Distinct myeloid cell subpopulations in controlling systemic candidiasis

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**DISTINCT MYELOID CELL SUBPOPULATIONS IN
CONTROLLING SYSTEMIC CANDIDIASIS**

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DISTINCT MYELOID CELL SUBPOPULATIONS IN CONTROLLING SYSTEMIC CANDIDIASIS

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Table of Content

Acknowledgement.....	I
Table of Content.....	III
Table of Figures	VIII
Abbreviations.....	XI
ABSTRACT	XIII
Chapter 1: INTRODUCTION.....	1
1.0.1 Candida spp.	2
1.0.2 Candidiasis	2
1.1 Candida Albicans	3
1.1.1 Pathogenic mechanisms used by C.albicans.....	4
1.1.2 Systemic Candidiasis	5
1.1.3 Animal Model for Systemic Candidiasis	7
1.1.4 Kidney - the main target organ for invasive C.albicans infection	8
1.2 Host Immunity against invasive C.albicans infection.....	9
1.2.1 Host Immunity - Pattern Recognition Receptors (PRRs).....	9
1.2.1.1 Host Immunity - Recognition of C.albicans by PRRs	10
1.2.2 Host Immunity - Macrophages and Monocytes.....	13
1.2.2.1 Kidney tissue-resident macrophages.....	13
1.2.2.2 Host Immunity - PRRs exploited by macrophages to detect C.albicans.....	15
1.2.2.3 Host Immunity - Antifungal mechanisms elicited by macrophages	17
1.2.2.4 Host Immunity - Roles of monocytes in systemic candidiasis ...	17
1.2.2.5 Host Immunity - Roles of macrophages in kidney immunity against C.albicans	18
1.2.2.6 Host Immunity - Interaction between macrophages and endothelial cells in kidney	20
1.2.3 Host Immunity - Dendritic cells (DCs)	21
1.2.3.1 Conventional dendritic cells (cDCs).....	22
1.2.3.2 Plasmacytoid dendritic cells (pDCs)	22
1.2.3.3 Host Immunity - Roles of DCs during invasive candidiasis	24

1.2.3.4 Host Immunity - Crosstalk between DCs and T-helper (Th) cells during Candida infection	25
1.2.4 Host Immunity - Polymorphonuclear Neutrophils (PMNs)	28
1.2.4.1 Host Immunity - Roles of PMNs during invasive candidiasis.....	28
1.3 Conditional Ablation of Specific Cell Subsets using the Diphtheria Toxin Receptor-Diphtheria Toxin (DTR-DT) system.	31
1.3.1 Advantage of the DTR-DT system	31
1.3.2 Underlying mechanism of the DTR-DT system.....	31
1.3.3 DTR Transgenic Mouse Strains	33
1.3.3.1 Clec9A-DTR mouse	33
1.3.3.2 Clec4a4-DTR mouse.....	33
1.3.3.3 Siglec H-DTR mouse	34
1.3.3.4 CD169-DTR mouse.....	34
1.4 AIMS OF PROJECT	36
Chapter 2: MATERIALS AND METHODS	37
2.1 Materials.....	37
2.1.1 Mice	37
2.1.2 Fungal strains	38
2.1.3 Media, buffers and solutions	38
2.1.4 Chemicals, reagents and kits	39
2.1.5 Commercial Antibodies.....	39
2.1.6 Computer software	39
2.2 Methods	39
2.2.1 C.albicans and mouse model of systemic candidiasis.....	39
2.2.2 Depletion of target cell population by DT and α Gr-1 antibody	40
2.2.3 Cell counting	40
2.2.4 Fungal burden analyses	40
2.2.5 Tissue collection, processing and isolation of single-cell suspension	41
2.2.6 Cell stainings for flow cytometry analyses	41
2.2.7 T cell stimulation for intracellular detection of cytokines	42
2.2.8 [H3]-Thymidine incorporation assay - in vitro T-cell proliferation assay.....	42
2.2.9 Cryosection Immunofluorescence staining	43

2.2.10 ELISA measurement of serum cytokines	43
2.2.11 Serum Creatinine.....	43
2.2.12 Measurement of vascular permeability (Evans Blue).....	43
2.2.13 Histological analysis	44
2.2.13.1 Tissue embedding and sectioning	44
2.2.13.2 Hematoxylin and Eosin (H&E) staining	44
2.2.13.3 Periodic Acid Schiff (PAS) staining	45
2.2.14 Quantitative real-time PCR (qPCR)	45
2.2.15 Statistical Analysis.....	46
Chapter 3: Results I.....	47
3. Understanding the host immune responses during acute systemic candidiasis	47
3.1 Infection dose determination for acute invasive candidiasis.....	47
3.2 Expansion of splenic leukocytes during systemic candidiasis.....	50
3.3 Expansion of renal leukocytes during systemic candidiasis.	53
Chapter 4: Results II.....	56
4. Neutrophils are indispensable in defending against acute disseminated candidiasis.	56
4.1 Candida growth kinetics in the spleen and kidneys of infected mice.	56
4.2 Efficient recruitment of neutrophils and monocytes to the kidneys.	58
4.3 Pronounced susceptibility to acute systemic candidiasis in mice lacking neutrophils.....	60
4.4 Establishment of infection and DT treatment scheme.	65
Chapter 5: Results III.....	68
5. Individual DC subset is dispensable in the host immunity against invasive candidiasis.	68
5.1 Conditional ablation of different DC subsets in vivo using the DTR-DT system.	69
5.2 The depletion of distinct DC subsets in the mice does not exacerbate their susceptibility nor protect them towards systemic candidiasis.....	71
5.3 Absence of CD8 ⁺ DCs or CD11b ⁺ DCs does not affect the general cellular infiltration in the spleen during invasive candidiasis.....	73
5.4 Disparity in the infiltration of NK, NKT and CD8 ⁺ T cells into the kidneys of Clec4a4-DTR and Clec9A-DTR mice.	73

5.5 Comparable ability of CD8 ⁺ DCs and CD11b ⁺ DCs in inducing CD4 ⁺ T cell proliferation in the presence of Candida antigens.....	76
5.6 Differential cytokines production by CD4 ⁺ T cells in the absence of CD8 ⁺ DCs or CD11b ⁺ DCs.	76
5.7 Rag2 ^{-/-} mice are less vulnerable towards systemic Candida infection.	77
Chapter 6: Results IV	79
6. CD169 ⁺ F4/80 ⁺ tissue resident macrophages are essential for kidney immunity against systemic candidiasis	79
6.1 Majority of the CD169 ⁺ F4/80 ⁺ tissue resident macrophages are localized in the renal outer medulla region.	79
6.2 Ablation of CD169 ⁺ macrophages does not alter the anatomical structure of the kidneys.	83
6.3 Splenic F4/80 ^{hi} macrophages are efficiently depleted in CD169-DTR mice.	83
6.4 Renal F4/80 ^{hi} macrophages are partially depleted in both DT-treated CD169-DTR and CX3CR1 ^{gfp/gfp} mouse strains.	85
6.5 CD169-DTR and CX3CR1-deficient mice show different susceptibilities towards disseminated Candida infection.	87
6.6 Quantification of kidney fungal burdens of CX3CR1 ^{gfp/gfp} and CD169-DTR mice.....	87
6.7 A unique renal subset of macrophages provides first-line defense against the initial assault by C.albicans.	89
6.8 CD169-DTR mice are unable to clear C.albicans in the kidneys at the later stage of infection.	92
6.9 Ablation of CD169 ⁺ macrophages does not affect the infiltration of myeloid cells into the kidneys during infection.	94
6.10 Absence of CD169 ⁺ macrophages leads to a reduction in the recruitment of NK and NKT cells into the kidneys.	96
6.11 Augmented vascular leakiness in the organs of Candida-infected CD169-DTR mice.	97
6.12 Progressive renal damage in CD169-DTR mice during Candida infection.	99
6.13 Kidneys of CD169-DTR mice are severely compromised at D10p.i.	102
6.14 Persistent accumulation of C.albicans in the renal pelvis of CD169-DTR mice.....	104

6.15 Few CD45 ⁺ CD11b ⁺ cells are detected in the renal collecting ducts.	107
6.16 CD169-DTR mice exhibit higher levels of pro-inflammatory cytokines and chemokines than the control mice during Candida infection.	109
Chapter 7: Discussion	112
Chapter 8: Conclusion.....	127
REFERENCES.....	129
Appendix	143
1.1 Media, Buffers and Solutions.....	143
1.2 Chemicals, reagents and kits.....	145
1.3 Antibodies	146
1.4 qPCR primer sequences.....	147
Conferences	149

Table of Figures

Introduction

Figure 1.1: Different morphological forms of <i>C.albicans</i>	3
Figure 1.2: Multiple organs are affected in invasive candidiasis.	6
Figure 1.3: Composition of <i>Candida albicans</i> cell wall.	10
Figure 1.4 Various PRRs expressed by innate immune cells to recognize <i>Candida</i>	12
Figure 1.5: Individual DC subset triggers different adaptive immune response.	23
Figure 1.6: Effective host defense against <i>Candida</i> infection requires the collaboration between the innate and adaptive immunity.	27
Figure 1.7: Conditional ablation of target cell population using the DTR-DT system	32
Figure 1.8: Various transgenic DTR mouse strains that target different myeloid cell subpopulations.	35
Figure 2.1 Targeting construct for the generation of Clec4a4-DTR knock-in mouse.....	38

Results

Figure 3.1 Infection dose titration and organ fungal burdens in murine experimental model of systemic candidiasis.....	49
Figure 3.2 Kinetics of myeloid cells infiltration in the spleen during <i>Candida</i> infection.	51
Figure 3.3 Kinetics of lymphoid cells infiltration in the spleen during <i>Candida</i> infection.	52
Figure 3.4 Kinetics of myeloid cells infiltration in the kidney during <i>Candida</i> infection.	54
Figure 3.5 Kinetics of lymphoid cells infiltration in the kidney during <i>Candida</i> infection.	55
Figure 4.1 Kinetics of <i>C.albicans</i> load in the spleen and kidneys during the first 24hrs of <i>Candida</i> infection.	57
Figure 4.2 Kinetics of neutrophils and monocytes influx in the spleen and kidneys during the first 24hrs of <i>Candida</i> infection.	59

Figure 4.3 Depletion of neutrophils significantly increases the susceptibility of mice towards invasive candidiasis.	63
Figure 4.4 Profiles of neutrophils and monocytes in the spleen and kidneys of <i>Candida</i> -infected CD11b-DTR and α Gr-1 treated mice.....	64
Figure 4.5 Higher neutrophil number reduction in α Gr-1 treated mice than in CD11b-DTR mice.....	65
Figure 4.6 DT treatment and infection scheme - bypassing DT-induced neutrophilia and monocytosis.	67
Figure 5.1 Depletion Profiles of target DC subsets in DTR transgenic mice.	70
Figure 5.2 Individual DC subset is dispensable in the host immunity against <i>Candida</i> infection.	72
Figure 5.3 Profiles of leukocytes in the spleen of <i>Candida</i> -infected Clec9A-DTR and Clec4a4-DTR mice.	74
Figure 5.4 Profiles of leukocytes in the kidneys of <i>Candida</i> -infected Clec9A-DTR and Clec4a4-DTR mice.	75
Figure 5.5 Absence of either Clec9A ⁺ DCs or Clec4a4 ⁺ DCs during <i>Candida</i> infection drastically reduces IFN γ -producing CD4 ⁺ T cell populations.....	78
Figure 6.0: Histology image of kidney.	80
Figure 6.1 Kidney CD169 ⁺ F4/80 ⁺ macrophages are found in the renal medulla during steady state.	81
Figure 6.2 Depletion of renal CD169 ⁺ F4/80 ⁺ macrophages does not affect the kidney structural integrity during steady state.....	82
Figure 6.3 Depletion Profiles of splenic macrophages in WT, CD169-DTR and CX3CR1 ^{gfp/gfp} mice.	84
Figure 6.4 Depletion Profiles of renal macrophages in WT, CD169-DTR and CX3CR1 ^{gfp/gfp} mice.	86
Figure 6.5 Both CD169-DTR and CX3CR1 ^{gfp/gfp} mice display increased susceptibility, with the latter showing the highest sensitivity towards systemic <i>Candida</i> infection.....	88
Figure 6.6 Profiles of myeloid cells in the kidneys of <i>Candida</i> -infected CD169-DTR and CX3CR1 ^{gfp/gfp} mice at D1p.i.	91
Figure 6.7 Kidneys of CD169-DTR mice are the only organs incapable of clearing <i>C. albicans</i> as the disease progresses.	93
Figure 6.8 Kinetics of myeloid cells infiltration in the kidneys of WT and CD169-DTR mice during <i>Candida</i> infection.....	95
Figure 6.9 Kinetics of lymphoid cells infiltration in the kidneys of WT and CD169-DTR mice during <i>Candida</i> infection.....	96

Figure 6.10 Endothelial linings in the organs are less intact in CD169-DTR mice than in control mice during <i>Candida</i> infection.	98
Figure 6.11 CD169-DTR mice show progressive kidney damage, while the structural integrity of infected control kidneys is more preserved during systemic candidiasis.	101
Figure 6.12 Kidneys of CD169-DTR mice are severely damaged at D10p.i.	103
Figure 6.13 <i>C.albicans</i> accumulate at the renal collecting ducts of control and CD169-DTR mice during infection.	107
Figure 6.14 CD45 ⁺ immune cell population in the renal collecting ducts is considerably little as compared to other renal regions during steady state.	108
Figure 6.15 Elevated levels of inflammation are observed in CD169-DTR mice during <i>Candida</i> infection.	111

Abbreviations

APC	allophycocyanin (fluorophore)
AMPs	antimicrobial peptides
Batf3	basic leucine zipper transcription factor, ATF-like 3
BMDC	bone marrow derived dendritic cells
BMDM	bone marrow derived macrophages
CARD	caspase activation and recruitment domain
CD	Cluster of distribution
CFU	colony forming unit
CTL	C-type lectin receptor
CCL	Chemokine (C-C motif) ligand
CR	complement receptor
CXCL	Chemokine (C-X-C motif) ligand
CX3CR	Chemokine (C-X3-C motif) receptor
DC	Dendritic cell
DC-SIGN	dendritic cell specific intercellular adhesion molecule 3 grabbing non-integrin
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DT	diphtheria toxin
DTR	diphtheria toxin receptor
EGF	epidermal growth factor
FACS	fluorescent-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage-colony stimulating factor
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
i.p	intraperitoneal

i.v	intravenous
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
IMDM	Iscoe's modified Dulbecco's medium
MHC	major histocompatibility complex
MINCLE	macrophage inducible Ca^{2+} -dependent lectin
MMR	macrophage mannose receptor
MyD88	myeloid differentiation primary response 88
NK	natural killer
NKT	natural killer T
P.I	post-infection
PAMPs	pathogen associated molecular patterns
PGE	prostaglandin E
PRRs	pattern recognition receptors
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PFA	paraformaldehyde
PRRs	pattern recognition receptors
RBC	red blood cell
ROS	reactive oxygen species
SPF	specific pathogen-free
Syk	spleen tyrosine kinase
SSC	side scatter
Th	T-helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
VCAM	vascular cell adhesion protein
WT	wild type
YPD	yeast peptone dextrose
YPAD	yeast peptone adenine dextrose

ABSTRACT

Systemic Candidiasis is one of the most frequent nosocomial infections to date, with a mortality rate as high as 40%. Despite continual efforts in antifungal therapy and hygienic measures in hospitals, the incidence of such invasive infection escalates, owing to the expanding population of immunocompromised patients and the increasing resistance against common antifungal chemotherapeutics. Over the past decades, host immunity against systemic candidiasis has been the focus of numerous studies with an aim to explore potential cure and in search of effective vaccine candidates. Although the importance of neutrophils in this infection is well-established, the role of mononuclear phagocytic subsets, which includes DCs and macrophages, during invasive *Candida* infection, is still unclear due to the lack of appropriate tools to specifically ablate these individual mononuclear phagocytic cell subpopulations. To this end, we exploited four different in house-generated Diphtheria Toxin Receptor (DTR) transgenic mouse strains to study the roles of CD8⁺/CD103⁺ DCs (Clec9A-DTR), CD11b⁺ DCs (Clec4a4-DTR), pDCs (Siglec H-DTR) and CD169⁺ macrophages (CD169-DTR) in the host immunity against disseminated candidiasis, in which depletion of target cell populations *in vivo* can be achieved upon DT injection. Noteworthy, we consistently detected highest and persisting fungal load in the kidneys, as compared to the other vital organs in our murine model of systemic candidiasis using *Candida albicans* SC5314 strain, indicating that kidney is the main target organ. Here, we reiterated the pivotal role of neutrophils in the host defense against invasive *Candida* infection, where ablation of neutrophils *in vivo* caused rapid death of the mice as soon as 2 days post infection. Correspondingly, these mice displayed significantly higher fungal burden in the kidneys when compared to the control mice. Next, our data demonstrated that individual DC subset (CD8⁺/CD103⁺ DCs, CD11b⁺ DCs and pDCs) is dispensable for the host immunity against invasive *Candida* infection, in which mice depleted either of these DC subsets displayed similar mortality, susceptibility and organ fungal burdens as that of the infected controls. In the subsequent part of our experiments, we showed that kidney CD169⁺F480⁺ macrophages are

absolutely required for the host defense against systemic candidiasis. CD169-DTR mice consistently exhibited higher susceptibility and their kidney fungal burden was significantly higher at the later stage of infection when compared to the control mice. In addition, kidneys of CD169-DTR mice appeared to be more damaged and inflamed than the control kidneys, as evidenced by the kidney histology and higher expression of KIM-1, endothelial adhesion molecules, pro-inflammatory cytokines and chemokines. Interestingly, based on our kidney histological observations, the absence of CD169⁺ macrophages seemed to adversely influence the neutrophils candidacidal capacity. Further investigations are warranted to unravel the mechanisms behind the modulation of *Candida* immunity by CD169⁺ macrophages. Our data suggest that these macrophages may likely be involved in restraining inflammation or in potentiating neutrophils candidacidal abilities during systemic candidiasis.

Chapter 1: INTRODUCTION

Humans have been regularly exposed to fungi since birth, from inhalation of spores to fungal colonization on the skin or in the gastrointestinal flora. Fungi, being an adept sensor and responder to the environmental cues, establish different kinds of relationships with plants, animals and humans either symbiotically, commensally, latently or pathologically. Out of all the fungi species known, only approximately 0.1% of them are pathogenic to humans. Even though the likelihood of these fungi in causing infections to immuno-competent individuals is relatively low, whenever opportunity arises, they can cause a spectrum of diseases ranging from superficial epidermal diseases (e.g. cutaneous lesions) to life-threatening systemic infections (e.g. sepsis) [1, 2].

In recent years, owing to medical and technological advances, the overall survival rates and quality of life for hospitalized inpatients have improved tremendously. This, in turn, increases the population of immuno-compromised individuals (e.g. patients suffering from AIDS, cancer, organ failure, trauma and others), which inevitably accompanied with increasing cases of opportunistic invasive fungal infections in hospitals; with *Candida* and *Aspergillus* species as the most common nosocomial fungal causative agents. Nonetheless, the introduction of antifungal therapy and hygienic measures in the hospitals over the years decreases the mortality rate associated with invasive *Aspergillus*. Yet, *Candida spp.* remain as the major cause of fungal severe sepsis in intensive care unit (ICU) patients, with mortality rate as high as 40-60% [3].

To date, *Candida spp.* are the fourth most common causative agents of nosocomial infection [4, 5] and "the second leading cause of infectious disease-related death in premature infants" [6].

1.0.1 *Candida* spp.

Candida spp. are opportunistic pathogens that are commonly found in human skin, vaginal and gastrointestinal flora. Under normal healthy circumstances, these commensal fungi are harmless to us, unless the balance between the host's immunity and the environment of the normal microbial flora is perturbed (e.g. broad-spectrum antimicrobial treatment), or the physical integrity of the skin or mucosal barrier is disrupted (e.g. wounds or use of catheters) [3]. Once the host's physical barrier is breached, *Candida* spp. can cause a wide range of infections, collectively known as Candidiasis.

1.0.2 Candidiasis

The term, Candidiasis, can be further sub-categorized into 1) mucocutaneous candidiasis, which includes thrush (oral candidiasis), vaginal and gastrointestinal candidiasis, 2) cutaneous candidiasis (skin), and 3) life-threatening systemic candidiasis (candidemia) [7], depending on the nature and the route of infection. Often, superficial infections on otherwise healthy individuals or immunity-weakened individuals, such as oral and vaginal candidiasis, are harmless, owing to the fact that superficial *Candida* infections are usually contained at the site of the infection. Strikingly, invasive candidiasis, which only occurs in immuno-compromised patients, affects all organs, and this is generally accompanied with high mortality rate despite the intervention of antifungal therapies in hospitals. Thus far, *Candida albicans* remains as the most common strain in causing candidiasis, in particular, candidemia, although there is an emerging trend that involves other *Candida* spp, such as *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* [5, 8, 9].

1.1 Candida Albicans

C.albicans is an obligate diploid, polymorphic fungus that is capable of transforming into the highest number of morphological forms known among its species, which comprise of yeast, pseudohyphae, true hyphae, opaque cells (mating-competent cells), and chlamydozoospores (large, thick-walled spherical cells) [Figure 1.1]. The yeast, pseudohyphae and hyphae are the most common forms observed during the infection, whereby upon encountering different environmental conditions (e.g. pH and temperature), *C.albicans* switches among these forms readily [10, 11]. On the other hand, chlamydozoospores has not been shown to be involved in *Candida* pathogenicity [12], while the involvement of opaque cells in candidiasis requires further investigations, though the latter has been shown by Kvaal al. et to be capable of colonizing the skin in a murine cutaneous infection model [13].

Figure 1.1: Different morphological forms of *C.albicans*

(A) Yeast; (B) True hyphae; (C) Pseudohyphae; (D) Chlamydozoospore (arrow); (E) Opaque cells. Scale = 10µm. Reprinted from [10].

1.1.1 Pathogenic mechanisms used by *C.albicans*

As an opportunistic pathogen, *C.albicans* is consistently adapting to its rapidly changing environmental conditions (e.g. pH and nutrients) by resorting to multiple virulence mechanisms to ensure its survival. Some of these virulence traits include dimorphism, biofilm formation, expression of adhesins and invasins, enzymes secretion, and stress adaptation [2, 14].

Dimorphism, the capacity to transform between the yeast and hyphae forms interchangeably, is regarded as one of the main virulence factors of *C.albicans* [10]. Both forms of *C.albicans* are necessary in conferring its virulence since the yeast form is essential for the dissemination of *C.albicans* to other organs, and the hyphae form is vital for tissue invasion and the evasion of host phagocytic immune surveillance [15]. This is further illustrated in several studies, where locking of *C.albicans* in either form attenuates its virulence in experimental systemic candidiasis [16-19].

Another virulence factor of *C.albicans* is its ability to form biofilms on both abiotic (e.g. catheters) and biotic (e.g. gut lumen) surfaces. The formation of biofilm involves the adherence of yeast cells to a surface, followed by massive cell proliferation, hyphae formation and eventually, dispersion of yeast cells from the mature biofilms. Notably, biofilm-tainted medical devices (e.g. catheters) have been a major cause of systemic candidiasis, especially when invasive medical procedures are involved. Biofilms have also been shown to enhance the resistance of *C.albicans* against antifungal drugs and host defense mechanisms that include antimicrobial peptides and phagocytic cells [20-22].

The highly invasive characteristics of *C.albicans* is highlighted by its ability to express an impressive array of proteins that facilitate adhesion and penetration to the host cells, which ultimately leads to extensive damage once it manages to breach the epithelial or endothelial barrier. This invasion is orchestrated by two key complementary steps, namely, induced endocytosis

and active penetration [23]. Induced endocytosis is a passive process that does not require viable fungi, where the expression of invasins by *Candida*, such as Als3 [24] and Ssa1 [25], triggers its own engulfment by the host cells readily. On the other hand, active penetration is an active process that requires viable fungal cells, though the molecules involved are still undefined. Nonetheless, the suggested list of candidate molecules includes proteins with reported adhesive function and those that are only expressed by hyphae, a form that fungi adopt to penetrate the host epithelial or endothelial barrier [2, 26].

The 3 classes of enzyme hydrolases secreted by *Candida*, namely proteases, phospholipases and lipases, have also been reported to be important *Candida* virulence factors. These secreted enzymes mediate critical functions that are associated with fungi well-being and invasion. For instance, they are required to breakdown extracellular nutrients for optimal fungal growth and they are also needed to disrupt host cell membrane for active penetration [2, 27, 28].

1.1.2 Systemic Candidiasis

Systemic candidiasis is a common nosocomial infection that was estimated to affect more than 250,000 ICU patients every year, with a mortality rate as high as 60%. Some of the major risk factors are neutropenia, cancers, broad-spectrum antibiotic treatment, surgery or the use of invasive medical devices. Of note, the genetic makeup of the individuals also plays a major determinant in conferring their susceptibility to this infection.

Invasive candidiasis occurs when *C.albicans* gains access to the host bloodstream, either by the penetration of physical barriers (e.g. skin or mucosal) or the external introduction of catheters or other surgical devices, where it is disseminated to other parts of the body and organs [Figure 1.2]. Patients suffering from candidemia often show septic shock-like symptoms, followed by multiple organ failure, with the major target organ being the kidney [9, 29-31].

Figure 1.2: Multiple organs are affected in invasive candidiasis.

Candida generally enters into the human bloodstream either through 1) gut colonization that eventually penetrates via translocation, 2) surgical incisions, or 3) from colonized indwelling catheters or other invasive medical devices. Once entered into the blood stream, the fungi is able to disseminate into other organs, such as bone, lungs, eyes, liver, spleen, kidneys and brain and cause deep-seated infections. Reprinted from [9].

1.1.3 Animal Model for Systemic Candidiasis

The investigation of experimental systemic candidiasis uses animal models extensively, as they show similar disease development as our human counterparts, with kidney being the main target organ. Of all the animal models, owing to its cost and reproducibility, murine model is the most preferred tool in the exploration of *C.albicans* pathogenicity and host immunity against *Candida* infection.

There are two ways to establish invasive candidiasis in the animal models. The most preferred method is through intravenous (i.v.) or intraperitoneal (i.p) injection, where a higher load of *C.albicans* is required for i.p. injection to exhibit similar pathology as that of i.v. injection. This route of infection is commonly adopted by researchers since the disease outcome induced is relatively consistent and it recapitulates the infection that is caused by *C.albicans*-manifested catheters or indwelling medical devices. Another route of infection is via gastrointestinal route, where *C.albicans* is first required to colonize the gut and subsequent successful colonization will then lead to the invasion of fungi into the bloodstream. However, this infection route is difficult to establish because even in the inbred mice, high variation in the disease progression is observed [10, 29].

1.1.4 Kidney - the main target organ for invasive *C.albicans* infection

During systemic infection, pathogens disseminate to all major organs through the blood circulation. Spleen, a secondary lymphoid organ, acts as the first line defense against these pathogens by constantly filtering the blood and mediates the corresponding adaptive immune response. In view of the anatomy of spleen and its function, researchers usually focus on spleen as the target organ for the investigation of bloodstream infections [32, 33]. To date, the majority of the reports on host immunity, especially adaptive immune response, against systemic *C.albicans* infections were documented based on spleen as the target organ. However, spleen is not the main target organ for systemic candidiasis. Nonetheless, the investigation on host adaptive immunity, such as cell-mediated immunity, has shed some light to the understanding of host defense against this infection.

Kidneys are the most affected organs in systemic candidiasis due to their incapability to contain and clear *Candida* load, hence the severity of kidney lesions is highly correlated with the host survivability and disease progression. Generally, once *C.albicans* enters into the bloodstream, this fungus rapidly lodges into every organ (e.g. lungs, kidney and spleen), as evidenced by the substantial organ fungal loads (e.g. lungs, kidney and spleen) and low to negligible *Candida* load in the blood as early as 5 h.p.i. As the disease progresses, *C.albicans* load in the kidneys continues to rise while the fungal load of other organs (e.g. lung, spleen, liver) decreases. Despite knowing the general trend of the fungal burden in various organs, the underlying mechanism that addresses the differential fungal load in different organs remains undefined [29, 30, 34, 35].

1.2 Host Immunity against invasive *C.albicans* infection

The host immune system employs a repertoire of defense mechanisms to fight against any foreign pathogens once the host epithelial barrier (e.g. skin) is breached. This system is subcategorized into the innate and adaptive immunity. The innate immunity, which comprises of myeloid (e.g. neutrophils, macrophages and dendritic cells) and innate lymphoid cells (e.g. natural killer cells), is the host's first line defence against any invading microbes. On the other hand, the adaptive immunity, which comprises of 'trained' lymphocytes (e.g. B and T cells) that express antigen-specific receptors, is essential for the establishment of host memory immunity and pathogen-specific immune responses. Unlike the adaptive immunity, detection of any microorganisms by immune cells from the innate counterpart is achieved by the presence of receptors that recognize the pathogen-associated conserved molecules. Upon sensing the pathogens, these innate immune cells trigger a series of host antimicrobial defence mechanisms, which include the induction of adaptive immune responses [36, 37].

1.2.1 Host Immunity - Pattern Recognition Receptors (PRRs)

Pattern recognition receptors (PRRs) are a set of germ-line encoded receptors that recognize pathogen-associated molecular patterns (PAMPs) expressed by the invading foreign pathogens. Sensing of PAMPs by PRRs triggers a series of downstream signaling pathways that activate the immune cell microbial killing responses, such as phagocytosis and cytokines secretion. Mainly expressed by myeloid cells (e.g. dendritic cells, neutrophils and macrophages), PRRs can be classified into several families - Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding domain, leucine-rich repeat - containing (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and absent-in-melanoma 2 (AIM2)-like receptors (ALRs), depending on the matching ligands, downstream pathways and localization.

Specifically, TLRs and CLRs are membrane-bound receptors that are involved in detecting extracellular PAMPs (e.g. mannan), whereas NLRs, RLRs and ALRs are cytosolic receptors, which are usually responsible in identifying intracellular PAMPs (e.g. nucleic acid) [37, 38].

1.2.1.1 Host Immunity - Recognition of *C.albicans* by PRRs

Because *C.albicans* can exist in multiple morphological forms, it expresses a variety of PAMPs. Reciprocally, the host innate immune compartment expresses a range of PRRs that enable them to detect these PAMPs and initiate corresponding protective immunity against different *Candida* forms. Generally, the main *Candida* PAMPs identified by host PRRs are mannans and β -glucans, the crucial components of *C.albicans* cell wall. Mannans are specifically found on the outer layer of the *Candida* cell wall, whereas β -glucans lies between the outer and the chitin innermost layer [Figure 1.3][39]. Of all the PRR families, the roles of TLRs and CLRs in *C.albicans* infection are the most extensively studied [40, 41], with CLRs being the most important receptor family involved in *Candida* immunity. As the list of PRRs in recognizing *C.albicans* is extensive [42], only some of the prominent PRRs will be discussed.

Figure 1.3: Composition of *Candida albicans* cell wall.

There are 3 layers in *C.albicans* cell wall; the outer layer (mainly mannan), inner layer (mainly β -glucan) and the innermost layer (mainly chitin). Reprinted from [39].

Mannans, which emerge in different structural forms, such as O-linked mannan and α -mannan, have been shown to be recognized by a number of CLRs and TLRs. Among those which have been widely studied include Mannose receptor (MR) [43-45], Dendritic cell (DC)-associated C-type lectin-2 (Dectin-2) [46, 47], Galectin 3 [48-51], DC-specific ICAM3-grabbing non-integrin (DC-SIGN) [52, 53], TLR2 [54, 55] and TLR4 [56]. On the other hand, two main receptors have been identified to recognize β -glucans. They are Dectin-1, a CLR [57-59], and complement receptor 3 (CR3) [60-63]. Notably, owing to its anatomic location in the *Candida* cell wall, β -glucans are usually hidden from the host immune surveillance, unless *C.albicans* undergoes budding [64, 65]. However, once exposed, β -glucans, in particular β -1, 3 glucans, interact with Dectin-1 to evoke a strong host immunological response against *C.albicans* [66-69]. A brief illustration on the type of PRRs expressed by various myeloid cells and their downstream signaling pathways are neatly summarized by Netea et al., as depicted in Figure 1.4.

The importance of TLR/MyD88 in anti-*Candida* immunity has been illustrated in several studies, where receptor knockout mice (e.g. TLR2^{-/-}) or mice deficient in MyD88 exhibit enhanced susceptibility towards systemic candidiasis, owing to the impairment in pro-inflammatory cytokines and chemokines secretions, as well as reduced fungi killing mechanisms (e.g. phagocytosis and neutrophils recruitment) [54, 56, 70]. Similarly, the indispensable role of CLR/Syk/Card9 in the host immunity against *Candida* infection has also been demonstrated in numerous studies on several important CLR knockout models and Card9-deficient mice, where mice with defects in the Syk/Card9 signaling pathways are indefensible against *Candida* infection [45-47, 49, 52, 59, 68, 69]. Of note, during infection, recognition of *Candida* by host innate immune cells involves the collaboration of several PRRs to synergistically elicit effective antifungal responses against *C.albicans*. Correspondingly, depending on the type of immune cells (and their respective PRR expressions) and the type of PAMPs expressed by *C.albicans*, different immune responses will be initiated.

Figure 1.4 Various PRRs expressed by innate immune cells to recognize *Candida*.

(A) The host innate immune system engages various types of PRRs to efficiently recognize different *Candida* PAMPs and to elicit the corresponding signaling pathways required for anti-*Candida* immunity. Most of these PRRs belong to the CLR family, where they (e.g. Dectin-1, Fc receptors, Dectin-2, Dectin-3) induce antifungal immune responses through the spleen tyrosine kinase (Syk)-mediated signaling pathways. Of note, it has been shown that some PRRs synergistically collaborate with other PRRs for the induction effective antifungal immunity (e.g. Dectin-1 and TLR2). While Fc receptors depend on opsonins to recognize *Candida*, complement receptor 3(CR3) can identify *Candida* directly. Activation of these PRRs triggers downstream signaling pathways that are important for the secretion of pro-inflammatory cytokines and chemokines and the initiation of *Candida* killing. CARD9, caspase activation and recruitment domain-containing 9; NF- κ B, nuclear factor- κ B; PAMP, pathogen-associated molecular pattern; PKC δ , protein kinase C δ ; ROS, reactive oxygen species. Reprinted from [71]. (B) Different immune cells express different PRRs on their cell surface. Reprinted from [72].

1.2.2 Host Immunity - Macrophages and Monocytes

Macrophages are key innate cells that serve as first line immune sentinels and are integral in the maintenance of tissue homeostasis. Using various fate mapping strategies, lineage-tracing studies revealed diverse ontogeny of tissue-resident macrophages [73]. With the exception of brain microglia that is yolk sac-derived, most tissue-resident macrophages originate from and are self-maintained by fetal hematopoietic precursors, with minimal contribution from the adult bone-marrow derived monocytes [74]. Nonetheless, upon infection or inflammation, the massive number of monocytes recruited to the tissue may differentiate into macrophage-like cells, which eventually contribute to the resident macrophage pool. Aside from being developmentally diverse, the close macrophage-tissue interaction and niche-specific signals also mould the macrophage population into various subsets with disparate functions [75]. For instance, in the spleen, CD169⁺ marginal metallophilic macrophages can rapidly respond to various blood-borne pathogens, due to its strategic location in the marginal zone, where this unique anatomic compartment is flooded by open, reduced blood-flow circulation. On the other hand, the liver macrophages, also known as kupffer cells, and splenic red pulp macrophages are specialized in the phagocytosis of damaged or senescent erythrocytes. In the kidney, macrophages are important in maintaining renal tubular integrity, promoting tubular repair, and displaying both pro- and anti-inflammatory roles depending on the context [75].

1.2.2.1 Kidney tissue-resident macrophages

In the kidney, mononuclear phagocytes, which comprise of dendritic cells (DCs) and macrophages, form a dense network surrounding the renal nephrons [76]. Unfortunately, to date, it is not yet possible to distinguish these 2 mononuclear phagocytes in the kidneys, owing to the overlapping expression of DC and macrophage markers. Specifically, the common markers, CD11c and F4/80, which are normally employed to differentiate between DCs and macrophages, are co-expressed by 70-90% of the renal mononuclear phagocytes [77]. In

addition, the general consensus of classifying CD11c^{hi} cells as classical DCs does not apply to kidney DCs since cell population expressing CD11c^{hi} and CD11c^{int} are indistinguishable. In other words, the relative expression level of CD11c cannot be used to differentiate between renal macrophages and DCs [78].

Nonetheless, kidney macrophages are generally defined by the expression of F4/80^{hi}, CD11b^{lo} and a recently introduced macrophage marker, CD64. CD64, an Fcγ receptor, was demonstrated by Schilitzer et al. to be generally expressed by macrophages, but not DCs, that reside in the peripheral tissues, such as lung and small intestine [79]. However, despite the addition of CD64, a partial population of renal F4/80^{hi} CD11b^{lo} CD64⁺ cells, which are supposedly macrophages, showed dendrite morphology and are capable of presenting antigen to T cells [78]. Therefore, owing to the difficulty in delineating different mononuclear phagocytic subsets in the kidney, understanding of these renal mononuclear phagocytic subpopulations is limited.

Tissue-resident macrophages are heterogeneous. This heterogeneity of macrophage population can be exemplified in spleen, where various splenic macrophages, including white pulp macrophages, red pulp macrophages and marginal zone macrophages, can be defined by certain unique markers [75]. A number of studies have since attempted to subcategorize renal F4/80^{hi} CD11b^{lo} macrophage population and it has been shown that these F4/80^{hi} CD11b^{lo} macrophages in the kidney predominantly co-express CD169, a marker that is also expressed by other tissue-resident macrophages, such as splenic marginal metallophilic macrophages [80-82].

1.2.2.2 Host Immunity - PRRs exploited by macrophages to detect *C.albicans*

Studies on the function of macrophages in *Candida* immunity has mostly been done in *in vitro* experiments that involve the use of human or mouse macrophage-like cell line and monocytes isolated from the human patients. To date, through these *in vitro* studies, a wide array of PRRs, of which mostly recognize *Candida* cell wall components, have been identified on the macrophages. Because each of the PRRs seems to play a prominent role in *Candida* immunity, a brief update on the function of macrophage PRRs will be discussed.

Some of these PRRs that are expressed by macrophages are Dectin-1, Dectin-2, macrophage mannose receptor (MMR), SIGN-R1 and MINCLE, all of which belong to the CLR family that is pivotal for *Candida* recognition. Engagement of β -glucans/Dectin-1 induces phagocytosis of un-opsonized *C.albicans* by macrophages and is required for the increased production of pro-inflammatory cytokines such as TNF α and IL6 [58, 66, 83]. Even though *Candida*-stimulated macrophages secrete relatively low level of IL10 and IFN γ , as compared to that of TNF α and IL6, the secretions of IL10 but not IFN γ is dependent on Dectin-1 [58, 84]. On the other hand, MMR, which is primarily expressed on macrophages, is critical for the host induction of pro-inflammatory cytokines (e.g. IL6, IL1 β and GM-CSF) that are essential for antifungal protective response but not for the production of neutrophil chemokines (e.g. CXCL1, CXCL2) [85]. The significance of MMR lies in the ligand it recognizes, fungal mannan, which can be easily found on the outer layer of the *Candida* yeast cell wall. In stark contrast, as aforementioned, β -glucans is hidden and unexposed on live *Candida* yeast unless *Candida* is heat-killed or undergoes budding. Therefore, majority of the Dectin-1 functional studies have to be interpreted with care since heat-killed *Candida* was used as stimulants in these studies.

Unlike Dectin-1 that recognizes β -glucans, Dectin-2 can be activated by fungal α -mannans, which are mainly expressed by *C.albicans* hyphae [46, 86]. Dectin-2 activation on macrophages has been shown to induce the secretion of TNF α and IL1 receptor antagonist [86]. In addition, SIGN-R1, a mouse homologue of human DC-SIGN that recognizes fungal cell-wall mannans [71], coordinates with Dectin-1 to enhance superoxide anions and ROS production by the macrophages for efficient killing of phagocytosed *Candida* [87]. Another important member of CLRs on macrophages is MINCLE, where its expression has been reported to be strongly induced by live *Candida*. Specifically, macrophages from MINCLE-deficient mice produce reduced amount of TNF α and these KO mice are highly susceptible to disseminated candidiasis [88].

Recently, NLRs has also been illustrated to be heavily involved in anti-fungal immunity. Upon engagement of MMR by fungal chitin, it has been shown that subsequent signaling involving intracellular NOD2 and TLR9 is required to induce the production of anti-inflammatory IL10. This involvement of MMR, NOD2 and TLR9 in inducing IL10 production indicates cross-talk among different class of innate receptors [89]. Another example of these collaborative efforts involves Dectin-1 and TLR2, whereby these receptors synergistically enhance the production of cytokine, TNF α , by the macrophages [90, 91].

Given the vast numbers of *Candida*-recognizing PRRs expressed by the macrophages, it is thus essential to understand how this innate population interacts with *C.albicans*. In one study conducted by Ghosh et al., they reported an increased secretion of TNF α , IL6, IFN β and IL10 by RAW264.7 macrophage cell line as early as 1-3 hours after live *C.albicans* challenge [92]. In addition, they discovered that RAW264.7 cells produced substantial amount of IL6 when cultured with both *Candida*-secreted molecule, farnesol, and cell-wall component, zymosan, though farnesol or zymosan alone cannot stimulate IL6 production in macrophages. This demonstrates that not only are macrophages capable of responding very rapidly, their activation requires a combination of stimuli that possibly involves different PRRs to initiate effective *Candida* immunity [92].

1.2.2.3 Host Immunity - Antifungal mechanisms elicited by macrophages

There are generally two facets on how macrophages defend against disseminated candidiasis. First is the elimination of *Candida* through direct phagocytosis and secondly, the secretion of pro-inflammatory cytokines (e.g. IL1 β , IL6 and TNF α) and chemokines (e.g. CCL2) to potentiate the surrounding immune cells and to recruit other leukocytes [71, 93]. Upon binding of *Candida* yeast to Dectin-1, phagocytosis can be swiftly triggered and subsequent phagosome-lysosome fusion leads to the formation of phagolysosome, where the engulfed yeasts are degraded [84, 94, 95]. Of note, the β -glucan bound Dectin-1 receptor-mediated internalization depends on the morphology of the *C.albicans*. Unlike the yeast form, β -glucan expressed by *Candida* hyphae is in a closed configuration and thus less likely to be detected by Dectin-1, indicating that the invasiveness of the *Candida* hyphae is partly due to the resistance to phagocytosis-mediated killing [65, 96]. In addition, though macrophages are frequently described as important innate cells that recognize and phagocytose *Candida* yeast via Dectin-1, the yeast-to-hyphae transformation of the engulfed *C.albicans* enables them to escape from the phago-lysosome and thus resistant to this killing mechanism [97, 98]. Hence, direct phagocytic killing of *Candida* may not be the predominant mechanism by which the macrophages use to control fungal growth. In contrast, it is the neutrophils that are highly efficient and play a more significant role in phagocytic killing of *C.albicans* [99]. The macrophages, however, are instrumental in the induction of rapid recruitment of neutrophils to the site of infection by producing pro-inflammatory cytokines and chemokines such as IL1 β , CXCL1, CXCL2 and CCL2 [100, 101].

1.2.2.4 Host Immunity - Roles of monocytes in systemic candidiasis

Current understandings on the functional roles of monocytes in disseminated candidiasis are mainly derived from human *in vitro* studies. The major *Candida*-recognizing PRRs that are expressed by this mononuclear phagocytic population are Dectin-1, CR3, TLR2, TLR4 and MINCLE [56, 102-

104]. Similar to macrophages, Dectin-1 activation also triggers the release of TNF α by monocytes and this release of TNF α can be enhanced through the concomitant activation of TLR2 and Dectin-1 [91]. Expression of CR3 on the monocytes may also play a role in phagocytosis and clearance of opsonized-*C.albicans* [93, 102, 105]. Even though the fungal ligand that activates MINCLE has yet been identified, its expression is required for monocytic release of pro-inflammatory cytokines. However, the phagocytic and candidacidal capacity of monocytes are inversely correlated with their expression of MINCLE [104].

The importance of monocytes remains controversial. In one study, mice depleted with monocytes via VP-16 neutralizing antibodies showed equivalent resistance to *Candida* infection as the control mice, suggesting a dispensable role of monocytes [106]. On the other hand, depletion of monocytes in CCR2-DTR mice showed enhanced susceptibility towards this infection [107]. However, in another experiment that used CCR2^{-/-} mice to assess monocytes function in systemic candidiasis, these mice were not as susceptible towards this infection as the CCR2-DTR mice [82]. Nonetheless, a recent study demonstrated that prior non-lethal *Candida* challenge is able to confer memory protection against lethal *Candida* infection, in which the memory protection is monocyte-dependent [108]. In addition, pro-inflammatory monocytes that are recruited during the early stage of disseminated candidiasis seem important for the host resistance, where reduction in this population results in increased kidney fungal burden [107].

1.2.2.5 Host Immunity - Roles of macrophages in kidney immunity against *C.albicans*

The importance of kidney tissue resident macrophages in *Candida* immunity *in vivo* has recently been demonstrated by Lionakis et al. Taking advantage of the functional importance of CX3CR1/fractalkine in maintaining the homeostatic number of macrophage population in the kidneys, whereby the renal macrophage numbers in these knockout mice are greatly reduced at

steady state, they utilized CX3CR1^{-/-} mice to study experimental disseminated candidiasis. In their study, CX3CR1^{-/-} mice exhibited increased susceptibility as compared to the wild type mice, with distinctly high kidney fungal burden [82].

Besides promoting fungal clearance, the importance of macrophages in reducing kidney damage during *Candida* infection has been demonstrated by Tran et al., where adoptive transfer of *in vitro*-polarized M2 macrophages improved the survival and decreased kidney immunopathology of *Candida*-infected mice [109]. Tran et al. reported that administration of exogenous IL33 increased the host tolerance towards disseminated candidiasis and this was attributed to the expanded M2 macrophage population induced by elevated level of IL13 [109]. Another corroborating study regarding the protective role of IL-13 was illustrated in IL-13-activated peritoneal macrophages, in which these macrophages displayed enhanced phagocytosis and candidacidal activity, as well as increased production of ROS [110]. The beneficial effects of M2-polarized macrophages are similarly shown in both ischemia-reperfusion-induced (IRI) and cisplatin-induced acute kidney injury models. In these two acute kidney injury models, administration of clondronated liposomes after the induction of kidney injury led to prolonged recovery and severe inflammation [111-114]. However, given that IRI and cisplatin-induced kidney injury are both sterile kidney injury models, there is likely an additional layer of complexity regarding the macrophages function in the *C.albicans*-induced kidney injury. For instance, depending on the environmental cues, it is believed that macrophages, in both IRI and cisplatin-induced acute kidney injury, are initially inflammation promoting and subsequently switching to anti-inflammatory and tissue repair-promoting phenotype [111-114]. Hence, the effect of macrophages in *C.albicans*-induced kidney immunopathology may similarly depend on the disease progression.

1.2.2.6 Host Immunity - Interaction between macrophages and endothelial cells in kidney

In 2006, Soos et al. demonstrated that renal mononuclear phagocytes form an extensive network throughout the kidney interstitial and mesangial space, suggesting the close proximity of macrophages and endothelial lining [76]. In the event of disseminated *Candida* infection, endothelial cells are the first to encounter the blood-borne *Candida* when the fungus seeds into the organ. Upon sensing *C.albicans*, endothelial cells secrete pro-inflammatory cytokines and chemokines, produce prostaglandins PGE-2, and increase the expression of ICAM-1 and VCAM-1 [115-117]. These *Candida*-activated products released by endothelial cells influence the local environment where macrophages reside. A number of studies have demonstrated that macrophages can be modulated by PGE-2 to increase IL6 production [118, 119]. Reciprocally, cytokines such as TNF α , IL1 α and IL1 β that are produced by *Candida*-activated macrophages can induce VCAM-1 expression by the endothelial cells to promote leukocytes recruitment [115]. These studies highlighted the potential role of macrophages during invasive *C.albicans* infection, in which macrophages can be rapidly activated by endothelial-associated products before direct encountering with *C.albicans* via PRRs. Also, *Candida*-activated macrophages can further potentiate the recruitment of leukocytes by secreting cytokines that promote endothelial VCAM-1 expression.

Because of its strategic location, its expression of an extensive array of PRRs, its capacity to produce multiple cytokines/chemokines, and its interaction with endothelial cells, renal tissue resident macrophages are important first-line defense for kidney immunity against bloodstream systemic *Candida* infection. However, given that majority of the findings are based on *in vitro* experiments, correlating these findings with *in vivo* work is warranted.

1.2.3 Host Immunity - Dendritic cells (DCs)

Dendritic cells (DCs) are heterogeneous hematopoietic cell populations that bridge the innate and adaptive immune responses. DCs constantly survey the environment (e.g. blood, lymph nodes and peripheral tissues) for self- and foreign-associated antigens, ingest, process and present them to T cells through a process called antigen presentation. DCs are superior antigen presenting cells, where the processed antigen can be delivered to either major histocompatibility complex (MHC) class I or II molecules and these MHC class I-peptide and MHC class II-peptide complexes on the DC cell surface will subsequently be presented to CD8⁺ and CD4⁺ T cells respectively. Like other innate cells, DCs sense intruding pathogens with their wide range of PRRs. Engagement of pathogen PAMPs activates and triggers DCs to release cytokines that are important to activate and induce specific T cell response that is tailored for the pathogen. Hence DCs, aside from its key role in the induction and modulation of host immunity against infections, are indispensable in the generation of effective memory responses.

Generally, DCs are categorized into two main subsets, namely conventional DCs (cDCs) and plasmacytoid DCs (pDCs), where cDCs can be further subdivided into CD8⁺/CD103⁺DCs and CD11b⁺DCs. The relevant immune responses triggered by different DC subsets with their respective cytokines secretion are briefly illustrated in Schlitzer et al., as displayed in Figure 1.5 [120-123].

1.2.3.1 Conventional dendritic cells (cDCs)

cDCs comprise of all DCs ($CD8^+/CD103^+$ DCs and $CD11b^+$ DCs) except pDCs, and they are usually characterized by their high expression level of MHCII and the integrin, CD11c. The morphology of cDCs is dendrite-like and they are found in all lymphoid and non-lymphoid organs. Being highly phagocytic, cDCs are superior antigen presenting cells that are representatives of classical DC paradigm, whereby they constantly sample the environment and process any antigen they encounter, regulating the balance between the host immunity and tolerance.

$CD8^+$ DC and $CD103^+$ DC subsets constitute a distinct DC lineage that is proficient in cross-presentation. These DC subsets are important in stimulating $CD8^+$ T cell immune response and promoting Th1 differentiation through the secretion of IL12. The only difference between these two subsets is their location, where $CD8^+$ DCs reside in the lymphoid organs (e.g. spleen and lymph nodes) and $CD103^+$ DCs reside in the non-lymphoid organs (e.g. kidney).

$CD11b^+$ DCs are located in both lymphoid and non-lymphoid organs. This DC subset is a strong inducer in stimulating $CD4^+$ T cell immune response and is important in promoting Th2 and Th17 differentiation, depending on the cytokines they secrete [122, 123].

1.2.3.2 Plasmacytoid dendritic cells (pDCs)

pDCs are mainly found in the blood and lymphoid organs at steady state. They are usually characterized by the expression of Siglec H, B220/CD45RA and low expression level of CD11c (in murine). Known for its role in antiviral immunity, pDCs secrete large amount of type I interferon when stimulated. In contrast to cDCs, the morphology of pDCs is similar to plasma cells. Due to their low phagocytic nature, low expression of MHCII and co-stimulatory

molecules at steady state, pDCs are not the main players in antigen presentation [123, 124].

Figure 1.5: Individual DC subset triggers different adaptive immune response.

Activated DC subpopulation induces the corresponding immune responses through the type of cytokines they secrete. Reprinted from [123].

1.2.3.3 Host Immunity - Roles of DCs during invasive candidiasis

In recent years, the revelation about CLRs being abundantly expressed by DCs has prompted vigorous investigations on the potential crosstalk between DCs and other innate cells, as well as how DCs respond to *Candida* and subsequently mould the adaptive immune response in the host defense against disseminated candidiasis. The PRRs that DCs use to identify *C.albicans* are Dectin-1, Dectin-2, DC-SIGN, TLR2, TLR4, CR3 and Fc-receptors.

Among all the PRRs, the CLR/Syk/Card9 (e.g. Dectin-1 and Dectin-2) signaling pathway seems to play a prominent role for DCs in recognizing *Candida* and eliciting antifungal immune responses [42, 125]. Specifically, mice deficient in Card9 are highly susceptible to systemic candidiasis, as evidenced with high mortality and fungal burden observed in *Candida*-infected Card9^{-/-} mice. This is attributed by the abrogation of Syk/Card9 signaling pathway that undermine the ability of DCs in secreting TNF α , IL6 and IL1 β , in which these cytokines are important for antifungal responses [68, 126-128]. The indispensable role of DCs in regulating the host innate immunity is further illustrated by Whitney et al., where they showed that activation of DCs by Dectin-1/ β -glucan interaction induced the secretion of IL23p19 through the Syk-dependent signaling pathway. This, in turn, triggers transient secretion of GM-CSF by NK cells, which subsequently potentiates the neutrophils antifungal ability [129]. Similar to the observations in murine model, it has been documented that patients with Card9 mutation have increased likelihood of getting systemic candidiasis, owing to the inability of myeloid cells to secrete pro-inflammatory cytokines as well as impaired *Candida* killing of their neutrophils [130-132].

On the other hand, the TLR/MyD88 signaling pathway in mice also plays an important role in inducing host immune response (e.g. cytokines production) against *Candida* infection, most likely through synergistic collaboration with Dectin-1 or other CLRs, to increase the efficiency of target identification and

enhance CLR-induced host antifungal immunity [90, 133]. In contrast to what have been observed in murine models, patients with MyD88 deficiency does not seem to be vulnerable towards systemic candidiasis [134].

Apart from its role in the regulation of host innate immunity, DCs also play an important part in orchestrating and educating T cell effectors and memory response against *Candida* infection [135]. To this end, DCs, like other innate cells (e.g. macrophages), employ a plethora of PRRs that allow them to differentiate different *Candida* morphological forms, through the synergistic collaboration of several PRRs, in order to elicit appropriate Th responses through cytokines production and antigen presentation. At present, Dectin-1 seems to predominantly recognize *Candida* yeast [96] and promote Th1 and Th17 immune responses [136], while Dectin-2 seems to identify *Candida* hyphae [86, 96, 137, 138] and induce a Th17 response [136, 139].

Of note, the significance of DCs in priming T cells, or rather, the role of the host antifungal Th responses in defending against acute systemic *Candida* infection seems dispensable as it has been demonstrated that mice which are deficient of either T cells or B cells remain resistant to systemic candidiasis [7, 29, 140-142]. These observations suggest that the host defense against acute disseminated candidiasis depend mainly on the host innate immunity, instead of the adaptive counterpart, to control the infection. Nonetheless, understanding how DCs shape the adaptive immune system against *Candida* infection is of utmost importance because of the potential DCs may have in the vaccination against or prophylactic treatments of this infection.

1.2.3.4 Host Immunity - Crosstalk between DCs and T-helper (Th) cells during *Candida* infection

The adaptive immune responses that are protective against fungal infection belong to the Th1- and Th17-type immunity. Differentiation of naive CD4⁺ T cells to CD4 Th1 cells is governed by IL12 from the DCs. These Th1 cells produce IFN γ , which is essential to stimulate *Candida* killing in neutrophils.

The importance of IFN γ in invasive candidiasis has been illustrated in some knockout studies, wherein IFN γ ^{-/-} mice are more vulnerable to acute invasive candidiasis and IFN γ R^{-/-} mice are not protected from systemic *Candida* re-infection after being exposed to gastrointestinal candidiasis [143, 144]. The lack of IFN γ in mice impairs the killing capacity of phagocytic cells [145], whereby administration of exogenous rIFN γ enhances these phagocytes' *Candida* killing (e.g. reactive oxygen species (ROS) production), thus improves the prognosis of *Candida*-infected mice [146-149].

Th17 immunity has been generally accepted to be an integral part of *Candida* immunity, especially in the mucosal immunity, because of the cytokines i.e. IL17 and IL22, which are essential to potentiate and activate antifungal mechanisms. The induction of Th17 cell differentiation is strictly controlled by CLR-induced secretion of IL1 β , IL6, TGF β and IL23 by DCs. Specifically, the main role of IL17 is to recruit and stimulate neutrophil antifungal mechanisms, whereas for IL22, it is essential for the induction of antimicrobial peptides (AMPs) production by the epithelial and endothelial cells [42, 150-152]. Though DCs do prime Th17 response during systemic candidiasis in both *in vitro* and *in vivo*, however, the more crucial question is how robust Th17 response is relative to other Th responses. This question has yet been fully addressed and until then, the role of Th17 response in *Candida* immunity requires further investigations.

Nevertheless, the high susceptibility of IL17RA^{-/-} mice towards invasive candidiasis underscores the indispensable role of IL17 in *Candida* immunity, in which these mice exhibited impaired neutrophils recruitment and antifungal mechanisms. Furthermore, wild-type mice treated with mIL17A show greater resistance against disseminated *Candida* infection than the control mice [153, 154]. Moreover, besides invasive candidiasis, the protective role of Th17 response has been shown to be irreplaceable for the host defense against experimental mucosal candidiasis [155, 156]. Importance of Th17 response is further documented in patients suffering from chronic mucocutaneous candidiasis, where they are often associated with defective Th17 immunity,

with prominent reduction of IL17 and IL22 production [157]. In addition, patients with CARD9 defects have also been associated with the impairment of IL17-induced antifungal responses [130, 131].

All in all, DCs sense the presence of *C.albicans* through a repertoire of PRRs. Engagement of PRRs by *C.albicans* activates DCs, where they ingest, process and present the *Candida* antigens to their MHCII molecules. Activated DCs then migrate to the secondary lymphoid organs (e.g. lymph nodes) and prime the naive CD4⁺ T cells to differentiate into either Th1 or Th17 cells, depending on the cytokines that DCs secrete. After which, the activated T effector cells migrate to the site of infection through chemokine gradient, and release the corresponding cytokines, such as IFN γ or IL17, to further enhance antifungal killing of phagocytes (e.g. neutrophils), as well as, the secretion of AMPs by the epithelial cells [Figure 1.6].

Figure 1.6: Effective host defense against *Candida* infection requires the collaboration between the innate and adaptive immunity.

DCs are essential for serving as the first line defense in recognizing *C.albicans* and eliciting adaptive immune response against the invading *C.albicans*. Once *Candida* is detected by DCs, DCs phagocytose *Candida*, process, and present the *Candida* antigen to the naive CD4⁺ T cells in the draining lymph nodes. Depending on the cytokines DCs secrete, primed CD4⁺ T cells will differentiate to either Th1 or Th17 CD4⁺ T cells. Activated Th1 or Th17 T cells will then migrate to the site of infection and secrete the relevant cytokines that are crucial for effecting anti-*Candida* immunity. Reprinted from [42].

1.2.4 Host Immunity - Polymorphonuclear Neutrophils (PMNs)

Neutrophils are first line defense against most intruding pathogens. Being the most abundant hematopoietic cells circulating in the peripheral blood, PMNs are immediately recruited to the site of infection by the endothelial cells during infection, where they detect any microbes through their expression of PRRs and perform a spectrum of mechanisms to kill microbes, which include phagocytosis, secretion of antimicrobial peptides, formation of reactive oxygen species (ROS) and release of neutrophil extracellular traps (NETs). Besides direct killing, neutrophils also secrete cytokines and chemokines to mediate and recruit other immune cells, including monocytes, DCs and more neutrophils, to the site of infection [158, 159].

1.2.4.1 Host Immunity - Roles of PMNs during invasive candidiasis

Neutrophils are indispensable in the host defense against systemic candidiasis. Thus far, only a few PRRs that PMNs use to recognize *C. albicans* have been identified, which include TLR2, TLR4, Dectin-1, Dectin-2, MINCLE and CR3. Of these PRRs, CR3, also known as CD11b/CD18 or $\alpha M\beta 2$, has been illustrated to be the major receptor used by human neutrophils to identify *Candida* yeast β -glucan [60] and hyphae Pra-1 [160]. Consequently, activation of CR3 on human neutrophils has been shown to enhance *Candida* killing by inducing the formation of neutrophil extracellular traps (NETs) and phagocytosis [160-163].

The importance of neutrophils in this infection was established in several studies where functional impairment or absence of neutrophils, such as granulocytopenic and myeloperoxidase-deficient mice, drastically increases the susceptibility of these mice suffering from invasive candidiasis, as evidenced with higher kidney fungal load and mortality rate as compared to the immuno-competent controls [164-167]. Similar to the observations in the murine model, patients with prolonged neutropenia or defective neutrophil function are predisposed to systemic candidiasis [5, 31, 168, 169]. Moreover,

human PMNs have been illustrated to be the only immune cells capable of inhibiting the *Candida* yeast-to-hyphae transition, an undisputable virulence factor of *C.albicans* [170-172].

Importantly, the protective effect of neutrophils in containing the spread of *C.albicans* infection is greatly affected by the presence or rapid recruitment of functional neutrophils to the site of infection, which is illustrated by Romani et al., where the eradication of host neutrophils prior to the infection leads to increased fungal burden across all organs investigated (e.g. kidneys, spleen). Delayed recruitment of neutrophils to the site of infection impairs neutrophil antifungal protection whereas accumulation of neutrophils at the late stage of infection seems to be detrimental for the host [173-175].

Recruitment and activation of PMNs during *Candida* infection depends on several important cytokines and chemokines, such as IL17, IL6, IL1 β , TNF α and IL8. Pro-inflammatory cytokine IL1 β is required to induce the influx of neutrophils, to trigger the production of superoxide by PMNs and to promote IL17 responses during disseminated candidiasis [176, 177]. IL1 β -deficient mice exhibited greater susceptibility towards disseminated candidiasis when compared to the controls [177]. On the other hand, mice deficient in IL6 or TNF α displayed increased mortality and uncontrolled *Candida* growth upon infection, with a distinct reduction in the infiltration of neutrophils to the site of infection [165, 178-181]. IL6 is required for efficient neutrophil *Candida* killing function and the induction of IFN γ production [178, 179]. It has also been reported that rIL-6 play a big role in promoting neutrophils oxidative burst in human patients [182]. Though not involved in mediating neutrophil killing capacity, TNF α is important for neutrophil phagocytic ability [181, 183].

The neutrophil chemo-attractant, IL8, which is a human homologue of murine CXCL1/KC, CXCL2/MIP-2 and CXCL5, is important for neutrophils recruitment to the site of infection [184]. Accordingly, IL8 can also be secreted by activated human neutrophils in response to *Candida*, which indicates a feed-forward mechanism by these PMNs to attract more neutrophils to the site of infection

[171]. In addition, growth factors such as granulocyte colony-stimulating factors (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are vital in increasing neutrophil numbers, potentiating neutrophil fungicidal abilities and enhancing their recruitment during acute systemic candidiasis [185-188].

Taken together, the antifungal mechanisms elicited by neutrophils are strictly regulated by the cytokine milieu they are in. Hence, for effective *Candida* immunity, the collaboration between other innate immune cells and neutrophils is essential. For instance, cytokines and chemokines secreted by macrophages and DCs are able to regulate the recruitment of neutrophils and modulate the activation and *Candida* killing capacity of neutrophils.

1.3 Conditional Ablation of Specific Cell Subsets using the Diphtheria Toxin Receptor-Diphtheria Toxin (DTR-DT) system.

1.3.1 Advantage of the DTR-DT system

The DTR-DT system was first introduced by Saito et al. in 2001 to circumvent a quandary that underlies the use of transgenic knockout mice (e.g. RAG2^{-/-}), whereby the host physiology could be altered due to some compensatory mechanisms triggered by the constitutive loss of a specific cell population. As will be further described below, the affinity of human DTR to DT is 1000 times higher than that of the mouse DTR. Hence, DTR-DT system works by DT-mediated ablation of murine cells that express human DTR, whereas murine cells that do not express human DTR will be spared. The susceptible cells undergo apoptosis, a mechanism that does not trigger inflammation. When the mouse is genetically modified such that the expression of human DTR is driven by the cell-specific promoter, it will allow the depletion of this target cell population without affecting other non-targeted cells in mice *in vivo*, therefore the toxicity elicited by DT treatment to the transgenic DTR mice should be minimal.

1.3.2 Underlying mechanism of the DTR-DT system

DT is a potent heterodimeric (subunit A and B) exotoxin produced by *Corynebacterium diphtheriae*. The cytotoxicity elicited by DT is strictly dependent on the binding of the DT subunit B to the DTR that is expressed on the mammalian cells. Upon binding, the DTR, which is known as precursor of heparin-binding epidermal growth factor receptor (hbEGF), induces receptor-mediated endocytosis. Once internalized, the DT subunit A causes rapid cell death via apoptosis, by blocking cellular protein synthesis through the inactivation of elongation factor 2. Owing to the difference in three amino acids

at the binding site of murine hbEGF, the DT/DTR binding affinity in mice is much lower than that in the humans. This confers murine cells 10^3 - 10^5 times more resistant to this toxin than the human counterparts. Hence, conditional ablation of a specific target population in the mice can be achieved with genetic modification of target cells to express the high affinity human hbEGF [Figure 1.7][189-192].

Figure 1.7: Conditional ablation of target cell population using the DTR-DT system

(A) Administration of DT into wild-type mouse does not induce cellular apoptosis, since murine cells do not express high-affinity DTR on their cell surface, thereby preventing DT from binding to the cells efficiently. (B) Target cell population undergo apoptosis when DT was administered into mice that are genetically engineered to express the high affinity human/simian DTR on a particular cell population. Adapted from [189].

1.3.3 DTR Transgenic Mouse Strains

In this project, four different in house-generated DTR transgenic mouse strains were used to study the roles of different mononuclear phagocytic subsets in the host defense against disseminated candidiasis. Briefly, for the study of DC subsets, we exploited Clec9A-DTR, Clec4a4-DTR and Siglec H-DTR mouse strains, which allow the depletion of CD8⁺/CD103⁺ DCs, CD11b⁺ DCs and pDCs respectively [123, 193, 194]. On the other hand, the study of CD169⁺ macrophages was carried out in CD169-DTR mice [Figure 1.8].

1.3.3.1 Clec9A-DTR mouse

Clec9A (DNDR-1) is a member of CLR family that is largely expressed by DCs, specifically on lymphoid tissue CD8⁺ cDCs, some pDCs and non-lymphoid tissue CD103⁺ DCs. Monocytes, macrophages, T cells as well as monocyte-derived inflammatory DCs generally show no expression of Clec9A [195]. Clec9A encodes a transmembrane receptor harboring a single C-type lectin domain in its extracellular region and in its cytoplasmic tail, a single tyrosine residue within an ITAM-like sequence that has been demonstrated to signal via Syk kinase [196]. Clec9A has been demonstrated to be involved in dead cell/pathogen antigen cross presentation, as well as being an endocytic receptor that is involved in regulating humoral and cell-mediated responses, hence an important target for vaccine enhancement [195, 197, 198].

1.3.3.2 Clec4a4-DTR mouse

Clec4a4 (DCIR2), also a member of CLR family, are predominantly expressed on DC population, in particular, CD4⁺ cDCs in the lymphoid organ but absent on the lymphoid CD8⁺ cDC and non-lymphoid CD103⁺ DC subsets [199, 200]. Splenic CD4⁺ cDC subset, which highly express Clec4a4, excels in producing peptide loaded-MHC class II, which is in stark contrast to splenic CD8⁺ cDCs, where the latter are specialized in cross-presenting antigens on the MHC class I [201]. This differential antigen presentation by CD8⁺ and CD4⁺ cDCs is due to

the increased expression of proteins involving MHC class I and MHC class II processing respectively [201]. Though harboring similar structure as Clec9A in the extracellular domain, the cytoplasmic tail of Clec4a4 is an ITIM motif, and the inhibitory functions for this receptor molecule [202] has been illustrated in a recent study, where Clec4a4 was reported to be important in dampening inflammation [199].

1.3.3.3 Siglec H-DTR mouse

Siglec H is a member of CD33-related siglecs (sialic acid-binding immunoglobulin-like lectin) that belong to the immunoglobulin superfamily (IgSF). Predominantly expressed by pDCs, Siglec H contains a short cytoplasmic tail that lacks ITIM. Nonetheless, Siglec H depends on the adaptor DAP12, an ITAM, to mediate its surface expression on pDCs and cell signaling. In particular, Siglec H has been shown to be involved in mediating endocytosis and is essential for dampening type-1 IFN responses [194, 203-206]. Ligand of Siglec H has yet been identified.

1.3.3.4 CD169-DTR mouse

CD169, also known as Siglec-1 or Sialoadhesin (Sn), is a sialic acid binding receptor that is expressed exclusively by most of the tissue-resident macrophages. Similar to Siglec H, CD169 belongs to IgSF and has 17 immunoglobulin-like extracellular domains [207]. Although it recognizes a variety of sialylated glycoconjugates, CD169 preferentially binds to $\alpha 2,3$ -linked sialic acid (i.e. Neu5Ac $\alpha 2,3$ Gal) [208]. Because of its conserved sequence pattern at the extracellular region and the lack of ITIM in its short cytoplasmic tail, CD169 is believed to be involved in mediating macrophage cell-cell and cell-extracellular matrix interactions. In addition, CD169 has been reported by Schadee-Eestermans to be found within the endocytic vesicles of rodent lymph node subcapsular sinus macrophages, indicating a possible involvement of CD169 in endocytosis [209-211].

Notably, expression of CD169 on tissue resident macrophages has been shown to be organ-dependent. For instance, CD169 has been illustrated to be highly expressed by lymphoid tissue resident macrophages residing in bone marrow and lymph node [212-214]. In the spleen, CD169 is highly expressed on the marginal zone metallophilic macrophages and at low level on the red pulp, while splenic marginal zone and white pulp macrophages do not express CD169 [215, 216]. In non-lymphoid organs, CD169 has been shown to be expressed on liver kupffer cells, lung alveolar macrophages (but not lung interstitial macrophages), gut serosal macrophages (but not lamina propria macrophages) and kidney medullary macrophages (but not renal cortical macrophages). On the other hand, thymus macrophages, peritoneal macrophages and brain microglia do not express CD169 [75, 81, 216, 217].

Figure 1.8: Various transgenic DTR mouse strains that target different myeloid cell subpopulations.

This figure illustrates the ablation of target myeloid cell subsets in the respective transgenic DTR mice when DT is administrated.

1.4 AIMS OF PROJECT

Numerous studies have highlighted the importance of host innate immunity against systemic candidiasis. While neutrophils being the integral immune effector cells in *Candida* immunity are indisputable, roles of other myeloid subpopulations (e.g. DCs and macrophages) in the host defense against experimental systemic candidiasis is not well understood, owing to the lack of appropriate tools to specifically ablate mononuclear phagocytic cell subpopulations *in vivo*. In this project, we aimed to first revisit the topic of neutrophils in disseminated candidiasis. Subsequently, we investigated the *in vitro* and *in vivo* roles of individual DC subsets using various transgenic mouse models that were developed in-house. Lastly, taking advantage of the existing CX3CR1^{gfp/gfp} mouse model and our CD169-DTR mouse model, we conducted comprehensive studies on the contribution of tissue-resident macrophages in *Candida* immunity.

Chapter 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Mice

Wild type BALB/c and C57BL/6 mice were purchased from ASTAR animal facility or procured from NTU School of Biological Science animal facility. CX3CR1^{gfp/gfp} mice were purchased from The Jackson Laboratory or procured from NTU School of Biological Science animal facility. CD169-DTR, Clec9A-DTR, Siglec H-DTR were developed in BALB/c genetic background as described previously [218, 219]. These DTR mouse strains in C57BL/6 background were generated by backcrossing DTR BALB/c to B6 for at least 10 generations. OTII TCR transgenic RAG^{-/-} mouse strain is a generous gift from Asst Prof. Su Ishin, NTU. Age and gender-matched mice were used for all experiments. Transgenic mice were bred and maintained under specific pathogen-free (SPF) conditions in NTU animal facility. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC).

Clec4a4-DTR mouse strain was generated using the gene targeting strategy. Briefly, the targeting construct contains the IRES-DTR cassette followed by the removable selection marker (PGK-NeoR). This targeting construct was inserted after the stop codon of Clec4a4 gene (Figure 2.1). After electroporation of this targeting construct, several BALB/c ES colonies carrying desired DTR insertion after the stop codon of Clec4a4 could be obtained. These ES colonies were selected with antibiotics and the surviving colonies were used for blastocyst microinjection. Successful microinjection will give rise to chimeric animals and ultimately germ line transmission of the modified allele. Obtained Clec4a4-DTR knock-in mice are viable, fertile and phenotypically indistinguishable from the WT mice

Figure 2.1 Targeting construct for the generation of *Clec4a4*-DTR knock-in mouse.

2.1.2 Fungal strains

The *C. albicans* strains used in this project are SC5314 and YJB11522. The clinical isolate strain SC5314 is a gift from Prof. Wang Yue from Institute of Molecular and Cell Biology [IMCB], ASTAR; and the strain YJB11522 is a gift from Adj. Assistant Prof. (NTU) Norman Pavelka from Singapore Immunology Network [SIgN], ASTAR. YJB11522 is a recombinant *C. albicans* strain that was genetically modified as described in [220] by Kaplan's group from the WT SC5314 to express ovalbumin (OVA) on the cell surface. Permission to use the recombinant strain YJB11522 was generously granted by Associate Professor Daniel H Kaplan from University of Pittsburgh and Professor Judith Berman from Tel Aviv University. SC5314 was used for all experiments except for T-cell proliferation assay. YJB11522 was used for T-cell proliferation assay.

2.1.3 Media, buffers and solutions

Please refer to Appendix 1.1

2.1.4 Chemicals, reagents and kits

Please refer to Appendix 1.2

2.1.5 Commercial Antibodies

Please refer to Appendix 1.3

2.1.6 Computer software

Flow cytometry data analyses were done using Flowjo VX software (TreeStar Inc, Ashland, OR). Graphs and statistical analyses were generated via Graphpad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Image processing was done by ImageJ 1.48 software (Wayne Rasband, National Institutes of Health, USA).

2.2 Methods

2.2.1 *C.albicans* and mouse model of systemic candidiasis

C.albicans was cultured on YPD plate for 24 hr at 30°C, followed by 16-18 hr in YPD (SC5314) or YPAD (YJB11522) media, in a shaking incubator. *C.albicans* suspension was centrifuged, washed thrice with phosphate-buffered saline (PBS) and counted. For most of the infection experiments, 5×10^4 cfu *C.albicans* per mouse were intravenously (i.v.) injected into the mice. Mice were monitored daily during the course of infection. For heat-inactivation of *C.albicans* YJB11522, washed *C.albicans* were resuspended into 10^6 cfu/20 μ l and incubated for 1hr at 65°C. (Heat-killed *C.albicans*)

For blocking IFNAR signaling, 100 μ l MAR1-5A3 antibody was injected intraperitoneally daily to WT mice, starting from day-1 post infection.

2.2.2 Depletion of target cell population by DT and α Gr-1 antibody

For most of the experiments, DTR mice were injected with 10ng/gBW DT intraperitoneally (i.p.). 10ng/bwg DT was prepared by diluting 2000-fold from 2mg/ml DT stock. Dilution was prepared in sterile PBS supplemented with 1:100 mouse serum. DT injection scheme is shown in the result section.

α Gr-1 antibody (clone: RB6-8C5) is an in-house generated antibody targeting neutrophils. For depletion of neutrophils, α Gr-1 antibody was injected i.p. into WT mice daily, starting from day -1 post infection.

2.2.3 Cell counting

To prepare for viable cell counting, 20ul was pipetted to Neubauer chamber (10 μ l of cell suspension was mixed with 10 μ l of trypan blue). All 4 corner squares of the chamber were counted and number of viable cells can be calculated by the formula:

Total cells in 4 squares/ (4 * 2 * 10000) = number of cells in 1ml

Similar method was done for counting *C.albicans* for infection, except for the inclusion of trypan blue:

Total cells in 4 squares/ (4 * 10000) = number of cells in 1ml

2.2.4 Fungal burden analyses

Mice were culled by cervical dislocation. Brain, heart, lungs, liver, kidneys, spleen were harvested at time point of infection as indicated in result section, weighed and minced before incubating with 1mg/mL Collagenase D in digestion medium for 1.5 hour at 37 °C with agitation. Samples were resuspended repeatedly until no visible aggregates. Serial dilution was conducted and plated on YPD plates. The plates were incubated for 48hrs at

30°C. After which, the CFU was determined through counting the colonies. Fungal burden was expressed as CFU per gram of organ.

2.2.5 Tissue collection, processing and isolation of single-cell suspension

Mice were culled by cervical dislocation. Spleen and kidney were harvested and minced before incubating with 1mg/mL Collagenase D in digestion medium for 1 hour at 37 °C with agitation. Subsequently, these minced organs were resuspended repeatedly until no visible aggregates. After which, these single cell suspensions were centrifuged at 330g for 5 mins. Spleen sample supernatants were discarded and cell pellets were resuspended with 10ml RBC lysis buffers. 10 mins later, cell suspensions were centrifuged and resuspended with PBS 2%. As for the kidney sample, supernatants were discarded and cell pellets were resuspended with 5ml 35% Percoll™ followed by centrifugation at 600g for 15 mins with reduced deceleration. Supernatants were discarded and cell pellets were resuspended with 5ml RBC lysis buffers. 10 mins later, cell suspensions were centrifuged and resuspended with PBS 2%. The single cell suspensions were stored at 4 °C until use.

For cell sorting, splenic DCs, from the cell suspension prepared above, were isolated using DC enrichment gradient and the relevant reagent preparations were done in accordance to AXIS-SHIELD's application sheet C20, 6th edition. (Ruedl, 2011) The cells harvested were washed with 2%FCS IMDM and resuspended in appropriate amount before cell counting was done. After staining, filtered stained cells were sent for cell sorting, using BD Biosciences FACSAria cell sorter.

2.2.6 Cell stainings for flow cytometry analyses

Single cell suspensions of kidney and spleen were prepared as described in Section 2.2.5. To stain for cell surface antigen, fluorochrome-labelled antibodies were incubated with the cells at 4°C. After 20 mins, the cells were

spun at 500g for 2 mins and resuspended with PBS 2%. The cells were again centrifuged at 500g for 2 mins and subsequently resuspended in PBS 2% for analyses. For detection of intracellular cytokine IFN γ , IL17 and IL22, the procedure was done according to manufacturer's instructions (eBioscience). Briefly, after staining for cell surface antigens, cells were subsequently fixed with 1% formaldehyde at room temp in the dark. After 45 mins, the cells were spun at 550g for 3 mins and resuspended with 0.5% saponin. The cells were again centrifuged at 550g for 3 mins and subsequently incubated with α - IFN γ , α -IL17a or α -IL22 antibodies for 20 mins at room temperature, after which the cell pellets were resuspended in PBS 2% for analyses. Antibodies targeting cell surface antigens were prepared in PBS 2% whereas α - IFN γ , α -IL17a or α -IL22 antibodies were prepared in 0.5% saponin.

2.2.7 T cell stimulation for intracellular detection of cytokines

Single cell suspensions from uninfected control, infected control and DTR mice were restimulated with heat-killed *C.albicans* for 3 hours and subsequently, Brefeldin A (10ug/ml) was added. After another 3 hours, cells were harvested and stained as described above.

2.2.8 [H3]-Thymidine incorporation assay - in vitro T-cell proliferation assay

Single cell suspensions of spleen were prepared as described in Section 2.2.5. Cells were counted and incubated with appropriate concentration of Miltenyl Biotec CD4 microbeads in IMDM 2% for 15mins at 4°C. Cells were then washed and filtered before transferring to the equilibrated 25 LS column by MACS Separation Columns. The column was washed thrice and the cells trapped in the column were collected. Cell counting was done.

Sorted DC subsets were incubated with either OVA peptide (323-339) or heat-killed *C.albicans* for 2hrs in IMDM 2% for cell pulsing. The cells were then

washed, resuspended and counted. After which, DCs were aliquot to 96-well plate, followed by the addition of 300,000 purified OTII CD4⁺ T-cells into each well. The cells were then incubated for 2 days before adding PerkinElmer tritiated thymidine (H³) for another incubation of 16-18 hrs. Cells were harvested and analyzed with PerkinElmer Cell Harvester and Luminescence Counters respectively.

2.2.9 Cryosection Immunofluorescence staining

Mice were culled by cervical dislocation. Spleen and kidney were harvested and embedded in Optimal Cutting Temperature compound (O.C.T. Tissue Tek), and stored at -80°C. Sections of six-micron thick were cut and fixed in acetone for 10-15 mins. Sections were incubated with Fc-block for 20 mins and washed once with PBS before incubating with fluorescent antibodies diluted in PBS 2% for 1 hour. Washed once with PBS and sections were overlaid with DAKO fluorescent mounting medium. Images were obtained using a Nikon Eclipse 80i microscope at 10x and 20× objective magnifications.

2.2.10 ELISA measurement of serum cytokines

Serum TNF- α , and IL-6 levels were quantified using the ELISA MAXTM Standard kit (BioLegend, USA), according to the manufacturer's instructions.

2.2.11 Serum Creatinine

Serum creatinine levels were quantified using the Mouse Cr (Creatinine) ELISA Kit (Elabscience, China), according to the manufacturer's instructions.

2.2.12 Measurement of vascular permeability (Evans Blue)

Blood vessel permeability was assessed by Evans blue dye assay, in accordance to [221]. Briefly, this method is based on intravenous injection of

200 µL of 0.5% Evans blue/PBS into uninfected control and *Candida*-infected control and CD169-DTR mice. The animals were sacrificed 30 mins later and their organs were collected, weighed, photographed, and transferred into a 2ml eppendorf tube containing 500ul of 100% formamide for 48 h to extract the Evans blue dye from the organs. Absorbance of the formamide extraction was measured at 610 nm and normalized by the weight of the organs.

2.2.13 Histological analysis

2.2.13.1 Tissue embedding and sectioning

Mice were perfused with 30ml 4% paraformaldehyde (Sigma Aldrich, USA) (PFA)/PBS from the heart. Kidneys were harvested and incubated in 10ml of 4%PFA/PBS at room temperature, with gentle agitation. 24 hours later, 4% PFA/PBS was decanted, and replaced with 70% ethanol for long term storage.

Kidneys were transferred into embedding cassettes (Hurst Scientific, Australia) and dehydrated in 95% ethanol for 45minutes, 100% ethanol for 45 minutes, and cleared in xylene (Sigma Aldrich, USA) for 1 hour. Kidneys were then incubated in Paraplast[®] Plus[™] (Leica, USA) at 60°C for 16 hours. Followed by embedding into blocks and chilled using the Leica EG1150H (Leica, USA) and Leica EG1150C (Leica, USA) respectively. Blocks were then sectioned with the Leica RM2265 microtome (Leica, USA) into 6µm slices, which were floated on a 37°C water bath to remove wrinkles, and captured onto Superfrost Plus microscope slides (Fisher Scientific, USA), and incubated at 37°C overnight to dry.

2.2.13.2 Hematoxylin and Eosin (H&E) staining

Slides were deparaffinized in xylene (Sigma Aldrich, USA) for 20 minutes, rehydrated in 100%, 90%, 80% ethanol respectively for 2 minutes each, followed by distilled water for 2minutes. Slides were then stained in Modified Mayer's Solution Hematoxylin (Abcam, Singapore) for 3 minutes, bluing in tap

water for 5minutes, counterstained with Eosin (Sigma Aldrich, USA) for 1minutes, dehydrated in 95% and 100% ethanol respectively for 2minutes, and cleared in xylene (Sigma Aldrich, USA) for 10minutes. Slides were lastly mounted with DPX Mountant (Sigma Aldrich, USA).

2.2.13.3 Periodic Acid Schiff (PAS) staining

Slides were deparaffinized in xylene (Sigma Aldrich, USA) for 20 minutes, rehydrated in 100%, 90%, 80% ethanol respectively for 2 minutes each, followed by distilled water for 2minutes. Slides were incubated at room temperature with periodic acid solution (Abcam, Singapore) for 10minutes, rinsed in distilled water four times, stained with Schiff's solution (Abcam, Singapore) for 30minutes, followed by soaking in hot tap water 5minutes, counterstained with Modified Mayer's Solution Hematoxylin (Abcam, Singapore) 3minutes, bluing in tap water 5minutes, dehydrated in 95% and 100% ethanol respectively for 2minutes, and cleared in xylene (Sigma Aldrich, USA) for 10minutes. Slides were lastly mounted with DPX Mountant (Sigma Aldrich, USA).

All H&E and PAS Slides were viewed under the Eclipse 80i microscope (Nikon, USA), captured with the Digital Sight DS-U3 (Nikon, USA) and NIS-Elements D software (Nikon, USA).

2.2.14 Quantitative real-time PCR (qPCR)

Kidneys were first harvested from the mice and immediately homogenized in Trizol using a homogenizer (CAT X360). 2ml of Trizol was used per kidney. Kidney homogenates were subsequently passed through a 27g needle a few times after which they were centrifuged at 10,000g for 5mins at 4 degrees. Supernatants were then transferred to new 2ml RNase-free eppendorf tubes. RNAs were purified from the supernatants using the column according to the manufacturer instruction (TIANGEN – RNAsimple total RNA kit). Quantity of the purified RNAs was measured by Thermo Scientific Nanodrop 2000. A total

of 2ug from each sample was used to synthesize the first-strand cDNA according to the manufacturer instruction (Promega M-MLV reverse transcriptase). Triplicates of each sample were done for each target gene and were normalized to β -actin. The RT-PCR was set-up according to the manufacturer instruction and in this experiment 25ng template was used for each reaction (Precision™ 2X qPCR Mastermix).

Please refer to Appendix 1.4 for primer sequences.

2.2.15 Statistical Analysis

Statistical significance was tested by two-tailed Student's t-test, 1-way ANOVA with Bonferroni's multiple comparison tests and 2-way ANOVA with Bonferroni posttests as stated accordingly in the figure legends with Graphpad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Survival curves were analyzed by the Mantel-Cox long-rank test. Statistical significance was accepted if p value < 0.05.

Chapter 3: Results I

3. Understanding the host immune responses during acute systemic candidiasis

To have a better understanding on the host defense against disseminated candidiasis, we examined the host immune response during acute systemic candidiasis by monitoring the immune cells infiltration and quantifying *Candida* load in the respective organs in a temporal manner.

3.1 Infection dose determination for acute invasive candidiasis.

Before the commencement of the project, we sought to determine a suitable *C.albicans* infection dose. Ideally, we aimed for an infection dose that is sublethal to the control mice while avoiding lethal dose that could mask the phenotype eventually displayed by the mutant mice. To this end, we infected control WT mice with 3 different *C.albicans* doses- 5×10^4 cfu, 10^5 cfu and 3×10^5 cfu [Figure 3.1A]. Mice infected with 10^5 cfu and 3×10^5 cfu *C.albicans* succumbed to the infection within 11 days after infection, while about 80% of those infected with 5×10^4 cfu *C.albicans* survived till the end of the experiment (D18p.i.). This suggests that there is a threshold in the amount of *Candida* load that the host can resist systemically (e.g. 5×10^4 cfu). Hence, 5×10^4 cfu may represent an optimal dose that allows us to examine the host immune response during invasive *C.albicans* infection.

Kidneys have been shown to be the prominent organs affected by systemic candidiasis, where its inability in clearing *Candida* burden correlates with the severity of renal failure, and thus the host survivability [30, 34, 35]. To have a better assessment on a suitable infection dose, we conducted fungal burden analyses in all major organs from mice infected with 10^5 cfu and 5×10^4 cfu *Candida* at Day 1, 3 and 6 post infections (p.i.). As expected, mice infected

with a lethal dose of 10^5 cfu *C.albicans* displayed uncontrolled fungal burden in the kidneys, while the other major organs investigated (e.g. brain, lung, liver and spleen) were able to contain the *Candida* load by D6p.i. [Figure 3.1B]. Since mice infected with the lowest dose (5×10^4 cfu) survived throughout the infection, we questioned whether the dose was too low to cause any pathogenesis during the progress of infection. Surprisingly, these mice exhibited similar organ-specific fungal burdens as those infected with 10^5 cfu, with kidneys being the only organs with increasing *Candida* burden [Figure 3.1B, C]. Taken together, the dose, 5×10^4 cfu, appears to be suitable for our experimental set-ups, given that this amount of *Candida* load does not cause rapid deaths, while concurrently, able to mimic the pathogenesis caused by *C.albicans*. Hence, we decided to use 5×10^4 cfu *C.albicans* as the infection dose for subsequent experiments, unless otherwise stated.

Figure 3.1 Infection dose titration and organ fungal burdens in murine experimental model of systemic candidiasis.

(A) Survival curves of WT mice infected with 5×10^4 cfu, 10^5 cfu and 3×10^5 cfu *C. albicans* [$n = 6$ per group]. Statistical significance was analyzed using Mantel-Cox log-rank test, $**p < 0.01$. (B and C) Fungal burdens were analyzed at D1, D3 and D6p.i. for vital organs in WT mice infected with (B) 10^5 cfu and (C) 5×10^4 cfu *C. albicans* [$n = 3-5$ per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; ns, not significant. Data are representative of at least 2 independent experiments.

3.2 Expansion of splenic leukocytes during systemic candidiasis.

Spleen is the first line defense for many bloodstream pathogens because it constantly surveys for any blood-borne microbes and subsequently mediates the appropriate immune responses [32, 33]. Due to the fact that spleen is able to restrain *Candida* growth [Figure 3.1B, C], we decided to conduct preliminary investigations on splenic immunity during the infection.

Various splenic leukocyte populations were assessed from the spleens of infected mice on D0, D3, and D6p.i. During the infection, total splenic cells (CD45⁺ cells) increased significantly and this expansion was observed in all types of cells investigated i.e. myeloid cells (CD11b⁺ cells), DCs, T cells and NK cells [Figure 3.2 and 3.3]. Specifically, the numbers of neutrophils in the spleen first increased significantly by 3-fold at D3p.i., followed by a rapid and dramatic decrease of 70% at D6p.i. Similar trend was observed for monocytes in the same period [Figure 3.2C]. On the other hand, other cells such as DCs, macrophages, NK cells and NKT cells showed delayed amplification in numbers at D6p.i. Though not significant, similar trends were also detected for CD4⁺ and CD8⁺ T cells [Figure 3.2C and 3.3C]. This indicates that neutrophils and monocytes are the first to respond during the assault of *C.albicans* in the spleen. Since neutrophils are the prominent effector cells in killing *C.albicans* [164-166], neutrophil recruitment to the spleen at the early stage of infection (D3p.i.) may be one of the determining factors in containing and clearing *Candida* burden in this organ. Correspondingly, once fungal load in the spleen was cleared at D6p.i. [Figure 3.1C], the numbers of neutrophils and monocytes also subsided.

Figure 3.2 Kinetics of myeloid cells infiltration in the spleen during *Candida* infection. WT mice were intravenously (i.v.) injected with 5×10^4 cfu *C.albicans*. (A and C) Absolute numbers of individual splenic myeloid cell subset at D0, D3 and D6p.i. [n = 3-4 per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments. (B) Representative FACS plots showing the percentage of corresponding splenic myeloid cell subpopulations.

Figure 3.3 Kinetics of lymphoid cells infiltration in the spleen during *Candida* infection. WT mice were intravenously (i.v.) injected with 5×10^4 cfu *C.albicans*. (A and C) Absolute numbers of individual splenic lymphoid cell subset at D0, D3 and D6p.i. [$n = 3-4$ per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Data are representative of at least 2 independent experiments. (B) Representative FACS plots showing the percentage of corresponding splenic lymphoid cell subpopulations.

3.3 Expansion of renal leukocytes during systemic candidiasis.

An uncontrolled accumulation of *C.albicans* was only observed in the kidneys [Figure 3.1C] and this has been shown to correlate with the host survivability and disease severity [34, 35]. Here, we asked if this discrepancy between the spleen and kidney fungal burdens was attributed by the difference in their kinetics of immune cells infiltration. Similar to the spleen, renal infiltrating cells increased distinctly during the course of infection (between D0 to D6p.i.). In fact, almost all the immune cell subsets investigated showed drastic augmentation only at D6p.i. [Figure 3.4 and 3.5]. Though not significant, expansion of renal neutrophil population could already be detected (12-fold) at 3 days after infection [Figure 3.4C]. However, the expanded neutrophil population in the kidneys did not manage to control *Candida* infection [Figure 3.1C], and this could be a consequence of an insufficient amount of recruited neutrophils in the kidneys to effectively clear *C.albicans*. Indeed, at D3p.i., the number of splenic neutrophils was approximately 6×10^6 , which was 60 times higher than those in the kidneys (10^5) [Figure 3.2C]. Whether this drastic difference in the number of neutrophils between spleen and kidneys was due to the delay or impairment in neutrophil recruitment in the kidneys will be discussed later in greater details.

In contrast to the spleen, where there was no distinct accumulation of T cells at D6p.i., the T cell population expanded remarkably in the kidneys [Figure 3.5A, C]. Since kidneys were the only organs incapable of clearing *C.albicans* [Figure 3.1B, C], it is possible that the accumulation of *C.albicans* in the kidneys increases the likelihood of DCs in acquiring the *Candida* antigens and hence priming the expansion and accumulation of T cells. Another explanation could be that the presence of *C.albicans* in the kidneys may continuously stimulate the innate, endothelial and epithelial cells via the PRRs. Ligand-bound PRRs not only trigger the release of multiple chemokines and cytokines, but also increase the surface expression of ICAM-1 that is known to facilitate the recruitment of activated T cells to the site of infection.

Taken together, our results illustrated leukocytosis in both kidneys and spleen during systemic candidiasis, in which the influx of various immune cell subsets was vastly dynamic. In addition, the resistance and the capacity of an individual organ in eliminating *Candida* presence is likely to be determined by the initial influx and the size of neutrophil population in that particular organ.

Figure 3.4 Kinetics of myeloid cells infiltration in the kidney during *Candida* infection. WT mice were intravenously (i.v.) injected with 5×10^4 cfu *C. albicans*. (A and C) Absolute numbers of individual renal myeloid cell subset at D0, D3 and D6p.i. [n = 3-4 per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments. (B) Representative FACS plots showing the percentage of corresponding renal myeloid cell subpopulations.

Figure 3.5 Kinetics of lymphoid cells infiltration in the kidney during *Candida* infection. WT mice were intravenously (i.v.) injected with 5×10^4 cfu *C. albicans*. (A and C) Absolute numbers of individual renal lymphoid cell subset at D0, D3 and D6p.i. [n = 3-4 per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments. (B) Representative FACS plots showing the percentage of corresponding renal lymphoid cell subpopulations.

Chapter 4: Results II

4. Neutrophils are indispensable in defending against acute disseminated candidiasis.

Numerous studies have illustrated the essential role of neutrophils in defending against *Candida* infection. Correspondingly, lack of neutrophils or neutrophil defects has been known to be one of the predisposing factors towards systemic candidiasis [164-166]. In this section, we reiterate that neutrophils are the pivotal cells capable of protecting the host from the assault of *C.albicans*. We posit that situation where neutrophils are prevented from exerting its full candidacidal ability would render the mice vulnerable to this invasive fungal infection.

4.1 *Candida* growth kinetics in the spleen and kidneys of infected mice.

According to Paul A. Iaizzo, the average percentage of overall blood flow into the kidneys is 22%, which indicates that kidneys receive one of the highest proportion of blood as compared to the other vital organs at any given moment [222]. In view of the mode of infection that we used (iv.) and that kidneys had the highest fungal load among all the organs investigated as early as one day after infection [Figure 3.1C], we sought to understand whether the high fungal burden observed in the kidneys was simply because *C.albicans* has easier access to the kidneys, which ultimately led to more *C.albicans* lodging in the kidneys at the initial assault. To address this question, we carried out more detailed fungal burden analyses of spleen and kidneys at 3hrs, 5hrs, 8hrs, 16hrs and 24hrs after infection. Interestingly, both kidneys and spleen showed similar amount of *Candida* load at the initial phase of infection, specifically from 3h to 8h.p.i. [Figure 4.1]. At 16h.p.i, there was distinct fungal burden amplification in the kidneys, followed by constant increasing *Candida* load [Figure 4.1B and 3.1C]. In contrast, spleen fungal burden remained relatively

consistent throughout the same period of observation [Figure 4.1A]. Thus, once *C.albicans* gains access to the bloodstream, the fungus disseminates evenly to all organs and its distribution is not affected by the differential blood flow from the host cardiac output.

Figure 4.1 Kinetics of *C.albicans* load in the spleen and kidneys during the first 24hrs of *Candida* infection.

WT mice were intravenously (i.v.) injected with 10^5 cfu *C.albicans*. (A and B) Fungal burdens were analyzed at 3h, 5h, 8h, 16h and 24h.p.i. for (A) spleen and (B) kidney in infected WT mice [n = 3-6 per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

4.2 Efficient recruitment of neutrophils and monocytes to the kidneys.

Next, we asked if the uncontrolled kidney fungal burden was due to the slow recruitment of neutrophils into the kidneys during *Candida* infection. Similar to the fungal burden analyses, frequency of neutrophils and monocytes infiltrating into the spleen and kidneys were assessed at 0h, 3h, 5h, 8h, 16h and 24h.p.i. Interestingly, both spleen and kidneys displayed comparable dynamics in the recruitment of neutrophils and monocytes [Figure 4.2]. Essentially, at 16h.p.i., the frequency of neutrophils in the kidneys augmented by 20-fold [Figure 4.2C, D] while those in spleen increased by 5-fold [Figure 4A, B], when compared to the respective uninfected organs. Similar trend was observed in monocytes, though the expansion of monocyte population in both organs was only observed at 16h.p.i., whereby this amplification could already be seen as early as 8h.p.i. for neutrophils [Figure 4.2]. This observation illustrated that a relatively larger proportion of neutrophils and monocytes were actually recruited to the kidneys during *Candida* infection when compared to the initial steady-state renal neutrophil and monocyte populations. However, the additional recruitment of these cells into the kidneys during infection did not seem to be quantitatively sufficient in eliciting *Candida* killing effectively [Figure 3.4C]. The inefficient *Candida* killing observed in the kidneys could be attributed by the distinctly little renal neutrophil populations ($\sim 10^4$) in the healthy kidneys, wherein this neutrophil populations was 150 times lower than those in the spleen ($\sim 1.5 \times 10^6$) [Figure 3.2C and 3.4C]. As it seemed that the host innate immune response took more than 5hrs to respond and recruit neutrophils to the infected organs, it is possible that the intrinsic amount of available neutrophils responding to the initial assault by *C.albicans* in each organ determines whether *C.albicans* could be contained at the early stage of infection.

Figure 4.2 Kinetics of neutrophils and monocytes influx in the spleen and kidneys during the first 24hrs of *Candida* infection.

WT mice were intravenously (i.v.) injected with 10^5 cfu *C.albicans*. (A and C) Representative FACS plots showing percentage of neutrophils and monocytes in (A) spleen and (C) kidney at 0h, 3h, 5h, 8h, 16h and 24h.p.i. (B and D) Frequency of neutrophils and monocytes in (B) spleen and (D) kidney at the indicated time points of infection [n = 3-6 per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

4.3 Pronounced susceptibility to acute systemic candidiasis in mice lacking neutrophils.

To verify the non-redundant role of neutrophils in the host defense against invasive *Candida* infection, we took advantage of CD11b-DTR mice to deplete neutrophils *in vivo*. CD11b is an integrin that is widely expressed by myeloid cells. Hence, theoretically, administration of DT to CD11b-DTR mice depletes all myeloid cells *in vivo*, such as neutrophils, monocytes and macrophages. To ensure efficient ablation of myeloid cells in CD11b-DTR mice, these mice were treated with two consecutive days of 20ng/gBW DT, before the collection of spleen and kidneys for FACS analysis. Here, we showed that, in our DT treated CD11b-DTR mice, monocytes were efficiently ablated in both organs, whereas depletion of neutrophils was only partial (spleen: ~50%; kidney: ~66%) [Figure 4.3A, middle panel].

Next, we infected CD11b-DTR and control mice with the sublethal dose of 5×10^4 cfu *C.albicans*. To ensure continual depletion of targeted cell population, DT was re-administered in every 3 to 4 days. As expected, the controls survived throughout the observation period, while all CD11b-DTR mice succumbed to this infection dose within six days after infection [Figure 4.3B]. The susceptibility of CD11b-DTR mice was probably due to its incapability in controlling *C.albicans* proliferation, as depicted in Figure 4.3D, where CD11b-DTR mice displayed significantly higher kidney and liver fungal burdens than the controls.

Because most of the myeloid cells are depleted in CD11b-DTR mouse strain, we could not conclude whether its susceptibility observed in systemic candidiasis was attributed solely or predominantly by the overall reduction of neutrophils. Hence, we designed another experiment to circumvent this issue by using an antibody that specifically targets neutrophils, α Gr-1. Again, we tested the efficiency of α Gr-1 antibody in depleting neutrophils *in vivo* in wild-type mice, and we illustrated that α Gr-1 treatment induced full ablation of

neutrophils in both spleen and kidneys. Notably, monocytes in α Gr-1 treated mice remained intact [Figure 4.3A, right panel].

To have a better understanding on the function of neutrophils in this infection model, we sought to investigate if neutrophils are required only at the initial assault of *C.albicans*, or if they are essential throughout the infection. To this end, we treated wild-type mice with α Gr-1 antibody at -24h, 3h, 6h and 24h.p.i. and control mice with isotype IgG antibody at -24h.p.i. To ensure continual depletion of neutrophils, α Gr-1 treated mice and controls were treated daily with α Gr-1 and isotype IgG antibodies respectively. Interestingly, our data demonstrated that neutrophils were required throughout the infection, at least within the initial 24h.p.i. [Figure 4.3C]. In addition, as expected, mice treated with α Gr-1 antibody showed distinct higher fungal burdens in both kidneys and spleen as compared to the controls [Figure 4.3E]. Hence, neutrophils are indispensable for the host resistance in the first 24 hours of *Candida* infection since removal of neutrophils within this period would ultimately lead to complete susceptibility to the infection.

Considering that DT treated CD11b-DTR mice depletes more myeloid cell subsets than α Gr-1 treated mice, we questioned why α Gr-1 treated mice showed greater susceptibility than CD11b-DTR mice during *Candida* infection [Figure 4.3B, C]. To address this question, we went on to study cellular infiltration in the spleen and kidneys of both CD11b-DTR and α Gr-1 treated mice during the infection. Indeed, there was a distinct reduction in the total CD45⁺ infiltrating leukocytes in CD11b-DTR mice as compared to the controls, and this was due to the depletion of myeloid cells (CD11b⁺), such as neutrophils and monocytes, during DT treatment [Figure 4.4A, B]. In contrast, there was no significant difference in the total number of CD45⁺ infiltrating leukocytes between α Gr-1 treated mice and their corresponding controls [Figure 4.4C, D]. In fact, the only cell population that remained drastically low in infected α Gr-1 treated mice was the neutrophils. Because α Gr-1 treated mice exhibited greater susceptibility towards acute systemic candidiasis, and that α Gr-1-treatment only depleted neutrophils and not other myeloid cells, we

concluded that the lack of neutrophils, but not other innate cells, undermined the host defense of α Gr-1 treated mice against this infection. The relative fold reduction of neutrophils in α Gr-1 treated and CD11b-DTR mice is depicted in Figure 4.5, where distinctively more neutrophils in α Gr-1 treated mice were depleted than in CD11b-DTR mice. As a consequence, α Gr-1 treated mice were more vulnerable towards invasive *C.albicans* infection than CD11b-DTR mice. Collectively, our results emphasize the indispensable role of neutrophils in the host resistance against *Candida* infection as already postulated by others [164-167, 169, 186].

Figure 4.3 Depletion of neutrophils significantly increases the susceptibility of mice towards invasive candidiasis.

(A) Representative FACS plots showing the ablation profiles of myeloid cells (middle panel) and neutrophils (right panel) in the spleen and kidney of CD11b-DTR and α Gr-1 treated mice. Prior to the assessment of depletion profiles, CD11b-DTR mice and α Gr-1 treated mice were treated with two consecutive days of 20ng/gBW DT and α Gr-1 antibody respectively. (B and C) Survival curves of (B) DT-treated CD11b-DTR and (C) α Gr-1 treated mice infected with 5×10^4 cfu *C. albicans*. [n = 4-5 per group]. Statistical significance was analyzed using Mantel-Cox log-rank test, *p<0.05; **p<0.01. (D) Fungal burdens were analyzed at 24h.p.i. for vital organs of CD11b-DTR mice [n = 5-6 per group]. (E) Fungal burdens were analyzed at 18h.p.i. for kidney and spleen in α Gr-1 treated mice [n = 3-5 per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using two-tailed Student's t-test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

Figure 4.4 Profiles of neutrophils and monocytes in the spleen and kidneys of *Candida*-infected CD11b-DTR and αGr-1 treated mice.

Absolute numbers of (A and C) splenic and (B and D) renal CD45⁺ cells, CD11b⁺ cells, neutrophils and monocytes in (A and B) CD11b-DTR and (C and D) αGr-1 treated mice [n = 3-5 per group] at 24h.p.i. Data are illustrated as mean±SEM. Statistical significance was determined using two-tailed Student's t-test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

Figure 4.5 Higher neutrophil number reduction in αGr-1 treated mice than in CD11b-DTR mice.

Fold reduction of neutrophil numbers in spleen and kidney of αGr-1 treated and CD11b-DTR mice. Fold reduction was normalized to WT steady state neutrophil number.

4.4 Establishment of infection and DT treatment scheme.

The DTR-DT system has been a useful inducible cell depletion tool that allows study of the physiological function of a particular cell of interest *in vivo*. Unfortunately, the *in vivo* ablation of certain myeloid cell populations induces a systemic transient increase in the numbers of neutrophils (neutrophilia) and monocytes (monocytosis), a side effect that should be carefully taken into account during experimental procedures involving bacteria and fungi infections [129, 223]. In particular, in the case of systemic candidiasis, a transient neutrophilia may provide an additional protection to the DTR mice, since neutrophils play a critical role in host defense against *C.albicans* [Figure 4.3]. For this reason, we aimed to establish a suitable experimental protocol that enables us to circumvent this byproduct of the DTR-DT system.

To study the temporal event of the expansion of neutrophils and monocytes in DT-treated DTR mice, we harvested spleen and kidneys at different time points as displayed in Figure 4.6A. Both spleen and kidneys showed similar kinetic influx of neutrophils and monocytes. In particular, neutrophilia was observed immediately after the first DT dose and this phenomenon subsided two days after the first DT treatment [Figure 4.6B, C, left panel]. On the other hand, there was a delayed onset in monocytosis, where its transient

augmentation was detected between Day 1 to 3 post DT injection [Figure 4.6B, C, right panel].

As it is necessary to treat the DTR mouse strains with DT in a regular interval to ensure continual ablation of the target cell subset, there is a possibility that neutrophilia and monocytosis may recur whenever DT is administered. To address this issue, we re-injected DT to the mice in a 4-day interval and harvested their spleen and kidneys to assess for any expansion in the neutrophils and monocytes [Figure 4.6A]. Our study revealed that subsequent DT treatments (Day 4 and 8) to the DTR mice did not induce significant neutrophilia and monocytosis [Figure 4.6B, C].

All in all, neutrophilia and monocytosis occurred only after the first DT treatment and the amount of neutrophils and monocytes after subsequent DT treatments remained relatively similar as during steady state [Figure 4.6B, C]. Hence, by bypassing the initial neutrophilia and monocytosis, we should be able to use the DTR-DT system to investigate the role of the target cell population in disseminated *Candida* infection model.

Our improvised experimental scheme is depicted in Figure 4.6D. Briefly, 10ng/gBW DT was given to the DTR mouse strains in every 3-day intervals to maintain continual depletion of the target cell population. To bypass neutrophilia and monocytosis, we infected the mice with *C.albicans* 2 days after the second dose of DT.

Figure 4.6 DT treatment and infection scheme - bypassing DT-induced neutrophilia and monocytosis.

(A) Experimental setup to assess the kinetics of DT-induced neutrophilia and monocytosis in the spleen and kidneys of DTR mice. (B and C) Frequency of neutrophils and monocytes in (B) spleen and (C) kidney at the indicated time points after DT treatment [$n = 3-6$ per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Data are representative of at least 2 independent experiments. (D) Infection scheme for all DTR mouse strains.

Chapter 5: Results III

5. Individual DC subset is dispensable in the host immunity against invasive candidiasis.

Broadly, the investigation on the role of DCs in disseminated candidiasis revolves around the functions and downstream signaling pathways of different PRRs upon *Candida* engagement and the type of Th immunity primed by DCs in the presence of different *Candida* morphological forms. Among all the techniques used to elucidate DC functions in invasive candidiasis, most of the studies employed the use of receptor knockout mice (e.g. Clec4n^{-/-} [46], TLR9^{-/-} [224]), murine bone-marrow derived DCs and human monocyte-derived DCs [53, 225]. Although current knowledge about the role of DCs were based on the mortality of these receptor knockout mice observed and their corresponding *in vitro* BMDCs cultures, one cannot deny the possibility that the susceptibility observed in these transgenic knockout mice could be attributed by the impaired function of other cells, such as macrophages, or a collective defect in the immune system since these receptors are not exclusively expressed by DCs.

To the best of our knowledge, the role of DC subsets during systemic candidiasis has not been well investigated, owing to the lack of appropriate tools in ablating these DC subpopulations and the byproducts of the DTR-DT system (neutrophilia, monocytosis) as mentioned earlier. With the help of our optimized infection scheme, as well as the availability of a series of new transgenic DTR mice that allow the depletion of different DC subsets, we attempted to investigate the *in vivo* role of various DC subsets in the immunity against systemic candidiasis.

5.1 Conditional ablation of different DC subsets *in vivo* using the DTR-DT system.

To reaffirm the ablation efficiency of specific DC subsets *in vivo* in our transgenic mice, Clec9A-DTR, Clec4a4-DTR and Siglech-DTR mice were treated with two consecutive days of 10ng/gBW DT. The depletion profiling of these transgenic DTR mouse strains were assessed in the spleen and kidneys. As shown in Figure 5.1A, administration of DT induced the ablation of CD8⁺ DCs, CD11b⁺ DCs and pDCs in the spleen of Clec9A-DTR, Clec4a4-DTR and Siglech-DTR mice respectively. Notably, conditional ablation of Clec9A-DCs partially depletes pDCs since Clec9A is expressed at low level on these cells [193].

Correspondingly, in the kidneys [Figure 5.1B], depletion of CD103⁺ DCs was observed in Clec9A-DTR mice, while there was only partial ablation of CD11b⁺ DCs in Clec4a4-DTR mice. The incomplete depletion of CD11b⁺ DCs in the kidneys could be due to the differential expression of Clec4a4 on CD11b⁺ DCs in the lymphoid and those in the peripheral organs such as kidneys. Clec4a4 expression was originally found in the splenic CD11b⁺ DCs [226]. In addition, Clec4a4 mRNA expression was found predominantly in lymphoid tissue such as spleen and lymph nodes, but at a substantially lower level in non-lymphoid tissue such as kidneys [227]. Consistent with the observations by other groups, we did not observe any pDCs in the kidneys during steady state [228, 229].

Figure 5.1 Depletion Profiles of target DC subsets in DTR transgenic mice.

Representative FACS plots showing the profiles of (A) spleen and (B) kidney of WT, Clec9A-DTR, Clec4a4-DTR and Siglec H-DTR mice after treated with 2 consecutive doses of 10ng/gBW DT [n = 3 per group]. Relative percentage of individual DC subpopulation in the respective DTR mouse strains is depicted as above. Data are representative of at least 2 independent experiments.

5.2 The depletion of distinct DC subsets in the mice does not exacerbate their susceptibility nor protect them towards systemic candidiasis.

To address the roles of DC subsets in the host defense against disseminated candidiasis, we monitored the survival of different DTR mouse strains, namely, Clec9A-DTR, Clec4a4-DTR and Siglec H-DTR, after a sublethal infection of 5×10^4 cfu *C.albicans*. Unexpectedly, all DC-DTR strains exhibited similar mortality as the controls [Figure 5.2A]. In addition, these DTR strains displayed comparable spleen and kidney fungal burdens as the controls (D6.p.i.) [Figure 5.2B, left]. Considering that the infection dose used could be too low to assess the functions of DCs in these mice, we infected a different batch of DC-DTR mouse strains with a lethal dose of 10^5 cfu *C.albicans* [Figure 3.1A] and examined their spleen and kidney fungal burdens at D6p.i. Similar to those infected with 5×10^4 cfu, we observed an identical trend in the spleen and kidney fungal loads across all the strains tested [Figure 5.2B, right].

In recent studies, the involvement of type I interferons (IFNs) in the recruitment of monocytes and neutrophils during *Candida* infection has been postulated [230, 231]. Since pDCs are major type I IFN producers and they also express *Candida*-sensing PRRs such as Dectin-1 and Dectin-2 [232], it was surprising that pDCs-depleted SiglecH-DTR mice displayed comparable survivability and fungal burdens as the controls [Figure 5.2A, right panel]. Hence, to further elucidate the importance of type I IFNs for this infection, we injected the mice with MAR1, a blocking monoclonal antibody that binds to the IFN α/β receptor subunit 1 (IFNAR-1), in the attempt to neutralize the type I IFN signaling processes [233]. However, we did not observe any difference in their mortality and physical health between the groups [Figure 5.2C]. Collectively, our data suggest that type I IFNs do not play a major role in the host defense against sublethal invasive *Candida* infection.

Figure 5.2 Individual DC subset is dispensable in the host immunity against *Candida* infection.

(A) Survival curves of DT-treated Clec9A-DTR (left panel), Clec4a4-DTR (middle panel) and Siglec H-DTR (right panel) mice infected with 5x10⁴cfu *C.albicans* [n = 28-30 per group]. Data are a combination of 5 independent experiments. Statistical significance was analyzed using Mantel-Cox log-rank test; ns, not significant. (B) Fungal burdens were analyzed at D6p.i. for kidney and spleen in all 3 DTR mouse strains infected with 5x10⁴cfu (left panel) and 10⁵cfu *C.albicans* (right panel)[n = 4-5 per group]. Data are illustrated as mean±SEM. Statistical significance was determined using two-tailed Student's t-test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments. (C) Survival curve of α-IFNAR treated mice infected with 5x10⁴cfu *C.albicans* [n = 5 per group]. Statistical significance was analyzed using Mantel-Cox log-rank test; ns, not significant. (D) Splenic CD11b⁺ DCs and CD8⁺ DCs were pulsed with heat-killed recombinant *C.albicans* YJB11522 and co-cultured with naive OTII CD4⁺ T cells [n = 3-4 per group]. Proliferation of OTII CD4⁺ T cells was measured based on the relative uptake of thymidine (H³). Data are illustrated as mean±SEM. Statistical significance was determined using two-tailed Student's t-test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments. Neg control, unpulsed DCs; Pos control, OVA-pulsed DCs; c.p.m., counts per minute.

5.3 Absence of CD8⁺ DCs or CD11b⁺ DCs does not affect the general cellular infiltration in the spleen during invasive candidiasis.

Because DCs have been suggested to be important regulators in the host innate immunity against *Candida* infection through the secretion of pro-inflammatory cytokines [126-129, 234], we aimed to understand whether the absence of either DC subsets affects the infiltration of leukocytes into the spleen and kidneys during disseminated *Candida* infection. To this end, splenic and renal infiltrating leukocytes of infected Clec9A-DTR, Clec4a4-DTR and control mice were compared at D6p.i. Depletion efficiency of specific DC subsets throughout the infection was verified in the spleen and kidneys of the respective DTR mice [Figure 5.3A and 5.4A, 3rd row]. Despite the ablation of target DC subsets, infected DTR mice displayed matching expansion of total splenic cells. No significant difference in the populations of neutrophils, monocytes, NK cells and T cells was detected across the strains [Figure 5.3].

5.4 Disparity in the infiltration of NK, NKT and CD8⁺ T cells into the kidneys of Clec4a4-DTR and Clec9A-DTR mice.

Analogous to the spleen, the infiltration of neutrophils and monocytes in the kidneys, during infection, were not affected by the absence of either CD103⁺ DCs or CD11b⁺ DCs [Figure 5.4A]. However, in Clec4a4-DTR kidney, there was a significant reduction in the numbers of NK and NKT cells, while in Clec9A-DTR kidney, CD8⁺ T cells were distinctively lower in numbers [Figure 5.4B]. Nonetheless, the reduction of these cells did not seem to affect the kidney defense against *Candida* infection since the kidney fungal burden and mortality of these mice were comparable to the infected controls [Figure 5.2A, B].

Figure 5.3 Profiles of leukocytes in the spleen of *Candida*-infected Clec9A-DTR and Clec4a4-DTR mice.

Clec9A-DTR and Clec4a4-DTR mice were intravenously (i.v.) injected with 5×10^4 cfu *C. albicans*. Absolute numbers of various splenic (A) myeloid and (B) lymphoid cells at D6p.i. [n = 3-5 per group]. Data are illustrated as mean ± SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

Figure 5.4 Profiles of leukocytes in the kidneys of *Candida*-infected Clec9A-DTR and Clec4a4-DTR mice.

Clec9A-DTR and Clec4a4-DTR mice were intravenously (i.v.) injected with 5×10^4 cfu *C. albicans*. Absolute numbers of various renal (A) myeloid and (B) lymphoid cells at D6p.i. [$n = 3-5$ per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Data are representative of at least 2 independent experiments.

5.5 Comparable ability of CD8⁺ DCs and CD11b⁺ DCs in inducing CD4⁺ T cell proliferation in the presence of *Candida* antigens.

CD8⁺ DCs are known to promote Th1 differentiation through the secretion of IL12, whereas CD11b⁺ DCs preferably prime Th2 and Th17 responses [123]. Since the protective role of IFN γ - and IL17-mediated immunity for systemic candidiasis has been demonstrated by many studies [143, 144, 153, 154], we explored whether CD8⁺ DCs and CD11b⁺ DCs displayed different abilities in priming the naive CD4⁺ T cells. In this experiment, the recombinant *C.albicans*, YJB11522, was used. YJB11522 is a recombinant *C.albicans* that is genetically modified from the wild-type *C.albicans*, SC5314, to express ovalbumin (OVA) antigen on their cell surface [220]. Splenic CD8⁺ DCs and CD11b⁺ DCs were first pulsed with heat-inactivated recombinant *C.albicans* and subsequently co-cultured with OVA-specific OT2 CD4⁺ T-cells, where their proliferation were assessed using [³H]-Thymidine incorporation assay. Here, we showed that CD8⁺ DCs and CD11b⁺ DCs exhibited parallel ability in inducing T-cell proliferation *in vitro* [Figure 5.2D].

5.6 Differential cytokines production by CD4⁺ T cells in the absence of CD8⁺ DCs or CD11b⁺ DCs.

Taking into account of the possibility that Clec9A-DTR and Clec4a4-DTR mouse strains employed different Th-type immunity against *Candida* infection, we proceeded to examine the type of Th response initiated in the absence of the corresponding DC subsets. Considering that IFN γ - and IL17-mediated immunity are protective against *Candida* infection [143, 144, 153, 154], production of IFN γ , IL17 and IL22 by activated splenic CD4⁺ T cells of infected Clec9A-DTR and Clec4a4-DTR mice were evaluated [Figure 5.5A, B]. To ensure that CD4⁺ T cells were primed and activated *in vivo*, we harvested the spleens from these mice 10 days after infection.

The ablation of CD8⁺ DCs in Clec9A-DTR mice resulted in significantly lesser IFN γ -producing CD4⁺ T cells and relatively more IL17-producing ones. Surprisingly, absence of CD11b⁺ DCs in Clec4a4-DTR mice led to a decrease in both CD4⁺ T cells that produced IFN γ and IL17 [Figure 5.5A, B]. Based on the polarization of CD4⁺ T cells in these mouse strains, the resistance of Clec9A-DTR mice against disseminated candidiasis may be due to the inclination of their immune response towards Th17 immunity. On the other hand, more investigations are required to explain the resistance displayed by Clec4a4-DTR mice against *Candida* infection since they produced lower levels of both IFN γ - and IL17- secreting CD4⁺ T cells. Nevertheless, the resistance observed in both Clec9A-DTR and Clec4a4-DTR mice could be attributed by their innate immune responses, since T cell-mediated immunity has been demonstrated to be redundant in acute systemic candidiasis [7, 29, 140-142].

5.7 Rag2^{-/-} mice are less vulnerable towards systemic *Candida* infection.

To reaffirm that the role of T cells are expendable for this infection model, we infected Rag2^{-/-} mice with the lethal dose of 10⁵cfu *C.albicans*. Similar to the observations made by other studies [140-142], Rag2^{-/-} mice were prominently more resistant against systemic candidiasis [Figure 5.5C]. However, the resistance observed in Rag2^{-/-} mice could be attributed by possible neutrophilia in the host system to compensate for the loss of T- and B- cells. More investigations are required to verify this hypothesis.

Taken together, based on our DTR-DT model, the role of individual DC subset seems to play a minor role in the host defense against invasive candidiasis. However, we could not rule out the importance of DCs in mediating the host innate immunity against systemic *C.albicans* infection, since there is a possibility that the integral function of a particular DC subset was masked by some compensatory mechanisms elicited by the remaining DC pool in each DT-depleted DTR strain. Hence, there could be a redundancy in the function of each DC subset in anti-*Candida* immunity.

Figure 5.5 Absence of either Clec9A⁺ DCs or Clec4a4⁺ DCs during *Candida* infection drastically reduces IFN γ -producing CD4⁺ T cell populations.

(A and B) Splenic cells were harvested from the respective mice groups, 10 days after 5×10^4 cfu *C. albicans* infection. Splenic cells were then re-stimulated with heat-killed *C. albicans* for 24 hours before assessing the polarization of CD4⁺ T cells. (A) Gating strategy and representative FACS plots (right) showing the percentage of IFN γ -, IL17a- and IL22-producing CD4⁺ T cells. (B) Frequency of IFN γ -, IL17a-, IL22-, and IL22/IL17-double producing CD4⁺ T-cells from the respective mice groups [n = 4 per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments. (C) Survival curve of Rag2^{-/-} mice infected with 10^5 cfu *C. albicans* [n = 21-27 per group]. Data are a combination of 3 independent experiments. Statistical significance was analyzed using Mantel-Cox log-rank test; **p<0.01.

Chapter 6: Results IV

6. CD169⁺ F4/80⁺ tissue resident macrophages **are essential for kidney immunity against** **systemic candidiasis**

The integral roles of macrophages in defending against *Candida* infection have been illustrated in numerous studies, in which upon *Candida* recognition, macrophages mediate *Candida* killing either through direct phagocytosis or through the secretion of neutrophil-recruiting chemokines and cytokines. Most of the investigations on the function of macrophages were examined in *in vitro* cultures, where macrophage-like cell lines or monocyte-derived macrophages were used. Because of their inferior capacity in *Candida* killing as compared to neutrophils, role of macrophages during disseminated candidiasis *in vivo* has been less appreciated until a recent publication by Lionakis et al., where they elegantly demonstrated the importance of renal tissue resident macrophages as the first line defense for this infection model[82].

To date, the role of CD169⁺ macrophages in defense against systemic *Candida* infection has not been illustrated. Therefore, in this section, we aimed to examine their contribution in the host resistance against acute invasive candidiasis.

6.1 Majority of the CD169⁺ F4/80⁺ tissue resident macrophages are localized in the renal outer medulla region.

Kidneys are the main target organs affected in disseminated candidiasis. Unlike spleen, the anatomic structure of kidney is not homogeneous, where different layers of kidneys constitute different parts of the filtration system. Since CD169⁺ macrophages are a subpopulation of macrophages, we first

sought to identify the localization of these cells in the kidneys, which may help to glean some insights on the role of renal CD169⁺ macrophages.

The structure of kidney is divided into renal cortex, medulla and pelvis. The kidney basic functional unit, also known as the nephron, spans across the cortical and medullar region, where the renal corpuscles are located in the renal cortex. The medullar region can be subdivided into outer medulla and inner medulla. In this report, in order to avoid confusion, the outer medulla will be stated as renal medulla, while the inner medulla will be mentioned as the collecting duct [Figure 6.0][235, 236].

Figure 6.0: Histology image of kidney.

Picture showing different kidney regions. 1) Cortex; 2) Medulla; 3) Pelvis; 4) Collecting Duct. Reprinted from [236].

To explore the position of CD169⁺ F4/80⁺ macrophages in the kidney, we stained the uninfected kidney sections with immunofluorescence antibodies specific for F4/80-a pan macrophage marker, CD31-an endothelial cell marker [237] and CD169 respectively. Here, we demonstrated that most of the CD169⁺ F4/80⁺ macrophages resided in the outer medullar region of the kidneys, while F4/80⁺ cells were randomly distributed across the cortical and medullar region. Kidney glomeruli are depicted as a cluster of green cells that were stained for CD31-positive in the renal cortex [Figure 6.1]. To our surprise, no F4/80⁺ cells were detected in the collecting duct [Figure 6.1, last panel].

Kidney (200X)

Cortex

Medulla

Collecting Duct

Figure 6.1 Kidney CD169⁺ F4/80⁺ macrophages are found in the renal medulla during steady state.

Representative cryo-sections of kidney from WT mice. Sections were stained with immunofluorescence antibodies specific for F4/80, CD169 and CD31. Different regions of the kidneys were illustrated (cortex, medulla and collecting ducts) to define the localization of renal CD169⁺ F4/80⁺ macrophages. Magnification, 200x; thickness, 6μm.

Figure 6.2 Depletion of renal CD169⁺ F4/80⁺ macrophages does not affect the kidney structural integrity during steady state.

(A and B) Representative cryo-sections of (A) spleen and (B) kidney from WT and DT-treated CD169-DTR mice. (A) Spleen sections were stained with immunofluorescence antibodies specific for F4/80 and CD169. Magnification, 40x. (B) Kidney sections were stained with F4/80, CD169 and CD31. Magnification, 200x. (C) Representative histological H&E sections of different kidney regions from WT and CD169-DTR mice. Magnification, 200x; Scale bars, 100µm.

6.2 Ablation of CD169⁺ macrophages does not alter the anatomical structure of the kidneys.

Expectedly, administration of DT to CD169-DTR mouse strain depleted CD169⁺ macrophages in both spleen and kidneys [Figure 6.2A, B]. In view of the report illustrated by Soos et al. that CX3CR1⁺ F4/80⁺ mononuclear phagocytes form an extensive network surrounding renal nephrons [76], we aimed to investigate whether the ablation of CD169⁺ macrophages, which reside in the medullar interstitium, would affect the overall integrity of the kidney structure. To this end, we performed histology on the kidneys harvested from DT-treated control and CD169-DTR mice. We observed no prominent difference between the kidneys of control and CD169-DTR mice, indicating that depletion of CD169⁺ macrophages does not significantly affect the anatomical structure of the kidneys [Figure 6.2C].

6.3 Splenic F4/80^{hi} macrophages are efficiently depleted in CD169-DTR mice.

In the study conducted by Lionakis group, mice deficient of CX3CR1 are highly vulnerable towards systemic candidiasis, owing to their decreased macrophage population in the kidney during steady and infection state [82]. Noteworthy, the dependency of CX3CR1 for the maintenance of mononuclear phagocyte populations is only restricted to the kidneys and small intestine, possibly due to the high expression of its ligand, fractalkine, in their niches [238, 239]. Since CX3CR1-deficient mice are highly susceptible to invasive *Candida* infection and kidney macrophages are predominantly affected in this knock-in mouse, we set out to conduct experiments with both CX3CR1-deficient and CD169-DTR mouse strains. Here, we used CX3CR1^{gfp/gfp} mouse strain, whereby the first 390bp of the coding exon in both alleles are replaced by the GFP reporter gene [240]. Consequently, CX3CR1^{gfp/gfp} knock-in mouse lacks CX3CR1 expression and hence no CX3CR1-mediated signaling. For easier reference, we termed F4/80^{hi} CD11b^{lo} cells as Fraction I macrophages and F4/80^{int} CD11b^{hi} cells as Fraction II population.

In CD169-DTR mice, we observed that DT treatment significantly ablated splenic Fraction I macrophages [Figure 6.3A, B, D]. Unlike the kidneys, the low level of CX3CR1 expression in the spleen suggested that the maintenance of mononuclear phagocytes is independent of CX3CR1 signaling [Figure 6.3E]. Hence expectedly, Fraction I macrophage population in the spleen remained intact in CX3CR1^{gfp/gfp} mice. As for Fraction II population in the spleen, there was no significant reduction in both CD169-DTR and CX3CR1^{gfp/gfp} mice [Figure 6.3A, B]. In addition, neither the ablation of CD169⁺ macrophages nor the deficiency in CX3CR1 substantially affected the total splenic leukocyte population [Figure 6.3C].

Figure 6.3 Depletion Profiles of splenic macrophages in WT, CD169-DTR and CX3CR1^{gfp/gfp} mice.

(A) Representative FACS plots showing the percentage of splenic Fraction I and II populations. (B and C) Absolute numbers of splenic (B) Fraction I, II populations, and (C) CD45⁺ cells. (D) Frequency of splenic Fraction I and II populations [n = 3-5 per group]. Data are illustrated as mean±SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. (E) Representative histogram showing the expression level of CX3CR1 on splenic Fraction I population from CX3CR1^{gfp/+} mice. Data are representative of at least 2 independent experiments.

6.4 Renal F4/80^{hi} macrophages are partially depleted in both DT-treated CD169-DTR and CX3CR1^{gfp/gfp} mouse strains.

Unlike the spleen where we observed full ablation, F4/80^{hi} Fraction I macrophages in the kidneys were partially depleted in both CD169-DTR and CX3CR1^{gfp/gfp} mice [Figure 6.4A]. In fact, flow cytometry analysis showed that only macrophages expressing slightly lower levels of F4/80 and CD11b were totally ablated, whereas macrophages expressing the highest levels were still detectable. Based on the depleted and non-depleted Fraction I populations, we further subcategorized the kidney Fraction I population into Fraction Ia (F4/80⁺⁺CD11b⁺) and Fraction Ib (F4/80⁺⁺⁺CD11b⁺⁺) [Figure 6.4A]. In the kidneys of CD169-DTR mice, although there was a slight reduction in the Fraction Ib population, administration of DT appeared to deplete mainly the Fraction Ia population. Absolute numbers of Fraction Ia and Fraction Ib populations are illustrated in Figure 6.4C. On the other hand, aside from the reduced number of Fraction Ia macrophages, Fraction Ib population was also affected in CX3CR1-deficient mice [Figure 6.4C]. While both Fraction Ia and Ib populations expressed high level of CX3CR1, there was also a slight difference in the CX3CR1 expression level between these 2 fractions [Figure 6.4B]. High CX3CR1 expression suggests CX3CR1-dependent maintenance and therefore both Fraction Ia and Ib populations were highly affected in the CX3CR1-deficient mice. Similar to the observation in the spleen, total leukocyte number and Fraction II population were unaffected in the kidneys of both CD169-DTR and CX3CR1^{gfp/gfp} mouse strains [Figure 6.4C, D].

All in all, our data depicted that the treatment of DT to CD169-DTR mice primarily depleted Fraction Ia population in the kidneys, whereas the deficiency of CX3CR1 resulted in the reduction of both Fraction Ia and Fraction Ib populations.

Figure 6.4 Depletion Profiles of renal macrophages in WT, CD169-DTR and CX3CR1^{gfp/gfp} mice.

(A) Gating strategy and representative FACS plots showing the percentage of renal Fraction Ia, Ib and II populations from WT, CD169-DTR and CX3CR1^{gfp/gfp} mice. (B) Representative histogram showing the expression level of CX3CR1 on renal Fraction Ia and Ib population from CX3CR1^{gfp/+} mice. (C and D) Absolute numbers of renal (C) Fraction Ia, Ib, II populations, and (D) CD45⁺ cells. (E) Frequency of renal Fraction Ia, Ib and II populations. [n = 3-5 per group]. Data are illustrated as mean±SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

6.5 CD169-DTR and CX3CR1-deficient mice show different susceptibilities towards disseminated *Candida* infection.

To assess the importance of CD169⁺ macrophages, CD169-DTR mice, together with CX3CR1^{gfp/gfp} mice, were infected with a sublethal dose of 5x10⁴cfu *C.albicans* and their survivability was monitored. Mice depleted of CD169⁺ macrophages were distinctively more vulnerable than the infected controls [Figure 6.5A]. Furthermore, consistent with the observations reported by Lionakis et al. that the expression of CX3CR1 is essential for the survival of the mice upon *Candida* infection [82], our results showed that infected CX3CR1^{gfp/gfp} mice displayed 100% mortality rate. Interestingly, one copy of the *Cx3cr1* gene was insufficient to rescue the phenotype, since CX3CR1^{+/gfp} mice also exhibited enhanced susceptibility when compared to the infected controls [Figure 6.5B]. Taken together, these data showed that while CD169-DTR mice exhibited increased sensitivity towards systemic *C.albicans* infection as compared to the WT mice, CX3CR1^{gfp/gfp} mice displayed the highest susceptibility and lethality towards *Candida* infection.

6.6 Quantification of kidney fungal burdens of CX3CR1^{gfp/gfp} and CD169-DTR mice.

Next, we addressed the question whether the vulnerability observed in CD169-DTR and CX3CR1^{gfp/gfp} mouse strains was caused by an uncontrolled *Candida* growth at the initial stage of infection due to the absence of tissue resident macrophages. Since macrophages were depleted systemically in CD169-DTR mice and CX3CR1 deficiency may affect macrophages in other organs, we conducted fungal burden analyses on all vital organs at D1p.i. Intriguingly, as compared to the infected controls, CD169-DTR mice did not display any significant increase in the fungal burdens across all organs, though the fungal load in the kidneys was slightly higher. Strikingly, kidney fungal load of CX3CR1^{gfp/gfp} mice was exceedingly higher than those of both the infected control and CD169-DTR mice, suggesting that CX3CR1 deficiency greatly impairs the host capacity to restrict *Candida* proliferation in the kidneys even at

the very early stage of infection [Figure 6.5C]. Of note, the brain fungal burden of CX3CR1^{gfp/gfp} mice was also significantly higher than that of the infected control and CD169-DTR mice [Figure 6.5C].

Figure 6.5 Both CD169-DTR and CX3CR1^{gfp/gfp} mice display increased susceptibility, with the latter showing the highest sensitivity towards systemic *Candida* infection.

(A and B) Survival curves of (A) DT-treated CD169-DTR and (B) CX3CR1^{gfp/gfp} mice infected with 5x10⁴cfu *C.albicans* [n = 7-22 per group]. Data are a combination of 2-3 independent experiments. Statistical significance was analyzed using Mantel-Cox log-rank test; **p<0.01; ***p<0.001; ns, not significant. (C) Fungal burdens were analyzed at D1p.i. for vital organs in WT, CD169-DTR and CX3CR1^{gfp/gfp} mice [n = 3-4 per group]. Data are illustrated as mean±SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

6.7 A unique renal subset of macrophages provides first-line defense against the initial assault by *C.albicans*.

Macrophages comprise of a heterogeneous family of phagocytic cells. It is widely accepted that adult tissue-resident F4/80^{hi} macrophages are established prenatally and display self-renewing capacity, with the exception of dermal, renal, cardiac and intestinal resident MHCII^{hi} F4/80^{hi} macrophages, which are progressively replenished by circulating adult blood monocytes. During infection or inflammation, massive influx of monocytes occurs, in which these monocytes differentiate to become either DCs or macrophages. Specifically, these monocyte-derived macrophages can also be known as inflammatory macrophages. Inflammatory macrophages are distinctive from the tissue-resident macrophages as these inflammatory infiltrates are entirely derived from bone-marrow derived monocytes. Of note, the Fraction I population illustrated in Figure 6.4A was mainly the tissue-resident macrophages, while monocytes and monocyte-derived macrophages constituted the Fraction II population. Monocytes and monocyte-derived macrophages populations can be segregated using the monocyte-waterfall model as proposed by Tamoutounour et al. [241], which is depicted in Figure 6.6B.

Given that kidneys from CX3CR1-deficient mice showed remarkably high fungal load while those from CD169-DTR mice displayed similar fungal load as the infected controls, we sought to understand whether this unexpected phenotypes can be explained by the dynamics of the immune cell infiltration at D1p.i. CD169-DTR mice displayed similar number of infiltrating neutrophils and monocytes as the infected controls [Figure 6.6A]. Consistently, Fraction Ia population was ablated in the infected CD169-DTR mice while leaving the Fraction Ib population relatively intact [Figure 6.6C]. In addition, the number of Fraction II population in CD169-DTR mice did not differ much as compared to the infected controls [Figure 6.6D, E].

In stark contrast, the numbers of infiltrating neutrophils and monocytes in CX3CR1-deficient mice were exceedingly higher than in the infected control and CD169-DTR mice [Figure 6.6A]. Strikingly, the deficiency of CX3CR1 affected all macrophage populations (both Fraction I tissue-resident macrophages and Fraction II inflammatory macrophages) in the kidneys during the infection [Figure 6.6C, E]. The lack of inflammatory macrophages was not due to the impairment of monocyte infiltration as there was distinctly more monocytes accumulated in CX3CR1-deficient mice [Figure 6.6E].

All in all, consistent with the findings reported by Lionakis et al. [82], the lack of renal macrophages in CX3CR1^{gfp/gfp} mice seemed to be the main cause of its enhanced susceptibility towards invasive candidiasis. Despite having excessive infiltrating neutrophils and monocytes, CX3CR1-deficient kidneys exhibited significantly high *Candida* burden. This reiterated that the renal macrophages are essential in conferring host resistance against systemic candidiasis, possibly in collaboration with the infiltrating neutrophils and monocytes, thus suggesting the requirement of a balanced contribution from multiple innate myeloid effector cells in eliciting the protective *Candida* immunity.

In addition, our data suggest that of all the cellular infiltrates examined at D1p.i, Fraction Ib population may have important functional significance at early stages of *Candida* disease progression, based on the fact that this macrophage sub-population remained intact in the infected CD169-DTR mice but was absent in the infected CX3CR1^{gfp/gfp} mice.

Figure 6.6 Profiles of myeloid cells in the kidneys of *Candida*-infected CD169-DTR and CX3CR1^{gfp/gfp} mice at D1p.i.

(A) Absolute numbers of renal CD45⁺ cells, CD11b⁺ cells, neutrophils and monocytes from WT, CD169-DTR and CX3CR1^{gfp/gfp} mice at D1p.i. (B) Gating strategy and representative FACS plots showing renal Fraction II monocytes and Fraction II inflammatory macrophages from uninfected WT mice. (C-E) Absolute numbers of renal (C) Fraction Ia, Ib, (D) II, (E) II monocytes and II inflammatory macrophages from WT, CD169-DTR and CX3CR1^{gfp/gfp} mice at D1p.i. [n = 3-5 per group]. Data are illustrated as mean±SEM. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

6.8 CD169-DTR mice are unable to clear *C.albicans* in the kidneys at the later stage of infection.

Though CD169⁺ macrophages may not play a major role in the early stage of infection as demonstrated in the earlier section, however, in view of the drastic vulnerability displayed by CD169-DTR mice towards a sublethal *C.albicans* infection, we continued to monitor the fungal burdens and renal cellular infiltration in the CD169-DTR mice for a longer period, i.e. D3, D6 and D10p.i. Interestingly, even though CD169⁺ macrophages were ablated in all organs investigated [242], these organs, with the exception of kidneys, from CD169-DTR mice displayed similar fungal burdens as the infected controls, which suggests kidney-restricted dependence of CD169⁺ macrophages in fungal clearance [Figure 6.7A-C]. Specifically, all the organs investigated, except kidneys, in CD169-DTR mice seemed capable of clearing *C.albicans* as most of the *Candida* was not recoverable by D10p.i. [Figure 6.7C].

In the kidneys, similar fungal load was observed on D1, D3, and D6p.i. in CD169-DTR and control mice [Figure 6.7D]. Strikingly, the disparity in kidney fungal burdens between the control and CD169-DTR mice was only detected at D10p.i., where the fungal load of CD169⁺ macrophages-depleted kidneys continued to escalate while in the controls, *Candida* load seemed to be contained [Figure 6.7C, D].

Altogether, it appeared that the cause of susceptibility observed in CD169-DTR and CX3CR1-deficient mice were different. CX3CR1-deficient mice were unable to contain the *Candida* growth at the beginning of the infection [Figure 6.5C], whereas in CD169-DTR mice, the kidney fungal burden only appeared to be uncontrollable at the later stage [Figure 6.7D]. Therefore, our results suggest that CD169⁺ renal macrophages are likely to be involved in the later stage of infection, seemingly between D6p.i. to D10p.i., where the absence of these macrophages resulted in uncontrolled fungal growth in the kidneys.

Figure 6.7 Kidneys of CD169-DTR mice are the only organs incapable of clearing *C.albicans* as the disease progresses.

(A-C) Fungal burdens were analyzed at D3, D6 and D10p.i. for vital organs in WT and CD169-DTR mice infected with 5×10^4 cfu *C.albicans* [n = 3-6 per group]. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using two-tailed Student's t-test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments. (D) Compilation of kidney fungal burden analyses from CD169-DTR and WT mice at D1, D3, D6 and D10p.i. Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using 2-way ANOVA, followed by Bonferroni posttests, *p<0.05; ns, not significant.

6.9 Ablation of CD169⁺ macrophages does not affect the infiltration of myeloid cells into the kidneys during infection.

In this section, we investigated the infiltration of various myeloid cell populations in the kidneys at D1, D3, D6 and D10p.i. From D1 to D6p.i., both control and CD169⁺ macrophages-depleted kidneys showed comparable numbers of total leukocytes, neutrophils, and inflammatory macrophages, except for monocytes whereby there were significantly more monocytes infiltrating in CD169⁺ macrophages-depleted kidneys at D6p.i. [Figure 6.8A, D]. At D10p.i., all infiltrating cell populations in the control kidneys, which include myeloid and lymphoid cells, subsided [Figure 6.8 and 6.9], probably due to the clearance of *Candida* load [Figure 6.7D]. In contrast, the total number of infiltrating cells in CD169⁺ macrophages-depleted kidneys continued to rise [Figure 6.8A], possibly because of the persisting fungal presence that triggered the recruitment of immune cells [Figure 6.7D].

Ablation of Fraction Ia population was maintained throughout the infection as depicted in Figure 6.8B and C. The increase of Fraction Ia population observed in CD169-DTR mice at D10p.i. [Figure 6.8B] was likely due to the expansion of total leukocyte numbers in the kidney at this time point, as demonstrated in the earlier section. More importantly, the ratio of Fraction Ia / Fraction Ib at D10p.i. remained as low as the Fraction Ia / Fraction Ib ratio observed in other time points [Figure 6.8C]. On the whole, depletion of CD169⁺ macrophages did not affect the recruitment of myeloid cells, in particular, neutrophils, to the kidneys during *Candida* infection.

Figure 6.8 Kinetics of myeloid cells infiltration in the kidneys of WT and CD169-DTR mice during *Candida* infection.

Mice were intravenously (i.v.) injected with 5×10^4 cfu *C.albicans*. (A, B, D) Absolute numbers of renal (A) CD45⁺ cells, CD11b⁺ cells, neutrophils, monocytes, (B) Fraction Ia, Ib, (D) Fraction II, II monocytes and II inflammatory macrophages of WT and CD169-DTR mice at D0, D1, D3, D6 and D10p.i. [n = 3-6 per group]. Statistical significance was determined using 2-way ANOVA, followed by Bonferroni posttests, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. (C) Graph showing the ratio of renal Fraction Ia/Fraction Ib from WT and CD169-DTR mice at D0, D1, D3, D6 and D10p.i. Statistical significance was determined using 1-way ANOVA, followed by Bonferroni's multiple comparison test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are illustrated as mean±SEM. Data are representative of at least 2 independent experiments.

Figure 6.9 Kinetics of lymphoid cells infiltration in the kidneys of WT and CD169-DTR mice during *Candida* infection.

Mice were intravenously (i.v.) injected with 5×10^4 cfu *C. albicans*. Absolute numbers of renal (A) NK cells, NKT cells, (B) T cells, CD8⁺ T cells and CD4⁺ T cells of WT and CD169-DTR mice at D0, D1, D3, D6 and D10p.i. [n = 3-6 per group]. Data are illustrated as mean ± SEM. Statistical significance was determined using 2-way ANOVA, followed by Bonferroni posttests, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

6.10 Absence of CD169⁺ macrophages leads to a reduction in the recruitment of NK and NKT cells into the kidneys.

On the contrary, depletion of CD169⁺ macrophages appeared to affect the influx of NK and NKT cells into the kidneys during the infection. Specifically, at D6p.i., the numbers of NK and NKT cells augmented distinctly in control kidneys. However, NK and NKT cell populations in CD169⁺ macrophages-depleted kidneys generally remained low throughout the infection [Figure 6.9A]. From D1 to D6p.i., infiltration of T cells into the kidneys of CD169-DTR mice, in particular CD8⁺ T cells, followed the same trend as that in the control kidneys. On the other hand, the number of CD4⁺ T cells in CD169⁺ macrophages-depleted kidneys seemed to be lesser than those in the control kidneys at D6p.i., though its number increased at D10p.i. [Figure 6.9B]. In summary, our

data suggest the involvement of CD169⁺ macrophages in the recruitment of NK and NKT cells into the kidneys during *Candida* infection.

6.11 Augmented vascular leakiness in the organs of *Candida*-infected CD169-DTR mice.

Considering that *C.albicans* disseminates to all organs during systemic candidiasis and endothelium integrity can be influenced by local inflammatory milieu, we attempted to examine the endothelium intactness in all the vital organs of infected control and CD169-DTR mice on D6 and D12p.i. To this end, we utilized the Evans Blue vascular permeability assay. Evans blue is a dye that binds to sera albumin. Under normal physiological conditions, this albumin protein is confined within the blood vessels due to the intact vascular integrity of the endothelium. In the event of inflammation, infection or endothelial damage, the endothelial cell barrier becomes disrupted, which results in the extravasation of the Evans blue bound albumin into the tissue. Therefore, the degree of endothelial damage in the organs correlates with the amount of Evans Blue trapped within the tissue interstitium [221].

At D6p.i., significantly higher amount of Evans Blue could already be detected in CD169⁺ macrophages-depleted spleen and kidneys [Figure 6.10A]. The overall conditions of CD169-DTR mice continued to aggravate by D12p.i. as evidenced by the increased amount of Evans Blue in all organs [Figure 6.10B].

This suggests that even though CD169⁺ macrophages-depleted kidneys had equivalent fungal load as the controls at D6p.i. [Figure 6.7B], kidneys depleted with CD169⁺ macrophages appeared to be more damaged than the controls. In addition, it is likely that the early damage in the kidneys may have compromised its function and this progressive loss of renal function caused by the accumulative *Candida*-induced damage subsequently affected the remaining organs, suggesting that these mice could die of progressive sepsis [243].

Figure 6.10 Endothelial linings in the organs are less intact in CD169-DTR mice than in control mice during *Candida* infection.

(A and B) Vascular leakiness were analyzed at D6 and D12p.i. for vital organs in WT and CD169-DTR mice infected with 5×10^4 cfu *C.albicans* [n = 3-4 per group]. Relative degree of leakiness was measured based on the optical density (OD) of Evans blue extracted from per gram organ (OD_{620}/g). Data are illustrated as mean $\pm$ SEM. Statistical significance was determined using two-tailed Student's t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Data are representative of at least 2 independent experiments.

6.12 Progressive renal damage in CD169-DTR mice during *Candida* infection.

To have a better overview on the conditions of the control and CD169⁺ macrophages-depleted kidneys during *Candida* infection, we performed detailed kidney histology on D3, D6 and D10p.i.

As early as D3p.i., early signs of acute tubular necrosis (ATN) and spots of hemorrhages could be detected in CD169⁺ macrophages-depleted kidneys, whereas in the infected controls, renal tubules appeared to be healthy and there was no signs of hemorrhages [Figure 6.11A].

At D6p.i., the conditions of CD169⁺ macrophage-depleted kidneys continued to aggravate, as evidenced with progressive ATN and hemorrhages. Correspondingly, the control kidneys started to show signs of renal tubular damages. Spots of hemorrhages were infrequent [Figure 6.11B].

At D10p.i., kidneys of CD169-DTR mice had developed severe ATN, as illustrated by the shrinkage of tubular epithelial cells throughout the kidneys. Furthermore, in CD169⁺ macrophages-depleted kidneys, distinct clusters of cellular infiltrations in the lumen of renal tubules were spotted and patches of hemorrhages could be frequently seen. Additionally, fibrotic tissues were prominent in CD169⁺ macrophages-depleted kidneys. In contrast, the renal tubules in the infected control kidneys seemed intact and signs of recovery were detected [Figure 6.11C].

Figure 6.11 CD169-DTR mice show progressive kidney damage, while the structural integrity of infected control kidneys is more preserved during systemic candidiasis.

Mice were intravenously (i.v.) injected with 5×10^4 cfu *C.albicans*. (A-C) Representative histological H&E sections of kidneys from uninfected control, WT and CD169-DTR mice at D3, D6 and D10p.i.; Magnification, 40x, 100x and 200x; Scale bars, 500 μ m, 200 μ m and 100 μ m respectively. Black arrow, leukocyte infiltration; Red arrow, tubular necrosis; Green arrow, fibrotic tissue.

6.13 Kidneys of CD169-DTR mice are severely compromised at D10p.i.

The severity of the damage observed in CD169⁺ macrophages-depleted kidneys at D10p.i. was further demonstrated in Figure 6.12A. Specifically, in CD169⁺ macrophages-depleted kidneys, we noticed an enlargement in the urinary space between the glomerulus and Bowman's capsule, with prominent shedding of tubular epithelial cells and clusters of leukocytes within the lumen of some renal tubules [Figure 6.12A, right panels]. In contrast, these observations were not found in the infected control kidneys [Figure 6.12A, middle panels].

Next, we attempted to quantify the severity of kidney damage observed by assessing the expression of Kim-1 at D6 and D10p.i. Kim-1, known as kidney injury molecule-1, is a type-1 transmembrane protein expressed by renal proximal tubular epithelial cells. During steady state, Kim-1 expression in the kidneys is undetectable, where its expression will only be prominent after injury [244-248]. As expected, at D10p.i., the expression of Kim-1 mRNA in CD169⁺ macrophages-depleted kidneys was drastically higher than that in the controls [Figure 6.12B], a time point when severe kidney damage was observed [Figure 6.11C and 6.12A].

Parallel with the kidney damage analyses, we examined the renal functionality through serum creatinine assay at D10p.i. Creatinine is a metabolite by-product of creatine, where the latter is broken down by skeletal muscles. As kidneys constantly filter creatinine out from the blood to the urine, an elevated level of blood serum creatinine will be indicative for the loss of kidney functionality [249, 250]. Indeed, CD169-DTR mice showed significantly higher serum creatinine level as compared to the uninfected and infected control mice [Figure 6.12C].

Figure 6.12 Kidneys of CD169-DTR mice are severely damaged at D10p.i.

Mice were intravenously (i.v.) injected with 5×10^4 cfu *C.albicans*. (A) Representative histological H&E sections of renal glomeruli and renal tubules from uninfected control, WT and CD169-DTR mice at D10p.i.; Magnification, 1000x; Scale bars, 20μm. (B) Relative quantification of KIM-1 expression in whole kidney homogenates from WT and CD169-DTR mice at D6 and D10p.i. [n = 3-5 per group]. Data was normalized to β-actin and are illustrated as fold induction (mean±SEM). (C) Serum creatinine levels from uninfected control, WT and CD169-DTR mice at D10p.i. [n = 3-5 per group]. Data are illustrated as mean±SEM. Statistical significance was determined using two-tailed Student's t-test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

6.14 Persistent accumulation of *C.albicans* in the renal pelvis of CD169-DTR mice.

In conjunction with kidney histology, we also attempted to pinpoint the location of *C.albicans* in the infected control and CD169⁺ macrophages-depleted kidneys by performing the Periodic Acid Schiff (PAS) staining. Here, we focused on the collecting ducts and renal pelvis since this was the area where most of the *C.albicans* was found.

At D3p.i., *C.albicans* yeast seemed to adhere to the collecting duct epithelial cells of CD169⁺ macrophages-depleted kidneys. In addition, signs of disruption in the structural integrity of these collecting ducts were detected, accompanied by distinct leukocyte infiltration. On the contrary, collecting ducts in the control mice showed no or minimal signs of *C.albicans* as well as leukocytes. The integrity of these collecting ducts also seemed intact [Figure 6.13A].

At D6p.i., we noted a substantial population of *Candida* yeast and hyphae accumulating at the lower end of the collecting duct of CD169-DTR mice, where severe damage and disintegration of the duct epithelial cells was observed. Leukocytes in the CD169⁺ macrophages-depleted renal pelvis seemed to be surrounding the *C.albicans* but in a dispersed manner. In stark contrast, a dense population of leukocytes appeared to be surrounding the collecting duct of the infected control mice, where minor signs of damage and epithelial cells degradation were detected. *Candida* yeast was found lining along or within the collecting duct epithelial cells of the control mice, though sights of *Candida* hyphae were infrequent [Figure 6.13B].

At D10p.i., accumulation of infiltrating leukocytes, along with a dense population of *Candida* hyphae, was detected in the renal pelvis of CD169-DTR mice. Distinctively, most of the collecting ducts appeared to have disintegrated, with only some remnants of the duct epithelial cell clusters. Remarkably, the renal pelvis of the infected controls seemed to have recovered from *C.albicans*

infection, where there was no sign of *C.albicans*. The collecting duct appeared to be healthy and minimal leukocyte infiltration was detected [Figure 6.13C].

To sum up, kidneys depleted with CD169⁺ macrophages showed signs of damage as early as D3p.i., where disruption of renal epithelial and endothelial cells could be detected throughout the organ. The condition of CD169⁺ macrophages-depleted kidneys aggravated as the disease progressed, accompanied by the accumulation of *C.albicans* in the renal collecting ducts. Despite the accumulation of leukocytes in the collecting ducts during the infection, CD169⁺ macrophages-depleted kidneys appeared to be incapable of clearing *C.albicans*. Given that the collecting ducts were the most severely damaged region in the kidneys, it is probable that the deterioration of the tubular cells in the cortical and medullar region was a causal sequence of the disintegration of the collecting ducts. Following the structural destruction of renal tubular epithelial cells, the overall function of the kidneys was compromised. No sign of recovery was detected in infected CD169-DTR mice.

In contrast, only at D6p.i., we noticed minor signs of damage in the control kidneys, where the containment of *C.albicans* seemed to be attributed by an organized, well-coordinated leukocyte infiltration in the collecting ducts. By D10p.i., *C.albicans* were cleared and most of the renal regions seemed to have recovered.

Figure 6.13 *C.albicans* accumulate at the renal collecting ducts of control and CD169-DTR mice during infection.

Mice were intravenously (i.v.) injected with 5×10^4 cfu *C.albicans*. (A-C) Representative histological PAS sections of renal collecting ducts from uninfected control, WT and CD169-DTR mice at D3, D6 and D10p.i.; Magnification, 40x, 100x and 200x; Scale bars, 500 μ m, 200 μ m and 100 μ m respectively. Black arrow, *C.albicans*; Red arrow, leukocyte infiltrates; Green arrow, epithelial cell shedding.

6.15 Few CD45⁺ CD11b⁺ cells are detected in the renal collecting ducts.

Because renal collecting ducts seemed to be the major site where *C.albicans* accumulated and we previously did not observe any F4/80⁺ cells in that region [Figure 6.1 and 6.14A], we sought to investigate if there were immune cells lurking in this region during steady state. Interestingly, visibly very little CD45⁺ CD11b⁺ cells were detected in the collecting ducts of uninfected kidneys, where the location of these cells was only found in the region near to the renal medulla [Figure 6.14B]. Therefore, it is probable that the lack of immune cells in the collecting ducts render kidneys as the main target organs for this infection, since this may provide *C.albicans* an upper hand in assembling and proliferating before the recruitment of innate effectors to this region.

Figure 6.14 CD45⁺ immune cell population in the renal collecting ducts is considerably little as compared to other renal regions during steady state.

Representative cryo-sections of kidney from WT mice. (A) Sections were stained with immunofluorescence antibodies specific for F4/80, CD169 and CD31; Magnification, 200x; thickness, 6 μ m. (B) Sections were stained with immunofluorescence antibodies specific for CD45, CD11b and CD31; Magnification, 100x; thickness, 6 μ m.

6.16 CD169-DTR mice exhibit higher levels of pro-inflammatory cytokines and chemokines than the control mice during *Candida* infection.

Given that most of the organs in CD169-DTR mice displayed more severe vascular leakiness at D12p.i. [Figure 6.10B], we examined whether this observation was associated with a systemic inflammation in the mice. Indeed, at D10p.i., levels of IL6 and TNF α were significantly elevated in the blood sera of CD169-DTR mice when compared to those of the uninfected and infected controls [Figure 6.15A].

Macrophages have been illustrated to be essential mediators in regulating neutrophil recruitments and neutrophil antifungal ability through cytokines and chemokines production. Since absence of CD169⁺ macrophages in the kidneys resulted in uncontrolled *Candida* growth at the later stage of infection, we asked if CD169⁺ macrophages were important in regulating the recruitment and antifungal mechanisms of neutrophils. To this end, we monitored the expression of endothelial cell adhesion molecules, antimicrobial peptides as well as some signature chemokines and cytokines that are important for neutrophils and monocytes recruitment into the kidneys of control and CD169-DTR mice at D6 and D10p.i.

In both D6 and D10p.i., the expression levels of CXCL1, CXCL2 (neutrophil chemokines), CCL2 (monocyte chemokines), TNF α and ICAM-1 were remarkably higher in CD169⁺ macrophages-depleted kidneys as compared to those in the infected controls [Figure 6.15B, C, E]. In addition, other pro-inflammatory cytokines (e.g. GM-CSF and G-CSF) and chemokines (e.g. CCL5), where their expression levels in CD169⁺ macrophages-depleted kidneys were initially similar to the infected controls at D6p.i., displayed drastic increase at D10p.i. [Figure 6.15B, C]. In contrast to the heightened pro-inflammatory cytokines observed at D10p.i., the level of IL10 was relatively low in CD169⁺ macrophages-depleted kidneys [Figure 6.15D]. This suggests that kidneys lacking of CD169⁺ macrophages were more inflamed than the control kidneys at D6p.i. and this inflammation continued to exacerbate as the disease

progressed. Strikingly, the amount of antimicrobial peptides, such as S100A8 and S100A9, appeared to be lower in CD169⁺ macrophages-depleted kidneys than in the control kidneys, suggesting a less effective *Candida* killing when CD169⁺ macrophages were absent [Figure 6.15F].

Interestingly, depletion of CD169⁺ macrophages seemed to affect the expression level of CX3CL1 in the kidneys during infection, a ligand that has been reported to be essential for the maintenance of renal macrophage numbers, whereby the control kidneys displayed higher amount of CX3CL1 than CD169⁺ macrophages-depleted kidneys at D6p.i. [Figure 6.15B].

Taken together, the severity of the kidneys of CD169-DTR mice seemed to be associated with progressive kidney inflammation, as CD169⁺ macrophages-depleted kidneys displayed higher expression levels of pro-inflammatory cytokines, chemokines and endothelial adhesion molecules. Despite the enhancement of innate effector cells recruitment through cytokines and chemokines secretion, the host candidacidal killing seemed to be impaired in absence of CD169⁺ macrophages. In addition, our histological data suggested a less coordinated influx of leukocytes in the collecting ducts of CD169-DTR mice, which could impede the efficiency of host *Candida* killing. More investigations are required to assess the involvement of CD169⁺ macrophages, whether they are required for the coordination of *Candida* killing or the regulation of neutrophil *Candida* killing ability.

Figure 6.15 Elevated levels of inflammation are observed in CD169-DTR mice during *Candida* infection.

Mice were intravenously (i.v.) injected with 5×10^4 cfu *C. albicans*. (A) Blood sera IL6 and TNF α levels from uninfected control, WT and CD169-DTR mice at D10p.i. [n = 3-5 per group]. Data are illustrated as mean $\pm$ SEM. (B-F) Relative quantification of (B) neutrophils- and monocytes chemoattracts, (C) pro-inflammatory cytokines, (D) anti-inflammatory cytokines, (E) endothelial cell adhesion molecules and (F) antimicrobial peptides expression in whole kidney homogenates from WT and CD169-DTR mice at D6 and D10p.i. [n = 3-5 per group]. Data was normalized to β -actin and are illustrated as fold induction (mean $\pm$ SEM). Statistical significance was determined using two-tailed Student's t-test, *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Data are representative of at least 2 independent experiments.

Chapter 7: Discussion

To date, systemic candidiasis is and remains as the 4th most common nosocomial infection, despite the hospital antifungal therapies and hygienic measures. Though the indispensable role of neutrophils being the main effectors in *Candida* immunity has been established over the years, current understanding on the roles of different mononuclear phagocytic subsets in the host defense against invasive *C.albicans* infection has been lacking, owing much to the lack of tools to selectively target individual mononuclear phagocytic subpopulations *in vivo*. In this report, we attempted to identify the roles of various mononuclear phagocytes in *Candida* immunity, namely CD8⁺/CD103⁺ DCs, CD11b⁺ DCs, pDCs and CD169⁺ macrophages, through the use of our four in-house generated DTR transgenic mouse strains, i.e. Clec9A-DTR, Clec4a4-DTR, Siglec H-DTR and CD169-DTR mice respectively.

In order to have a clearer understanding on the host defense against disseminated *Candida* infection, we first thoroughly examined the disease progression of wild-type mice infected with the sublethal dose of *C.albicans*. Here, we demonstrated that kidneys were the only organs incapable of clearing or containing *C.albicans* growth at the early phase of the infection (D1-D6p.i.) and this was most likely attributed by the intrinsic low numbers of immune cells in the kidneys at steady state.

Consistent with what we observed in this study, several other papers have also previously described kidneys as the main target organs for systemic candidiasis [30, 34, 35]. Aside from the organs we investigated for fungal burden analyses, one study by MacCallum et al. also included blood and thigh muscles in their assessment of fungal spread. Parallel to our observations, they observed persisting fungal load in the kidneys and more importantly, kidneys were the only organs displaying positive correlation with the disease progression, regardless of the amount of *C.albicans* used for the infection [34].

Lionakis et al. is one of the pioneering group that conducted detailed investigation on the kinetics of cellular infiltration in the kidney, brain, liver, spleen and blood of infected WT mice during systemic candidiasis [30]. In their experimental setup, they infected the mice with a fatal dose of 2.5×10^5 cfu *C.albicans* and assessed the temporal events of cellular infiltrations in the respective organs at D1, D4 and D7p.i. Analogous to our findings, they observed gradual accumulation of infiltrating immune cells, which mainly comprised of neutrophils, in all the organs they examined during the one-week period. In their study, Lionakis et al. reasoned that the uncontrolled *Candida* growth in the kidneys was attributed by the slow or delayed recruitment of neutrophils into the kidneys during the first 24h.p.i., which rendered the kidneys in a 'neutropenic' state as compared to the other organs in that period [30]. Indeed, when we traced the absolute number of neutrophils in the spleen and kidneys at D0, D3 and D6p.i., we found that neutrophils recruitment in the spleen was more rapid and drastic, whereas neutrophils amassed gradually in the kidneys. Hence, we asked whether this observation could mean defective neutrophils recruitment in the kidneys, as one could reason that the gradual increase in renal neutrophil populations could be due to the low starting number of renal leukocytes. This could be true, considering the fact that leukocytes account for the majority of neutrophil chemo-attractants production and that the total number of kidney leukocytes at steady state is 150 times lower than that of the spleen. Hence, the comparison using the fold of increase in terms of neutrophils frequency, rather than the absolute number, between the kidneys and spleen may better reflect the dynamics of neutrophils recruitment.

In our study, we demonstrated that the recruitment of neutrophils and monocytes into the kidneys was comparable to their influx into the spleen, where the increase of neutrophil frequency in both organs could be observed as early as 8h.p.i. Strikingly, when we examined the fold of increase for neutrophils and monocytes frequencies during the first 16h of infection between kidneys and spleen, the kidneys recruited proportionately more neutrophils and monocytes than the spleen. In line with our observations, a

study by MacCallum et al showed that production of KC/CXCL1 and MCP-1/CCL2 in the kidney homogenates could be detected by 12h.p.i., suggesting that rapid recruitment of innate cells could be attainable as early as 8 to 12h.p.i. [251]. Hence, the uncontrolled kidney fungal burden is unlikely due to the inefficient recruitment of neutrophils and monocytes into the kidneys during *Candida* infection.

Nonetheless, despite the heightened recruitment of innate effectors observed in the kidneys as early as 8h.p.i., the kidney fungal burden persisted and began to increase by 16h.p.i., suggesting that the leukocytes influx into the kidney is insufficient and that the starting low number of immune cells in the healthy kidneys may be the determining factor. Collectively, since the total number of immune cells in the kidneys were drastically lower than those in the spleen during steady state and the recruitment of innate immune effectors into the organs required no less than 5hrs in both spleen and kidneys, we postulate that the kidneys were most probably overwhelmed by the initial assault of *C.albicans*, due to the insufficient number of resident neutrophils or immune cells in countering *C.albicans* infection.

As aforementioned, neutrophils are the pivotal host immune effectors against invasive candidiasis, where they are necessary to elicit effective *Candida* killing. Any defect in neutrophil functions or absence of neutrophils would greatly undermine the host defense against this infection [164-167, 169, 186]. Likewise, in our study, enhanced susceptibility in both neutrophils-depleted CD11b-DTR and α Gr-1 treated mice was observed when challenged with the sublethal dose of *C.albicans*. The importance of neutrophils was further underscored wherein mice depleted with neutrophils at any time within the first 24 hours of infection showed 100% mortality. Coupled our observation that CD11b-DTR mice were more resistant to systemic candidiasis than α Gr-1-treated mice with our flow-cytometry analyses showing that neutrophils depletion in CD11b-DTR mice was not as complete as α Gr-1 antibody treatment, we posit that the amount of neutrophils available in defending against the initial assault of *C.albicans* determines the overall disease

prognosis. In line with our speculation, Romani et al. also pointed out the importance of neutrophils, in particular, at the initial phase of *Candida* infection as the deciding factor for effective *Candida* immunity. In that study, they showed that mice depleted with neutrophils at D1p.i. were highly susceptible towards systemic candidiasis, while mice depleted with neutrophils at D7p.i. showed improved disease conditions [174].

Considering that the presence of neutrophils during the initial assault of *Candida* infection affects the overall disease progression, one would need to take into consideration of the side effect of the DTR-DT system (DT-induced neutrophilia) when using DTR mice to study fungal infection models [223, 252]. The main concern on the use of DTR-DT system is neutrophilia, especially when the disease model of interest involves neutrophils as the main immune effectors in conferring resistance. This was highlighted in a study by Tittel et al., in which they showed that the better clearance of bacterial infection pyelonephritis in the kidneys of DT-treated CD11c-DTR mice was due to the expansion of neutrophils induced by the ablation of CD11c-expressing cells [223]. In another example, Whitney et al. observed that DT-treated CD11c-DTR mice were highly resistant to systemic *Candida* infection, even though CD11c Δ Syk mice were highly susceptible [129]. However, these seemingly contradicting conclusions can be reconciliated by taking into account on the effect of DT-induced neutrophilia.

Because of the side effect of the DTR-DT system and the lack of alternative tools to conditionally ablate specific mononuclear phagocytic subsets *in vivo*, the roles of various mononuclear phagocytic subpopulations in the host defense against systemic candidiasis has not been clearly elucidated. Hence, taking this byproduct of DTR-DT system into consideration, we have established an infection and appropriate DT injection scheme that serves to minimize the effect of DT-induced neutrophilia and monocytosis. In brief, this scheme involves delaying the time period between the infection and the first DT administration, so as to bypass the transient neutrophilia and monocytosis caused by the initial administration of DT.

In the exploration of the functions of different DC subsets in *Candida* immunity, it was surprising that mice depleted with either DC subsets exhibited similar susceptibility and fungal burdens (spleen and kidneys) as the infected controls. In addition, there was no distinct difference in the infiltration of myeloid cells in both spleen and kidneys of infected Clec9A-DTR and Clec4a4-DTR when compared to the infected control mice. Hence, our data suggest that these DC subsets are dispensable in the host immunity against systemic candidiasis.

Nonetheless, the essential roles of DCs in *Candida* innate immunity have recently been highlighted by several studies. In 2011, Bourgeois et al. illustrated that BMDCs, but not BMDMs, are the innate immune cells capable of producing type 1 interferons (IFN-I) when co-cultured with *Candida* [253]. The induction of IFN-I in response to *Candida* infection was also reported by Smeekens et al., whereby human peripheral blood mononuclear cells expressed IFN-I when stimulated with *C.albicans* [254]. In addition, the functional role of IFN-I during systemic candidiasis was further demonstrated by two independent studies, although with conflicting observations. Notably, both groups utilized identical *C.albicans* strain (SC5314), infection dose (1×10^5 cfu) and murine strains (C57BL/6 WT and *Ifnar1*^{-/-}) [230, 231]. According to del Fresno et al., the abrogation of IFN-I signaling pathway in *Ifnar1*^{-/-} mice led to reduced cellular infiltration in the kidneys during *Candida* infection and consequently resulted in higher susceptibility (higher kidney fungal burden and 100% mortality). They concluded that DCs, upon activated by *C.albicans*, produce IFN-I and subsequently, IFN-I potentiates neutrophils recruitment to the kidneys by inducing the recruited neutrophils to produce more neutrophil chemokines - CXCL1 and CXCL2, in a feed-forward manner [230]. On the other hand, study by Majer et al. showed that *Ifnar1*^{-/-} mice were more resistant towards systemic *Candida* infection. They reasoned that IFN-I signaling is detrimental for the host immunity against disseminated candidiasis because it drives excessive host inflammatory response by recruiting overwhelming numbers of monocytes and neutrophils into the kidneys [231]. Although both groups reported contradictory observations, the conclusions regarding the role of IFN-I in *Candida* immunity in both papers are, in fact,

congruent. It appears that IFN-I signaling is involved in inducing the production of neutrophil chemo-attractants, in particular CXCL1 and CXCL2, thereby regulating neutrophils recruitment to the kidneys during the infection.

Considering that pDCs are major type 1 IFN producers [123, 124, 232] and that IFN-I regulates neutrophils recruitment during systemic candidiasis, it was surprising that Siglec H-DTR mice behaved similarly as the control mice during systemic candidiasis, thus suggesting the redundancy of pDC-mediated function in *Candida* immunity. The dispensable role of pDCs was previously suggested in the study by Biondo et al., whereby they depicted that cDCs, but not macrophages or pDCs, are responsible for IFN-I production when challenged with *C.albicans* in *in vitro* cultures [255]. Indeed, all the studies mentioned above utilized BMDCs, and not pDCs, to assess the production of IFN-I.

In our attempts to verify the role of IFN-I, we treated the mice with α -IFNAR-1 blocking antibody in order to neutralize IFN-I signaling. However, we did not observe any difference between the infected control and α -IFNAR treated mice. Nonetheless, we could not rule out the possibility that the endogenous IFN-I was produced in excess during *Candida* infection, thereby out-competed and displaced the blocking antibody for IFNAR. In addition, the disparate observations could be due to different murine backgrounds. Since all the papers mentioned above used *lfnar1*^{-/-} mice to investigate the role of IFN-I, the physiological condition of these mice could be different from our WT mice, considering that some unknown compensatory mechanisms could be induced by the constitutive loss of IFNAR in the *lfnar1*^{-/-} mice. In line with this, Majer et al. reported a discrepancy between *lfnar1*^{-/-} BMDCs and α -IFNAR1 treated BMDCs when stimulated with *C.albicans*. It was found that blocking of IFN-I signaling in WT BMDCs did not reduce the secretion of neutrophil chemokines when simulated with *C.albicans*, despite the fact that secretion of these chemokines was greatly reduced in *lfnar1*^{-/-} BMDCs. Their results suggested that a functional IFNAR could be more important than IFN-I signaling processes, where they could be involved in other signaling pathways, most

likely through collaboration, to elicit effective neutrophil chemokines production [231].

As previously mentioned in the introduction, Whitney et al. depicted that DCs are important in potentiating neutrophil *Candida* killing by inducing NK cells to secrete GM-CSF. In their study, they concluded that the increased susceptibility observed in CD11c Δ Syk mice was attributed by the loss of DC function [129]. Since in peripheral organs such as kidney, where both DCs and macrophages similarly express CD11c [77], the impairment of Syk signaling may also affect macrophages and not DCs alone. In other words, the phenotype observed in CD11c Δ Syk mice could be due to the loss of function in both DCs and macrophages. Indeed, Whitney et al. did acknowledge that CD11c is not a DC-restricted marker and this explained why they used Clec9a Δ Syk mice in one subsequent experiment, whereby they reported higher renal fungal burden in Clec9a Δ Syk mice as compared to the control mice [129]. However, in a separate study by Break et al., they reported that infected Batf3^{-/-} mice showed similar kidney fungal burden as the infected controls [256]. Because Batf3 transcription factor is strictly required for the development of Clec9A-expressing CD8⁺ DCs and CD103⁺ DCs [122], Batf3^{-/-} mouse is devoid of these DC populations. That is to say, if Clec9A-expressing cells are involved in the host *Candida* immunity, similar phenotype should be observed, regardless of using Clec9a Δ Syk or Batf3^{-/-} mice. Similar to the observation by Break et al., Kashem et al. reported that kidney fungal burden between Batf3^{-/-} and control mice was not distinctly different after systemically challenged with *C.albicans* [257]. Correspondingly, our observation is in favor of what had been observed in Batf3^{-/-} mice, in which our infected Clec9A-DTR mice did not display significant increase in fungal burdens and susceptibility as compared to the infected controls.

Based on our *in vitro* stimulation experiments, we identified that both DC subsets displayed equivalent capacity in priming T cells proliferation and they were also capable of priming IFN γ -producing CD4⁺ T cell response. Noticeably, the induction of IFN γ response seemed to be more prominent than that of IL17

response in the infected mice during systemic candidiasis, whereby higher proportion of IFN γ -producing CD4⁺ T cells than IL17-producing ones were observed. Consistent with our findings, LeibundGut-Landmann et al. also reported similar observations, in which they found that splenic cells produced higher amount of IFN γ than IL17 when re-stimulated with heat-killed *Candida* [258]. Comparatively, it appears that IFN γ -mediated immunity is more pronounced in systemic candidiasis. Another evidence that corroborated the beneficial role of IFN γ in *Candida* immunity was derived from a publication by Kashem et al. In their study, they demonstrated that the adoptive transfer of IFN γ -producing Th1 cells conferred protection to the mice against systemic candidiasis but not IL17-producing Th17 cells [257].

Nonetheless, the importance of Th17 in systemic *Candida* immunity was brought into light when Drummond et al. illustrated that if mice were pretreated with OVA⁺ liposomes conjugated with anti- α 8-integrin antibody to promote aggregation of antigen-specific CD4⁺ T cells in the kidney before infection, there will be less morbidity and reduced kidney fungal load in these mice. More importantly, these protective antigen-specific CD4⁺ T cells predominantly expressed Th17-restricted transcription factor ROR γ t [259]. The significance of IL17 was further illustrated in studies that observed increased susceptibility of cytokine-deficient IL17a^{-/-} and receptor knock-out IL17RA mice after systemically challenged with *Candida* [46, 154]. The importance of Th17-mediated protection against systemic candidiasis was further exhibited in the context of vaccination. In a study by Bar et al., they discovered a dominant CD4⁺ T cell specific *Candida* peptide, pALS. Bar et al. reported that when mice were immunized with pALS, together with the adjuvant, curdlan, they were less susceptible to systemic *Candida* challenge and this protection was attributed by curdlan-induced pALS-specific IL17 response. Interestingly, this protection was not observed in mice vaccinated with pALS/CpG, in which Th1 response was induced [260]. On the other hand, according to Lin et al., the induction of both Th1 and Th17 response in mice immunized with rAls3p-N/AlOH₃ was necessary to elicit protection from lethal *Candida* infection. Absence of either IFN γ - or IL17-mediated signaling abolished the protective effect elicited by the

vaccine [261]. Taken together, it appears that both Th1 and Th17 response are important for the host defense against systemic candidiasis.

The importance of macrophages in the host immunity against disseminated candidiasis *in vivo* was believed to be first highlighted by Qian et al. in 1994 [262]. However, for almost 2 decades, their observations were not followed up till a paper published by Lionakis et al. in 2013 [82]. In the study by Qian et al., they reported that mice depleted with macrophages were highly susceptible towards invasive *Candida* infection. The depletion of macrophages *in vivo* was achieved by i.v. administration of liposome-entrapped dichloromethylene diphosphonate (L-Cl₂MDP) into the mice. More importantly, Qian et al. stated that this treatment ablated splenic and liver macrophages but not neutrophils *in vivo*. The authors reasoned that the observed phenotype is due to the binding of *C.albicans* to the splenic marginal zone macrophages and depletion of these macrophages renders the host incapable of entrapping *C.albicans* in the spleen, hence leading to an increase in the kidney fungal burdens of macrophages-depleted mice [262]. However, given that the mice were treated with L-Cl₂MDP through i.v. route, there is a possibility that tissue-resident macrophages in all organs, including kidneys, were also depleted. Hence, the enhanced susceptibility of these macrophages-depleted mice could be due to the depletion of renal macrophages. Indeed, they observed that kidneys of these L-Cl₂MDP-injected mice exhibited drastic increase of *Candida* load, but not in the spleen and liver, as compared to that of the infected controls [262].

A more detailed investigation was then comprehensively carried out by Lionakis et al., who demonstrated the indispensable role of renal macrophages during systemic candidiasis through the use of CX3CR1^{-/-} mice [82]. CX3CR1^{-/-} mouse is a very useful mouse model to study kidney macrophage function because macrophages in the kidneys, but not in other organs with the exception of the small intestine, is drastically reduced due to the absence of CX3CR1/fractalkine interaction. Essentially, this is because the dependency on CX3CR1/fractalkine interaction for the maintenance of macrophage population appears to be restricted to the kidneys and small

intestine [238, 239]. In their study, Lionakis et al. put forward the idea that selective absence of macrophages in the kidneys of CX3CR1^{-/-} mice was the main driver behind the observation that kidney was the only organ with exceedingly high fungal burden. They concluded that the presence of renal tissue-resident macrophages was vital for serving kidney first line defense during the early phase of *Candida* infection, by preventing the spread of *C.albicans* into the renal tubules. This is important because macrophages or monocytes do not seem to enter renal tubules during the infection, suggesting that they may not be the innate cells directly involved in killing *C.albicans* within the renal tubules. In contrast, neutrophils can be found co-localizing with *Candida* within the tubules during the infection, thus further demonstrating the strategic position of tissue-resident macrophages as well as cementing the *Candida* killing role of neutrophils [82, 263].

Herein, we identified CD169⁺ macrophages as a subpopulation of kidney macrophages and these CD169⁺ macrophages are essential in the kidney immunity against invasive *Candida* infection. Depletion of CD169⁺ macrophages in CD169-DTR mice exacerbated their susceptibility towards sublethal systemic candidiasis. Intriguingly, despite the ablation of CD169⁺ macrophages in all organs [242], kidneys of CD169-DTR mice were the only organs incapable of controlling *Candida* growth, while other organs tested showed clearance of *C.albicans* by D10p.i.

To the best of our knowledge, renal CD169⁺ tissue resident macrophages were first reported by Karasawa et al. in 2015 [81]. In their study, Karasawa et al. identified and localized these renal CD169⁺ macrophages through the use of their CD169-Cre-YFP mice. Consistent with their findings, we found that kidney CD169⁺ macrophages mainly reside in the renal medullar region and these CD169⁺ macrophages constitute a subpopulation of the renal F4/80^{hi} CD11b^{lo} macrophages. As mentioned previously, for easier reference, we coined these F4/80^{hi} CD11b^{lo} macrophages as Fraction I population. Based on our FACS analysis, we noticed that ablation of kidney CD169⁺ macrophages in CD169-DTR mice mainly depleted a subpopulation of Fraction I macrophages

that expressed F4/80⁺⁺CD11b⁺ (Fraction Ia), while those expressing F4/80⁺⁺⁺CD11b⁺⁺ (Fraction Ib) remained relatively intact. Therefore, taken together, our data suggest that kidney CD169⁺ macrophages are a unique macrophage subset of Fraction I population that mainly reside in the renal medullar interstitium.

Interestingly, renal CD169⁺ macrophages did not appear to be essential for the host defense against initial *C.albicans* assault (~D1-2p.i.), as our data showed that kidney fungal burdens of both infected control and CD169-DTR mice were comparable from D1 to D6p.i. Furthermore, there was no difference in the overall structural integrity of both infected control and CD169⁺ macrophages-depleted kidneys at D1p.i. (data not shown) This is in stark contrast to CX3CR1-deficient mice, in which kidney fungal burdens of these mice at D1p.i. were already drastically higher than that of both infected control and CD169-DTR mice. Because the loss of CX3CR1/fractalkine interaction resulted in a drastic reduction of both Fraction Ia and Ib population in CX3CR1^{-/-} kidneys and that depletion of CD169⁺ macrophages in the kidneys of CD169-DTR mice mainly ablated Fraction Ia population, we speculate that Fraction Ib could be crucial in providing first line defense against the initial assault of *C.albicans*.

Since the difference between the kidney fungal burdens of CD169-DTR and control mice only became significant at D10p.i., it is likely that CD169⁺ macrophages are involved in *Candida* immunity at the later stage of infection. Notably, though the ablation of these CD169⁺ macrophages did not affect the recruitment of neutrophils during *Candida* infection, there was significantly less recruitment of NK cells to the kidneys of CD169-DTR mice as compared to the infected controls. NK cells have been demonstrated to be crucial for *Candida* immunity, owing to its capacity to produce GM-CSF that functions to potentiate neutrophil antifungal ability [129, 234]. Moreover, it has also been shown that *C.albicans* activates NK cells to produce IFN γ [264], a cytokine that can potentiate macrophage and neutrophil candidacidal activities [145, 147-149]. Similar findings have been reported in human NK cells studies, wherein these NK cells recognize *C.albicans* and elicit antifungal response by modulating

neutrophil candidacidal activities through the secretion of GM-CSF, TNF α and IFN γ [265-267]. Because the number of NK cells infiltrating into CD169⁺ macrophages-depleted kidneys was generally lower than that in the infected controls, and since NK cells potentiate neutrophils function, it is likely that defective neutrophil antifungal activities could be the reason why CD169-DTR mice exhibited enhanced susceptibility.

The suggestion that antifungal activity of neutrophils may be aberrant in the CD169⁺ macrophages-depleted kidneys is supported by our observations in the kidney histology. At D6p.i., despite both the kidneys of infected control and CD169-DTR mice displayed similar amount of leukocyte infiltrations, the tip of the collecting duct of CD169-DTR mice was surrounded by a pronounced aggregate of *Candida* hyphae, whereas *Candida* hyphae was rarely found in the infected control kidneys. In addition, infiltration of leukocytes in the collecting duct of the infected control mice appeared to be well organized and proficient in containing *Candida* growth. In stark contrast, the infiltrating leukocytes in the collecting duct of CD169-DTR mice were scattered amidst the overwhelming presence of *Candida* hyphae. These observations suggest that neutrophil antifungal function may be compromised in the absence of CD169⁺ macrophages.

The increased severity of kidney damage in CD169-DTR mice corresponded with the period when exceedingly high amount of *Candida* hyphae was observed. In particular, at D6p.i., collecting duct of CD169-DTR mice exhibited prominent renal papillary necrosis, whereby *Candida* hyphae appeared to be extending into the collecting tubules. On the other hand, the collecting duct of the infected control mice remained relatively unscathed, with mainly *Candida* yeast lining along or hiding within the collecting duct epithelial cells. The kidney damage in CD169-DTR mice became more extensive at D10p.i. and by this period, there was severe disintegration of collecting duct epithelial cells accompanied by the formation of *Candida* fungus ball within the renal pelvis, which could ultimately lead to urinary obstruction [268-270]. Other kidney pathologies observed in CD169-DTR mice that could be associated with

urinary obstruction include dilatation of renal tubules, sloughing of tubular epithelium, enlargement in the urinary space and higher KIM-1 expression levels [271]. On the contrary, kidneys of infected control mice appeared to have recovered from the fungus assault by D10p.i. In addition, in line with our histology data, our serum creatinine assay result indicated that kidney function in CD169-DTR mice, but not in the infected controls, was compromised.

Apart from *Candida*-induced damage, over-inflammation may play a part in the pathogenesis of kidney damage in CD169-DTR mice. In particular, the kidneys of CD169-DTR mice were already more inflamed than the control kidneys at D6p.i., based on the higher expression levels of pro-inflammatory chemokines and cytokines, such as CXCL1, CXCL2, CCL2 and TNF α . Notably, kidney inflammation continued to exacerbate in CD169-DTR mice at D10p.i., possibly due to the persisting presence of *C.albicans*. Parallel to our findings, this positive correlation between the severity of kidney lesions and the expression levels of inflammatory cytokines and chemokines, such as IL6, TNF α , CXCL1, CXCL2 and CCL2, has also been reported by MacCallum et al. [251]. The high numbers of leukocyte infiltrates, in particular neutrophils, in the kidneys of CD169-DTR mice at D6p.i. and D10p.i. may account for the aggravated kidney damage observed. In support of this notion, studies by both Romani et al. and Lionakis et al. demonstrated that immunopathology of the kidneys could be predominantly attributed by the excessive infiltration of neutrophils at the later stage of the infection [174, 272].

Though the excessive inflammation could be the host compensatory response to restrain the increasing *Candida* load in the kidneys, continual uncontrolled inflammation may exacerbate the disease progression and lead to the development of sepsis [273, 274]. In a study by Spellberg et al., they investigated the cause of death of mice suffering from invasive candidiasis by assessing their hemodynamic and blood chemistry parameters. They reported that *Candida*-infected mice died of progressive sepsis due to failing kidney function and this was highly correlated with kidney fungal burden [243]. Similarly, in our study, the drastic high levels of pro-inflammatory cytokines,

IL6 and TNF α , in the blood sera of CD169-DTR, but not in the control mice, at D10p.i. suggested that the infected CD169-DTR mice were likely debilitated with systemic inflammation. Moreover, drastic increase in vascular leakiness was observed in all the vital organs of CD169-DTR mice at D12p.i., as compared to the infected controls, and this could be a consequence of systemic inflammation that leads to vasodilation in multiple organs [275].

Considering that CD169-DTR mice displayed more severe renal inflammatory response during disseminated candidiasis, renal CD169⁺ macrophages may play a role in modulating kidney inflammation during *Candida* infection, possibly by suppressing excessive inflammation. In fact, the anti-inflammatory role of renal CD169⁺ macrophages has recently been reported by Karasawa et al., whereby they examined the function of CD169⁺ macrophages in a sterile kidney injury model - renal ischemia-reperfusion injury (IRI). Similar to our findings, higher expression of pro-inflammatory chemokines and cytokines was detected in the injured kidneys depleted of CD169⁺ macrophages. In their study, they suggested that CD169⁺ macrophages may be M2-like macrophages in the IRI injury model because the depletion of CD169⁺ macrophages led to the increased expression of ICAM-1 on vascular endothelial cells during inflammation and this subsequently resulted in excessive infiltration of neutrophils and elevated renal damage. Karasawa et al. proposed that CD169⁺ macrophages are perivascular macrophages, wherein the direct interaction of CD169⁺ macrophages and endothelial cells mediates vascular homeostasis [81]. Parallel to their observations, besides the heightened pro-inflammatory cytokines and chemokines, we also observed higher expression of ICAM-1 in the infected CD169⁺ macrophages-depleted kidneys when compared to the infected controls. The protective role of M2 macrophages against invasive *Candida* infection was recently described by Tran et al., wherein the induction of renal M2 macrophage polarization by the administration of IL33 at the later stage of infection reduced murine susceptibility towards systemic candidiasis. In addition, they demonstrated that mice were more resistant when they were adoptively transferred with M2 macrophages, as evidenced with lower kidney fungal burdens, renal pro-

inflammatory cytokines production (i.e. IL6, TNF α and IL1 β) and serum creatinine levels [109]. Hence, a thorough investigation on CD169⁺ macrophages will certainly facilitate a more detailed understanding of *Candida* immunity.

Lastly, it is surprising that only few CD45⁺ CD11b⁺ cells and no F4/80⁺ cells, were detected in the renal collecting duct during steady state, a region where most of the *C.albicans* eventually accumulated as the disease progressed. Though the unique kidney environment (e.g. urea and osmolality) could favor *C.albicans* growth [276, 277], the underlying reason on why kidneys being the main target organs remains undefined. Given that *C.albicans* eventually accumulated in the collecting duct, the lack of immune cells in the collecting duct could be one of the determining factors on why kidneys are the main target organs for *Candida* infection.

Chapter 8: Conclusion

In this project, we investigated the roles of various myeloid subpopulations, namely, 1) neutrophils, 2) DCs (i.e. CD8⁺/CD103⁺ DCs, CD11b⁺ DCs, pDCs) and 3) CD169⁺ macrophages, in controlling systemic candidiasis. In our assessment of neutrophils role in systemic candidiasis, besides verifying that ablation of these cells would drastically undermine the host resistance, we also demonstrated that neutrophils are absolutely required at least for the first 24hours of infection. Because DT treatment to DTR mice tends to induce neutrophilia, we improvised a DT injection scheme that would allow us to minimize this by-product. As such, we can reduce the risk of DT-induced neutrophils from interfering with the outcomes derived from our DTR mice studies.

Through our side-by-side comparison of Clec9A-DTR, Clec4a4-DTR, Siglec H-DTR mice, we illustrated that the ablation of individual DC subsets did not significantly affect the host resistance against invasive *Candida* infection. However, more investigations will be required before any conclusion can be drawn regarding the role of DCs in this infection model because these DC may have as-yet-unidentified redundant function. In other words, other non-targeted DCs may compensate for the ablated DC function and mask the phenotype. Henceforth, a more comprehensive approach to cross-examine DC function is required; for instance, ablate 2 or more DC subsets at the same time to minimize the likelihood of compensation elicited by the remaining non-targeted DC populations.

In the last part of this project, we discovered that renal CD169⁺ macrophages are absolutely essential in *Candida* immunity, wherein CD169-DTR mice displayed increased sensitivity towards *Candida* infection accompanied by exceedingly high kidney *Candida* burden. In addition, observations from CD169⁺ macrophages-depleted kidney histology suggest inefficient killing of *C.albicans*, hence hinting a possible role of CD169⁺ macrophages in mediating

or potentiating neutrophils antifungal capacity. Moreover, the heightened expression of inflammation-associated molecules in the kidneys of CD169-DTR mice pointed out a possible role of CD169⁺ macrophages in regulating renal inflammation, probably by dampening the excessive inflammation induced during the infection.

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Appendix

1.1 Media, Buffers and Solutions

Red blood cells (RBC) lysis buffer

0.89% ammonium chloride
Distilled H₂O

IMDM 2%

Iscove's Modified Dulbecco's Medium (IMDM)
2% Fetal Calf serum (FCS)

PBS 2%

1X PBS
2% FCS

Cell fixation buffer

1X PBS
1% paraformaldehyde
pH adjusted to 7.4

Permeabilization Buffer

PBS 2%
0.5% saponin

Diphtheria Toxin (DT)

1X PBS
1% mouse serum
2 ng/μl DT

35% Percoll solution

10X PBS, 17.5 ml
Percoll, 157.5 ml
IMDM, 325ml

ELISA wash buffer

1X PBS
0.05% Tween-20

ELISA assay buffer

1X PBS
10% FCS

1% acid ethanol solution

100% Ethanol

1% HCL

YPD agar

1% Bacto yeast extract

2% Bacto peptone

2% Glucose

2% Bacto agar

YPD agar

1% Bacto yeast extract

2% Bacto peptone

2% Glucose

YPAD agar

1% Bacto yeast extract

2% Bacto peptone

2% Glucose

0.004% Adenine sulfate

Solution C

0.88% NaCL

1mM EDTA

0.5% BSA

10nM HEPES-NaOH, pH7.4

Evans Blue solution

0.5% Evans blue

1x PBS

Fixation buffer

1X PBS

4% paraformaldehyde

pH adjusted to 7.4

1.2 Chemicals, reagents and kits

Name	Manufacturer
Ammonium chloride	Sigma, St. Louis, MO, USA
Acetone	Fisher Scientific, Longhborough, UK
Brefeldin A	Sigma, St. Louis, MO, USA
Collagenase D	Roche, Basel, Switzerland
Dimethyl Sulfoxide (DMSO)	Sigma, St. Louis, MO, USA
DMEM	Gibco, Grand Island, NY, USA
DT	Sigma, St. Louis, MO, USA
Ethanol	Merck KGaA, Darmstadt, Germany
IMDM	Gibco, Grand Island, NY, USA
NaCl	Merck KGaA, Darmstadt, Germany
Paraformaldehyde (PFA)	Sigma, St. Louis, MO, USA
Percoll TM	GE Healthcare, Uppsala, Sweden
Saponin	Fluka Chemie AG, Buchs, Switzerland
Trypan blue	Sigma, St. Louis, MO, USA
Tween 20	Sigma, St. Louis, MO, USA
BSA	Sigma Aldrich, St. Louis, MO, USA
D-(+)-Glucose	Sigma Aldrich, St. Louis, MO, USA
DPX Mountant for histology	Sigma Aldrich, St. Louis, MO, USA
EDTA	USB, Cleveland, OH, USA
Eosin	Merck KGaA, Darmstadt, Germany
Ethanol	Merck KGaA, Darmstadt, Germany
Evans Blue	Sigma Aldrich, St. Louis, MO, USA
FCS	Chemicon International, CA, USA
Fluorescence mounting medium	Dako, Golstrup, Denmark
HCl	Merck KGaA, Darmstadt, Germany
Hematoxylin	Merck KGaA, Darmstadt, Germany
Mouse TNF α ELISA MAX	BioLegend, San Diego, CA, USA
Mouse IL6 ELISA MAX	BioLegend, San Diego, CA, USA
NaOH	Merck KGaA, Darmstadt, Germany
N, N-dimethyl formamide	Sigma Aldrich, St. Louis, MO, USA
Paraffin	Leica Microsystems, Wetzlar, Germany
PBS	Gibco, Grand Island, NY, USA
Sodium bicarbonate	Sigma Aldrich, St. Louis, MO, USA
Tissue-Tek OCT compound	Sakura Finetek, Torrance, CA, USA
TMB Substrate Reagent Set	BioLegend, San Diego, CA, USA
Xylene	Sigma Aldrich, St. Louis, MO, USA
Bacto Yeast Extract	BD Diagnostic Systems, Sparks, USA
Bacto peptone	BD Diagnostic Systems, Sparks, USA
Bacto Agar	BD Diagnostic Systems, Sparks, USA
Periodic Acid Schiff (PAS) stain kit	Abcam, Cambridge, UK
Adenine Hemisulfate Dihydrate	MP Biomedicals, Solon, Ohio, USA
Mouse Cr (Creatinine) ELISA kit	Elabscience, Wuhan, China

RNAsimple Total RNA kit	Tiagen, Beijing, China
M-MLV Reverse transcriptase	Promega, Madison, USA
PrecisionFAST qPCR mastermixes	Primerdesign, Chandler's Ford, UK
Thymidine [methyl- ³ H]	PerkinElmer, MA, USA

1.3 Antibodies

Anti-mouse Antibody	Label	Clone	Company
CD11c	PE-Cy7	N418	Biolegend
I-A/I-E	Pacific Blue	M5/114.15.2	Biolegend
CD103	APC	2E7	Biolegend
CD11b	APC-Cy7	M1/70	Biolegend
CD31	FITC	390	Ebioscience
B220	APC	RA3-6B2	Biolegend
Ly6G	APC	1A8	Biolegend
CD4	FITC	H129.19	Biolegend
CD8	APC	53-6.7	Biolegend
IFN- γ	PE	MP6-XT22	Biolegend
IL17a	FITC	TC11-18H10-1	Biolegend
IL22	APC	IL22J0P	Ebioscience
Thy1.2	PE	30-H12	Biolegend
CD3	FITC	17A2	Biolegend
SiglecH	FITC	551	Biolegend
CD45	BUV737	104	BDbioscience
CD8	Percp-Cy5.5	53-6.7	BDbioscience
CD11b	BUV395	M1/70	BDbioscience
Ly6C	Percp-Cy5.5	HK1.4	Ebioscience
CD8	FITC	53-6.7	Ebioscience
panNK/CD49b	APC	DX5	Ebioscience
F4/80	PE	BM8	Ebioscience
CD169	APC	REA197	Miltenyi
α IFNAR	na	MAR1-5A3	BioXcell
α Gr-1	na	RB6-8C5	Home-made

1.4 qPCR primer sequences

Gene	Forward primer	Reverse primer
CXCL1	5' ACTGCACCCAAACCGAAGTC	5' TGGGGACACCTTTTAGCATCTT
CXCL2	5' ACAGAAGTCATAGCCACTCTC	5' CCTTGCCTTTGTTCAGTATC
CCL2	5' CATCCACGTGTTGGCTCA	5' GATCATCTTGCTGGTGAATGAGT
CCL5	5' GCAGCAAGTGCTCCAATCTT	5' ACTTCTTCTCTGGGTTGGCA
CX3CL1	5' CATCCGCTATCAGCTAAACCA	5' CAGAAGCGTCTGTGCTGTGT
GM-CSF	5' GCATGTAGAGGCCATCAAAGA	5' CGGGTCTGCACACATGTTA
G-CSF	5' GTGCTGCTGGAGCAGTTGT	5' TCGGGATCCCCAGAGAGT
ICAM-1	5' AGTCCGCTGTGCTTTGAG	5' AGGTCTCAGCTCCACACT
IL-10	5' CAGAGCCACATGCTCCTAGA	5' TGTCCAGCTGGTCCTTTGTT
KIM-1	5' AGATACCTGGAGTAATCACACTGAAG	5' TGATAGCCACGGTGCTCA
NGAL	5' CCATCTATGAGCTACAAGAGAACAAT	5' TCTGATCCAGTAGCGACAGC
S100A8	5' TCCTTGCGATGGTGATAAAA	5' GGCCAGAAGCTCTGCTACTC
S100A9	5' AATGGTGGAAGCACAGTTGG	5' GCTCAGCTGATTGTCCTGGT
TGF- β 1	5'TGGAGCAACATGTGGAATC	5' GTCAGCAGCCGGTTACCA
TNF- α	5' TCTTCTCATTCTGCTTGTGG	5' GGTCTGGGCCATAGAACTGA
VCAM-1	5' TCTTACCTGTGCGCTGTGAC	5' ACTGGATCTTCAGGGAATGAGT
β -actin	5' AAGGCCAACCGTGAAAAGAT	5'CCTGTGGTACGACCAGAGGCATACA

Conferences

6th International Singapore Symposium of Immunology, Singapore,
5-6 June 2013. (Participant)

4th Singaporean Immunology PhD student Retreat

29 August 2014

Poster presentation: 'Dendritic Cells are dispensable in defense against acute systemic candidiasis'

Yi Juan TEO, Christiane RUEDL

The 4th SigN-IFReC NIF Winter School 2015 on Advanced Immunology

18-23 January 2015

Oral and poster presentation: "Roles of dendritic cell subsets in acute *Candida Albicans* infection"

Yi Juan TEO, Christiane RUEDL

6th Congress of the Federation of Immunological Societies of Asia Oceania (FIMSA2015)

30 June-3 July 2015

Poster presentation: "Host Immunity in acute systemic *Candida Albicans* infection"

Yi Juan TEO, Christiane RUEDL

International Congress of Immunology 2016 (ICI 2016)

21-26 August 2016

Poster presentation: "Role of renal CD169+ F480+ resident macrophages in acute systemic candidiasis"

Yi Juan TEO, Christiane RUEDL

-attended Clinical Immunology Course (21 August 2016)