curdlan, 0.5 μg/ml CpG oligonucleotide 1668 or IL-23 (10ng/ml).

Th17 control consisted of $CD4^+GFP^-CD25^-$ T cells from DEREG mice or DEREG bone marrow chimeras cocultured with BMDCs in presence of rhTGF-β1 (10ng/ml), IL-6 (20ng/ml) and neutralizing antibodies against IFN-γ (2μg/ml) and IL-4 (2μg/ml). "Treg" control consisted of $CD4^+GFP^+CD25^+$ cells from DEREG mice or DEREG bone marrow chimeras cocultured with BMDCs in presence of 100IU/ml of rhIL-2. On day 5 or day 7, cells were stimulated with PMA, ionomycin and brefeldin A for 4h. Intracellular staining for Foxp3 and IL-17 was analyzed by flow cytometry as described on section 2.6.2 and 2.6.3. Alternatively, half of the content of each well were stimulated on day 5 on plate bound anti-CD3ε (5μg/ml) for 48 h and cytokines in the supernatant were analyzed by sandwich ELISA as described in section 2.5.

2.5 Sandwich ELISA for detection of mouse cytokines

For detection of cytokines present in supernatants of stimulated cells, MAXISORP 96-well immunoplates (Nunc 4-39454) are coated with 50μl of a solution containing capture antibodies diluted in ELISA coating buffer. Plates are sealed and incubated for 6hr at room temperature or overnight at 4°C in a humidified chamber. Then plates are washed 3 times with ELISA wash and blocked with 200μl ELISA block for 2 hrs at RT. After blocking, plates are washed 3 times with ELISA wash and 50μl of experimental supernatants and cytokine standards are added to the wells. Sealed plates are incubated overnight at 4°C in a humidified chamber following by 3 washes with ELISA wash. 50μl of detection antibodies diluted in ELISA block are added to the wells and plates are incubated 1hr at RT. Plates are washed 6 times with ELISA wash and incubated

with 50µl of Extravidin-Alkaline Phosphatase (Sigma) diluted 1:5000 in ELISA block for 1hr at RT. Then plates are washed 6 times with ELISA wash and 100µl of Alkaline Phosphatase substrate (Sigma FAST tablet set, pNPP, Sigma) is added to the wells. 405nM Absorbance is measured after 15min, 1hr and 2hrs using a SpectraMax 190, Molecular Devices, UK and data is analyzed using SoftMax Pro software (Molecular Devices) and GraphPad Prism software.

	IL-2	**IL12 p40**	**TNF**	**IL-10**	**IL-6**
Capture antibody	JES6-1A12 (4µg/ml) BD Pharmingen	C15.6 (5µg/ml) BD Pharmingen	AF-410-NA (2µg/ml) R&D	JES5-2A5 (4µg/ml) BD Pharmingen	MP5-20F3 (4µg/ml) BD Pharmingen
Top standard	5 ng/ml	10ng/ml	15 ng/ml	15 ng/ml	10ng/ml
Detection antibody	JES6-5H4-Biotin (1µg/ml) BD Pharmingen	C17.8 Biotin (1µg/ml) BD Pharmingen	BAF410-Biotin (250ng/ml) R&D	SXC-1-Biotin (1 µg/ml) BD Pharmingen	MP5-32C11-Biotin (1 µg/ml) BD Pharmingen

	IFN-γ	**IL-17**
Capture antibody	R4-6A2 (5µg/ml) BD Pharmingen	TC11-18H10 (2µg/ml) BD Pharmingen
Top standard	20ng/ml	10ng/ml
Detection antibody	XMG1.2 Biotin	TC11-8H4.1 Biotin

	(0.5μg/ml) BD Pharmingen	(1μg/ml) BD Pharmingen

2.6 Flow cytometry

2.6.1 Surface staining of cells

Cell suspensions were washed once in ice-cold FACS buffer and labelled with surface staining antibodies for 20 min at 4ºC on the dark. Data were acquired on a FACSCalibur (BD Biosciences) or LSRII (BD Biosciences) and analyzed using FlowJo software (Treestar).

All antibodies were purchased from BD pharmingen unless otherwise indicated and used at a concentration of 1:100.

Antibody	**Clone**	**Antibody**	**Clone**
Anti-CD4	RM4-5	Anti-CD45.1	A20
Anti-CD8α	53-6.7	Anti-CD62L	MEL-14
Anti-CD11b	M1/70	Anti-TCRβ	H57-597
Anti-CD11c	HL3	Anti-FR4 (eBioscience)	12A5
Anti CD16/CD32	2.4 G2	Anti-Ly6G/C	RB6-8C5
Anti-CD25	PC61	Anti-B220	RA3-6B2
Anti-CD44	IM7		

2.6.2 Intracellular staining for T cell cytokines/transcription factors

Intracellular antibodies used in this study

Antibody	**Clone**	**Company**	**Antibody**	**Clone**	**Company**
Anti-IL-17	TC11-18H10.1	eBioscience	Anti-IL-17	TC11-18H10.1	BD Pharmingen
Anti-Foxp3	FJK-16s	eBioscience	Anti-IFN-γ	XMG1.2	BD Pharmingen

Cells suspensions were restimulated for 4h with phorbol 12-myristate 13-acetate (PMA)(10ng/ml; Sigma), ionomycin (1μg/ml; Calbiochem) and brefeldin A (5μg/ml; Sigma) or Golgiplug (1:1000; BD pharmingen). Cells are washed once with ice-cold FACS buffer and stained with surface antibodies. After 20 min incubation at 4ºC, cells were washed in ice-cold FACS buffer and fixed with "Fix and Perm" Reagent A (Caltag laboratories) for 30 min at room temperature. Cells were washed once in ice-cold FACS buffer and resuspended in "Fix and Perm" Reagent B (Caltag laboratories) containing fluorochrome-conjugated intracellular antibodies. After incubation for 20 min at 4ºC, cells were washed and resuspended in ice-cold FACS buffer containing a defined number of Calibrite beads (BD pharmingen).

All antibodies were used at a concentration of 1:100.

2.6.3 Intracellular staining of Foxp3

Cells suspensions were restimulated, stained and washed as described above. Cells were fixed using a Fixation/Permeabilization solution from mouse/rat Foxp3 staining set (eBioscience) for 30-45 min at 4ºC. After incubation, cells were washed in permeabilization buffer 1X from mouse/rat Foxp3 staining set (eBioscience) and resuspended in permeabilization buffer 1X containing fluorochrome-conjugated intracellular Foxp3 and IL-17 antibodies. After incubation for 30 min at 4ºC, cells were washed with permeabilization buffer 1X and resuspended in ice-cold FACS buffer containing a defined number of Calibrite beads (BD pharmingen).

2.7 Western Blot for Syk kinase (appendix section)

Splenic DCs from CD11cΔSyk mice and WT littermates were purified with CD11c beads (described in section 2.2.4) and sorted into $CD11c^+CD4^+$, $CD11c^+CD8^+$ and $CD11c^+CD8^-CD4^-$ population by flow cytometry. As a positive control, B220 expressing cells were sorted by flow cytometry. Cells were washed in PBS and suspended in 60μl of RIPA buffer (1% Nonidet-P40, 0.5% deoxycholic acid, 0.1% SDS, 150mM NaCl, 10nM NaF, 1mM Na_3VO_4, 2nM $Na_4P_2O_7$ and a cocktail of protease inhibitors (Roche). Samples are incubated in ice for 30 min followed by

centrifugation at 13000rpm for 10 min. Supernatants containing the protein fraction are carefully transferred into new tubes containing SDS loading buffer. Protein extracts were separated on 4-20% acrylamide gradient, Tris-glycine (Novex, Invitrogen), in Tris-Glycine-SDS running buffer (Invitrogen) and subsequently transferred into a polyvinyldifluoride membrane (Millipore) using methanol transfer buffer (48mM Tris-base, 39nM Glycine, 1.3 mm SDS, 20% MeOH). Membrane was blocked in PBS-1%Tween-20 containing 5% w/v milk for 1 hour at RT. Syk protein was detected using a anti-Syk rabbit serum (serum "2131" raised against the peptide 318-330 of murine Syk and described in (Turner et al., 1995), kindly provided by Dr V. Tybulewicz). Serum 2131 was diluted 1:1000 in PBS-1%Tween-20 containing BSA and membranes were incubated overnight at 4ºC with shaking. The membrane was then washed 3 times for 15 min in 15ml PBS-1%Tween-20 at RT with shaking. HRP-goat-anti-rabbit HRP was diluted 1:5000 in PBS-1%Tween-20 containing BSA and membrane was incubated for 1hr at RT with shaking. After the incubation, the membrane was washed 3 times for 15 min in 15ml PBS-1%Tween-20 at RT with shaking and incubated for 5 min with Supersignal WestPico Chemiluminescent substrate (Pierce). The membrane was exposed to X-ray film for developing.

2.8 Molecular Biology and cloning

2.8.1 Transformation of competent *E. Coli*

1µl of plasmid DNA was added to 60µl of One Shot TOP10® chemically competent bacteria *E. Coli* (Invitrogen) and samples were incubated 30 min on ice. Tubes were transferred to a 42ºC water bath for 1 minute and then were returned to ice for 10 min. 500µl of SOC medium (Invitrogen) was added to the samples and tubes were incubated in a shaker at 1050 rpm, 1 hr and 37ºC. Cells were centrifuged at 2000 rpm for 2 min and suspended in 200µl SOC medium. Cells were plated into selective agar plates containing 100µg/ml Carbenicillin (Sigma) following by incubation at 37ºC, overnight.

2.8.2 Plasmid isolation

Resistant clones were picked using sterile tips into 5ml LB containing 100µg/ml carbenicilin and incubated at 37ºC, 220rpm overnight. Plasmid isolations were performed by the miniprep service provided by the equipment park (Cancer Research

UK). For large-scale plasmid isolation, 1 ml of the cultures was added to 200ml of LB containing carbenicilin and incubated at 37ºC, 220 rpm overnight. Cells were centrifuged at 3500 rpm for 5 minutes and pellets were processed using HiPure Plasmid Filter Maxiprep Kit (Invitrogen) according manufacturer's instructions.

2.8.3 Restriction enzymes reaction

All enzymes, buffers and BSA were purchased from New England Biolabs (UK) and used according to manufacturer's instructions. Briefly, 1μg of plasmid was added to 2μl of 10x buffer, ± 0.2μl BSA, 1μl of restriction enzyme and ddH_20 to a final volume of 20μl. Samples were incubated for 1-1.5 hours at 37ºC.

2.8.4 Gel Electrophoresis

DNA samples were prepared on ddH_20 and mixed with DNA loading buffer (Sigma) in ratio 6:1. 1% Agarose gels were prepared in TAE buffer and melted in a microwave oven. 1μg/ml of ethidium bromide (Sigma) was added to the gel before pouring it into a gel tray. The solidified gel was placed onto a gel chamber filled with TAE buffer and a voltage of 80-120 V was applied for 30-45 min.

2.8.5 Gel purification of DNA fragments

Bands were excised by using a sterile scalpel and cleaned using QIAGEN gel purification kit according to manufacturer's instructions.

2.8.6 Purification of DNA fragments from solution

DNA purification was carried out using QIAquick PCR purification kit (QIAGEN) according to manufacturer's intructions. DNA was routinely eluted in 30μl of ddH_20.

2.8.7 Isolation of RNA from T cells

$CD4^+$ T Cells were harvested on day 5 or 7, washed on ice-cold PBS and supernantats were discarded. Total RNA was prepared by adding 1 ml of TRIzol reagent (Invitrogen) and 5 min incubation at room temperature. 200μl of chloroform was added to each sample and tubes were shaken vigorously by hand for 20 sec. Samples were incubated at room temperature for 3 min and then centrifuged at 12,000 g, 15 min, 4ºC. The upper phase (600μl approx) was transferred into a new eppendorf tube and 500μl isopropanol plus 5μg glycogen was added to the samples. Tubes were mixed by inversion and

incubated for 10 minutes at room temperature. Then samples were centrifuged at 12,000 g for 15 minutes at 4ºC supernatants were discarded. RNA was washed with 500µl of 70% ethanol and centrifuged at 7,500 g for 5 min at 4ºC. Supernatants were discarded and RNA was air-dried at room temperature. Samples were resuspended in 50µl ddH_2O.

2.8.8 Isolation of RNA from BMDC stimulated with *C. albicans*

Total RNA from BMDCs was extracted after 3 hrs of stimulation with yeast or hyphae using the RNeasy Mini Kit (QIAGEN) following manufacturer's instructions. As an additional step, contaminating DNA was removed from the samples by using RNase-Free DNase set (QIAGEN) according to manufacturer's instructions.

2.8.9 Reverse transcription reaction

cDNA was synthesized from total RNA with random hexamers and Superscript II following manufacturer's instructions. Briefly, 11.5µl of RNA solution were incubated with 1µl of random hexamers 50µM (Invitrogen) for 10 min at 70ºC. Tubes were placed on ice and 7.5µl per reaction of a Superscript II containing mix (see below) was added to the samples.

Superscript II-containing mix (all from Invitrogen):

5x first strand buffer	4µl
0.1M DTT	2µl
10mM dNTP	1µl
SuperscriptII	0.5µl
Total volume per 1 sample	7.5µl

Samples were incubated for 60 min at 42ºC. Then samples were incubated 15 min at 70ºC and left at 4ºC.

20µl of cDNA generated above was diluted by adding 40µl of ddH_2O plus 0.1µg/ml glycogen to each tube.

2.8.10 Real-time RT-PCR

5µl/well of the cDNA solution prepared as above was added to a MicroAmp™ Optical 96-well reaction plate (Applied Biosystems). Quantitative real-time PCR was carried out using Platinum SYBR green pPCR Supermix-UDG with ROX (Invitrogen).

Reaction protocol per well:

2x SYBRgreen pre-mix	10µl
ddH_20	4.8µl
Primer mix (stock 10µM)	0.2µl
Template	5µl
Total volume per 1 sample	20µl

Measurements were performed in duplicate wells using the ABI PRISM 7700 sequence detection system (Applied Biosystems) and analysed using SDS 1.9.1 and Microsoft Excel.

List of real time PCR primers used in this thesis:

Gene name	**Primer name**	**Sequence**	**Reference**
IL-17F fwd	C135	GAGGATAACACTGTGAGAGTTGAC	(Ivanov et al., 2006)
IL-17F rev	C136	GAGTTCATGGTGCTGTCTTCC	
RORγt fwd	C137	CCGCTGAGAGGGCTTCAC	(Ivanov et al., 2006)
RORγt rev	C138	TGCAGGAGTAGGCCACATTACA	
IL-23R fwd	C139	GCCAAGAAGACCATTCCCGA	(Mangan et al., 2006)
IL-23R rev	C140	TCAGTGCTACAATCTTCTTCAGA GGACA	
IL-23 fwd	A583	TGCTGGATTGCAGAGCAGTAA	(Holscher et al., 2001)
IL-23 rev	A584	GCATGCAGAGATTCCGAGAGA	

18S fwd	C66	CGGCTACCACATCCAAGGAA	-
18S rev	C67	GCTGGAATTACCGCGGCT	

GADPH primers were bought commercially from Applied Biosystems.

Normalization was performed using 18S rRNA or GADPH as a reference and results are shown as relative mRNA quantities.

2.9 Treg control of *Helicobacter hepaticus*-triggered intestinal inflammation

H. hepaticus infections were carried out at the laboratory of Dr Fiona Powrie (Oxford University, UK) as previously described (Maloy et al., 2003), and were done in conjunction and under supervision of Philip Ahern, senior graduate student in that laboratory. Briefly, *H. hepaticus* was grown in blood agar plates under microaerophilic conditions and checked for viability prior to infection. *129SvEvRag2*$^{-/-}$ female mice were infected by oral gavage four times on alternate days with $5x10^7$–$1x10^8$ CFU *H. hepaticus.* In addition, mice were reconstituted on the first day of infection with $1x10^5$ $CD4^+CD45RB^{low}CD25^+$ T cells intraperitoneally (purified by cell sorting facility at Oxford university).

Alternatively, a group of mice was additionally fed with 5mg curdlan in PBS by oral gavage on a weekly basis starting on the first day of infection. Weight loss was monitored throughout the course of the experiment and mice were sacrificed 8 weeks after the first *H. hepaticus* inoculation. Spleen, mesenteric lymph node and colonic lamina propria were removed for T cell analysis and histological score was performed by facilities at Oxford University.

2.10 Construction of *C. albicans* strains

2.10.1 *C. albicans* preparations

Strains of *C. albicans* were routinely grown at 30ºC on YPD media overnight in a water bath with agitation with the exception of CAI4 strain, which was grown on YPD-Uri^+ media. For cell stimulations or infections, cells were washed twice with sterile PBS before use. Cells were used either live or heat-killed by boiling for 30-45 minutes.

For hyphal induction, cells were suspended in RPMI containing 10% FCS at a concentration of 10^7 cells/ml. 5 ml of the cell suspension was seeded on a petri dish and incubated overnight at 37ºC, 5% CO_2. After washing in PBS, hyphae was used as live or heat-killed for stimulations. Morphology was verified by differential interference contrast microscopy using a 40x objective (Plan Fluor; Nikon) and analyzed with ImageJ program.

2.10.2 *C. albicans* strains generated in this thesis

The strains of *C. albicans* used on this study are listed in the following table:

Strain	Parent	Genotype	Reference
SC5314	Wild type		
CAI4	CAF2-1	*ura3::λimm434/ura3::λimm434*	(Fonzi and Irwin, 1993)
pACT1-GFP	CAI4	*ura3::λimm434/ura3::λimm434 RPS10/RPS10 ::CIp10-ACT1p-GFP*	(Barelle et al., 2004)
pACT1- GFP-$OVA_{323-339}$	CAI4	*ura3::λimm434/ura3::λimm434 RPS10/RPS10 ::CIp10-ACT1p-GFP*-$OVA_{323-339}$	This study
pACT1- GFP-SIINFEKL	CAI4	*ura3::λimm434/ura3::λimm434 RPS10/RPS10 ::CIp10-ACT1p-GFP*-SIINFEKL	This study

2.10.3 Plasmids for *C. albicans* transformation

pACT1-GFP (Barelle et al., 2004) is a vector derived from the integrative plasmid CIp10 (Murad et al., 2000) expressing a codon optimised yeast enhanced version of GFP (described in(Cormack et al., 1997)) under the promoter region (-2 to-1019) of the *Ca*ACT1 gene (Barelle et al., 2004).

The fusion GFP-$OVA_{323-339}$ was generated by PCR from pACT1-GFP vector using the oligonucleotides FO 8_5' and FO 23_3' and then subcloned at the *HindIII* and *NheI* sites in pACT1-GFP vector in place of GFP to generate pACT1-GFP-$OVA_{323-339}$.

The fusion GFP-SIINFEKL was generated by PCR as described above for GFP-$OVA_{323-339}$ but using the oligonucleotides FO 8_5' and FO 22_3'.

The sequences encoding $OVA_{323-339}$ and SIINFEKL peptides were designed for optimal codon utilisation in *C. albicans* according to Brown et al (Brown et al., 1991).

Oligonucleotide name	**Sequence (5'-3')[a,b]**
FO 23 (*C. albicans* optimized $OVA_{323-339}$ –NheI) antisense	TAGCGCTAGCTTAAACAACTTCTCTACC AGCTTCATTAATTTCAGCATGAGCAGCA TGAACAGCTTGTGAAATTTTAAGGTCGA CTTTGTACAATTCATCCATACC
FO 22 (*C. albicans* optimized SIINFEKL –NheI) antisense	TAGCGCTAGCTTAAGTCAATTTTTCAAAA TTAATAATACTCTCAAGGTCGACTTTGTA CAATTCATCCATACC
FO 8 (located upstream of GFP ORF)	CTCCTGGTTTTCTTTCTTTC

[a] black underline indicates NheI restriction site
[b] blue underline indicates SalI restriction site

Fusion sequences were checked by digestion with the restriction endonucleases HindIII/NheI and HindIII/SalI and analyzed by sequencing.

2.10.4 Sequencing of PACT1-GFP, PACT1-GFP-$OVA_{323-339}$ and PACT1-GFP-SIINFEKL

DNA sequencing was performed by the equipment park at Cancer Research UK. Briefly, 8µl of BigDye Terminator mix (Cancer Research UK) was mixed with 2µl of sequencing primer (3.2 pmol) and 10ul of a solution containing 150 ng of plasmid in ddH_20. The PCR reaction was carried out using the following program:

Step 1	Denature	96ºC	30s
Step 2	Anneal	50ºC	15s
Step 3	Extension	60ºC	4mins
Step 4	Return to step 1	x 25	

Step 5	Chill	4ºC	Forever

The primers FO 10 and FO 21 were used to confirm plasmid integrity

FO 10: sense - GTCTTGTTACCAGACAACC it binds within GFP region

FO 21: antisense – CTTGAGTTGGATCTACGCG it binds downstream of GFP region in PACT1-GFP vector

2.10.5 *C. albicans* transformation

Before transformation, all plasmids were linearized with the blunt restriction endonuclease StuI (New England Biolabs) following by 30 min at 65ºC to allow enzyme inactivation.

C. albicans was transformed using a modified version of the lithium-acetate procedure (Sanglard et al., 1996). A single colony of *C. albicans* CAI4 (Uridine auxotroph, kindly provided by Dr. Alistair Brown, Aberdeen, UK) was grown for 24 hrs on YPD-Uri^+ media in a water bath with agitation. 200µl of the culture was centrifuged and resuspended in 100µl of Osmotic Stabilizing Buffer (OSB mix described above). 1µg of the linearized vector was added to the cell suspension and tubes were vortexed to allow mixing. Cells were incubated for 30-45 min at 43.5ºC and plated out onto selective SC-URI media. Few transformants were observed 3-4 days after transformation. Single colonies were grown on YPD (Uri^-) media overnight and frozen stocks were kept at -80ºC.

2.10.6 Isolation of *C. albicans* genomic DNA

The procedure for isolation of yeast genomic DNA has been described previously (Harju et al., 2004). A single colony of *C. albicans* was inoculated into YPD and incubated overnight at 30ºC in a water bath with agitation. 1.5 ml of liquid culture was transferred to a microcentrifuge tube and centrifuged at 13000 x g for 5 min. Cells were resuspended in 200µl of lysis buffer (2%Triton X-100, 1%SDS, 100mM NaCl, 10mM Tris-HCl, pH 8.0, 1mM EDTA pH 8.0). The tubes were placed in -80ºC freezer for 2 minutes (until completely frozen) following by 1 min incubation at 95ºC. This process

was repeated once and the tubes followed by vortex for 30 seconds. 200µl Chloroform was added to the tubes and followed by vortex for 2 minutes and centrifugation at room temperature 13000 x g, 3 min. The aqueous phase was transferred to a microcentrifuge tube containing 400µl ice-cold 100% ethanol and the samples were incubated 5 minutes at room temperature and then centrifuged at 13000 x g for 5 minutes. Supernatants were discarded and pellets were washed with 500µl of 70% ethanol. Samples were incubated 5 minutes at 60ºC and resuspended in 25-50µl ddH_20 for PCR analysis.

2.10.7 Plasmid integration into RPS10 locus in *C. albicans*

Integration into the *C. albicans* RPS10 locus was confirmed by PCR using the following primers described in (Barelle et al., 2004)

Oligonucleotide name	**Sequence**
FO 24 (located on chromosomal RPS10 locus, outside of plasmid sequences) (Barelle et al., 2004)	CGTATTCACTTAATCCCACAC
FO 25 (located in the GFP ORF) (Barelle et al., 2004)	CCAATTGGTGATGGTCC

PCR reaction was performed in a 50µl reaction containing 50pmol of primers using the following program:

Step 1	Denature	95ºC	7 mins
Step 2	Denature	95ºC	1 min
Step 3	Anneal	60ºC	45s
Step 4	Extension	72ºC	1min
Step 5	Return to step 2	x 29	
Step 5	Chill	4ºC	forever

GFP expression in *C. albicans* integrants was confirmed by western blot.

2.10.8 *C. albicans* protein extraction protocol

A single colony of *C. albicans* was inoculated into YPD and grown overnight at 30ºC in a water bath with agitation. 10^6 cells were washed with ddH_2O and cells were suspended on 100µl of ddH_2O. 100µl of NaOH 0.2M was added to the cell suspension following

by vortex and 5 minute incubation at room temperature. After centrifugation at 13000xg for 1 min, media was carefully removed and 50μl of 2x loading buffer containing DTT was added to the protoplasts. Then the suspension was incubated for 5 min at 95ºC and centrifuged at 10000xg for 10 min. Supernatants were collected and 5μl were loaded on an acrylamide gel for western blot analysis.

2.10.9 Western blot for detection of GFP in *C. albicans* transformants

Protein extracts were resolved by SDS PAGE (4-20% acrylamide gradient, Tris-glycine; Invitrogen). Proteins were transferred to a polyvinyldifluoride membrane (Millipore) as in section 2.7. GFP (FL) protein was detected by incubation with an anti-GFP rabbit polyclonal IgG antibody (Santa cruz, sc-8334, used 1:1000) and a HRP Goat anti-rabbit IgG (H+L) antibody (Invitrogen, used 1:5000). GFP was visualized using a chemoluminescence kit (Pierce).

2.10.10 Microscopy

C. albicans morphology was verified by differential interference contrast microscopy using a 40x objective (Plan Fluor, Nikon).

For analysis of GFP expression, cells were grown in synthetic defined media to avoid autofluorescence and GFP fluorescence was analysed using a LSM 510 confocal microscope (Zeiss).

2.11 In vitro T cell differentiation assays to *Candida*-associated antigens

2.11.1 Assays containing $CD4^+$ OT-II T cells

10^4 BMDC were incubated in a p96 U-bottom plate for 2 hrs with various doses of heat-killed or live *C. albicans*-GFP-$OVA_{323-229.}$ When using live organisms, fungizone was added at the end of the incubation time. Then $5x10^4$ naïve OT-II T cells labelled with CFSE were added to the wells. As a positive control, $OVA_{323-339}$ peptide was used at the indicated concentrations and *C. albicans*-GFP (9/1 *candida*/DC) was used as a negative control. On day 5, half of each well was analyzed and counted by FACS. The second half was restimulated for analysis of cytokine production by plating on immobilised

anti-CD3 (5μg/ml) for 48 hours in a p96 flat bottom plate. Cytokines were quantified by sandwich ELISA as described in section 2.5.

2.11.2 Assays containing $CD8^+$ OT-I T cells

10^4 BMDC were incubated in a p96 U-bottom plate for 2 hrs with various doses of heat-killed or live *C. albicans*-GFP-SIINFEKL. When using live organisms, fungizone was added at the end of the incubation time. Then $5x10^4$ OT-I T cells labelled with CFSE were added to the wells. As a positive control, SIINFEKL peptide was used at the indicated concentrations and *C. albicans*-GFP (9/1 *candida*/DC) was used as a negative control. On day 5, half of each well was analyzed and counted by FACS. The second half was restimulated for analysis of cytokine production by plating on immobilised anti-CD3 (5μg/ml) for 48 hours in a p96 flat bottom plate. IFN-γ production was quantified by sandwich ELISA as described in section 2.5.

2.12 Systemic *C. albicans* infection model

2.12.1 Study of Dectin-2 function during systemic *C. albicans* infection

These experiments were performed in the laboratory of Dr Philip Taylor (Cardiff University, UK). Wild-type or *Clec7a* $^{-/-}$ (129/Sv background) female mice aged 12-16 weeks were injected with 200μg anti-Dectin-2 (D2.11E4) or isotype matched control (OX11), intraperitoneally. After 6h, mice were injected i.v. with $3x10^4$ live *C. albicans* yeast. On day 2 and 4 after infection, mice were further injected i.p. with 200μg of anti-Dectin-2 or isotype control. Mice were weighed on a daily basis and on day 7, they were sacrificed. Total splenocytes were harvested and seeded in a 96-well U bottom plate at $2x10^6$ cells/well. Splenocytes were restimulated with medium alone, 10^5 or 10^6 heat-killed *C. albicans* per well for 2 days. IL-17 and IFN-γ on supernatants were measured by sandwich ELISA as described in section 2.5.

2.12.2 Analysis of antigen-specific $CD4^+$ T cell responses to *C. albicans*-associated antigens

These experiments were performed at Imperial college and Cancer Research UK. Naïve OT-II cells are isolated from congenic B6.SJL.CD45.1 OT-II mice as described in 2.2.5 and labelled with CFSE. $1x10^6$ OT-II cells were transferred i.v. into wild-type

(C57BL/6) female mice aged 12-18 weeks. One day later, mice were infected i.v. with $1x10^5$ or $5x10^4$ *C. albicans*-GFP-$OVA_{323-339}$ or *C. albicans*-GFP. Mice were sacrificed on day 5 or alternatively, on day 7. OT-II proliferation was analyzed on day 5 in Spleen, mesenteric LN, renal LN, brachial LN and inguinal LN by gating on $CD4^+CD45.1^+$ cell population. For intracellular cytokine staining, renal LN cells were stimulated with PMA and Ionomycin in the presence of BFA as described in 2.6.2.

2.12.3 Analysis of antigen-specific $CD8^+$ T cell responses to *C. albicans*-associated antigens

These experiments were performed at Imperial college and Cancer Research UK. Naïve OT-I cells are isolated from congenic B6.SJL.CD45.1 OT-I mice as described in 2.2.6 and labelled with CFSE. $1x10^6$ OT-I cells were transferred i.v. into wild-type (C57BL/6) female mice aged 12-18 weeks. One day later, the mice were infected i.v. with $5x10^4$ *C. albicans*-GFP-SIINFEKL or *C. albicans*-GFP. Mice were sacrificed on day 4. OT-I cell proliferation and cytokine production were analyzed by FACS in kidneys, spleen and renal LN. For intracellular FACS staining in the OT-I cell population, $2x10^6$ splenocytes and half of a renal LN were pulsed with 1μM SIINFEKL peptide for 1 hour following by the addition of BFA for 3 additional hours.

2.12.4 Systemic infection in CD11cΔSyk mice (appendix section).

These experiments were carried out at Cancer Research UK. *cd11c* cre^+ x $syk^{fl/fl}$ (CD11cΔSyk) or *cd11c* cre^- x $syk^{fl/fl}$ (wild type littermate) female mice aged 12-18 weeks were injected i.v. with $5x10^4$ live *C. albicans* yeast. Mice were weighed on a daily basis and were sacrificed at day 6-7 or when the human end point established in the national and institutional guidelines for animal care (Animal Scientific Procedures Act 1986, UK) was reached.

2.12.5 Quantification of fungal burden

Kidneys, lung and liver were aseptically removed, weighed and disrupted in 0.5ml PBS using an electrical homogenizer (T 25 digital ULTRA-TURRAX®, IKA®). 20μl of serial dilutions (0, 1/10, 1/100, 1/1000 and 1/10000) were plated on agar plates in 20μl drops in duplicate. Plates were incubated overnight at 37ºC and the total number of colonies was counted and calculated back to colony forming units.

2.12.6 Histopathology (appendix)

Samples were fixed in 10% Neutral Buffered Formalin and analyzed by the histopathology laboratory at Cancer Research UK. Samples were dehydrated with ethanol and embedded in paraffin. Periodic Acid Schiff (PAS) and hematoxylin and eosin (H&E) were used to assess fungal invasion and leukocyte infiltration respectively.

2.12.7 Statistical analysis

The statistical significance of the study described in 2.12.1 was determined by one-way analysis of variance with Tukey multiple comparison of all pairs for posttest analysis.

The statistical significance of the experiments described in section 2.9 and 2.12.4 (appendix section) was determined by Mann-Whitney U test. All other values are graphed as mean ± SD unless otherwise stated.

Chapter 3. Dectin-1 in the induction of IL-17-producing $CD4^+$ T cells.

3.1 Introduction

3.1.1 Curdlan is an adjuvant for the induction of Th17 responses.

Before this project was initiated it was established that Dectin-1 was a PRR that directed signalling via the Spleen tyrosine kinase Syk ((Rogers et al., 2005, Underhill et al., 2005), for details see introduction). Syk deficiency abrogated the production of IL-2 and IL-10 in zymosan-stimulated DCs and partially decreased uptake of fungal particles (Rogers et al., 2005). Furthermore, the adaptor CARD9 was identified as a downstream effector of Dectin-1/Syk signalling by activating NF-κB in response to fungal infection (Gross et al., 2006).

Those studies demonstrated that the Dectin-1/Syk/CARD9 axis constituted a novel innate signalling pathway that can act independently of TLR/MyD88 stimulation. Nonetheless, it remained unclear whether Dectin-1/Syk/CARD9 signalling was capable of inducing cytokines other than IL-2 and IL-10 on DCs. Moreover, it was undefined whether activation via Dectin-1 rendered DCs competent to prime effector T cell responses.

To determine the impact of Dectin-1/Syk signalling pathway on phenotype and function of DCs, it was necessary to identify a selective Dectin-1 agonist. Dr Salomé LeibundGut-Landmann (former member of the Immunobiology Lab, currently based at ETH zurich, Switzerland) validated the β-(1,3)-glucan preparation known as curdlan (for details see introduction) as a purified Dectin-1 agonist. Stimulation of bone marrow derived DC (BMDC) with curdlan led to the induction of the costimulatory molecules CD86, CD80 and CD40, activation of the MAPKs p38, Erk and Jnk and activation of NF-κB (LeibundGut-Landmann et al., 2007). Furthermore, curdlan-activated BMDC produced a subset of cytokines including IL-2, IL-10, TNF, IL-6, IL-12p40 and IL-23 but very little IL-12 p70 (LeibundGut-Landmann et al., 2007). Importantly, all cytokines triggered by the Dectin-1 agonist were dependent on Dectin-1, Syk and CARD9 and were independent of MyD88 and TRIF (LeibundGut-Landmann et al.,

2007). Thus, this work demonstrated for first time that selective activation of Dectin-1/Syk/CARD9 signalling pathway in isolation was sufficient to induce full DC activation (LeibundGut-Landmann et al., 2007).

Notably, curdlan-stimulated BMDCs produced high levels of the cytokines involved in Th17 cell induction including IL-6, TNF and IL-23 (Aggarwal et al., 2003, Veldhoen et al., 2006a, Bettelli et al., 2006, Mangan et al., 2006, Harrington et al., 2005). This distinctive cytokine signature elicited by this Dectin-1 agonist suggested that curdlan could be used as an adjuvant for the induction of Th17 responses. Thus, Salomé LeibundGut-Landmann first showed that when curdlan-stimulated BMDCs were cultured with transgenic OT-II $CD4^+$ T cells in the presence of specific antigen, an accumulation of IL-17-producing $CD4^+$ T cells was observed (LeibundGut-Landmann et al., 2007). The production of IL-17 was dependent on Syk signalling in BMDCs and was independent of MyD88 (LeibundGut-Landmann et al., 2007). These series of experiments elegantly demonstrated that the Dectin-1/Syk/CARD9 axis constitute an innate signalling pathway leading to the induction of Th17 effector responses (LeibundGut-Landmann et al., 2007). Remarkably, depletion of $CD4^+CD25^+$ T cells from the $CD4^+$ transgenic T cell preparation in curdlan-containing cocultures reverted this effect (LeibundGut-Landmann et al., 2007), as previously reported for TLR-containing cultures (Veldhoen et al., 2006a). Only when $CD4^+CD25^+$ T cells were present, the production of IL-17 was restored and transcripts encoding RORγt, IL-17A and IL-17F were detected (LeibundGut-Landmann et al., 2007).

In vivo, curdlan could also be used as an adjuvant for inducing Th1 and Th17 responses and antibody production against ovalbumin (LeibundGut-Landmann et al., 2007). In sum, these results demonstrate that activation of the Dectin-1/Syk/CARD9 innate signalling pathway results in the instruction of adaptive immunity.

The aim of work described in this chapter is to identify the mechanism involved in the induction of Th17 responses upon activation of the Dectin-1 signalling pathway in DCs.

3.2 Results

3.2.1 Curdlan-activated BMDCs trigger IL-17 production by $CD4^+$ T cells at various doses of antigen or stimulus.

The remarkable superiority of curdlan over CpG at inducing Th17 responses (LeibundGut-Landmann et al., 2007) raises the question as to whether this faculty reflects qualitative differences between both innate signalling pathways or is the result of a quantitative effect. Factors such as the antigen dose and concentration of microbial stimuli can influence the ability of DCs to direct Th1 and Th2 development (Boonstra et al., 2003). First, we decided to evaluate whether the efficiency of curdlan-stimulated BMDCs to induce Th17 responses was subjected to the concentration of antigen present in the cultures. $CD4^+$ T cells were isolated from OT-II transgenic mice, which express a TCR specific for the peptide 323-339 from ovalbumin. $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from OT-II mice were cultured in a mixture 2:1 in the presence of BMDCs stimulated with curdlan or CpG plus different concentrations of $OVA_{323\text{-}339}$ peptide for 5 days. The production of IL-17 and IFN-γ was measured by sandwich ELISA after two days of restimulation with plate-bound α-CD3 antibody. Results are shown in Figure 3.1.a. When no stimulus was present, lower peptide concentrations resulted in low production of IFN-γ and IL-17 whereas higher doses of peptide led to the production of IFN-γ as previously described (Boonstra et al., 2003). The presence of CpG enhanced the production of IFN-γ at 2nM of $OVA_{323\text{-}339}$ peptide and elicited little production of IL-17 at various doses of antigen. The presence of curdlan did not promote the secretion of IFN-γ compared to unstimulated cultures but led to the production of high levels of IL-17. These results indicate that variations on antigen concentration do not modify the quality of the $CD4^+$ effector response induced by DCs activated with the Dectin-1 agonist (LeibundGut-Landmann et al., 2007).

To determine whether the concentration of stimuli modifies the ability of BMDCs to induce Th1 or Th17 cells, titration of both stimuli was performed. $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from OT-II mice plus BMDCs were cultured with 2nM of $OVA_{323\text{-}339}$ peptide in the presence of various doses of stimuli and the production of IL-17 and IFN-γ was measured after restimulation. Results are shown in Figure 3.1b. Whereas various doses of CpG led to the production of high levels of IFN-γ and very little IL-17

from CD4$^+$ T cells, titration of curdlan in the cocultures resulted in the production of high levels of IL-17 and little IFN-γ. These results indicate that curdlan is a bona fide Th17 inducer and its effect is a consequence of qualitative effects on DC activation, as titration of both stimuli does not induce interconversion of their effect (LeibundGut-Landmann et al., 2007).

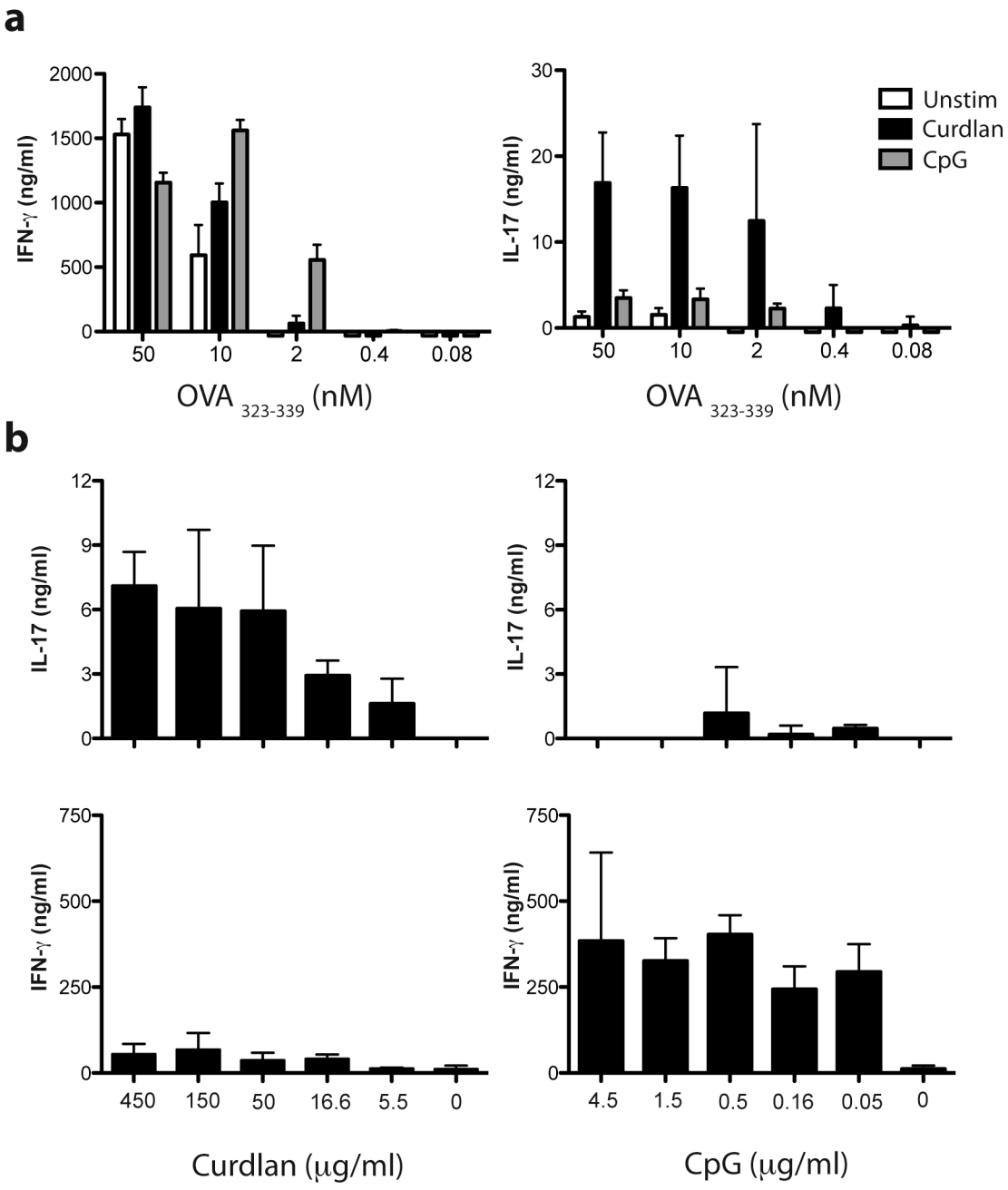


Figure 3-1: Dendritic cells stimulated with curdlan preferentially induce Th17 cells.
(**a**) $5x10^4$ $CD4^+CD25^-$ OT-II T cells and $2.5x10^4$ $CD4^+CD25^+$ OT-II T cells were cocultured with $1x10^4$ C57BL/6 plus 50μg/ml curdlan or 0.5μg/ml CpG and the indicated amounts of $OVA_{323-339}$. At day 5, cells were restimulated for 2 extra days in the presence of 5μg/ml coated a-CD3e. (**b**) Cells were cultured as in (**a**) in the presence of 2nM $OVA_{323-339}$ plus the indicated amounts of curdlan or CpG. Cytokines were measured by sandwich ELISA and data shown are mean + s.d. of triplicate wells.

3.2.2 Curdlan-activated BMDCs promote development of IL-17-producing $CD4^+$ T cells in a mechanism involving IL-23, TNF, TGF-β and $CD4^+CD25^+$ T cells.

As mentioned above, curdlan-stimulated BMDCs produced cytokines involved in the development of Th17 cells such as IL-6, TNF and IL-23 (LeibundGut-Landmann et al., 2007). Thus we sought to identify whether those factors are required for the generation of IL-17-producing $CD4^+$ T cells in response to Dectin-1–activated BMDCs. Neutralizing antibodies specific for IL-23 and TNF were added to cocultures containing the $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells mixture, BMDCs plus $OVA_{323-339}$ peptide in the presence of curdlan or CpG.

Figure 3.2a shows that in curdlan-containing cocultures, IL-23 plays a non-redundant role in promoting the generation of Th17 cells as the addition of a blocking antibody against the p19 subunit of IL-23 abrogated the production of IL-17 when compared to an isotype-matched control.

As previously described for TLR containing cocultures (Veldhoen et al., 2006a), the addition of an anti-TNF antibody to curdlan-containing cocultures slightly reduced the production of IL-17 (Fig 3.2b), reinforcing the notion that this cytokine has an accessory role by augmenting the frequency of IL-17 producers.

We attempted to determine the role of IL-6 in the differentiation of Th17 cells from cocultures containing the Dectin-1 agonist. The addition of a neutralizing antibody to IL-6 did not modify the production of IL-17 in cocultures containing curdlan as stimulus (data not shown). Is important to note that there may be caveats with this experiment associated with the efficiency of the blocking antibody to neutralize cytokine bioactivity. In agreement with previous reports (Bettelli et al., 2006, Veldhoen et al., 2006a), IL-6 signalling might be crucial for Th17 induction in conditions of Dectin-1 stimulation and this would be clarified by using IL-6 deficient BMDCs.

Considering the relevance of TGF-β signalling in the process of Th17 differentiation (reviewed in (Weaver et al., 2007, Korn et al., 2009)), we sought to determine the involvement of this factor in Dectin-1-associated Th17 responses. The addition of a neutralizing antibody to TGF-β to curdlan-containing cocultures abrogated the production of IL-17 after restimulation (Fig 3.2.c), in agreement with previous publications (Veldhoen et al., 2006a, Bettelli et al., 2006, Mangan et al., 2006).

Interestingly, accumulation of IL-17-producing cells in cocultures containing the Dectin-1 agonist has shown to be directly proportional to the amount of $CD4^+CD25^+$ OT-II T cells present initially in the well (LeibundGut-Landmann et al., 2007). In this set of experiments, Salomé LeibundGut-Landmann showed that Tregs did not only act to prevent Th1 differentiation, as their effect could not be mimicked by the addition of an IFN-γ neutralizing antibody (LeibundGut-Landmann et al., 2007). In addition, it is known that Tregs produce robust amounts of TGF-β for Th17 differentiation in cultures containing TLR agonists (Veldhoen et al., 2006a). In order to test whether TGF-β could substitute for the Treg presence in cocultures containing curdlan, $CD4^+CD25^+$ T cells from OT-II mice were replaced with 1 or 10ng/ml of hTGF-β1 in cocultures containing $CD4^+CD25^-$ OT-II T cells, BMDCs, $OVA_{323-339}$ peptide in the presence of curdlan or CpG. Results are shown in Figure 3.2.d. The addition of exogenous hTGF-β1 to curdlan containing cocultures supported the differentiation of Th17 cells albeit at very low levels. Broader titration of TGF-β was performed with similar results (data not shown) indicating that the presence of Tregs in the culture results in greater Th17 skewing. These results are particularly intriguing as they suggest that Tregs may not only act as TGF-β producers under this condition of culture. One explanation to these findings is that $CD4^+CD25^+$ T cells serve in part as a source for TGF-β but they also provide additional signals that support the induction of Th17 cells.

In this context I sought to further investigate the role of $CD4^+CD25^+$ T cells in the induction of Th17 by curdlan-stimulated BMDCs.

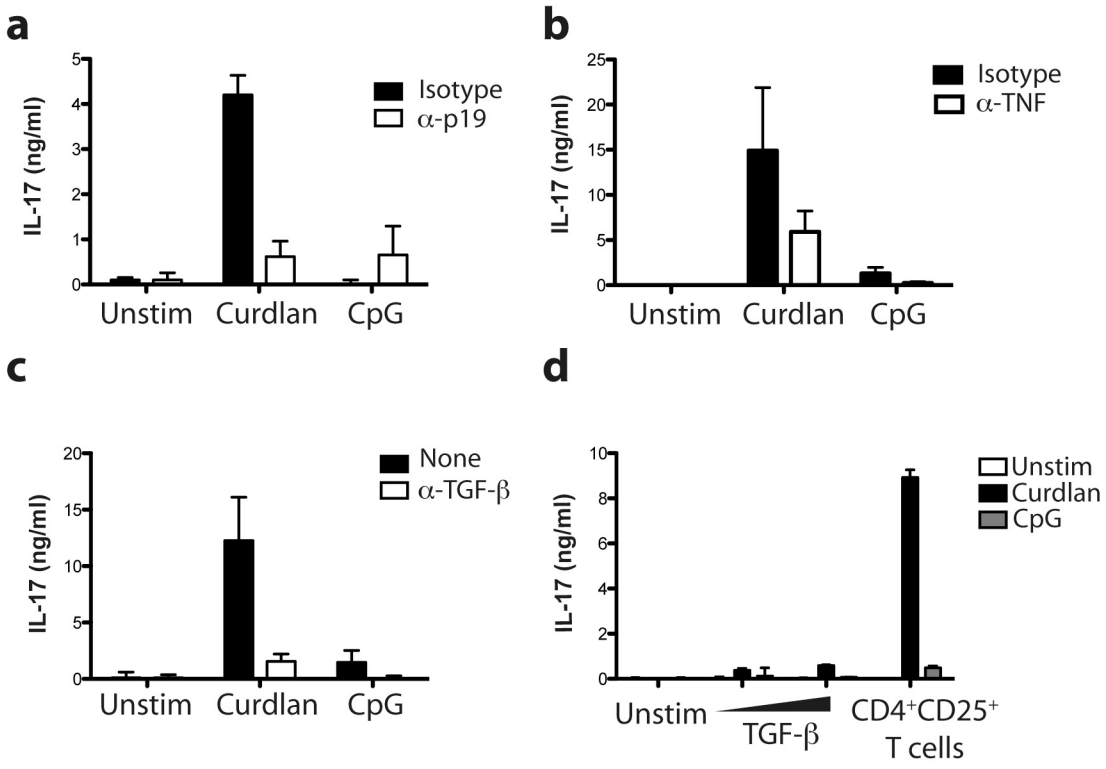


Figure 3-2: Dectin-1 activated BMDCs induce IL-17-producing CD4$^+$ T cells through a mechanism involving IL-23, TNF and TGF-β.
(**a**) 5x10^4 CD4$^+$CD25$^-$ OT-II T cells and 2.5x10^4 CD4$^+$CD25$^+$ OT-II T cells were cocultured with 1x10^4 C57BL/6 plus 50μg/ml curdlan or 0.5μg/ml CpG and 2nM OVA$_{323-339}$. On days 0 and 3, 10μg/ml anti-IL-23p19 (α-p19) or Isotype control was added to the cultures. At day 5, cells were restimulated for 2 extra days in the presence of 5μg/ml coated a-CD3e. (**b**) as in (**a**) with the addition of 10μg/ml anti-TNF (α-TNF) or Isotype matched control on day 0. (**c**) as in (**a**) with the addition of 10μg/ml anti-TGF-b (α-TGF-b) on day 0. (**d**) Cells were cultured as in (**a**) with the addition of increasing doses of recombinant human TGF-β1 (1 and 10ng/ml) on day 0. Cytokines were measured by sandwich ELISA and data shown are mean + s.d. of triplicate wells. Data are representative of two to three independent experiments.

3.2.3 CD4$^+$CD25$^+$ T cells cultured with curdlan-activated BMDCs become IL-17 producers.

To further investigate the role of CD4$^+$CD25$^+$ T cells in the induction of curdlan-associated Th17 responses, we dissected our original three-cell system consisting of CD4$^+$CD25$^-$ T cells, CD4$^+$CD25$^+$ T cells and BMDCs described above. We cocultured, separately, naïve T cells (purified as CD4$^+$CD25$^-$CD62L^{high}CD44^{low}) or CD4$^+$CD25$^+$ T cells from C57BL/6 mice with BMDCs plus soluble α-CD3 mAb in the presence of curdlan or CpG in order to identify the cell source of IL-17. In Figure 3.3a, cultures

were restimulated at day 5 of culture for 2 extra days in the presence of coated α-CD3 mAb (5μg/ml) and the production of IFN-γ and IL-17 was evaluated by capture ELISA. In cultures in which naïve T cells were the starting population, the presence of CpG led to the secretion of IFN-γ rather than IL-17. The inverse was observed when curdlan was present, as previously shown in Figure 3.1 and in (LeibundGut-Landmann et al., 2007). Remarkably, in cultures containing $CD4^+CD25^+$ T cells as the starting population, neither curdlan nor CpG induced the secretion of IFN-γ but curdlan induced the production of high levels of IL-17.

To determine the production of IL-17 on a per-cell basis I employed intracellular staining for cytokines. Cultures containing naïve or $CD4^+CD25^+$ T cells (as described above) were analyzed after 5 days by restimulation with PMA, Ionomycin and Brefeldin A for 4 hrs and stained with a surface antibody to CD4 and antibodies to intracellular IL-17 and IFN-γ. Results are shown in Fig 3.3b. In agreement with the ELISA data, the presence of CpG in cultures containing naïve T cells led to the development of cells producing IFN-γ. On the other hand, the presence of curdlan in the same cultures induced a small population of cells producing IL-17. Notably, in cocultures containing $CD4^+CD25^+$ T cells as the starting population, the presence of curdlan promoted the appearance of a large frequency of IL-17 producing cells. The total number of IL-17 producers and total $CD4^+$ T cells at the end of the culture were calculated by adding a fixed amount of Calibrite beads during acquisition. Figure 3.3c illustrates that the effect induced by curdlan in cultures containing $CD4^+CD25^+$ T cells is accompanied by an increase in the absolute number of IL-17 producing cells. In addition, there is a greater expansion of total $CD4^+CD25^+$ T cells when compared to cultures containing CpG or lacking any innate stimulus suggesting that factors produced by BMDCs in response to curdlan favour proliferation /survival of $CD4^+CD25^+$ T cells and promote the development of an IL-17 producing population.

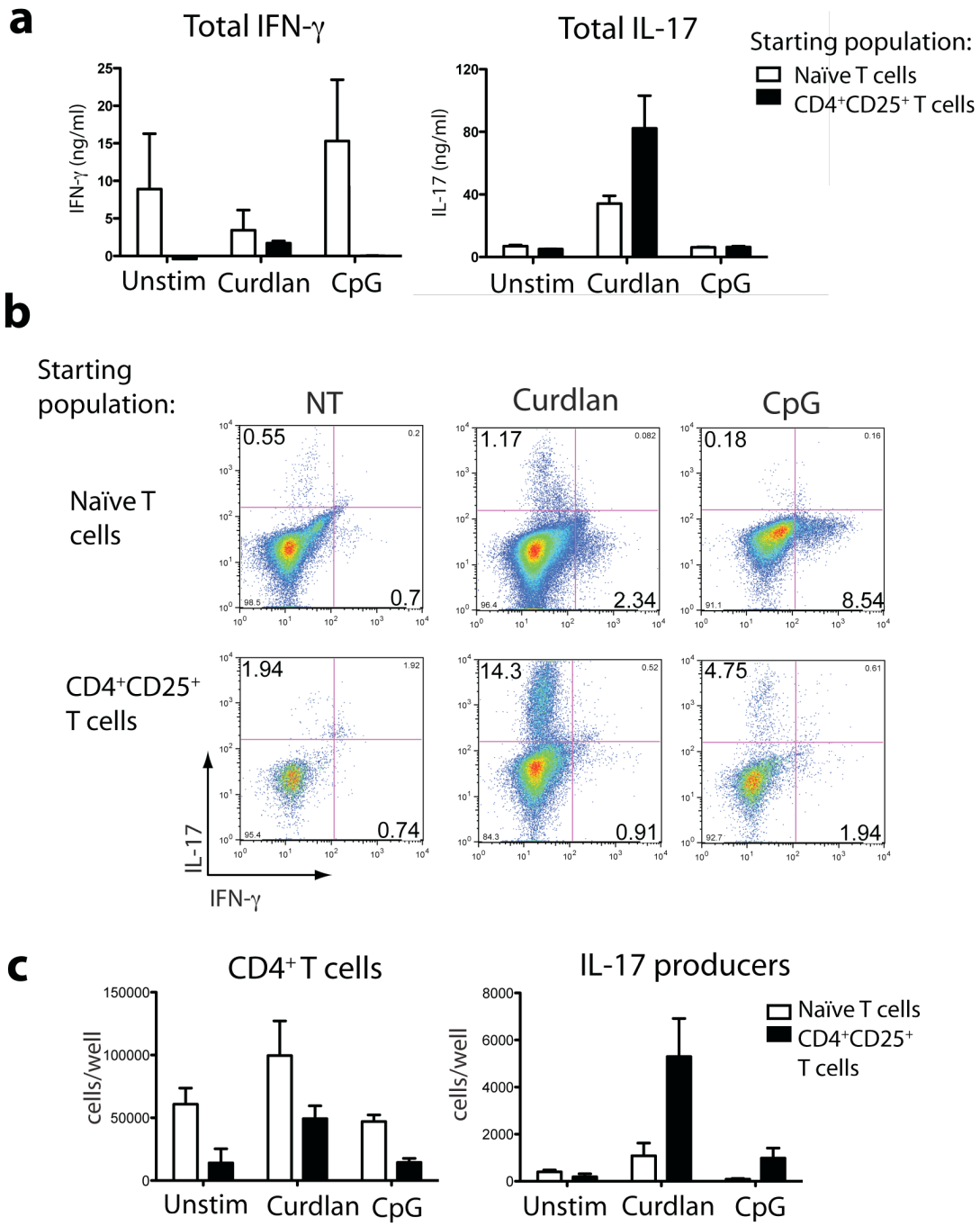


Figure 3-3: Curdlan stimulated BMDCs promote IL-17 production by CD4$^+$CD25$^+$ T cells.
(**a**) FACS-sorted naïve T cells (CD4$^+$CD25$^-$CD62^{high}CD44^{low}) or CD4$^+$CD25$^+$ T cells from C57BL/6 mice were cocultured for 5 days with C57BL/6 BMDCs and soluble α-CD3e (0.2µg/ml) in medium alone or in the presence of curdlan (50µg/ml) or CpG (0.5µg/ml). Half of the content of each well was restimulated on day 5 for 48 hr with coated α-CD3 (5µg/ml) and cytokine production was determined by sandwich ELISA. (**b**) As in (**a**) but cells were restimulated on day 5 with PMA (10ng/ml), ionomycin (1µg/ml) and brefeldin A (5µg/ml) for 4 hr and the presence of intracellular cytokines was analyzed by flow cytometry. Data show IFN-γ and IL-17 after gating on CD4$^+$ T cells. (**c**) Total numbers of CD4$^+$ T cells or CD4$^+$IL-17$^+$ T cells obtained on day 5 in cultures containing naïve or CD4$^+$CD25$^+$ T cells. Graphs on (**c**) show mean + SEM of three independent experiments. Data are representative of two to six independent experiments.

3.2.4 Curdlan-activated BMDCs induce the generation of $Foxp3^+IL\text{-}17^+$ T cells

Natural Tregs are characterized by expressing constitutive levels of CD25, CTLA-4, GITR, CD152 and CD103. However none of these markers are exclusively expressed by Tregs and they can be expressed on conventional T cells upon activation (reviewed in (Sakaguchi, 2004)). Foxp3, a transcription factor involved in the development and function of Tregs is the only marker restricted to this T cell population (Hori et al., 2003, Fontenot et al., 2003, Khattri et al., 2003). To work with a highly purified population of Tregs, we isolated $Foxp3^+$ T cells from the bacterial artificial chromosome-transgenic mice termed "depletion of regulatory T cell" (DEREG) mice (kindly donated by Dr. Tim Sparwasser, Hannover), which express GFP fused to the primate diphteria toxin receptor under the foxp3 gene regulatory regions (Lahl et al., 2007). Foxp3 positive T cells were sorted from DEREG mice (or from radiation chimeras bearing bone marrow from DEREG mice) based on the expression of CD4, GFP and CD25. To analyze whether curdlan has an effect on proliferation of Tregs, $CD4^+CD25^+GFP^+$ T cells were labelled with CFSE and were cocultured with BMDCs plus α-CD3 mAb and cells were analyzed on day 5 by flow cytometry. As the geometric mean value of the GFP^+ population in FL1 is dim (it ranges between 30-50 as quantified in a FACS Calibur, data not shown), this allows the use of CFSE to track cell proliferation. Results are shown in Figure 3.4a. As for the total $CD4^+CD25^+$ T cells used previously, $CD4^+CD25^+GFP^+$ DEREG cells proliferated more extensively when curdlan was present. As a positive control for Treg proliferation, we compared curdlan to IL-2 (called "Treg" control), which acts a Treg mitogen (reviewed in (Sakaguchi, 2004)) and independently analyzed the blast and resting T cell population for Foxp3 expression and production of IL-17. As a positive control for Th17 differentiation, a cocktail of cytokines containing IL-6 and TGF-β plus blocking antibodies to IFN-γ and IL-4 ("Th17 control") was added to cocultures of naïve T cells and BMDCs. Blast and non blast cells were selected by forward and side-scatter criteria, whereas Foxp3 expression was assessed by nuclear staining with a specific mAb to directly measure the expression of the protein at the time of analysis. Results are shown in Figure 3.4b. As expected, $Foxp3^+$ T cells expanded when cultured under "Treg" conditions and IL-17-

producing cells were readily detected in "Th17 control" cultures. Notably, in curdlan-containing cocultures of Foxp3$^+$ T cells and BMDCs, an accumulation of Foxp3$^+$-IL-17$^+$ double-positive cells emerged, which were especially noticeable in the blast fraction. T cells producing IL-17 and coexpressing Foxp3 in curdlan containing cocultures continued to accumulate over time and represented one fourth of the cells at day 7 (Fig 3.4c). At both time points, we detected the presence of transcripts related with Th17 cell fate such as RORγt, IL-23R and, to a lesser extent, IL-17F. We conclude that in response to BMDC activated by curdlan, Foxp3$^+$ T cells can give rise to T cell population capable of coexpressing Foxp3 and IL-17, which constitutes a distinct phenotype from classical Th17 or Tregs.

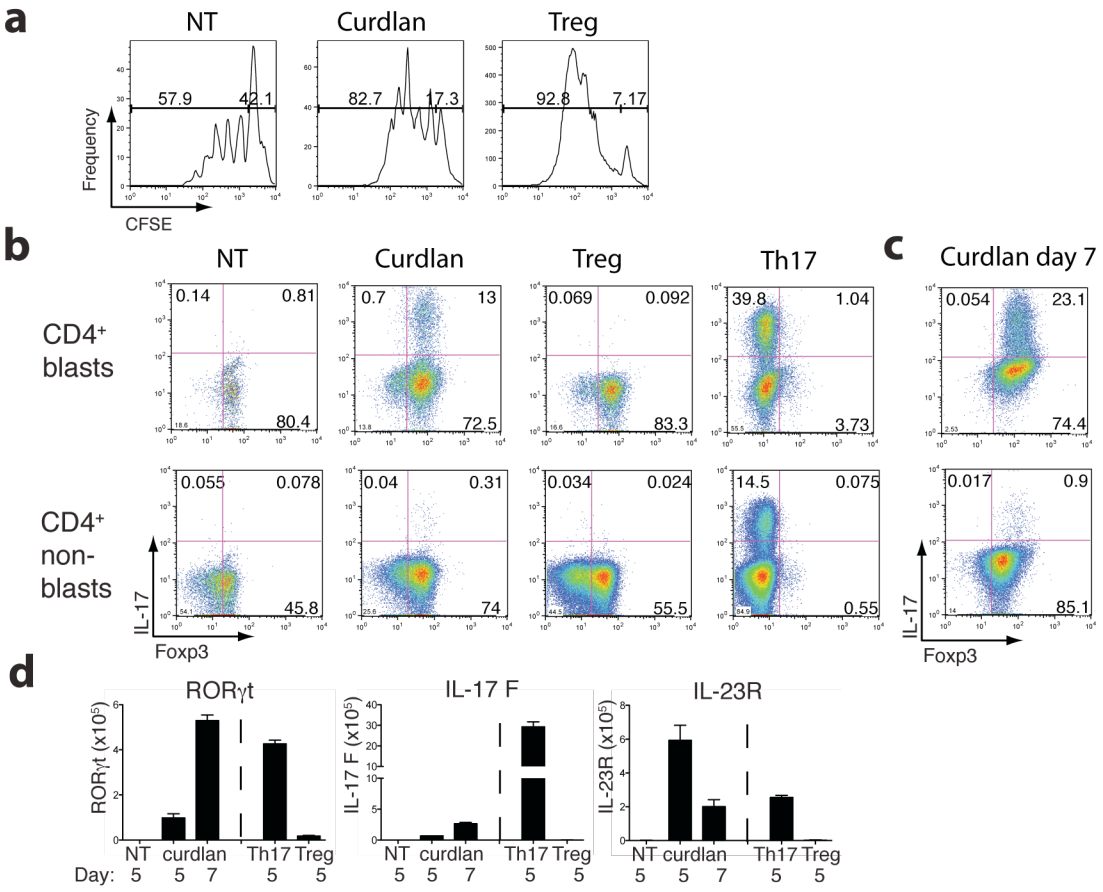


Figure 3-4: Dectin-1 activated BMDCs induce the generation of Foxp3$^+$IL-17$^+$ T cells.
(**a**) 5x10^4 CFSE-labelled CD4$^+$CD25$^+$GFP$^+$ DEREG T cells were cultured for 5 days with 2x10^4 wild-type C57BL/6 BMDCs and soluble α-CD3 (0.2μg/ml) in the absence (NT) or presence of curdlan (50μg/ml) or rhIL-2 (100IU/ml) ("Treg"), as indicated. Plots show CFSE profile after gating on CD4$^+$ cells. (**b**) As in (**a**) but with the addition of a "Th17" control consisting of 5x10^4 CD4$^+$GFP$^-$CD25$^-$ T cells cultured with 2x10^4 BMDC, α-CD3 and IL-6 (20ng/ml)+TGF-β (10ng/ml) in the presence of neutralizing antibodies to IFN-γ (2μg/ml) and IL-4 (2μg/ml). All cells were restimulated on day 5 with PMA, ionomycin and brefeldin A for 4 hrs and the expression of Foxp3 and IL-17 was analyzed by gating separately on CD4$^+$

cell blasts or non-blasts by flow cytometry. (**c**) Analysis of parallel cultures from (**b**) on day 7. (**d**) RT-PCR analysis of transcripts for RORγt, IL-17F and IL-23R in the indicated cultures at 5 and 7 days. Data are representative of two to six independent experiments.

3.2.5 Foxp3$^+$RORγt$^+$ but not Foxp3$^+$RORγt$^-$ become Foxp3$^+$IL-17$^+$ T cells in response to curdlan-stimulated BMDC

As previously mentioned in this chapter, Foxp3$^+$ T cells are a heterogeneous population and include a fraction that coexpresses RORγt and produces little or no IL-17 (Zhou et al., 2008a, Lochner et al., 2008). Given the importance of RORγt in the differentiation of conventional Th17 cells (Ivanov et al., 2006), we sought to determine whether Foxp3$^+$RORγt$^+$ might represent the source of Foxp3$^+$IL-17$^+$ double positive cells observed in curdlan-containing cocultures. We therefore compared RORγt$^+$ and RORγt$^-$ Tregs isolated from chimeric mice bearing bone marrow from *Rorc(γt)Gfp*^{TG} mice in which GFP reports RORγt expression (Lochner et al., 2008). To obtain a highly purified population of Tregs, cells were sorted based on the expression of CD4, CD25 and folate receptor 4, a membrane receptor that closely mirrors Foxp3 expression (Yamaguchi et al., 2007). To confirm that the combination of these 3 antibodies accurately identifies Tregs in our system, we sorted CD4$^+$CD25$^+$FR4$^+$ T cells from C57BL/6 mice and then stained for intracellular Foxp3 after sorting. As shown in Figure 3.5a and as previously described, the CD4$^+$CD25$^+$FR4$^+$ T cell fraction constitutes a population of cells expressing high levels of Foxp3. We then prepared donor origin CD4$^+$CD25$^+$FR4$^+$ cells from *Rorc(γt)Gfp*^{TG} chimeric mice, which were subdivided into GFP$^+$ or GFP$^-$ fractions and cultured separately with BMDCs, α-CD3 mAb in the presence of curdlan for 5 days. Remarkably, only CD25$^+$ FR4$^+$GFP$^+$ T cells but not CD25$^+$ FR4$^+$GFP$^-$ T cells gave rise to IL-17 producing cells in response to curdlan stimulated BMDC. The majority of these cells coexpressed Foxp3 although some IL-17 single positives could be observed, probably reflecting expansion of contaminating Foxp3$^-$RORγt$^+$ T cells present at the beginning of the culture. We conclude that Foxp3$^+$RORγt$^+$ but not Foxp3$^+$RORγt$^-$ T cells can be induced to produce IL-17 after culture with curdlan activated BMDC.

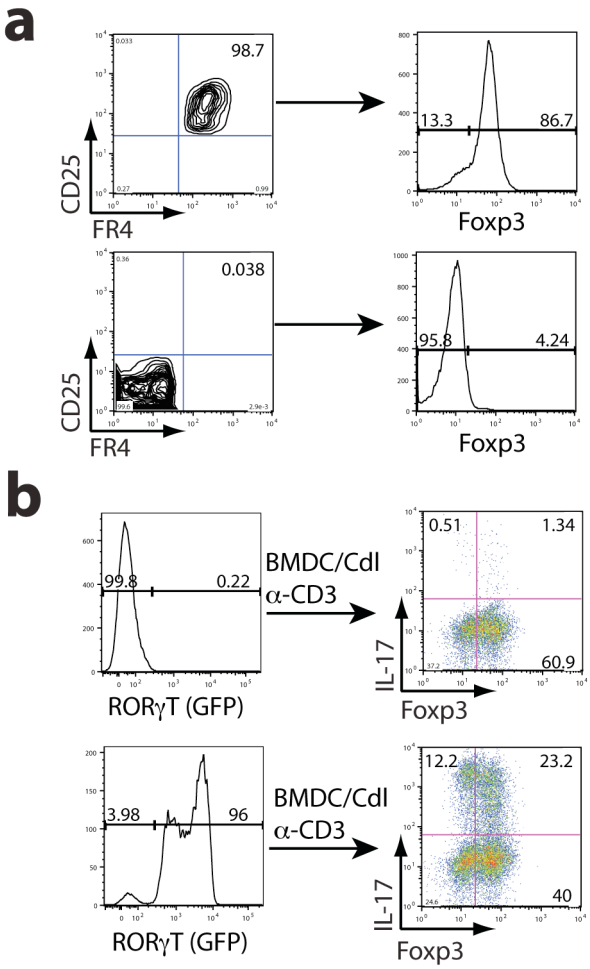


Figure 3-5: Foxp3^{+}RORγt^{+} but not Foxp3^{+}RORγt^{-} T cells become Foxp3^{+}IL-17^{+} T cells in response to curdlan-activated BMDCs.
(**a**) FACS-sorted CD4^{+}CD25^{+}FR4^{+} and CD4^{+}CD25^{-}FR4^{-} T cells from C57BL/6 mice stained for intracellular Foxp3. (**b**) FACS-sorted CD4^{+}CD25^{+}FR4^{+}GFP^{+}CD45.1^{-} and CD4^{+}CD25^{+}FR4^{+}GFP^{-}CD45.1^{-} T cells from Rorc(γt)-Gfp^{TG} chimeras were cocultured with wild-type BMDCs and soluble α-CD3 in the presence of curdlan (Cdl). Cells were restimulated on day 5 with PMA, ionomycin and brefeldin A for 4 hrs and the expression of Foxp3 and IL-17 on CD4^{+} T cell blasts was analyzed by flow cytometry. Data are representative of two independent experiments.

3.2.6 IL-23 drives the generation of Foxp3$^+$IL-17$^+$ T cells

As previously shown in this chapter, IL-23 was required to induce IL-17-producing cells from cultures containing naïve T cells, CD4$^+$CD25$^+$ T cells and curdlan-activated BMDCs. To address the role of IL-23 in the induction of Foxp3$^+$IL-17$^+$ T cells, we cultured IL-23 deficient (*p19*$^{-/-}$) or wild type BMDC with Foxp3$^+$ T cells from DEREG mice in the presence of curdlan. Notably, the frequency of Foxp3$^+$IL-17$^+$ T cells was drastically reduced when *p19*$^{-/-}$ cells were used as a source of APC (Figure 3.6a). This observation also correlated with the decrease in absolute numbers of Foxp3$^+$IL-17$^+$ T cells while total cell numbers remained unaffected (Figure 3.6b). These results indicated that *p19*$^{-/-}$ BMDC are not impaired at inducing T cell proliferation/survival of Foxp3$^+$ T cells but they failed to promote the development of Foxp3$^+$IL-17$^+$ T cells. The appearance of RORγt and IL-23R transcripts in curdlan containing cocultures were also IL-23 dependent indicating that IL-23 is necessary to switch to the IL-17 associated program in Foxp3$^+$ T cells (Figure 3.6c). Given that IL-23 is required for the development of Foxp3$^+$IL-17$^+$ T cells, we wondered if that cytokine was sufficient to drive the generation of those cells. To address that question, curdlan was replaced with IL-23. Although IL-23 was able to induce similar frequencies of Foxp3$^+$IL-17$^+$ T cells when compared to curdlan, T cell expansion was reduced resulting in lower number of Foxp3$^+$IL-17$^+$ T cells at the end of the culture (Figure 3.6d-e). These data indicate that although IL-23 is necessary for the production of IL-17 in Foxp3$^+$ T cells, it is not sufficient for the maintenance of Foxp3$^+$IL-17$^+$ T cells and other factors might be required to support the phenotype and/or survival of this T cell population.

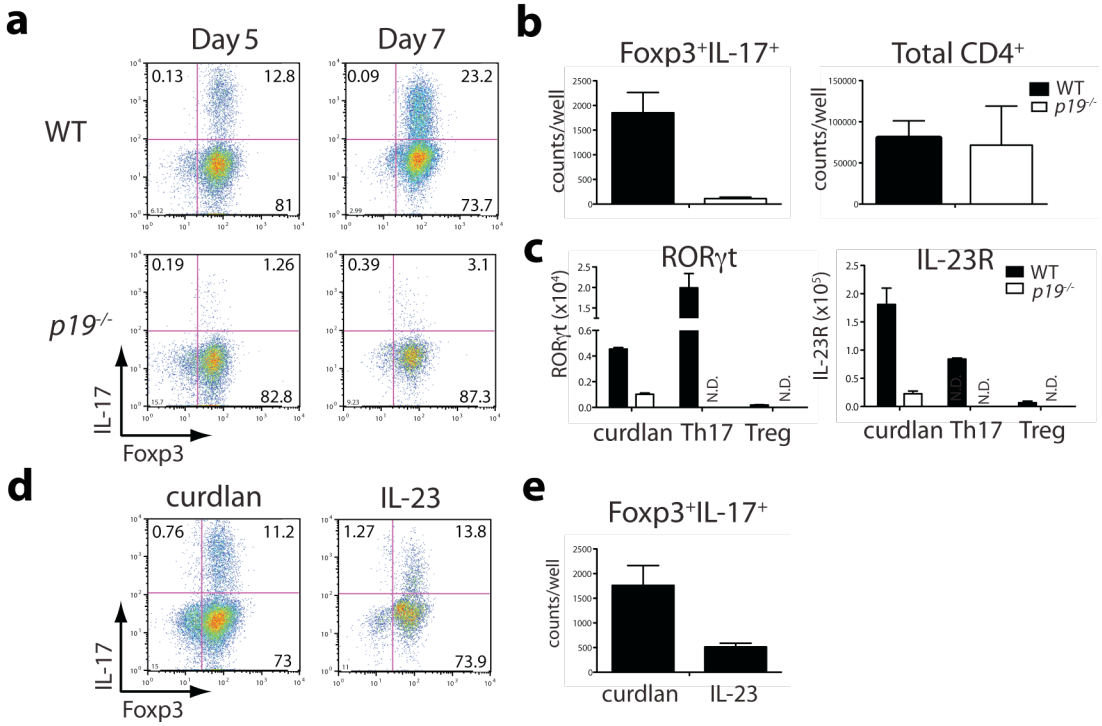


Figure 3-6: IL-23 drives the generation of Foxp3$^+$IL-17$^+$ T cells in response to curdlan-activated BMDCs.
(**a**) FACS-sorted CD4$^+$CD25$^+$GFP$^+$ DEREG T cells were cultured with wild-type or *p19*$^{-/-}$ BMDCs plus soluble α-CD3 in the presence of curdlan. Cells were restimulated on day 5 and 7 with PMA, ionomycin and brefeldin A for 4 hrs and the expression of Foxp3 and IL-17 on CD4$^+$ cell blasts was analyzed by flow cytometry. (**b**) Total numbers of Foxp3$^+$IL-17$^+$ T cells and CD4$^+$ T cells from cultures as in (**a**) on day 5. (**c**) RT-PCR analysis of transcripts encoding RORγt, and IL-23R in cultures as in (**a**) analyzed on day 5. (**d**) CD4$^+$CD25$^+$GFP$^+$ DEREG T cells were cultured for 5 days with wild-type BMDCs plus soluble α-CD3 in the presence of curdlan or IL-23 (10ng/ml). Cells were restimulated on day 5 with PMA, ionomycin and brefeldin A for 4 hrs and the expression of Foxp3 and IL-17 on CD4$^+$ cell blasts was analyzed by flow cytometry. (**e**) Numbers of Foxp3$^+$IL-17$^+$ T cells obtained in cultures as in (**d**). Data in (**b**) and (**e**) are mean + SEM of three independent experiments. Data are representative of two to four independent experiments. N.D., not done.

3.2.7 Foxp3$^+$IL-17$^+$ T cells emerge in vivo but their frequency is not increased upon curdlan administration

Small numbers of Foxp3$^+$RORγt$^+$ T cells are found in the lamina propria of mice and have been shown to produce little or no IL-17 (Lochner et al., 2008, Zhou et al., 2008a). In addition, a small percentage of Foxp3 and IL-17F coexpressors can be detected in *IL-17f*^{rfp}*Foxp3*^{gfp} dual reporter mice upon immunisation with OVA plus CFA (Yang et al., 2008b). To determine whether Foxp3$^+$IL-17$^+$ cells arise in vivo and evaluate the impact of the Dectin-1 agonist on these responses, we analyzed the coexpression of IL-17 and

Foxp3 during Treg-control of colitis induced by *Helicobacter hepaticus*. This T-cell independent model of colitis is induced in RAG2 deficient mice infected with *H. hepaticus*, which leads to severe intestinal inflammation and is prevented by coadministration of $CD4^+CD45RB^{low}CD25^+$ T cells (Maloy et al., 2003). In this experimental setting, we sought to determine whether curdlan administration (by oral gavage) could promote the appearance of $Foxp3^+IL\text{-}17^+$ cells from the $CD4^+CD45RB^{low}CD25^+$ T cell compartment according to the experimental design shown in figure 3.7a.

This experiment was executed in the Laboratory of Dr Fiona Powrie and it was set up and analyzed by myself together with Philip Ahern, senior graduate student from Powrie's Laboratory and must be considered as preliminary data as it was performed once with 6 mice per experimental group.

All mice were sacrificed when infected animals without T cell reconstitution showed signs of disease, which was 8 weeks later. All mice that received Treg cells did not develop intestinal inflammation as expected (Maloy et al., 2003), and the administration of curdlan had no effect on the resolution of the disease (according to histological score assessed by pathologists at Oxford University, data not shown).

On week 8, $CD4^+$ T cells were analyzed in spleen, mesenteric lymph node and colonic lamina propria and were analyzed for the expression of IL-17 and Foxp3. Results are shown in Figure 3.7b-c. Unexpectedly, a noticeable population of $Foxp3^+IL\text{-}17^+$ cells was clearly found in spleen, mesenteric lymph node and lamina propria from Treg reconstituted mice. The Foxp3 and IL-17 coexpressors constituted a considerable fraction of the total $CD4^+IL\text{-}17^+$ pool found under this experimental setting and accounted for 16% of the total IL-17 produced by $CD4^+$ T cells approximately. Notably, the presence of IL-17-producers was not associated with immunopathology as T-cell reconstituted mice did not develop intestinal inflammation (according to histological score by pathologists at Oxford University, data not shown). Administration of the Dectin-1 agonist did not increase the frequency of $Foxp3^+IL\text{-}17^+$ cells in any of the organs analyzed and it reduced the percentages of $Foxp3^+IL\text{-}17^+$ T cells found in the

lamina propria (Figure 3.7c). In addition, the frequency of Foxp3 or IL-17 single positives was not modified by oral administration of curdlan (Figure 3.7c).

These results indicate that $Foxp3^+IL\text{-}17^+$ T cells is a cell population that can be naturally found *in vivo* in the context of Treg control of innate colitis. In addition, these results indicate that curdlan administration had a negative effect of this response noticed in the lamina propria but not in spleen or mesenteric lymph node. Whether *H. Hepaticus* itself contain a Dectin-1 ligand that promotes the generation of $Foxp3^+IL\text{-}17^+$ T cells is unknown. Moreover, it is unclear whether $Foxp3^+IL\text{-}17^+$ T cells are beneficial or detrimental in the context of intestinal inflammation.

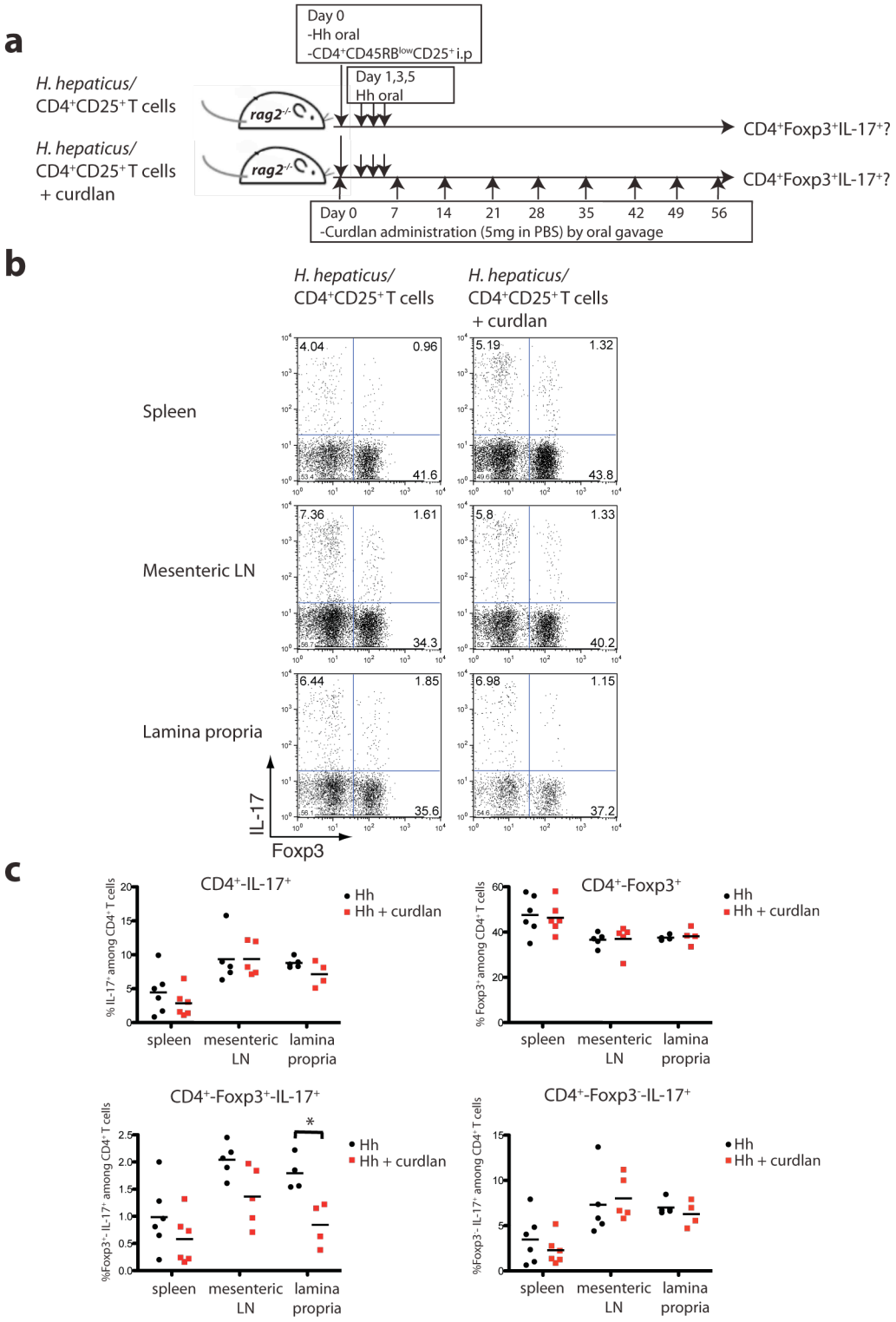


Figure 3-7:Foxp3^{+}IL-17^{+} T cells emerge during Treg control of *H. hepaticus*-mediated colitis, but their frequency is not increased upon curdlan administration.
(**a**) Schematic representation of the experimental procedure. (**b**) Spleen, mesenteric lymph node and large intestine lamina propria were collected from *H. hepaticus*-infected mice reconstituted with

$CD4^+CD25^+CD45RB^{low}$ T cells in presence or absence of curdlan administration. Cells were restimulated with PMA, ionomycin and brefeldin A for 4 hrs and the expression of Foxp3 and IL-17 on $CD4^+$ T cells was analyzed by flow cytometry. (**c**) Frequencies of $CD4^+$-$IL\text{-}17^+$, $CD4^+$-$Foxp3^+$, $CD4^+$-$Foxp3^+$-$IL\text{-}17^+$ and $CD4^+$-$Foxp3^-$-$IL\text{-}17^+$ analyzed at the end of the experiment. Each point represents one mouse. Statistically different groups are indicated (*, $0.01 < p < 0.05$).

3.3 Discussion

Innate recognition of pathogens by the immune system results in the initiation of adaptive responses that will be matched to the nature of the offending microbe. Identifying qualitative differences between innate signalling pathways in the outcome of adaptive immunity is essential to understand the initiation of effector T cell responses against infection.

3.3.1 The induction of IL-17-producing $CD4^+$ T cells by curdlan-activated DCs requires IL-23 and Tregs

Work from Salomé LeibundGut-Landmann demonstrated that activation of the Dectin-1/Syk/CARD9 signalling pathway leads to activation of BMDCs suited to generate Th17 responses (LeibundGut-Landmann et al., 2007). Results shown Fig 3.1a-b demonstrate that activation of the Dectin-1 signalling pathway leads to the production of IL-17 by T cells response at different doses of antigen or stimuli.

Although IL-17 producing T cells have been previously noticed to develop in cultures containing DC plus TLR agonists (Veldhoen et al., 2006a), results presented in this chapter together with the work from Salomé LeibundGut-Landmann (LeibundGut-Landmann et al., 2007), indicate that activation of the Dectin-1 pathway induces greater Th17 skewing (Fig 3.1b) (LeibundGut-Landmann et al., 2007).

BMDCs stimulated with CpG produce both IL-23 and IL-12p70 whereas BMDCs activated by curdlan produce mainly IL-23 (LeibundGut-Landmann et al., 2007). The lack of IL-12 p70 and the presence of IL-10 in curdlan-containing cocultures might inhibit the development of Th1 cells (Harrington et al., 2005). Notably, data presented in this chapter showed that IL-23 is required for the production of IL-17 in cocultures containing $CD4^+CD25^-$, $CD4^+CD25^+$ T cells and curdlan-activated BMDCs (Fig 3.2a and (LeibundGut-Landmann et al., 2007)). This is an intriguing observation, as IL-23 has shown to be dispensable for the differentiation of IL-17 producing cells in cultures containing naïve T cells and TLR-activated BMDCs (Veldhoen et al., 2006a). Furthermore, the T cell targets of IL-23 *in vitro* arise mainly from the effector and memory T cell compartment (Langrish et al., 2005) (Aggarwal et al., 2003) with no

effect in the differentiation of naïve T cells (Bettelli et al., 2006, Mangan et al., 2006, Veldhoen et al., 2006b). This result suggests that IL-17 production in response to curdlan-activated DCs may arise from a non-naïve T cell.

In agreement with previous publications (Mangan et al., 2006, Bettelli et al., 2006, Veldhoen et al., 2006a), the development of IL-17-producing T cells upon Dectin-1 stimulation of BMDCs requires TGF-β (Fig 3.2c). Notably, Tregs are essential but this cannot be fully explained by TGF-β production (Fig 3.2d). These results suggested that Tregs themselves might respond to Dectin-1 activated BMDCs to produce IL-17.

3.3.2 Dectin-1 signalling renders BMDCs competent to convert Tregs into $Foxp3^+IL\text{-}17^+$T cells

Experiments presented in Figures 3.1 and 3.2 were done using a mixture of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells as previously described for *in vitro* differentiated Th17 cells in cultures containing TLR agonists (Veldhoen et al., 2006a). Experiments showed in Fig 3.3a-b demonstrate that Curdlan-stimulated BMDCs promote the appearance of a large frequency of IL-17-producing T cells in cultures containing $CD4^+CD25^+$ T cell as the starting population. Notably, a small population of naïve T cells (0.5%) differentiate into Th17 cells in response to Dectin-1 activated BMDCs in absence of exogenous TGF-β (Fig 3.3a-b), suggesting that both subsets of T cells can differentiate to produce IL-17 in this condition of innate challenge, albeit at different levels. Along these lines, Olivier Joffre (former member of the Immunobiology lab, Cancer Research UK) has shown that antigen targeting to DNGR-1 leads to Th17 differentiation of adoptively-transferred naïve T cells when curdlan is used as adjuvant *in vivo* (Joffre et al., 2010).

In addition, it is important to note that the experiments presented in this chapter have been performed using RPMI as culture media. It has been recently noted that Iscove's modified Dulbecco medium (IMDM) is an optimal culture media for *in vitro* generated Th17 cells due to the presence of an endogenous AhR ligand (Veldhoen et al., 2009). Therefore, changing culture media might further increase the frequencies of IL-17 producers noticed in my experiments.

My data demonstrate that Dectin-1 signalling renders BMDCs competent to convert $CD25^+Foxp3^+$ T cells into $Foxp3^+IL\text{-}17^+$ T cells. Previous work (Xu et al., 2007, Zheng et al., 2008a, Yang et al., 2008b) showed that $CD4^+CD25^+$ T cells can be converted into IL-17 producers *in vitro* and *in vivo* but this process is accompanied by downregulation of Foxp3. A distinct hallmark of my study is that $CD25^+Foxp3^+$ T cells maintain the expression of Foxp3 while coexpressing IL-17.

Why Dectin-1 activated BMDCs are particularly suited to induce $Foxp3^+$-$IL\text{-}17^+$ T cells? In Fig 3.2a, I showed that in conditions of curdlan stimulation, IL-23 is essential to induce IL-17-producing T cells from cultures containing $CD4^+$ $CD25^-$ and $CD4^+CD25^+$ T cells. In addition, IL-23 signalling is also necessary to induce the generation of $Foxp3^+$-$IL\text{-}17^+$ T cells (Fig 3.6a-c). Nonetheless, the pure presence of IL-23 in those cultures is not sufficient to sustain the phenotype and/or survival of those cells (Fig 3.6 d-e) indicating that other factors present in curdlan-containing cocultures such as IL-23 and TNF may act synergistically. On the other hand, Dectin-1 triggering on BMDCs leads to the production of considerable levels of IL-2, which could contribute to the generation of $Foxp3^+$-$IL\text{-}17^+$ T cells. Although IL-2 has been described as a negative regulator of Th17 differentiation from naïve T cells (Laurence et al., 2007), it potentiates Foxp3 expression on Tregs (Fontenot et al., 2005). I propose that the unique combination of cytokines produced by Dectin-1 activated BMDCs may be particularly suited to the generation of $Foxp3^+$-$IL\text{-}17^+$ T cells, with IL-23, IL-6 and TNF favouring IL-17 production and IL-2 maintaining Foxp3 expression. IL-10, which is also present on these cultures, may help sustaining Treg markers or activity.

Notably, we have found that $Foxp3^+$-$IL\text{-}17^+$ T cells arise from the $Foxp3^+$-$ROR\gamma t^+$ T cell population and not from $Foxp3^+$-$ROR\gamma t^-$ T cell counterparts (Fig 3.5). Although $Foxp3^+$-$ROR\gamma t^+$ T cells have been shown not to produce IL-17 in the steady state (Lochner et al., 2008, Zhou et al., 2008a), results presented in this chapter suggest that the $Foxp3^+$-$ROR\gamma t^+$ T cell population can produce IL-17 in some conditions of immune challenge. In addition, these results suggests that $Foxp3^+$ T cells are not being genetically reprogrammed to become IL-17 producers but that the appropriate milieu triggers the production of IL-17 in cells already precommitted to perform that function.

The Foxp3$^+$-IL-17$^+$ T cell is a population that defies classification into Tregs or Th17 cells. Notably, a population of human Foxp3$^+$ T cells producing IL-17 have been recently identified in peripheral blood (Ayyoub et al., 2009) and tonsils (Voo et al., 2009). Those cells share some common features with Th17 cells such as expression of CCR6 but produce little IL-22 and conserve suppressive activity *in vitro* (Voo et al., 2009, Ayyoub et al., 2009).

The fact that fungal stimuli lead to the development of both Th17 and Foxp3$^+$-IL-17$^+$ T cell responses may have important implications. IL-17 RA deficient mice are highly susceptible to systemic and oropharyngeal candidiasis (Huang et al., 2004, Conti et al., 2009) and human memory CD4$^+$ T cells specific for *C. albicans* have a Th17 phenotype (Acosta-Rodriguez et al., 2007). Whether Foxp3$^+$-IL-17$^+$ T cells participate in anti-fungal immunity or maintain their regulatory activity remains to be determined. Along these lines, Zymosan administration has been associated with the generation of cells with regulatory activity and also with the induction of Th17 cells (Dillon et al., 2006, Veldhoen et al., 2006b). By developing techniques for Foxp3$^+$IL-17$^+$ T cell isolation, hopefully the questions regarding their role could begin to be addressed.

3.3.3 Foxp3$^+$-IL-17$^+$ T cells coexist with Foxp3$^+$-IL-17$^-$ and Foxp3$^-$-IL-17$^+$ T cells in vivo

At present, there is evidence demonstrating Treg adaptation to microbe-exposed tissues (reviewed in (Wohlfert and Belkaid, 2010)). Foxp3$^+$ T cells coexpressing T-bet and producing IFN-γ have been found in mice acutely infected with *T.gondii* (Oldenhove et al., 2009), although is not year clear whether they emerge as a cause or a consequence of the excessive immunopathology. Along these lines, Foxp3$^+$-IL-17$^+$ T cells are detected during Treg suppression of *H. Hepaticus* (Figure 3.7). These results suggest that this cell population might emerge only under conditions of immune challenge as very low frequencies of Foxp3$^+$-IL-17$^+$ T cells are found in steady state (Zhou et al., 2008a) (Lochner et al., 2008).

Whether the Foxp3$^+$-IL-17$^+$ T cell population contribute to the inhibition of intestinal inflammation is at present unclear. One experimental approach to address this question would be to test the suppressive functions of Foxp3$^+$ cells isolated from RORγt deficient

mice. Those Tregs could be compared to wild type Tregs in the control of innate colitis. This might be a good experimental approach to elucidate the relevance of IL-17 produced by the $Foxp3^+$ compartment during an inflammatory response.

The fact that curdlan had no effect in increasing the frequency of CD4-IL-17 producers does not mean that the Dectin-1/Syk/CARD9 pathway is dispensable for the induction of a type 17 response *in vivo*. One explanation is that the effect of curdlan was simply masked by the continuous presence of *H. Hepaticus* in the gut. It has been shown that the Treg presence controls intestinal pathology but does not influence the levels of pathogen colonisation (Maloy et al., 2003). In addition, is possible that PAMPs derived from *H. Hepaticus* activate the Syk/CARD9 pathway and favour the induction of Th17 and $Foxp3^+$-IL-17^+ T cells via additional Syk-coupled PRRs. It is important to note that some bacteria express 1,3 β-glucans and that curdlan is isolated from the gram-negative bacteria *Alcaligenes faecalis*.

The role of Syk in the generation of Th17 responses in the gut is entirely unknown. Furthermore, the signalling requirements for the induction of Th17 cells in the intestine have proven to be MyD88, Trif and RIP-2 independent (Atarashi et al., 2008, Ivanov et al., 2008, Ivanov et al., 2009). Whether Syk signalling has a predominant role in driving Th17 responses in the gut is at present unknown but will be certainly tested in the near future.

Chapter 4. Dectin-2 is a Syk-coupled PRR involved in the initiation of Th17 responses to fungal infection

4.1 Introduction

4.1.1 Pattern recognition receptors and fungi

Innate cells express multiple PRRs that recognize fungal signatures including TLR2, TLR4, TLR6, TLR9, mannose receptor, Dectin-1, DC-SIGN, SIGN-R1, Dectin-2, Fcγ receptor, Mincle, Galectin-3, SCARF1 and CD36 among others (Netea et al., 2008, van de Veerdonk et al., 2008, Wells et al., 2008, Bugarcic et al., 2008, Means et al., 2009).

Although many of these PRRs signal independently of Syk and CARD9, the Syk/CARD9 axis plays a non-redundant role in DC responses to fungal stimuli. *Syk*$^{-/-}$ DCs are unable to produce IL-2 and IL-10 in response to zymosan (Rogers et al., 2005) and *Card9* $^{-/-}$ DCs are impaired at producing of IL-2, IL-6 and TNF in response to zymosan and *C. albicans* (Gross et al., 2006). Interestingly, Dectin-1 is not responsible for these responses, as the production of IL-2 and IL-10 to zymosan is preserved in *Dectin-1*$^{-/-}$ DCs (Saijo et al., 2007, Taylor et al., 2007).

Notably, Th17 responses to *C. albicans* are preserved in Dectin-1-deficient mice and yet these responses are absent in CARD9-deficient mice (LeibundGut-Landmann et al., 2007). These results suggest the existence of additional Syk/CARD9-coupled PRRs involved in DC activation by fungal stimuli. It has been recently reported that the mannose receptor could contribute to human Th17 responses to *C. albicans* (van de Veerdonk et al., 2009), although there is no evidence linking this receptor to the Syk/CARD9 pathway.

Altogether, these observations indicate that, although Dectin-1 is a fungal PRR capable of coupling innate into adaptive immunity, is probably not the only Syk-CARD9 coupled PRR involved in DC activation by fungi and induction of Th17 responses to *C. albicans.*

The aim of work descrbed in this chapter was to identify an additional Syk/CARD9 PRR involved in coupling DC activation to Th17 responses to *C. albicans.*

4.2 Results

4.2.1 *C. albicans* preparations and comparison of antifungal agents

As previously mentioned in this thesis, observations by the Immunobiology Laboratory defined a novel innate signalling pathway in DCs, which is activated in response to zymosan and involves Syk kinase (Rogers et al., 2005). DCs activated with zymosan produce IL-2 and IL-10 in a Syk-dependent manner but produce IL-12/23 p40 independently of Syk via the TLR pathway, suggesting that Dectin-1/Syk and TLR2/MyD88 signalling pathways can operate independently (Rogers et al., 2005).

I decided to test whether those previous observations might be valid in the recognition of a potential fungal pathogen. The model organism used is *C. albicans*, a human commensal that becomes highly pathogenic in immunocompromised individuals (Sable et al., 2008, Crameri and Blaser, 2002). *Candida* is a dimorphic fungus and coexists as unicellular blastopores (yeast) or multicellular germ tubes (hyphae) (Molero et al., 1998).

The morphology from yeast and hyphae was analyzed by microscopy. Due to the lack of a microscope located within the containment room, images of live organisms could not be generated. Figure 4.1a shows images of heat-killed yeast and hyphae. These images indicate that both preparations are highly pure as contaminating hyphae was never observed in yeast preparations and yeast presence was minor in the hyphae form. To prevent fungal outgrowth, antifungal agents were added to the cultures 2 hours after live *C. albicans* stimulation. I tested two classes of antifungal compounds in assays containing BMDCs and live fungi. Fungizone (Amphotericin B); a polyene antimycotic used as a standard treatment for invasive candidiasis and Caspofungin (Cancidas, Merck); an echinocandin used for the treatment of invasive *Aspergillus* infections and candidemia (Sable et al., 2008, Mora-Duarte et al., 2002). Fungizone associates with membrane components such as ergosterol and results in altered cell wall permeability (Sable et al., 2008, Ghannoum and Rice, 1999). Echinocandins interfere with glucan

synthesis by inhibiting β-(1,3)-glucan synthase (Sable et al., 2008, Ghannoum and Rice, 1999).

Results are shown in Figure 4.1 b. Neither the presence of Fungizone nor Caspofungin on their own elicited cytokine production by BMDCs in absence of stimuli. In addition, the presence of antifungal compounds did not modify the pattern of cytokines induced by curdlan. Notably, live yeast and hyphae trigger strong production of IL-2, IL-10 and TNF by BMDCs. Although the addition of Fungizone can lead to higher levels of IL-2, IL-10 and TNF compared to Caspofungin, the nature of the antifungal agent did not modify the quality of the responses observed.

I chose to use Fungizone throughout the course of this chapter, as it is a compound accessible commercially and, unlike Caspofungin that affects β-glucan, it does not interfere directly with the nature of the Dectin-1 ligand.

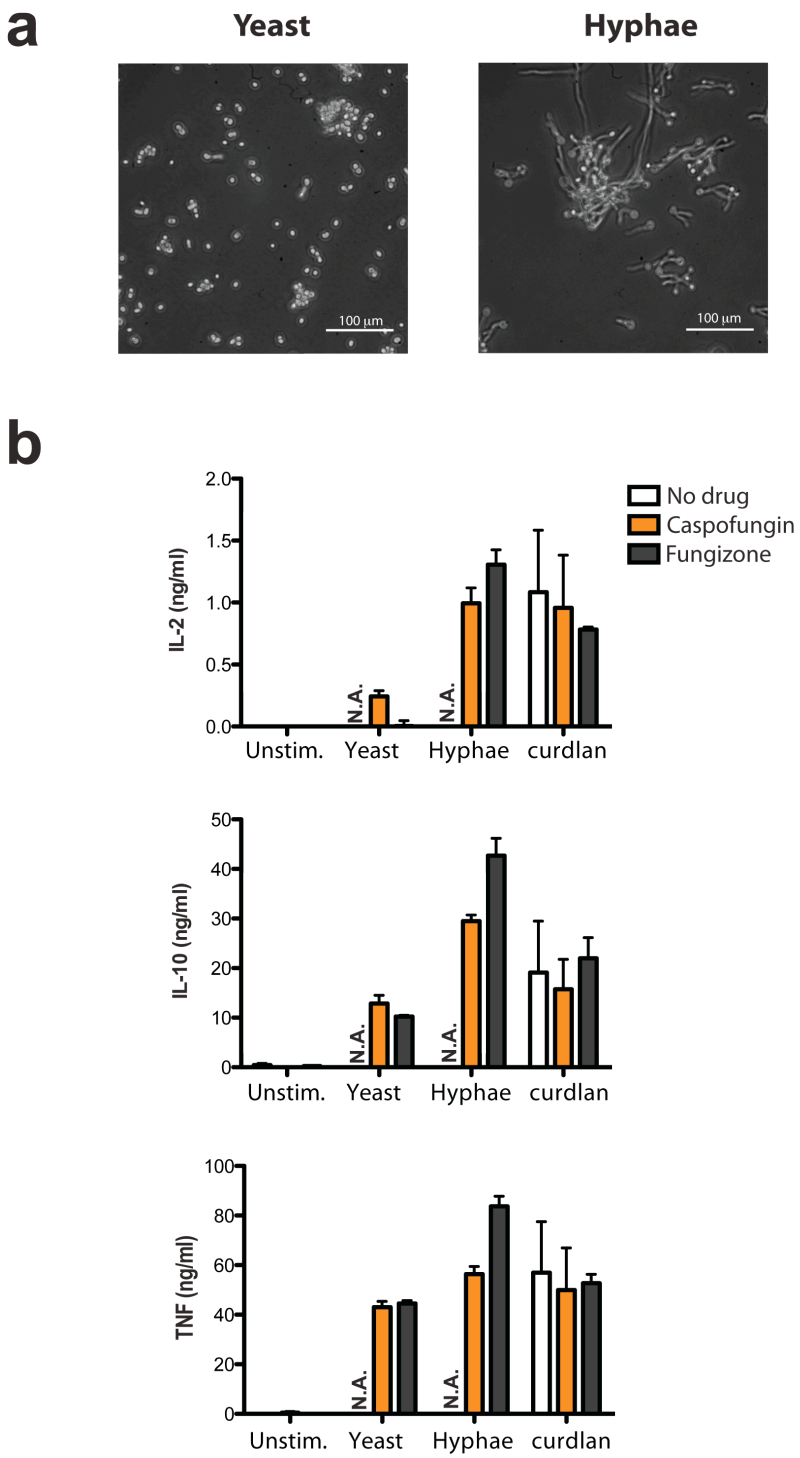


Figure 4-1: Antifungal drugs have minor effects on the cytokine respose to *C. albicans* or to curdlan.

(**a**) Morphology of heat-killed yeast and heat-killed hyphae. White bar denotes 100µm. (**b**) $1x10^5$ BMDCs from wild type mice were stimulated with $1x10^5$ live *C. albicans* yeast or hyphae or with 100µg/ml curdlan. Caspofungin (50ng/ml) or Fungizone (2.5µg/ml) were added 2hr later and cytokine levels in the supernatants were measured after overnight incubation. Data are mean +/- SD of duplicate wells and are representative of 3 independent experiments. n.a. indicates not applicable.

4.2.2 The induction of IL-2, IL-10 and TNF by *C. albicans* in BMDCs is dependent on Syk kinase

To determine the relevance of Syk signalling in response to *C. albicans* stimulation, BMDCs derived from wild type mice, MyD88-TRIF double knock out mice and Syk knock out chimeras were cultured overnight with heat-killed or live preparations of the organism. As an internal control, cells were routinely tested for their ability to respond to a pure Dectin-1/Syk agonist (curdlan) and a pure TLR agonist (CpG) (Fig 4.2 a). As expected, Syk deficient BMDCs do not produce TNF in response to curdlan and *Myd88*$^{-/-}$*Trif*$^{-/-}$ BMDCs do not produce IL-12 p40 after CpG stimulation.

For cultures containing fungal preparations, BMDCs were cultured overnight with live or heat-killed organisms in a ratio 1:1 and 5:1 (*Candida*: BMDC) and cytokines present in supernatants were analyzed by ELISA. As previously observed with zymosan (Rogers et al., 2005), recognition of live and heat-killed *C. albicans* by BMDCs leads to the production of significant levels of IL-2, IL-10 and TNF (figure 4.2b-c). The production of these three cytokines was completely dependent on Syk irrespective of the morphologic form or whether heat-killed or live organisms were used (Fig 4.2 b-c). In contrast, the production of IL-12/23 p40 in response to heat-killed and live organisms was dependent on the adaptors MyD88/TRIF and was largely independent of Syk (Fig 4.2 d and not depicted).

Interestingly, IL-2, IL-10 and TNF were induced at higher levels by hyphae than yeast, especially in cultures containing heat-killed organisms (Fig 4.2 b-c).

Athough the Syk dependence for IL-2, IL-10 and TNF in all conditions is evident, a contribution by the MyD88-TRIF signalling pathway can be observed in two situations: First, the production of IL-2 in response to heat-killed hyphae reflects a dual dependence on Syk and MyD88-TRIF (Figure 4.2c). This observation has been previously noted for zymosan (Rogers et al., 2005) and may reflect exposition of TLR ligands during denaturation as previously reported for *Candida*-derived β-glucans (Gantner et al., 2005). Second, the production of TNF in response to live organisms also reveals partial dependence on TLR signalling (Figure 4.2b) which is also consistent with alterations in PAMP display caused by heat denaturation.

In contrast, the production of IL-10 remained MyD88/TRIF independent in most conditions as previously noted for zymosan (Rogers et al., 2005). In summary, I conclude that Syk signalling in BMDCs is essential for the production of IL-2, IL-10 and TNF to *C. albicans* despite some possible contribution from TLR pathways. These three factors (termed "Syk-dependent cytokines") can be used to monitor the activity of Syk-coupled PRRs for fungi in BMDCs.

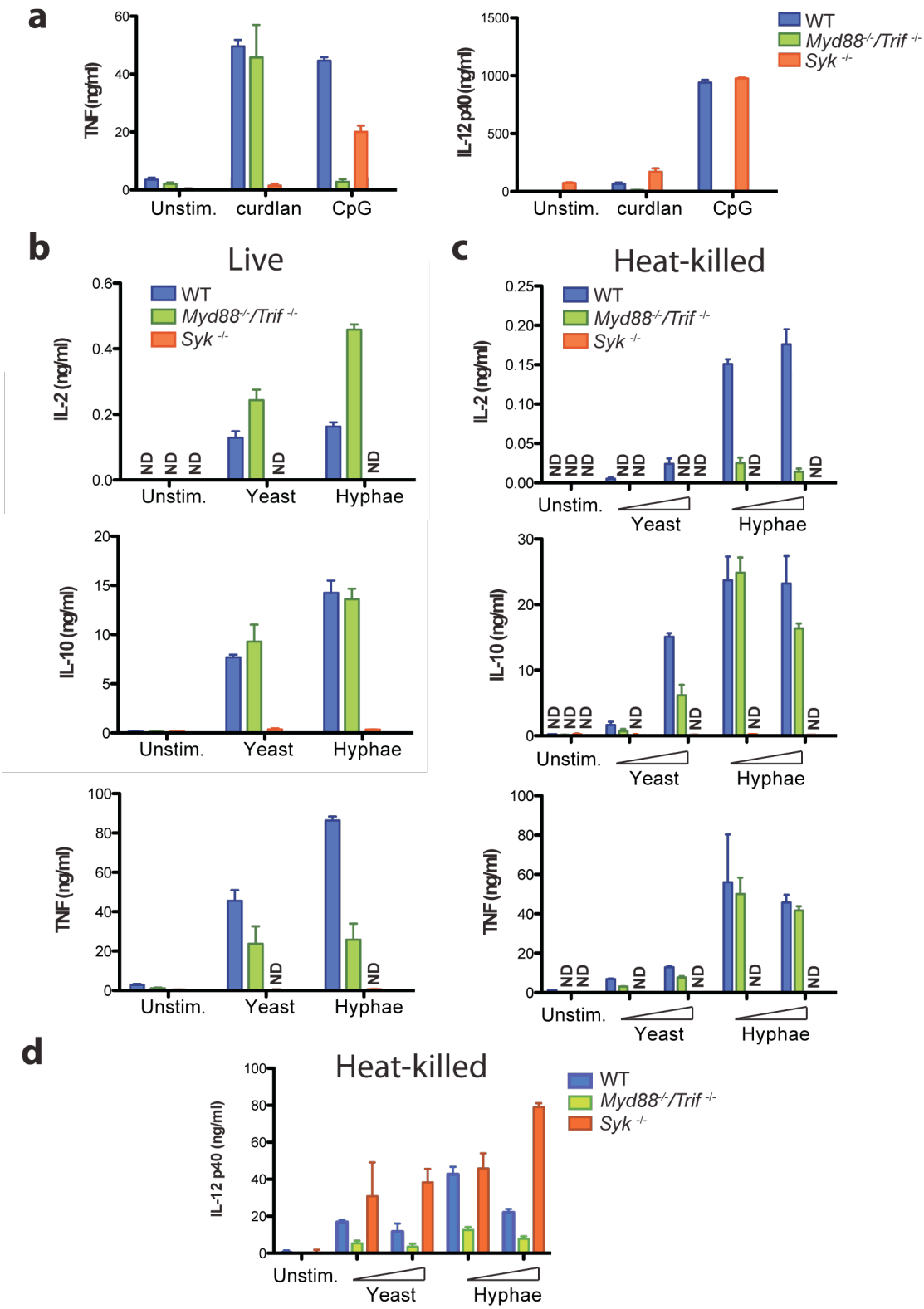

a
TNF (ng/ml)
IL-12 p40 (ng/ml)
WT
Myd88-/-/Trif -/-
Syk -/-
Unstim.
curdlan
CpG
b
Live
c
Heat-killed
IL-2 (ng/ml)
IL-10 (ng/ml)
TNF (ng/ml)
ND
Unstim.
Yeast
Hyphae
d
Heat-killed
IL-12 p40 (ng/ml)

Figure 4-2: The contribution of Syk and TLR signalling to cytokine induction by *C. albicans*. (**a**) BMDCs from C57BL/6 wild type (WT, blue bars), *Myd88*$^{-/-}$/*Trif*$^{-/-}$ (green bars) or *Syk*$^{-/-}$ chimeric (orange bars) mice were stimulated with curdlan (50ug/ml) or CpG (0.5μg/ml) and TNF and IL-12p40 were detected after overnight incubation. (**b**) BMDCs as in (**a**) were stimulated with 10^5 live *C. albicans* yeast or hyphae. Fungizone was added 2hr later and cytokine levels in the supernatants were measured after overnight incubation. (**c**) BMDCs as in (**a**) were stimulated overnight with 10^5 or 5 x 10^5 heat-killed *C. albicans*. (**d**) IL-12p40 production from BMDCs stimulated as in (**c**). Data are mean +/- SD of duplicate wells and are representative of at least 3 independent experiments. ND indicates Not Detectable.

4.2.3 The production of IL-2, IL-10 and TNF in response to *C. albicans* is independent of Dectin-1

As Dectin-1 is the archetypical example of a C-type lectin receptor that signals via Syk for gene induction and cytokine production (LeibundGut-Landmann et al., 2007), we analyzed whether Dectin-1 accounts for the production of the Syk-dependent cytokines to *C. albicans.* BMDCs from Dectin-1 deficient mice were generated and compared to wild type cells in their ability to produce IL-2, IL-10 and TNF to the organism. Results are shown in Figure 4.3. Dectin-1 deficient BMDCs produce similar levels of these three factors response to heat-killed or live (not shown) *Candida*. This is consistent with the notion that the production of IL-2, IL-10 and TNF is only partially affected in Dectin-1 deficient BMDCs stimulated with zymosan (Dr Matthew Robinson, Immunobiology lab, (Robinson et al., 2009)). In agreement with these observations, previous reports have shown that Dectin-1 expression in BMDCs is dispensable for zymosan recognition (Saijo et al., 2007, Taylor et al., 2007).

The strict Syk dependence but Dectin-1 independence of IL-2, IL-10 and TNF produced by BMDC in response to *C. albicans* stimulation suggests the existence of additional Syk-coupled PRRs in DCs that remain active in the absence of Dectin-1. The aim of this chapter is to identify such a receptor.

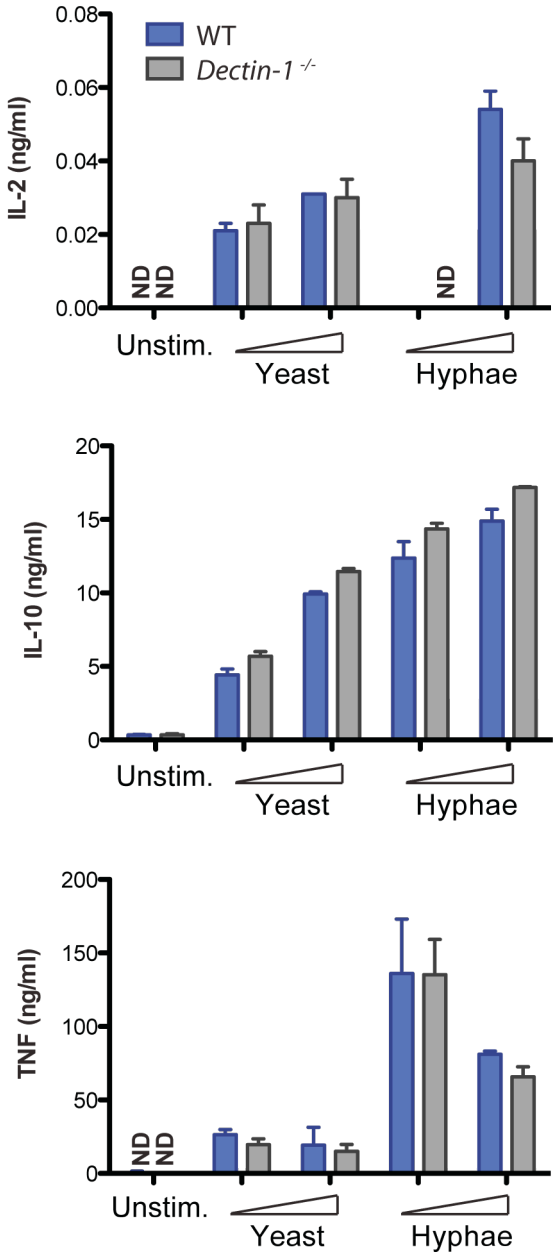


Figure 4-3: The contribution of Dectin-1 to cytokine production by *C. albicans*.
BMDCs from C57BL/6 wild type (WT, blue bars) or *Clec7a*$^{-/-}$ chimeric (Dectin-1 KO; grey bars) mice were stimulated overnight with 10^5 or 5 x 10^5 heat-killed *C. albicans* yeast or hyphae. Data are mean +/- SD of duplicate wells and are representative of at least 3 independent experiments. ND indicates Not Detectable.

4.2.4 Dectin-2 is a Syk-coupled receptor in DCs

Dectin-2 is a C-type lectin receptor that binds to zymosan, *C. albicans*, *M. tuberculosis*, *P. brasiliensis*, *H. capsulatum, M. audouinii, T. rubrum* and house dust mite allergens (McGreal et al., 2006, Barrett et al., 2009, Sato et al., 2006). For *C. albicans* recognition, soluble Dectin-2 receptor binds preferentially to the hyphae form over the yeast (Sato et al., 2006) and its expression pattern is restricted to myeloid cells, particularly to some subsets of macrophages and inflammatory monocytes (Taylor et al., 2005). Despite some reports showing limited expression of Dectin-2 in DCs (Taylor et al., 2005), we reevaluated the expression of Dectin-2 in BMDCs and spleen DCs by FACS analysis. Results are shown in Fig 4.4a-b. Splenic DCs or BMDCs were stained with 10μg/ml of an anti Dectin-2 antibody (Clone 11E4, kindly provided by Dr Philip Taylor, Cardiff University) or isotype control for 30 min following by anti-Rat IgG-PE. Dectin-2 expression was clearly observed in $CD4^+$, $CD8^+$ and double negative subsets of splenic DCs (Fig 4.4a). As expected, BMDCs also expressed high levels of the receptor (Fig.4.4 b). As previously reported (Leibundgut-Landmann et al., 2008), Dectin-1 is also expressed in the three main subsets of splenic DCs and its expression pattern is similar to that of Dectin-2 (Fig 4.4c) (Robinson et al., 2009), indicating that splenic DCs express both fungal CLRs.

Whether Dectin-2 is a Syk-coupled PRR involved in DC activation and gene induction was unknown. Work from Dr Matthew Robinson first demonstrated that Dectin-2 signals via Syk in DCs (Robinson et al., 2009). To trigger selective Dectin-2 stimulation, BMDCs were cultured with anti-Dectin-2 antibody (or Fab preparations) or isotype control immobilized on plastic. Contact of BMDCs with anti-Dectin-2 surfaces led to phosphorylation of Syk and MAPK cascades and production of cytokines (Robinson et al., 2009). Remarkably, Dectin-2 cross-linking triggered accumulation of IL-2, IL-10 and TNF in a manner dependent on Syk and CARD9 but independent of MyD88-TRIF (Robinson et al., 2009) (Fig 4.4d produced by Matthew Robinson and reproduced with kind permission here, and not depicted). These experiments demonstrate that triggering endogenous Dectin-2 in BMDCs results in Syk and CARD9 activation and cytokine production independently of TLR stimulation (Robinson et al.,

2009). In addition, Dectin-2 can couple to Syk to trigger the production cysteinyl leukotrienes in response to house dust mites (Barrett et al., 2009).

In contrast to Dectin-1, Dectin-2 does not contain any ITAM or hemITAM motif within its short intracellular tail that would enable direct interaction with Syk. However, it has been reported that Dectin-2 over expressed in RAW and COS cells coimmunoprecipitates with γ chain of Fc receptor, which contains a conventional ITAM motif (Sato et al., 2006). Furthermore, the expression of Dectin-2 is enhanced when cotransfected with FcRγ chain (Barrett et al., 2009). Work from Matthew Robinson further demonstrated that the presence of FcRγ chain is required for appropriate surface expression of Dectin-2 in DCs and showed that its ITAM motif is responsible for Dectin-2 signalling (Robinson et al., 2009). Altogether, these experiments identified Dectin-2 as a second Syk-coupled PRR involved in DC activation.

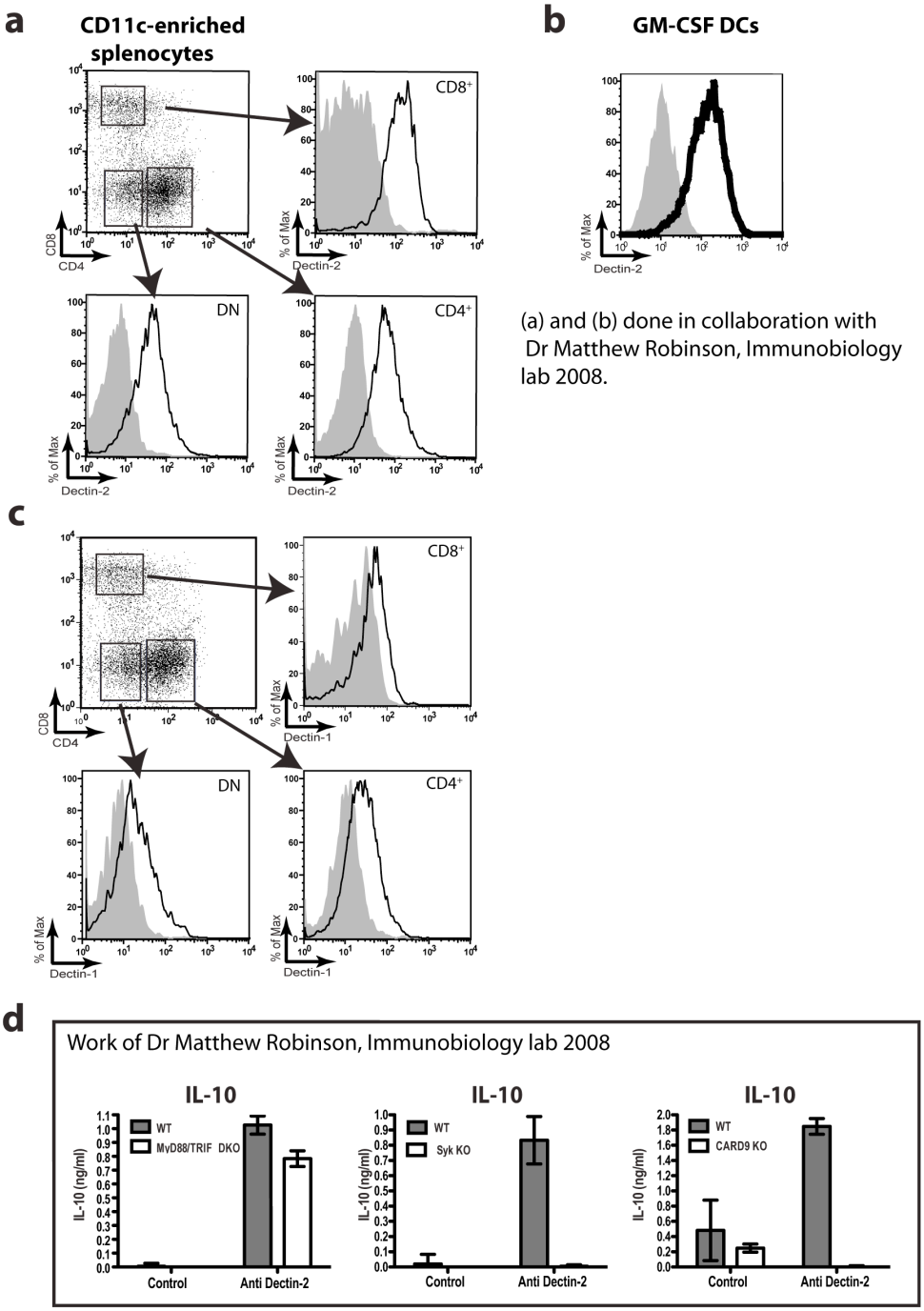


Figure 4-4: Dectin-2 is expressed by DCs and signals via Syk and CARD9.
(**a**) CD11c enriched splenocytes were stained for CD11c, CD4, CD8 and anti-Dectin-2 or isotype-matched control. The upper left panel shows gating of $CD11c^{hi}$ conventional DC populations into $CD4^{+}$, $CD8^{+}$ and double negatives. Other panels are histograms of anti-Dectin-2 (black line) or isotype control (shaded area) staining in the gated subpopulations. (**b**) Staining of GMCSF-derived BMDCs with anti-Dectin-2 (black line) or isotype control (shaded area). (**c**) As in A except that cells were stained with anti-Dectin-1. Data are representatives of 3 (**b**) or 2 (**a** and **c**) independent experiments. (**d**) Work of Dr Matthew Robinson. Wild type C57BL/6 (WT; grey bars), *Myd88*$^{-/-}$/*Trif*$^{-/-}$ (MyD88/TRIF DKO; white bars), *Syk*$^{-/-}$ chimeric (Syk KO; white bars) and *Card9*$^{-/-}$ (Card9 KO; white bars) BMDCs were stimulated overnight with plated anti-Dectin-2 or control Fab as above and IL-10 levels in the supernatants were

measured. Representatives of at least 3 independent experiments are shown. Cytokine data are mean +/- SD of duplicate wells. ND indicates Not Detectable.

4.2.5 Induction of the Syk-dependent cytokines in DCs requires Dectin-1 and Dectin-2

The results discussed above suggest that in the context of fungal stimulation, Dectin-2 might act in conjunction with Dectin-1 to promote Syk-CARD9–dependent responses in DCs. To test this hypothesis, WT and Dectin-1 deficient BMDCs were incubated with soluble anti-Dectin-2 antibody to abrogate receptor function, or with an isotype control antibody. In contrast to the cell activation method triggered by anti-Dectin-2 immobilized on plastic described above (Robinson et al., 2009), incubation of BMDCs with anti-Dectin-2 antibody in solution can be used as a blocking reagent, as previously noticed for Dectin-1 (Herre et al., 2004). Effective blockade of Dectin-2 was achieved by preincubating BMDCs with anti-Dectin-2 or isotype control antibody for 2 hrs before stimulation with fungi. WT and *Dectin-1*$^{-/-}$ BMDCs with or without Dectin-2 function were incubated with *C. albicans* overnight and cytokines were measured in supernatants.

Results are shown in Figure 4.5. As previously shown in Figure 4.3, wild type and Dectin-1 deficient BMDCs produce similar levels of IL-2, IL-10 and TNF in response to heat-killed and live forms of the organism. Although a partial contribution of Dectin-1 can be observed for IL-2 and IL-10 to heat-killed *Candida*, this effect was modest and variable and was not observed in stimulations with live organisms. Is important to note that fresh fungal preparations and newly generated BMDCs were used for each independent experiment described on this thesis and this might account for the subtle differences observed between experiments. Nonetheless, these data confirms that Dectin-1 deficiency has no major effect in Syk-dependent responses to *C. albicans*.

Similar results were obtained after blockade of Dectin-2 function in wild type cells: modest and variable reductions in IL-2, IL-10 and TNF were observed as compared to wild type cells.

However, blockade of Dectin-2 in Dectin-1 deficient BMDCs led to a profound reduction in the production of the three Syk-dependent cytokines, mimicking the

decrease observed in Syk deficient BMDCs (Figure 4.2). This observation is seen for heat-killed and live organisms and was highly reproducible. The exception was TNF produced in response to live *C. albicans*, which was only partially dependent on the two CLRs, probably reflecting the TLR dependency observed in Figure 4.2. As expected, absence of Dectin-1 and Dectin-2 did not reduce IL-12/23p40 in response to live *C. albicans* and heat killed hyphae albeit a small reduction was observed with heat-killed yeast (Fig 4.5 d).

These results indicate that Dectin-1 and Dectin-2 are not primarily involved in the induction of IL-12/23 p40 but are required for the induction of IL-2, IL-10 and TNF in response to *C. albicans.*

Notably, Matthew Robinson obtained similar results using zymosan as stimulus: loss of each receptor individually led to modest reductions in the Syk-dependent cytokines. However, dual absence of Dectin-1 and Dectin-2 in BMDCs led to a drastic abrogation of IL-2, IL-10 and TNF, approaching previous observations noted in Syk deficient BMDCs (Robinson et al., 2009, Rogers et al., 2005). Together, these results indicate that Dectin-1 and Dectin-2 are required for the induction of the cytokines controlled by Syk signalling.

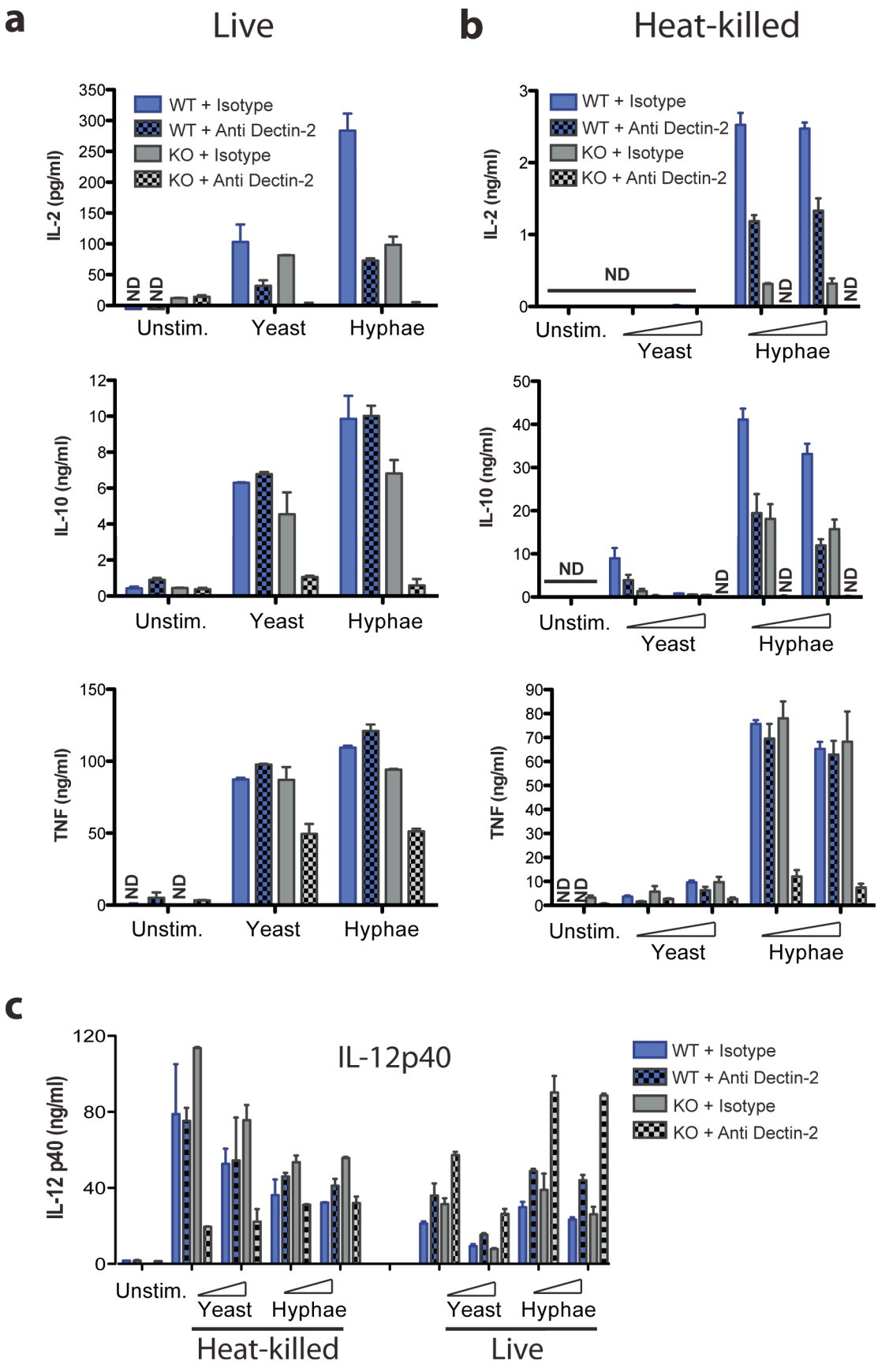

a
Live
b
Heat-killed
WT + Isotype
WT + Anti Dectin-2
KO + Isotype
KO + Anti Dectin-2
IL-2 (pg/ml)
IL-2 (ng/ml)
IL-10 (ng/ml)
TNF (ng/ml)
ND
Unstim.
Yeast
Hyphae
c
IL-12p40
IL-12 p40 (ng/ml)
Heat-killed
Live

Figure 4-5: Dectin-1 and -2 mediate Syk-dependent responses to *C. albicans*.
(**a**) BMDCs from wild type C57BL/6 (WT) or *Clec7a*$^{-/-}$ chimeric (Dectin-1 KO) mice were treated with 10μg/ml anti-Dectin-2 or isotype control for 2 hr prior to stimulation with 10^5 live *C. albicans* yeast or hyphae. Fungizone was added 2hr after stimulation, and the cytokines levels in the supernatants measured after overnight incubation. (**b**) BMDCs pretreated with mAbs as in (**a**) were stimulated overnight with 10^5 or 5 x 10^5 heat-killed *C. albicans*. (**c**) BMDCs pretreated with mAbs as in (**a**) were stimulated overnight with 10^5 or 5 x 10^5 heat-killed or live *C. albicans* and IL-12p40 production was measured after overnight stimulation. Data are mean +/- SD of duplicates wells and representative of at least 3 experiments. ND indicates Not Detectable.

Triggering Dectin-1 in isolation results in the production of IL-23p19 but little IL-12p35 by BMDCs (LeibundGut-Landmann et al., 2007). Thus, I sought to investigate whether Dectin-1 and Dectin-2 could regulate the production of IL-12p35 and IL-23p19 in BMDCs activated with fungi. RNA from cells was extracted 3 hrs after stimulation with *C. albicans.* Results are shown in Fig 4.6. Whereas IL-12p35 was at the limit of detection (data not shown), the induction of transcripts encoding IL-12p19 was also dually dependent on Dectin-1 and Dectin-2. Notably, blockade of Dectin-2 in wild type cells led to a considerable reduction in the levels of IL-12 p19 transcript, particularly in response to the yeast form, suggesting that Dectin-2 might have a predominant role in coordinating the induction of IL-23 in response to the organism.

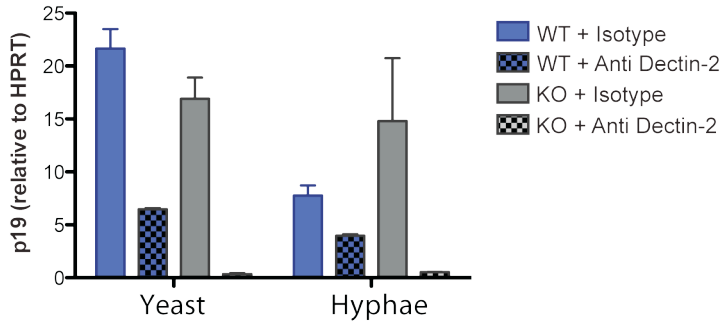


Figure 4-6: Dectin-1 and -2 mediate *C. albicans* induction of IL-23.
4x10^5 BMDCs from wild type C57BL/6 (WT) or *Clec7a*$^{-/-}$ (Dectin-1 KO) mice were treated with 10μg/ml anti-Dectin-2 or isotype control for 2 hr prior to stimulation with 4x10^5 live *C. albicans* yeast or hyphae. IL-23 p19 mRNA was quantitated by quantitative PCR after 3hrs. Fungizone was added 2hr for the final 1 hr of stimulation. Data are mean +/- SD of duplicate wells and representative of 2 independent experiments.

To address the role of Dectin-2 in fungal recognition by an independent means, I used short hairpin RNA (shRNA) to knock down Dectin-2 expression in BMDCs. The shRNA construct for mouse Dectin-2 was chosen based on a previous publication (Barrett et al., 2009). Lentiviral particles containing Dectin-2 or scramble shRNA used in this study were obtained through collaboration with Dr Luis F Moita (Instituto de medicina molecular, Universidade de Lisboa, Portugal). Infection of bone marrow from wild type and Dectin-1 deficient mice with shRNA-containing lentiviruses was performed on day 2 of culture and cells were stimulated on day 6. BMDC populations are shown on Figure 4.7a. Infection with a lentivirus containing the Dectin-2 shRNA construct resulted in similar frequencies of viable CD11c-expressing cells as compared to scramble shRNA control. After puromycin selection, CD11c-expressing cells infected with Dectin-2 shRNA-containing lentivirus expressed lower levels of the receptor and reduced the mean fluorescence intensity of Dectin-2 to 56.8 ± 9.16% as compared to scramble shRNA (Figure 4.7b-c).

Lentivirus-infected cells were stimulated with heat-killed *C. albicans* and the production of IL-2, IL-10 and TNF was assessed by ELISA after overnight stimulation. Results are shown in Fig 4.7d. As previously observed with the blocking anti-Dectin-2 antibody, knockdown of Dectin-2 in wild type cells led to similar levels of the Syk-dependent cytokines compared to scramble shRNA control. Notably, knock down of Dectin-2 in Dectin-1 deficient cells resulted in marked reduction of IL-2, IL-10 and TNF to the organism, in agreement with results showed in Fig 4.5. Overall, these results indicate that Dectin-1 and Dectin-2 account for the Syk-dependent cytokines in BMDCs.

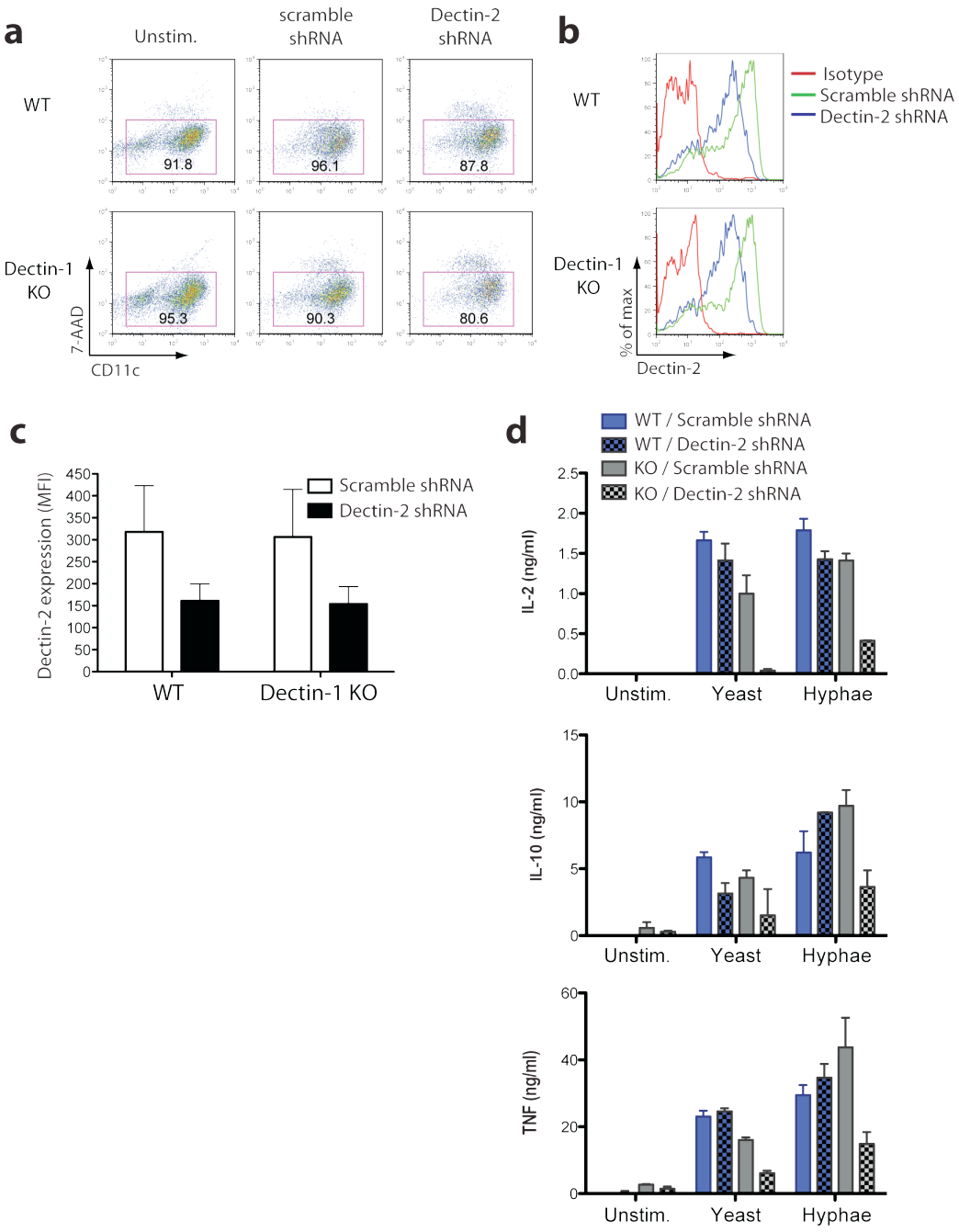


Figure 4-7: Lentiviral knockdown of Dectin-2 in Dectin-1 deficient DCs abrogates Syk-dependent responses to *C. albicans*.
(**a**) BMDCs from wild type 129/Sv (WT) or *Clec7a*$^{-/-}$ (Dectin-1 KO) mice were infected with lentivirus encoding Dectin-2 shRNA or a scrambled control shRNA. After puromycin selection (except for uninfected samples, left panel), cells were stained for CD11c and 7AAD and analyzed by FACS on day 6. (**b**) BMDCs infected with lentivirus as in (**a**) assessed for Dectin-2 levels by FACS on day 6. (**c**) Geometric mean fluorescence in FL2 from cells described in (**b**) was plotted for WT and Dectin-1 KO cells infected with scramble shRNA or Dectin-2 shRNA-containing lentiviruses. Results are represented as mean+ SEM from three independent experiments. (**d**) BMDCs infected as in (**a**) were stimulated with 10^5 heat-killed *C. albicans* and cytokines levels in the supernatants measured after overnight incubation. Data are mean +/- SD of duplicates wells and representative of 3 independent experiments.

Notably, Matthew Robinson observed that binding of fluorescent zymosan was greatly decreased when Dectin-2 was blocked in Dectin-1 deficient BMDCs. This result indicates that in addition to the loss of the Syk-dependent cytokines, Dectin-1 and Dectin-2 are the primary binding receptors for fungal stimuli. This raises a caveat with the interpretation that signals derived from Dectin-1 and -2 cause Syk-dependent DC activation. In turn, the loss of cytokines observed with Dectin-2 blockade in Dectin-1 deficient cells could be caused by lack of binding of fungal particles, which would prevent engagement of additional Syk-coupled PRRs. To address this point, Matthew Robinson transduced Dectin-1 deficient BMDCs with a Y15F mutant form of Dectin-1, which binds avidly to zymosan but does not signal via Syk (Rogers et al., 2005). Whereas the blockade of Dectin-2 in Y15F-transduced cells did not inhibit binding to zymosan, the production of IL-2, IL-10 and TNF was markedly decreased. Blockade of Dectin-2 in Y15F mutant cells or in mock-transduced Dectin-1 deficient BMDCs led to a comparable reduction in the levels of the Syk-dependent cytokines (Robinson et al., 2009). This result demonstrates that Dectin-2 nearly blocks Syk-dependent cytokine responses in a scenario in which Dectin-1 promote high degree of fungal particles but is unable to signal. This demonstrates that the requirement of Dectin-1 and -2 in Syk-dependent DC activation by fungi is caused by signals propagated by these receptors and not just by their ability to bind to fungal stimuli (Robinson et al., 2009).

4.2.6 Dectin-2 is crucial for Th17 responses to *Candida* infection

In light of our *in vitro* observations with BMDCs, we sought to determine the role of Dectin-2 in the induction of T helper responses during *C. albicans* infection. As Dectin-2 deficient mice were unavailable at that time, we blocked Dectin-2 *in vivo* by administering the anti-Dectin-2 antibody intraperitoneally. Wild type and Dectin-1 deficient mice were injected i.p. with anti-Dectin-2 or isotype matched control 6 hrs before, 2 and 4 days after systemic (i.v.) infection with *C.albicans*. The dose of pathogen used was $3x10^4$ colony forming units (CFU), which is sublethal in wild type mice and is tolerated by CARD9 deficient mice within the first 4 days of infection (Gross et al., 2006). To assess the nature of the T cell response generated in response to the organism, splenocytes from *Candida*-infected mice were collected on day 7 after infection and restimulated with different doses of heat killed *C. albicans* for 2 days. The presence of Th1 and Th17 cells was monitored by measuring IFN-γ and IL-17

respectively. Is important to note that this method of restimulation is strictly dependent on the presence of $CD4^+$ T cells in the well, as IL-17 and IFN-γ are not detected when using CD4-depleted splenocytes from infected mice as the responding population (LeibundGut-Landmann et al., 2007). These experiments were performed together with Matthew Robinson and were executed in the Laboratory of Dr Philip Taylor in Cardiff University (Cardiff, Wales), who provided reagents and the infection facility required to carry out these types of experiments.

Results are shown in Figure 4.8. No cytokines were detected in wells containing splenocytes from infected mice cultured in medium only (Figure 4.8a). After restimulation with 10^5 heat-killed *C. albicans* per well, IL-17 and IFN-γ were readily detected in splenocytes from wild type infected mice treated with isotype control (Figure 4.8b). Remarkably, anti-Dectin-2 administration into wild type mice led to a drastic reduction in the levels of IL-17 produced in response to *C. albicans*. This observation was also seen at a higher dose of the organism used for restimulation (Figure 4.8c), indicating that Dectin-2 plays a non-redundant role in coordinating Th17 responses to *C. albicans*. Notably, anti-Dectin-2 treatment in wild type mice did not modify the Th1 response to *Candida* (Figure 4.8b and 4.8c).

Comparison between wild type and Dectin-1 deficient mice treated with isotype control showed no differences in the overall Th17 and Th1 response to the organism, confirming previous findings indicating Dectin-1 redundancy in T cell responses to *C. albicans* (LeibundGut-Landmann et al., 2007). Nonetheless Dectin-1 is not completely dispensable for T cell immunity to *Candida* in these experimental settings, as Dectin-1 deficient mice without Dectin-2 function produce significantly less IFN-γ when restimulating with 10^5 particles per well (Figure 4.8b). This decrease is no longer observed in cultures restimulated with higher doses of the pathogen (Figure 4.8c) suggesting these two CLRs may modulate Th1 responses in a quantitative manner. It is possible that additional PRRs contribute to the initiation of this type of response at saturating concentrations of the organism.

We conclude that Dectin-2 is crucial for the initiation of Th17 responses to *Candida* infection and together with Dectin-1, can modulate Th1 responses to the organism.

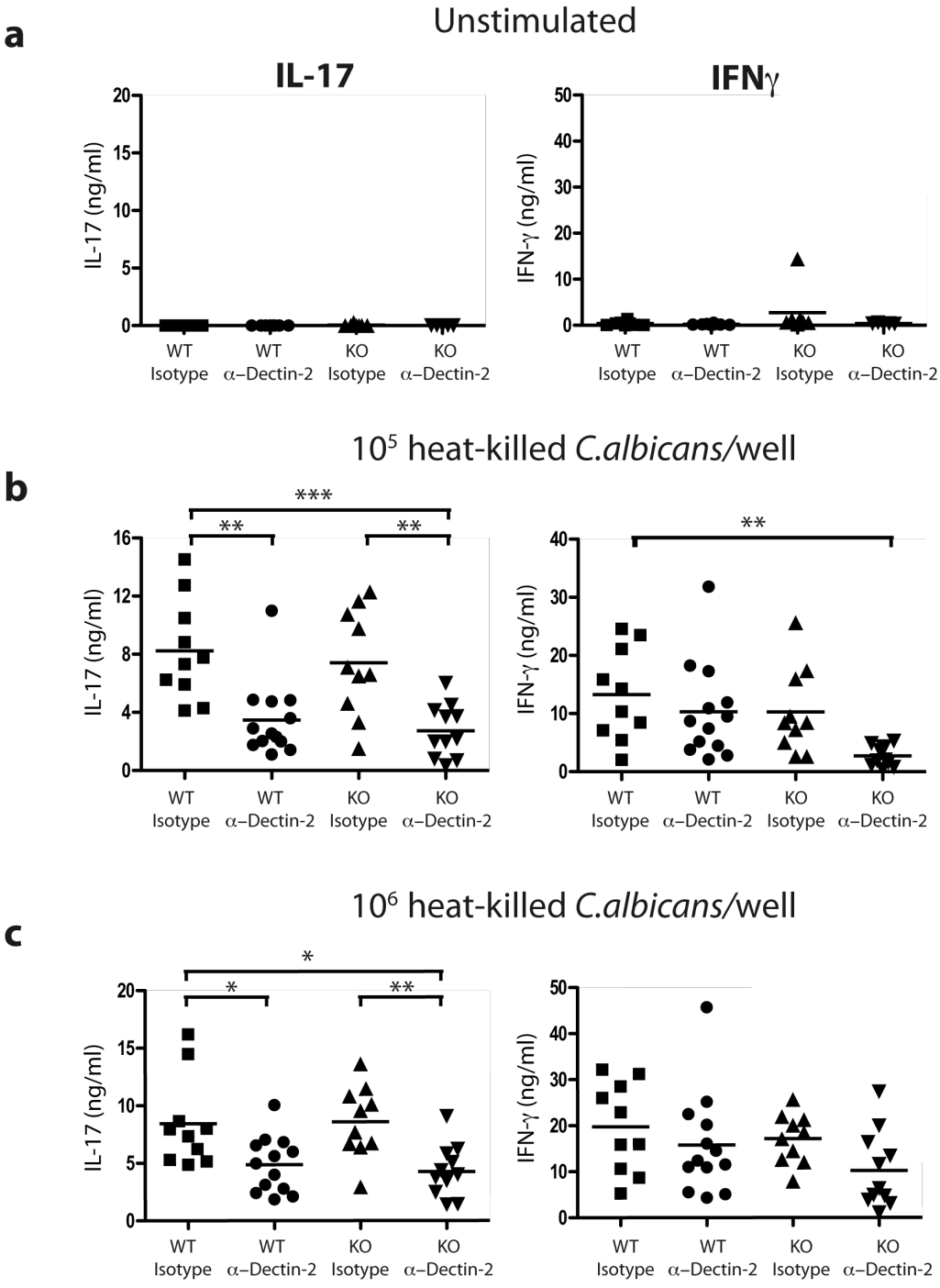


Figure 4-8: Dectin-2 is required during systemic *C. albicans* infection for Th17 responses
Wild type 129/Sv (WT) or *Clec7a*$^{-/-}$ (Dectin-1 KO) mice were given anti-Dectin-2 or isotype-matched control mAb intraperitoneally before and after intravenous *C. albicans* infection. After 7 days, splenocytes were restimulated with (**a**) medium alone, (**b**) 10^5 heat-killed *C. albicans* or (**c**) 10^6 heat-killed *C. albicans* for 2 days. IFNγ and IL-17 production in the supernatants was measured by ELISA. The data are pooled from 2 independent experiments and each symbol represents the mean of triplicate stimulations from an individual mouse. Statistically different groups are indicated by * $0.01<P<0.05$; ** $0.001<P<0.01$ and *** $P<0.001$.

4.2.7 Blockade of Dectin-2 does not lead to impairment of innate immunity to *C. albicans*

During the course of infection, mice were weighed on a daily basis and at the end of the experiment, and fungal growth was quantified in kidneys. Dr Philip Taylor kindly measured these parameters. Results are shown in Figure 4.9. Despite the dramatic effect of Dectin-2 in the initiation of Th17 responses to *Candida*, wild type mice treated with anti-Dectin-2 showed similar fungal burdens and body weight as compared to isotype control treated wild type mice. The same observation was made in Dectin-1 deficient mice, independently of Dectin-2 function. These results indicate that, at day 7 after infection, loss of Dectin-1 and -2 does not impair resistance to *C. albicans* and suggest that there might be additional PRRs involved in coordinating innate immunity to *Candida*. These observations will be clarified in Dectin-2 deficient animals.

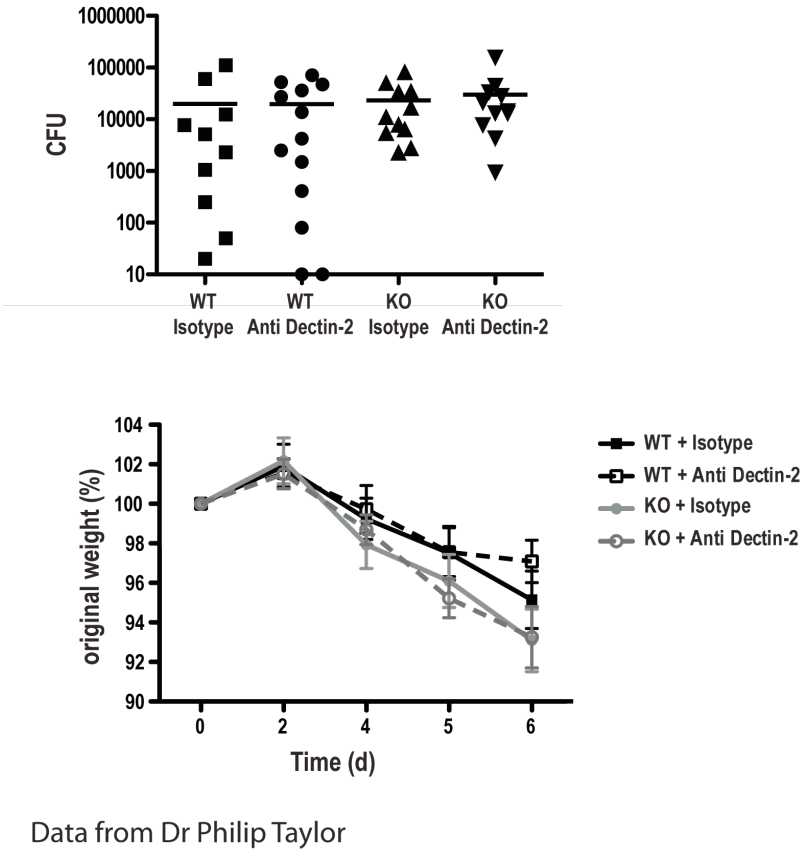


Figure 4-9: Dectin-2 is not required for containing early renal fungal burden in systemic *C. albicans* infection.
Wild type 129/Sv (WT) or *Clec7a*$^{-/-}$ (Dectin-1 KO) mice were treated with anti-Dectin-2 or isotype-matched control mAb and infected with *C. albicans* intravenously. After 7 days, mice were sacrificed. (**a**) Kidney fungal burden and (**b**) percentage of original weight at time of sacrifice.

4.3 Discussion

4.3.1 Dectin-2 is a Syk-coupled CLR involved in DC activation by fungi.

Upon pathogen recognition, PRRs trigger intracellular signalling events that result in gene induction and cytokine/chemokine production and initiation of immunity. Members of the TLR, NLR or RLR families are well accepted PRRs but Dectin-1 was the only member of the CLR family fulfilling the criterion of a signalling PRR. My work, together with the work of Matthew Robinson, showed that Dectin-2 is an additional Syk/CARD9-coupled CLR that signals for DC activation.

Matthew Robinson showed that triggering of this receptor in isolation leads to cytokine production by DC in FcRγ chain/Syk and CARD9-dependent manner and results in MAPK activation (Robinson et al., 2009). My results demonstrate that Dectin-2 accounts for the Syk-dependent yet Dectin-1-independent responses of DCs stimulated with fungi. Although macrophages express Dectin-1 and Dectin-2 (Taylor et al., 2002, Brown et al., 2002, Taylor et al., 2005), they depend mainly on Dectin-1 for the production of IL-10 and IL-12p40 in response to zymosan (Taylor et al., 2007) indicating that in the context of microbial sensing, the usage of PRRs might be cell-type specific. Along these lines, it has been shown that DCs from mesenteric lymph nodes stimulated with *C. albicans* produce IL-10 in a TRIF dependent manner (De Luca et al., 2007). In the case of BMDCs, I have showed that Dectin-1 and Dectin-2 are responsible for the production of IL-2, IL-10 and TNF to fungal stimuli. Notably, these receptors are expressed in $CD4^+$, $CD8^+$ and double negative subsets of spleen DCs (fig 4.4) suggesting that conventional DCs might also depend on Dectin-1 and -2 to sense fungal stimuli.

Is important to note that Dectin-1 and Dectin-2 are remarkably different; they belong to different CLR groups and present a full sequence homology of only 19.6% (Robinson et al., 2006, Ariizumi et al., 2000a). Furthermore, these receptors recognize ligands of diverse nature. Whereas the CTLD of Dectin-1 binds to 1,3 β-glucans in a calcium-independent manner (Brown and Gordon, 2001), the carbohydrate recognition domain of Dectin-2 binds to high mannose structures in a calcium-dependent mode (McGreal et al., 2006). Binding of the CRD of Dectin-2 to zymosan is inhibited by the

monosaccharides fucose and mannose whereas binding to *C. albicans* is inhibited by mannans but not galactans (McGreal et al., 2006). This evidence suggests that the CRD of Dectin-2 has specificity for high mannose structures such as mannans.

Although many PRRs have been loosely classified as mannan-specific, they recognize different signatures within the polysaccharide structures. O-linked yeast mannans are short linear chains consisting of one to five α-linked mannoses that can be recognized by TLR4 (Netea et al., 2006) (reviewed in (Netea et al., 2008)). Alternatively, N-linked mannans are long-chain and highly branched residues that can be sensed by multiple receptors such as mannose receptor, SIGNR1, DC-SIGN, TLR2 and Dectin-2 (Netea et al., 2006) (reviewed in (Netea et al., 2008)). Nonetheless, competition studies have demonstrated that the lectin activity of Dectin-2 is different from that of SIGNR1 and mannose receptor (McGreal et al., 2006), indicating that these receptors bind to different residues within the oligomannose chains. Indeed, a recent publication has demonstrated Dectin-2 is a receptor specific for α-linked mannans derived from *C. albicans* (Saijo et al., 2010).

Previous publications have suggested that Dectin-1 and Dectin-2 recognize different morphological stages of *C. albicans*. β-glucan exposition during yeast but not hyphae cell division favours detection by Dectin-1 (Gantner et al., 2005) whereas Dectin-2 have shown to bind preferentially to the hyphae form of *C. albicans* (Sato et al., 2006). Although results presented in this chapter show that the hyphal form elicits higher cytokine response than yeasts, my results clearly demonstrate that Dectin-2 mediates cytokine production to yeasts and that Dectin-1 also participates in hyphal sensing. These two independent sets of data might seem contradictory at first glance, however the use of soluble Dectin-1 and -2 receptors as tools for ligand identification might not necessarily predict the outcome of the cytokine response in DC activated with fungi. In addition, McGreal et al has shown that soluble Dectin-2-Fc can bind to *C. albicans* yeast (McGreal et al., 2006).

The generation of the Dectin-2 deficient mice has been recently described (Saijo et al., 2010). That report shows that the production of IL-6, TNF, IL-1β, IL-10, IL-23 and IL-12p70 by BMDCs stimulated with heat-killed yeast is dependent on Dectin-2 (Saijo et

al., 2010). Whether the Dectin-2-dependency of those cytokines is caused by lack of signals propagated by Dectin -2 or is the consequence of a decrease in fungal binding is unclear.

Data by Saijo et al also indicates that Dectin-2 acts as a yeast receptor, in agreement with our previous findings. In response to hyphal stimulation, *Dectin-2* $^{-/-}$ BMDCs are only partially impaired at producing the same subset of cytokines (Saijo et al., 2010), probably reflecting Dectin-1 activity as proposed throughout this chapter. Notably, this report illustrates that IL-2, IL-10, TNF and IL-23 are just a fraction of the spectrum of cytokines regulated by Dectin-2 (Saijo et al., 2010).

In contrast to the data provided by Saijo et al, my results indicate that Dectin-2 is not the only Syk/CARD9 PRR for yeast as functional blockade of this receptor or shRNA gene silencing in wild type cells does not markedly affect the production of IL-10 and TNF to heat-killed yeast (Figs 4.5 and 4.7). One plausible explanation to this discrepancy might be the use of different strains of *C. albicans* and the effectiveness of the receptor inhibition achieved in my experiments. In addition, the contribution of Dectin-2 to the recognition of live organisms was not provided in the paper of Saijo et al (Saijo et al., 2010).

The hallmark cytokines controlled by the Syk/CARD9 pathway to fungal stimuli presented in this chapter and elsewhere are IL-2, IL-10 and TNF ((Rogers et al., 2005, Robinson et al., 2009) and this chapter). As mentioned above, these factors represent only a small fraction of the cellular aspects controlled by Syk signalling in response to fungal challenge. I showed that transcripts encoding IL-23 are regulated by Dectin-1 and -2 (Fig 4.6) and it is known that Syk signalling controls synthesis and processing of IL-1β in response to *Candida* (Gross et al., 2009). In addition, Dectin-2 mediates the production of cysteinyl leukotrienes in response to house dust mite allergens (Barrett et al., 2009). These results indicate that the "Syk signature" in DCs might include multiple factors that will have to be analyzed by using genome-wide screens.

4.3.2 Dectin-2 is essential for Th17 generation to *Candida* infection

At the time of starting this project, the role of Dectin-2 in the context of fungal infection was not elucidated. Results presented in figure 4.8 first demonstrated that Dectin-2 is

essential to coordinate Th17 responses to *C. albicans* infection. This result raises the question as to why Dectin-2 is crucial to generate a Th17 response to *Candida*.

Although my results indicate that the Syk-dependent cytokines IL-2, IL-10 and TNF are dually dependent on Dectin-1 and Dectin-2, I have observed that the transcripts encoding IL-23 are markedly affected in the absence of Dectin-2 (Fig 4.6). In addition, others have reported that the production of IL-6, IL-1β and IL-23 in *Candida*-stimulated BMDCs is largely dependent on Dectin-2 (Saijo et al., 2010). All those cytokines have a strong impact on the generation of Th17 responses *in vitro* and *in vivo* (Bettelli et al., 2006, Veldhoen et al., 2006a, Langrish et al., 2005, Chung et al., 2009).

The analysis of the Dectin-2 deficient mice has also showed that this C-type lectin is required for the initiation of Th17 responses to *C. albicans* (Saijo et al., 2010), confirming our previous findings (Fig 4.8). Nonetheless, the precise mechanism of Th17 induction is still unresolved.

Despite the marked effect of Dectin-2 on the generation of Th17 responses to *Candida in vivo*, our data indicate that Dectin-2 blockade did not lead to an increase in kidney fungal burden (Fig 4.9), not even in absence of Dectin-1. These results contrast with the CARD9 deficiency (Gross et al., 2006), and indicate that Dectin-1 and -2 are redundant for host innate defense within the first seven days of infection. Consistent with this notion, Dectin-1 deficient mice infected i.v. with *C. albicans* do not display higher fungal burden in the kidney early in infection (Taylor et al., 2007).

In the absence of Dectin-2 deficient mice available at that time, we were restricted to do seven-day infection experiments with an anti-Dectin-2 antibody used as a functional blocking reagent. In this experimental setting, any beneficial or detrimental effect of Th17 cells might be masked by innate resistance mechanisms. Nevertheless, Saijo et al (Saijo et al., 2010) have recently reported the role of this receptor in host defense to *C. albicans*. Although *Dectin-2*$^{-/-}$ mice show increased susceptibility to systemic infection with the organism, the differences in fungal burden and survival only become apparent at day 10-11 after systemic infection (Saijo et al., 2010). These data are highly consistent with our results showing a lack of effect by the anti-Dectin-2 antibody treatment at day 7 after systemic infection (Fig 4.9).

The fact that Dectin-2 is involved in host defense to *C. albicans* is an important statement and distinguishes it from that of the mannose receptor, which is reported to coordinate human Th17 responses to *Candida* (van de Veerdonk et al., 2009) but has no impact in host defense during fungal infection in mice (Lee et al., 2003).

Whether the deficiency in the Th17 compartment observed in Dectin-2 deficient mice is responsible for the increased susceptibility to infection is at present unclear. Furthermore, the role of Th17 cells during fungal infections is not clearly elucidated. Mice deficient in IL-17RA are susceptible to systemic and oropharyngeal candidiasis (Huang et al., 2004, Conti et al., 2009) and human memory $CD4^+$ T cells specific for *C. albicans* have a Th17 phenotype (Acosta-Rodriguez et al., 2007, Zhou et al., 2008b). Nonetheless, the Th17 responses have also been argued to promote inflammation and susceptibility to an intragastric model of candidiasis (Zelante et al., 2007). Along these lines, I have demonstrated that activation of the Dectin-1 pathway in BMDCs results in $Foxp3^+$-$IL\text{-}17^+$ T cell generation (Osorio et al., 2008), a cell type with an undefined function during immune challenge.

A clearer picture of the role of Th17 responses during fungal infections comes from studies in humans. Chronic mucocutaneous candidiasis (CMC) is commonly associated with T cell immunodeficiency. Interestingly, patients with mutations in *STAT3* fail to generate Th17 cells and suffer from recurrent CMC and *S. aureus* infections (Ma et al., 2008, Milner et al., 2008, de Beaucoudrey et al., 2008). Similarly, APECED patients with CMC display high titers of autoantibodies against IL-17A, IL-17F and IL-22 (Puel et al., 2010, Kisand et al., 2010), highlighting the role of IL-17 immunity to the control of mucosal *Candida* infection.

The connection between the C-type lectin receptor pathway and susceptibility to fungal infections in humans has recently been reported. Patients with an early stop mutation in Dectin-1 or a homozygous point mutation in CARD9 are more susceptible to CMC (Ferwerda et al., 2009, Glocker et al., 2009). In sum, our findings together with the evidence in humans strongly indicate that signalling downstream of fungal C-type lectin receptors is an essential innate axis for the establishment of antifungal Th17 responses.

Chapter 5. Tools to study antigen-specific T cell responses to *C. albicans*-associated antigens

5.1 Introduction

By analyzing endogenous responses to *C. albicans*, results from the Immunobiology Laboratory demonstrated that the generation of Th17 responses to the organism is dependent on the adaptor CARD9 (LeibundGut-Landmann et al., 2007). Furthermore, we have identified Dectin-2 as an essential member of the Syk/CARD9 pathway responsible for the induction of Th17 responses during systemic *C. albicans* infections (Figure 4.8, (Robinson et al., 2009)). Although those experiments provided invaluable information regarding the role of Syk-coupled CLRs in the coordination of effector T cell responses, many questions remain unresolved. What is the role of anti-fungal Th17 cells to the resolution of infection? Are the signals derived from the CLR/Syk/CARD9 pathway relevant for priming of naïve $CD4^+$ T cells? What is the impact of innate signalling pathways to the expansion and trafficking of T cells to the site of infection?

If we were able to study T cell immunity to *C. albicans* in an antigen-specific manner, those questions could be easily addressed. The study of defined T cell populations is a very powerful approach to dissect the contribution of innate signalling pathways in recruitment, priming, expansion and trafficking of cells during immune challenge.

One example comes from studies of the immune response to *A. fumigatus*, in which the generation of an *A. fumigatus* specific $CD4^+$ TCR-transgenic mouse helped revealing the kinetic of fungus-specific $CD4^+$ T cells during the course of the infection (Rivera et al., 2006). In that model, MyD88-dependent signals contributed to the production of IFN-γ but not to proliferation and trafficking of *A. fumigatus*-specific $CD4^+$ T cells (Rivera et al., 2006).

In the absence of a *C. albicans*-specific $CD4^+$ TCR-transgenic mouse, an alternative approach to study antigen-specific T cell dynamics to the organism would be to design a transgenic strain of *Candida* expressing a model antigen. Conventional genetic analysis in *C. albicans* is difficult due to its unusual parasexual cycle, its diploid genome and the usage of a non canonical genetic code, in which CUG codons are translated into serine

rather than leucine ((Santos and Tuite, 1995) and reviewed in (Noble and Johnson, 2007)). Nonetheless, technological advances have substantially improved our current understanding of *C. albicans* basic biology. The development of transformation techniques has enabled genetic manipulation such as gene deletions, fusions or insertions. The instability of episomal plasmids has been circumvented by the development of integrative vectors, which promote efficient gene expression and stability (Murad et al., 2000). In addition, auxotrophic strains of *C. albicans* have become useful reagents for genetic transformation (Fonzi and Irwin, 1993). To date, some reporter genes including GFP, luciferase or the β-galactosidase *lacZ* gene have been optimized for gene expression studies in *C. albicans* and have proven to be effective in infection settings (Uhl and Johnson, 2001, Barelle et al., 2004, Enjalbert et al., 2009).

Given these advances, we decided to generate transgenic strains of *C. albicans* expressing peptides from the model antigen ovalbumin.

The aim of this chapter is to generate tools that allow the visualization, priming and trafficking of T cells specific for a *C. albicans*–derived antigen. The main purpose behind the generation of these strains is to pinpoint the contribution of Dectin-1 and -2/Syk/CARD9 to antigen-specific $CD4^+$ and $CD8^+$ T cell responses to fungal infection.

5.2 Results

5.2.1 Development of transgenic *C. albicans* strains expressing $OVA_{323-339}$ and SIINFEKL peptides derived from Ovalbumin.

Due to the lack of suitable tools to study natural *C. albicans* $CD4^+$ and $CD8^+$ T cell immunodominant epitopes, I sought to generate two model transgenic organisms expressing the peptides from ovalbumin $OVA_{257-264}$ and $OVA_{323-339.}$ $OVA_{257-264}$ is the MHC class I H-$2K^b$-restricted SIINFEKL peptide that can be detected by OT-I transgenic $CD8^+$ T cells and $OVA_{323-339}$ is a MHC class II H-2^b and H-2^d epitope that is recognized by both OT-II and DO11.10 transgenic $CD4^+$ T cells (Robertson et al., 2000).

To generate these new strains, we based our strategy in a previously described strain of *C. albicans* expressing a yeast-enhanced version of GFP (Barelle et al., 2004). This strain was generated by transformation of the *C. albicans* strain CAI4 with the vector pACT1-GFP. CAI4 is a ura3$^-$ derivative of the strain Ca5314 generated by targeted mutagenesis, in which the coding region of the URA3 gene was replaced with a 3kb fragment of the *imm4* region of λgt10 (*ura3*Δ::*imm434/ ura3*Δ::*imm434*) (Fonzi and Irwin, 1993). URA3 encodes the enzyme orotidine-5'-phosphate (OMT) decarboxylase, which is involved biosynthesis of pyrimidine ribonucleotides by converting OMT into uridine monophosphate (Flynn and Reece, 1999). Thus, CAI4 is an auxotrophic strain that requires medium supplemented with uridine for appropriate growth.

The vector pACT1-GFP is derived from the integrative vector CIp10 (Candida Integrating plasmid 10)(Murad et al., 2000) and contains the marker CaURA3, the ribosomal coding protein 10 region (RPS10) that provides homology to target chromosomal integration, and a codon-optimized yeast enhanced GFP (Cormack et al., 1997) driven by the actin (ACT1) promoter region (Barelle et al., 2004).

A schematic representation of the pACT1-GFP vector is illustrated in figure 5.1a.

The pACT1-GFP vector (kindly provided from Dr Alistair Brown, University of Aberdeen, Aberdeen UK) was used as a template to generate the vectors pACT1-GFP-$OVA_{323-339}$ and pACT1-GFP-SIINFEKL.

The gene fusions GFP-$OVA_{323-339}$ and GFP-SIINFEKL were generated by PCR using a sense oligonucleotide that binds upstream of the GFP coding region and antisense oligonucleotide that contain the full coding sequence of $OVA_{323-339}$ or SIINFEKL peptides respectively (for sequence details refer to materials and methods). During the oligonucleotide design, I selected those codons that are preferentially used by *C. albicans* (Brown et al., 1991) and avoided the use of CTG codons, as they are translated into serine instead of leucine by the organism (Santos and Tuite, 1995). The gene fusions GFP-$OVA_{323-339}$ and GFP-SIINFEKL were cloned as HindIII/NheI fragments and placed in place of GFP in the plasmid pACT1-GFP to generate pACT1-GFP-$OVA_{323-339}$ and pACT1-GFP-SIINFEKL (Figure 5.1a). The integrity of the newly

generated vectors was assessed by digestion with restriction enzymes and by DNA sequencing.

The vectors described above were linearised by digestion with the blunt endonuclease StuI and used to transform the strain CAI4 (kindly provided from Dr Alistair Brown, University of Aberdeen, Aberdeen UK) using the lithium acetate protocol. Uridine prototrophs were selected on minimal synthetic medium (full details are described in materials and methods section).

As pACT1-GFP is a CIp10 derivative, genomic integration occurs at the RPS10 locus. Proper genomic integration was assessed by genomic PCR and is illustrated in Figure 5.1b. As previously described for *C. albicans* expressing pACT1-GFP (Barelle et al., 2004), pACT1-GFP-SIINFEKL and pACT1-GFP-$OVA_{323-339}$ -containing cells have integrated the plasmid within the RPS10 region as demonstrated by the presence of an amplification product. A second PCR reaction designed to detect tandem integrated plasmids exclusively (Barelle et al., 2004) was carried out with no amplification product detected (data no shown), indicating that the transformants integrated a single copy of the plasmid.

The expression of GFP in the newly generated strains was assessed by western blot (Figure 5.1c). Cells expressing the $OVA_{323-339}$ or SIINFEKL peptides fused to GFP expressed similar levels of the protein as to compared to *C. albicans* pACT1-GFP.

The activity of these GFP fusions was assessed by flow cytometry and fluorescence microscopy and illustrated in figure 5.1d-e. After fixation with PFA, *C. albicans* expressing GFP, GFP-$OVA_{323-339}$, and GFP-SIINFEKL expressed higher levels of GFP fluorescence than the parental strain CAI4 as assessed by FACS (Figure 5.1d). Fluorescence microscopy of live organisms also indicates strong GFP fluorescence in all transgenic strains, although the fluorescence level produced by the GFP-SIINFEKL strain was slightly lower than the two counterparts (Fig 5.1 d-e). Nonetheless the three strains emitted a positive fluorescence for GFP.

Altogether, these results indicated that two new strains of *C. albicans* expressing peptides for MHC-I and MHC-II presentation have been successfully generated and were ready to be tested in assays containing antigen-specific T cells.

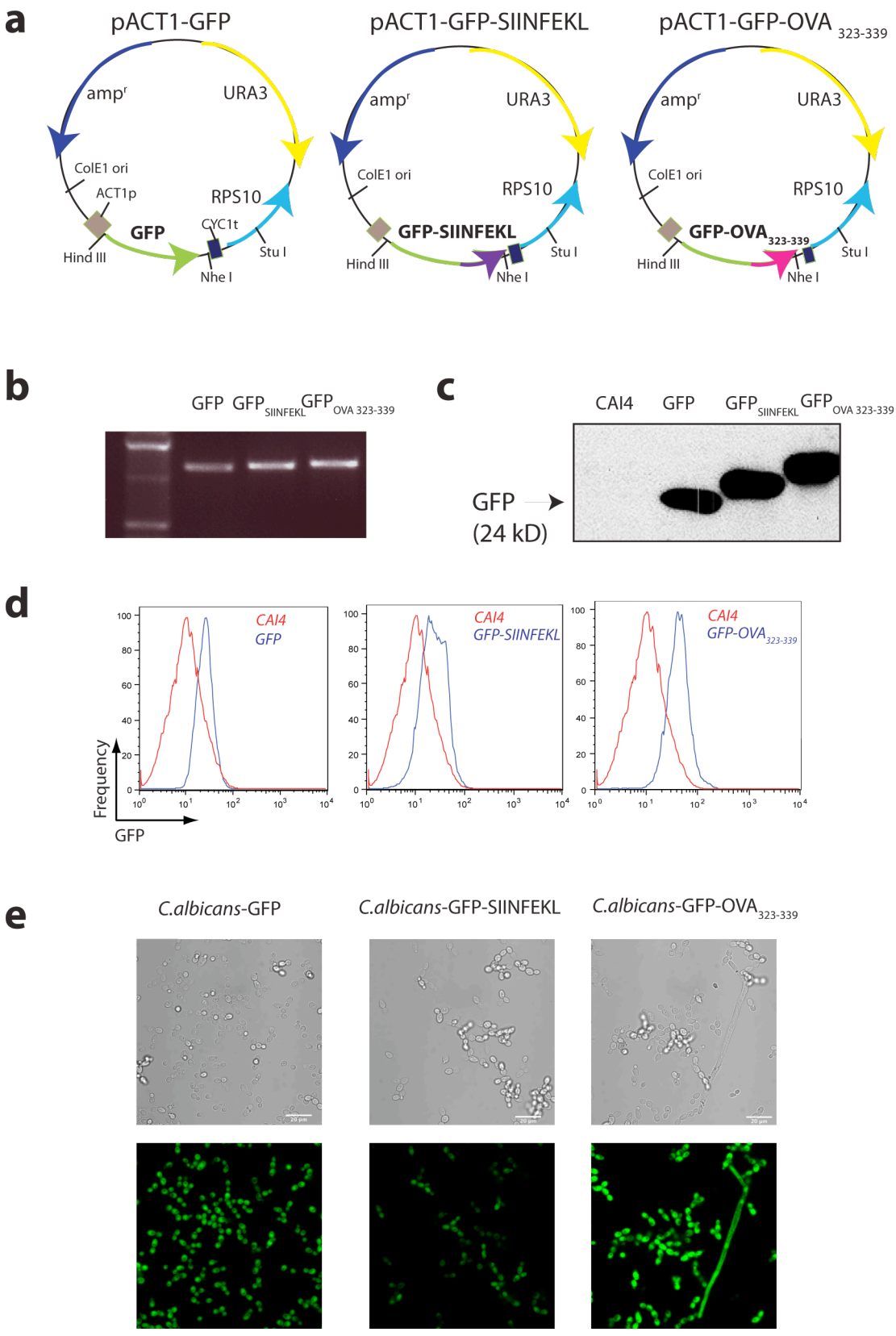


Figure 5-1: Generation of *Candida albicans* strains expressing $OVA_{323-339}$ and SIINFEKL peptides.

(**a**) Schematic representation of pACT1-GFP, pACT1-GFP-SIINFEKL and pACT1-GFP-$OVA_{323-339}$. pACT1-GFP is a CIp10 derivative that contains the *C. albicans URA3* transformation marker. Upon digestion with the restriction enzyme Stu I, pACT1-GFP integrates at the *C. albicans* locus RPS10. The gene fusions GFP-SIINFEKL or GFP-$OVA_{323-339}$ were generated by PCR and cloned as a HindIII/NheI fragment in place of GFP. (**b**) Plasmid integration at the RPS10 locus was confirmed by PCR using the oligonucleotides described in (Barelle et al., 2004). (**c**) GFP expression in *C. albicans* strains was confirmed by western blot using an anti-GFP polyclonal antibody. (**d**) FACS analysis of *C. albicans* strains carrying various GFP constructs. Prior to acquisition, cells were fixed in 4%PFA. (**e**) Microscopic analysis of live *C. albicans* strains carrying pACT1-GFP, pACT1-GFP-SIINFEKL and pACT1-GFP-$OVA_{323-339}$ constructs. Scale bar represents 20μm.

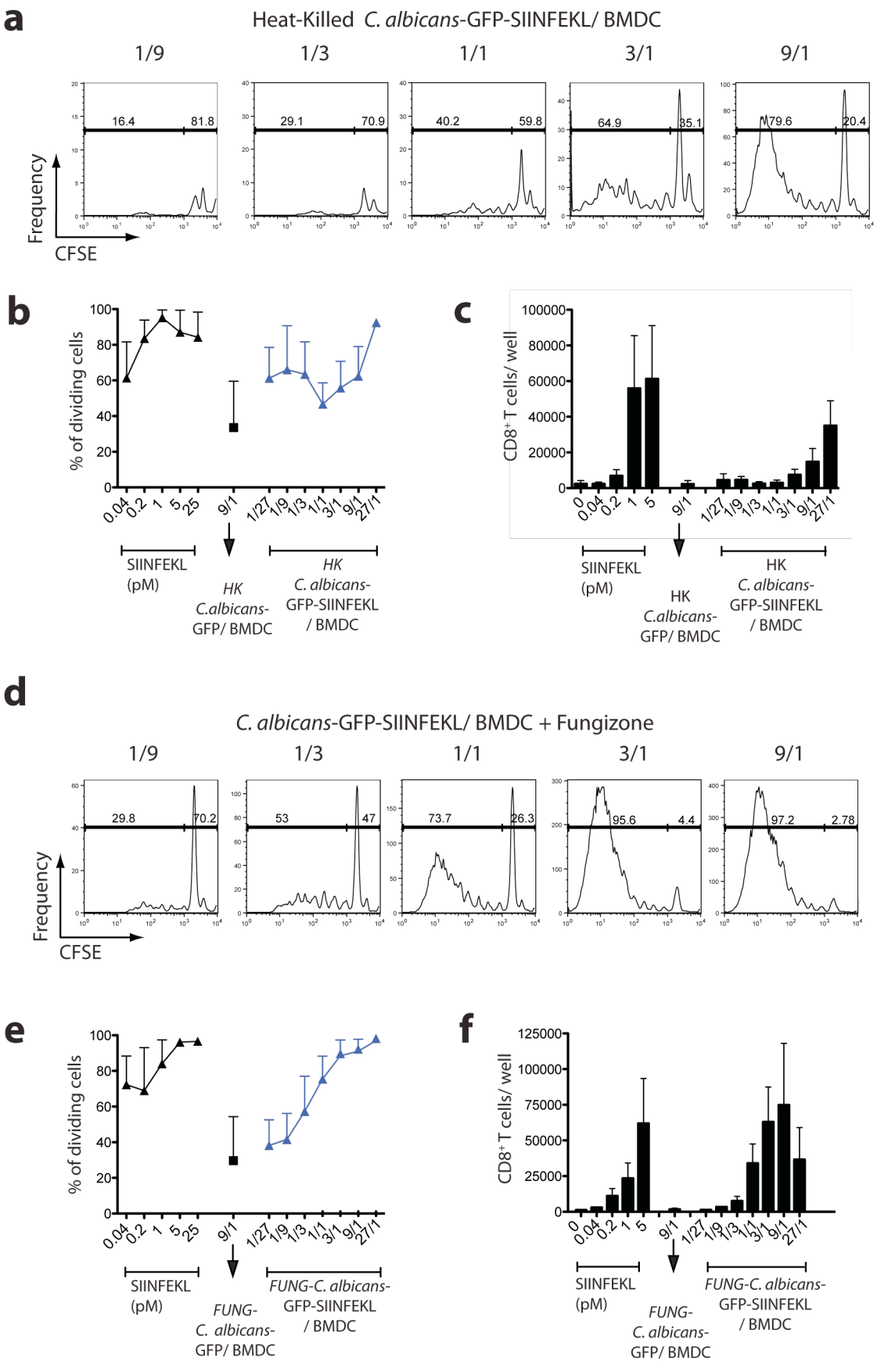
5.2.2 C. albicans expressing GFP-SIINFEKL is a useful tool to measure proliferation and priming of OT-I $CD8^+$ T cells *in vitro*

To test whether BMDCs process and present antigens derived from SIINFEKL-expressing *C. albicans* to antigen-specific $CD8^+$ T cells, I carried out a T cell proliferation assay in vitro. BMDCs were incubated two hours with various doses of heat-killed *C. albicans* GFP-SIINFEKL, *C. albicans* GFP, or SIINFEKL peptide followed by the addition of CFSE-labelled OT-I T cells. $CD8^+$ T cell proliferation was used to measure antigen processing and presentation. Results are shown in figure 5.2 a-c. BMDCs stimulated with heat-killed *Candida* expressing SIINFEKL induced proliferation of OT-I cells, albeit at low levels and only in wells containing high doses of the organism (Fig 5.2a). This effect was antigen-specific, as it was not observed in wells containing *C. albicans* GFP (Fig 5.2 b-c). In contrast, maximal OT-I proliferation and expansion was observed in wells containing high doses of SIINFEKL peptide (Fig 5.2 b-c). These results indicate that GFP-SIINFEKL associated with heat-killed *C. albicans* is not a potent inducer of $CD8^+$ T cell proliferation.

To determine whether processing of live organisms leads to strong $CD8^+$ T cell responses, BMDCs were exposed to live *C. albicans* GFP-SIINFEKL for 2 hours prior to the addition of fungizone and CFSE-labelled OT-I T cells. In contrast to cultures containing heat-killed organisms, BMDCs activated with SIINFEKL-expressing *Candida* plus fungizone triggered strong proliferation of OT-I T cells (Figure 5.2 d-e). This was observed at doses as low as 0.33 *Candida* per BMDC and was accompanied by great expansion of $CD8^+$ T cells (Fig 5.2 e-f). As a result, high levels of IFN-γ were detected in cultures containing live organisms (Fig 5.2 g-h). The remarkable differences

between heat-killed and live organisms as source of antigen for OT-I proliferation and intracellular IFN-γ are illustrated in figure 5.2 i.

Altogether these results indicate that antigens derived from live *C. albicans* are efficiently presented by BMDCs to $CD8^+$ T cells *in vitro*.


a
Heat-Killed C. albicans-GFP-SIINFEKL/ BMDC
1/9
1/3
1/1
3/1
9/1
16.4
81.8
29.1
70.9
40.2
59.8
64.9
35.1
79.6
20.4
Frequency
CFSE
b
% of dividing cells
SIINFEKL (pM)
HK C.albicans-GFP/ BMDC
HK C. albicans-GFP-SIINFEKL / BMDC
c
CD8+ T cells/ well
SIINFEKL (pM)
HK C.albicans-GFP/ BMDC
HK C. albicans-GFP-SIINFEKL / BMDC
d
C. albicans-GFP-SIINFEKL/ BMDC + Fungizone
1/9
1/3
1/1
3/1
9/1
29.8
70.2
53
47
73.7
26.3
95.6
4.4
97.2
2.78
Frequency
CFSE
e
% of dividing cells
SIINFEKL (pM)
FUNG-C. albicans-GFP/ BMDC
FUNG-C. albicans-GFP-SIINFEKL / BMDC
f
CD8+ T cells/ well
SIINFEKL (pM)
FUNG-C. albicans-GFP/ BMDC
FUNG-C. albicans-GFP-SIINFEKL / BMDC

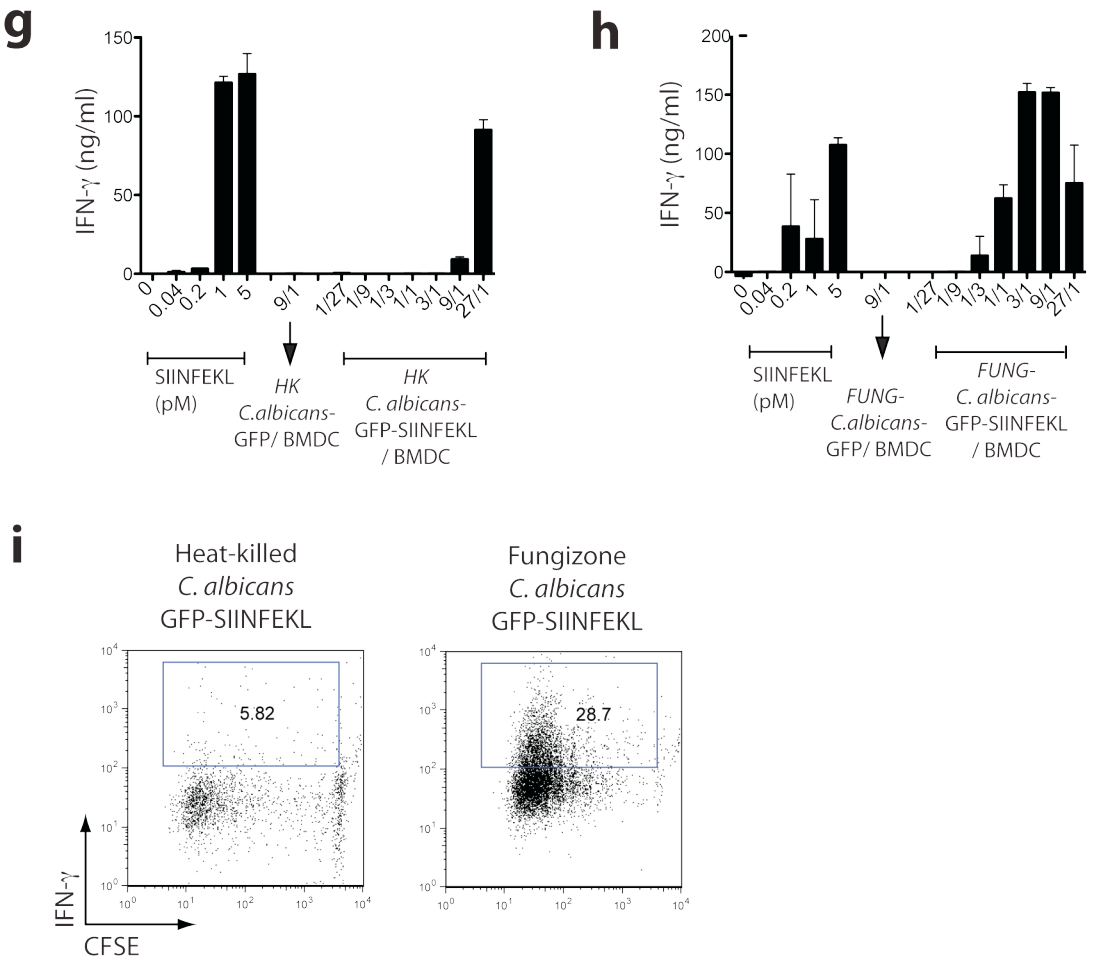


Figure 5-2: DCs pulsed with *C. albicans* GFP-SIINFEKL stimulate CD8$^+$ OT-I T cell proliferation in vitro.
(**a**) 1x10^4 wild-type C57BL/6 BMDCs were stimulated for 2 hrs with various doses of heat-killed *C. albicans* GFP-SIINFEKL and cultured with 5x10^4 CFSE-labeled OT-I T cells. Histograms show CFSE dilution on day 5 of culture. (**b**) BMDCs as in (**a**) were stimulated for 2 hrs with various doses of heat-killed *C. albicans* GFP-SIINFEKL, *C. albicans* GFP or SIINFEKL peptide and cultured with 5x10^4 CFSE-labeled OT-I T cells. Graph shows the percentage of dividing CD8$^+$ T cells at day 5. (**c**) CD8$^+$ T cell counts per well from the experiment shown in (**b**). (**d**) BMDCs as in (**a**) but stimulated with live *C. albicans* GFP-SIINFEKL for 2 hrs prior to the addition of fungizone. Then cells were cultured with 5x10^4 CFSE-labeled OT-I T cells. Histograms show CFSE dilution on day 5 of culture. (**e**) Percentage of dividing CD8$^+$ T cells obtained on day 5 from cultures containing *C. albicans* GFP-SIINFEKL, *C. albicans* GFP or various doses of SIINFEKL peptide in presence of fungizone. (**f**) CD8$^+$ T cell counts per well from the experiment shown in (**e**). (**g**) Cells as in (**b**) were restimulated on day 5 for 48 hr with coated α-CD3 (5μg/ml) and IFN-γ production was determined by sandwich ELISA. (**h**) Cells as in (**e**) were restimulated as in (**g**). (**i**) CD8$^+$ T cells from cultures containing heat killed or live *C. albicans* GFP-SIINFEKL in a ratio 9/1 (*Candida*/BMDC) were restimulated with PMA, ionomycin and BFA and the presence of IFN-γ was analyzed by flow cytometry. Data in (**g**) and (**h**) are mean + SD of duplicate wells. Data in (**b**), (**c**), (**e**) and (**f**) are mean + SEM of three independent experiments. Data are representative of three independent experiments.

5.2.3 Antigens derived from *C. albicans* GFP-SIINFEKL are presented to OT-I $CD8^+$ T cells during the course of systemic infection.

To determine whether systemic infection with *C. albicans* leads to proliferation and priming of $CD8^+$ T cells, C57BL/6 mice that had received CFSE-labelled OT-I T cells were subsequently infected with *C. albicans* GFP-SIINFEKL or *C. albicans* GFP intravenously. Spleen, renal lymph node (RLN) and kidney were harvested 5 days after infection and OT-I T cell proliferation was measured. OT-I T cells were found to proliferate extensively in RLN of mice infected with *Candida* expressing SIINFEKL (Figure 5.3 a). Highly proliferating cells were detected in the spleen and kidney as expected, albeit at lower levels than in RLN. To analyse whether proliferating OT-I cells have become IFN-γ producers after infection, spleen and RLN were taken and were pulsed with 1μM of SIINFEKL peptide *ex vivo* in presence of brefeldin A. The production of intracellular IFN-γ was analyzed by FACS. Notably, OT-I T cells that proliferate in response to systemic infection with *C. albicans* GFP-SIINFEKL produce large amounts of IFN-γ (Figure 5.3b). Overall these results show that *C. albicans* expressing GFP-SIINFEKL is a useful tool to track antigen-specific OT-I T cells during the course of systemic infection.

This *C. albicans* strain could also be used to monitor endogenous SIINFEKL-specific $CD8^+$ T cells by using a H-$2K^b$ SIINFEKL tetramer.

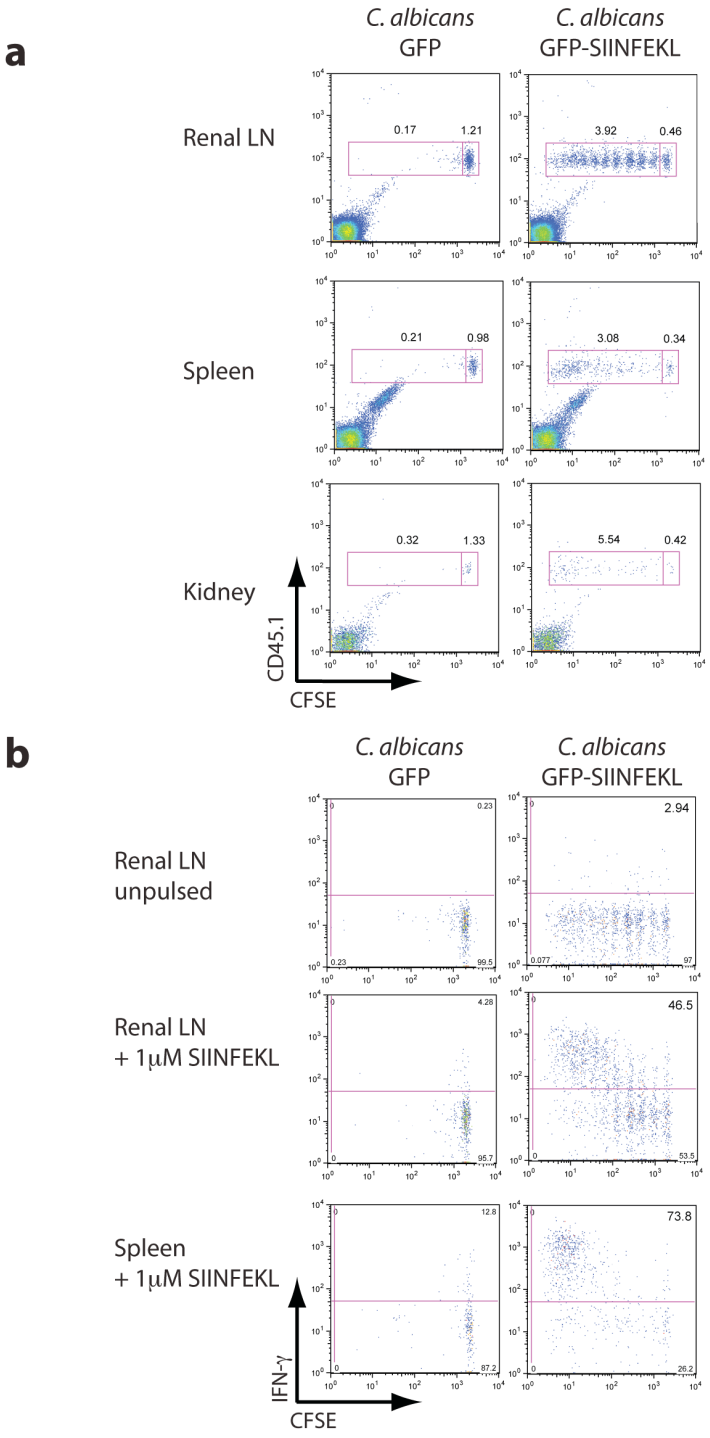


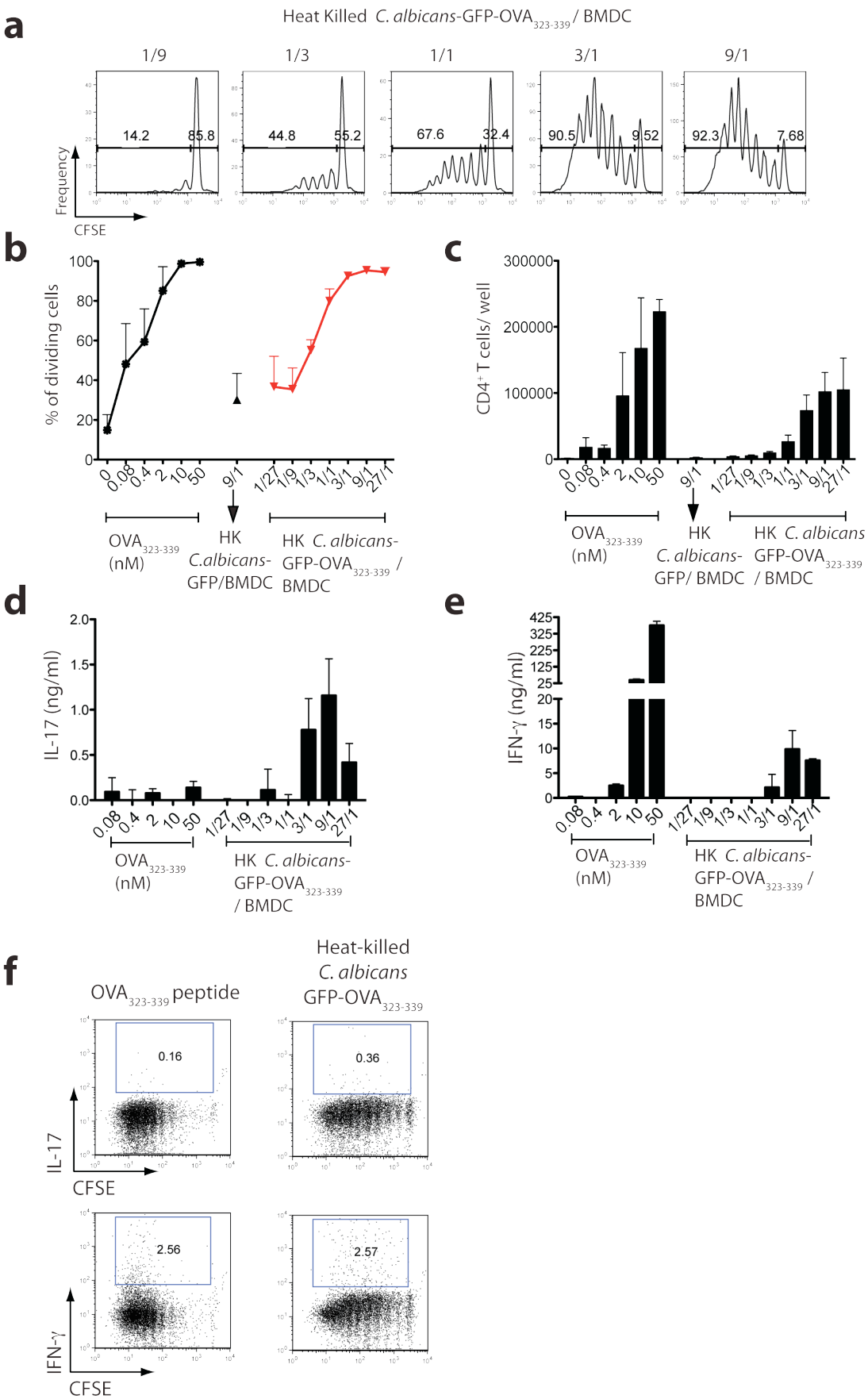
Figure 5-3: Infection with *C. albicans* GFP-SIINFEKL leads to proliferation and priming of CD8$^+$ OT-I T cells.
(**a**) 1x10^6 CFSE-labelled OT-I CD45.1 T cells were transferred i.v. into Wild-type C57BL/6 (WT) mice. One day later, mice were infected intravenously with 5x10^4 *C. albicans* GFP or *C. albicans* GFP-SIINFEKL. On day 5 after infection, OT-I cells from spleen, renal LN and kidney analyzed by FACS. Graphs show CFSE dilution versus CD45.1 in CD8$^+$ population. (**b**) Cells from renal LN and spleen shown in (**a**) were pulsed for 3 hrs with medium alone or 1μM SIINFEKL in the presence of BFA. Graphs show CFSE dilution versus intracellular IFN-γ.

5.2.4 Antigens derived from heat-killed *C. albicans* GFP-OVA$_{323-339}$ are efficiently presented to OT-II CD4$^+$ T cells *in vitro*

I sought to determine the ability of BMDC to present antigens derived from *C. albicans* GFP-OVA$_{323-339}$ to OT-II CD4$^+$ T cells. As previously shown for OT-I T cells, BMDCs were exposed for 2 hrs to various doses of heat-killed *C. albicans* GFP-OVA$_{323-339,}$ following by the addition of CFSE-labelled naïve OT-II T cells. Results are illustrated in figure 5.4 a-c. BMDCs that had phagocytosed heat killed OVA$_{323-339}$ –containing fungi induce strong proliferation and expansion of OT-II T cells as depicted in the CFSE profiles and cell numbers obtained at the end of the culture. This was accompanied by production of IL-17 and IFN-γ after restimulation with α-CD3 for 48hr (Figure 5.4 d-e). Is important to note that the levels of cytokine are modest, as low levels of IL-17 and IFN-γ are detected at on a per cell basis (Figure 5.4 f). In addition, no IL-4 was detected on cultures containing the fungus (data not shown). These results indicate that processing of antigens associated to heat-killed *C. albicans* by BMDCs leads to proliferation and priming of naïve CD4$^+$ T cells *in vitro*.

In contrast to cultures containing heat-killed organisms, BMDCs stimulated with live *Candida*-OVA$_{323-339}$ plus fungizone induced CD4$^+$ T cell proliferation and but failed to induce T cell expansion (Figure 5.4 g-i). This effect is not associated with fungizone toxicity as control wells containing OT-II T cells, BMDCs and OVA peptide were not affected by addition of the antifungal agent (Figure 5.4 i, j). As a consequence, no effector cytokines are detected by ELISA (data not shown) or intracellular staining (Figure 5.4 j).

These results indicate that BMDCs stimulated with live *C. albicans* expressing a cytosolic antigen are not competent to promote CD4$^+$T cell expansion and priming in vitro.


a
Heat Killed C. albicans-GFP-OVA323-339 / BMDC
1/9
1/3
1/1
3/1
9/1
14.2
85.8
44.8
55.2
67.6
32.4
90.5
9.52
92.3
7.68
Frequency
CFSE
b
% of dividing cells
0
0.08
0.4
2
10
50
9/1
1/27
1/9
1/3
1/1
3/1
9/1
27/1
OVA323-339 (nM)
HK C.albicans-GFP/BMDC
HK C. albicans-GFP-OVA323-339 / BMDC
c
CD4+ T cells/ well
HK C. albicans-GFP/ BMDC
HK C. albicans GFP-OVA323-339 / BMDC
d
IL-17 (ng/ml)
HK C. albicans-GFP-OVA323-339 / BMDC
e
IFN-γ (ng/ml)
HK C. albicans-GFP-OVA323-339 / BMDC
f
OVA323-339 peptide
Heat-killed C. albicans GFP-OVA323-339
0.16
0.36
IL-17
CFSE
2.56
2.57
IFN-γ
CFSE

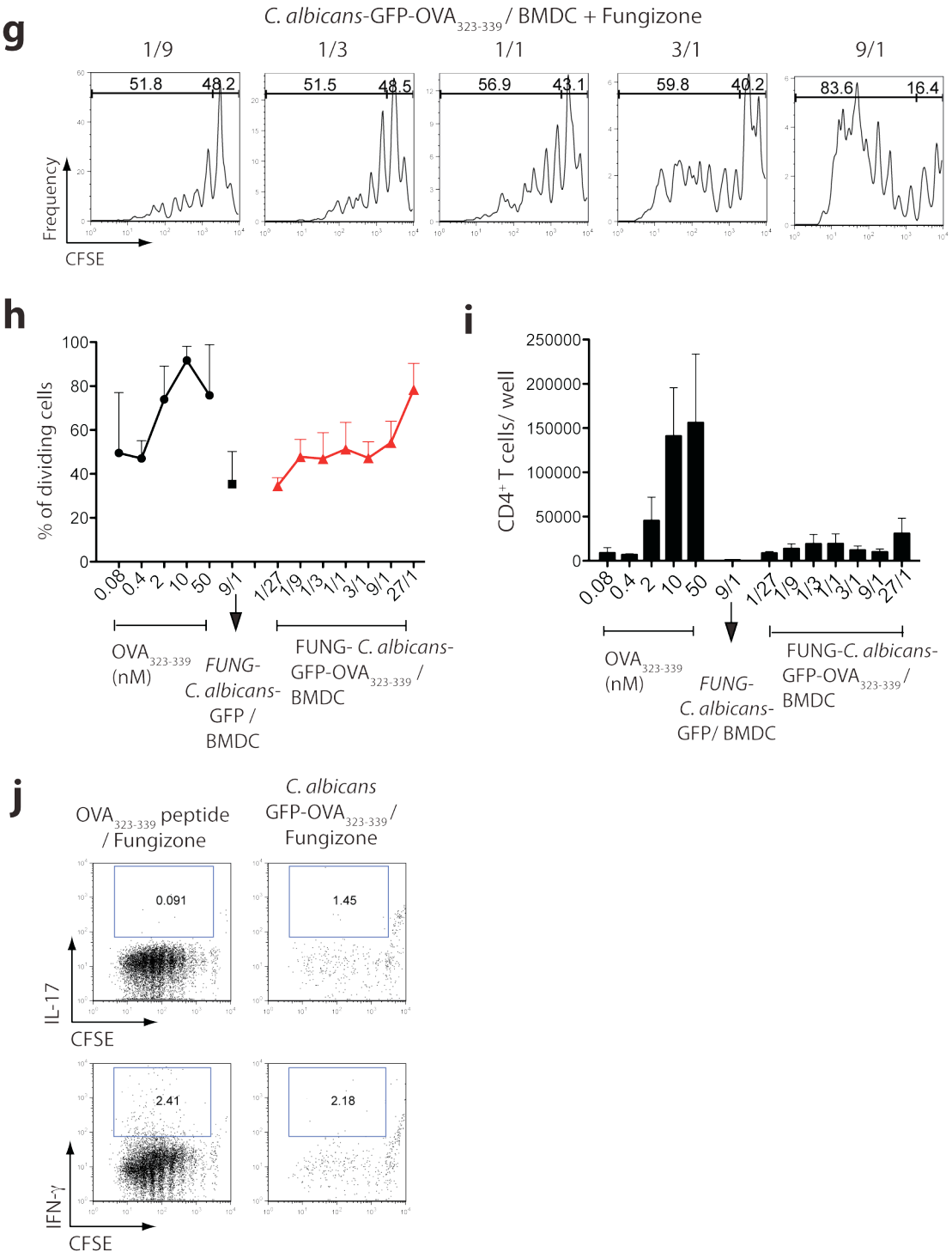


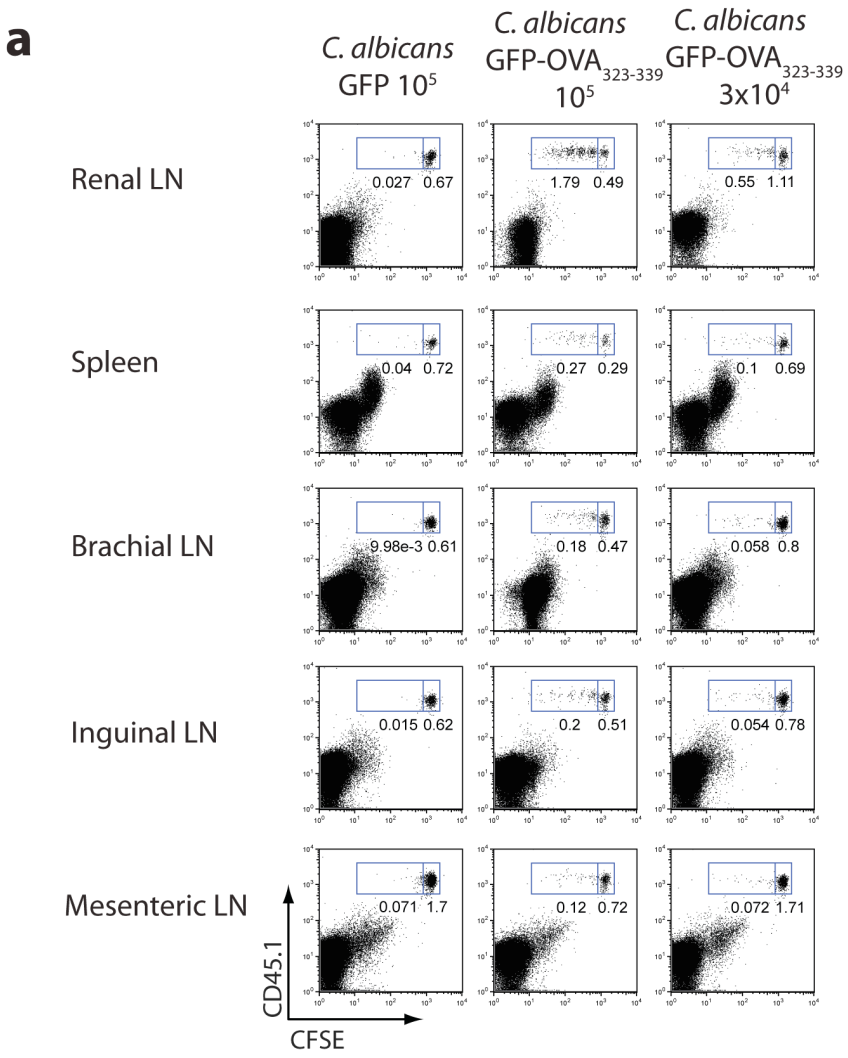
Figure 5-4: DCs stimulated with heat-killed *C. albicans* GFP-OVA323-339 induce priming of CD4$^+$ OT-II T cells in vitro.
(**a**) 1x10^4 wild-type C57BL/6 BMDCs were stimulated for 2 hrs with various doses of heat-killed *C. albicans* GFP-OVA $_{323-339}$ and cultured with 5x10^4 CFSE-labeled OT-II T cells. Histograms show CFSE dilution on day 5 of culture. (**b**) BMDCs as in (**a**) were stimulated for 2 hrs with various doses of heat-killed *C. albicans* GFP-OVA $_{323-339}$, *C. albicans* GFP or OVA $_{323-339}$ peptide and cultured with 5x10^4 CFSE-labelled OT-II T cells. Graph shows the percentage of dividing CD4$^+$ T cells at day 5. (**c**) CD4$^+$ T cell counts per well from the experiment shown in (**b**). Cells as in (**a**) were restimulated on day 5 for 48

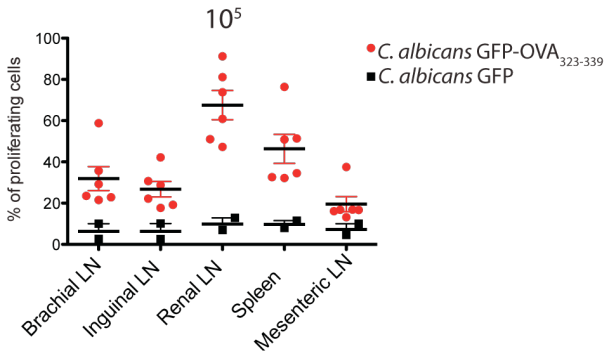
hr with coated α-CD3 (5μg/ml) and the production IFN-γ (**d**), and IL-17 (**e**), was determined by sandwich ELISA. (**f**) Cells as in (**b**) were restimulated on day 5 with PMA, Ionomycin and BFA and the presence of intracellular IFN-γ and IL-17 was analyzed by flow cytometry.
(**g**) BMDCs as in (**a**) but stimulated with live *C. albicans* GFP-OVA $_{323-339}$ for 2 hrs prior to the addition of fungizone. Then $5x10^4$ CFSE-labeled OT-II T cells were added to the cultures. Histograms show CFSE dilution on day 5 of culture. (**h**) Percentage of dividing $CD4^+$ T cells obtained on day 5 from cultures containing various doses of *C. albicans* GFP-OVA $_{323-339}$, *C. albicans* GFP or OVA $_{323-339}$ peptide in presence of fungizone. (**i**) $CD4^+$ OT-II T cell counts per well from the experiment shown in (**h**). (**i**) Cells as in (**g**) were restimulated on day 5 with PMA, Ionomycin and BFA and the presence of intracellular IFN-γ and IL-17 was analyzed by flow cytometry. Data in (**d**) and (**e**) are mean + SD of duplicate wells. Data in (**b**), (**c**), (**h**) and (**i**) are mean + SEM of three independent experiments. Data are representative of three independent experiments.

5.2.5 OT-II T cells proliferate primarily in the renal lymph node of *C. albicans* infected mice.

To track antigen specific $CD4^+$ T cells during the course of *C. albicans* systemic infection, CFSE-labelled naïve OT-II T cells were transferred into C57BL/6 mice that were subsequently infected with *C. albicans* GFP-OVA$_{323-339}$ or *C. albicans* GFP. On day 5 after infection, spleen, renal, brachial, inguinal and mesenteric lymph nodes were collected and OT-II $CD4^+$ T cell proliferation was assessed by FACS. In response to infection with *Candida*-expressing OVA$_{323-339}$, a small but noticeable population of proliferating OT-II T cells can be detected in spleen, brachial, inguinal and mesenteric lymph nodes (Figure 5.5.a). Notably, a high proportion of proliferating T cells accumulate primarily on the renal lymph node of infected mice (Figure 5.5 a) indicating the kidney-draining lymph node is the primary site for T cell priming to *Candida*-associated antigens. This effect is dependent on the infection dose, as the percentage of dividing cells decreases when infecting with a lower dose of the pathogen (Fig 5.5 b-c).

This experiment demonstrates that antigens derived from *C. albicans* are presented to $CD4^+$ T cells *in vivo*. Furthermore, this strain of *Candida* allowed the identification of the renal lymph node as the primary site of antigen presentation during the course of a systemic infection.


a
C. albicans GFP 10^5
C. albicans GFP-OVA323-339 10^5
C. albicans GFP-OVA323-339 3x10^4
Renal LN
Spleen
Brachial LN
Inguinal LN
Mesenteric LN
0.027 0.67
1.79 0.49
0.55 1.11
0.04 0.72
0.27 0.29
0.1 0.69
9.98e-3 0.61
0.18 0.47
0.058 0.8
0.015 0.62
0.2 0.51
0.054 0.78
0.071 1.7
0.12 0.72
0.072 1.71
CD45.1
CFSE


b
10^5
C. albicans GFP-OVA323-339
C. albicans GFP
% of proliferating cells
100
80
60
40
20
0
Brachial LN
Inguinal LN
Renal LN
Spleen
Mesenteric LN

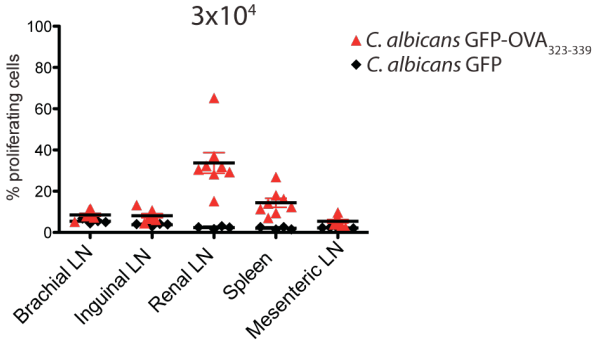

c
3x10^4
C. albicans GFP-OVA323-339
C. albicans GFP
% proliferating cells
100
80
60
40
20
0
Brachial LN
Inguinal LN
Renal LN
Spleen
Mesenteric LN

Figure 5-5: OT-II T cells accumulate primarily in the renal lymph node after systemic infection with *C. albicans* $OVA_{323-339}$.
(**a**) $2x10^6$ CFSE-labelled naïve OT-II $CD45.1^+$ $CD4^+$ T cells were transferred i.v. into wild-type C57BL/6 (WT) mice. One day later, mice were infected intravenously with $1x10^5$ or $3x10^4$ *C. albicans* GFP-$OVA_{323-339}$ or $3x10^4$ *C. albicans* GFP. On day 5 after infection, OT-II T cells were analyzed by FACS. Graphs show CFSE dilution versus CD45.1 gated in the $CD4^+$ population. Percentage of dividing cells in response to infection with $1x10^5$ (**b**) or $3x10^4$ (**c**) *C. albicans*. The data in (**b**) and (**c**) are mean +SEM of two independent experiments and each dot represents one mouse.

5.2.6 The frequency of antigen-specific $CD4^+$ T cells decreases during the course of infection.

To have a better understanding of the kinetics of the $CD4^+$ T cell response during *C. albicans* infections, I carried out a time course experiment. Mice that received naïve OT-II T cells were infected with *C. albicans* GFP-$OVA_{323-339}$ and were sacrificed at day 4, 8 and 12 post infection. Lymph nodes, kidney and spleen from each mouse were processed for flow cytometric comparison. On day 4, $CD4^+CD45.1^+$ double positive OT-II T cells are readily detected in most lymphoid tissues with particular accumulation in the renal lymph node (Fig 5.6 a-b). By day 8, the frequency of OT-II T cells had a remarkable contraction in most organs analyzed (Fig 5.6 a). Notably, whilst proliferating OT-II clearly accumulate in renal lymph nodes by day 4-5 (Fig 5.5), migrating OT-II T cells were never found in kidneys of infected mice on day 4 or 8 (Fig 5.6 b). By day 12, OT-II T cells were almost undetectable (Fig 5.6 a).

To test whether OT-II T cell contraction correlates with clearance of *C. albicans* GFP-$OVA_{323-339}$, fungal colonies were quantified in kidneys from infected mice (Fig 5.6 c). Presence of the pathogen was detected on day 4, 8 and 12 indicating that, despite the continuous presence of the fungal organism, the OT-II T cell response contracts and declines over time.

These results indicate that a weak and transient OT-II T cell response is generated to a strain of *C. albicans* expressing intracellular $OVA_{323-339}$.

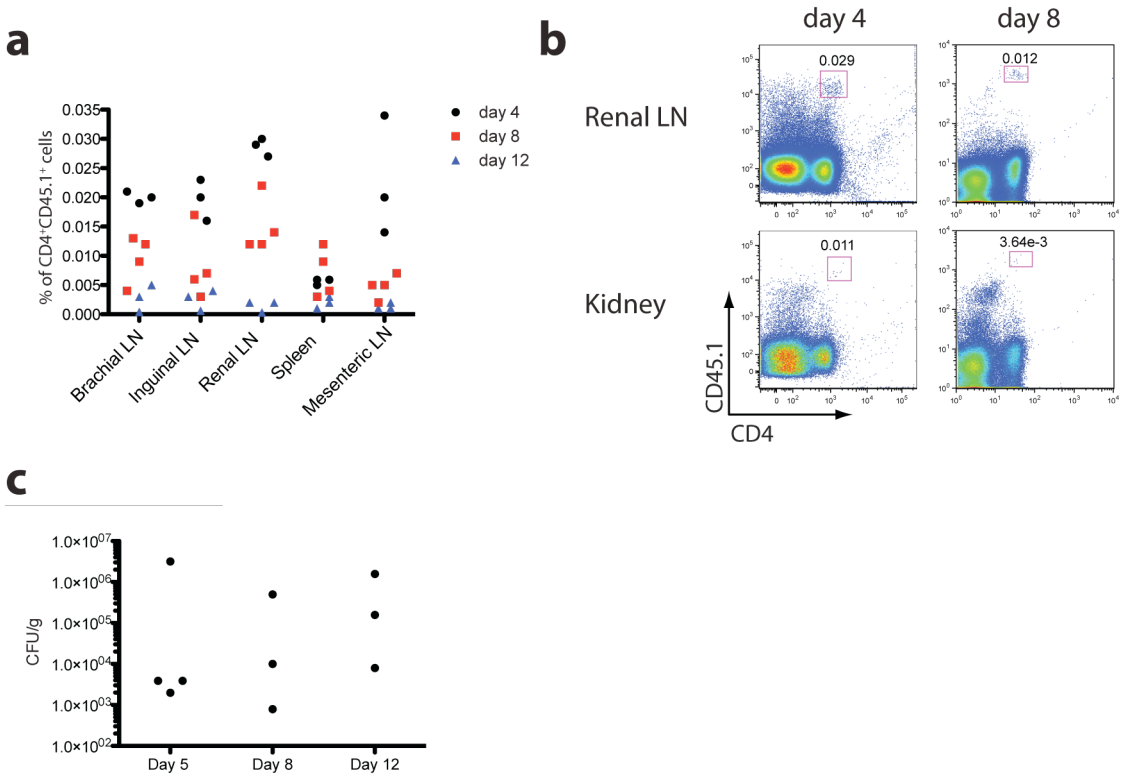


Figure 5-6: Frequency of OT-II CD4$^+$ T cells decline during the course of *C.albicans* GFP-OVA$_{323-339}$ infection.
(**a**) 1.5x10^6 CFSE-labelled naïve OT-II CD45.1$^+$ CD4$^+$ T cells were transferred i.v. into wild-type C57BL/6 (WT) mice. One day later, mice were infected intravenously with 1x10^5 *C. albicans* GFP-OVA $_{323-339}$. The graph shows the percentage of CD4$^+$CD45.1$^+$ OT-II T cells within FSC/SSC gate on day 4, 8 or 12 after infection. Each dot represents one mouse. (**b**) CD4$^+$CD45.1$^+$ OT-II T cells in renal lymph node and kidney on day 4 and 8. (**c**) Fungal burden measured of mice analyzed in (**a**) at different time points.

5.2.7 OT-II T cells do not produce detectable levels of IFN-γ, IL-17 or Foxp3 after systemic infection with *C. albicans* GFP-OVA$_{323-339}$.

Having framed the kinetics of OT-II T cell responses during *Candida* infection, I decided to assess whether antigen-specific CD4$^+$ T cells acquire an effector phenotype. CFSE-labelled OT-II T cells present in renal lymph nodes from infected mice were analyzed at day 5 and cytokine production was measured by FACS after restimulation with PMA/Ionomycin and brefeldin A. Results are shown in figure 5.7. As previously noted (Figure 5.5), dividing OT-II CFSE$^+$ T cells do not undergo more than four division cycles and do not produce detectable levels of IL-17 and IFN-γ as measured by intracellular staining (Figure 5.7a-b). In contrast, endogenous CD4$^+$ CFSE$^-$ T cells from mice infected with *Candida*-GFP or *Candida*- GFP-OVA$_{323-339}$ produce notable levels

of both cytokines with this method of restimulation (Figure 5.7a-b). Furthermore, OT-II T cells do not become regulatory T cells under these experimental conditions as assessed by Foxp3 staining (Figure 5.7 c)

These results indicate that, upon infection with a strain of *C. albicans* expressing an intracellular antigen, antigen specific $CD4^+$ T cells do not proliferate extensively and do not acquire a measurable effector function.

In sum, these results suggest that the tools generated here are not optimal to study the contribution of innate signalling pathways to the coordination of adaptive immunity to *C. albicans.* Improvements to this current approach will be proposed in the discussion section of this chapter.

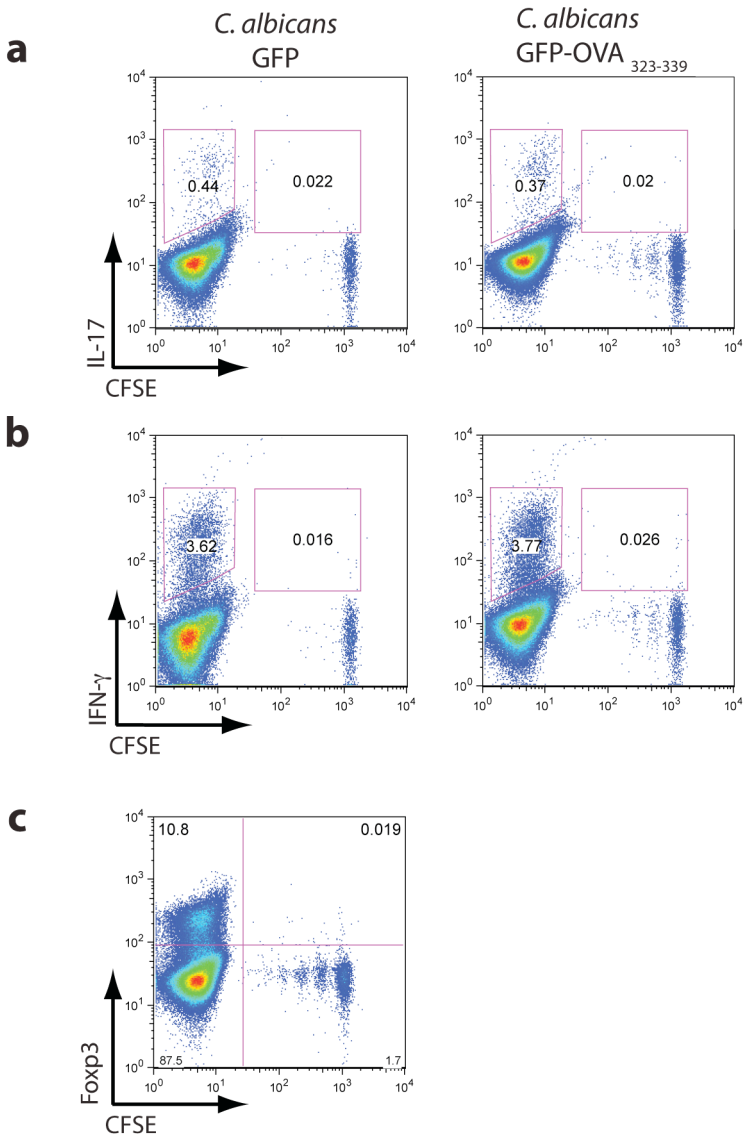


Figure 5-7: OT-II T cells do not express detectable levels of IL-17, IFN-g or Foxp3 after systemic infection with *C. albicans* GFP-OVA$_{323-339}$.
2x10^6 CFSE-labelled naïve OT-II CD45.1$^+$ CD4$^+$ T cells were transferred i.v. into wild-type C57BL/6 (WT) mice. One day later, mice were infected intravenously with 5x10^4 *C. albicans* GFP-OVA $_{323-339}$ or *C. albicans* GFP. On day 5 after infection, OT-II T cells were analyzed by FACS. Graphs show CFSE dilution versus IL-17 (**a**), IFN-γ (**b**) or Foxp3 (**c**), gated in the CD4$^+$ population.

5.3 Discussion

In this chapter, I have generated two transgenic strains of *C. albicans* for the study of $CD4^+$ and $CD8^+$ T cell responses during fungal infections. This is, to my knowledge, the first attempt to study antigen-specific T cell immunity to this organism. Nonetheless, analysis of T cell responses during infection with transgenic strains of *Candida* requires further improvement, as the approach provided in this chapter is not fully effective to study $CD4^+$ T cell dynamics to the organism.

Our strategy was based on the use of the pACT1-GFP integrative vector, in which the actin promoter region drives the expression of intracellular GFP (Barelle et al., 2004). Expression of GFP fused to either SIINFEKL or $OVA_{323-339}$ peptides in the newly generated strains of *C. albicans* was successful as confirmed by PCR, sequencing, western blot, FACS analysis, fluorescence microscopy and T cell proliferation analysis.

5.3.1 C. albicans GFP-SIINFEKL is a potent source of antigen for proliferation and priming of $CD8^+$ T cells *in vitro* and *in vivo*

Although the reason for studying $CD8^+$ T cells during the context of fungal infections may not seem straightforward, activation of Dectin-1 by β-glucans leads to efficient priming of cytotoxic T cell responses (Leibundgut-Landmann et al., 2008) and CD8 deficient mice are highly susceptible to oropharyngeal candidiasis (Conti et al., 2009). Furthermore, antifungal $CD8^+$ T cells to *H. capsulatum* or *C. neoformans* can limit fungal replication and confer protection in absence of $CD4^+$ T cells. These responses are thought to be IFN-γ-mediated (Lin et al., 2005, Lindell et al., 2005). These data suggest that $CD8^+$ T cells might play an important role in the immune control of *C. albicans* infection and future work is needed to elucidate their function.

My results indicate that BMDCs exposed to live *Candida*-SIINFEKL (followed by the addition of fungizone) are more efficient than those exposed to heat-killed organisms to promote OT-I T cell activation. This marked distinction between heat-killed and live organisms in the activation of $CD8^+$ T cells have been previously noted for other pathogens including the parasites *Toxoplasma gondii* and *Leishmania major* (Goldszmid et al., 2009, Bertholet et al., 2005). Although *L. major* and *T. gondii* gain

access to the MHC I pathway by different mechanisms (reviewed in (Goldszmid and Sher, 2010)), infection with live parasites is necessary in both cases or efficient cross-presentation of pathogen-associated antigens (Goldszmid et al., 2009, Bertholet et al., 2005).

In contrast to intracellular protozoan parasites, *C. albicans* has an extracellular life style. Nonetheless, others and myself have observed that DCs are fully competent to phagocytose yeast and establish contacts with hyphae (data not shown and (d'Ostiani et al., 2000)). These observations suggest that whilst the mechanisms of antigen presentation between intracellular parasites and extracellular fungi might seem obviously different, DC interaction with live fungus might also divert the antigenic cargo and favour access to the MHC I pathway. Along these lines, $CD8^+$ T cells were efficiently primed during systemic infection with *Candida*-SIINFEKL (Fig 5.2). Whether functional memory $CD8^+$ T cells arise as consequence of *C. albicans* infection remains an open question that is beyond the scope of this thesis. Nonetheless this strain could be used for future studies of CTL responses to *C. albicans*. Those experiments are not provided in this thesis, as I was more interested in the induction of $CD4^+$ T cell responses to the organism.

5.3.2 *C. albicans* expressing a model antigen expressed in the cytosol does not trigger an efficient $CD4^+$ T cell response

In contrast to the strong $CD8^+$ T cell responses observed against live fungus, processing of heat-killed but not live *C.albicans*-GFP-$OVA_{323-339}$ induced efficient $CD4^+$ proliferation in vitro. These results suggest that antigens derived from dead pathogens might be more accessible to the MHC-II pathway. In favour of this notion, dead *T. gondii* has also been shown to be potent antigenic source for $CD4^+$ T cells (Goldszmid et al., 2009).

Early after systemic infection with *C. albicans*-GFP-$OVA_{323-339}$, $CD4^+$ OT-II T cells accumulate in the renal lymph node. However, they do not proliferate extensively and do not become effector T cells. There may be various reasons that explain why this strain induces poor $CD4^+$ T cell responses. Live organisms may produce factors that inhibit processing and presentation of *Candida*-derived antigens to $CD4^+$ T cells. An

additional possibility is that the cytosolic localisation of $OVA_{323-339}$ is not an efficient source of antigen. It is worth noticing that most transgenic parasites used in studies of $CD4^+$ T cell immunity deliberately express model antigens as membrane-bound or as secreted forms (reviewed in (Goldszmid and Sher, 2010)). In the case of *T. gondii,* only OVA secreted to the parasitophorus vacuole but not a cytosolic OVA form is efficiently presented on MHC II molecules (Pepper et al., 2004). Similar observations have been described for *L. major*: surface localisation of OVA is strictly required to drive an effective OT-II $CD4^+$ response whereas processing of cytosolic antigens is a highly inefficient process (Prickett et al., 2006). Although is well established that most endogenous $CD4^+$ T cells specific for *L. major* recognize a single peptide derived from the cytosolic protein LACK (reviewed in (Sacks and Noben-Trauth, 2002)), is important to note that this peptide is edited by the nonclassical MHC class II molecule DM (Kamala and Nanda, 2009). In addition, for intracellular bacteria such as *Listeria monocytogenes*, peptides derived from the secreted proteins lysteriolysin o and p60 are immunodominants epitopes for $CD4^+$ and $CD8^+$ T cells (Geginat et al., 2001).

Altogether, this evidence indicates that for pathogens-derived antigens, the subcellular localisation might determine the outcome of a T cell response. Proteins located on the cell surface might gain rapid access to the endosomal compartment responsible for antigen processing, facilitating the presentation process.

5.3.3 Revised strategy for the generation of transgenic strains of *C. albicans* to study optimal T cell responses.

At the time of generating the strain of *C. albicans* expressing cytosolic GFP-$OVA_{323-339}$, we were restricted to use the vector pACT1-GFP. Nonetheless, novel approaches for the study of fungal infections have been developed in recent times. In this context, a reporter system designed for imaging *C. albicans* infections has been published recently (Enjalbert et al., 2009). In that system, a codon-optimized version of luciferase was targeted to the *C. albicans* cell surface to favour accessibility to its substrate coelenterazine (Enjalbert et al., 2009). The strategy takes advantage of the PGA59 gene, which encodes the small GPI-linked cell wall protein Pga59 (Moreno-Ruiz et al., 2009). Fungal GPI-anchored proteins are inserted in the outer layer of the cell wall through their glycolipid region. These proteins are characterized by the presence of a signal

sequence that directs the protein into the endoplasmic reticulum, a mature domain, and a C-terminal region involved in GPI-anchoring (Mayor and Riezman, 2004). The GPI anchor of Pga59 is covalently linked to β-(1,6)-glucans present in the cell wall of *C. albicans* and the protein is evenly distributed in the cell surface of yeast and hyphae (Moreno-Ruiz et al., 2009). Due to its small size (113 aminoacids), Pga59 is a protein that can be easily manipulated. In the work reported by Enjalbert et al, the luciferase gene (without the stop codon) was cloned within the Pga59 gene, between the sequence encoding for the second and third aminoacid of the mature Pga59 (Enjalbert et al., 2009). Therefore, the luciferase gene was placed in frame downstream of the Pga59 signal sequence and upstream of the Pga59 GPI-containing region (Enjalbert et al., 2009). This gene fusion was subsequently cloned in the vector CIp10, downstream the pACT1 promoter region to allow efficient expression. This was done following the same cloning strategy to that I provided in this chapter. This reporter strain express high levels of luciferase at the cell wall and has proven to be a highly convenient and sensitive tool for *in vivo* imaging of *C. albicans* infections (Enjalbert et al., 2009).

To circumvent the limitations of my current approach and to direct antigens to the cell wall, I propose to clone the gene fusions GFP- $OVA_{323-339}$ and GFP-SIINFEKL downstream of the Pga59 signal sequence following the same strategy provided by Enjalbert et al (Enjalbert et al., 2009) (Figure 5.8). The constructs Pga59-GFP-$OVA_{323-339}$ and Pga59-GFP-SIINFEKL can be subsequently cloned as HindIII/NheI fragments in pACT1-GFP vector in place of GFP. This will allow high expression of the gene fusions.

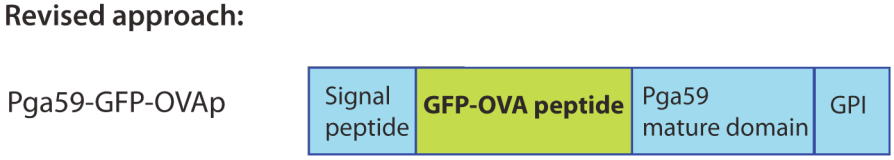


Figure 5-8: Revised strategy for the generation of *C. albicans* strains expressing OVA peptides targeted to the cell surface.
Similar to the strategy provided in (Enjalbert et al., 2009), the gene fusions GFP- $OVA_{323-339}$ and GFP-SIINFEKL (without the stop codon) can be cloned in frame within the PGA59 gene between the sequence encoding the aminoacids 2 and 3 of the mature Pga59 protein. As the GFP gene fusions lack the stop codon, the resulting Pga59-GFP-OVA peptide construct will contain the signal peptide, the Pga59 mature domain, and the GPI-anchor region.

I hypothesize that the OVA peptides derived from *Candida* strains expressing Pga59-GFP-$OVA_{323-339}$ and Pga59-GFP-SIINFEKL would be efficiently presented to antigen-specific T cells during infection. In histopathological sections of *C. albicans* infected mice, all *C. albicans* forms (yeast, pseudohyphae and hyphae) can be detected (Romani, 2004), albeit the hyphae form is predominant in my experiments (unpublished observations). Therefore, DCs might process material derived from both forms for antigen presentation to T cells. In this scenario, the fact that Pga59 is expressed at the cell wall of yeast and hyphae might favour the presentation of *C. albicans* derived antigens during infections.

Altogether, this revised approach may substantially facilitate the study of T cell responses to the *C. albicans* and eventually, the contribution of Syk/CARD9 signalling pathway to the coordination and priming of adaptive immunity to the organism.

Chapter 6. Final discussion and future perspectives.

Results presented in this thesis unveiled the mechanisms by which Dectin-1-activated DCs initiate a type 17 response. The purified agonist curdlan has proven to be a very useful reagent to delineate the contribution of Dectin-1 in isolation to the coordination of adaptive immunity. Having defined the requirements for the development of IL-17-producing cells in response to Dectin-1 ligation in DCs (Chapter 3), we decided to investigate the contribution of additional Syk-coupled CLRs to Th17 responses during fungal infections. To address this question in such a complex scenario, it was first required the identification of an additional Syk-coupled PRR that accounted for the Syk dependent but Dectin-1 independent aspects of fungal recognition by DCs. Results presented in this thesis in collaboration with those generated by Dr Matthew Robinson revealed that Dectin-2 is the additional Syk-coupled PRR that fulfils this criteria. We found that Dectin-2 is not redundant for the initiation of anti-fungal Th17 responses during systemic *C. albicans* infection. Finally, this thesis presents a strategy to study antigen-specific T cell responses to *C. albicans.* Although this plan requires further refinement, these tools will provide invaluable information regarding priming and trafficking of antigen-specific T cells to the site of infection. In this section, I will discuss some of the questions that remain open and will provide my views about the future perspectives in the field.

6.1 Dectin-1/Syk signalling in DCs couples to the induction of IL-17-producing $CD4^+$ T cells.

Dectin-1, the archetypal CLR that signals via Syk and CARD9 for gene induction and cytokine production in DCs (LeibundGut-Landmann et al., 2007), induces IL-17-producing $CD4^+$ T cells in an IL-23 dependent mechanism (Chapter 3, Fig 3.2 and 3.6). β-glucan stimulation leads to the production of large amounts of IL-23 and decreased amounts of IL-12 by DCs (LeibundGut-Landmann et al., 2007, Gerosa et al., 2008). This differential regulation of IL-23 over IL-12 is observed in stimulations with Dectin-1 and TLR2 agonists and contributes to the coordination of adaptive immunity (Gerosa et al., 2008).

Although Dectin-1 ligation leads to the induction of a small fraction of Th17 cells differentiated from naïve precursors (Figure 3.3 and (LeibundGut-Landmann et al., 2007)), a large proportion of IL-17 producers emerge from the $CD4^+CD25^+$ T cell population (Fig 3.3). Further characterization of the responding population *in vitro* demonstrated that $Foxp3^+ROR\gamma t^+$ T cells, a cell subset that produce low levels of IL-17 in steady state (Lochner et al., 2008, Zhou et al., 2008a), are a main source of IL-17 upon culture with Dectin-1 activated DCs. Because most of our studies were performed *in vitro*, it was unclear whether our findings fully represented an *in vivo* situation. For this reason we decided to evaluate the T cell behaviour during Treg control of murine colitis induced by *H. Hepaticus*. Notably, $Foxp3^+IL\text{-}17^+$ T cells were clearly noticed under this experimental setting and coexisted with $Foxp3^+IL\text{-}17^-$ T cells *in vivo*, indicating that the former population can emerge under some conditions of immune challenge. The fact that $Foxp3^+IL\text{-}17^+$ T cells were present in mice that suppressed the development of intestinal inflammation, suggests that $Foxp3^+IL\text{-}17^+$ T cells may not be detrimental for the resolution of the disease (Figure 3.7). Further studies are required to address this point, as $Foxp3^+IL\text{-}17^+$ T cells constitute a small fraction of the total $Foxp3^+$ population, which complicates functional studies. As discussed in chapter 3, the fact that curdlan administration did not alter the frequency of IL-17-producing cells does not indicate that the Dectin-1 innate signalling pathway does not play a role in this response. This aspect can be further studied in CD11cΔSyk mice (for a detailed description, see below).

The production of IL-17 by $Foxp3^+$ cells is an intriguing finding that highlights the plasticity of T cell subsets at microbe-exposed sites. The outcome of this 'adaptation' during an immune response is not fully understood. The acquisition of transcription factors and cytokines previously attributed to effector T helper subsets might render Tregs competent to control a particular type of immune response. In favour of this notion, it has been recently reported that Tregs require signalling via Stat3 to control excessive Th17 responses (Chaudhry et al., 2009). In addition, IL-17 produced by $Foxp3^+$ T cells might contribute to processes including tissue repair and the restore of cellular homeostasis. Results presented in this thesis indicate that Tregs and naïve T cells are responsive to the same environmental cues. This might confer Tregs the ability

to migrate and survive in the same location with the cells they must regulate (reviewed in (Wohlfert and Belkaid, 2010)).

Notably, Dectin-1 ligation by β-glucans is the first innate recognition system that triggers IL-17 production from various T cell subsets in mice and also by human $CD4^+$ T cells (LeibundGut-Landmann et al., 2007, Osorio et al., 2008, Martin et al., 2009, Gerosa et al., 2008). *In vivo,* this pathway may operate in sites where $Foxp3^+$ T cells and Th17 are highly abundant such as the gut. Although β-(1,3)-glucan has been recognized as an essential constituent of fungal cell walls, this molecule is also found in the periplasmic space of some proteobacteria species (Lee et al., 2009). As such, β-(1,3)-glucans contained in commensal or pathogenic organisms could be sensed by Dectin-1 expressing DCs, which may promote IL-17 production from three different $CD4^+$ T cell subsets (Figure 6.1). Dectin-1 activated DCs can prime a small fraction of naïve T cells via the production of IL-6 and IL-23 plus TGF-β present in the environment (point 1, figure 6.1). Secondly, Dectin-1 activated DCs might induce the production of IL-17 from $Foxp3^+ROR\gamma t^+$ T cells in an IL-23 dependent manner. In this setting, IL-2 signalling might help maintaining the expression of Foxp3 (see point 2, Fig 6.1). Finally, a considerable amount of IL-17 might be derived from effector T cells, which express CD25 and are IL-23 responsive (point 3, Fig 6.1).

In addition to $CD4^+$ T cells, Dectin-1 ligation can also trigger the production of IL-17 from an innate source. A subset of γδ T cells express Dectin-1 and sense β-(1,3)-glucans directly to produce IL-17 (Martin et al., 2009). Whether they utilise the Syk and CARD9 axis to produce IL-17 is currently unclear. Altogether, the production of IL-17 from various cell sources might be an efficient manner to coordinate the immune responses to microbes. Whether the findings reported in this thesis represent an *in vivo* situation, particularly at mucosal sites, remains to be addressed.

An important statement derived from this thesis is that some CLRs compensate for each other by engaging the same protein cascade, which generates a high degree of redundancy at the receptor level. Such is the case of Dectin-1 and Dectin-2 in the context of fungal recognition. Is it is known that Dectin-1 deficiency does not affect the development of Th17 responses during fungal infections (LeibundGut-Landmann et al.,

2007). It is therefore conceivable that the coordination of more than one signalling CLR contribute to the initiation of Th17 responses to commensals or to pathogenic organisms. Thus, a relevant tool to continue the findings presented in this thesis will be to study the development of Th17 responses in CD11cCre x *Syk*$^{fl/fl}$ mice (named CD11cΔSyk mice and described in the appendix section). The Immunobiology Laboratory has recently obtained this strain of mice and my results indicate that Syk is specifically ablated in the CD11c compartment (See appendix figure 7.1). These mice might be a useful tool to dissect the contribution of Syk and CLR signalling by DCs in the initiation of adaptive Th17 cells, which was shown to be a MyD88, TRIF and RIP-2 independent process (Atarashi et al., 2008, Ivanov et al., 2008, Ivanov et al., 2009). The contribution of Syk to the initiation of Th17 cells in the gut is a rather unexplored area that requires further investigation.

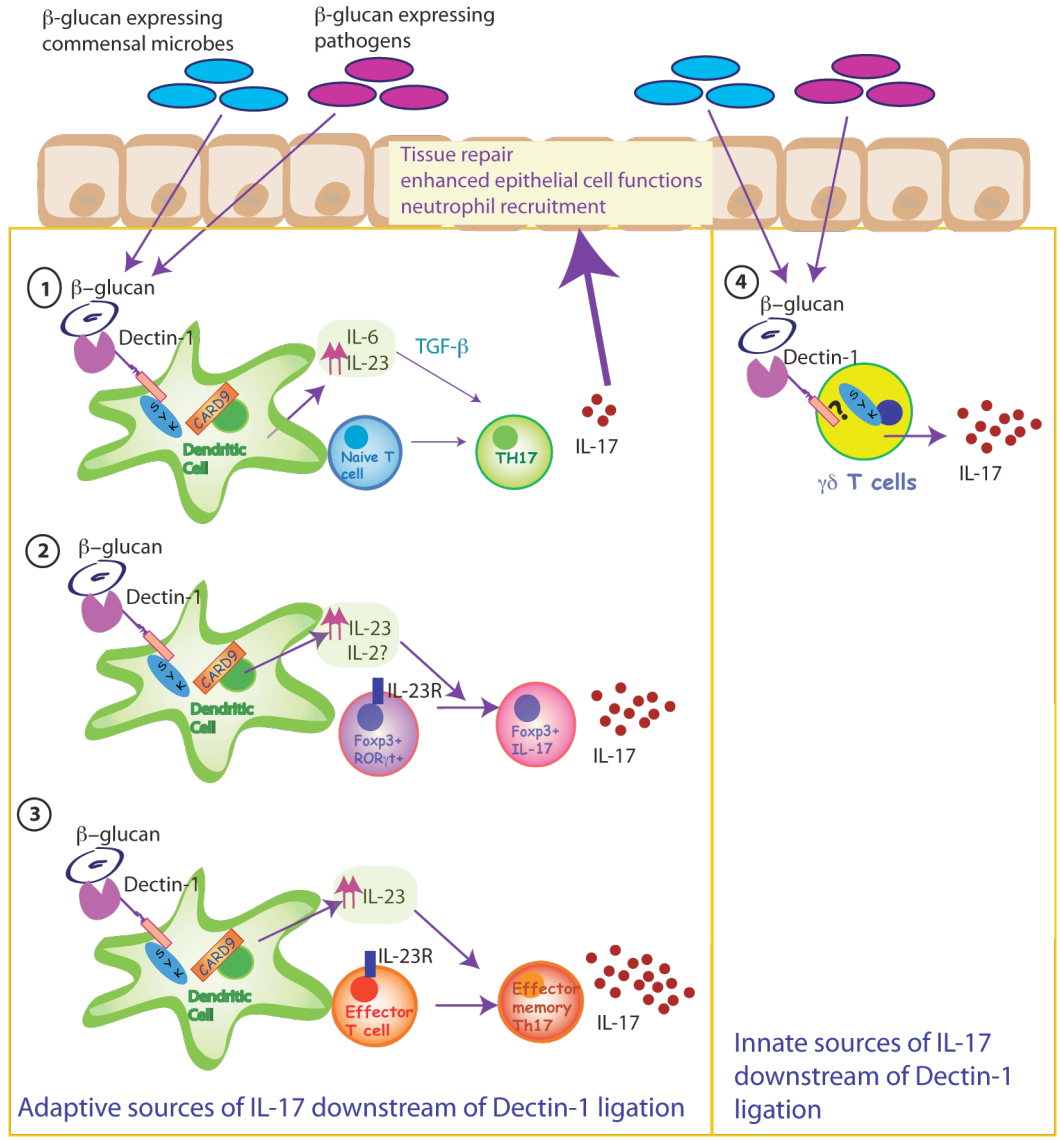


Figure 6-1:Proposed model of IL-17 production *in vivo* in response to Dectin-1 ligation. β-(1,3)-glucan derived from microbes can be sensed by Dectin-1 expressing DCs, which may activate 3 adaptive sources of IL-17. (1) Dectin-1 activated DC prime a small fraction of naïve T cells via IL-6 and IL-23 and TGF-β present in the environmental milieu (LeibundGut-Landmann et al., 2007, Joffre et al., 2010). (2) Dectin-1 activated DCs induce the production of IL-17 from $Foxp3^{+}ROR\gamma t^{+}$ T cells present at mucosal sites. This process requires IL-23 but might need additional factors such as IL-2 in order to sustain and enhance Foxp3 expression and promote cell survival/expansion (Osorio et al., 2008). (3) Effector $CD4^{+}$ T cells are IL-23 responsive and could respond to Dectin-1 activated DCs by producing IL-17. IL-17 produced by all these cell types might contribute to tissue repair mechanisms and homeostasis. (4) In addition to the adaptive sources of IL-17, Dectin-1 signalling can activate a subset of γδ T cells directly (Martin et al., 2009). Additional studies are required to demonstrate the contribution of CLR signalling to the development of IL-17 producing cells *in vivo*.

6.2 Dectin-2 is essential for the initiation of Th17 responses to *C. albicans.*

Results presented in this thesis revealed that Dectin-2 is a second Syk-coupled CLR involved in fungal recognition by DCs and initiation of anti-fungal Th17 responses. Dectin-1 and Dectin-2 couple to the same innate signalling pathway and control the production of the cytokines IL-2, IL-10, TNF and IL-23 (Fig 5.5 to 5.7 and Figure 6.2). Furthermore, Dectin-2 is essential for the induction of Th17 cells during infection with *C. albicans* (Figure 5.8, (Robinson et al., 2009)). These findings have been confirmed in the recently generated Dectin-2 deficient mice, which do not generate Th17 responses to *Candida* and are susceptible to infection with the same organism (Saijo et al., 2010).

Experiments presented in chapter 4 have opened new avenues for the study of fungal Syk-coupled CLRs. Many interesting questions remain unanswered. Dectin-2, in contrast to Dectin-1, is not redundant for the establishment of a Th17 response to systemic infection with *C. albicans.* This is an intriguing finding that suggests that there might subtle differences in the signalling requirements downstream of hemITAM or ITAM-coupled CLRs, which may result in differential immune outcomes. Saijo et al recently reported that Dectin-2 but not Dectin-1 deficient DCs fail to produce IL-23 to fungal stimulation (Saijo et al., 2010). Although these differences are not apparent in the experiments conducted in this thesis with a blocking anti-Dectin-2 antibody, it would be interesting to formally characterize the proximal signalling events downstream of hemITAM and ITAM-dependent CLRs. It has been recently shown that α-mannan derived from the *C. albicans* cell wall acts as an agonist for Dectin-2 (Saijo et al., 2010). If this molecule is further validated as a purified Dectin-2 agonist in the near future, it will become an important reagent for studying ITAM-coupled CLRs. Analysis of signalling protein cascades and gene transcription programs induced in response to Dectin-1 or Dectin-2 ligation will provide better insights in the understanding of C-type lectin receptor biology.

Syk deficient DCs do not produce IL-2, IL-10 and TNF in response to fungal stimulation (Figure 4.2). Furthermore, our results clearly indicate that Dectin-1 and Dectin-2 are required for this process. An additional unanswered question derived from these studies is to identify what are the additional Syk-dependent aspects of CLR

ligation in DCs. As previously mentioned, IL-2, IL-10, TNF, IL-1β and IL-23 are probably a small fraction of the Syk-dependent signature induced by DCs in response to fungal challenge. One approach is to carry out a microarray-based genome-wide screen of WT and Syk deficient DCs stimulated with fungal particles. Additionally, MyD88-TRIF doubly deficient DCs could be included in this analysis to distinguish the profile of genes expressed in absence of TLR signalling. Nonetheless, the fact that Syk controls cellular aspects at multiple levels including survival, proliferation, differentiation, cytokine production and cytoskeletal rearrangements among others indicate that a genetic profile will not provide a complete answer to this question. Syk activation in response to CLR ligation might also influence phagocytosis, migration, processing and presentation of fungal-derived antigens, and ROS production. Each one of these aspects requires a particular experimental setting. On-going work is examining these possibilities.

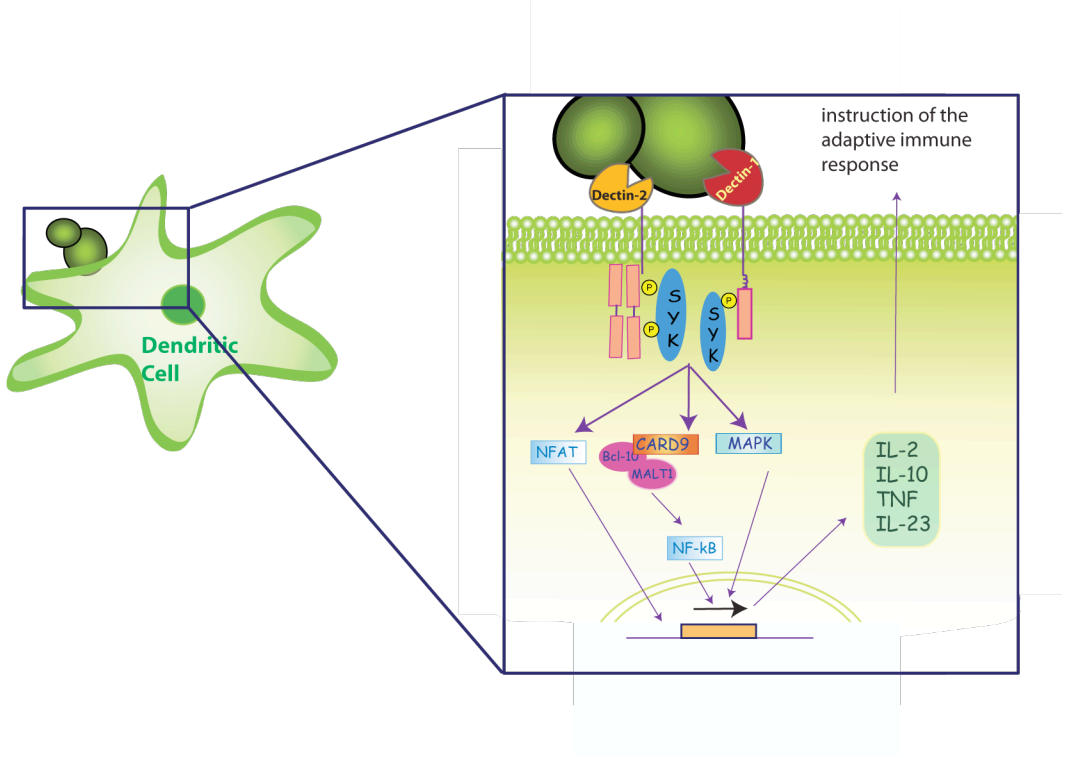


Figure 6-2: In response to fungal recognition, Dectin-1 and Dectin-2 couple to Syk for activation of CARD9, NFAT and MAPK and production of the Syk dependent cytokines in DCs.
Summary of the results obtained in Chapter 5 together with data from Dr Matthew Robinson (Immunobiology Laboratory, Cancer Research UK)(Robinson et al., 2009). Study of the signalling requirements downstream of hemITAM and ITAM coupled CLRs will provide better insights in the study of CLR biology.

The experiments provided in this thesis together with those from another group elucidated the role of Dectin-2 in the generation of an anti-fungal Th17 response (Robinson et al., 2009, Saijo et al., 2010). As the Dectin-2 deficiency affected every Dectin-2 expressing cell in the organism, a caveat derived from those studies is that they do not allow addressing the relevance of Dectin-2-dependent signalling in DCs. To address the contribution of the CLR signalling in DCs to the generation of immune responses to fungi, infection studies in CD11cΔSyk mice will of vital relevance. This strain of mice could be subjected to systemic candidiasis, but perhaps more importantly, to the experimental model of oropharyngeal candidiasis (OPC) that is a model where adaptive T cells play a predominant role (Conti et al., 2009). We are currently trying to obtain the animal license to be able to establish this model in the Immunobiology Laboratory, and we are also collaborating with Dr Salomé LeibundGut-Landmann (ETH Zurich, Switzerland) to answer some of those questions.

6.3 Syk signalling in the innate control of antifungal immunity.

CARD9 deficient mice are much more susceptible to systemic candidiasis than Dectin-1 or Dectin-2 deficient mice (Gross et al., 2006, Saijo et al., 2007, Taylor et al., 2007, Saijo et al., 2010), suggesting that these receptors might be redundant for early antifungal resistance to *C. albicans.* Indeed, our data indicate that even the absence of both Dectin-2 and Dectin-1 function does not recapitulate the phenotype observed in CARD9 knock out mice, which have significantly impaired innate resistance to *C. albicans* (Figure 4.9). An important question derived from these studies is to address whether Syk signalling in DCs accounts for the CARD9 dependent aspects of antifungal defense. Remarkably, results presented in the appendix section demonstrate that Syk kinase expressed by the CD11c compartment is necessary for the control of disseminated candidiasis (Figure 7.2, appendix). Due to the early stage of this project, these results do not constitute part of the thesis body. CD11cΔSyk mice succumb within 5 days to systemic *Candida* infection and have large increases in fungal burden, resembling the phenotype observed in *Card9*$^{-/-}$ mice (Gross et al., 2006). Furthermore, heavy fungal colonisation and a high degree of tissue damage are detected in kidneys of CD11cΔSyk mice infected *C. albicans* (Figure 7.2, appendix). These results indicate that although Dectin-1 and Dectin-2 were redundant for antifungal resistance during the

first week of infection, absence of Syk in the CD11c compartment renders mice highly susceptible to systemic infection with the same organism.

Thus, a single non-TLR innate signalling pathway in a defined population of cells is key for the establishment of antifungal immunity. Clearly, these are preliminary observations and much remains to be addressed. Syk signalling in $CD11c^+$ cells might be involved in the regulation of many functions that could result in impaired antifungal immunity. Those aspects vary from the production of a soluble mediator involved in immune activation, to the production of components with direct antifungal activity (e.g. ROS) by CD11c-expressing cells. In addition, Syk insufficient $CD11c^+$ cells might be less competent to phagocytose fungal pathogens at early time points, allowing fungal spread and invasion. An additional aspect that requires investigation is to demonstrate that the CD11c-expressing cell responsible for anti-*Candida* immunity is indeed a DC. CD11c is mainly expressed by conventional DCs but is also detected in a small subset of NK cells, macrophages and monocyte-derived DCs (Caton et al., 2007). Reconstitution of CD11cΔSyk mice with various types of wild type $CD11c^+$ cells will permit the identification of the cell subset responsible for antifungal resistance. On-going work is currently trying to address all those possibilities.

Throughout this thesis, I have presented evidence demonstrating that the members of the CLR innate signalling pathway Dectin-1, Dectin-2 and Syk coordinate different levels of the anti-fungal immune response. Dectin-1 and Dectin-2 are expressed in DCs and are closely involved in the regulation of adaptive immunity to *Candida*. On the other hand, Syk decodes the information triggered by multiple PRRs and its functions within the CD11c compartment also encompass innate resistance to fungi. Altogether, results presented in this thesis underscore the relevance of this innate recognition system in the coordination of innate and adaptive immunity to fungi.

Appendix

Syk kinase in the CD11c compartment mediates antifungal immunity.

Results presented in this section do not constitute an integral part of the thesis body. Nonetheless, I considered they are of relevance for a deep understanding of the role of Syk in the initiation of antifungal defense. These data should be considered as 'work in progress' as they belong to my current project in the laboratory.

To address the relevance of Syk in DCs and its contribution to an antifungal immune response, the laboratory has crossed the strain of mice *Syk*$^{fl/fl}$ to mice carrying the CD11c Cre^{+} transgene, which is highly expressed in DCs (Caton et al., 2007). *Syk*$^{fl/fl}$ x CD11cCre^{+} mice will be referred to here as CD11cΔSyk mice whereas the littermates *Syk*$^{fl/fl}$ x CD11cCre^{-} will be referred as wild type littermates.

CD11cΔSyk mice have normal frequencies of DC subsets in the spleen (Figure 7.1a). In addition, no gross abnormalities have been detected in the CD4^{+}, CD8^{+}, NK1.1^{+}, and B220^{+} populations in the spleen (data not shown). To verify deletion of Syk protein in the CD11c compartment, spleen DC subsets were enriched using CD11c beads (Miltenyi Biotec) and were subsequently sorted into CD11c^{+}CD4^{+} (CD4^{+} DC), CD11c^{+}CD8^{+} (CD8^{+} DC) and CD11c^{+}CD4^{-}CD8^{-} (double-negative DC) subsets. The expression of Syk protein was assessed by Western blot. As a positive control, B220-expressing B cells were isolated in the same experiment. Results are shown in figure 7.1b. Whereas high levels of Syk protein are detected in the three main subsets of splenic DCs isolated from wild type littermates, Syk protein was drastically reduced in DC subsets isolated from CD11cΔSyk mice. This observation was less obvious in the double negative subset from CD11cΔSyk mice, which expressed low levels of the protein (Figure 7.1b). B220^{+} cells from wild type and CD11cΔSyk mice express similar levels of Syk indicating that Syk deletion is restricted to the CD11c compartment. To obtain a functional readout of Syk deficiency, GM-CSF-derived BMDC from WT and CD11cΔSyk mice were stimulated with curdlan, LPS and CpG and the production of the Syk dependent cytokines IL-2, IL-10 and TNF was analyzed. As expected, wild type

BMDCs produce large amounts of IL-2, IL-10 and TNF in response to curdlan stimulation. The production of IL-10 and TNF in response to the Dectin-1 agonist was abrogated in CD11cΔSyk BMDC indicating effective Syk deletion. Surprisingly, CD11cΔSyk and wild type BMDC stimulated with curdlan produced similar levels of IL-2. One reason accounting for this difference might be that Syk is not entirely deleted in GM-CSF cultures, which might affect IL-2 production particularly. Altogether, Syk protein is efficiently deleted from the CD11c compartment of CD11cΔSyk mice validating this strain as a good tool to study antifungal immunity.

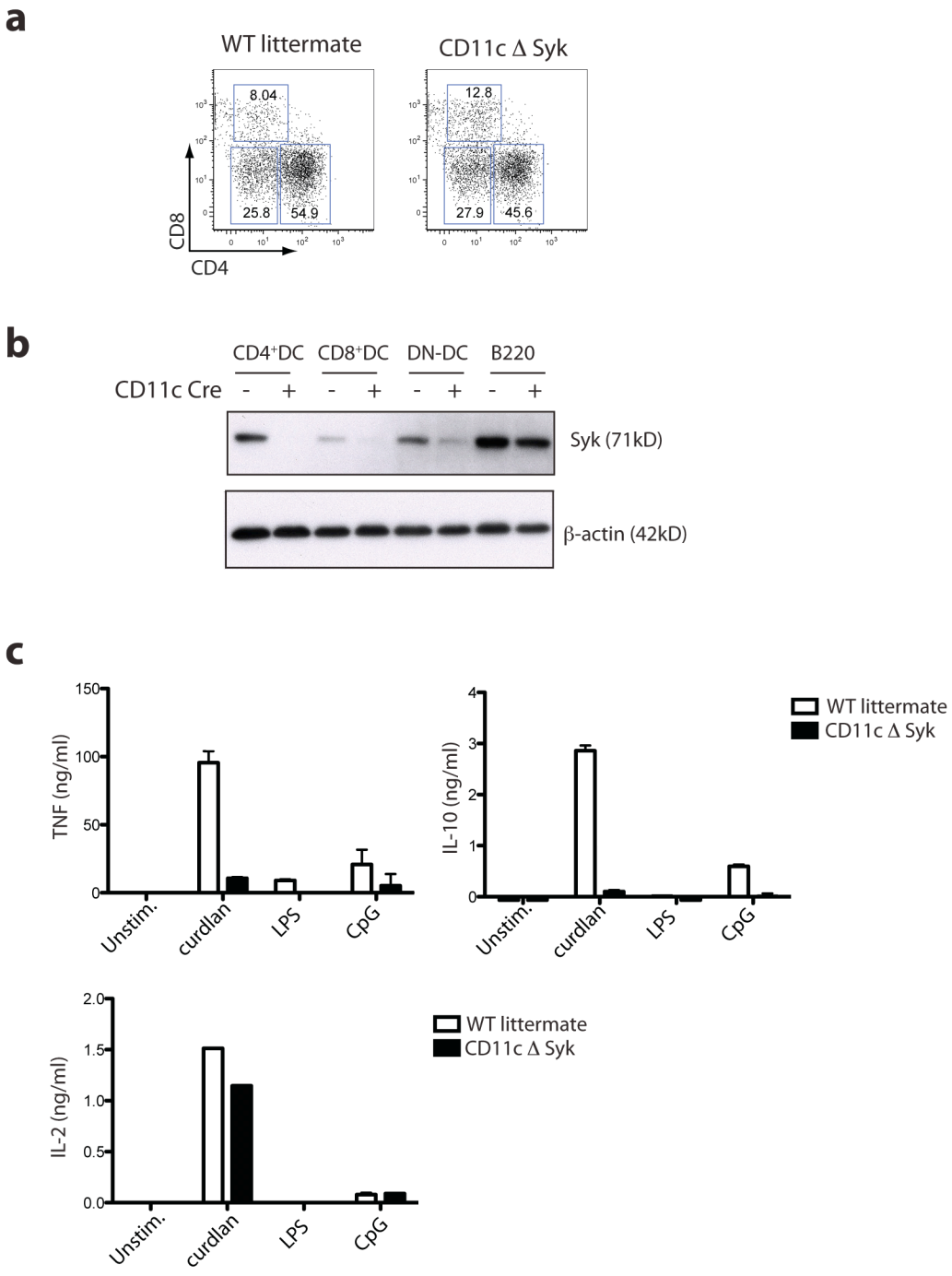


Figure 7-1: Deletion efficiency in CD11cΔSyk mice.
(**a**) CD11c enriched splenocytes from WT littermate and CD11cΔSyk were labelled with CD11c, CD8 and CD4 antibodies and analyzed by FACS. (**b**) Splenic DC subsets and B220 expressing cells from WT littermate and CD11cΔSyk were lysed and immunoblotted for total Syk. Syk protein levels are compared to β-actin. (**c**) BMDC from WT littermate and CD11cΔSyk were stimulated o.n. with curdlan, LPS or CpG and cytokines were quantified by sandwich ELISA.

CD11cΔSyk mice succumb to systemic *C. albicans* infection.

Dectin-1 and Dectin-2 do not account for the CARD9 dependent aspects of antifungal innate immunity (chapter 5). One possibility is that additional Syk-CARD9 fungal PRRs expressed by additional cells including neutrophils, granulocytes and macrophages may be involved in early anti-fungal immunity. Another possibility is that Dectin-1 and Dectin-2 do not fully account for the Syk-dependent aspects in DCs. In fact, the contribution of DCs to innate resistance to fungal infection has not been addressed so far. To address whether Syk signalling in DCs contributes to early resistance to disseminated candidiasis, we infected CD11cΔSyk mice and wild type littermates with $5x10^4$ *C. albicans* i.v. and compared their susceptibility to infection (Figure 7.2). Surprisingly, all CD11cΔSyk mice reached the end point within 5-6 days of infection whereas most wild type counterparts survived. Data from 2 independent experiments is depicted in figure 7.2a. Higher fungal burden was found in kidney, lung and liver from CD11cΔSyk mice compared to wild type littermates (Figure 7.2b). This phenotype is highly reminiscent to that observed in CARD9 knock out mice (Gross et al., 2006). The Histopathology Laboratory at Cancer Research UK performed histological analysis of kidneys from infected mice. Periodic acid Schiff staining (PAS) was used to detect *C. albicans* and Hematoxylin and Eosin (H&E) staining was used to detect leukocyte infiltration. Kidneys from infected CD11cΔSyk mice showed heavy fungal abscesses localized to the cortical zone that were accompanied by enhanced leukocyte infiltration (Figure 7.2c, see PAS and H&E). In contrast, kidneys from wild type counterparts were largely free of hyphae and when pathogen was found, it was completely restricted to the urinary tract (data not shown). Data in Figure 7.2d shows a high degree of hyphal infiltration and tissue disruption in infected CD11cΔSyk mice. Collectively these data indicates that Syk kinase in the CD11c compartment is strictly required for innate antifungal immunity.

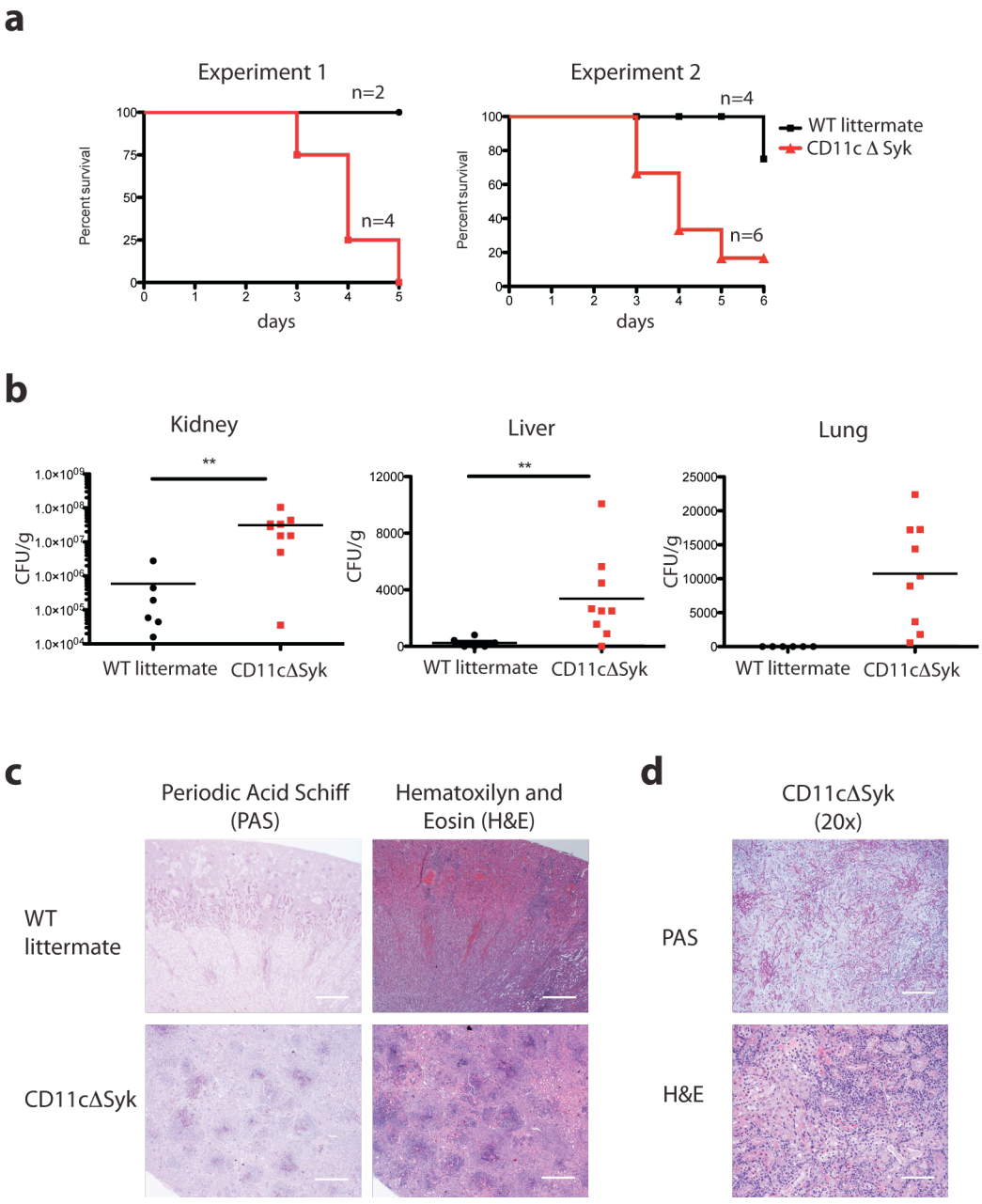


Figure 7-2: CD11c Syk mice show defective anti-fungal immunity.
(**a**) WT (*Syk*$^{fl/fl}$ x CD11cCre^{-}) and CD11cΔSyk (*Syk*$^{fl/fl}$ x CD11cCre^{+}) mice were infected with 5x10^{4} *C. albicans* i.v. Mice were monitored on a daily basis for survival. (**b**) Kidney fungal burden from mice in (**a**) was quantified. The data are pooled from 2 independent experiments depicted in (**a**) (**, 0.001< P<0.01)(**c-d**) histological analysis of kidneys of *C. albicans*-infected mice on day 4 (CD11cΔSyk) or day 6 (WT). Histology is representative of 5 CD11cΔSyk mice and 3 WT mice. White bars indicate 500μm (**c**) or 100μm(**d**).

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Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection

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Innate immune cells detect pathogens via pattern recognition receptors (PRRs), which signal for initiation of immune responses to infection. Studies with Dectin-1, a PRR for fungi, have defined a novel innate signaling pathway involving Syk kinase and the adaptor CARD9, which is critical for inducing Th17 responses to fungal infection. We show that another C-type lectin, Dectin-2, also signals via Syk and CARD9, and contributes to dendritic cell (DC) activation by fungal particles. Unlike Dectin-1, Dectin-2 couples to Syk indirectly, through association with the FcRγ chain. In a model of *Candida albicans* infection, blockade of Dectin-2 did not affect innate immune resistance but abrogated *Candida*-specific T cell production of IL-17 and, in combination with the absence of Dectin-1, decreased Th1 responses to the organism. Thus, Dectin-2 constitutes a major fungal PRR that can couple to the Syk–CARD9 innate signaling pathway to activate DCs and regulate adaptive immune responses to fungal infection.

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Abbreviations used: BMDC, bone marrow–derived DC; CLR, C-type lectin receptor; ITAM, immunoreceptor tyrosine-based activation motif; NLR, NOD-like receptor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; RLR, RIG-I–like receptor; shRNA, short hairpin RNA; TLR, Toll-like receptor.

The sensing of pathogens by innate immune cells is mediated by germline-encoded pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs; Janeway, 1989). Although some PRRs may be involved primarily in phagocytic clearance of invading organisms, others engage a plethora of signaling pathways that lead to the expression of genes that encode chemokines, cytokines, and other mediators of innate immune responses to infection. In addition, PRR signaling in DCs renders them competent to prime T cells, thereby initiating adaptive immunity (Reis e Sousa, 2004). Established families of PRRs regulating both innate and adaptive immunity include the Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I–like receptors (RLRs; Akira et al., 2006; Lee and Kim, 2007; Pichlmair and Reis e Sousa, 2007). However, recent work by us and others has indicated that Dectin-1 and related members of the C-type lectin receptor (CLR) family may also function as PRRs that can signal to regulate immunity (Brown, 2006; Robinson et al., 2006).

Dectin-1 is expressed primarily by neutrophils, macrophages, and DCs, and recognizes β-glucans present in cell walls of fungi and some bacteria (Brown and Gordon, 2001;

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Dennehy and Brown, 2007). The intracellular tail of Dectin-1 contains a single YxxL motif, termed a hemITAM, that is able to recruit and activate Syk upon engagement by agonist ligands (Rogers et al., 2005; Underhill et al., 2005). Dectin-1 coupling to Syk leads to downstream activation of MAPKs, NFAT, and, via the adaptor CARD9, NF-κB, which coordinate the transcription of innate response genes (Gross et al., 2006; Goodridge et al., 2007; LeibundGut-Landmann et al., 2007). In DCs, the pattern of cytokines triggered by selective Dectin-1 engagement is somewhat different from that induced by agonists of TLR, RLR, or NLR pathways, and is typified by robust induction of proinflammatory cytokines such as TNF, IL-6, and IL-23 accompanied by high levels of IL-2 and IL-10 (LeibundGut-Landmann et al., 2007). Consistent with its stimulatory effects on DCs, a Dectin-1 agonist acts as an adjuvant in vivo to induce adaptive immune responses against a coadministered model antigen, resulting in induction of antigen-specific Th1 and Th17 cells, as well as CTL and antibody responses (LeibundGut-Landmann et al., 2007; LeibundGut-Landmann et al., 2008). In addition, Dectin-1–activated DCs are able to convert regulatory T cells into IL-17 producers (Osorio et al., 2008). In view of its immunomodulatory activities, Dectin-1 is therefore thought to modulate both the innate and adaptive responses to fungal infection. In line with this notion, Dectin-1–deficient mice display increased susceptibility to *Candida albicans* and *Pneumocystis carinii* infection in some models (Saijo et al., 2007; Taylor et al., 2007).

Innate immune cells express many receptors that can potentially recognize fungi, including TLR-2, TLR-4, Dectin-2, mannose receptor, DC-SIGN, SIGN-R1, and Mincle (Netea et al., 2008; Willment and Brown, 2008). Although many of these receptors signal independently of Syk, Syk-deficient DCs fail to produce IL-2 and IL-10 upon stimulation with zymosan, a heat-killed and trypsinized preparation of *Saccharomyces cerevisiae* (Rogers et al., 2005). This would be consistent with a dominant role for Dectin-1 in DC responses to fungi, as we have observed in macrophages (Taylor et al., 2007). However, surprisingly, Dectin-1–deficient DCs display only a mild impairment in their ability to produce the same two cytokines in response to zymosan (LeibundGut-Landmann et al., 2007; Saijo et al., 2007; Taylor et al., 2007). Along the same lines, *Card9*$^{-/-}$ mice appear to be much more susceptible to infection by *C. albicans* (Gross et al., 2008) than Dectin-1–deficient mice (Saijo et al., 2007; Taylor et al., 2007). In addition, CARD9-deficient mice fail to mount a *Candida*-specific Th17 response, yet this response is preserved in mice lacking Dectin-1 (LeibundGut-Landmann et al., 2007). Finally, a recent paper suggests that the mannose receptor contributes to human Th17 responses to *C. albicans* (van de Veerdonk et al., 2009), although there is as yet no evidence linking that receptor to the Syk–CARD9 pathway. Collectively, these different observations imply that Dectin-1 is not the only Syk–CARD9–coupled fungal PRR in DCs and suggest that multiple receptors capable of activating this signaling pathway could regulate DC-dependent Th17 responses to fungal infection.

Dectin-2 is a type II transmembrane CLR that was originally cloned from a DC line (Ariizumi et al., 2000a) but is most abundantly expressed on tissue macrophages and inflammatory monocytes and has specificity for high mannose structures (Taylor et al., 2005; McGreal et al., 2006). Dectin-2 lacks any obvious signaling motif within its short cytoplasmic domain and, despite its name, is only 20–25% homologous to Dectin-1 (Ariizumi et al., 2000a). Nevertheless, like Dectin-1, Dectin-2 can bind to zymosan and to many fungi, including *C. albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Microsporum audouinii*, and *Trichophyton rubrum*, displaying a preference for the hyphal form over yeast (McGreal et al., 2006; Sato et al., 2006). Notably, Dectin-2 can associate with the immunoreceptor tyrosine-based activation motif (ITAM)–bearing FcRγ chain (Sato et al., 2006; Barrett et al., 2009), and Dectin-2–transfected macrophages respond to hyphal stimulation with protein tyrosine phosphorylation, NF-κB activation, and secretion of TNF and IL-1ra (Sato et al., 2006). Whether this reflects signaling by Dectin-2 or is a consequence of fungal binding allowing engagement of other signaling receptors has not been explored (Sato et al., 2006) but led us to hypothesize that Dectin-2 might substitute for Dectin-1 in activating Syk and CARD9 in DCs in response to fungal presence.

In this paper, we demonstrate that Dectin-2, together with Dectin-1, explains most of the Syk- and CARD9-dependence of DC responses to fungal stimuli. We show that triggering of endogenous Dectin-2 in DCs activates Syk and the MAPK cascades, and leads to CARD9- and Syk-dependent cytokine production. Furthermore, by blocking Dectin-2 in vivo, we identify a role for this CLR in the induction of adaptive immune responses, particularly Th17, in a model of systemic *C. albicans* infection. These results not only confirm Dectin-2 as a PRR for fungi but also describe it as another Syk-coupled myeloid CLR involved in regulating DC function and the nature of the adaptive immune response to fungal infection.

RESULTS

Induction of IL-2, IL-10, and TNF by fungal stimuli in DCs is dependent on Syk but not on Dectin-1

In previous studies using DCs stimulated with zymosan, we found that production of IL-10 and IL-2 but not production of IL-12/23 p40 depends on Syk (Rogers et al., 2005). In more recent experiments, we have found that zymosan-induced production of TNF is also Syk dependent and independent of MyD88 and TRIF, two adaptors essential for TLR signaling (Fig. S1 A). To extend those studies to a potential fungal pathogen, we compared the response of GM-CSF–cultured bone marrow–derived DCs (BMDCs) to different preparations of *C. albicans* (Fig. S2 A). As for zymosan, both live and heat-killed yeast and hyphal forms of *C. albicans* elicited production of IL-2, IL-10, and TNF by BMDCs. Production of these cytokines was not greatly affected by the antifungal agents used to prevent overgrowth in cultures with

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live *C. albicans*, although slight variations between samples treated with fungizone and with caspofungin were noted (Fig. 1 and Fig. S2 B). However, none of the antifungal agents stimulated cytokine production on their own or altered the response to curdlan, a selective Dectin-1 agonist (Fig. S2 B). Notably, IL-2, IL-10, and TNF were strictly dependent on Syk whether live or heat-killed organisms were used (Fig. 1). In contrast, the production of IL-12/23 p40 and IL-6 was dependent on MyD88/TRIF adaptors and largely independent of Syk (not depicted). Interestingly, the three Syk-dependent cytokines were more strongly induced by the hyphal than the yeast form of *C. albicans*, especially when the organisms were subjected to heat inactivation (Fig. 1). The latter also unmasked a MyD88/TRIF dependence for IL-2 induction, which was not seen with live organisms (Fig. 1, A and B). The dual dependence of IL-2 on Syk and MyD88/TRIF pathways has been previously noted for zymosan (Rogers et al., 2005), and may reflect denaturation of some PAMPs and exposure of others during the heat inactivation process (Gantner et al., 2005). Consistent with PAMPs being altered by boiling, the MyD88/TRIF adaptors contributed partially to TNF induction by live but not heat-inactivated *C. albicans* (Fig. 1). IL-10 remained

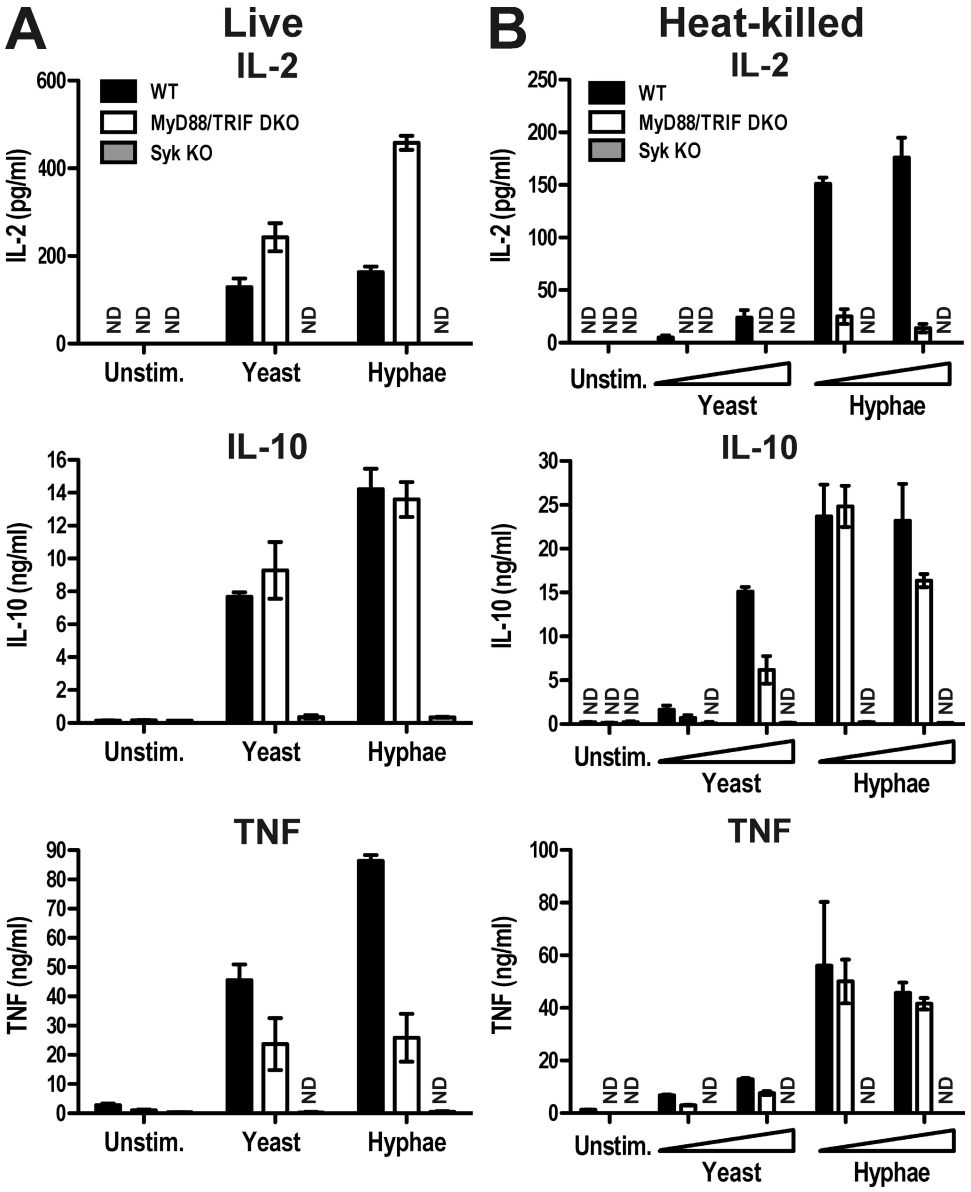

Figure 1. The contribution of Syk and TLR signaling to cytokine induction by *C. albicans*. (A) BMDCs from C57BL/6 wild-type (WT, black bars), *Myd88*$^{-/-}$*Trif*$^{-/-}$ (MyD88/TRIF DKO, white bars), or *Syk*$^{-/-}$ chimeric (Syk KO, gray bars) mice were stimulated with 10^5 live *C. albicans* yeast or hyphae. Fungizone was added 2 h later, and cytokine levels in the supernatants were measured after overnight incubation. (B) BMDCs as in A were stimulated overnight with 10^5 or 5×10^5 heat-killed *C. albicans*. Data are means ± SD of duplicate wells and are representative of at least three independent experiments. ND, not detectable.

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largely MyD88/TRIF independent regardless of the fungal stimulus (Fig. 1), consistent with our data using zymosan (Rogers et al., 2005). We conclude that, independently of possible contributions from TLR pathways, IL-2, IL-10, and TNF production by BMDCs in response to fungal stimuli requires signaling via Syk and can be used to monitor the activity of Syk-coupled PRRs for fungi that are expressed on DCs.

Dectin-1 can trigger the production of IL-2, IL-10, and TNF upon engagement by agonistic β-glucans (Yoshitomi et al., 2005; LeibundGut-Landmann et al., 2007), so we next analyzed whether the Syk-dependent responses were attributable to Dectin-1. Compared with wild-type cells, Dectin-1–deficient BMDCs produced similar levels of IL-2, IL-10, and TNF in response to heat-killed (Fig. 2) or live *C. albicans* (not depicted). Similarly, in response to zymosan, the induction of the Syk-dependent cytokines was only marginally reduced by Dectin-1 deficiency (Fig. S1 B). This is consistent with previous observations made in DCs (LeibundGut-Landmann et al., 2007; Saijo et al., 2007; Taylor et al., 2007) but, interestingly, contrasts with data indicating that in macrophages induction of TNF by zymosan and *Candida* is predominantly mediated by Dectin-1 (LeibundGut-Landmann et al., 2007; Saijo et al., 2007; Taylor et al., 2007). The near complete Syk dependence and the relative Dectin-1 independence of TNF, IL-2, and IL-10 responses to fungal stimuli suggests the existence of additional Syk-coupled fungal PRRs in DCs that remain active in triggering this signaling pathway in the absence of Dectin-1.

Dectin-2 is a Syk-coupled receptor in DCs

Dectin-2 is capable of binding both *C. albicans* and zymosan (McGreal et al., 2006) and could therefore potentially act as a PRR for fungi. Despite previous reports of limited expression of Dectin-2 on DCs (Taylor et al., 2005; Carter et al., 2006), using a more sensitive detection method we found surface expression by the $CD4^+$, $CD8^+$, and double-negative subsets of splenic DCs (Fig. S3 A). As reported recently (Barrett et al., 2009), BMDCs also expressed Dectin-2 (Fig. S3 B). This broad expression of Dectin-2 on conventional DCs is similar to that of Dectin-1 (Fig. S5 C; LeibundGut-Landmann et al., 2007; Saijo et al., 2007; Taylor et al., 2007). Next, we cultured BMDCs with anti–Dectin-2 immobilized on plastic to assess whether selective stimulation through Dectin-2 alone was sufficient to promote DC activation. $F(ab')_2$ preparations of secondary antibody and Fab preparations of anti–Dectin-2 were used to avoid Fc receptor engagement, but in all cases, similar data were obtained with intact antibodies (unpublished data). Contact of DCs with the anti–Dectin-2 surfaces induced phosphorylation of Syk and of ERK, p38, and JNK MAPKs, whereas no increase above basal phosphorylation was observed with control antibodies (Fig. 3 A). In addition, overnight stimulation with plated anti–Dectin-2 induced accumulation of high levels of IL-2, IL-10, and TNF in culture supernatants (Fig. 3, B–D). This was not caused by contamination of the Fab/$F(ab')_2$ preparations with TLR agonists, because a similar response was observed with BMDCs doubly deficient in MyD88 and TRIF (Fig. 3 B). We therefore explored the notion that Dectin-2 might signal via the Syk–CARD9 pathway. Consistent with that hypothesis, Dectin-2 cross-linking completely failed to induce IL-2, IL-10, and TNF from Syk- or CARD9-deficient BMDCs (Fig. 3, C and D) despite the fact that those cells expressed normal levels of Dectin-2 and responded normally to stimulation with TLR agonists (not depicted). We conclude that triggering of endogenous Dectin-2 in DCs is sufficient to activate Syk and downstream signaling pathways in DCs, leading to TLR-independent production of cytokines.

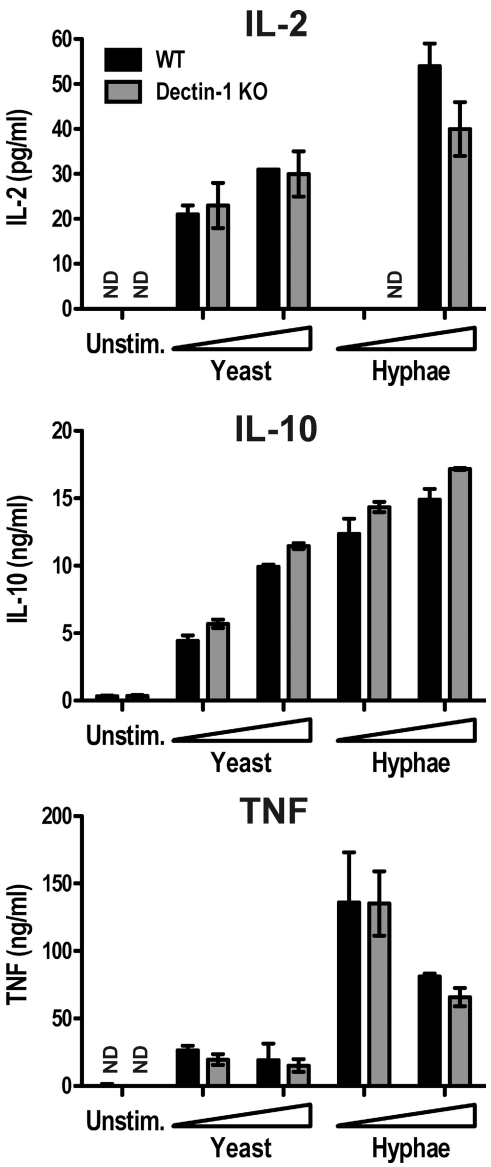

Figure 2. The contribution of Dectin-1 to cytokine induction by *C. albicans*. BMDCs from C57BL/6 wild-type (WT, black bars) or *Clec7a*$^{-/-}$ chimeric (Dectin-1 KO, gray bars) mice were stimulated overnight with 10^5 or 5×10^5 heat-killed *C. albicans* yeast or hyphae. Data are means ± SD of duplicate wells and are representative of at least three independent experiments. ND, not detectable.

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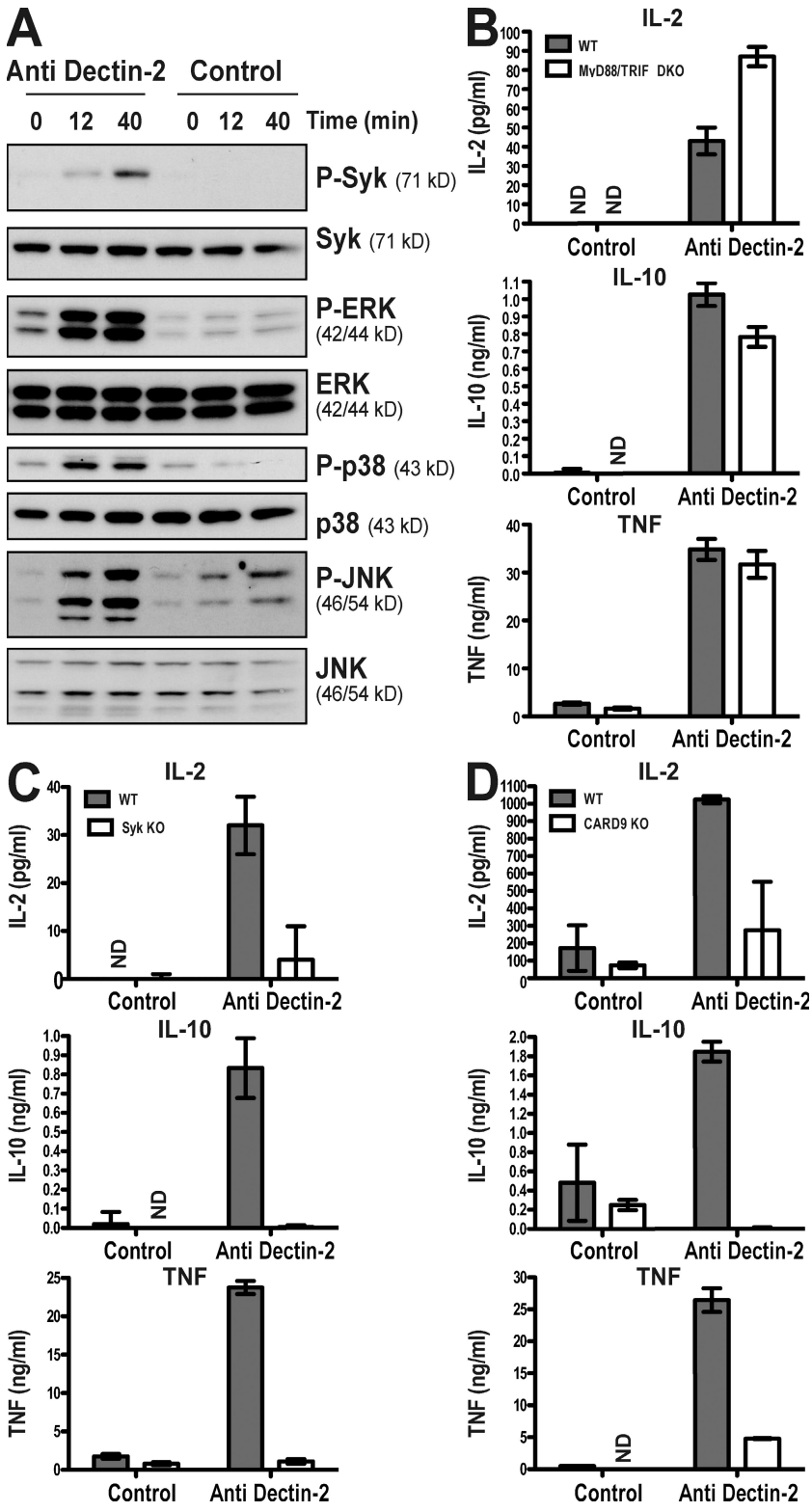

Figure 3. Syk- and CARD9–dependent activation of DCs by Dectin-2. (A) Activation of Syk and MAPK cascades by plated anti–Dectin-2. C57BL/6 BMDCs were centrifuged onto wells coated with anti–Dectin-2 Fab or control Fab. After the indicated times, the cells were lysed and immunoblotted for active (phosphorylated) and total Syk, p38, ERK, or JNK. (B) Wild-type C57BL/6 (WT, gray bars) or *Myd88*$^{-/-}$*Trif*$^{-/-}$ (MyD88/TRIF DKO, white bars) BMDCs were stimulated overnight with anti–Dectin-2 or control Fab as in A, and cytokines levels in the supernatants were measured. (C and D) BMDCs from *Syk*$^{-/-}$ chimeric (Syk KO, white bars; C) and *Card9*$^{-/-}$ (Card9 KO, white bars; D) mice were stimulated as in B, and cytokine levels were compared with those made by WT cells (gray bars). Representatives of at least three independent experiments are shown. Cytokine data are means ± SD of duplicate wells. ND, not detectable.

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Dectin-2 signals through association with FcRγ chain

Unlike Dectin-1, Dectin-2 does not have an intracellular hemITAM motif that would allow it to couple directly to Syk. However, exogenously expressed Dectin-2 in myeloid cells coimunoprecipitates with the ITAM-bearing γ chain of the Fc receptor (Sato et al., 2006), and its surface expression is increased by FcRγ cotransfection (Barrett et al., 2009). To analyze the interaction between Dectin-2 and possible signaling adaptors, including FcRγ chain, DAP-10, or DAP-12, we used B3Z T hybridoma cells, which express a β-galactosidase NFAT reporter of ITAM signaling (Karttunen et al., 1992). Dectin-2–transduced B3Z cells expressed modest amounts of receptor at the cell surface that were not significantly increased by previous transduction with DAP-10 or DAP-12 (Fig. 4 A). However, in B3Z cells coexpressing FcRγ chain, cell-surface staining of Dectin-2 was greatly enhanced (Fig. 4 A). This increase in surface expression conferred the ability to bind fluorescent zymosan, which was not detectable in the other B3Z cell lines (Fig. 4 A). To analyze whether endogenous Dectin-2 expression similarly required FcRγ chain, BMDCs from wild-type and FcRγ chain–deficient mice were analyzed. Surface expression of Dectin-2 was not detectable on FcRγ chain–deficient BMDCs (Fig. 4 B), which also failed to respond to plated anti–Dectin-2 Fab stimulation (Fig. S4). Collectively, these results demonstrate that association with FcRγ chain is necessary for functional surface expression of Dectin-2.

To analyze the ability of FcRγ chain to mediate Dectin-2 signaling, we stimulated B3Z cells with zymosan and measured activation of the NFAT reporter. Reporter activity was seen in B3Z cells cotransduced with Dectin-2 and FcRγ chain but not in B3Z cells expressing either protein alone (Fig. 4 C). To dissect the role of the ITAM motif of FcRγ chain on Dectin-2 signaling, we mutated the two key tyrosines to phenylalanines. The double tyrosine mutation did not impair the ability of FcRγ chain to promote Dectin-2 cell-surface expression or zymosan binding (Fig. S5). However, B3Z cells expressing the mutant FcRγ chain and Dectin-2 were unable to signal in response to zymosan, as determined by induction of the NFAT reporter (Fig. 4 C). We conclude that the ITAM motif of the FcRγ chain mediates signaling by Dectin-2.

Induction of Syk-dependent cytokines by fungal stimuli requires Dectin-1 and -2

These results suggested that, through its association with FcRγ chain, Dectin-2 might potentially function as a Syk-coupled PRR and contribute, in conjunction with Dectin-1, to Syk–CARD9–dependent DC activation by fungal stimuli. To test this hypothesis, we compared fungal stimulation of Dectin-1–sufficient and –deficient BMDCs with or without Dectin-2 function. As has been observed for many antibodies against other ITAM-coupled receptors, anti–Dectin-2 did not trigger activation unless immobilized on plastic or beads (unpublished data). Instead, in solution, the mAb caused down-regulation of Dectin-2 and could consequently be used as a blocking reagent (unpublished data). Therefore, we treated BMDCs with anti–Dectin-2 or an isotype-matched irrelevant antibody control for 2 h before stimulation with zymosan. As with loss of Dectin-1, down-regulation of Dectin-2 in wild-type DCs only modestly reduced the levels of IL-2, IL-10, or TNF produced in response to zymosan (Fig. 5, A and B). However, blockade of Dectin-2 in Dectin-1–deficient BMDCs led to a profound reduction in the same cytokines (Fig. 5, A and B), approaching the decrease observed in Syk-deficient cells (Fig. S1 A; Rogers et al., 2005). Similar results were obtained using live *C. albicans*: blockade of Dectin-2 in wild-type BMDCs or genetic loss of Dectin-1 led to modest and variable reductions in the levels of IL-2 and IL-10, but blockade of Dectin-2 in Dectin-1–deficient BMDCs caused an almost complete loss in production of all three Syk-dependent cytokines (Fig. 6 A). The exception was TNF, which was only partially dependent on the two CLRs (Fig. 6 A), possibly reflecting the TLR contribution noted in Fig. 1 A. Induction of IL-2, IL-10, and TNF by heat-killed *C. albicans* was mediated by Dectin-1 and -2, as seen with zymosan. We also analyzed the induction of IL-12 and IL-23 and found that IL-12 p35 mRNA was at the limit of detection (unpublished data), whereas IL-23p19 mRNA was elicited by *Candida* stimulation in a manner dually dependent on Dectin-1 and -2 (Fig. S6). To verify the contribution of Dectin-2 by an independent means, we used short hairpin RNA (shRNA) to knock down Dectin-2 expression (Barrett et al., 2009). Infection of BMDCs with a lentivirus encoding Dectin-2 shRNA but not with a lentivirus encoding a scrambled control insert reduced the surface expression of Dectin-2 by >50% as determined by flow cytometry (not depicted). Notably, knockdown of Dectin-2 in Dectin-1–deficient cells almost completely prevented the induction of IL-2, IL-10, and TNF by heat-killed *C. albicans* (Fig. 6 C). These results suggest that signaling by Dectin-1 and -2 together could account for the Syk–CARD9 dependence of DC activation by fungi.

In addition to the loss of Syk-dependent cytokines, we noted that binding of fluorescent zymosan was markedly decreased when Dectin-2 function was blocked in Dectin-1–deficient BMDCs (Fig. S7). These results indicate that the primary binding receptors for zymosan on DCs are Dectin-1 and -2. At the same time, they raise a caveat with the interpretation that Syk-dependent DC activation is caused by signals propagated by Dectin-1 and -2 because the loss of cytokines seen with Dectin-2 blockade in Dectin-1–deficient cells could, at least in principle, be caused by lack of binding of fungal particles, which would then prevent triggering of any other Syk-coupled receptors. To address this possibility, we transduced Dectin-1–deficient BMDCs with a Y15F mutant form of Dectin-1 that allows restoration of zymosan binding without Syk signaling (Rogers et al., 2005). The transduced cells bound zymosan even more avidly than wild-type controls, and in this setting, the binding was only marginally decreased by Dectin-2 blockade (Fig. S7). Yet, production of IL-2, IL-10, and TNF was profoundly compromised by the same Dectin-2 blockade (Fig. 5 B). Indeed, anti–Dectin-2 blocked cytokine production by Y15F-transduced cells to the same extent as in Dectin-1–deficient

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mock-transduced cells (Fig. 5 B). Thus, anti–Dectin-2 nearly completely blocks Syk-dependent cytokine responses in a condition in which Dectin-1 is unable to signal but promotes a high degree of binding of fungal particles. This, in turn, argues against a major contribution from other signaling receptors and indicates that the requirement for Dectin-1 and -2 in Syk-dependent DC activation by fungi is caused by signals propagated by these receptors and not just by their ability to mediate binding of fungal particles.

Dectin-2 and -1 direct the Th cell response to fungal infection

In light of our in vitro results with DCs, we assessed the contribution of Dectin-2 to adaptive immunity to *Candida* infection

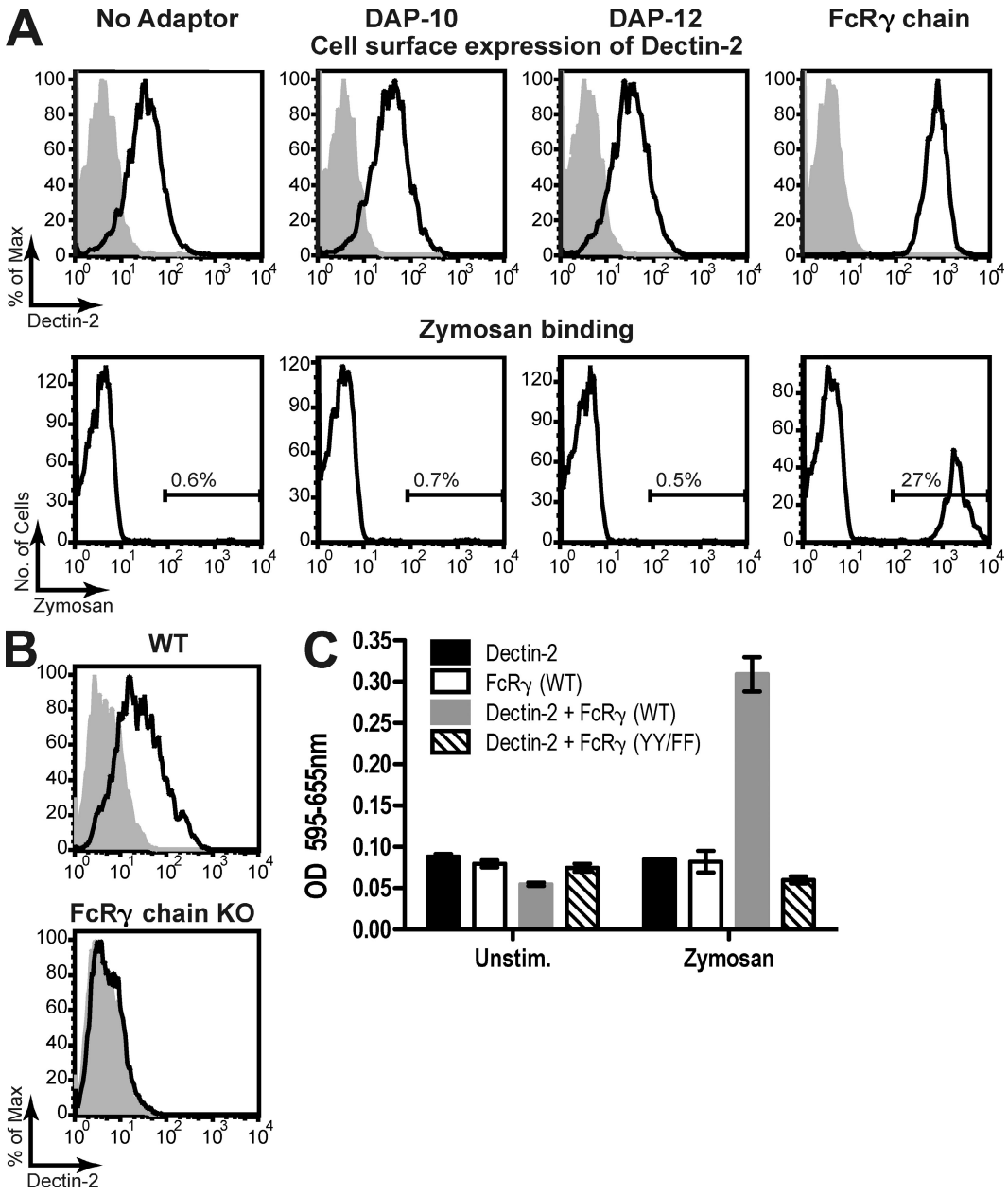

Figure 4. Dectin-2 requires FcRγ chain for surface expression and signaling. (A) B3Z cells (No Adaptor) or sublines derived by transduction with DAP-10, DAP-12, or FcRγ chain were subsequently transduced with Dectin-2–IRES–EGFP. (top) Anti–Dectin-2 staining of GFP-positive parental, DAP-10, DAP-12, or FcRγ chain (open histogram) cell lines compared with isotype control (shaded histogram). (bottom) Cy5-zymosan binding to the same cell lines. (B) BMDCs from C57BL/6 (WT) or *Fcer1g*$^{-/-}$ (FcRγ chain KO) mice stained with anti–Dectin-2 (open histogram) or an isotype-matched control mAb (shaded histogram). (C) B3Z cells expressing Dectin-2, wild-type FcRγ chain (FcRγ (WT)), Dectin-2 and FcRγ chain (Dectin-2 + FcRγ (WT)), or Dectin-2 and a signaling-deficient mutant of FcRγ chain (Dectin-2 + FcRγ (YY/FF)) were stimulated with or without 100 µg/ml zymosan. NFAT activity was measured using a β-galactosidase reporter. Data are means ± SD of duplicate wells (C), and are representative of two (C) or at least three (A and B) independent experiments.

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in vivo. As seen in vitro, anti–Dectin-2 antibody was able to promote clearance of Dectin-2 from the cell surface of DCs in vivo (unpublished data). Wild-type and Dectin-1–deficient mice were therefore injected with the mAb 6 h before and 2 and 4 d after systemic infection with a sublethal dose of *C. albicans* and were compared with mice injected with an isotype-matched irrelevant control mAb. Th1 and Th17 responses were monitored by measuring IFN-γ and IL-17, respectively, after restimulation of splenocytes with heat-killed *C. albicans* for 2 d (LeibundGut-Landmann et al., 2007). Remarkably, the Th17 response to *C. albicans* was markedly reduced in mice treated with anti–Dectin-2, independent of Dectin-1 deficiency (Fig. 7). This was seen even at a higher dose of *C. albicans* used for restimulation (Fig. S8 A), which indicates that, in this model, Dectin-2 is required for driving the Th17 response to the organism. In contrast, little effect of Dectin-2 blockade was apparent on the Th1 response to the same pathogen, although when anti–Dectin-2 was used in combination with Dectin-1 deficiency, a statistically significant decrease in IFN-γ production was observed in cells restimulated with the lower but not the higher dose of heat-killed *C. albicans* (Fig. 7 and Fig. S8 A). Splenocytes from Dectin-1–deficient mice given isotype-matched control mAb produced similar levels of IFN-γ and IL-17 upon restimulation as similarly treated wild-type animals (Fig. 7 and Fig. S8 A), consistent with previous data (LeibundGut-Landmann et al., 2007). Therefore, Dectin-2

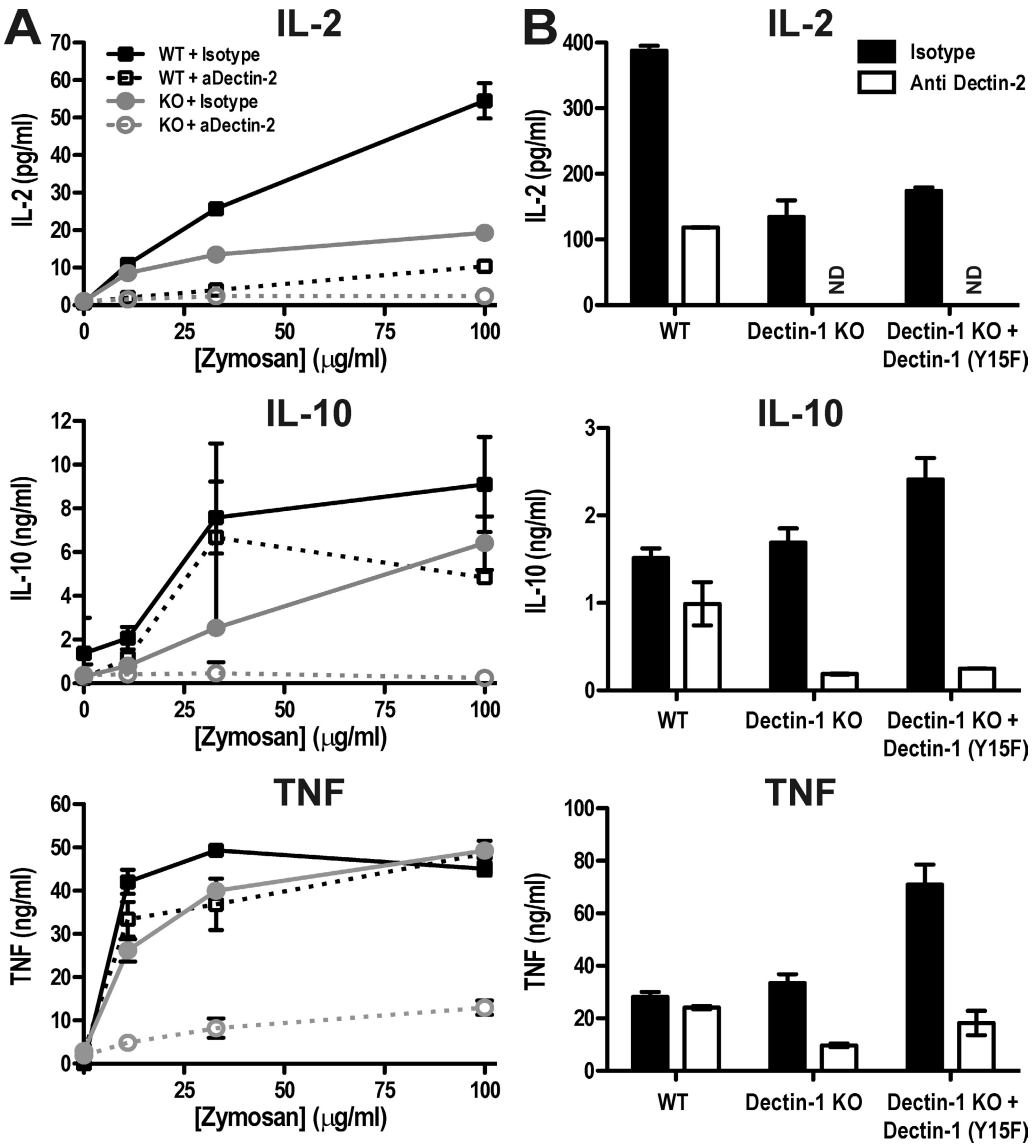

Figure 5. Dectin-1 and -2 mediate Syk-dependent responses to zymosan. (A) BMDCs from wild-type C57BL/6 (WT) or *Clec7a*$^{-/-}$ chimeric (Dectin-1 KO) mice were treated with 10 μg/ml anti–Dectin-2 or isotype-matched control mAb for 2 h before stimulation with increasing doses of zymosan overnight. (B) BMDCs from *Clec7a*$^{-/-}$ chimeric mice were transduced with signaling-deficient Dectin-1 (Dectin-1 KO + Dectin-1 (Y15F)) or mock transduced (Dectin-1 KO). These cells, as well as mock-transduced C57BL/6 BMDCs (WT) were then stimulated overnight with 33 μg/ml zymosan. Cytokines in the supernatants were quantified by ELISA. Data are means ± SD of duplicate wells and are representative of at least three independent experiments. ND, not detectable.

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is required for Th17 responses to *Candida* and, with Dectin-1, contributes to Th1 cell differentiation in response to the same organism.

Dectin-2 blockade does not impair innate resistance to *C. albicans* infection

Despite the marked impact of Dectin-2 blockade on Th17 responses to *C. albicans*, mice receiving anti–Dectin-2 showed similar kidney fungal burdens at day 7 after infection as mice receiving isotype-matched control antibody (Fig. S8 B). This was true even in mice deficient for Dectin-1 (Fig. S8 B). In addition, weight loss during the course of infection was also not affected by Dectin-2 blockade independently of Dectin-1 sufficiency or deficiency (Fig. S8 C). Therefore, in this model of systemic disease, Dectin-2 blockade does not impair resistance, and Dectin-2 may therefore be redundant for innate immunity to *C. albicans* infection.

DISCUSSION

In the broadest sense of the term, PRRs include all receptors that detect PAMPs (Janeway, 1989). However, a subset of PRRs possesses the ability to trigger intracellular signaling pathways that lead to expression of innate response genes, including those encoding chemokines and cytokines. These types of PRRs play a key role in orchestrating the innate and adaptive immune responses to infection, and have previously

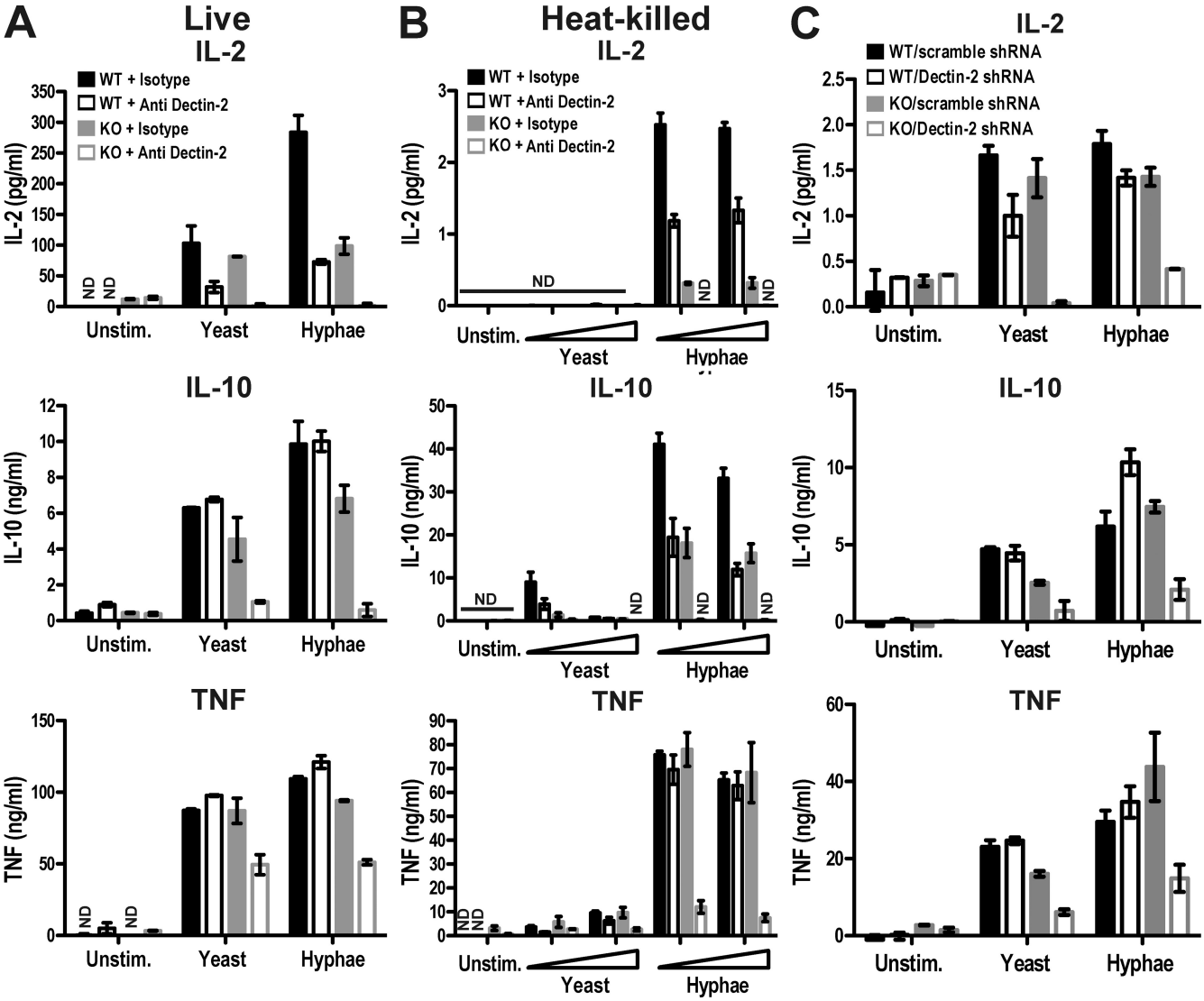

Figure 6. Dectin-1 and -2 mediate Syk-dependent responses to *C. albicans*. (A) BMDCs from wild-type C57BL/6 (WT) or *Clec7a*$^{-/-}$ chimeric (Dectin-1 KO) mice were treated with 10 µg/ml anti–Dectin-2 or isotype control for 2 h before stimulation with 10^5 live *C. albicans* yeast or hyphae. Fungizone was added 2 h after stimulation, and the cytokines levels in the supernatants were measured after overnight incubation. (B) BMDCs pretreated with mAbs as in A were stimulated overnight with 10^5 or 5×10^5 heat-killed *C. albicans*. (C) BMDCs from wild-type 129/Sv (WT) or *Clec7a*$^{-/-}$ (Dectin-1 KO) mice were infected with lentivirus encoding Dectin-2 shRNA or a scrambled control. After puromycin selection, cells were stimulated overnight with 10^5 heat-killed *C. albicans*. Data are means ± SD of duplicate wells and are representative of at least three (A and B) or two (C) independent experiments. ND, not detectable.

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been described as belonging to the TLR, RLR, and NLR families. The C-type lectin Dectin-1 constituted a notable exception, as it is not a member of any of these families yet it is able to induce immunity via a Syk and CARD9-dependent pathway. In this study, we identify Dectin-2 as an additional Syk–CARD9–coupled C-type lectin that mediates DC activation by fungi. Notably, we show that Dectin-2 accounts for the seemingly paradoxical Syk- and CARD9-dependent yet Dectin-1–independent aspects of DC activation by fungal stimuli. This is in contrast to most macrophages that respond to *C. albicans* primarily through Dectin-1 (Taylor et al., 2007) and to some types of DCs that may rely primarily on TLR signaling via TRIF to respond to the same organism (De Luca et al., 2007). The importance of Dectin-2 in DC function is underscored by the fact that in vivo blockade of Dectin-2 severely reduces Th17 responses to *C. albicans* infection and, in the context of Dectin-1 deficiency, affects Th1 immunity to the same organism. Thus, Dectin-2 constitutes a major receptor regulating T cell responses to fungi by engaging the Syk–CARD9 pathway in DCs.

The fungal PAMP recognized by Dectin-2 has not been fully identified. The C-type lectin domain of Dectin-2, unlike that of Dectin-1, possesses a classical carbohydrate recognition site that coordinates Ca^{2+} and specifically recognizes high mannose structures (McGreal et al., 2006). Binding of this domain to zymosan is blocked by mannose, fucose, or mannan, suggesting that the yeast ligand is probably a high-mannose–like structure such as mannan (McGreal et al., 2006). Both O- and N-linked yeast mannans possess immunostimulatory properties, and the activity of the former has been ascribed to its ability to engage TLR4 (Netea et al., 2006). In contrast, many innate receptors, including SIGNR-1, DC-SIGN, mannose receptor, and TLR2, have been proposed to bind structures within N-mannan (Jouault et al., 2003; Taylor et al., 2004; Cambi et al., 2008). Dectin-2 may fall in the same group, although competition studies suggest that the molecular pattern recognized by Dectin-2 is distinct from that bound by SIGNR-1 and mannose receptor (McGreal et al., 2006). Thus, different receptors may sense different forms of mannan, which could be differentially expressed during the fungal life cycle. Indeed, Sato et al. (2006) have shown that Dectin-2 preferentially binds the hyphal form of *C. albicans* and suggested that the ligand may not be expressed or could be concealed in the yeast form. Our results, however, show that, although hyphae induce the Dectin-1/2–dependent cytokines more strongly than yeasts, Dectin-2 is also involved in the sensing of the latter. Consistent with the notion that Dectin-2 can act as a yeast receptor, we have previously reported that the recombinant C-type lectin domain of Dectin-2 binds to zymosan and to the yeast form of *C. albicans* (McGreal et al., 2006). Finally, the PAMPs recognized by Dectin-2 may not be restricted to fungi, as this receptor appears to play a role in the response to house dust mite allergens (Barrett et al., 2009) and the extracellular domain of Dectin-2 is capable of binding bacterial polysaccharides (McGreal et al., 2006).

Stimulation through Dectin-2 defines a new mechanism for DC activation by a PRR. We show that Dectin-2 requires association with the FcRγ chain to be expressed on the surface of cells but, more critically, we provide evidence that the ITAM of this adaptor is required for signaling. Cross-linking of Dectin-2 triggered the activation of Syk and the MAPK cascades, leading to cytokine production. The latter process was dependent on CARD9, the adaptor necessary for induction of NF-κB downstream of Dectin-1 and other ITAM-signaling myeloid receptors (Gross et al., 2006; Hara et al., 2008). However, in contrast to Dectin-1 that binds Syk directly, Dectin-2 couples to Syk in trans, through association

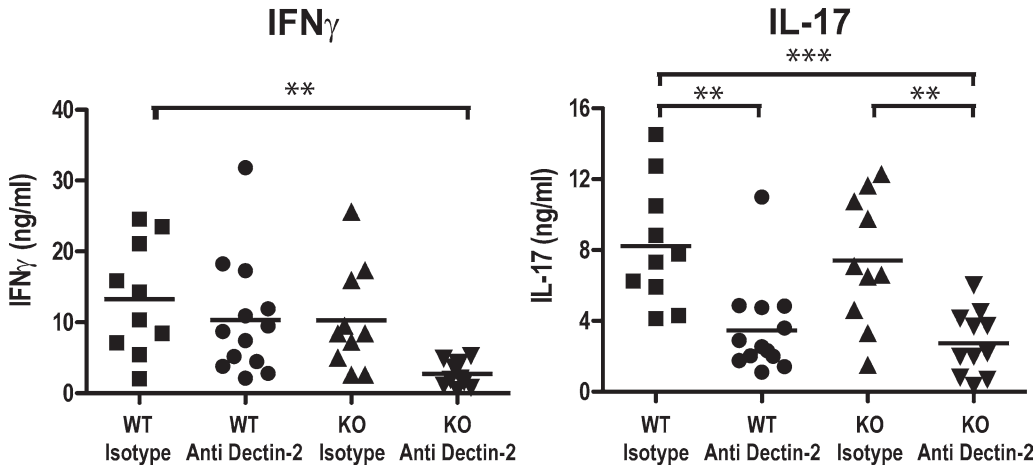

Figure 7. Dectin-1 and -2 are required during systemic *C. albicans* infection for subsequent splenocyte recall responses. Wild-type 129/Sv (WT) or *Clec7a*$^{-/-}$ (Dectin-1 KO) mice were given anti–Dectin-2 or isotype-matched control mAb intraperitoneally before and after i.v. *C. albicans* infection. After 7 d, splenocytes were restimulated with heat-killed *C. albicans* for 2 d, and IFN-γ and IL-17 accumulation in the supernatants was measured by ELISA. The data are pooled from two independent experiments, and each symbol represents the mean of triplicate stimulations from an individual mouse. Statistically different groups are indicated (**, $0.001 < P < 0.01$; and ***, $P < 0.001$).

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with FcRγ chain. In this regard, Dectin-2 behaves like lymphocyte antigen receptors and activatory Fc receptors, all of which engage Syk or ZAP-70 indirectly, through association with ITAM-bearing signaling chains (Isakov, 1997). Likewise, other myeloid receptors, including Mincle, MAIR-II, TREM-1, TREM-2, OSCAR, and DCAR, engage ITAM pathways through association with FcRγ chain or DAP12 (Bouchon et al., 2001; Kanazawa et al., 2003; Hara et al., 2007; Yamasaki et al., 2008). All of these receptors can be easily triggered by means of antibody cross-linking, as shown in this study for Dectin-2. In contrast, antibody cross-linking does not induce measurable Dectin-1–mediated cytokine responses. Thus, although our study highlights the equivalence of Dectin-1 and -2 in activating the Syk–CARD9 pathway, hemITAM and ITAM receptors may have distinct signaling requirements, which could lead to subtle differences in downstream responses not apparent in our study.

As shown in this paper, IL-2, IL-10, and TNF, the hallmark cytokines triggered by either Dectin-1 or -2 engagement in DCs, are the same cytokines that are critically dependent on Syk and CARD9 for induction in response to fungal stimuli. Why TLR signaling in response to agonists present in fungal particles (Hohl et al., 2006; Netea et al., 2008) is unable to mimic Syk-dependent signals in eliciting IL-2, IL-10, and TNF but can induce IL-6 and IL-12 p40 is at present unclear. This phenomenon may be DC subtype dependent, as TLR signaling via TRIF can also lead to IL-10 production by mesenteric lymph node DCs in response to *Candida* (De Luca et al., 2007). However, it is consistent with our previous study showing a marked dependence of zymosan-induced IL-2 and IL-10 on ERK, which was predominantly activated via Syk but not MyD88 (Slack et al., 2007). Also, *C. albicans* and zymosan activate NFAT via Dectin-1 and not via TLRs in macrophages, and this NFAT activation is required for IL-2 and IL-10 production (Goodridge et al., 2007). Therefore, a robust engagement of the ERK and NFAT pathways by Dectin-1 and -2 may underlie the induction of the Syk-signature cytokines.

It is important to note that IL-2, IL-10, and TNF may be only part of the Syk–CARD9 "signature" of DC activation by fungi, which will need to be investigated fully using genome-wide screens. Interestingly, a recent report indicates that, in DCs, Dectin-2/Syk signaling can additionally couple to the generation of cysteinyl leukotrienes (Barrett et al., 2009). The contribution of Dectin-2 to DC activation by fungi is not fully elucidated, but in vivo, it maps out at the level of the Th17 response to the organism. This follows from previous work in which we found that systemic infection with *C. albicans* induces potent Th1 and Th17 responses and that the latter but not the former are completely abrogated in CARD9-deficient mice (LeibundGut-Landmann et al., 2007). In this paper, we show that Dectin-2 is the major regulator of the Th17 response to systemic *Candida* infection and confirm earlier evidence that Dectin-1–deficient mice show little or no impairment in this response in the absence of concomitant Dectin-2 blockade (LeibundGut-Landmann et al., 2007). However, combined Dectin-2 blockade and Dectin-1 deficiency impaired the Th1 response to the organism, indicating that Dectin-1 is not fully dispensable for adaptive immunity to *Candida*. This is an identical phenotype to that seen previously in the CARD9-deficient mice (LeibundGut-Landmann et al., 2007), implying that Dectin-1 and -2 account for most of the CARD9-dependent adaptive immune response to *Candida* infection.

In contrast to Th1 and Th17 induction, Dectin-1 and -2 do not fully account for the CARD9-dependent innate immune response to *C. albicans*. Indeed, we observed no significant increase in renal fungal burden, even when Dectin-2 was blocked in Dectin-1–deficient mice, which indicates that Dectin-2 function may be redundant for antifungal resistance during the first week of systemic infection. This is consistent with previous data showing that fungal burden in the kidneys of Dectin-1–deficient mice infected i.v. with similar doses was significantly increased only in those mice that succumbed to the infection and not at these early time points (Taylor et al., 2007). In contrast, *Card9*$^{-/-}$ mice are highly susceptible to a similar dose of the fungus and have large increases in kidney mass and fungal burden as early as 4 d after infection (Gross et al., 2006). As it is believed that resistance to disseminated candidiasis in acute infection primarily reflects the activity of innate immunity (Romani, 2008), it seems likely that additional Syk–CARD9–coupled receptors are expressed by other immune cells, such as granulocytes, which are involved in the killing of *Candida* organisms during the acute phase of the antifungal response. A distinct question is whether the adaptive Th17 response to *Candida* infection is involved in protection from infection. In favor of that notion, a recent paper has shown that mice deficient in IL-23p19, a critical cytokine for Th17 responses, or in IL-17RA are very susceptible to oropharyngeal candidiasis (Conti et al., 2009). This is in agreement with earlier observations that IL-17RA–deficient mice are very susceptible to the organism (Huang et al., 2004). Notably, human memory CD4^{+} T cells specific for *C. albicans* are skewed toward Th17 (Acosta-Rodriguez et al., 2007; Zhou et al., 2008), and STAT3-mediated Th17 deficiency in humans is associated with increased susceptibility to *C. albicans* infection (Ma et al., 2008). However, Th17 responses have also been argued to contribute not to protection but to *Candida*-associated immunopathology (Zelante et al., 2007). In addition, we have recently found that IL-17 responses initiated by Dectin-1–activated DCs can involve FoxP3^{+} cells that, by other criteria, would be classified as regulatory and thought to have a down-modulatory effect on immunity (Osorio et al., 2008). Therefore, the protective role of type 17 immunity in fungal infection remains controversial (Zelante et al., 2009). It should be noted that any protective effect of Th17 immunity might only be apparent in a longer term follow up of animals infected with sublethal doses of *C. albicans*. Unfortunately, in the absence of a Dectin-2–deficient mouse, we are restricted to short-term experiment infection with mAb blockade such as described in this paper, in which any protective (or detrimental) effect of Th17 cells may be masked by innate resistance mechanisms.

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Dectin-1 was previously considered an exception as it does not belong to the three most prominent families of PRRs, namely the TLRs, NLRs, and RLRs. Given our present and past findings, we propose that Dectin-1 and -2 may represent a novel class of myeloid innate receptors characterized by Syk–CARD9 coupling that can function to initiate immunity. It is important to note that Dectin-1 and -2 possess structurally different C-type lectin domains, fall into different subgroups of the CLR family, and use markedly different strategies for engaging the Syk pathway. Therefore, members of this class of CLRs will need to be defined by reference to function rather than structure. A possible candidate to be included in this family is Clec5a, which has recently been shown to be a receptor for Dengue virus involved in the induction of proinflammatory cytokines, including TNF (Chen et al., 2008). Clec5a associates with DAP-12 (Bakker et al., 1999), an ITAM-bearing adaptor similar to the FcRγ chain, and therefore may link to Syk. In addition, Mincle, an inducible myeloid CLR, was recently implicated in macrophage sensing of *C. albicans* and *Malassezia* species and the subsequent induction of proinflammatory mediators (Wells et al., 2008; Yamasaki et al., 2009). Interestingly, Mincle has also been shown to associate with the FcRγ chain and signal via CARD9 in response to self-ligands such as the ribonucleoprotein SAP130 released by necrotic cells (Yamasaki et al., 2008). Notably, both Dectin-1 and -2 have also been reported to possess an endogenous ligand (Ariizumi et al., 2000b; Aragane et al., 2003), and DNGR-1, an additional hemITAM-bearing CLR, mediates Syk-dependent crosspriming responses to dead cells (Sancho et al., 2009). Thus, Syk-coupled CLRs may have additional functions not only as PRRs but also as innate receptors for initiating proinflammatory responses to sterile injury.

MATERIALS AND METHODS

Mice. Wild-type C57BL/6 and *Myd88*$^{-/-}$/*Trif*$^{-/-}$ mice were bred at Cancer Research UK in specific pathogen-free conditions. The bone marrow from *Clec7a*$^{-/-}$ mice (Taylor et al., 2007) and fetal liver cells from *Syk*$^{-/-}$ embryos were used to generate radiation chimeras in C57BL/6 hosts, as described previously (Rogers et al., 2005). *Card9*$^{-/-}$ mice (Gross et al., 2006) were bred at the Technische Universität München, and *Fcer1g*$^{-/-}$ (FcRγ chain knockout) mice (Park et al., 1998) were bred at the Leiden University Medical Center. All mice were on a C57BL/6 background with the exception of those used for knockdown and infection studies. For these, both *Clec7a*$^{-/-}$ and wild-type mice were on a 129/Sv genetic background with breeding, and the in vivo experiments were performed at Cardiff University. All animal protocols were approved by the London Research Institute Animal Ethics Committee or the Cardiff University Local Ethical Review Process, and were performed under the authority of the Animal Scientific Procedures Act 1986 (UK).

Cells, fungal stimuli, constructs, and antibodies. Culture medium was RPMI 1640 supplemented with glutamine, penicillin, streptomycin, 2-mercaptoethanol (all from Invitrogen), and 10% heat-inactivated fetal calf serum (Autogen Bioclear). Mouse BMDCs were generated using GM-CSF (Inaba et al., 1992) and purified from bulk cultures by magnetic selection with anti-CD11c microbeads. This routinely gave purities of >98%. Retroviral transduction of BMDCs has previously been described (LeibundGut-Landmann et al., 2007). Spleen cells were prepared by digestion with liberase and DNase, followed by enriching for conventional DCs by positive selection with anti-CD11c microbeads.

A single colony of *C. albicans* strain SC5314 was grown overnight at 30°C in yeast peptone dextrose media. The cells were washed twice with PBS before use as live yeasts or were heat-killed by boiling for 30–45 min. For the hyphal forms, the washed yeast were resuspended at 10^7 cells/ml in RPMI 1640 with 10% FCS and grown overnight. After washing in PBS, the hyphae were used for live stimulations or were heat killed as described. The morphology was verified by differential interference contrast microscopy using a 40× objective (Plan Fluor; Nikon; Fig. S2). Zymosan was purchased from InvivoGen and labeled with Cy5 as previously described (Rogers et al., 2005). Curdlan was purchased from Wako Chemicals USA, Inc.

The cDNA for FcRγ chain, DAP-10, and DAP-12 was amplified from either the RAW264.7 macrophage cell line or C57BL/6 bone marrow cells and cloned into the retroviral vector pMX-IP (a gift from T. Kitamura, University of Tokyo, Tokyo, Japan; Kitamura et al., 2003). The signaling-defective FcRγ chain was made by mutating tyrosines 65 and 76 to phenylalanine by standard molecular biology techniques and verified by sequencing. The full-length α isoform of Dectin-2 was cloned from wild-type BMDC cDNA by PCR, sequenced, and subcloned into pFB-IRES-EGFP (donated by R. Germain, National Institute of Allergy and Infectious Diseases, Bethesda, MD).

B3Z cells containing a reporter plasmid for NFAT coupled to β-galactosidase activity have been previously described (Karttunen et al., 1992) and were a gift from N. Shastri (University of California, Berkeley, Berkeley, CA). B3Z cells were retrovirally transduced with the pMX-IP vectors separately and selected under 10 μg/ml puromycin (Sigma-Aldrich) for at least 7 d. These lines were subsequently retrovirally transduced with pFB–Dectin-2–IRES–EGFP or the empty vector. For functional assays, the cells were FACS sorted based on EGFP expression.

The anti–Dectin-2 mAb, clone D2.11E4 (Taylor et al., 2005), and a rat IgG2a isotype-matched control (BD) were used in vitro. For the in vivo model of *C. albicans* infection, the rat IgG2a clone OX11 was used as the isotype-matched control. F(ab′) preparations of D2.11E4 and control antibody (Y13-259, a rat IgG1 raised against p21 V-ras; CRUK Monoclonal Antibody Service) were made using immobilized papain digestion followed by protein G depletion of Fc and undigested antibody (Thermo Fisher Scientific). All immunoblotting antibodies were rabbit polyclonal antibodies purchased from Cell Signaling Technology, with the exception of those against phospho-JNK (Promega) and total Syk (Turner et al., 1995).

DC stimulations. For analysis of cytokine production, 10^5 BMDCs were cultured overnight in a 96-well U-bottomed plate with 10^5 live *C. albicans*, 10^5 or 5×10^5 heat-killed *C. albicans*, or increasing doses of zymosan. In blocking experiments, the BMDCs were preincubated for 2 h with 10 μg/ml D2.11E4 or isotype-matched control mAb before addition of the stimuli. 2.5 μg/ml fungizone (Invitrogen) or 50 ng/ml caspofungin (Merck) was added to the cultures 2 h after live *C. albicans* stimulation. Cytokine levels in the supernatant were measured by sandwich ELISA.

For stimulation with plated antibodies, UV-irradiated MaxiSorp plates (Thermo Fisher Scientific) were coated overnight with 40 μg/ml goat $F(ab')_2$ anti–rat $F(ab')_2$ (Jackson ImmunoResearch Laboratories) in PBS. After washing, the plates were incubated with 10 μg/ml of control or anti–Dectin-2 F(ab′) for 1–3 h at 37°C before washing with PBS. 2×10^5 BMDCs were incubated on the plate overnight and cytokines were measured as described.

Immunoblotting. BMDCs were stimulated by plated F(ab′) as described, except that 2.5×10^5 cells were spun at 233 *g* onto the coated plate. At the times after centrifugation indicated in the figures, the medium was removed and the cells were lysed for 30 min on ice in RIPA buffer (50 mM Tris [pH 7.5], 1% Nonidet-P40, 0.5% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, and a mixture of protease inhibitors; Roche). Insoluble material was discarded and a fixed amount of lysate was mixed with sample buffer (LeibundGut-Landmann et al., 2007) before separation by SDS-PAGE. After transfer to a polyvinyldifluoride membrane (Millipore), proteins were analyzed by immunoblotting and visualized by enhanced chemiluminescence (Thermo Fisher Scientific).

FACS staining, zymosan binding, and B3Z cell stimulation. Cell suspensions were stained in ice-cold FACS wash (PBS containing 5mM EDTA and 1% FCS), which was further supplemented with 1% normal mouse serum when staining for Dectin-2. BMDCs, splenic DCs, and B3Z cells were stained with 10 μg/ml D2.11E4, 2A11 (anti–Dectin-1), or isotype-matched control mAb for 30 min, followed by anti–rat IgG-PE (Jackson ImmunoResearch Laboratories) and, in the case of DCs, anti–CD8-FITC, anti–CD4-PerCP, and/or anti–CD11c-allophycocyanin (BD).

For zymosan binding experiments, 2×10^5 B3Z cells or 10^5 BMDCs were pretreated or not for 2 h at 37°C with 10 μg/ml anti–Dectin-2 or an isotype-matched antibody control. After cooling on ice, the cells were incubated with 10–100 μg/ml Cy5-zymosan for 10–90 min and washed three times with ice-cold media before flow cytometric analysis.

10^5 B3Z cells/well in a 96-well plate were incubated overnight with 100 μg/ml zymosan. LacZ activity was measured by lysis in CPRG-containing buffer (Roche). OD 595 was measured using OD 655 as a reference.

Lentiviral knockdown. The shRNA construct for mouse Dectin-2 in a pLKO.1 lentiviral vector (TRCN0000066785; Open Biosystems) was used for this study based on a previous publication (Barrett et al., 2009). A scrambled shRNA sequence was used as the control. Infectious viral particles were generated after cotransfection of 293 T cells with the PLKO.1 construct, the packaging vector Δ8.9, and the envelope vector VSV-G.

10^5 bone marrow precursors/well in complete media containing GM-CSF were seeded into a 96-well plate. On day 2, the media was removed and replaced by the viral stocks in the presence of 8 μg/ml hexadimethrine bromide. The cells were centrifuged at 1,130 *g* for 90 min, after which the virus was replaced by fresh media. 5 μg/ml puromycin was added on day 4. Cells were harvested on day 6 and stimulated as described. In each experiment, Dectin-2 expression was analyzed by flow cytometry and the median fluorescence was reduced by at least 50% as compared with the scrambled control.

Systemic *C. albicans* infection model. Female mice aged 12–16 wk were injected with 200 μg D2.11E4 or control mAb, OX11, intraperitoneally. After 6 h, the mice were infected i.v. with 3×10^4 live *C. albicans* yeast. The mice were further injected with mAbs 2 and 4 d after infection. After a total of 7 d, the mice were sacrificed. Total splenocytes were harvested and re-stimulated at 2×10^6 cells/well in a 96-well U-bottomed plate with 10^5 heat-killed *C. albicans* for 2 d. ELISA was used to measure the IL-17 and IFN-γ levels in the supernatant. The kidneys were removed and disrupted in 0.5 ml PBS using a Dounce homogenizer. Serial dilutions were plated on agar plates, and the total number of colonies was counted and calculated back to colony forming units.

Statistical analysis. Statistical significance of the infection study was determined by one-way analysis of variance with Tukey multiple comparison of all pairs for posttest analysis.

RNA isolation and quantitative RT-PCR. Total RNA from BMDCs was extracted 3 h after stimulation with live yeast or hyphae and prepared with an RNeasy Mini Kit (QIAGEN). cDNA was synthesized from total RNA with random hexamer primers and Superscript II RT (Invitrogen). Quantitative real-time PCR was performed using SYBR green incorporation. Measurements were performed in duplicate wells using a sequence detection system (ABI PRISM 7700; Applied Biosystems). Normalization was performed against HPRT and results are shown as relative mRNA quantities.

Online supplemental material. Fig. S1 demonstrates that zymosan induction of cytokines from DCs is dependent on Syk but not on MyD88, TRIF, or Dectin-1. Fig. S2 shows the morphology of the *C. albicans* preparations and demonstrates that the use of fungizone or caspofungin, two distinct antifungals, does not substantially alter the Syk-dependent cytokine response to *C. albicans* or to curdlan. Fig. S3 depicts Dectin-2 expression on conventional DC subpopulations. Fig. S4 demonstrates the requirement for FcRγ chain in anti–Dectin-2 triggering of cytokine production. In Fig. S5, cell-surface expression of Dectin-2 and the subsequent binding of fluorescent zymosan is similarly enhanced by expression of either wild-type or an ITAM-mutated FcRγ chain. Fig. S6 demonstrates that combined loss of Dectin-1 and blockade of Dectin-2 abolishes the up-regulation of IL-23p19 mRNA by heat-killed *C. albicans*. Fig. S7 shows that both Dectin-1 and -2 contribute to the binding of fluorescent zymosan by BMDCs and that this is increased by transduction of the signaling-defective Dectin-1. Fig. S8 shows that anti–Dectin-2 mAb treatment of *C. albicans*–infected mice, but not Dectin-1 deficiency, compromises IL-17 but not IFN-γ production in an assay in which splenocytes are restimulated with a high dose of C. *albicans*. It further shows that the kidney fungal burden and mouse weight loss are not significantly different in wild-type and Dectin-1–deficient mice with and without anti–Dectin-2 treatment early after *C. albicans* infection. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082818/DC1.

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Frontline

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DC activated *via* dectin-1 convert Treg into IL-17 producers

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Th cells producing IL-17 play a pro-inflammatory role at mucosal surfaces. Treg at the same sites dampen inflammation and prevent immunopathology. Th cells producing IL-17 (Th17) and Treg are thought to be distinct populations defined by expression of the transcription factors ROR-γt and Foxp3, respectively. Here, we show that mouse CD25$^+$Foxp3$^+$ Treg can be converted into a hybrid T-cell population characterized by the expression of Foxp3 and ROR-γt and the production of IL-17. Conversion was observed upon coculture with DC selectively activated *via* dectin-1, a C-type lectin receptor involved in fungal recognition, and depended on IL-23 produced by DC. Within the Foxp3$^+$ population, only Foxp3$^+$ROR-γt$^+$ T cells but not Foxp3$^+$ROR-γt$^-$–T cells become Foxp3$^+$IL-17$^+$ T cells. These results indicate that some Foxp3$^+$ T cells can produce IL-17 while retaining Foxp3 expression and suggest that Treg could play an unexpected pro-inflammatory role in some settings.

Key words: DC · Dectin-1 · Th17 · Treg

Introduction

Our immune system identifies pathogens in part by recognizing signatures present in microbes, so-called PAMP, *via* specific germline encoded PRR [1]. Recognition of PAMP by PRR leads to an immediate innate response designed to contain infection and may also result in the mobilization of adaptive defense mechanisms. The translation of PAMP presence into adaptive immunity is carried out by specialized leukocytes called DC and results in distinct responses matched to the nature of the offending microbe. For CD4$^+$ T cells, infection with intracellular bacteria tends to induce responses dominated by Th1 cells, whereas extracellular parasites often promote Th2-biased responses. Some bacteria and fungi promote a third type of response defined by the induction of Th cells producing IL-17 (Th17) [2–4]. Th17 cells are pro-inflammatory cells characterized by the expression of IL-17A, IL-17F, IL-21, IL-22, IL-23R and the transcription factors ROR-γt and ROR-α [5–7]. They have been implicated in several models of autoimmunity [8–11] but under normal conditions may play a role in protection from infection at mucosal surfaces [2]. IL-6 and TGF-β are key cytokines required for lineage commitment of murine Th17 [2, 8, 12] whereas IL-23 is a critical factor for sustaining these cells and is required for the acquisition of their pathogenic function *in vivo* [9, 13].

Treg are important regulators of potentially detrimental responses against normal self-constituents or commensal microbes and loss of Treg function leads to autoimmune and inflammatory diseases [14, 15]. Treg are characterized by the expression of CD25 and Foxp3, the latter being a transcription

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factor required for the maintenance of the Treg lineage [15, 16]. Like Th1, Th2 and Th17 cells, Foxp3$^+$ Treg can be generated in the periphery from newly activated CD4$^+$ T cells. The differentiation pathways of such inducible Treg and those of Th17 cells are closely related as both processes require the presence of TGF-β [8]. Notably, it has recently been found that fully differentiated Treg can themselves be converted into Th17 cells in mice [17–19] and humans [20]. This process is accompanied by extinction of Foxp3 expression and upregulation of ROR-γt [18]. Interestingly, some cells in the mouse can coexpress Foxp3 and ROR-γt [21, 22] and could represent an intermediate in this process or, alternatively, an intermediate cell in the differentiation of naïve T cells into Th17/Treg. Foxp3$^+$ROR-γt$^+$ cells behave as *bona fide* Treg, like their ROR-γt$^-$ counterparts, and produce IL-10 but not IL-17 [22]. Thus, the available data suggest that, although Foxp3 and ROR-γt can be coexpressed in CD4$^+$ T cells, Foxp3 expression and IL-17 production are mutually exclusive. This has led to the notion that regulatory and inflammatory T-cell programs are antagonistic [18, 21].

Dectin-1 is a Syk-coupled C-type lectin receptor for β-glucans involved in the innate recognition of fungi and some bacteria. Activation of the dectin-1 pathway in DC leads to the generation of Th17 cells both *in vitro* and *in vivo* [4]. Here, we show that dectin-1-activated DC can directly interact with CD25$^+$Foxp3$^+$ Treg and instruct them to become IL-17-producers. Notably, in contrast to previous studies, we find that such Treg do not extinguish the expression of Foxp3. Instead, they become a population of Foxp3$^+$IL-17$^+$ T cells that defies classification as either Treg or Th17 and could play an important role in inflammation.

Results

CD4$^+$CD25$^+$ T cells become IL-17 producers in response to curdlan-stimulated DC

We have previously reported the induction of Th17 cells in cocultures of CD4$^+$ T cells and DC stimulated with the dectin-1 agonist, curdlan [4]. The emergence of a substantial fraction of IL-17-producing cells required the presence of both CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells [4]. To further investigate the role of CD4$^+$ CD25$^+$ cells, we purified naïve (CD4$^+$CD25$^-$CD62L^{hi}CD44^{lo}) or CD4$^+$CD25$^+$ T cells from C57BL/6 mice and cultured each population individually with BM-derived DC (BMDC) plus α-CD3 mAb in the presence of curdlan or CpG (a TLR9 agonist). In cultures in which naïve T cells were the starting population, inclusion of CpG induced development of T cells that preferentially produced the Th1 cytokine IFN-γ rather than IL-17 upon restimulation (Fig. 1A and B). The inverse was observed in the presence of curdlan (Fig. 1A and B), as previously reported [4]. Notably, in CD4$^+$CD25$^+$ T-cell cultures, neither curdlan nor CpG led to the development of IFN-γ-producing cells but curdlan promoted the appearance of a large frequency and absolute number of cells able to produce IL-17 upon restimulation (Fig. 1A–C). This was accompanied by greater expansion of total CD4$^+$CD25$^+$ T cells in curdlan-containing cultures when compared with cultures containing CpG or lacking any innate stimulus (Fig. 1C), suggesting that factors produced by DC in response to curdlan favor proliferation and/or survival of CD4$^+$CD25$^+$ T cells and induce development of an IL-17-producing population.

Curdlan-activated DC induce the generation of Foxp3$^+$IL-17$^+$ T cells

CD4$^+$CD25$^+$ T cells include activated T cells as well as a large population of Treg expressing Foxp3 [23]. We therefore set up cultures with a pure Foxp3$^+$ T-cell population isolated by cell sorting from depletion of regulatary T-cell (DEREG) mice (or from radiation chimeras bearing BM from DEREG mice), which express GFP fused to the primate diphteria toxin receptor under the control of *foxp3* gene regulatory regions [24]. As for the total CD4$^+$CD25$^+$ T cells used previously, CD25$^+$GFP$^+$ DEREG T cells proliferated more extensively when curdlan was included in the cultures than when no innate stimulus was present (Fig. 2A). To account for a possible role of proliferation, we compared curdlan with IL-2 (Fig. 2A, Treg control), which acts as a Treg mitogen, and independently analyzed the blast and the resting T-cell population for expression of Foxp3 and the ability to produce IL-17 upon restimulation. Blast and non-blast cells were selected by forward- and side-scatter criteria, whereas Foxp3 expression was assessed by nuclear staining with a specific mAb to directly measure the presence of the protein at the time of analysis. As expected, we observed Foxp3$^+$IL-17$^-$ cells in Treg control cultures and Foxp3$^-$IL-17$^+$ cells in Th17 control cultures. However, in curdlan-containing cultures of Foxp3$^+$ T cells and DC, we observed an accumulation of Foxp3$^+$IL-17$^+$ double-positive cells, which were especially noticeable in the blast fraction (Fig. 2B). T cells producing IL-17 and coexpressing Foxp3 continued to accumulate over time in curdlan-containing cultures and represented one-fourth of the cells by day 7 (Fig. 2C). At both time points, we detected the presence of transcripts associated with Th17 cell fate such as ROR-γt, IL23R and, to a lesser extent, IL-17F (Fig. 2D). Foxp3 transcripts were also found in these cultures, consistent with the staining data (data not shown). We conclude that, in response to BMDC stimulated with curdlan, Foxp3$^+$ cells can give rise to a T cell capable of coexpressing Foxp3 and IL-17, which constitutes a distinct phenotype from classical Th17 or Treg.

Foxp3$^+$ROR-γt$^+$ but not Foxp3$^+$ROR-γt$^-$ T cells become Foxp3$^+$IL-17$^+$ T cells

Foxp3$^+$ Treg are heterogeneous and include a fraction that coexpresses ROR-γt and produce little or no IL-17 [21, 22]. Given the importance of ROR-γt in differentiation of conventional Th17 CD4$^+$ T cells [5], we wondered whether Foxp3$^+$ROR-γt$^+$

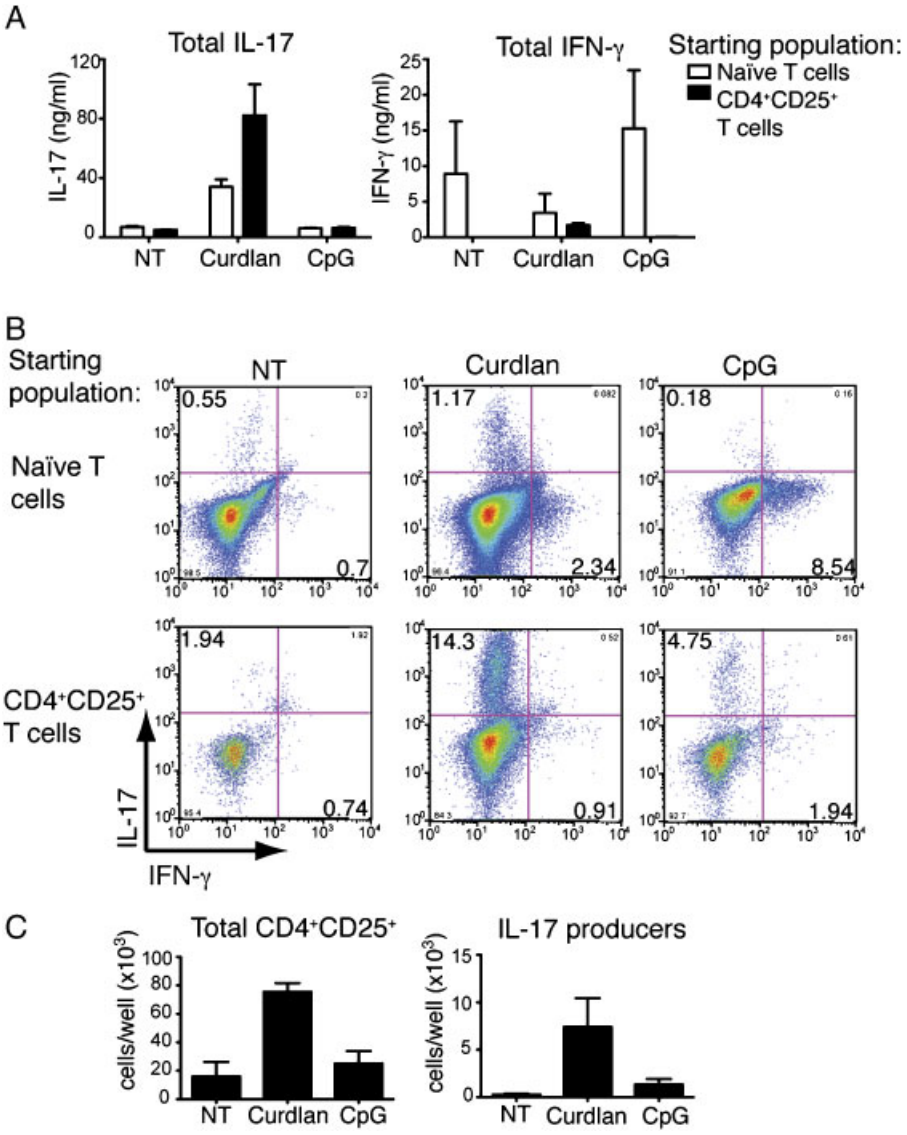

Figure 1. Curdlan-stimulated DC promote IL-17 production by $CD4^+CD25^+$ T cells. (A) FACS-sorted naïve T cells or $CD4^+CD25^+$ T cells from C57BL/6 mice were cocultured for 5 days with BMDC and soluble α-CD3 in medium alone or in the presence of curdlan or CpG. Half of the content of each well was restimulated on day 5 for 48 h with coated α-CD3 and cytokine production was determined by sandwich ELISA. (B) same as part A, but cells were restimulated on day 5 with PMA, ionomycin and brefeldin A for 4 h and the presence of intracellular cytokines was analyzed by flow cytometry. Data show IFN-γ and IL-17 after gating on $CD4^+$ cells. (C) Total numbers of $CD4^+IL\text{-}17^+$ T cells or $CD4^+$ T cells obtained on day 5 in cultures containing $CD4^+CD25^+$ T cells. Graph shows mean+SEM of three independent experiments. Data are representative of two to six independent experiments. N.D., not done.

cells might represent the source of $Foxp3^+IL\text{-}17^+$ double-positive cells. We therefore compared ROR-γt^+ and ROR-γt^- Treg isolated from chimeric mice reconstituted with BM from *Rorc(γt)-Gfp*^{TG} mice, in which GFP reports ROR-γt expression [22]. To obtain Treg from those mice, we sorted cells based on the expression of CD4, CD25 and folate receptor 4, which closely mirrors Foxp3 expression [25]. We confirmed that the majority of sorted $CD25^+FR4^+$ $CD4^+$ T cells express Foxp3 (Fig. 3A). We then prepared donor origin $CD25^+FR4^+$ T cells from *Rorc(γt)-Gfp*^{TG} chimeric mice, which were sub-divided into GFP^+ and GFP^- fractions and separately cultured with BMDC and α-CD3 in the presence of curdlan (Fig. 3B). Notably, only $CD25^+FR4^+GFP^+$ and not $CD25^+FR4^+GFP^-$ fractions were able to generate IL-17-producing cells (Fig. 3B). The majority of these cells coexpressed Foxp3 (Fig. 3B) as observed in experiments with DEREG T cells (see above). In addition, a proportion of $IL\text{-}17^+$ cells were negative for Foxp3 (Fig. 3B), probably reflecting expansion of contaminating ROR-γt^+Foxp3^- cells present in the starting population, which correspond to Th17 cells (Fig. 3A). We conclude that the $Foxp3^+$ROR-γt^+ but not the $Foxp3^+$ROR-γt^- subset can be induced to produce IL-17 after culture with DC in the presence of a dectin-1 agonist.

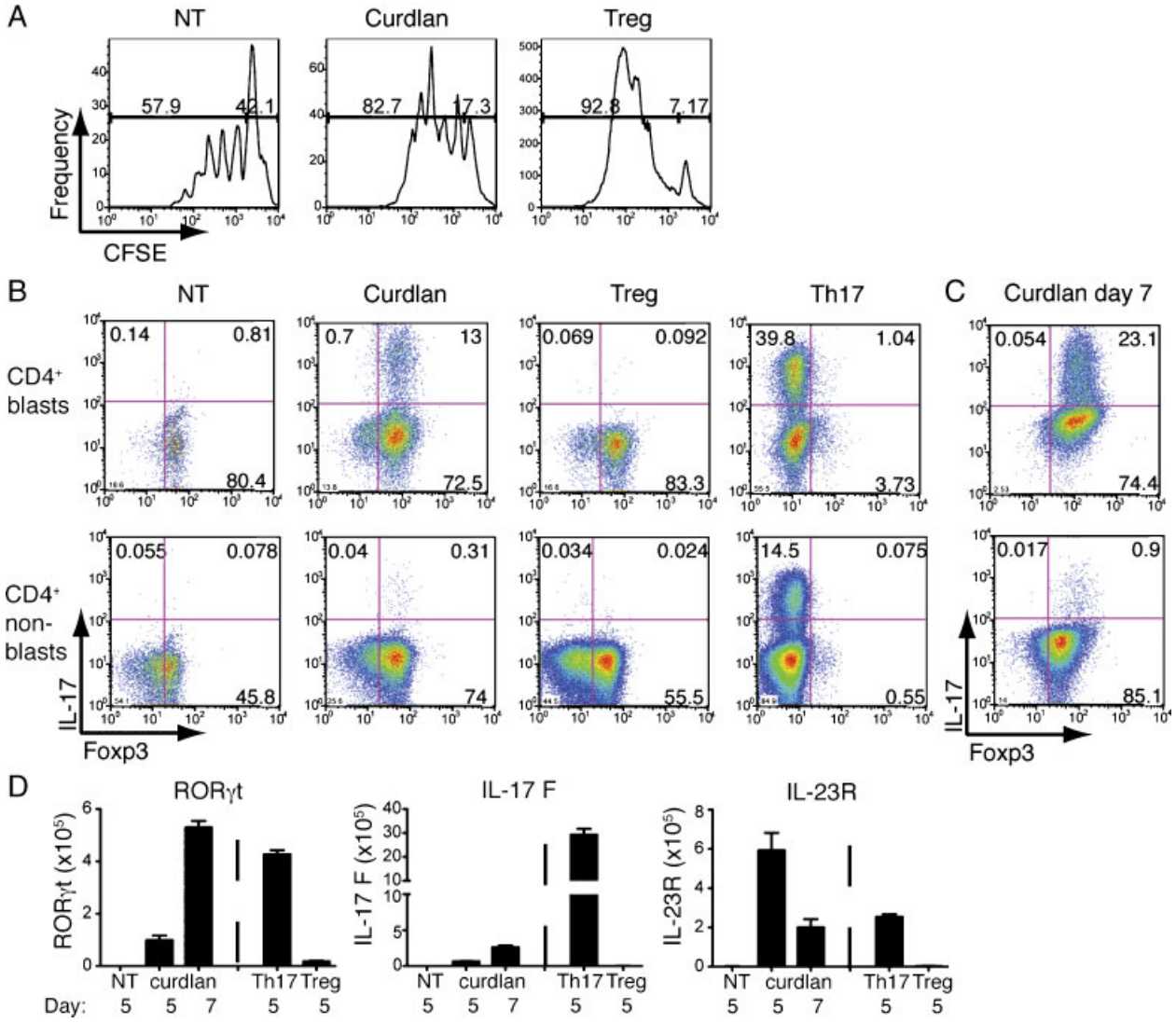

Figure 2. Curdlan-stimulated DC induce the generation of Foxp3$^+$IL-17$^+$ T cells. (A) CFSE-labeled CD4$^+$CD25$^+$GFP$^+$ DEREG T cells were cultured for 5 days with wild-type BMDC and soluble α-CD3 in the absence (NT) or presence of curdlan or rhIL-2 ("Treg"), as indicated. Plots show CFSE profile after gating on CD4$^+$ cells. (B) As in (A) but with the addition of a "Th17" control consisting of CD4$^+$GFP$^-$CD25$^-$ T cells cultured with BMDC, α-CD3 and IL-6+TGF-β in the presence of neutralizing antibodies to IFN-γ and IL-4. All cells were restimulated on day 5 with PMA, ionomycin and brefeldin A for 4 h and the expression of Foxp3 and IL-17 was analyzed by gating separately on CD4$^+$ cell blasts or non-blasts by flow cytometry. (C) Analysis of parallel cultures from part B on day 7. (D) RT-PCR analysis of transcripts for ROR-γt, IL-17F and IL-23R in the indicated cultures at 5 and 7 days. Data are representative of two to six independent experiments.

IL-23 drives the generation of Foxp3$^+$IL-17$^+$ T cells

We have previously shown that curdlan stimulation of DC leads to production of IL-23 but not IL-12 p70 [4]. To address the role of IL-23 in the generation of Foxp3$^+$IL-17$^+$ T cells, we cultured IL-23-deficient ($p19^{-/-}$) or wild-type BMDC with Foxp3$^+$ T cells from DEREG mice in the presence of curdlan. Notably, the frequency and number of Foxp3$^+$IL-17$^+$ T cells was markedly decreased when $p19^{-/-}$ BMDC were used as APC (Fig. 4A and B) while CD4$^+$ T-cell expansion was unaffected (Fig. 4B). The appearance of ROR-γt and IL-23R transcripts in curdlan-containing cultures was also IL-23 dependent (Fig. 4C). When curdlan was replaced with IL-23, we obtained similar frequencies of Foxp3$^+$IL-17$^+$ T cells (Fig. 4D) but T-cell expansion was reduced, resulting in lower numbers of Foxp3$^+$IL-17$^+$ T cells at the end of the culture (Fig. 4E). These data suggest that IL-23 is necessary but not sufficient for maintaining the phenotype and/or survival of Foxp3$^+$IL-17$^+$ T cells.

Discussion

Innate signals play a key role in determining the class of adaptive immune response against infection. In this study, we have shown that innate stimulation *via* the dectin-1 pathway allows DC to

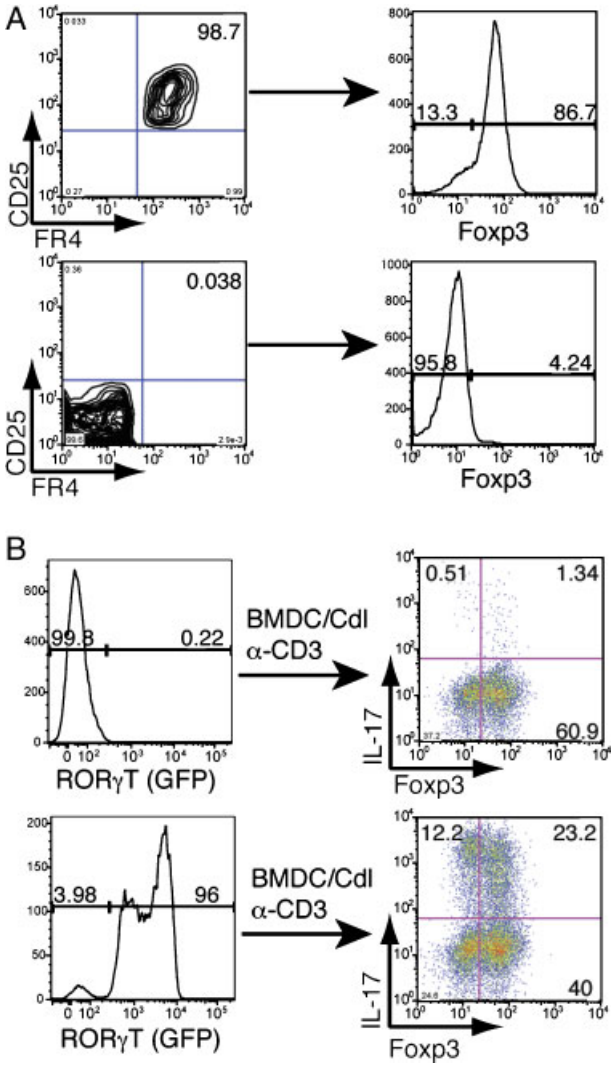

Figure 3. Foxp3$^+$ROR-γt$^+$ but not Foxp3$^+$ROR-γt$^-$ T cells become Foxp3$^+$IL-17$^+$ T cells in response to curdlan-activated BMDC. (A) FACS-sorted CD4$^+$CD25$^+$FR4$^+$ and CD4$^+$CD25$^-$FR4$^-$ T cells from C57BL/6 mice stained for intracellular Foxp3. (B) FACS-sorted CD4$^+$CD25$^+$FR4$^+$GFP$^+$ and CD4$^+$CD25$^+$FR4$^+$GFP$^-$ T cells from *Rorc*(γt)-*Gfp*^{TG} chimeras were cocultured with wild-type BMDC and soluble α-CD3 in the presence of curdlan. Cells were restimulated on day 5 with PMA, ionomycin and brefeldin A for 4 h and the expression of Foxp3 and IL-17 on CD4$^+$ cell blasts was analyzed by flow cytometry. Data are representative of two independent experiments.

instruct CD25$^+$Foxp3$^+$ T cells to become Foxp3$^+$IL-17$^+$ T cells. This process is distinct from the differentiation of naïve T cells in curdlan-containing cocultures [4], which generates Th17 cells that do not express Foxp3 (data not shown). Previous reports have demonstrated that Foxp3$^+$ T cells can be converted into Th17 cells *in vitro* and *in vivo* under certain conditions [17–20]. In those studies, Foxp3$^+$ T cells may pass through a transient stage characterized by the coexpression of Foxp3 and IL-17 before downregulating Foxp3. A distinct hallmark of the cells described in this study is that they maintain or even increase the expression of Foxp3 while acquiring the ability to produce IL-17.

Dectin-1 signaling in DC induces the production of several cytokines, including IL-2, IL-6, IL-10, TNF-α and IL-23 [4]. We have shown that IL-23 is essential to induce but not sufficient to sustain the generation of Foxp3$^+$IL-17$^+$ T cells. IL-23 might act synergistically with IL-6 and TNF-α, both of which contribute to the generation of Th17 cells [12]. Dectin-1-activated DC also produce IL-2, which could contribute to the generation of Foxp3$^+$IL-17$^+$ T cells. Indeed, although IL-2 acts as an inhibitor of Th17 differentiation from naïve CD4$^+$ T cells [26], it potentiates the expression of Foxp3 in Treg [27]. We propose that the unique balance of cytokines induced in DC by dectin-1 stimulation may be particularly suited to the generation of Foxp3$^+$IL-17$^+$ T cells, with IL-23, TNF-α and IL-6 favoring IL-17 production and IL-2 sustaining Foxp3 expression. IL-10, which is also produced at high levels by DC stimulated *via* the dectin-1/Syk pathway [4, 28, 29], could potentially help maintain Treg markers and/or Treg activity.

Foxp3$^+$IL-17$^+$ T cells arise from the Foxp3$^+$ROR-γt$^+$ Treg subset and not the Foxp3$^+$ROR-γt$^-$ population, indicating that the Foxp3$^+$ROR-γt$^+$ population possesses the ability to produce IL-17 under certain conditions of innate immune challenge. In turn, this implies that Foxp3$^+$ T cells are not being genetically reprogrammed to become IL-17 producers but that the appropriate cytokine milieu can trigger IL-17 production in cells that already express the genetic machinery required to perform that function.

The fact that conditions of innate stimulation with fungal products favor the development of a novel population of Foxp3$^+$IL-17$^+$ cells may have important implications. Fungal infections have been linked to IL-17 responses [4] and IL-17-deficient mice are more susceptible to infection with *Candida albicans* [30]. In addition, human memory CD4$^+$ T cells specific for *C. albicans* are skewed toward Th17 [3, 31] and STAT3-mediated Th17 deficiency in humans is associated with increased susceptibility to *C. albicans* infection [32]. Whether Foxp3$^+$IL-17$^+$ T cells participate in anti-fungal immunity or whether they retain regulatory activity that could counteract the detrimental effects of overexuberant Th17 responses [33] remains to be determined. Consistent with the latter possibility, it has previously been reported that DC activated by yeast particles induce T cells with regulatory activity [29]. Notably, small numbers of cells coexpressing Foxp3, ROR-γt and IL-17 have been previously noticed in mouse gut lamina propria [21]. By developing techniques for enriching and isolating Foxp3$^+$IL-17$^+$ T cells from animals and patients, we hope that multiple questions regarding their role can begin to be addressed.

Materials and methods

Mice

C57BL/6 mice were obtained from Charles River. DEREG mice [24] were bred at Technische Universität München or at Cancer

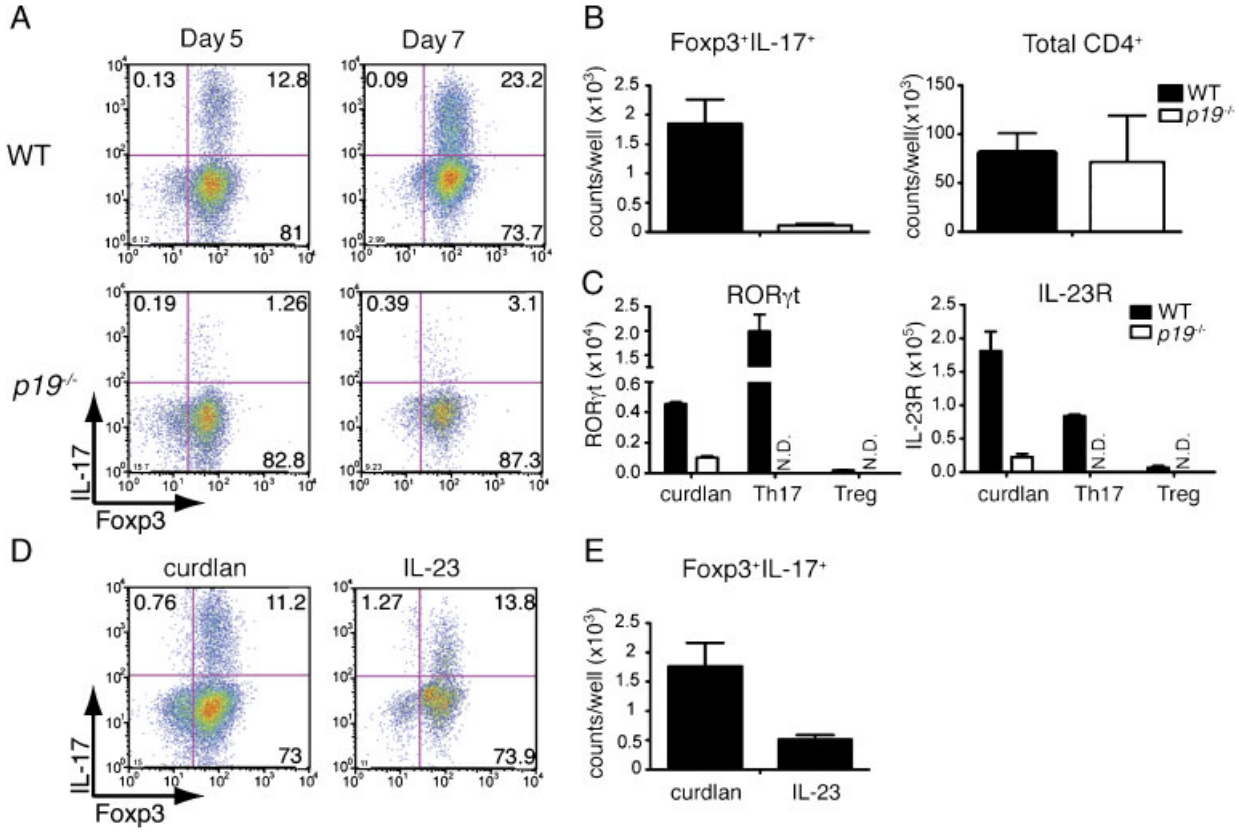

Figure 4. IL-23 drives the generation of Foxp3$^+$IL-17$^+$ T cells in response to curdlan-activated BMDC. (A) FACS-sorted CD4$^+$CD25$^+$GFP$^+$ DEREG T cells were cultured with wild-type or *p19*$^{-/-}$ BMDC plus soluble α-CD3 in the presence of curdlan. Cells were restimulated on days 5 and 7 with PMA, ionomycin and brefeldin A for 4 h and the expression of Foxp3 and IL-17 on CD4$^+$ cell blasts was analyzed by flow cytometry. (B) Total numbers of Foxp3$^+$IL-17$^+$ T cells and CD4$^+$ T cells from cultures as in (A) on day 5. (C) RT-PCR analysis of transcripts encoding ROR-γt, and IL-23R in cultures as in part A and analyzed on day 5. (D) CD4$^+$CD25$^+$GFP$^+$ DEREG T cells were cultured for 5 days with wild-type BMDC plus soluble α-CD3 in the presence of curdlan or IL-23 (10 ng/mL). Cells were restimulated on day 5 with PMA, ionomycin and brefeldin A for 4 h and the expression of Foxp3 and IL-17 on CD4$^+$ cell blasts was analyzed by flow cytometry. (E) Numbers of Foxp3$^+$IL-17$^+$ T cells obtained in cultures as in part D. Data in part B and E are mean+SEM of three independent experiments. Data are representative of two to four independent experiments. N.D., not done.

Research UK in specific pathogen-free conditions. IL-23 p19-deficient mice [34] were provided by A. MacDonald (Edinburgh, UK) with kind permission from N. Ghilardi, Genentech, and were bred at Cancer Research UK in specific pathogen-free conditions. *Rorc*(γ*t*)-*Gfp*^{TG} mice [22] were bred at Institut Pasteur. Radiation chimeras using BM from DEREG or *Rorc*(γ*t*)-*Gfp*^{TG} mice were generated at Cancer Research UK. All animal experiments were performed in accordance with national and institutional guidelines for animal care.

In vitro T-cell assays

CD4$^+$CD25$^-$CD62L^{hi}CD44^{lo} (naïve) and CD4$^+$CD25$^+$ cells from C57BL/6 mice or CD4$^+$GFP$^+$CD25$^+$ and CD4$^+$GFP$^-$CD25$^-$ cells from DEREG mice or DEREG BM chimeras were purified from spleen and lymph nodes. CD4$^+$GFP$^+$CD25$^+$FR4$^+$CD45.1$^-$ and CD4$^+$GFP$^-$CD25$^+$FR4$^+$ CD45.1$^-$ cells from *Rorc*(γ*t*)-*Gfp*^{TG} BM chimeras or CD4$^+$CD25$^+$FR4$^+$ and CD4$^+$CD25$^-$FR4$^-$ cells from C57BL/6 mice were purified from mesenteric lymph node. Cell purification was performed by cell sorting using a MoFlo (Dako Cytomation) or a FACSAria cell sorter (BD Biosciences); 5×10^4 sorted T cells were cocultured with 2×10^4 BMDC generated with GM-CSF as described previously [4] and 0.2 μg/mL of soluble anti-CD3ε in the presence or absence of 50 μg/mL curdlan (Wako; suspended in PBS at 10 mg/mL), 0.5 μg/mL CpG oligonucleotide 1668 (CpG; Sigma) or 10 ng/mL IL-23 (eBiosciences).

Th17 control consisted of CD4$^+$GFP$^-$CD25$^-$ T cells from DEREG mice or DEREG BM chimeras cocultured with BMDC in the presence of 10 ng/mL of TGF-β (Sigma), 20 ng/mL of IL-6 (RnD systems) and neutralizing antibodies against IFN-γ (2 μg/mL) and IL-4 (2 μg/mL). Treg control consisted of CD4$^+$GFP$^+$CD25$^+$ cells from DEREG mice or DEREG BM chimeras cocultured with BMDC in the presence of 100 IU/mL of rhIL-2 (RnD systems). In some experiments, sorted CD4$^+$GFP$^+$CD25$^+$ T cells were labeled with 2 μM CFSE for 12 min at 37°C before culture.

Cells from cultures were restimulated on day 5 or 7 for 4 h with phorbol 12-myristate 13-acetate (10 ng/mL; Sigma), ionomycin (1 μg/mL; Calbiochem) and brefeldin A (5 μg/mL; Sigma).

Intracellular staining for Foxp3 and IL-17 was analyzed by flow cytometry. Alternatively, half of the content of each well were restimulated on day 5 on plate-bound anti-CD3ε (5 μg/mL) for 48 h before cytokines in the supernatant were analyzed by sandwich ELISA.

Flow cytometry

Antibodies specific for CD4 (RM4-5), CD25 (PC61), CD62L (MEL-14), CD44 (IM7) and CD3ε (145-2C11) were from BD Pharmingen. Antibodies against Folate receptor 4 (12A5), IL-17 (TC11-18H10.1) and Foxp3 (FJK-16s) were from eBiosciences. For intracellular cytokine staining, cells were stained with anti-CD4, fixed and stained with anti-mouse/rat Foxp3 staining set (eBiosciences) containing fluorochrome-labeled antibodies. Data were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Treestar).

RNA isolation and real-time RT-PCR

Total RNA was prepared with TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA with random hexamers and Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was carried out using SYBR green incorporation (*Il17f, ROR-*(γt), *Il23r*). For SYBR green reactions, primer sequences were as described previously [2, 5]. Measurements were performed in duplicate wells using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Normalization was performed using 18S rRNA as a reference (primers and probe from Applied Biosystems) and results are shown as relative mRNA quantities.

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Abbreviations: **BMDC:** BM-derived DC · **DEREG:** depletion of regulatory T cell · **Th17:** Th cells producing IL-17

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Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17

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The C-type lectin dectin-1 binds to yeast and signals through the kinase Syk and the adaptor CARD9 to induce production of interleukin 10 (IL-10) and IL-2 in dendritic cells (DCs). However, whether this pathway promotes full DC activation remains unclear. Here we show that dectin-1–Syk–CARD9 signaling induced DC maturation and the secretion of proinflammatory cytokines, including IL-6, tumor necrosis factor and IL-23, but little IL-12. Dectin-1-activated DCs 'instructed' the differentiation of CD4$^+$ IL-17-producing effector T cells (T_H-17 cells) *in vitro*, and a dectin-1 agonist acted as an adjuvant promoting the differentiation of T_H-17 and T helper type 1 cells *in vivo*. Infection with *Candida albicans* induced CARD9-dependent T_H-17 responses to the organism. Our data indicate that signaling through Syk and CARD9 can couple innate to adaptive immunity independently of Toll-like receptor signals and that CARD9 is required for the development of T_H-17 responses to some pathogens.

CD4$^+$ T cells are the main regulators of adaptive immunity. Responses by interferon-γ (IFN-γ)–producing CD4$^+$ T helper type 1 (T_H1) cells promote immunity to viruses, intracellular bacteria and protozoan parasites, whereas T_H2 cells making interleukin 4 (IL-4), IL-5 and IL-13 direct immunity to metazoan parasites[1,2]. Immunopathology is also often associated with particular classes of CD4$^+$ effector T cells: T_H2 cells are important in allergy, and T_H1 cells have been thought to drive autoimmunity. The last conclusion has been called into question by the finding that defects in IFN-γ signaling exacerbate autoimmune disease in some mouse models[3]. It is now recognized that many autoimmune diseases previously attributed to T_H1 cells are driven by a third class of effector CD4$^+$ T cells that produce IL-17 rather than IFN-γ and are therefore called 'T_H-17' cells[3,4]. IL-17 and/or its receptor have also been linked to resistance to infection by extracellular bacteria such as *Klebsiella pneumoniae* as well as by fungi such as *Candida albicans*[5,6]. However, the mechanisms leading to the generation of T_H-17 cells during infection remain poorly understood.

The differentiation of T_H1 or T_H-17 cells is determined by exposure to IFN-γ or to transforming growth factor-β (TGF-β) and IL-6, respectively[7–10]. These cytokines act on newly primed CD4$^+$ T cells to induce expression of the transcription factor T-bet, which promotes T_H1 responses[11], or RORγt, a transcription factor required for the differentiation of T_H-17 cells[12]. T-bet and RORγt control expression of the IL-12 and IL-23 receptors, respectively[12,13], and render T cells susceptible to IL-12 or IL-23, cytokines that sustain and amplify the T_H cell differentiation process. The critical importance of these cytokines is emphasized by the fact that mice lacking IL-12 or IL-23 cytokine subunits are unable to mount T_H1 or T_H-17 responses, respectively[3]. IL-12, IL-23 and other factors that influence T cell effector fate are produced by dendritic cells (DCs) that have been activated by signals from pattern-recognition receptors (PRRs), which recognize components of microbes or viruses[14]. Members of the Toll-like receptor (TLR) family have been linked to the induction of IL-12 production by DCs and the initiation of T_H1 responses[15]. Whether other pattern-recognition pathways control alternative forms of T_H cell differentiation is not clear.

An alternative pattern-recognition pathway initiated by the engagement of a C-type lectin, dectin-1, by fungal β-glucans has been described[16]. Dectin-1 signals through a 'hemITAM' motif, an immunoreceptor tyrosine-based activation motif–like sequence containing a single 'YxxL' motif (where 'x' is any amino acid)[16], that becomes phosphorylated by Src family kinases after receptor engagement[17]. This allows recruitment of the spleen tyrosine kinase Syk, which then activates 'downstream' signaling components, including the

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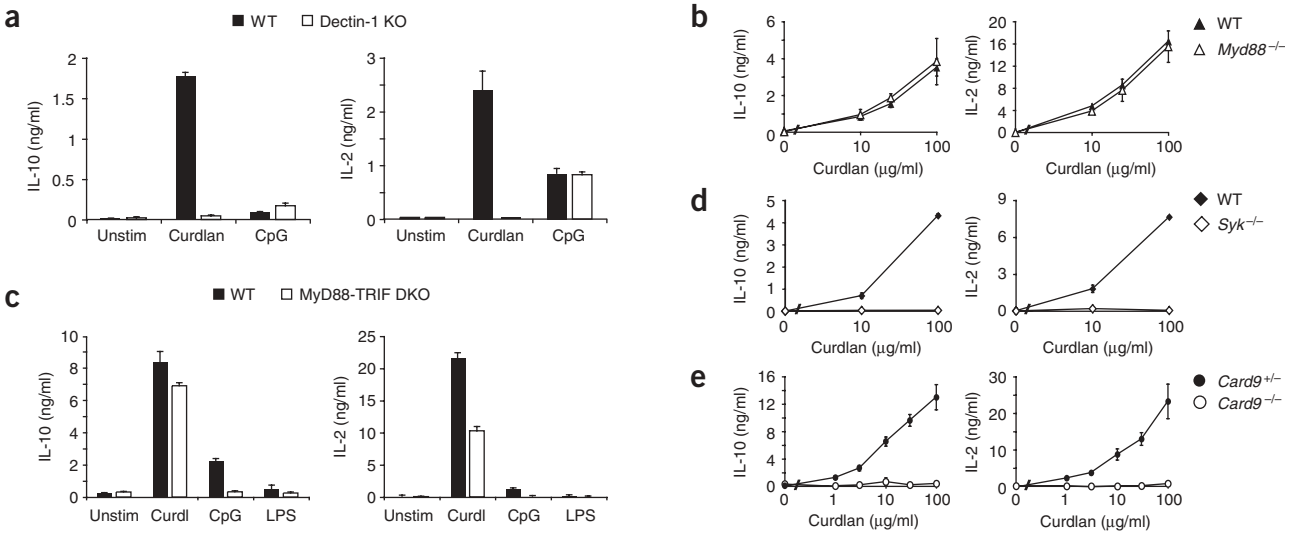

Figure 1 Production of IL-10 and IL-2 in response to curdlan depends on dectin-1, Syk and CARD9 but is independent of MyD88 and TRIF. ELISA of IL-10 and IL-2 production by BMDCs of various genotypes after stimulation with curdlan, CpG and/or LPS. (**a**) C57BL/6 wild-type (WT) or dectin-1-knockout (Dectin-1 KO) BMDCs left unstimulated (Unstim) or stimulated with curdlan or CpG. (**b**) C57BL/6 wild-type or *Myd88*$^{-/-}$ BMDCs stimulated with various amounts of curdlan (horizontal axis). (**c**) C57BL/6 wild-type or MyD88-TRIF–double-knockout (MyD88-TRIF DKO) BMDCs left unstimulated or stimulated with curdlan (Curdl), CpG or lipopolysaccharide (LPS). (**d**,**e**) C57BL/6 wild-type or *Syk*$^{-/-}$ BMDCs (**d**) or *Card9*$^{-/-}$ or *Card9*$^{+/-}$ BMDCs (**e**) stimulated with various amounts of curdlan (horizontal axis). Data are the mean ± s.d. of triplicate stimulations and are representative of two to six independent experiments.

transcription factor NF-κB[16–18]. Activation of NF-κB by dectin-1 requires the CARMA1-related adaptor protein CARD9, which binds the adaptors MALT1 and Bcl-10 and promotes activation of the IKK kinase complex[18].

Dectin-1–Syk signaling in DCs has thus far been associated only with the production of IL-10 and IL-2 (ref. 16), a cytokine pattern not generally associated with the induction of T_H cell responses. Therefore, it remains unclear whether dectin-1 acts in a way similar to TLRs, allowing the 'translation' of innate information into adaptive immunity or, alternatively, whether it acts mainly to modulate signals from other PRRs. Here we show that dectin-1–Syk–CARD9 signaling promoted DC maturation and induced the secretion of proinflammatory cytokines, including IL-23, but little IL-12. Notably, DCs activated by dectin-1 engagement strongly biased T_H cell differentiation to a T_H-17 fate, and the use of dectin-1 agonists as adjuvants promoted T_H-17 and T_H1 responses *in vivo*. Furthermore, T_H-17 cells developed naturally during experimental infection with *C. albicans* in wild-type but not *Card9*$^{-/-}$ mice. Thus, Syk-CARD9–coupled innate signaling pathways may promote DC activation independently of TLRs and may regulate T_H-17 responses to certain infections.

RESULTS

Dectin-1–Syk–CARD9 signaling activates DCs

Zymosan, a β-glucan-containing preparation of yeast cell walls, is widely used for 'probing' dectin-1-mediated innate responses. However, zymosan-induced production of IL-10 and IL-2 is almost normal in dectin-1-knockout DCs, indicating receptor redundancy[19,20] (data not shown). We therefore sought to identify a selective dectin-1 agonist. Curdlan, a pure β-glucan that has been shown to stimulate DCs deficient in the TLR adaptor MyD88 (*Myd88*$^{-/-}$ DCs)[21], elicited much more production of IL-10 and IL-2 from bone marrow–derived DCs (BMDCs) than did a selection of TLR agonists 'titrated' to induce similar quantities of IL-6 (**Supplementary Fig. 1** online). Notably, IL-10 and IL-2 production was mostly independent of the TLR adaptors MyD88 and TRIF but was dependent on dectin-1, Syk and CARD9, suggesting that curdlan acts as a selective agonist of the dectin-1 signaling pathway (**Fig. 1**). We therefore used curdlan to delineate the consequences of dectin-1–Syk–CARD9 signaling on DC phenotype and function.

Curdlan stimulation induced rapid Syk-dependent phosphorylation of the mitogen-activated protein kinases p38, Erk and Jnk and activation of NF-κB in BMDCs (**Fig. 2**). Consistent with the fact that mitogen-activated protein kinases and NF-κB regulate the expression of genes involved in the innate response, curdlan stimulation also led to Syk-dependent but MyD88-independent upregulation of the

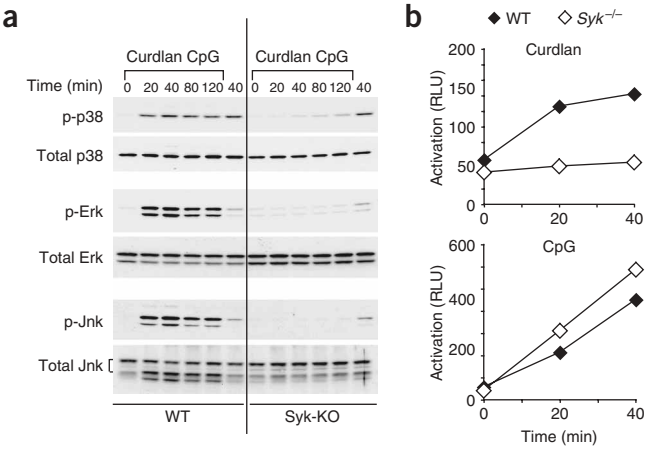

Figure 2 Curdlan stimulation promotes activation of mitogen-activated protein kinases and NF-κB. (**a**) Analysis of the activation of p38, Erk and Jnk by immunoblot of lysates of C57BL/6 wild-type or *Syk*$^{-/-}$ BMDCs stimulated with curdlan or CpG (time, above lanes), assessed with antibodies to phosphorylated forms of the kinases (p-), followed by reprobing of blots for total kinase content (loading control). (**b**) Luciferase assay of NF-κB activation in protein extracts from **a**. RLU, relative light units. Data are representative of at least two independent experiments.

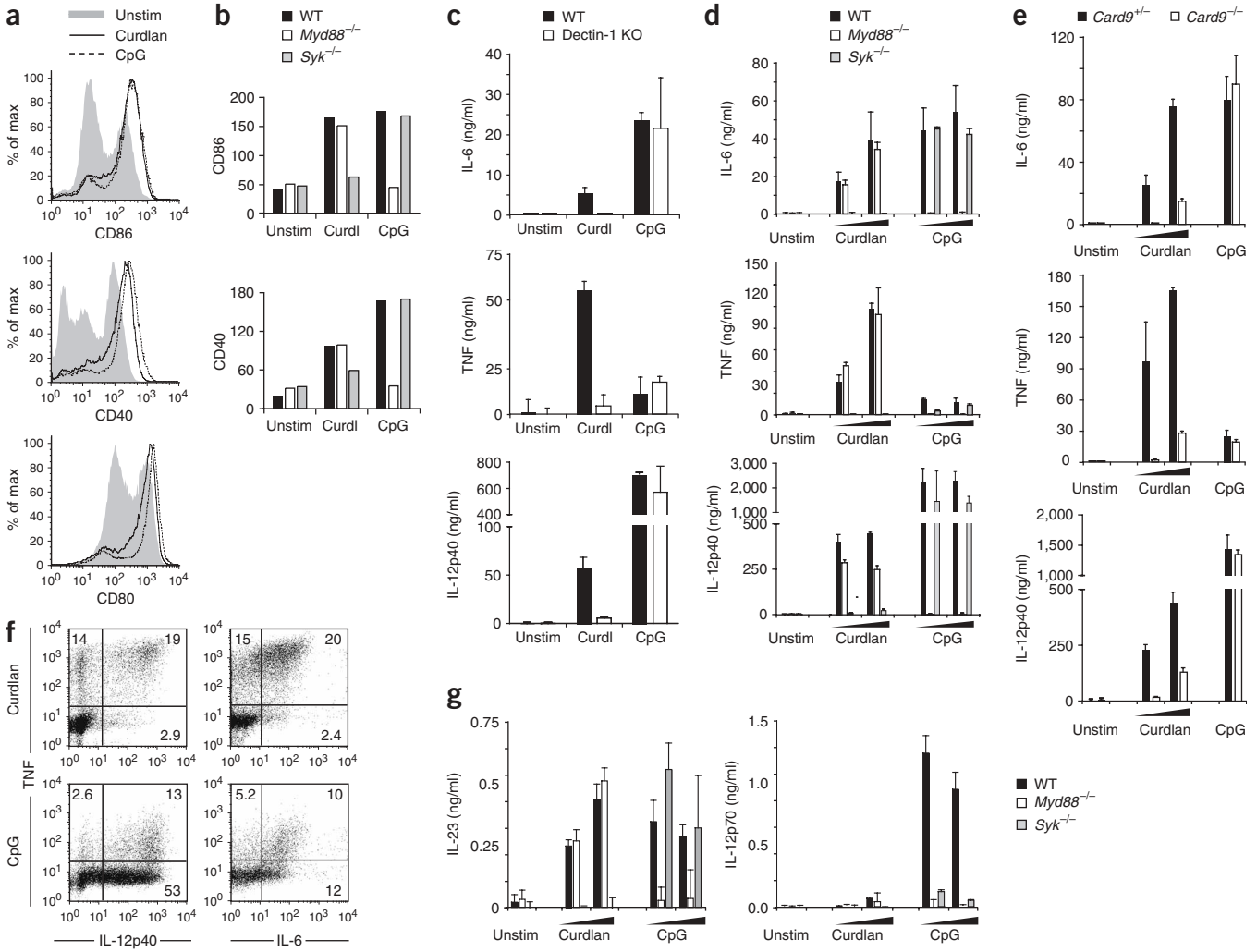

Figure 3 Selective dectin-1 signaling induces Syk- and CARD9-dependent DC maturation and cytokine production. (**a**,**b**) Flow cytometry for surface expression of CD86, CD40 and CD80 on C57BL/6 BMDCs after no stimulation or stimulation for 24 h with curdlan or CpG (**a**) or on C57BL/6 wild-type, *Myd88*$^{-/-}$ or *Syk*$^{-/-}$ BMDCs left unstimulated or stimulated with curdlan or CpG (**b**). (**c**–**e**) ELISA of supernatants of C57BL/6 wild-type or dectin-1-knockout BMDCs left unstimulated or stimulated with curdlan or CpG (**c**); C57BL/6 wild-type, *Myd88*$^{-/-}$ or *Syk*$^{-/-}$ BMDCs left unstimulated or stimulated with 10 or 100 μg/ml of curdlan or with 50 or 500 ng/ml of CpG (**d**); or *Card9*$^{+/-}$ or *Card9*$^{-/-}$ BMDCs left unstimulated or stimulated with 10 or 100 μg/ml of curdlan or with 5 μg/ml of CpG (**e**). (**f**) Flow cytometry of intracellular cytokine expression by C57BL/6 wild-type BMDCs stimulated with curdlan or CpG. Numbers in quadrants indicate percent cells in each. (**g**) ELISA of cytokine production by C57BL/6 wild-type, *Myd88*$^{-/-}$ or *Syk*$^{-/-}$ BMDCs stimulated with 10 or 100 μg/ml of curdlan or with 50 or 500 ng/ml of CpG. Wedges (**d**,**e**,**g**) indicate increasing concentrations of reagent. Data are the mean ± s.d. of triplicate stimulations (**c**–**e**,**g**) and are representative of two to six independent experiments.

CD86, CD40 and CD80 surface proteins, similar to that achieved by stimulation through the TLR9-MyD88 pathway with CpG oligodeoxynucleotides (**Fig. 3a**,**b**). In addition, curdlan stimulation led to the production of large quantities of proinflammatory cytokines, including IL-6, tumor necrosis factor (TNF) and IL-12p40 (a subunit of both IL-12 and IL-23; **Fig. 3c**–**e**). Notably, most of the DCs that produced IL-12p40 in response to curdlan simultaneously produced TNF and IL-6, whereas DCs producing only IL-12p40 predominated after CpG stimulation (**Fig. 3f** and data not shown). Induction of IL-12p40 by curdlan was accompanied by the production of transcripts encoding IL-12p19 (a subunit of IL-23) and secretion of IL-23 protein; however, in contrast to CpG stimulation, curdlan elicited little production of transcripts encoding IL-12p35 (a subunit of IL-12) or IL-12p70 protein (**Fig. 3g** and **Supplementary Fig. 2** online). Cytokine induction by curdlan was independent of MyD88 but depended on dectin-1, Syk and CARD9 (**Fig. 3c**–**e**,**g** and **Supplementary Fig. 2**). In contrast, CpG-induced cytokine production was dependent on MyD88 and independent of Syk, except for the production of IL-12p35 and IL-12p70, which was dependent on both MyD88 and Syk (**Fig. 3g** and **Supplementary Fig. 2**).

As an alternative approach to examine the effects of dectin-1 signaling, we transduced BMDCs with retroviruses encoding dectin-1 molecules bearing a streptavidin-binding peptide tag[17] and stimulated the cells with streptavidin-sepharose. Streptavidin-sepharose triggered phenotypic maturation of and cytokine production by BMDCs expressing full-length dectin-1 but not those expressing a signaling-deficient dectin-1 mutant (**Fig. 4**). Those responses were unaffected by lack of MyD88 and TRIF, which excluded the possibility of a contribution by contaminating TLR agonists and confirmed that triggering of dectin-1 alone was sufficient for DC activation.

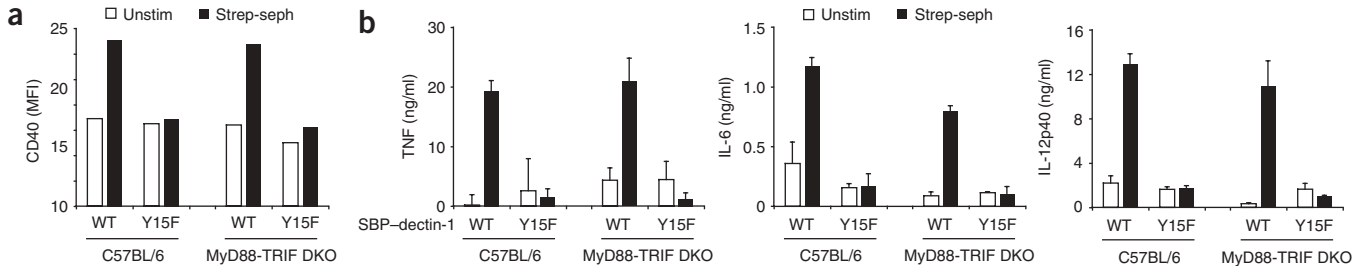

Figure 4 Triggering of dectin-1 alone is sufficient to induce DC maturation and cytokine production. (**a**) Flow cytometry of CD40 surface expression by C57BL/6 wild-type or MyD88-TRIF double-knockout BMDCs transduced with retrovirus encoding a streptavidin-binding peptide–tagged version of wild-type (WT) or mutant (Y15F) dectin-1 and then left unstimulated or stimulated for 24 h with streptavidin-sepharose (Strep-seph). MFI, mean fluorescence intensity. (**b**) ELISA of TNF, IL-6 and IL-12 p40 in supernatants of the cells in **a**. SPB, streptavidin-binding peptide. Data are the mean ± s.d. of triplicate stimulations and are representative of two to four independent experiments.

We conclude that, like TLR signaling, selective triggering of the dectin-1–Syk–CARD9 signaling pathway in DCs induces activation but results in an altered cytokine profile characterized by large amounts of IL-2, IL-10, IL-6 and TNF and a bias in production of IL-23 rather than IL-12.

Dectin-1-activated DCs prime T_H1 and T_H-17 cells

To assess whether dectin-1–stimulated DCs can 'instruct' $CD4^+$ T cell proliferation and effector differentiation, we cultured ovalbumin-specific OT-II $CD4^+$ T cells with antigen and BMDCs in the presence of curdlan or CpG. Both curdlan and CpG led to more T cell proliferation than did antigen alone (data not shown). However, whereas CpG induced differentiation skewed mainly toward the generation of IFN-γ-producing T_H1 cells, the presence of curdlan also led to the accumulation of IL-17-producing T cells (**Fig. 5a**). Compared with a range of specific TLR agonists, curdlan and, to a lesser extent, zymosan, induced a much higher ratio of IL-17 production to IFN-γ production (**Supplementary Fig. 3** online). Neither stimulus induced the development of IL-4-producing T cells (data not shown). The effect of curdlan and CpG was mediated by the antigen-presenting cells and not the T cells, as the effect was abrogated by the use of BMDCs lacking Syk or MyD88, respectively (**Fig. 5b**). In addition, the cytokine bias induced by curdlan versus CpG was not simply a reflection of quantitative differences in DC activation, as 'titration' of either compound did not allow interconversion of its effect (**Supplementary Fig. 4** online).

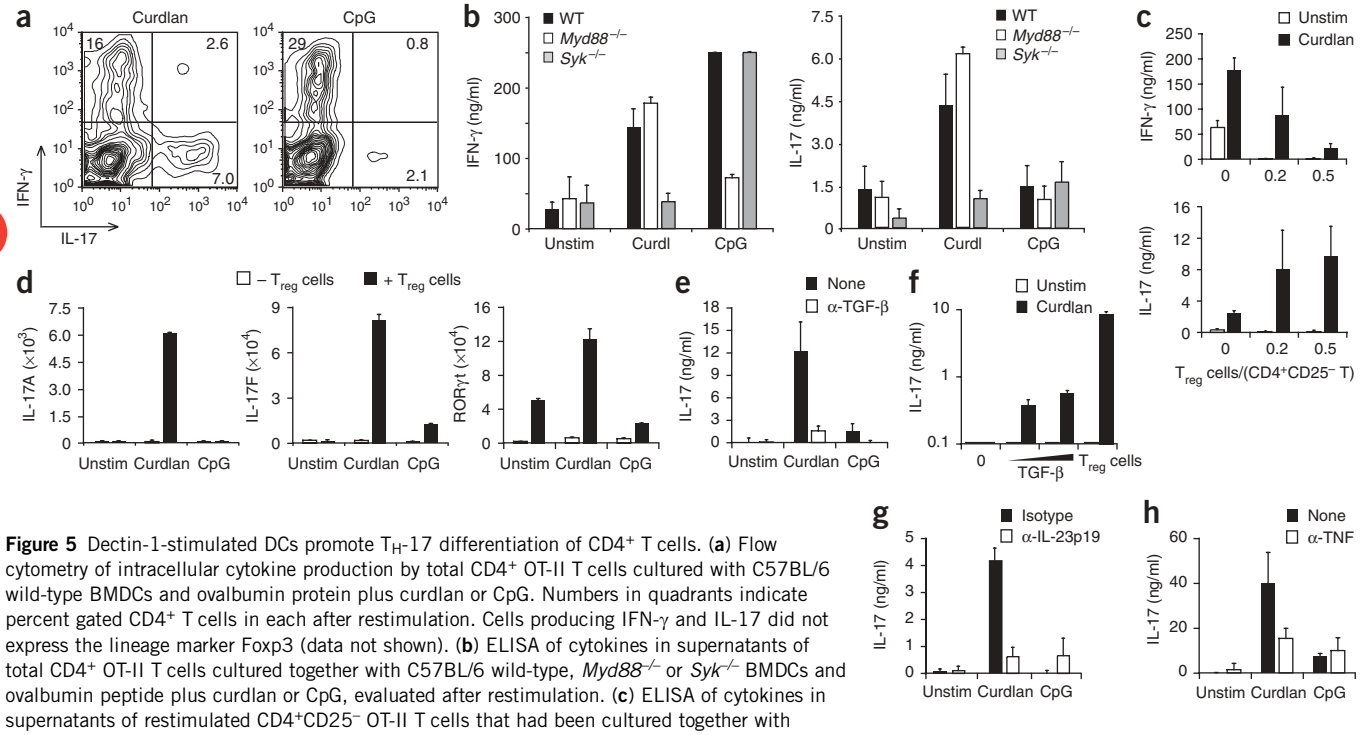

Figure 5 Dectin-1-stimulated DCs promote T_H-17 differentiation of $CD4^+$ T cells. (**a**) Flow cytometry of intracellular cytokine production by total $CD4^+$ OT-II T cells cultured with C57BL/6 wild-type BMDCs and ovalbumin protein plus curdlan or CpG. Numbers in quadrants indicate percent gated $CD4^+$ T cells in each after restimulation. Cells producing IFN-γ and IL-17 did not express the lineage marker Foxp3 (data not shown). (**b**) ELISA of cytokines in supernatants of total $CD4^+$ OT-II T cells cultured together with C57BL/6 wild-type, *Myd88*$^{-/-}$ or *Syk*$^{-/-}$ BMDCs and ovalbumin peptide plus curdlan or CpG, evaluated after restimulation. (**c**) ELISA of cytokines in supernatants of restimulated $CD4^+CD25^-$ OT-II T cells that had been cultured together with C57BL/6 wild-type BMDCs and ovalbumin protein plus curdlan together with $CD4^+CD25^+$ OT-II T_{reg} cells at various ratios (horizontal axis). (**d**) Real-time RT-PCR analysis of transcripts encoding IL-17A, IL-17F and RORγt in $CD4^+CD25^-$ OT-II T cells 5 d after culture with C57BL/6 wild-type BMDCs and ovalbumin peptide plus curdlan or CpG, with (+ T_{reg} cells) or without (– T_{reg} cells) the addition of $CD4^+CD25^+$ OT-II T_{reg} cells. (**e**–**h**) ELISA of cocultures as described in **b** but with the addition of anti-TGF-β (α-TGF-β) on day 0 (**e**); increasing concentrations (wedge) of recombinant TGF-β (**f**); anti-IL-23p19 (α-IL-23p19) or isotype control antibody (Isotype) on days 0 and 3 (**g**); or anti-TNF (α-TNF) on day 0 (**h**). Data are the mean ± s.d. of triplicate (**b**,**c**,**e**–**h**) or duplicate (**d**) cultures and are representative of two to four independent experiments.

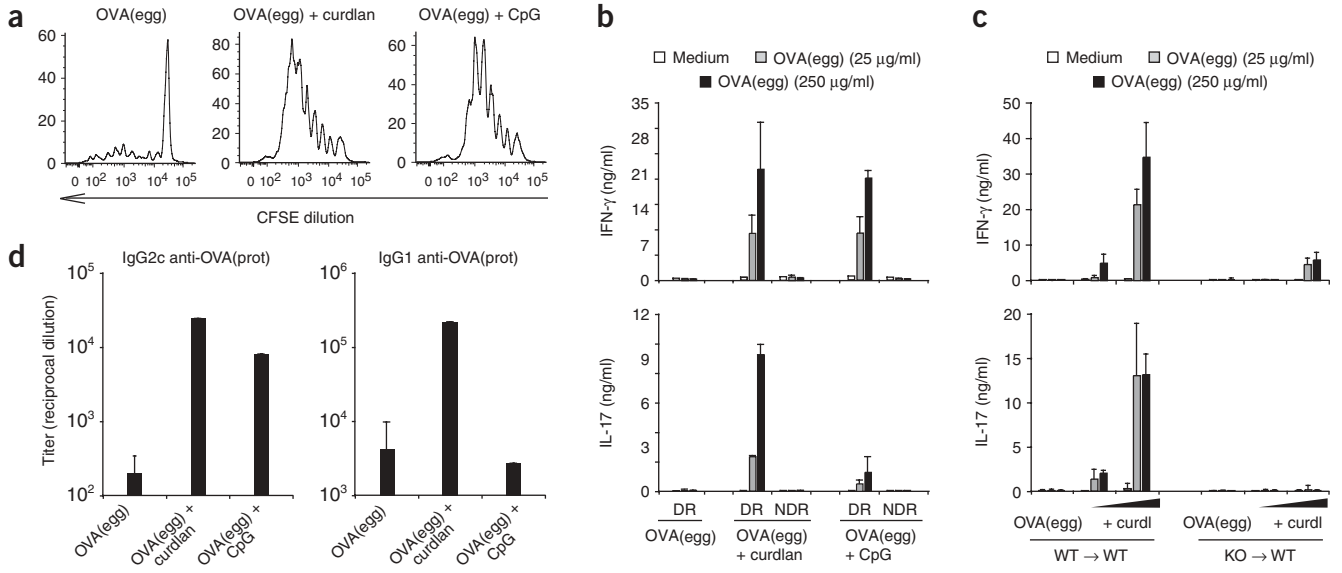

Figure 6 Curdlan acts as an adjuvant for T_H-17 and T_H1 responses and antibody production *in vivo*. (**a**) CFSE dilution analysis of OT-II T cells from the spleens of mice immunized with egg white (OVA(egg)) alone or in combination with curdlan or CpG. (**b**) ELISA of cytokine production by cells isolated from draining popliteal lymph nodes (DR) and nondraining lymph nodes (NDR) from mice that received OT-II T cells and then were immunized as described in **a** (below graph); cells were left unstimulated (Medium) or were restimulated with egg white (key). (**c**) ELISA of cytokine production by cells from wild-type recipients of dectin-1 wild-type bone marrow (WT→WT) or dectin-1-knockout bone marrow (WT→KO), immunized with egg white and increasing amounts (wedges) of curdlan and restimulated as described in **b**. (**d**) ELISA of ovalbumin-specific IgG2c and IgG1 in serum from wild-type mice at day 14 after two immunizations (day 0 and day 7) as described in **a** (horizontal axis). Data are mean ± s.d. of duplicate restimulations (**b**,**c**) or of two to three mice per group (**d**) and are representative of at least three independent experiments.

Notably, depleting the OT-II $CD4^+$ T cell preparation of $CD25^+$ regulatory T cells (T_{reg} cells) led to the differentiation of T_H1 cells even in the presence of curdlan (**Fig. 5c**). As reported for TLR-stimulated DCs[8], only when T_{reg} cells were 'added back' to the starting T cell population did the IFN-γ-dominated response subside and IL-17-producing cells emerge (**Fig. 5c** and **Supplementary Fig. 3**). Similarly, we detected transcripts encoding IL-17A, IL-17F and RORγt only in curdlan-containing cultures when T_{reg} cells were present (**Fig. 5d**). The effect of T_{reg} cells was not simply to prevent T_H1 cell differentiation, as it could not be mimicked by the neutralization of IFN-γ (**Supplementary Fig. 5** online). Instead, as reported before[8], T_{reg} cells served at least in part as a source of TGF-β; their effect could be blocked by antibody to TGF-β (anti-TGF-β) and could be mimicked, albeit only partially, by exogenous TGF-β (**Fig. 5e**,**f**). The differentiation of T_H-17 cells induced by curdlan and T_{reg} cells was also dependent on IL-23 and TNF, as this differentiation was blocked by antibodies neutralizing either cytokine (**Fig. 5g**,**h**). Finally, the addition of excess IL-12p70 prevented the differentiation of T_H-17 cells induced by curdlan-stimulated DCs, whereas the addition of IL-23 led to T_H-17 skewing in cultures of CpG-stimulated DCs (**Supplementary Fig. 6** online). We conclude that DCs stimulated with curdlan can bias the differentiation of $CD4^+$ T cells toward a T_H-17 fate through a pathway requiring T_{reg} cells and involving TGF-β, TNF and IL-23.

Dectin-1 agonists are adjuvants for T_H1 and T_H-17 priming

To assess whether dectin-1 stimulation also leads to the induction of T_H-17 responses *in vivo*, we tested curdlan as an adjuvant. Adoptively transferred OT-II cells proliferated more extensively in response to antigen mixed with curdlan or CpG than in response to antigen alone (**Fig. 6a**). Cells from draining lymph nodes of mice that had received antigen and CpG produced considerable IFN-γ but little IL-17 after restimulation with antigen *in vitro* (**Fig. 6b**). In contrast, substantial IL-17, as well as IFN-γ, was produced by cells from mice immunized with antigen and curdlan (**Fig. 6b**,**c**). Intracellular cytokine staining showed the presence of T_H1 and T_H-17 cells, which existed together as separate populations (data not shown). Notably, the T_H-17 and T_H1 responses induced by antigen and curdlan *in vivo* were dependent on dectin-1, as they were abrogated in chimeric mice containing dectin-1-knockout hematopoietic cells (**Fig. 6c**). In contrast, the T_H1 response induced by CpG was unaffected by dectin-1 deficiency (data not shown). Consistent with the finding that curdlan and CpG induced T_H1 cells, both stimuli triggered the production of ovalbumin-specific immunoglobulin G2c (IgG2c) antibodies (**Fig. 6d**). However, curdlan also promoted the production of high titers of ovalbumin-specific IgG1 antibodies (**Fig. 6d**). In addition, mice that received curdlan developed anti-β-glucan IgM, which could be used to stain *C. albicans* yeast (**Supplementary Fig. 7** online). We conclude that selective engagement of dectin-1 *in vivo* is able to link innate immunity with the induction of T cell and B cell responses.

CARD9 regulates T_H-17 responses to *C. albicans*

Finally, we sought to determine whether $CD4^+$ T_H-17 cell responses are induced during fungal infection and to what extent this depends on the dectin-1–Syk–CARD9 signaling pathway. Large amounts of IFN-γ and IL-17 were produced by $CD4^+$ spleen cells from mice infected with *C. albicans* after restimulation with heat-killed organisms (**Fig. 7a**). Dectin-1 was redundant for this response (data not shown), consistent with the fact that it is also not essential for DC responses to zymosan[19,20]. In contrast, CARD9 was indispensable for the T_H-17 response and partially contributed to the T_H1 response (**Fig. 7b**). We conclude that adaptive immune responses to *C. albicans* infection

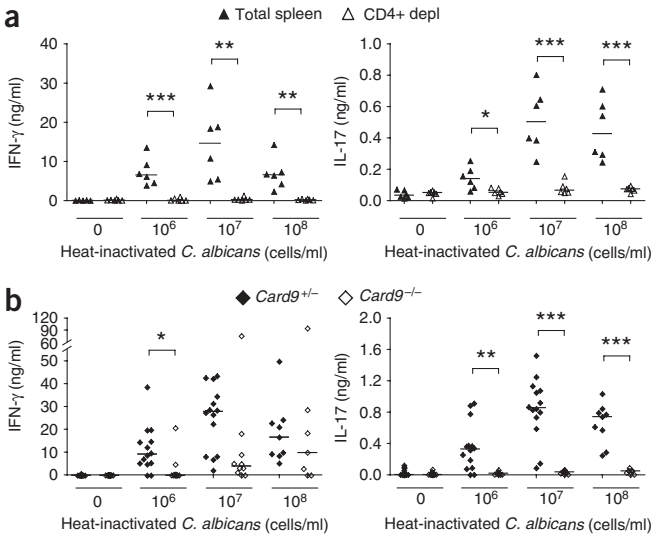

Figure 7 Infection with *C. albicans* induces CARD9-dependent T_H-17 antifungal responses. (**a**) ELISA of IFN-γ and IL-17 production by total splenocytes (Total spleen) or splenocyte samples depleted of $CD4^+$ cells ($CD4^+$ depl); cells were obtained from wild-type mice infected with *C. albicans* and were restimulated with heat-killed organisms. (**b**) ELISA of IFN-γ and IL-17 production by total splenocytes from *Card9*$^{-/-}$ or *Card9*$^{+/-}$ mice infected with *C. albicans* before restimulation of cells with heat-killed organisms. Each symbol represents one mouse (mean of triplicate restimulations); horizontal bars, median. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are from six to fourteen mice per group pooled from three independent experiments.

include the induction of T_H-17 cells and that this process is dependent on innate signaling via CARD9.

DISCUSSION

Pattern recognition is critical for the mobilization of defense mechanisms after invasion by potential pathogens[14]. In addition to stimulating the microbicidal effector functions of myeloid cells, some classes of PRRs can signal to regulate the expression of genes involved in the innate response and to facilitate the induction of adaptive immune responses by DCs[15]. However, of the many possible PRRs expressed at the cell surface, only the TLR family members have been conclusively proven to generate immunogenic DCs. Here we have added Syk-coupled C-type lectin PRRs to that list and have shown that dectin-1 signaling through the Syk-CARD9 pathway induced the maturation of DCs into effector antigen-presenting cells capable of eliciting the differentiation of T_H-17 and T_H1 cells. Furthermore, we have shown that the same signaling pathway was critical for the development of T_H-17 responses during infection with *C. albicans*. Thus, our results identify the Syk-CARD9 axis as a TLR-independent innate signaling pathway for activating DCs that is nonredundant for directing potent T_H-17 responses to infection.

Dectin-1 signaling in DCs resulted in maturation and the production of a distinct combination of cytokines, including considerable IL-2, IL-10, TNF and IL-23. All DC responses to dectin-1 agonists were strictly dependent on Syk, distinguishing them from responses to TLR signals, which are independent of Syk. One apparent exception was the induction of IL-12p35 and IL-12p70 by CpG, which seemed to be dependent on Syk. IL-12p35 production is regulated at many levels and is strongly enhanced by synergistic interaction between different TLRs, costimulation by IFN-γ or CD40 ligand, or type I interferon receptor signaling[22]. The Syk dependence of the production of IL-12p35 and IL-12p70 in response to CpG may therefore indicate involvement of a Syk-coupled cellular receptor that can act in synergy with TLR9 for IL-12p35 induction. In addition, dectin-1 signaling for cytokine production was dependent on CARD9, again distinguishing it from TLR signaling, which is CARD9 independent[18,23]. The CARD9 dependence of cytokine induction is consistent with the importance of this adaptor in coupling dectin-1–Syk signals to NF-κB activation[18]. However, CARD9 has also been associated with linking signaling by the receptor Nod2 to activation of p38 and Jnk[23]. It is therefore possible that CARD9 also coordinates activation of the mitogen-activated protein kinase cascade 'downstream' of dectin-1–Syk. Experiments with curdlan stimulation of CARD9-deficient cells will help address this issue.

TLR stimulation of DCs can induce T_H-17 responses[8], but activation through the dectin-1–Syk–CARD9 pathway induced greater T_H-17 skewing. However, in both cases the mechanism involved was similar, being dependent on the presence of T_{reg} cells and requiring TGF-β[8]. This is consistent with reports suggesting that TGF-β (in concert with IL-6) acts in the first step of T_H-17 instruction[8–10] and that IL-23 acts secondarily to support the proliferation and differentiation of T_H-17 cells[24,25]. DCs stimulated through the dectin-1–Syk–CARD9 pathway had a propensity to produce IL-23 rather than IL-12. The lack of IL-12, together with the production of IL-10, may limit T_H1 skewing, thereby diminishing the amount of negative feedback on T_H-17 cell differentiation. Indeed, the addition of IL-12p70 abrogated the induction of T_H-17 cells by curdlan-stimulated DCs, probably because IL-12 boosts the differentiation of T_H1 cells, which produce IFN-γ and inhibit the differentiation of T_H-17 cells[26,27]. However, the IL-23–versus–IL-12p70 balance is not sufficient to explain the effect of curdlan, as, for example, *Syk*$^{-/-}$ DCs stimulated with CpG, which also make IL-23 but little IL-12p70, elicited little T_H-17 cell differentiation. In this context, the finding that DCs produce large quantities of TNF, IL-12p40 and IL-6 after curdlan stimulation is notable, as IL-23, TNF and IL-6 all have synergistic effects on the induction of T_H-17 cells[8]. Consistent with that idea, blockade of TNF or IL-23 decreased the curdlan-dependent differentiation of T_H-17 cells. Notably, even though zymosan induces TGF-β production by macrophages[28] and DCs can secrete TGF-β[8,29], the amount of TGF-β induced by curdlan alone was insufficient to promote the differentiation of T_H-17 cells in the absence of T_{reg} cells. T_{reg} cells act by providing TGF-β or signals that potentiate the ability of DCs to secrete this cytokine[8]. In addition, curdlan-stimulated DCs produce considerable IL-2, which might help maintain T_{reg} cell function and establish a positive feedback loop[30,31]. The combination of all these factors may explain why DCs stimulated through the dectin-1–Syk–CARD9 pathway were more efficient than TLR-stimulated DCs at inducing the differentiation of T_H-17 cells.

The importance of adaptive immunity in protection from *C. albicans* is emphasized by the notable susceptibility of immunodeficient patients to this otherwise nonpathogenic commensal organism[32]. Furthermore, protective T cell responses to *Aspergillus fumigatus*[33–35] are partially MyD88 independent[36], consistent with the possibility that many antifungal responses may be induced through the Syk-CARD9 pathway. However, the induction of T_H-17 responses to fungal infection has not been reported before to our knowledge, even though both TGF-β and IL-17 are critical for resistance to *C. albicans* infection[6,37]. How T_H-17 cells might contribute to fungal clearance is unknown, but it is notable that IL-17 is a potent recruitment factor for neutrophils and that neutropenia is a chief

risk factor for invasive candidiasis[38]. Notably, the induction of T_H-17 cells through the Syk-CARD9 pathway may also explain why subclinical fungal infections and fungal β-glucans precipitate disease in mouse models of rheumatoid arthritis[21,39] and other autoimmune diseases[40,41]. How this conforms with the observation that zymosan can also promote immunological tolerance is unclear[28].

The finding that dectin-1–Syk–CARD9 signaling in DCs acts as an autonomous pattern-recognition pathway capable of linking the innate and adaptive immune system has notable implications. It is becoming increasingly apparent that dectin-1 is not the only C-type lectin that can signal by means of Syk[42]. The possibility that other Syk-coupled receptors act as PRRs in myeloid cells could help explain the development of T_H-17 responses not only during infection with fungi but also during infection with extracellular bacteria such as *K. pneumoniae* and *Citrobacter rodentium*[5,9]. All of these organisms engage many receptors, in particular those of the TLR family, so it will be useful to study how signaling by means of C-type lectins is integrated with signaling from other PRRs[42]. Nevertheless, the identification of a PRR family that accomplishes a function analogous to that of TLRs but more is powerful in 'instructing' T_H-17 cell development reinforces the idea that innate recognition is central to immune regulation and opens the possibility for therapeutic intervention. Thus, agonists of the Syk-CARD9 pathway might be useful in vaccine development, whereas antagonists might find a place in the treatment of immunopathology. Finally, the central function of Syk in DC activation by means of C-type lectin PRRs raises the issue of whether other receptors that engage Syk in myeloid cells, such as TREM proteins[43], Fc receptors[44] and integrins[45], can also couple innate and adaptive immunity. Syk coupling by myeloid receptors could therefore extend the function of immunoreceptor tyrosine-based activation motif signaling, the central pivot of adaptive immunity, to the earlier step of innate recognition[42].

METHODS

Mice. C57BL/6 mice were from Charles River. Mice lacking both MyD88 and TRIF were generated by intercrossing of MyD88-knockout and TRIF-knockout mice. OT-II TCR-transgenic mice on a B6.SJL background (congenic CD45.1$^+$) were generated by intercrossing of OT-II TCR-transgenic mice on C57BL/6 background with B6.SJL mice. All of these mice, as well as MyD88-knockout, TRIF-knockout and OT-II TCR-transgenic mice on a C57BL/6 background, were bred at Cancer Research UK in specific pathogen–free conditions. Dectin-1-knockout mice[19] were bred at the University of Cape Town, South Africa. CARD9-knockout mice[18] were bred at the Technische Universität München, Germany. Radiation chimeras were generated as described[16]. All animal experiments were in accordance with national and institutional guidelines for animal care.

Reagents. Culture medium was RPMI 1640 medium supplemented with glutamine, penicillin, streptomycin, 2-mercaptoethanol (all from Invitrogen) and 10% (vol/vol) heat-inactivated FCS (Bioclear). Curdlan (Wako) was suspended in PBS at a concentration of 10 mg/ml. Zymosan, MALP2, FSL and Pam_3CSK_4 were from Invivogen. CpG oligonucleotide 1668 was synthesized by Sigma. Streptavidin-sepharose was from GE Healthcare. Ovalbumin peptide (residues 323–339) was synthesized and purified by high-performance liquid chromatography at Cancer Research UK. Ovalbumin protein used for *in vitro* assays was from Calbiochem, and sterile-filtered egg white for *in vivo* use was prepared as described[46]. The amount of egg white presented here always represents the ovalbumin equivalent. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was made by the Cancer Research UK protein purification service and batches were 'titrated' to produce optimal growth conditions for BMDCs. Antibodies used for flow cytometry were from BD Pharmingen and included those specific for CD11c (clone HL3), CD86 (GL1), CD80 (16-10A1), CD40 (3/23), CD4 (RM4-5), CD25 (PC61), CD45.1 (A20), IFN-γ (XMG1.2) and IL-17 (TC11-18H10.1). Neutralizing antibodies were anti-TGF-β (1D11; R&D Systems), anti-p19 (G23.8; eBioscience), anti-TNF (MP6-XT3; BD Pharmingen) and anti-IFN-γ (XMG1.2; BD Pharmingen). Purified 2.4G2 (anti-FcγRIII/II used to block nonspecific antibody binding) was from the Cancer Research UK antibody production service. Antibodies used for immunoblot analysis were rabbit polyclonal antibodies, except the p38-specific antibody (L53F8), and were from Cell Signaling.

BMDC culture and stimulation. BMDCs were generated as described[47], and DCs were purified from bulk cultures with anti-CD11c microbeads before use (Miltenyi Biotec). BMDC purity was checked by flow cytometry and was routinely over 98% (data not shown). For expression of streptavidin-binding peptide–tagged dectin-1 (ref. 17) in BMDCs, the streptavidin-binding peptide tag[48] was fused to the carboxyl terminus of wild-type dectin-1 or dectin-1 with a substitution in its signaling domain (Y15F) and was cloned into the retroviral vector pFB-IRES-GFP. Retrovirus production and BMDC infection were done as described[49]. Of the CD11c$^+$ cells, 80–95% were routinely positive for green fluorescent protein (data not shown). For analysis of cytokine production and surface marker expression, 5×10^4 to 10×10^4 BMDCs per well were cultured for 18–24 h in 96-well round-bottomed plates in 200 μl culture medium containing GM-CSF in the presence of curdlan (100 μg/ml), CpG (500 ng/ml) and/or lipopolysaccharide (50 ng/ml) or as described in the legends to **Figures 1b,d,e** and **3d,e,g**. Streptavidin-sepharose was used at a concentration of 25 μl/ml. All stimuli were 'pretitrated' and were used near their optimal concentration, as determined by induction of maximum cytokine production and DC maturation. Cytokines in the supernatants were measured by sandwich ELISA. For RNA isolation, 4×10^5 BMDCs per well were cultured for 3 h in 48-well plates in 0.5 ml culture medium containing GM-CSF and various stimuli as described in the legend to **Supplementary Figure 2**. For biochemical assays, 1×10^6 to 2×10^6 BMDCs per well were cultured overnight in 24-well plates in 1 ml culture medium containing GM-CSF before stimulation for various times with or without curdlan (500 μg/ml) or CpG (10 μg/ml).

Flow cytometry. Cell suspensions were stained in ice-cold PBS supplemented with 2 mM EDTA, 1% (vol/vol) FCS and 0.02% (wt/vol) sodium azide. For intracellular cytokine staining, cells were stained with anti-CD4 or anti-CD11c, were fixed with Fix and Perm Reagent A (Caltag Laboratories) and then were resuspended in Fix and Perm Reagent B (Caltag Laboratories) containing labeled antibodies. Data were acquired on a FACSCalibur (BD Biosciences) and were analyzed with FlowJo software (TreeStar).

RNA isolation and real-time RT-PCR. Total RNA was prepared with TRIzol reagent (Invitrogen). The cDNA was synthesized from total RNA with random hexamers and Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was accomplished with SYBR Green incorporation (for transcripts encoding IL-17A, IL-17F, RORγt, IL-23p19, IL-2, IL-6 and TNF) or Taqman (for transcripts encoding IL-12p35, IL-12p40 and IL-10). For SYBR Green reactions, primer sequences were as described[12,50,51]; for IL-6, primer sequences were as follows: forward, 5′-GTTCTCTGGGAAATCGTGGA-3′; reverse, 5′-TGTACTCCAGGTAGCTATGG-3′. For Taqman reactions, primers and probes were from Applied Biosystems. Measurements were made in duplicate wells with the ABI PRISM 7700 sequence detection system (Applied Biosystems). Results were normalized to those obtained with 18S rRNA (primers and probe from Applied Biosystems) and results are presented as relative quantities of mRNA.

Immunoblot analysis and NF-κB assay. Cells were collected by being scraped into ice-cold PBS supplemented with 5 mM EDTA. After centrifugation, the cell pellet was lysed for 30 min on ice with buffer (50 mM Tris, pH 7.5, 1% (vol/vol) Nonidet-P40, 0.5% (wt/vol) deoxycholic acid, 0.1% (wt/vol) SDS, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4 and 2 mM $Na_4P_2O_7$, plus a mixture of protease inhibitors; Roche Molecular Biochemicals). Cell debris were removed by centrifugation and extracts were quantified by protein assay (Bio Rad). For immunoblot analysis, a fixed amount of total protein was mixed with sample buffer (0.125 M Tris, pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol) and was resolved by SDS-PAGE (4–20% acrylamide gradient, Tris-glycine; Invitrogen). After

transfer to a polyvinyldifluoride membrane (Millipore), proteins were analyzed by immunoblot and were visualized by enhanced chemiluminescence (Pierce). NF-κB was measured in the extracts by analysis of a fixed amount of total protein with the NF-κB p65 transcription factor assay kit according to the manufacturer's instructions (Pierce).

***In vitro* T cell differentiation.** $CD4^+$ T cells from the lymph nodes of OT-II mice (C57BL/6 background) were purified by a two-step procedure with MACS beads (Miltenyi Biotec), which included depletion of antigen-presenting cells followed by enrichment for $CD4^+$ cells. Alternatively, $CD4^+CD11c^-CD25^-$ and $CD4^+CD11c^-CD25^+$ cells were sorted from the spleens of OT-II mice (C57BL/6 background) by flow cytometry with a MoFlo (Dako Cytomation) or a FACSAria (BD Biosciences). Purified OT-II T cells (5×10^4) were cultured together with BMDCs (1×10^4) and 10–50 μg/ml of ovalbumin protein or 2–10 nM ovalbumin peptide (residues 323–339) in the presence or absence of curdlan (50 μg/ml) or CpG (0.5 μg/ml) in complete culture medium further supplemented with sodium pyruvate, nonessential amino acids and HEPES (all from Invitrogen). In some experiments, neutralizing antibodies (10 μg/ml) or recombinant cytokines (TGF-β, 1-10 ng/ml (Sigma); IL-12 p70, 5 ng/ml (Biosource); IL-23, 10 ng/ml (eBioscience)) were added. Cultures were split at a ratio of 1:2 on day 3 and were restimulated on day 5 for 5 h with phorbol 12-myristate 13-acetate (10 ng/ml; Sigma), ionomycin (1 μg/ml; Calbiochem) and GolgiStop (BD Pharmingen), and intracellular cytokines were analyzed by flow cytometry. Alternatively, the entire content of each well was restimulated on day 5 on plate-bound anti-CD3ε (Cancer Research UK antibody production service) for 48 h before cytokines in the supernatant were analyzed by sandwich ELISA.

Immunizations. Naive C57BL/6 mice were immunized intraperitoneally with 5 mg curdlan alone or with 50 μg egg white plus 2 mg curdlan or 5 μg CpG or were immunized in the footpads with 3 μg egg white plus 50–500 μg curdlan or 0.5–2.5 μg CpG. For T cell differentiation assays, $CD4^+$ OT-II populations (from OT-II mice on B6.SJL background; 1×10^6 to 4×10^6 cells per mouse), depleted of antigen-presenting cells and labeled with CFSE, were transferred into host mice by intravenous injection 1 d before immunization. CFSE dilution of $CD4^+CD45.1^+$ spleen cells was analyzed by flow cytometry 3 d after immunization. Alternatively, 1×10^6 to 2×10^6 total popliteal lymph node cells were restimulated for 48 h with egg white before cytokines in the supernatant were measured by sandwich ELISA.

Infection. *Card9*$^{-/-}$ mice and *Card9*$^{+/-}$ control mice were infected intravenously with 4×10^3 to 1.2×10^4 colony-forming units of *C. albicans* strain SC5314. On days 6–8 after infection, mice were killed and total splenocytes or splenocyte samples depleted of $CD4^+$ cells (2×10^6 cells per well) were restimulated in 96-well U-bottomed plates with heat-inactivated *C. albicans* (1×10^6 to 1×10^8 per ml). After 48 h, IFN-γ and IL-17 in the supernatants were measured by sandwich ELISA.

Antibody measurement. Relative titers of ovalbumin-specific IgG2c and IgG1 in serum samples from experimental mice were measured by capture ELISA with anti–mouse IgG1 (BD Pharmingen, clone A85-1) or anti–mouse IgG2c (Jackson Immunoresearch) on plates coated with ovalbumin protein. The β-glucan-specific immunoglobulins were detected by ELISA with anti–mouse immunoglobulin (Jackson Immunoresearch) and plates coated with curdlan made soluble in NaOH. For staining of *C. albicans*, heat-inactivated yeast cells were incubated with diluted serum followed by anti–mouse immunoglobulin. Confocal images were acquired on a Zeiss LSM510 microscope.

Statistics. Statistical significance was determined by a two-tailed unpaired *t*-test with Graphpad Prism (GraphPad Software).

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

S.LG.-L. and C.R.S. designed the study, analyzed data and wrote the paper; S.LG.-L. coordinated the study, did experiments and analyzed data; M.J.R., F.O. and E.C.S. contributed additional experiments and intellectual input; O.G. and J.R. did the experiments with CARD9-knockout cells and mice; S.V.T. and G.D.B. provided dectin-1-knockout bone marrow and infected dectin-1-knockout mice; and E.S. and V.T. provided Syk-knockout fetal livers.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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