CD5 as immunomodulatory agent in experimetal models of fungal infection

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CD5 AS IMMUNOMODULATORY AGENT IN EXPERIMETAL MODELS OF FUNGAL INFECTION

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A mis padres y a mi hermano

"Quien no está preso de la necesidad, está preso del miedo: unos no duermen por la ansiedad de tener las cosas que no tienen, y otros no duermen por el pánico de perder las cosas que tienen."

Eduardo Galeano

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ABBREVIATIONS

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A. fumigatus	*Aspergillus fumigatus*
Ab	Antibody
AICD	Activation-induced cell death
AIM	Apoptosis inhibitor of macrophages
ALR	AIM2-like receptor
APC	Antigen Presenting Cell
B-ALL	B-cell Acute lymphoblastic leukemia
BCMA	B cell maturation antigen
BCR	B cell receptor
C. albicans	*Candida albicans*
C. glabrata	*Candida glabrata*
C. neoformans	*Cryptococcus neoformans*
C57	C57BL/6
CamK2	Ca^{2+}/calmodulin-dependent kinase II
CAR	Chimeric antigen receptor
CBNK	Cord Blood derived natural killer cells
CCR2	C-C chemokine receptor 2
CD5-Fc	CD5-immunoglobuling fusion protein
CFU	Colony forming unit
CGD	Chronic granulomatous disease
CIA	Collagen-induced arthritis
CK2	Casein kinase 2
CLL	Chronic lymphocytic leukemia
CLR	C-type lectin receptor
CMAD	Cell Membrane Alloantigenic Determinant
CR3	Complement receptor 3
CSF	Colony stimulating factor
cSMAC	Central Supramolecular activation complex
CX_3CR1	CX_3C-chemokine receptor 1

DAMP	Damage-Associated Molecular Pattern
DC	Dendritic cells
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DP	Double positive
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalomyelitis
EAN	Experimental Autoimmune/Allergic Neuritis
EphA2	Ephrin type-A receptor 2
FBS	Fetal bovine serum
FcRγ	Fc receptor γ-chain
FDA	Food and Drug Administration
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GXM	Glucuronoxylomannan
H. capsulatum	*Histoplasma capsulatum*
HCV	Hepatitis C Virus
HSA	Human serum albumin
HSCT	Hematopoietic stem cell transplantation
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
IDDM	Insulin-dependent diabetes mellitus
IFI	Invasive fungal infection
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer cell immunoglobulin-like receptors
KIR	immunoglobulin-like receptors

KO	Knock-out
LPS	Lipopolysaccharide
LTA	Lipotheichoid acid
mAb	Monoclonal antibody
MAMP	Microbial-Associated Molecular Patterns
MAPK	Mitogen activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MDR	Multidrug resistant
MPO	Myeloperoxidase
MR	Mannose receptor
MS	Multiple Sclerosis
MyD88	Myeloid Differentiation primary response 88
Mϕ	Macrophages
NET	Neutrophil extracellular trap
NK	Natural killer
NLR	NOD-like receptor
o/n	Overnight
P. brasiliensis	*Paracoccidioides brasiliensis*
PAMP	Pathogen associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	programmed death-1
PFA	Paraformaldehyde
PGN	Peptidoglycan
PI3K	Protein Kinase 3
PKC	Protein kinase C
PLC	Phospholipase C
PRR	Pattern recognition receptor
r.p.m.	Revolutions per minute

RA	Rheumatoid arthritis
RLR	RIG-I-like receptor
ROS	Reactive oxygen species
rshCD5	Recombinant soluble human CD5
rshCD6	Recombinant soluble human CD6
RT	Room temperature
RTA	Ricin toxin A
S. cerevisiae	*Saccharomyces. cerevisiae*
s.c.	Subcutaneous
SIRS	Systemicinflammatory response syndrome
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SP-A	Surfactant protein A
SP-D	Surfactant protein D
SR	Scavenger receptor
SRCR	Scavenger receptor cysteine-rich
TCR	T cell receptor
Th	T helper
TILs	Tumour Infiltrating T lymphocytes
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing IFN-β
WT	Wild type
ZIGI	Zymosan-induced generalized inflammation

I. INTRODUCTION

1. THE IMMUNE RESPONSE TO FUNGAL INFECTIONS

Fungi are ubiquitous environmental organisms with special clinical relevance in immunocompromised individuals and hospitalized patients. The spectrum of fungal diseases in humans ranges from mild skin or mucosal infections to life-threatening invasive fungal infections (IFIs) (Romani, 2011). Mortality and morbidity rates due to fungal infections are still rising as a consequence of multiple factors, comprising: (a) the increasing number of surgical procedures and subsequent augment of intensive care units (ICU) admitted patients, (b) the emergence of multidrug resistant (MDR) pathogens, (c) clinical interventions triggering neutropenia, (d) the AIDS epidemics, and (e) population aging (Nami *et al.*, 2019). Current antifungal treatment effectiveness depends on drug selection and diagnosis. In this context, azoles, echinocandins and polyenes are the main antifungal agents used for IFIs treatment. More specifically, ergosterol and 1,3-β-glucan biosynthesis inhibitors, such as fluconazole and caspofungin, respectively, are used as first-line therapeutic agents (Nami *et al.*, 2019). However, these compounds have multiple side effects including cross-resistance, toxicity and drug interactions.

The integrity of the host's immune system is critical in fungal infections. The immune system is a combination of cells and soluble components acting together to maintain host integrity and respond to damage (exogenous or endogenous). These functions are achieved through two lines of defence: the innate immunity and the adaptive immunity. The **innate immunity** (also termed natural immunity) is the first line of defence of the immune system. It has been classically described as an unspecific system that responds quickly against

pathogens, with no need of previous instructive encounters and without keeping memory of such encounters. The **adaptive immunity** (also termed acquired immunity) stands as the second line of defence. It specifically requires prior exposure to the pathogen. This response increases with the successive encounters with the same pathogen, generating specific immune memory which protects against such a pathogen but no other even highly related pathogens. Both mechanisms are in continuous interaction to enable a successful immune response.

1.1. INNATE IMMUNE RESPONSE TO FUNGAL PATHOGENS

The first line of defense against infections is provided by the skin and the mucosal epithelial surfaces, which are constantly exposed to environmental microorganisms. Different adhesins expressed by fungal cells facilitate fungus and host's cells interaction. Host cell invasion involves two complementary mechanisms: fungal-induced endocytosis and active penetration (Sheppard and Filler, 2014) **(Figure I.1)**. Invasins Als3 and Ssa1 on *Candida albicans* surface, interact with E-cadherin and the heterodimer EGFR-HER2 on epithelial cell surfaces. Active penetration by *C. albicans* involves yeast germination forming hyphae and hydrolases secretion. Other medically important fungi, such as *Aspergillus fumigatus* and *Cryptococcus neoformans*, carry out similar processes but host cell invasion by *C. albicans* has been the most widely studied. Besides their role as physical barriers, the skin and the mucosal epithelia also actively participate in the response against pathogens by expressing a wide range of Pattern Recognition Receptors (PRRs). Therefore, epithelial cells sense pathogens through PRRs (Wells *et al.*, 2011; Zheng *et al.*, 2015). This pathogen-PRR interaction triggers antimicrobial peptide secretion and pro-inflammatory cytokines and

chemokines production, which promote immune cell activation (Naglik and Moyes, 2011; Swidergall and Ernst, 2014). Following this interaction, fungal bloodstream dissemination requires adhesion and invasion of the endothelia. Fungus and N-cadherins interaction promotes endocytosis by the endothelial cells (Phan *et al.*, 2005). In addition, different Toll-like receptors (TLRs) also recognise fungal pathogens and produce immune mediators (Zheng *et al.*, 2015).

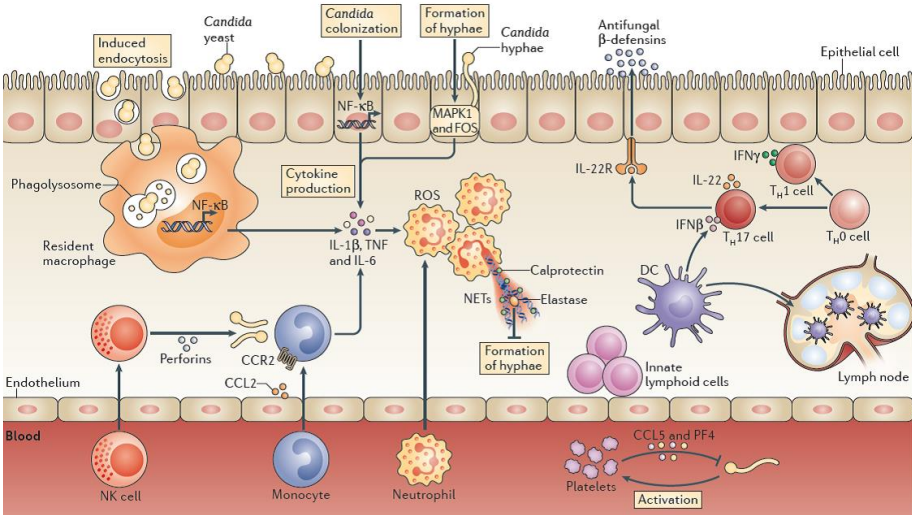

Figure I.1. Schematic representation of the antifungal immune response. Adopted from Netea *et al.*, 2015.

The main effector cells in innate immunity are those of hematopoietic origin and include myeloid cells (e.g., neutrophils, monocytes, macrophages, dendritic cells (DCs)) and innate-type lymphoid cells (e.g., natural killer (NK) cells, NKT cells, γδ T cells and innate lymphoid cells (ILCs)). Upon fungal recognition, PRRs on these cells signal pathogen uptake and killing, and trigger immunomediators production to modulate the immune response.

Neutrophils are the most important effector cells for the control of IFIs (Gazendam *et al.*, 2016). Consequently, neutropenia is a major risk factor in fungal infections (Gerson *et al.*, 1984; Farah *et al.*, 2001). Neutrophils are recruited to the site of infection by chemokines such as interleukin (IL)-8, chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, and CXCL5 produced by epithelial cells, tissue-resident macrophages, monocytes and DCs (Netea *et al.*, 2008; Gazendam *et al.*, 2016). Neutrophils' fungicide function takes place via three different mechanisms: oxidative mechanisms, non-oxidative mechanisms and NETosis. The oxidative mechanism is based on reactive oxygen species (ROS) production, which is mediated by NADPH oxidase and myeloperoxidase (MPO) (Amulic *et al.*, 2012). Indeed, susceptibility to IFIs in chronic granulomatous disease (CGD) results from NADPH oxidase deficiency (Brown, 2011). The non-oxidative mechanism consists on the release of granules containing antimicrobial peptides and proteins that act directly on microbes. These granules contain proteolytic enzymes (e.g. lysozymes, cathepsin-G, elastase), cationic peptides and proteins (e.g. defensins, histones, LL-37) and metal chelator proteins (e.g. lactoferrin, calprotectin) (Amulic *et al.*, 2012). Neutrophils also produce Neutrophil Extracellular Traps (NETs), which are fibrillar structures containing DNA, histones and antimicrobial proteins that directly interact with the pathogen (Amulic *et al.*, 2012).

In addition to neutrophils, **tissue-resident macrophages** and **circulating monocytes** are relevant effector cells in the anti-fungal response as illustrated in macrophage-depleted mice, which show increased susceptibility to invasive candidiasis (Qian *et al.*, 1994). The same effect was observed in mice deficient for CX_3C-chemokine receptor 1 (CX_3CR1), a receptor involved in the recruitment of monocyte-derived

macrophages to the site of infection (Lionakis *et al.*, 2013). Monocytes differentiate into macrophages once in the tissues, but they also possess antifungal activity. Monocyte-depleted mice, as well as C-C chemokine receptor 2 (CCR2)-deficient mice, have increased susceptibility to IFIs (Ngo *et al.*, 2014; Espinosa *et al.*, 2014). IL-15 production by $CCR2^{+}Ly6^{hi}$ monocytes is necessary for efficient activation and Granulocyte macrophage-colony stimulating factor (GM-CSF) release by NKs, and subsequent neutrophils antifungal activity (Domínguez-Andrés *et al.*, 2017). Moreover, macrophages develop into pro-inflammatory (M1) or anti-inflammatory (M2) macrophages, according to cytokine milieu, which modulate the immune response to fungi in opposite directions. For instance, increased susceptibility to *C. neoformans* infection has been associated to M2 phenotype differentiation (Arora *et al.*, 2011).

NK cells have been extensively studied in the context of tumours and viral infections though they also participate against fungal infection. NK cells release cytotoxic molecules, such as perforins and granzymes, and produce pro-inflammatory cytokines, such as interferon (IFN)-γ and GM-CSF, which potentiate antifungal host response (Schmidt *et al.*, 2017). Several receptors expressed by NK cells have been involved in their antifungal activity. NKp30 is used by NK cells to bind to β-glucans and directly kill *C. albicans* and *C. neoformans* (Li *et al.*, 2018). NKp46 and CD56 have also been involved in antifungal activity of NK cells (Vitenshtein *et al.*, 2016; Ziegler *et al.*, 2017).

Fungal recognition by **DCs** increases the expression of activation markers (mainly CD80 and CD86) as well as the production of IL-8 and IL-12p70 (Fidan *et al.*, 2014). Type I IFN-β production by DCs is also important for the host response against *C. albicans* (del Fresno *et al.*, 2013). The main functions of DCs during fungal infection are the

activation and differentiation of naive T cells towards different effector T cell subsets and tolerance induction. DCs also kill fungal pathogens directly though with lower efficiency than neutrophils and macrophages (Netea *et al.*, 2004).

In addition to the cellular component, the **humoral arm of the innate immunity** has also been involved in the host's defence against fungus. Soluble proteins such as collectins, pentraxins, natural antibodies or the complement system, among others, cooperate in the antifungal response. These molecules can signal the presence of fungi and consequently, regulate the cytokine secretion and expression of co-stimulatory molecules by phagocytes (Romani, 2004). Furthermore, protective effects have been observed after the administration of some of such soluble proteins, which will be further discussed in this thesis (Romani, 2004; Diniz *et al.*, 2004; Gaziano *et al.*, 2004; Farnworth *et al.*, 2008; Lo Giudice *et al.*, 2010).

The immunological memory has been classically described as an exclusive trait of the adaptive immune system. However, in the last years, the existence of a similar phenomenon in the innate immune system known as trained immunity has been proposed (Dominguez-Andres and Netea, 2019). The encounter with certain inflammatory stimuli promotes functional reprogramming of innate immune cells to deliver a faster and enhanced immune response to future challenges. This phenomenon is due to a long-term reprogramming at the epigenetic, metabolic and transcriptional level. In this context, modulation of hematopoietic stem and progenitor cells, and consequently peripheral myeloid cells response, has been reported after β–glucan and Bacillus Calmette-Guérin (BCG) exposure (Kaufmann *et al.*, 2018; Mitroulis *et al.*, 2018).

1.2. ADAPTIVE IMMUNE RESPONSE TO FUNGAL PATHOGENS

The initial interaction between fungi and innate immune system components allows the initiation of adaptive immune responses in which T cells are an essential component. Antigen Presenting Cells (APCs) cross-present fungal antigens to $CD4^+$ and $CD8^+$ T cells via MHC-II and MHC-I molecules, respectively. Due to the production of different cytokine profiles, DCs subsets promote differentiation of naïve $CD8^+$and $CD4^+$T cells into T cytotoxic (Tc) and T helper (Th) subtypes **(Figure I.2)**. $CD4^+$ T cells are the main players in the adaptive immune response against fungal infections. Indeed, HIV patients, who have low $CD4^+$ T cell counts, are highly susceptible to these infections (Armstrong-James *et al.*, 2014).

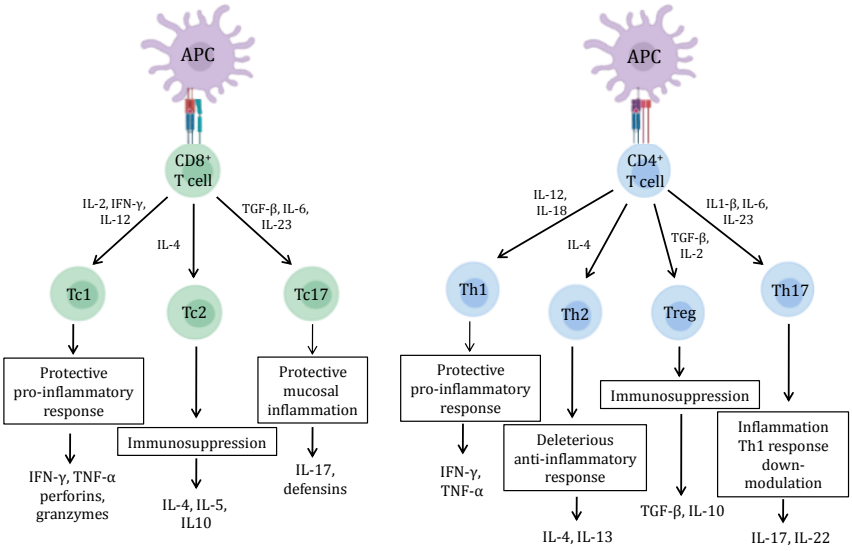

Figure I.2. Overview of adaptive immune T cell subsets involved in fungal infection.

DCs drive **Th1** subset polarization through IL-12 and IL-18 production, whose response correlates with protective antifungal immunity. Th1 cells are characterized by IFN-γ production, an essential

cytokine for phagocyte's antifungal activity (Nathan *et al.*, 1983; Shalaby *et al.*, 1985). Consequently, IFN-γ, IL-18 or IL-12 deficiencies correlate with an increased susceptibility to fungal infections (Balish *et al.*, 1998; Gwo-Hsiao Chen *et al.*, 2005; Scheckelhoff and Deepe, 2005). For instance, IL-18 and IL-12 deficient mice are susceptible to *C. albicans* and *C. neoformans* infections, respectively (Decken *et al.*, 1998; Netea *et al.*, 2003).

The **Th2** cell polarization is driven by IL-4 and IL-13, promoting deleterious responses against fungal pathogens such as *C. albicans*, *C. neoformans* and *Histoplasma capsulatum,* among others (Verma *et al.*, 2014). IL-13 and IL-4 promote alternative development of macrophages to M2 phenotype, which trigger an uncontrolled fungal growth (Verma *et al.*, 2014). Indeed, IL-4 and IL-13 deficient mice are resistant to *C. neoformans* infection (Decken *et al.*, 1998; Müller *et al.*, 2007).

Whereas the role of Th1 and Th2 cell responses has been well understood in the context of fungal infections, the role of **Th17** cells remains controversial. The TGF-β, IL-6 and IL-23 secretion induce Th17 cell polarization. These cells are a $CD4^+$ T cell subset characterized by the production of IL-17A, IL-17F and IL-22, which promote neutrophil mobilization, defensins production and enhancement of epithelial barrier function (Zúñiga *et al.*, 2013). Several studies have shown an increased susceptibility to oral and mucocutaneous *C. albicans*-infections as a consequence of deficiencies in the Th17 pathway, both in mice and humans (Verma *et al.*, 2014). However, unfavourable effects of Th17 response in fungal diseases have also been reported. For instance, an exacerbated Th17 immune response against *C. albicans* and *A. fumigatus* induced by IL-23 has been reported (Zelante *et al.*, 2007). In

turn, Th17 cells down-modulate Th1 responses that are essential to clear the infection (Zelante *et al.*, 2007).

Regulatory T cells (**Tregs**) play an important role in the down-regulation of the pro-inflammatory immune response, thus limiting host-collateral damage and restoring homeostasis. This $CD4^+$T cell subset controls different immunosuppressive mechanisms including the production of anti-inflammatory cytokines, the suppression of IL-2 secretion and the inhibition of APCs function. Thus, Tregs are able to promote either positive or negative responses, depending on the type or the stage of the infection. On one hand, Tregs activity can promote an increased susceptibility to fungal infection through the suppression of protective pro-inflammatory responses. For instance, *tlr2*$^{-/-}$ mice which have impaired anti-inflammatory responses (decreased IL-10 production and $CD4^+CD25^+$ T cells percentage) are less susceptible to *C. albicans* infection compared with their wild-type (WT) counterparts (Netea *et al.*, 2004).On the other hand, Tregs may exert a protective antifungal response by preventing excessive inflammation but enabling fungal persistence at the mucosal sites (Luca *et al.*, 2007). Likewise, *A. fumigatus* conidia-activation of Tregs suppresses potential deleterious effects of neutrophils and prevents allergic diseases (Montagnoli *et al.*, 2006).

In addition to $CD4^+$ T cells, $CD8^+$ T cells are also involved in antifungal immunity (Kumaresan et al., 2018). Cytokines and cytotoxic factors such as perforins, granulysins and granzymes produced by this T cell subset boost the innate and mucosal antifungal responses. It has also been reported that $CD8^+$ T cells inhibit *C. albicans* hyphae growth (Beno *et al.*, 1995).

1.3. FUNGAL RECOGNITION BY THE IMMUNE SYSTEM

Immune recognition of fungal pathogens relies on a limited number of germ-line encoded receptors (termed PRRs) that recognise conserved structures shared by different microbes. Such structures (termed Pathogen-Associated Molecular Patterns, PAMPs) are essential for the microorganism survival and are absent in the host. Additionally, PRRs may also recognize endogenous structures released during cell stress (named Damage-Associated Molecular Patterns, DAMPs) **(Figure I.3)**.

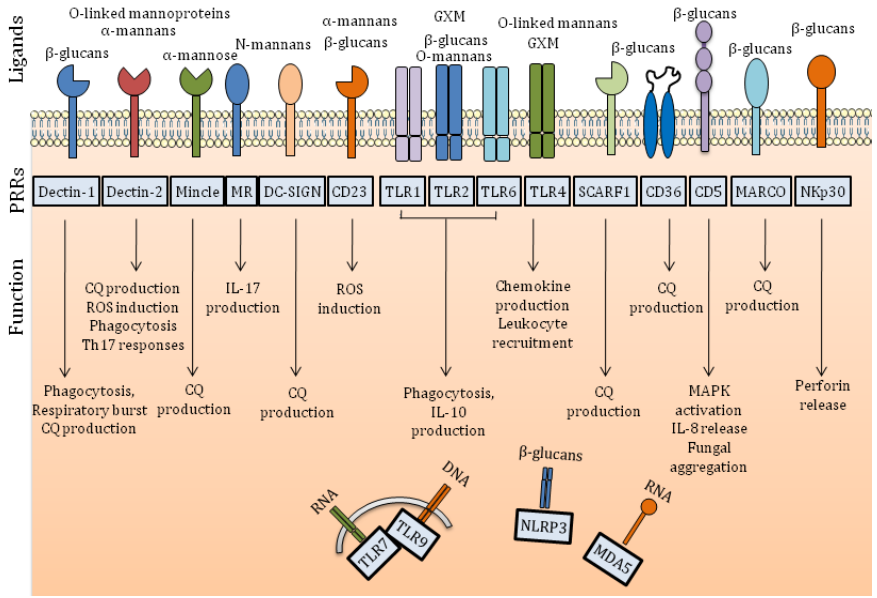

Figure I.3. Schematic representation of the PRRs involved in the anti-fungal immune response.

PRRs are non-clonally distributed receptors belonging to different structural families. They can be found as membrane-bound (e.g., TLRs or C-type lectin receptors) or soluble receptors (e.g., NOD-like, RIG-like or AIM2-like receptors) constitutively expressed by hematopoietic and non-hematopoietic-derived immune cells. After

binding to their respective ligands, PRRs trigger immediate pro-inflammatory responses, which contribute to further activation and ammunition of the innate and adaptive immune responses.

1.3.1 C-type lectin receptors (CLRs)

The most studied PRRs in antifungal immunity are CLRs, a superfamily of soluble and membrane-bound proteins characterized by the presence of at least one C-type lectin domain (Salazar and Brown, 2018). The best characterized CLR is **Dectin-1**, which is mainly expressed by monocytes, macrophages, DCs and granulocytes (Brown, 2006). The interaction of fungal β-1,3-glucans with Dectin-1 induces phagocytosis, respiratory burst and cytokine production by myeloid cells, promotes autophagy and prevents NETosis (Salazar and Brown, 2018). Dectin-1 deficiency results in increased susceptibility to fungal infection in different mouse models (Taylor *et al.*, 2007; Werner *et al.*, 2009; Viriyakosol *et al.*, 2013; Chen *et al.*, 2017). In agreement with such studies, *Dectin-1* polymorphisms have been associated with increased susceptibility to different fungal diseases in humans (Plantinga *et al.*, 2009; Ferwerda *et al.*, 2009; Sainz *et al.*, 2012; Skonieczna *et al.*, 2017).

Dectin-2 and **Mincle** are CLRs expressed by DCs, macrophages and neutrophils. Upon ligand recognition, both receptors associate with the immunoreceptor tyrosine-based activation motif (ITAM) containing the Fc receptor γ-chain (FcRγ)to induce intracellular signals (Ostrop and Lang, 2017). Dectin-2 recognizes fungal α-mannans and O-linked mannoproteins and induces cytokine and ROS production, phagocytosis and Th17 responses. In mouse models, Dectin-2 deficiency results in increased susceptibility to *C. albicans* infection (Saijo *et al.*, 2010; Ifrim *et al.*, 2014; Ifrim *et al.*, 2016). Furthermore, *Dectin-2* gene

polymorphisms have been associated with higher susceptibility to *C. neoformans* and *A. fumigatus* infections in humans (Hu *et al.*, 2015; Skonieczna *et al.*, 2017). Mincle is mainly involved in the immune response against *Malassezia*, by inducing cytokine production (Yamasaki *et al.*, 2009). Mincle interaction with *C. albicans* has also been reported, with Mincle deficient mice being more susceptible to candidiasis (Wells *et al.*, 2008).

The **DC-specific ICAM-3 grabbingnon-integrin** (DC-SIGN) and **mannose receptor** (MR) are CLRs mainly expressed by myeloid cells, including macrophages and DCs, which recognise fungal mannans (Patin *et al.*, 2019). DC-SIGN is not able to directly induce cellular responses, but modulates signalling pathways induced by other receptors such as TLRs (Gringhuis *et al.*, 2007). It has been suggested that *DC-SIGN* polymorphisms could be associated with increased risk of invasive pulmonary aspergillosis but further analyses are required (Sainz *et al.*, 2012). The MR has been involved in IL-17 production from human APCs after *C. albicans* recognition (van de Veerdonk *et al.*, 2009). Furthermore, it has been reported that MR-deficient mice are more susceptible to cryptococcosis and blastomycosis (Dan *et al.*, 2008; Wang *et al.*, 2016).

Collectins such as **mannose binding lectin** (MBL) and **surfactant proteins A** (SP-A) and **D** (SP-D) have also been implicated in the immune response against fungal pathogens. Complement activation by MBL increases fungal opsonization whereas SP-A and SP-D promote microbe agglutination in a complement-independent manner (Brummer and Stevens, 2010).

CD23 also plays a role in antifungal immunity by binding to α-mannans and β-glucans. CD23 is expressed by B cells, eosinophils, monocytes, activated macrophages, follicular DCs and keratinocytes (Guo *et al.*, 2018). Increased CD23 expression, in the absence of its negative regulator c-Jun N-terminal Kinase 1 (JNK1), induces fungal killing by ROS induction (Zhao *et al.*, 2017). CD23 deficient mice are more susceptible to fungal infection induced by *C. albicans* and *A. fumigatus*, but not by *C. neoformans* as it cannot bind to glucuronoxylomannans (GXM), one of the major capsular components of *Cryptococcus* (Guo *et al.*, 2018).

1.3.2. Toll-like receptors (TLRs)

TLRs are expressed by a wide range of immune and non-immune cells, and recognise different fungal structures. TLRs form homo- or hetero-dimers that signal intracellularly through different adaptor proteins, mainly Myeloid Differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing IFN-β (TRIF) (Patin *et al.*, 2019). The role of this family of receptors in fungal defence is complex since it depends on the pathogen and on the interactions between them as well as with other PRRs.

TLR2 is a cell surface receptor mainly expressed by myeloid cells and mast cells that binds to phospholipomannans, β-glucans and zymosan (Bellocchio *et al.*, 2004). There is some controversy on the role of TLR2 in antifungal immunity. TLR2 deficient mice (*tlr2*$^{-/-}$) were reported to have increased susceptibility to disseminated candidiasis (Villamón *et al.*, 2004),while *tlr2*$^{-/-}$ mice were also more resistant to *C. albicans* infection (Netea *et al.*, 2004). The use of different strains may be behind this contradiction. In the first case, increased susceptibility

was attributed to a decrease in tumour necrosis factor (TNF)-α and MIP-2 production and to a decrease in neutrophils recruitment. In the second case, the greater resistance was attributed to increased IL-10 release and to the generation of Treg cells. Regarding other fungal pathogens, *tlr*2$^{-/-}$ mice were not more susceptible to *A. fumigatus* infection (Bellocchio *et al.*, 2004). Moreover, polymorphisms of ***TLR1*** and ***TLR6***, two TLRs forming heterodimers with TLR2, have been associated with susceptibility to aspergillosis (Carvalho *et al.*, 2010). Although TLR2 binds to GXM, this binding is not necessary for *Cryptococcus* serum clearance *in vivo* (Campuzano and Wormley, 2018).

TLR4 is expressed at the cell surface and endosomal compartments of myeloid cells, mast cells, neutrophils, B lymphocytes and intestinal epithelium, and recognises O-linked mannosyl chains. Interaction of TLR4 with fungal mannans from *C. albicans* induces chemokine release and leukocyte recruitment (Netea *et al.*, 2002),while interaction with GXM promotes intracellular signalling but has no effect on cytokine production (Shoham *et al.*, 2001). TLR4 has been shown to play a protective role in *A. fumigatus* and *C. albicans* infections (Netea *et al.*, 2002; Bellocchio *et al.*, 2004). *TLR4* gene polymorphism have been associated with increased susceptibility to aspergillosis and candidiasis in humans (Carvalho *et al.*, 2010).

TLR7 and **TLR9** are endosomal receptors expressed by myeloid cells and B lymphocytes that sense fungal RNA and DNA, respectively (Ramaprakash *et al.*, 2009; Biondo *et al.*, 2012). Both receptors cooperate in the activation of the transcription factor IRF1. TLR7 and TLR9 deficient mice show higher susceptibility to *C. albicans* (Biondo *et al.*, 2012) but not to *A. fumigatus* infection (Bellocchio *et al.*, 2004). Gene

polymorphisms of *TLR7* and *TLR9* have been associated with aspergillosis in humans (Skonieczna *et al.*, 2017).

Finally, specific TLRs seem to be dispensable to the antifungal immune response against *C. neoformans* but this is not the case of the adaptor molecule MyD88. It has been reported that MyD88 deficient mice are highly susceptible to infections by different fungal pathogens such as *C. albicans*, *A. fumigatus*, *Coccidioides immitis*, *Paracoccidioides brasiliensis* or *C. neoformans* (Campuzano and Wormley, 2018).

1.3.3. NOD-like receptors (NLRs)

NLRs are a family of intracellular (cytoplasmic) receptors extensively studied in the context of bacterial infections. In fungal infections, the most important NLR is the **NLRP3,** an inflammasome component expressed by myeloid, lymphoid and epithelial cells, which recognise β-glucans. Mouse models of fungal infection have shown that NLRP3 plays an important role in the immune response against *C. albicans* (Hise *et al.*, 2009)and *P. brasiliensis* (Ketelut-Carneiro *et al.*, 2015). It has also been reported that this receptor is important in the host response against *A. fumigatus* (Saïd-Sadier *et al.*, 2010). *NLRP3* polymorphisms in humans are also associated with higher susceptibility to mucosal candidiasis (Lev-Sagie *et al.*, 2009).

1.3.4. RIG-like receptors (RLRs)

RLRs are a family of cytoplasmic receptors traditionally associated with antiviral immunity, but they also play a role in antifungal immunity. RLRs are expressed by immune and non-immune cells and sense foreign RNA. After ligand binding, these receptors promote the production of type-I and type III IFNs (Brubaker *et al.*,

2015). It has been reported that the **melanoma differentiation-associated protein 5** (MDA5) plays an important role in the immune response against *C. albicans* in humans and mice, and that *MDA5* gene polymorphisms are associated with increased susceptibility to this pathogen (Jaeger *et al.*, 2015).

1.3.5. Scavenger receptors (SR)

The SR constitute a structurally diverse superfamily of proteins involved in the recognition of a wide range of endogenous and exogenous structures (Pombinho *et al.*, 2018). Several members of this superfamily have been implicated in the recognition of fungi. The **scavenger receptor type F family member 1**(SCARF1) and **CD36** bind to β-glucans and induce cytokine production. Indeed, CD36 deficient mice show increased susceptibility to *C. neoformans* infection (Means et al., 2009). The **apoptosis inhibitor of macrophages** (AIM), a homologue of human Spα, binds to and aggregates pathogenic and saprophytic fungal pathogens such as *C. neoformans*, *C. albicans*, *S. pombe* and *Saccharomyces. cerevisiae* (Martinez et al., 2014). A role in antifungal immunity for the **macrophage receptor with collagenous structure** (MARCO) has been reported in a mouse model of cryptococcosis. Indeed, MARCO deficient mice displayed higher fungal growth accompanied by lower cell recruitment and pro-inflammatory cytokine production to *C. neoformans* infection (Xu *et al.*, 2017). Finally, it has also been reported that the SR **CD5** binds to and aggregates fungal pathogens such as *C. neoformans* and *C. albicans* (Vera *et al.*, 2009). The role of CD5 in fungal infections will be further discussed in this thesis.

1.3.6. Other fungal receptors

Other receptors involved in fungal PAMPs recognition include the **complement receptor 3** (CR3), a dimer consisting of the integrin chains CD11b and CD18. CR3 is expressed by myeloid cells and binds to β–glucans with high affinity, promoting fungal killing and phagocytosis in a complement-dependent manner (Goodridge *et al.*, 2009). It has also been shown that CR3 has a role in driving Th1 and Th17 responses during *A. fumigatus* infection (Gresnigt *et al.*, 2013).CR3 deficiency increases mortality rates in mouse models of infection induced by *C. albicans* and *Candida glabrata* (Tsoni *et al.*, 2009).

The epithelial receptor **ephrin type-A receptor 2** (EphA2) also recognize β-glucans and plays an important role in the immune response against *C. albicans* in a oropharyngeal model of infection in mice (Swidergall *et al.*, 2018).

Galectins are additional mannose-binding lectins expressed by myeloid, lymphoid and mast cells. Among them, **Galectin-3**(Gal-3) has been involved in the immune response against *C. albicans* and *C. neoformans*. (Becker *et al.*, 2015; Almeida *et al.*, 2017). Following β-mannan recognition, Gal-3 collaborates with TLR2 for the secretion of TNF-α inducing a protective response (Becker *et al.*, 2015). Enhanced neutrophils antifungal activity has been observed against *C. albicans* and *C. parapsilosis* after incubation with recombinant Gal-3. Furthermore, the effect was inhibited by the addition of anti-gal3 blocking mAb (Linden *et al.*, 2013). These results are in accordance with those obtained in a mice model of *S. pneumoniae* where administration of recombinant Gal-3 reduces lung injury and bacteremia by augmenting neutrophils function (Farnworth *et al.*, 2008). Moreover, increased

susceptibility to candidiasis and cryptococcosis has been reported in Gal-3 deficient mice (Becker *et al.*, 2015; Almeida *et al.*, 2017).

Recently, the NK cell receptor **NKp30** has been identified as a β-glucan receptor (Li *et al.*, 2018). NKp30 promotes Phosphoinositide 3-kinase 3 (PI3K) and Erk 1/2 activation, perforin release, and fungal cytotoxicity after *C. albicans* and *C. neoformans* recognition by NK cells (Li *et al.*, 2013). **NKp46**, and its mouse orthologue **NCR1**, are also NK cell receptors involved in fungal recognition through its interaction with the Epa1, Epa6, and Epa7 adhesins from *C. glabrata.* Thus, NKp46/NCR1 is essential for *in vitro C. glabrata*-killing and *in vivo* clearing systemic infection (Vitenshtein *et al.*, 2016).

Beneficial effects have been observed after the administration of recombinant human **Pexantrin-3** (rhPTX3)- a soluble PRR involved in the immune response against fungi-in rat and mouse models of aspergillosis (Diniz *et al.*, 2004; Gaziano *et al.*, 2004; Lo Giudice *et al.*, 2010).

2. THE SCAVENGER RECEPTOR CD5

CD5 is a lymphocyte surface receptor belonging to the Scavenger Receptor Cysteine-Rich superfamily (SRCR-SF), which is an ancient and highly conserved group of protein receptors characterized by: 1) the presence of one or several cysteine-rich domains called SRCR, and 2) their involvement in the regulation of innate and adaptive immune responses (Martinez *et al.*, 2011).Consequently, CD5 has been involved in the regulation of T and B cell development and function, as well as in microbial recognition. From the historical point of view, CD5 (Lyt-1) was one of the first lymphocyte receptors described in mice and humans

thanks to the advent of the mAb technology (Ledbetter *et al.*, 1980)(Boumsell *et al.*, 1980), thus allowing the characterization of T cell subpopulations and B-cell chronic lymphocytic leukemia (B-CLL) cells.

2.1. GENE AND PROTEIN STRUCTURE

The human *CD5* gene maps to 11q12.2, telomeric to *CD6* (a highly related member of the SRCR -SF) and encompasses 24.5 kb. It consists of 11exons: exons 1 and 2 encode the signal peptide leader, exons 3-6 the extracellular region, exon 7 the transmembrane region and exons 8-10 the intracellular region (Padilla *et al.*, 2000). The *CD5* gene is conserved across species as evidenced by its orthologue on mouse chromosome 19 (Lecomte *et al.*, 1996; Padilla *et al.*, 2000).

The *CD5* gene codes for a type I trans-membrane glycoprotein of 67 kDa. The extracellular region is composed by three SRCR domains: the most N-terminal domains (D1 and D2) are interspaced by a highly *O*-glycosylated Proline-, Serine- and Threonine (PST)-rich region. The most-membrane proximal domain (D3) is not glycosylated and directly linked to D2 (Rodamilans *et al.*, 2007; Garza-Garcia *et al.*, 2008). The cytoplasmic region is devoid of intrinsic catalytic activity but it has several residues (11 Ser, 4 Tyr and 4 Thre) suitable for phosphorylation by different Ser/Thre- (casein kinase 2, CK2; Protein Kinase C, PKC; Ca^{2+}/calmodulin-dependent kinase II, CaMK2) and Tyr- (Lck and Fyn) kinases (Burgess *et al.*, 1992; Gary-Gouy *et al.*, 1997; Bauch *et al.*, 1998; Calvo *et al.*, 1998; Vilà *et al.*, 2001a; Vilà *et al.*, 2001b; Bamberger *et al.*, 2011). Furthermore, some Tyr are embedded into a pseudo-immunoreceptor tyrosine–based inhibitory motif (pseudo-ITIM) or a pseudo-ITAM that allows its interaction with signalling mediators (i.e., Lck, Fyn, Ras-GAP, c-Cbl or SHP-1) thus modifying downstream events

of the T cell receptor (TCR) (Simarro *et al.*, 1999; Vilà *et al.*, 2001a) **(Figure I.4)**.

Genetic polymorphisms for CD5 have been reported in some mammalian species (McKenzie and Potter, 1979; Howard *et al.*, 1989; Starling *et al.*, 1997). In humans, a total of 27 single nucleotide polymorphisms (SNPs) have reported, with two of them present at relatively high frequencies in Caucasians: rs2241002 and rs2229177. The SNP rs2229177 involves an Alanine (Ala) to Valine (Val) substitution at the amino acidic position 471 (A471V), which is C-terminal to a cytoplasmic ITAM-like motif. The SNP rs2241002 involves a Proline (Pro) to Leucine (Leu) substitution at position 224 (P224L) in the second SRCR extracellular (D2) domain (Moreno-Estrada *et al.*, 2009; Carnero-Montoro *et al.*, 2012). The Ala to Val substitution has been described as a gene signature for positive selection in East Asian population, which would involve differential immune signalling of still undefined nature (Carnero-Montoro *et al.*, 2012).

2.2. EXPRESSION

CD5 is a lymphoid-specific marker mainly expressed on thymocytes (from early maturational stages) and all mature T cells (Reinherz *et al.*, 1979; Ledbetter *et al.*, 1980). In thymocytes, CD5 levels correlate with TCR expression and avidity: lower levels are found on double negative (DN, $CD4^-CD8^-$), increase on double positive (DP, $CD4^+CD8^+$), and achieve the higher levels on simple positive (SP $CD4^+$ or SP $CD8^+$) (Azzam *et al.*, 1998). On the other hand, Treg cells ($CD4^+$ $CD25^+$ $Foxp3^+$) show the highest expression levels (Ordoñez-Rueda *et al.*, 2009).

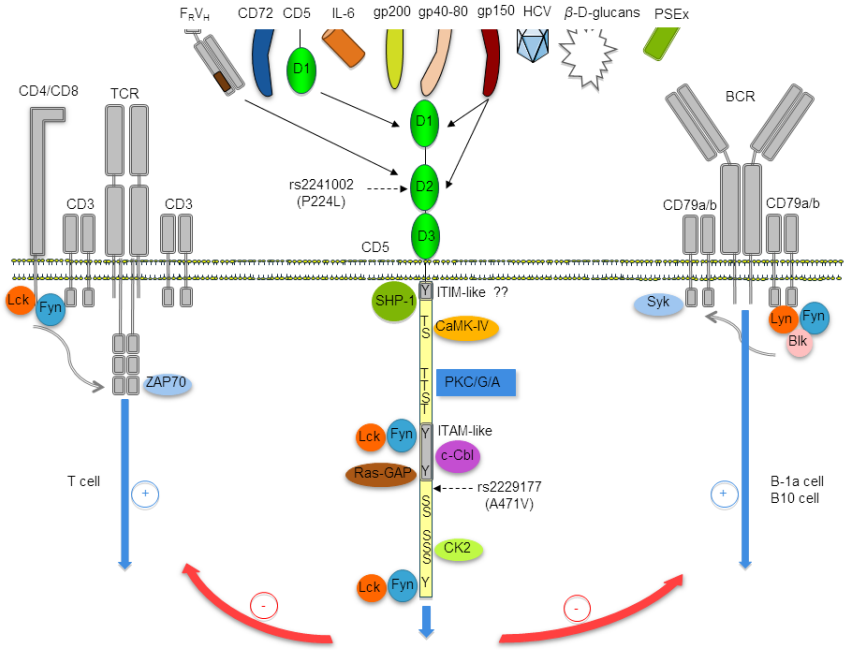

Figure I.4. Schematic representation of the extracellular and intracellular interactions mediated by CD5. Adapted from (Consuegra-Fernández *et al.*, 2015).

CD5 expression has been reported in B-CLL cells (Wang *et al.*, 1980) and the subpopulation of B cells named B1a cells (IgM^{hi}, IgD^{low}, $CD23^{-}$, $CD5^{+}$), the main source of polyreactive natural IgM antibodies (Berland and Wortis, 2002). High levels of CD5 are also expressed by mouse regulatory B cells (Breg or B10 cells), an IL-10-producing subpopulation involved in prevention of autoimmune diseases (Yanaba *et al.*, 2009)

CD5 is also expressed in extra-lymphoid cells, namely macrophages and DCs. Thus, CD5 expression has been reported in a mouse biphenotypic cell subset with normal B and macrophage function, which could play a dual role in the adaptive and innate immune response (Borrello *et al.*, 2001). Furthermore, low CD5 levels

have been reported in macrophages from *Theileria annulata* clinical isolates (Moreau *et al.*, 1999), in certain endothelial cells (Gogolin-Ewens *et al.*, 1989), in a subpopulation of vaginal DCs (De Bernardis *et al.*, 2006) and in a subpopulation of human skin DCs (Korenfeld *et al.*, 2017). The later would induce cytotoxic T cell and Th22 responses, playing an important role in the development of psoriasis (Korenfeld *et al.*, 2017). Recently, it has been reported the expression of CD5 by $CD11c^+$ DCs in lymphoid and non-lymphoid tissues from mice (Li *et al.*, 2019).

In addition to membrane-bound CD5, the presence of a circulating soluble form of CD5 has been reported in sera from healthy individuals at pico/nanomolar range resulting from proteolytic cleavage following lymphocyte activation (Calvo *et al.*, 1999a). Moreover, increased levels of soluble CD5 has also been detected in serum of patients with lymphocyte hyperactivation diseases, such as the Sjögren syndrome (Ramos-Casals *et al.*, 2001) and the Systemic Inflammatory Response Syndrome (SIRS) (Aibar *et al.*, 2015). Although the biological function of the soluble form of CD5 is still unknown, it has been proposed that it could act as a competitor with the membrane-bound CD5 for interaction with its ligands (Axtell *et al.*, 2004; Simões *et al.*, 2017).

2.3. LIGANDS

Despite the numerous studies published, the nature of the endogenous CD5 ligand/s still remains controversial and elusive since none of the different candidates proposed have been validated by independent research groups. The long list of reported CD5 ligands includes CD72 (de Velde *et al.*, 1991), the framework region of IgV_H

(Pospisil *et al.*, 2000), gp200 (Haas and Estes, 2001), gp40-80 (Biancone *et al.*, 1996; Bikah *et al.*, 1996), the CD5 itself (Brown and Lacey, 2010), gp150 (Calvo *et al.*, 1999b), and IL-6 (Masuda and Kishimoto, 2016; Zhang *et al.*, 2016). Some of these interactions have been mapped: D1 for CD5, D2 for the framework region of IgV_H, and D1-D2 for gp150 (**Figure. I.4**).

Regarding the possibility that CD5 could interact with exogenous ligands, our group has reported that the extracellular region of CD5 binds to β-glucans- a PAMP of fungal origin (Vera *et al.*, 2009). The affinity of this interaction (Kd =3.7 ± 0.2 nM) is in the same range of that reported for Dectin-1 -the main β-glucan receptor in myeloid mammalian cells (Adams *et al.*, 2008). Such interaction would be relatively specific since no binding to other fungal (mannans) or bacterial (lipopolysaccharide, LPS; lipotheichoid acid, LTA; peptidoglycan, PNG) PAMPs was observed (Vera *et al.*, 2009). Recently, the recognition of PAMPs by CD5 has been extended to viral and parasitic structures. It has been reported that CD5 facilitates Hepatitis C Virus (HCV) entry into T cells, which could act as reservoir for its persistence (Sarhan*et al.*, 2012). Furthermore, the interaction of CD5 with tegumental components of the cestode parasite *Echinococcus granulosus* has also been described (Mourglia-Ettlin *et al.*, 2018).

2.4. FUNCTION

Thanks to its ability to negatively modulate the intracellular signals delivered by the antigen receptor from T and B lymphocytes, CD5 plays a relevant role on the regulation of immune responses in both health and disease.

2.4.1. CD5 in health

2.4.1.1. CD5 in cell activation

CD5 is physically associated with the antigen-specific clonotypic receptor of T (TCR) and B1a (BCR) cells. In T cells, CD5 co-localizes with the TCR at the centre of the supramolecular activation complex (cSMAC), also named immunological synapsis (Beyers et al., 1992; Gimferrer et al., 2003; Brossard et al., 2003). Thus, CD5 is well positioned for modulating the activation or death cell signals generated following antigen-specific recognition. By using anti-CD5 mAbs, alone or in combination with anti-CD3, anti-CD4 or anti-CD28 mAbs, CD5 was initially reported in the 1980s as a positive regulator (co-stimulator) of TCR signalling (Ledbetter et al., 1985; Ceuppens and Baroja, 1986). Later on, the characterization of the CD5-deficient mice positioned this receptor as a negative modulator of activation and differentiation signals from T and B cells. Accordingly, thymocytes from *cd5*$^{-/-}$ mice showed to be hyperresponsive to TCR/CD3 cross-linking, as deduced by an enhanced proliferation, increased Ca^{2+} mobilization as well as phospholipase C (PLC)-γ1, TCRζ, LAT, and Vav phosphorylation (Tarakhovsky et al., 1995). Furthermore, the analysis of TCR-transgenic mice showed that CD5 negatively modulates the intensity of the TCR signalling, thus influencing thymocyte selection (Tarakhovsky *et al.*, 1995; Azzam *et al.*, 1998; Azzam *et al.*, 2001). Mechanistically, an increased phosphorylation of a negative regulatory tyrosine of Fyn after CD5 stimulation has been reported (Bamberger *et al.*, 2011).

Several works reported the ability of CD5 to generate TCR-independent events. The anti-CD5 TS 43 mAb alone is able to increase T cells proliferation as well as IL-2R expression, IL-2 production and Ca^{2+}

mobilization (Spertini *et al.*, 1991). Similarly, stimulation with the anti-CD5 Cris-1 mAb alone also resulted in T lymphoblast proliferation without triggering the canonical TCR signalling pathway (e.g., inositol phosphate metabolism or Ca^{2+} mobilization) but inducing phosphatidylcholine-specific phospholipase C (PC-PLC)-dependent activation of acidic sphingomyelinase (A-SMase) and protein kinase C zeta (PKC-ζ) (Alberola-Ila *et al.*, 1992; Simarro *et al.*, 1999). In both cases the presence of monocytes was necessary, so other co-stimulatory effectors may be involved in these effects.

As in the case of T cells, cross-linking of the BCR with anti-IgM antibodies showed enhanced B cells proliferation and an increase in Ca^{2+} mobilization in *cd5*$^{-/-}$ mice (Bikah *et al.*, 1996). BCR and CD5 co-ligation also reduced Ca^{2+} mobilization and ERK2 phosphorylation (Gary-Gouy *et al.*, 2000).

2.4.1.2. CD5 in cell survival

The first evidence that CD5 may regulate T and B-1a cell survival was the induction of ERK phosphorylation in thymocytes after mAb-induced CD3 and CD5 crosslinking, which resulted in the expression of the anti-apoptotic molecule Bcl-2 (Zhou *et al.*, 2000). The mechanism by which CD5 increases T cell survival has not been fully elucidated. Besides Bcl-2, the induction of CK2 and AKT as well as the inhibition of pro-apoptotic mediators such as caspases, Bid and FasL by CD5 has also been reported (Soldevila *et al.*, 2011). Indeed, gene-targeted mice in which the CK2-binding domain of CD5 was selectively ablated (CD5-ΔCK2BD) showed increased apoptosis and pERK levels sin double positive thymocytes, which resulted in altered positive and negative selection processes (Mier-Aguilar *et al.*, 2016). On the other hand, CD5-

deficient mice show enhanced resistance to experimental autoimmune encephalomyelitis (EAE) as a consequence of enhanced activation-induced cell death (AICD) of T cells (Axtell *et al.*, 2004).

With respect to B cells, it has been proposed that CD5 could play a role by regulating IL-10 production. After stimulation, $CD5^+$ B cells are less susceptible to apoptosis than $CD5^-$ B cells by producing higher amounts of IL-10 and reducing Ca^{2+} mobilization. Thus, CD5 could prevent AICD events maintaining B-1a cell homeostasis and supporting cell survival (Gary-Gouy *et al.*, 2002). Moreover, PKC activation has been reported in a subset of B-CLL cells after anti-CD5 cross-linking, being correlated with the Mcl-1 pro-survival intermediate (Perez-Chacon *et al.*, 2007).

2.4.1.3. CD5 in tolerance

The immune system has developed different mechanism to avoid self-reactivity. One of these mechanisms is anergy induction. In this context, over expression of CD5 correlates with anergy of $CD8^+$ T cells chronically exposed to their cognate antigen, with CD5 levels returning to normal following antigen removal (Stamou *et al.*, 2003). By using transgenic mice lacking the CD5-CK2 binding domain, it has been shown that this interaction is important for tolerance induction. Mice lacking the CD5-CK2 interaction showed a less severe and a delayed onset in EAE mice model as a consequence of enhanced AICD of T cells (Sestero *et al.*, 2012).

On the other hand, Tregs are characterized by high CD5 expression levels (Kuniyasu *et al.*, 2000; Ordoñez-Rueda *et al.*, 2009). *cd5*$^{-/-}$ Treg cells express higher mRNA levels of Foxp3 and CD5 deficient mice are less susceptible to dextran induced colitis (Dasu *et al.*, 2008).

Moreover, blocking of mTOR (mammalian target of rapamycin)-dependent signalling has been reported as a mechanism for Treg cell induction by CD5 (Henderson *et al.*, 2015).

Modulation of regulatory B cell function by CD5 has also been suggested. Indeed, increased Ca^{2+} mobilization and hyper-proliferation of anergic B cells from CD5 deficient mice has been reported (Hippen *et al.*, 2000). Moreover, transgenic mice overproducing a soluble form of human CD5 showed a reduction in the number of IL-10-producing B cells (also named B10) in spleen an peritoneal cavity (Fenutría *et al.*, 2014).

2.4.2. CD5 in disease

2.4.2.1. CD5 in infection

As mentioned earlier, CD5 can recognize pathogen-related structures of fungal, viral and parasitic origin. Though physiological relevance of these interactions has not been fully elucidated, it has been proposed that CD5 may help to prevent autoimmunity as well as to optimize antimicrobial immune responses (Lenz, 2009).

It has been demonstrated that expression of membrane-bound CD5 on 2G5 cells (a Jurkat T cell derivative) and HEK 293 (non-lymphoid cells) transfectants induces signalling events (namely, mitogen activated protein kinase (MAPK) phosphorylation) upon exposure to zymosan, a β- glucan-rich fungal particle (Vera *et al.*, 2009). This MAPK activation depends on the cytoplasmic tail integrity, as MEK and ERK1/2 phosphorylation was not observed when a truncated form of CD5 lacking the most C-terminal 88 amino acids of the protein was transfected. In the same way, exposure of HEK 293 transfectants

expressing the membrane form of CD5 to zymosan promotes a significant induction in IL-8 release compared with un-transfected cells or HEK 293 transfectants expressing the truncated form of CD5. Also, as a result of the β-glucan recognition, a recombinant soluble human form of CD5 (rshCD5) induces fungal aggregation in a dose-dependent manner avoiding pathogen dissemination and facilitating fungal clearance (Vera *et al.*, 2009).

Ex vivo assays challenging peritoneal cells from CD5-deficient mice with PSEx (a fraction of tegumental antigens from *E. granulosus*) induced increased TNF-α, but decreased IL-6 production compared with their WT counterparts. On the other hand, peritoneal cells from WT C57BL/6 (C57) mice stimulation with PSEx in the presence of increasing amounts of rshCD5 increase PSEx-induced TNF-α and IL-6 production. Thus, the absence of membrane-bound CD5 or the presence of soluble CD5 modulates cytokine production upon PSEx exposure. Furthermore, rshCD5 infusion reduced the proportion of infected mice, the number of hydatid cysts per mouse and the total wet weight of hydatid cysts per mouse (Mourglia-Ettlin *et al.*, 2018).

Aside from the interaction between CD5 and HCV above mentioned (Sarhan *et al.*, 2012), the possibility that CD5 could have a role in other viral infections has been documented or Hepatitis B virus (Sun *et al.*, 2013), Human Immunodeficiency Virus type 1 (Penney *et al.*, 2014), Equine Infectious Anemia (Tumas *et al.*, 1994) and Eipstein-Barr Virus-associated hemophagocytic lymphohistiocystosis (Karandikar *et al.*, 2004).

Finally, the fact that CD5 is expressed in some non-lymphoid cell subsets could influence the innate immune response against infection.

In this sense, a protective role of $CD5^+$ vaginal DCs has been described in a rat model of experimental *C. albicans* vaginitis (De Bernardis *et al.*, 2006).

2.4.2.2. CD5 in cancer

Several studies have positioned CD5, a negative modulator of T cell activation and differentiation, as a target for the development of anti-tumour immunotherapies. Positive results were early reported in the treatment of mouse tumour models (leukemia and Lewis Lung Carcinoma) by using the non-depleting anti-CD5 Lyt-1mAb (Hollander, 1984). Later, a phase I clinical trial was developed with an anti-CD5 T101 mAb in patients with B-CLL and cutaneous T-cell lymphomas (Dillman *et al.*, 1984). A second trial was performed with a radioimmunoconjugate version of the same mAb (^{90}Y-T101) (Foss *et al.*, 1998) resulting both studies in partial but transient responses.

Recently, CD5 expression levels have been inversely correlated with the anti-tumour activity of Tumour Infiltrating T lymphocytes (TILs) (Dorothée *et al.*, 2005). Indeed, low or undetectable CD5-levels have been related with increased AICD of TILs (Friedlein *et al.*, 2007). Moreover, TILs from CD5 deficient mice *s.c.* challenged with B16.F10 melanoma cells displayed a more activated phenotype and showed slower tumour growth (Tabbekh *et al.*, 2011). These results are in line with those reported for transgenic mice expressing a soluble form of human CD5, which showed slower tumour growth in mouse models of melanoma (B16.F0) and thymoma (EG7-OVA). The higher anti-tumour response of such transgenic mice was associated with a lower proportion of Treg and B10 cells, and a higher percentage of NKT cells (Fenutría *et al.*, 2014). Moreover, lower tumour growth was also

observed after peritumoural injection of rshCD5 protein to WT mice *s.c.* challenged with B16.F0 melanoma cells. These results could be correlated with NK cells cytotoxic activity, as lower intratumoural levels of IL-6 were observed in the transgenic mice and rshCD5-treated mice, and NK cell depletion with mAbs abrogated the anti-tumour effect (Simões *et al.*, 2017).

On the other hand, it has been shown that carriage of certain *CD5* gene variants may influence the outcome of cancer patients. Thus, the SNP A471V seems to correlate with progression-free survival in patients undergoing B-CLL (Sellick *et al.*, 2007; Delgado *et al.*, 2017) and with improved survival in melanoma (Potrony *et al.*, 2016).

2.4.2.3. CD5 in autoimmunity

Since both regulatory (T and B) cell subpopulations and autoantibody-producing B cells express high levels of CD5, several groups have investigated the role of this receptor in autoimmunity.

Controversy over the role of CD5 in Systemic lupus erythematosus (SLE) has been raised. On one hand, a relationship between $CD5^+$ circulating B cells and secretion of autoantibodies has been reported (Dauphinée *et al.*, 1988; Markeljević *et al.*, 1994; Böhm, 2004). This could be a consequence of uncontrolled Ig VDJ recombination resulting from the expression of recombination activation genes (*RAG 1* and *2*) (Morbach *et al.*, 2006; Hillion *et al.*, 2007). On the other hand, $CD5^+$ B cells from SLE patients show reduced levels of membrane-bound CD5 as a consequence of increased expression of a truncated CD5 isoform (CD5-E1B) that remains retained in the cytoplasm (Garaud *et al.*, 2008). Expression of CD5-E1B is induced by IL-6 (Garaud *et al.*, 2009) and accordingly, the blockade of anti-IL-6

receptor improves abnormal B and T cell homeostasis (Shirota *et al.*, 2013). In addition, another study demonstrated that $CD5^+$ B cells seems to be involved in IL-21 and granzyme B induction (Hagn *et al.*, 2010). Pilot studies treating SLE patients with a murine anti-CD5 mAb bound to ricin toxin A chain (RTA) (zolimomabaritox) have been performed. This immunoconjugate induces a persistent but modest T cell depletion and a transient decrease in $CD5^+$ B cells, in conjunction with high cytotoxic effects and multiple adverse reactions (Wacholtz and Lipsky, 1992; Stafford *et al.*, 1994).

As for SLE, there is controversy over the role of CD5 in Multiple Sclerosis (MS). In humans, a positive correlation between $CD5^+$ B cells percentage and higher MS risk and early disease onset has been reported (Correale *et al.*, 1991; Hardy and Hayakawa, 2001; Villar *et al.*, 2011). CD5 expression levels in B cells have been associated with the onset and duration of relapsing-remitting MS (Scott *et al.*, 1994; Bongioanni *et al.*, 1996; Seidi *et al.*, 2002). In contrast, another study showed higher percentages of intrathecal $CD5^-$ B cells (Sellebjerg *et al.*, 2002), and decreased CD5 expression levels in B cells seems to be associated with secondary progressive MS (Niino *et al.*, 2012). Experimental models of $CD4^+$ T cell-mediated MS have been used to study the role of CD5 in this disease. A protective role for CD5 or $CD5^+$ B cells has been reported in mice models of EAE (Hawiger *et al.*, 2004; Ochoa-Repáraz *et al.*, 2010; Begum-Haque *et al.*, 2011). However, the induction of EAE in *cd5*$^{-/-}$ deficient mice resulted in an increased resistance to the disease (Axtell *et al.*, 2004). Due to the interaction between CD5 and CK2, the authors proposed that the delayed EAE onset and decreased severity was a consequence of the pro-survival activity of CD5 on T cells resulted from the binding of CK2 to this receptor. This

was also supported by the results obtained from transgenic mice lacking the CD5-CK2 binding interaction site, which also showed resistance to EAE (Axtell *et al.*, 2006). The use of mAb for the treatment of EAE has been also explored. In this context, the administration of an anti-CD5 mAb (OX19, IgG_1) at the immunization time partly prevents clinical signs of Experimental Autoimmune/Allergic Neuritis (EAN), while its administration shortly before the expected onset of the disease or during its height promoted an exacerbation of the disease symptoms (Strigård *et al.*, 1988). An enhancement of EAE severity has also been observed in transgenic mice constitutively expressing a soluble form of CD5 as well as in WT mice when rshCD5 was repeatedly injected from the disease initiation (Fenutría *et al.*, 2014). However, Axtell and colleagues reported a recovery from EAE with the administration of adenovirus expressing CD5-immunoglobuling fusion protein (CD5-Fc) (Axtell *et al.*, 2004).

The role of CD5 in Rheumatoid arthritis (RA) has been studied for many years. Interestingly, the SNP rs229177 (Ala471Val) has been included within 14 new susceptibility variants described for RA in populations of European ancestry (Eyre *et al.*, 2012). Increased percentages and numbers of $CD5^+$ B cells have been reported in patients with RA (Hara *et al.*, 1988; Smith and Olson, 1990; Burastero *et al.*, 1993; Cantaert *et al.*, 2012). In these studies, higher levels of these cells have been correlated with increased amounts of polyreactive antibodies, increased levels of rheumatoid factor and clinical features of severe disease (Burastero *et al.*, 1990). However, another study failed to show such correlation (Sowden *et al.*, 1987), and a negative role for Breg populations has been reported in RA (Ma *et al.*, 2014; Daien *et al.*, 2014; Cui *et al.*, 2015). Clinical trials using an anti-CD5 mAb linked to ricin

toxin A chain (RTA) resulted in an inhibition of the IL-2-induced proliferation of synovial-fluid T cells in some RA patients (Verwilghen *et al.*, 1992; Olsen *et al.*, 1993; Strand *et al.*, 1993; Fishwild and Strand, 1994; Cannon *et al.*, 1995). Although positive results were initially observed, the use of depleting anti-CD5 mAbs was finally stopped as no beneficial effects or significant differences between groups were reported in a multicenter clinical trial (Olsen *et al.*, 1996; Lorenz and Kalden, 1998). In mouse models of collagen-induced arthritis (CIA), an experimental mouse model for human RA, the use of non-depleting anti-CD5 mAbs have resulted in a significant decrease in disease severity due to T-cell mediated mechanisms, as anti-native collagen II circulating levels were unaltered and the amelioration of disease severity appeared six days after mAb treatment (Plater-Zyberk *et al.*, 1994).

Increased proportions of $CD5^+$ B cells have been reported in early phases of Insulin-dependent diabetes mellitus (IDDM) (Lorini *et al.*, 1993; Muñoz *et al.*, 2008). Two studies have shown the utility of anti-CD5 mAbs in the treatment of IDDM although the mechanism underlying the observed protective effects need to be further investigated. A clinical trial was performed in patients with recent-onset IDDM in which administration of the immunoconjugate CD5-Plus® (anti-CD5 H65 mAb bound to RTA) resulted in reversible T-cell depletion and preservation of β-cell function in a dose-dependent manner (Skyler *et al.*, 1993). In a mouse model of IDDM, treatment with anti-Lyt-1 mAb linked to RTA promotes a dose-dependent protection against diabetes onset (Vallera *et al.*, 1992).

An inverse correlation has been reported between $CD5^+$ B cell number and treatment response in autoimmune nephropathy (Wu *et al.*, 2011; Kim *et al.*, 2011; Nagatani *et al.*, 2013; Wang *et al.*, 2014).

Furthermore, an anti-CD5 mAb (OX19) has been used as therapy in rats, in which reduced proteinuria and glomerular lessons amelioration was observed (Tipping *et al.*, 1996; Huang*et al.*, 1997; Ikezumi *et al.*, 2000). Infusion of CD5-Fc chimera in a mouse model of antibody-mediated membranous glomerulonephritis also resulted in beneficial effects (Biancone *et al.*, 1996).

Finally, the role of CD5 has been investigated in inflammatory bowel disease (IBD). It has been reported that *cd5*$^{-/-}$ mice are more resistant to dextran sulphate sodium (DSS)-induced colitis than their WT counterparts, (Dasu *et al.*, 2008). The authors observed increased levels of Foxp3 mRNA levels in colon an enhanced suppressive activity of *cd5*$^{-/-}$ Tregs. On the other hand, Yanaba and colleagues have shown that *cd19*$^{-/-}$ mice are more susceptible to DSS-induced colitis and that adoptive transfer of WT B10 cells (CD1d^{hi}CD5^{+}IL-10^{+}) to *cd19*$^{-/-}$ mice induced a less severe intestinal injury in a IL-10-dependent manner (Yanaba *et al.*, 2011).

3. IMMUNOTHERAPY AGAINST FUNGAL INFECTIONS

Treatment of IFIs is a worldwide problem as the overall mortality rates are still around 50%, despite advances in the development of new antifungal drugs (Drgona *et al.*, 2014; Bassetti *et al.*, 2014). This is due to the increasing number of immunocompromised patients, the limited number of effective antifungal drugs, toxicity, drugs interactions and the emergence of resistant strains (Nami *et al.*, 2019). These facts highlight the real need for the development of new alternative therapies. In this sense, immunotherapy could act as an alternative approach in the prevention and treatment of such infections, by modulating the host immune response to fungal pathogens. Different

immunotherapeutic strategies have been explored for the management of fungal diseases, both at host and pathogen levels **(Figure I.5)**.

3.1. VACCINES

The development of antifungal vaccines has been an important research area during the last years. However, immunocompromised host failed in mounting a proper immune response so this approach requires the use of immunoadjuvants. These patients are also at risk from live attenuated formulations. This strategy could be an attractive option in some particular situations, as is the case of immunocompetent individuals that will undergo severe immunosuppression like those waiting for solid organ transplantation.

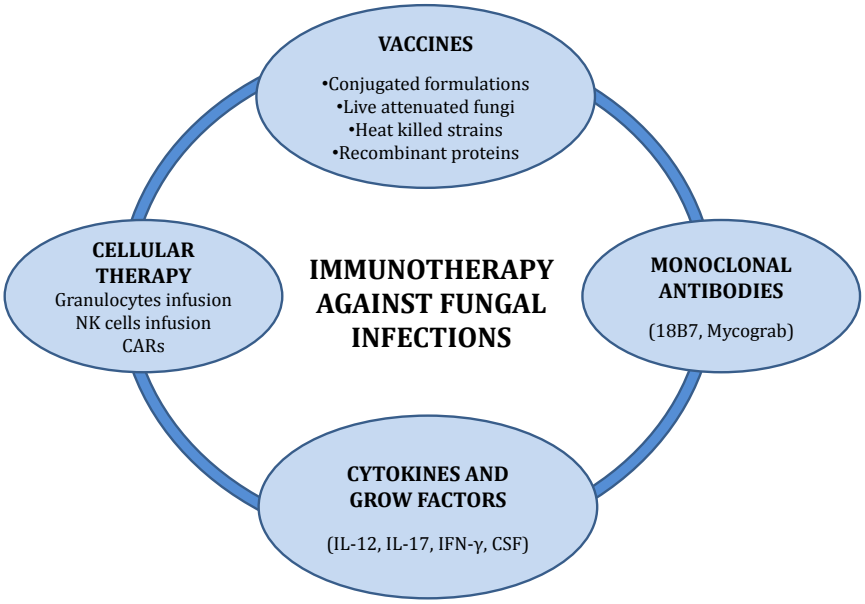

Figure I.5 Schematic representation of the immunotherapeutic strategies for the treatment of IFIs.

Ideally, fungal vaccines targeting antigens shared by most of the pathogenic fungi, such as the glycan β-1,3-glucan are the most attractive.

While this fungal cell wall component is poorly immunogenic, protective effects have been reported in mice immunized with laminarin conjugated to a diphtheria toxoid. This conjugate induces strong antibody responses and protection in mice models of candidiasis and aspergillosis (Torosantucci *et al.*, 2005).

Promising results have been shown with vaccines containing live attenuated fungi. An increased survival has been observed in a mice model of pulmonary cryptococcosis, upon immunization with a modified strain of *C. neoformans* (Wozniak *et al.*, 2012). However, these strains need to be sufficiently attenuated in order to avoid disease. Immunization with heat killed strains could solve this problem acting as panfungal vaccines. Protection against *Aspergillus*, *Coccidioides* and *Candida* infection has been shown after vaccination with heat killed *Saccharomyces* (Stevens *et al.*, 2011). Development of subunit vaccines has also been investigated. Vaccines containing recombinant proteins of *C. albicans* were also found to confer protection in mice models of infection and clinical trials (Lin *et al.*, 2009; De Bernardis *et al.*, 2012; Schmidt *et al.*, 2012).

3.2. MONOCLONAL ANTIBODIES

Protection conferred by vaccination-elicited antibodies has led to the development of therapeutic mAbs. Indeed, administration of mAbs against cell surface components of *C. albicans*, *A. fumigatus*, *C. neoformans* or *H. capsulatum* have shown protective effects in mouse models of infection (Dromer and Charreire, 1991; Han and Cutler, 1995; Chaturvedi *et al.*, 2005; Guimarães *et al.*, 2011).

Nevertheless, use of mAbs against fungal components is not a developedfield. 18B7 and Mycograb are the only two mAbs that have

been evaluated in clinical trials. 18B7 is a mAb with hydrolytic activity directed against GXM, one of the main components of *C. neoformans* capsule (Bowen *et al.*, 2017). A phase I trial on HIV-patients with cryptococcal meningitis has yielded positive results (Larsen *et al.*, 2005). Mycograb is a mAb directed to the fungal shock protein HSP90. Beneficial effects has been reported in patients with invasive candidiasis in combination with amphotericin B (Pachl *et al.*, 2006).

3.3. CYTOKINES AND GROW FACTORS

The use of recombinant cytokines and grow factors with the aim of modulating the host response against fungal pathogens has received much attention during the last years. Numerous promising studies have been developed based on the use of cytokines such as IL-12, IL-17, IFN-γ or Colony-Stimulating Factors (CSFs) in combination with classical antifungal treatments.

Being neutropenia one of the main risk factors in the development of IFIs, CSFs such as GM-CSF and granulocyte (G)-CSF were among the first to be tested as antifungal immunotherapy. G-CSF promotes antifungal activity of neutrophils as well as its proliferation and differentiation (Bendall and Bradstock, 2014). G-CSF-deficient mice are more susceptible to *C. albicans* infection than WT mice (Basu *et al.*, 2008). Furthermore, a protective effect of G-CSF administration in animal models of fungal-induced infection has been reported (e.g., *C. albicans* and *A. fumigatus*) (Polak-Wyss, n.d.; Lechner *et al.*, 1994; Deepe *et al.*, 1999; Patera *et al.*, 2004; Kasahara *et al.*, 2016). GM-CSF plays an important role in fungal infections by stimulating maturation, activation and migration of a wide range of immune cells (Shi *et al.*, 2006) and also enhances the antifungal immune response by inducing phagocytosis and

ROS production (Richardson *et al.*, 1992). GM-CSF-deficient mice are susceptible to different fungal pathogens including *A. fumigatus* and *H. capsulatum*. In humans, the use of GM-CSF and G-CSF as adjunctive therapies alongside conventional anti-fungal treatments have shown beneficial effects in different IFIs (van de Veerdonk *et al.*, 2012; Scriven *et al.*, 2017).

There is an important number of studies supporting the increased susceptibility to fungal infections associated with IFN-γ deficiency (Balish *et al.*, 1998a; Clemons *et al.*, 2000; Chen *et al.*, 2005b; Vinh *et al.*, 2009; Carreras *et al.*, 2018). This cytokine promotes anti-fungal response of macrophages and neutrophils (Schroder *et al.*, 2004). The administration of recombinant IFN-γ improves the immune response against *C. albicans*, *C. neoformans* and *A. fumigatus* among others (Kullberg *et al.*, 1993; Joly *et al.*, 1994; Nagai *et al.*, 1995; Carreras *et al.*, 2018). The use of this cytokine has been approved by the Food and Drug Administration (FDA) for the treatment of infections in patients with CGD. Several clinical trials have demonstrated the enhanced immune response after IFN-γ administration in patients infected by *C. albicans*, *C. neoformans* and *A. fumigatus* (Delsing *et al.*, 2014a; Coelho and Casadevall, 2016).

3.4. CELL THERAPY

Therapy involving cell infusion could be an alternative approach in the treatment of IFIs. Since the initial development of adoptive cell transfer for the treatment of T cell malignancies, progress has been made towards engineering T cells redirected to specific antigens. For the same reason as GM-CSF and G-CSF where among the first candidates to be tested, theoretically, granulocyte infusion should improve the host

response against fungi. In a review of 97 case reports, overall response rates between 50 and 100%were found (West *et al.*, 2017). Problems such as their short lifespan or the low cell counts precluded obtaining optimal and concluding results. However, the authors described an improvement in the yield of this cell type after the availability of G-CSF (West *et al.*, 2017).

NK cells do not induce graft-versus-host disease (GvHD), a fact that makes them interesting candidates for adoptive cell transfer. In mice models of infection by *C. neoformans* and *A. fumigatus*, NK cells administration promotes fungal clearance, although this effect is IFN-γ dependent (Schmidt *et al.*, 2017). Nowadays, the effect of NK cell administration as immunotherapy is being investigated in cancer clinical trials so further analysis is necessary regarding its use in IFIs.

Recently, a novel immunotherapeutic approach based on the infusion of autologous cells expressing Chimeric Antigen Receptors (CARs) has been proposed. These receptors have been mainly expressed on T lymphocytes which can therefore overcome some limitations of other T cell therapies as they act in a MHC-independent manner. These receptors can be classified in three main generations depending on the design. First generation CARs are constituted by a specific antigen-binding domain (a single-chain variable fragment, scFv, resulting from the fusion of the variable regions of the heavy (V_H) and light chains (V_L) of an specific antibody), a spacer region, a transmembrane domain and an intracellular domain capable to trigger cell signalling. The initial clinical trials using CAR-T cells for cancer treatment were disappointed because of problems such as poor expression and persistence, immunogenicity, toxicity or low efficacy. In an attempt to avoid T cell anergy and increase T cell activation, second generation CARs have been

designed by adding a co-stimulatory intracellular signalling domain. The CD28 signalling domain initially, and tumour necrosis factor receptor-members such as CD27, 4-1BB (CD137) or OX40 (CD134) have been used. Finally, third-generation CARs contains two co-stimulatory domains have been described (Barrett *et al.*, 2014; Chang and Chen, 2017). Nowadays, several clinical trials are on-going for testing second and third-generation CARs (Kohn *et al.*, 2011). In 2017, the first CAR-T cell therapy was approved by the FDA for B-cell acute lymphoblastic leukemia (B-ALL) (June *et al.*, 2018).

Most studies involving CAR-T cells are related to cancer immunotherapy, but some are now attempting to apply this strategy against infection. For instance, an increase in IFN-γ production *in vitro* as well as virus clearance *in vivo* has been reported by CAR T cells against the M2 membrane protein of the Influenza A virus (Talbot *et al.*, 2013). Another example is the development of CD4-based CARs for HIV-1 infection treatment (Maldini *et al.*, 2018). Regarding fungal infections, human T cells expressing a modified version of prototypical CD19-CAR accommodating the extracellular domain of human Dectin-1 (D-CAR) exhibited specificity to the glucose polymer laminarin, and led to damage and inhibition of *A. fumigatus* growth *in vitro* and *in vivo* (Kumaresan *et al.*, 2014).

Despite T cells constitute the main cell type chosen for the expression of CARs, other cell types, such as NK cells, are under evaluation for the expression of these chimeric receptors. Promising results have been reported in preclinical studies in haematological and solid cancer therapy (Fang *et al.*, 2017). Furthermore, in contrast to CAR-T cells, there are immortal NK cell lines available which possess cytotoxic activity and cytokine-production capacity. Among them, the

NK-92 cell line has been the most widely studied and its safety and effectiveness is being investigated in clinical trials. NK-92 cells lack almost all killer cell immunoglobulin-like receptors (KIRs), but express activating NK-cell receptors which together with the *in vitro* expansion possibilities, makes this cell line an attractive option for the development of CAR-based immunotherapies (Zhang *et al.*, 2017a; Liu *et al.*, 2017).

II. HYPOTHESIS AND OBJECTIVES

CD5 is a scavenger receptor mainly expressed on lymphoid (T and B1a) cells but also on some minor myeloid (Mϕ and DCs) cell subsets. It is long known to negatively modulate differentiation and activation signals mediated by the clonotypic antigen specific receptor complexes of T (TCR) and B1a (BCR) lymphocytes, both being an identity hallmark of the adaptive immune system (Burgueño-Bucio *et al.*, 2019). Recently, several reports have also shown its ability to recognise and signal the presence of PAMPs of fungal, viral and parasitic origin (Consuegra-Fernández *et al.*, 2015; Burgueño-Bucio *et al.*, 2019), which is a formal trait of PRRs expressed by the innate immune system's components (Salazar and Brown, 2018). In consequence, CD5 can be considered as a relevant immunomodulatory receptor at the interphase between the innate and adaptive immune responses.

IFIs have emerged in recent decades as a significant health problem associated with high morbidity, mortality, and economic burden (Klingspor *et al.*, 2015). Nowadays, only a few antifungal drugs are available and their use is limited by their associated side effects, making necessary the development of new alternative or complementary therapeutic strategies (Nami *et al.*, 2019). The discovery by our group that CD5 binds with relative high affinity to and signal the presence of β-glucans (Vera *et al.*, 2009) -a constitutive and highly conserved component of fungal cell walls -motivated our interest on exploring the CD5's physiological function and/or therapeutic potential in IFIs. In our view, the study of soluble and/or membrane-bound immune receptors involved in antifungal immunity, as it may be the case of CD5, could provide an important source of functional information to be translated into such a novel therapeutic approaches.

Based on the above mentioned premises, the specific objectives of this thesis have been the following:

- To study the influence of the mouse genetic background on fungal infection by analyzing the antifungal immune response of the inbred (C57) and outbred (CD1) mouse strains most widely used in basic and pharma-industry research.
- To study the influence of membrane-bound CD5 on fungal infection by analyzing the antifungal immune response of mice genetically deficient for CD5 (*cd5*$^{-/-}$).
- To study the therapeutic potential of soluble human CD5 administration (alone or in combination) in experimental models of fungal infection.
- To study the therapeutic potential of CD5-based adoptive cell transfer strategies by analysing the influence of immune cells transduced with membrane-bound chimerical CD5 receptors in pre-clinical models of fungal infection.

III. MATERIALS AND METHODS

1. MICE

Wild-type CD1, C57 and Balb/c mice were purchased from Charles River Laboratories (France) and quarantined for one week prior to experimental manipulation. Immunodeficient NSG (NOD/SCID IL-2Rγcnull) mice from Charles River Laboratories (France) were breeded and kept in individual ventilated cages under specific pathogen-free (SPF) conditions. CD5 deficient mice (*cd5*$^{-/-}$) in C57 background (Tarakhovsky *et al.*, 1995), kindly provided by Dr Chander Raman (Department of Medicine, University of Alabama at Birmingham), and their WT counterparts were breeded and kept in individual cages under SPF conditions.

Unless otherwise stated, animals of 8 to 12 weeks of age were used in all experimental procedures, which were approved by the Animal Experimentation Ethical Committee of the University of Barcelona and Generalitat de Catalunya.

2. PRODUCTION AND PURIFICATION OF RECOMBINANT PROTEINS

Production of purified rshCD5 (from R25 to D345; in phosphate-buffered saline (PBS) plus 10% glycerol, pH 7.4) was performed as previously described (Sarrias *et al.*, 2004) but using stably transfected SURE CHO-M Cell line™ clones developed at Selexis (SUREtechnology Platform™, Geneva, Switzerland). Serum-free culture supernatants were further subjected to size-exclusion chromatography protocols developed at PX´Therapeutics (Grenoble, France).

Recombinant Human Serum Albumin (HSA) and murine IFN-γ (mIFN-γ) were purchased from Sigma Aldrich (A9731), and Genscript (Z02916), respectively.

3. CELL SUSPENSIONS

Spleen and peritoneal cells from euthanized CD1, C57 and Balb/c mice were removed aseptically. Peritoneal cells were obtained by peritoneal lavage with 3 mL of saline (B/Braun). Splenocytes were incubated for 15 min at 37 °C in 4 mL of Hank's balanced salt solution medium (HBSS; LabClinics), containing 1 mg/mL collagenase D (11088866001; Roche) and 0.1 mg/mL DNAse I (10104159001; Roche). Following disaggregation through 40 µm cell strainers (Biologix) with a syringe plunger, the cells were washed with 10 mL of HBSS plus 10 % fetal bovine serum (FBS; BioWest). After supernatant discard, the cells were incubated at room temperature (RT) for 5 min with 3 mL of red blood cell lysis solution buffer (RBC; 00-4333-57, eBioscience). After a second wash, cells were counted and adjusted at the desired concentration in RPMI 1640 medium with L-glutamine (R8758-6X500ML; Sigma & Aldrich) plus FBS (10%), Hepes (10 mM; Life technologies), sodium pyruvate (1 mM; Gibco), 2-Mercaptoethanol (50 µM; Merck), penicillin (100 U/mL; 6191309, Lab EBN) and streptomycin (100 µg/mL; 624569, Lab Normon).

4. *EX VIVO* CELL STIMULATION ASSAYS AND MEASURE OF CYTOKINE LEVELS

Spleen and peritoneal cells (5 x 10^6 and 2 x 10^6 cells/mL, respectively) from CD1, C57 and Balb/c mice were plated in 48- or 96-well plates in RPMI 1640 medium with L-glutamine (supplemented as

described above). The cells were stimulated with zymosan (Zym; 0–75 μg/mL), anti-IL-12 antibody (1–5 μg/mL; C17.8, BD Biosciences), isotype control mAb (5 μg/mL), LPS (1 μg/mL; L2630, Sigma Aldrich), LTA (10 μg/mL; L2515, Sigma Aldrich), and anti-CD3 (0.1 μg/mL; 70-0031-M001, TONBO), either alone or in combinations. Mouse IFN-γ and IL-17A cytokine levels in culture supernatants were determined by ELISA (BD OptEIA-Mouse ELISA Sets, BD Biosciences Pharmingen) following the manufacturer's instructions.

Spleen cells (5 x 10^6 cells/mL) from CD1 mice were plated in 96-well plates in RPMI 1640 medium with L-glutamine plus FBS (10%) and 2-Mercaptoethanol (50 μM). Splenocytes were exposed to heat-killed *C. albicans* (0.5 x 10^6 CFUs/mL) in the presence or absence of rshCD5 (1 to 10 μg/mL) or vehicle. Heat killed *C. albicans* was obtained by incubation for 30 min at 100 ºC. Mouse IFN-γ, TNF-α, IL-1β and IL-10 inflammatory cytokine levels were assessed in 24 h-culture supernatants were determined by ELISA (BD OptEIA-Mouse ELISA Sets, BD Biosciences Pharmingen) following the manufacturer's instructions.

Spleen cells (2 x 10^5 cells/well) from *cd5*$^{-/-}$ and WT C57 mice were plated in 96-well plates in RPMI 1640 medium (supplemented as described above). IFN-γ, TNF-α, IL-12 and IL-6 levels were determined by ELISA in culture supernatants from splenocytes in basal conditions or exposed to heat-killed *C. albicans* or *C. neoformans* (1 x 10^5 CFUs/well) for 24 h.

5. *IN VITRO* KILLING ASSAYS

Splenocytes (1 x 10^6 cells/mL) from CD1 mice were suspended in RPMI 1640 medium with L-glutamine plus FBS (10%) and 2-

Mercaptoethanol (50 μM), and co-cultured for 2 h at 37 ºC and 5% CO_2 in 96-well plates with *C. albicans* or *C. neoformans* (0.5 x 10^6 cells/mL) in the presence of rshCD5 (1 to 10 μg/mL) or vehicle. Then, the cells were lysed with water and the number of viable CFUs was assessed by seeding and subsequent incubation for 48 h at 30ºC on sabouraud dextrose agar plates. Killing activity was calculated as percentage of non-viable CFUs in the presence of splenocytes compared with non-viable CFUs in absence of cells.

6. FUNGAL VIABILITY ASSAYS

C. albicans and *C. neoformans* (0.5 x 10^6 /mL) were suspended in RPMI 1640 medium (with L-glutamine plus FBS (10%) and 2-Mercaptoethanol (50 μM)), and cultured for 2 h at 37 ºC in 96-well plates in the presence of vehicle or rshCD5 (1 to 10 μg/mL). Then, fungal serial dilutions were seeded on Sabouraud dextrose agar plates and incubated for 48 h at 30 ºC for further viable CFUs determination. Viability was calculated as percentage of viable CFUs in the presence of rshCD5 compared with viable CFUs in absence of the protein.

7. *EX VIVO* LEUKOCYTE SUBPOPULATIONS ANALYSIS

7.1. ANALYSIS OF PRRS EXPRESSION ON MYELOID AND LYMPHOID CELL SUBPOPULATIONS.

Spleen cells (1 x 10^7 cells/mL) from euthanized CD1 and C57 mice were characterized in basal conditions by using the fluorescent-labelled mAbs listed in **Table II.1.** Before staining, 1 x 10^6 cell suspensions were incubated for 15 min at RT in blocking solution (PBS plus 2 % FBS and anti-mouse CD16/CD32; Fc Shield, clone 2.4G2, Tonbo

Bioscience). Mixes of mAbs were prepared in blocking solution and 50 µl of each mix was added to the cells. The samples were incubated for 20 min at 4 ºC in dark, and then centrifuged at 1500 r.p.m., washed twice with PBS and resuspended in fixing solution (PBS plus 1% paraformaldehyde (PFA)). Labelled cells were analyzed with a BD FACSCanto II flow cytometer (Becton Dickinson, US) and mean fluorescence intensity (MFI) data analyzed using FlowJo software (Tree Star, USA).

Table II.1 Specificity of the mAbs used for the PRRs expression characterization on leukocyte subpopulations by flow cytometry.

Specificity	Conjugate	Clone	Source
CD45R (B220)	violetFluor 450	RA3-6B2	Tonbo
CD45R (B220)	APC	RA3-6B2	Biolegend
CD3	PE-Cy7	145-2C11	Tonbo
CD11c	Percp-Cy5.5	N418	Tonbo
CD11c	eFluor 450	N418	eBioscience
F4/80	FITC	BM8.1	Tonbo
CD282 (TLR2)	PE	CB225	Biolegend
CD284 (TLR4)	APC	SA15-21	Biolegend
CD119 (IFNgR1)	PE	2E2	eBioscience
CD11b	PE	M1/70	Tonbo
Dectin-1	APC	bg1fpj	eBioscience
CD5	Percp-Cy5.5	53-7.3	Biolegend

7.2. ANALYSIS OF ACTIVATION AND APOPTOSIS T CELL MARKERS

Total splenocytes (2 x 10^5 cells/well) from *cd5*$^{-/-}$ and WT C57 mice were suspended in RPMI 1640 medium with L-glutamine plus FBS (10%) and 2-Mercaptoethanol (50 µM), and co-cultured for 24 h at 37 ºC and 5% CO_2 in 96-well plates with *C. albicans* or *C. neoformans* (1 x 10^5 CFUs/well). Then, the cell suspensions were incubated for 15 min at

RT in blocking solution (PBS plus 2 % FBS and anti-mouse CD16/CD32). T cell activation was determined by using the fluorescent-labelled mAbs listed in **Table II.2**. The cells were stained for the surface expression markers for 20 min at 4 ºC in dark. For intracellular IFN-γ staining the Fixation/Permeabilization Solution Kit (554714; BD Biosciences) was used according to the manufacturer's instructions. Finally, after the appropriate washes, the cells were analyzed in a FACS Canto II flow cytometer.

T cell apoptosis was measured with the Annexin/7AAD Kit (Immunostep) following manufacturer's indications. Once stained for surface expression markers, the cells were resuspended in 400 µL of Annexin buffer, 10 µL of Annexin and 10 µL of 7AAD. After incubating for 15 min at RT they were analyzed in the flow cytometer. The $Annexin_{+}/7AAD_{+}$ and $Annexin_{+}/7AAD_{-}$ cells were considered as late apoptotic and early apoptotic cells, respectively. The data analyzed with Flow Jo software (Tree Star).

Table II.2 Specificity of the mAbs used for $CD4^{+}$ and $CD8^{+}$ T cells activation and apoptosis by flow cytometry.

Specificity	Conjugate	Clone	Source
CD4	FITC	RM4-5	Tonbo
CD8	PE	53-6.7	Tonbo
CD69	APC	H1.2F3	eBioscience
CD25	Percp-Cy5.5	PC61.5	Tonbo
IFN	eFluor 450	XMG1.2	Tonbo
PD1	FITC	J43	eBioscience

8. GENERATION AND FUNCTIONAL CHARACTERIZATION OF HUMAN LYMPHOCYTES EXPRESSING A CD5-BASED CAR.

8.1. DESIGN AND CONSTRUCTION OF THE CD5-CAR LENTIVIRAL VECTOR

The lentiviral vector for expression of the CD5CAR construct was designed based on the pCCLsinPPT_EF1a_CART19 vector previously described (Milone *et al.*, 2009; Porter *et al.*, 2011; Castella *et al.*, 2019) **(Figure II.1A)**. Briefly, the coding sequence CD19-scFV sequence of pCCLsinPPT_EF1a_CART19 was replaced by that of the whole extracellular region of the human CD5 molecule (from R25 to D345), which was then preceded by the EF1α promoter and the CD8α signal peptide sequences, and followed by the CD8α transmembrane region and the cytoplasmic activating motifs of the 4-1BB/CD137 and CD3ζ receptors **(Figure II.1B)**. The entire CD5CAR sequence **(Annex I)** was gene synthesized and cloned as a *Mlu*I-*Bsp*EI fragment into the third generation lentiviral vector pCCLsinPPT_EF1a_CART19at GenScript (USA).

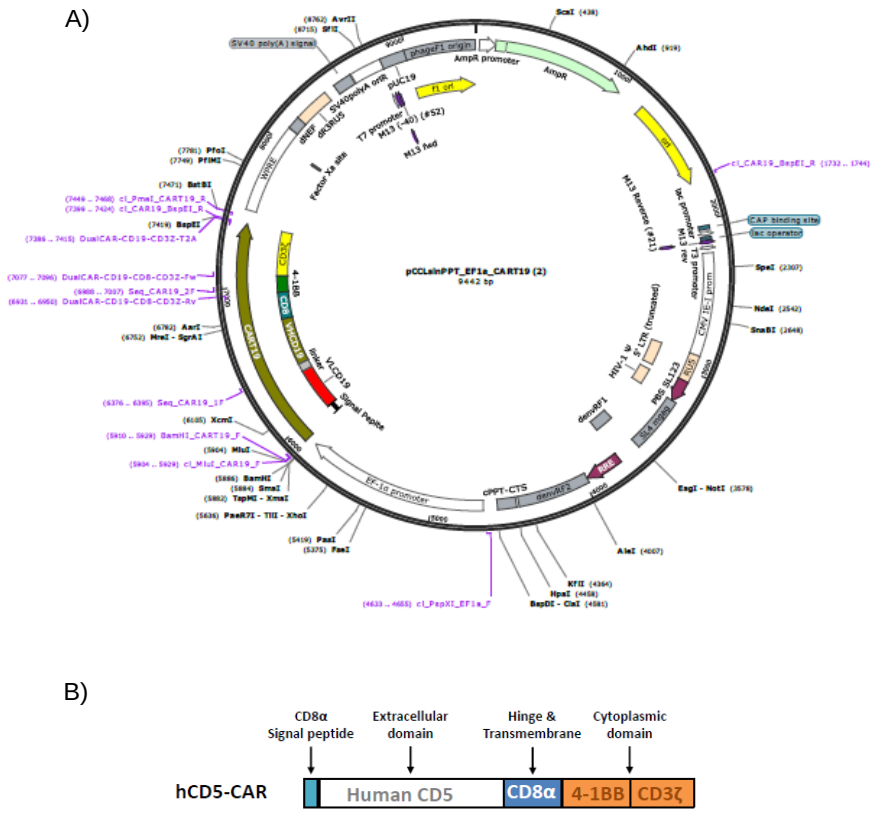

Figure II.1. CD5-CAR lentiviral vector design. A) Map of the lentiviral pCCLsinPPT_EF1a_CART19 vector. **B)** Schematic representation of the CD5CAR construct.

8.2. LENTIVIRUS PRODUCTION

HEK 293T cells (9 x 10^6 cells) were plated into 10-cm culture dishes 24 h before co-transfection with the CD5CAR-pCCL vector and the packaging plasmids pRSV-Rev (Addgene, 12253), and pMDLg-pRRE(Addgene, 12251) and the envelope plasmid pMD2-VSVG (Addgene, USA). At the transfection time, 16 µg of total DNA (8 µg pCCL-CD5CAR, 4 µg pMDLg/pRRE, 2 µg pRSV-Rev, and 2 µg pMD2.G) were diluted in serum-free DMEM (41966-052; GIBCO) and then mixed with 40 µg of linear Polyethylenimine (PEI) (23966-1; Polysciences) for 20

min at RT. Next, medium was replaced and DNA-PEI complexes were added into the cells. At 4 h after transfection, medium was replaced again. Complete lentiviral particles were collected from culture supernatants 72h later, passed through 0.45 µm filters and concentrated by centrifugation at 18000 rpm for 3h at 4ºC. Lentivirus-containing pellets were resuspended in DMEM and stored at -80°C until infection. Whole lentiviral particles from packaging HEK 293T cells were used to transduce previously activated primary human T cells or Cord blood derived NK cells (CBNKs). which were then subjected to different *in vitro* experimental procedures schematically shown in **Figure II.2.**

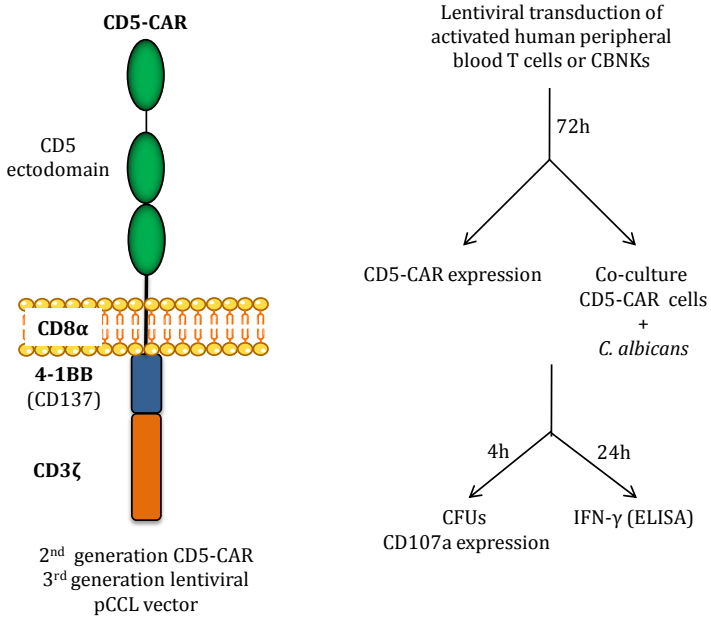

Figure II.2 Schematic diagram summarizing the generation of CD5CAR-transduced human T or CBNK cells for *in vitro* analyses.

8.3. CELL TRANSDUCTION AND CULTURE CONDITIONS

For isolation of human T cells, peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation over Ficoll

(10771-6X100ML; Sigma-Aldrich) from buffy coats of healthy donors from Banc de Sang iTexits (BST) of Generalitat de Catalonia, upon approval by the internal Ethical Committee. Monocytes were removed by adherence to plastic for 2 h at 37 ºC of PMBC cells suspended in RMPI 1640 medium with L-glutamine supplemented with 10% FBS, 100 UI/mL penicillin (6191309; Lab EBN), and 100 µg/mL streptomycin (624569; Lab Normon) and plated into 10-cm culture dishes. Non-adherent cells (1 x 10^6 cells/mL) were then activated and expanded for 72 h with Dynabeads™ Human T-Activator CD3/CD28 (111-32D; GIBCO) (at a bead to cell ratio of 1:1) in the same RPMI 1640 medium as above and further supplemented with 50 µM β-mercaptoethanol (31350-010; Thermo Fisher) and 30 IU/mL IL-2 (11011456001; Roche).

CBNKs from healthy donors, kindly provided by Dr. Álvaro Urbano-Ispizua (Hematology Department, Hospital Clinic, Barcelona, Spain), were isolated by magnetic depletion with the NK cell Isolation Kit (MiltenyiBiotec, San Diego, CA) following the manufacturer's instructions. Then, cells were expanded for 14 days by co-culture with K562-based antigen presenting cells expressing membrane bound IL-21 ("Clone 9.mbIL21") (Shah *et al.*, 2013) in 45 % RPMI-1640 and 45 % Click's media (Irvine Scientific, Santa Ana, CA) supplemented with 10 % human AB serum (Atlanta Biologicals, Lawrenceville, GA) plus IL-2 (400 IU/m; Proleukin; Chiron, Emeryville, CA) added every other day.

Lentiviral transduction of T or CBNK (1 x 10^6) cells was performed by 5 h-incubation at 37 °C and 5 % CO_2 in the presence of 0.5 µl/mL of polybrene (TR-1003-G; Merck Millipore) and centrifugation (2000 rpm) for 90 min at 32 ºC RPMI 1640 medium with L-glutamine in the case of T cells or 45 % RPMI-1640 and 45 % Click's mediain the case

of CBNK cells (supplemented as described above). Next, fresh medium was added to avoid polybrene toxicity and left in culture for 72h.

For cell surface analysis of CD5CAR expression on T or CBNK cells, both cell types as well as the untransduced controls were adjusted to the desired concentration (1 x 10^6 cells/mL) in staining solution (PBS plus 2% FBS) plus anti-human CD5 PercPCy5.5 (UCHT2; TONBO) and incubated for 20 min at 4ºC in dark. Next, cells were centrifuged at 1500 r.p.m., washed twice with PBS and resuspended in PBS plus 1% PFA. Labelled cells were then analyzed with a BD FACSCanto II flow cytometer and mean fluorescence intensity (MFI) or percentage of positive cells was assessed using FlowJo software (Tree Star, USA).

8.4. *IN VITRO* ASSAYS OF ANTIFUNGAL ACTIVITY

All functional assays with CD5CAR-T or -CBNK cells were performed at 72 h post-transduction. CD5CAR-T or -CBNK cells (and the corresponding un-transfected cells) were incubated with alive *C. albicans* at different effector: target (E:T) ratios (20:1, 10:1, 5:1 and 1:1), at 37 °C and 5 % CO_2. After 4 h of co-incubation, cells were stained with PE-labeled anti-human CD107a/LAMP1 (H4A3, BD Pharmigen) and co-culture supernatants collected and plated on Sabouraud dextrose agar plates. The number of CFUs was determined after 48 h of incubation at 30 °C. In parallel experiments, the production of IFN-γ after 24 h of co-incubation was determined by ELISA (BD OptEIA-Human ELISA Set) following the manufacturer's instructions.

9. EXPERIMENTAL MOUSE MODELS OF DISEASE

9.1. MURINE MODEL OF ZYMOSAN-INDUCED GENERALIZED INFLAMMATION (ZIGI).

The murine model of ZIGI was performed by intraperitoneal (*i.p.*) injection of Zym (100-1000 mg/kg; Z450, Sigma Aldrich) to CD1 and C57 mice according to previous reports (Volman *et al.*, 2005; Bian *et al.*, 2012; Jia *et al.*, 2013). Buprenorphine (0.1 mg/kg; INDIVIOR) was administered as analgesic every 12 h.

At the specified experiments, mIFN-γ (0.2 μg/kg), rshCD5 (0.7 mg/kg) and HSA (0.7 mg/kg) were *i.p.* administered alone or in combination, 1 h post-ZIGI induction. Animals were monitored daily for 2 weeks, and survival, weight lost, and clinical score evaluated.

Survival and body weight loss were monitored overtime. The clinical score was calculated as the mean of the individual scores (lethargy, diarrhoea, conjunctivitis, and fur appearance), each of them rated from 0 to 3.

9.1.1. Measurement of cytokine levels after ZIGI challenge.

Cytokine levels were determined in serum from CD1 and C57 mice *i.p.* injected with Zym (700 mg/kg). At 18 h post- challenge, mice were sacrificed and blood samples were obtained by cardiac puncture and stored on ice until centrifugation (2000 rpm) for 10 min at 4 °C. Serum was recovered and stored at -80 °C until used. Mouse IL-12 (p70), IL-6, TNF-α, IFN-γ, IL-10, IL-4, GM-CSF, IL-1β, IL-5, IL-2, and IL-17A cytokine levels were determined by commercially available ELISA kits (BD OptEIA-Mouse ELISA Sets, BD Biosciences Pharmingen)

or Mouse cytokine magnetic 10-plex panel (Invitrogen) following the manufacturer's instructions.

9.2. MURINE MODEL OF FUNGAL INFECTION BY *C. albicans*.

C. albicans (strain SC5314; ATCC MYA−2876), kindly provided by Dr. Oscar Zaragoza (Instituto de Salud Carlos III, Madrid), was grown for 48 h at 30 ºC on Sabouraud agar plates (01024_00; Conda). After that period, an isolated colony was took and grown o/n at 37 ºC in Sabouraud liquid medium (CM0147; Oxoid) under horizontal shaking at 180 rpm. The culture was washed with PBS and serial dilutions were done until achieving the desired concentration in saline for inoculum preparation depending on the mouse weight and model lethality.

CD1 mice infection was performed by injecting intravenously (*i.v.*; tail vein) 2.86 x 10^4 - 2.86 x 10^2 colony forming units (CFUs) per gram, in a final volume of 100µL. In the case of the fungal infection model developed in C57 WT, C57 *cd5*$^{-/-}$, Balb/c and NSG mice, the inoculums were the same as in the case of CD1 mice, but adjusted to the animal's weight (CFUs/gr). Survival and body weight loss were monitored daily.

At the indicated experiments, *C. albicans*-infected CD1, C57 *cd5*$^{-/-}$, Balb/c and NSG mice were treated with vehicle, HSA (1.25 mg/kg), rshCD5 (0.625 to 2.5 mg/kg) or mIFN-γ (0.2 or 10 µg/kg). The proteins were injected *i.v.* or *i.p* at different time points post-infection (1-48 h).

At the specified experiments, fluconazole (0.1-10 mg/kg; B/ Braun) was *i.p.* injected to CD1 mice according to previous reports (MacCallum and Odds, 2004). The drug was daily administered for a period of 7 days starting at 48 h post infection alone or in combination with rshCD5 (1.25 mg/kg; + 18h; *i.v.*).

9.2.1. Determination of fungal load in *C. albicans* infected mice.

CD1 mice were *i.v.* infected by *C. albicans* (2.86 x 10^3 CFU/gr) and *i.v.* treated with HSA or rshCD5 (1.25 mg/kg; +18 h). C57 WT and C57 *cd5*$^{-/-}$ mice were *i.v.* infected by *C. albicans* (2.86 x 10^3 CFU/gr). At the indicated times points post-infection, mice were euthanized, and spleen and kidney were aseptically removed. The organs were weighed and homogenized in sterile PBS using 40 μm cell strainers and a syringe plunger. Fungal burden was determined by plating serial dilutions of the homogenates on Sabouraud agar plates incubated for 48 h at 30 ºC for further CFUs/gr count.

9.2.2. Measurement of cytokine levels after *C. albicans* infection.

Cytokine levels were determined in serum and kidney from CD1 mice *i.v.* infected with *C. albicans* (2.86 x 10^3 CFU/gr) and treated via *i.v.* with HSA or rshCD5 (1.25 mg/kg; +18 h). At 72 h post-infection, mice were sacrificed and blood samples obtained by cardiac puncture and stored on ice until centrifugation (2000 rpm) for 10 min at 4 °C. Serum was recovered and stored at -80 °C until used. Kidney samples were homogenised with a tissue disrupter in PBS with 1x protease inhibitors cocktail (cOmplete™, 11697498001, Roche). After centrifugation at 12000 g for 10 min at 4 ºC the supernatant was recovered and stored at -80 °C until used. In both cases, mouse IL-6, IFN-γ, TNF-α, IL-1β and IL-10 cytokine levels were determined by commercially available ELISA kits.

9.2.3. Leukocyte subpopulations analysis in *C. albicans* infected mice.

Spleen and kidney leukocyte subpopulations from CD1 mice *i.v.* infected by *C. albicans* (2.86 x 10^3 CFU/gr) and treated via *i.v.* with HSA

or rshCD5 (1.25 mg/kg; +18 h) were analyzed at 72 h post-infection. Spleen and kidney from euthanized animals were removed and incubated for 20 min at 37ºC in PBS containing 0.5 (kidneys) to 1 (spleens) mg/mL collagenase D and 0.1mg/mL DNAse I. Following disaggregation through 40 μm cell strainers, cell suspensions were washed twice with PBS plus 2% FBS and erythrocytes lysed with 4 mL of RBC for 4 min at RT. After a second wash, cells were counted and adjusted to the desired concentration (1 x 10^7 cells/mL) in PBS plus FBS (2%).

Kidney and spleen leukocyte subpopulations were characterized using the fluorescent-labelled mAbs listed in **Table II.3**. Before staining, 1 x 10^6 cell suspensions were incubated for 15 min at RT in blocking solution (PBS plus 2 % FBS and anti-mouse CD16/CD32). Then the samples were stained for 30 min at 4 °C in the dark with the appropriate antibody mixes prepared in blocking solution. LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (L34976; Invitrogen) was used to exclude dead cells. After the appropriate washes cells were incubated o/n in fixing solution (PBS plus PFA /1%). Finally, leukocyte subpopulations were determined by a FACS Canto II flow cytometer and the data analyzed with Flow Jo software.

Table II.3. Specificity of the mAbs used for leukocyte subpopulations characterization in *C. albicans* infected CD1 mice.

Specificity	Conjugate	Clone	Source
CD45	FITC	30-F11	Biolegend
CD3	APC	145-2C11	Tonbo
CD45R (B220)	violetFluor 450	RA3-6B2	Tonbo
CD45R(B220)	APC	RA3-6B2	Biolegend
NK1.1	PE	PK136	BD Biosciences
Gr-1	APC	RB6-8C5	Tonbo
CD11b	PE	M1/70	Tonbo
CD11b	APC	M1/70	eBioscience
CD11c	Percp-Cy5.5	N418	Tonbo
CD11c	eFluor 450	N418	eBioscience

9.3. MURINE MODEL OF FUNGAL INFECTION BY *C. neoformans*.

C. neoformans, var *grubii* (serotype A; strain H99) (Perfect *et al.*, 1980), kindly provided by Dr. Oscar Zaragoza (Instituto de Salud Carlos III, Madrid), was grown for 48 h at 30ºC on Sabouraud agar plates. After that period, an isolated colony was took and grown in Sabouraud liquid medium, o/n at 30ºC under horizontal shaking at 180rpm. The culture was washed with PBS and serial dilutions were done in saline until achieving the desired concentration (3.3 x 10^7 CFUs/mL).

CD1, C57 WT and C57 *cd5*$^{-/-}$ mice infection was performed by intranasal (*i.n.*) administration of 2.86 x 10^4 CFUs/gr (in a final volume of 30 µL) to mice *i.p.* anesthetized with ketamine (Ketamidor; 100 mg/kg) and xilacine (Rompun; 10 mg/kg). At the specified experiments, *C. neoformans*-infected CD1 mice were *i.v.* treated with a single dose of HSA (1.25 mg/kg) or rshCD5 (0.625 - 2.5 mg/kg) at different time points post-infection (1-6 days). Survival and body weight loss were monitored daily.

9.3.1. Determination of fungal load in *C. neoformans* infected mice.

At the specified time point's post-infection, CD1 mice *i.n.* infected by *C. neoformans* (2.86 x 10^4 CFUs/gr) and treated with HSA or rshCD5 (1.25 mg/kg, + 3 days) were euthanized and lung and brain were aseptically removed. The organs were weighed and homogenized in sterile PBS using 40 µm cell strainers and a syringe plunger. Fungal burden was determined by plating serial dilutions of the homogenates on Sabouraud agar plates incubated for 48 h at 30 ºC for further CFUs/gr count.

9.3.2. Measurement of cytokine levels after *C. neoformans* infection.

Cytokine levels were determined in serum from CD1 mice *i.n.* infected by *C. neoformans* (2.86 x 10^4 CFUs/gr) and treated with HSA or rshCD5 (1.25 mg/kg, + 3 days). At the indicated time points post-infection, mice were sacrificed and blood samples obtained by cardiac puncture and stored on ice until centrifugation (2000 rpm) for 10 min at 4 °C. Serum was recovered and stored at -80 °C until used. Serum was recovered and stored at -80 °C until used. Mouse IFN-γ levels were determined by commercially available ELISA kits.

IV. RESULTS

1. ANALYSIS OF THE SUSCEPTIBILITY OF DIFFERENT MOUSE STRAINS TO EXPERIMENTAL FUNGAL SEPSIS.

Besides the advances made last few years in the understanding of the molecular and cellular basis of antifungal immunity, more efforts are necessary since the incidence of IFIs still continues rising. Animal models help in our understanding of fungal pathogenesis, host immune responses, diagnosis and antifungal treatment (Capilla *et al.*, 2007; Hohl, 2014). The septic shock-like syndrome induced by zymosan, also known as zymosan-induced generalized inflammation (ZIGI), has been extensively used as a model of fungal sepsis (Genovese et al., 2004; Volman et al., 2005). Zymosan is a β-glucan-rich particle derived from *S. cerevisiae*, which induces inflammatory mediator release leading to multiple organ dysfunction syndrome (MODS). The β-glucans are conserved structural PAMPs of fungal cell walls, which are sensed by PRRs (e.g., TLR2, Dectin-1, Langerin, CD23, CR3/CD11bCD18, CD36, SCARF1, and CD5) expressed by host innate and adaptive immune cells to mount protective responses (Vera *et al.*, 2009; Levitz, 2010; Latgé, 2010; Salazar and Brown, 2018).

Inbred (homozygous) and outbred (heterozygous) mouse strains commonly used in academic and industrial research have also been used to model fungal infections (Hohl, 2014). Differences regarding disease susceptibility/severity when inbred and outbred mouse strains are challenged with the same fungal species (e.g., *P. brasiliensis* or *C. neoformans*) have been reported (Calich *et al.*, 1985; Zaragoza *et al.*, 2007; García-Barbazán *et al.*, 2016). Such differences may provide insight into the genetic and mechanistic foundations, as well as into possible therapeutic approaches to fungal infections (Capilla

et al., 2007). C57 and CD1 are two of the most common mouse strains used for academic and industrial purposes. C57 is an inbred (homozygous) strain most commonly used for developing genetically modified mice to model human diseases (e.g., the Knockout Mouse Project initiative). CD1 is an outbred (heterozygous) strain (Aldinger *et al.*, 2009) commonly used in toxicology testing (safety and efficacy) by the pharmaceutical and chemical industry. To explore possible differences regarding susceptibility to ZIGI, mice from both strains were *i.p.* injected with identical single doses of zymosan (ranging from 100 to 1,000 mg/kg). These dose-response experiments showed a trend to higher susceptibility of C57 mice to ZIGI at all zymosan tested doses, which reached statistical significance only at 700 mg/kg (p<0.05) likely due to the low size (n) of most experimental groups analysed **(Figure IV.1)**.

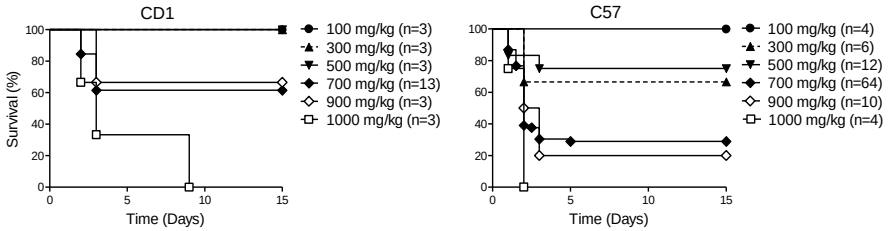

Figure IV.1 Dose-dependent survival of CD1 and C57 mice after zymosan challenge. Survival percentage of CD1 (left) and C57 mice (right) *i.p.* injected with increasing doses of zymosan (100 to 1000 mg/kg).

In light of this data and according to previous reports (Volman *et al.*, 2005; Bian *et al.*, 2012; Jia *et al.*, 2013), further comparative experiments were performed by using 700 mg/kg as the optimal zymosan dose. As illustrated by **Figure IV.2A**, under those conditions C57 mice underwent higher mortality, clinical score, and body weight lost after ZIGI.

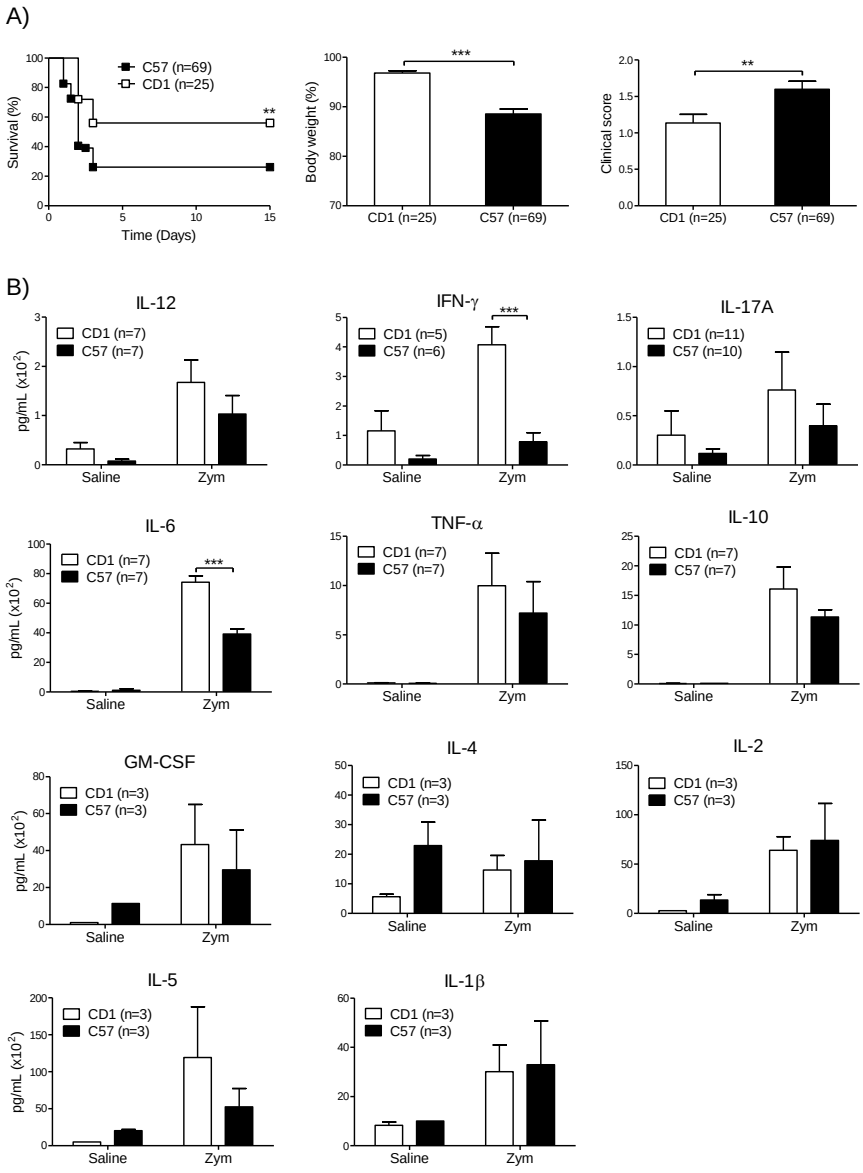

Figure IV.2 CD1 and C57 mice differ in their susceptibility to ZIGI. A) CD1 (n = 25) and C57 (n = 69) mice were *i.p.* injected with zymosan (700 mg/kg). Survival percentage (left) overtime. **, p < 0.01 (Log-rank Mantel–Cox test). Body weight percentage (middle) and clinical score (right) at 1 day post-zymosan challenge. **, p < 0.01 and ***, p < 0.001 (Unpaired t test). **B)** CD1 and C57 mice *i.p.* challenged with zymosan (700 mg/kg) or saline, and bled 18 h later for assessment of cytokine serum levels by ELISA or Multiplex. The number of mice from each strain goes from 3 to 11 depending on the cytokine. ***, p < 0.001 (Unpaired t test).

The higher ZIGI susceptibility of C57 versus CD1 mice was also evidenced by significantly lower serum levels of the pro-inflammatory IFN-γ and IL-6 cytokines at 18 h post zymosan injection **(Figure IV.2B)**. Non-significant differences were observed between the two mouse strains regarding the GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, and TNF-α serum levels.

IFN-γ is a prototypical Th1 cytokine produced by several innate and adaptive immune cells (macrophages, DCs, $CD4^+$ Th1 cells, CD8+ T cells, Tγδ cells and NK cells) and plays a key role in the immune control of fungal infections (Stevens *et al.*, 2006; Gozalbo *et al.*, 2014). Thus, IFN-γ levels were also measured in *ex vivo* cultures of C57 and CD1 splenocytes exposed to 75 μg/mL of zymosan (Zym), as determined by previous dose-response assays (data not shown). As illustrated by **Figure IV.3A (left)**, lower IFN-γ levels were detected in culture supernatants from C57 versus CD1 splenocytes. Similar results were observed when peritoneal cells were *ex vivo* exposed to the same Zym dose **(Figure IV.3A, right)**.

As IL-12 is an inducer of IFN-γ production (Trinchieri, 1995), their interdependence in response to zymosan was investigated. To this end, zymosan -stimulated splenocytes from CD1 mice were cultured for 20 h in the presence of different doses of a blocking anti-IL-12 mAb antibody or an isotype control. As shown in **Figure IV.3B**, zymosan-induced IFN-γ by CD1 splenocytes was only partly reduced by IL-12 blockade, meaning that other IL-12-independent IFN-γ-inducing factors (e.g., IL-18) could be also operating upon zymosan challenge (Fantuzzi *et al.*, 1998).

The lower *in vivo* and *ex vivo* IFN-γ responses of C57 versus CD1 mice challenged the classical view of C57 as a prototypical Th1-biased mouse strain (Watanabe *et al.*, 2004). Thus, we decided to compare zymosan-induced IFN-γ production by splenocytes from C57 and CD1 mice with that of Balb/c - a prototypical Th2-biased mouse strain. As expected, IFN-γ levels achieved by C57 splenocytes were 10-fold higher than those of Balb/c splenocytes **(Figure IV.3C)**. In turn, CD1 splenocytes produced 10-fold higher IFN-γ levels than C57 splenocytes **(Figure IV.3C).**

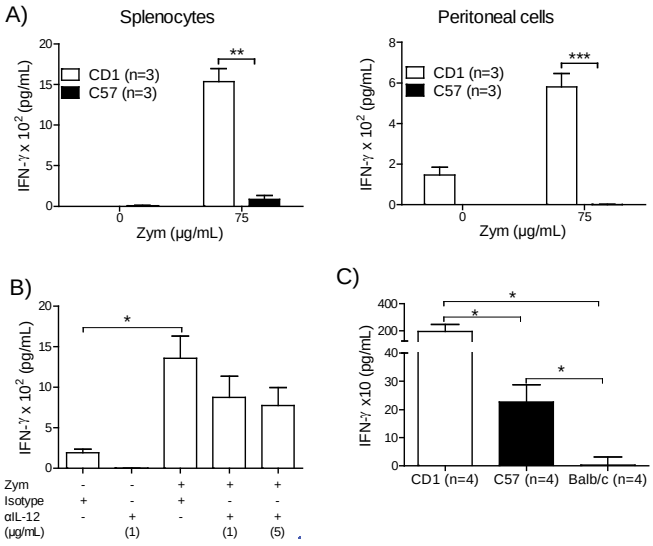

Figure IV.3 *Ex vivo* stimulated CD1 and C57 splenocytes differ in their IFN-γ response following exposure to Zym. A) IFN-γ levels measured by ELISA in supernatants from total splenocytes (5 x 10^6 cells/mL; left) and peritoneal cells (2 x 10^6 cells/mL; right) of CD1 and C57 mice exposed to Zym (75 µg/mL) for 18 h. **, $p < 0.01$ and ***, $p < 0.001$ (Mann–Whitney test). **B)** IFN-γ levels determined by ELISA in supernatants from total splenocytes (5 x 10^6 cells/mL) of CD1 mice exposed for 18 h to isotype control monoclonal antibody (5 µg/mL), blocking anti-IL-12 antibody (1 or 5 µg/mL) or Zym (75 µg/mL) either alone or in combination. *, $p< 0.05$ (Student *t* test). **C)** IFN-γ concentration in supernatants from total splenocytes (5 × 10^6 cells/mL) of CD1, C57and Balb/c mice exposed to Zym (75 µg/mL) for 18 h. *, $p < 0.05$ (Mann–Whitney test).

It was further investigated whether the reduced IFN-γ response of C57 versus CD1 splenocytes was specific to Zym by using alternative stimuli. As shown in **Figure IV.4A**, C57 splenocytes also produced lower IFN-γ levels than CD1 mice following stimulation with LTA (10 µg/mL) or LPS (1 µg/mL), two bacterial PAMPs binding to TLR2 and TLR4, respectively. In contrast, no differences were observed between C57 and CD1 splenocytes when exposed to an anti-CD3 mAb (0.1 µg/mL; **Figure IV.4B**), which excluded putative intrinsic *ifn-γ* gene expression defects.

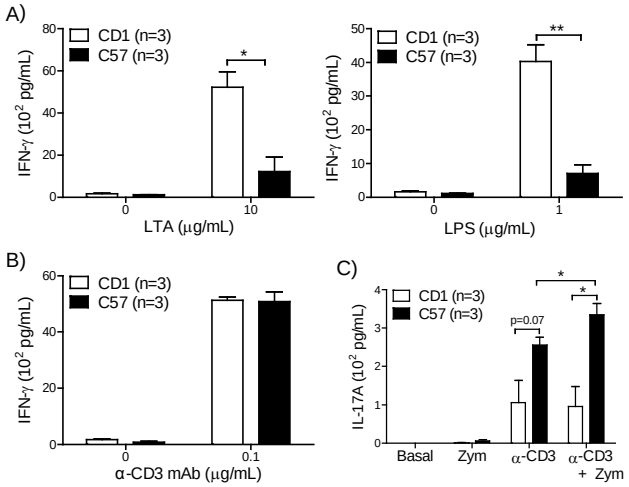

Figure IV.4. CD1 and C57 splenocytes differ in their IFN-γ and IL-17A responses following *ex vivo* exposure to different stimulatory conditions. A) IFN-γ levels in supernatants from total splenocytes (5 x 10^6cells/mL) of CD1 (n = 3) and C57 (n = 3) mice exposed to LTA (10 µg/mL) or LPS (1 µg/mL) for 18 h. **B)** IFN-γ levels in supernatants from total splenocytes (5 x 10^6 cells/mL) of CD1 (n = 3) and C57 (n = 3) mice exposed to anti-CD3 mAb (0.1 µg/mL) for 18 h. **C)** Splenocytes from C57 and CD1 mice (5 x 10^6 cells/mL) exposed for 18 h either to Zym (75 µg/mL), anti-CD3 (0.1 µg/mL) or a combination of both. IL-17A cytokine levels in supernatants were determined by ELISA *, $p < 0.05$ and **, $p < 0.01$ (Student *t* test).

In addition to IFN-γ, IL-17A has also been involved in the immune control of fungal infection, especially in epithelial and mucosal surfaces (Jin and Dong, 2013). In light of this, IL-17A production by splenocytes from both strains was assessed. As illustrated in **Figure**

IV.4C, *ex vivo* exposure of splenocytes to Zym alone (75 μg/mL) led to weak IL-17A production in both mouse strains. However, splenocytes from C57 mice produced higher IL-17A levels than CD1 mice after anti-CD3 mAb stimulation alone or in combination with Zym. This relative lower IL-17A production might be indicative of likely intrinsic defects in the *il-17a* gene expression pathway in CD1 mice.

It was further investigated whether C57 susceptibility to ZIGI could be ameliorated by IFN-γ replacement therapy. To this end, C57 mice were *i.p.* injected 1h post ZIGI-challenge with 0.2 μg/kg IFN-γ, a dose intended to allow C57 mice achieving similar serum levels in magnitude to those observed in CD1 mice **(Figure IV.2B)**. As illustrated by **Figure IV.5A (left)**, IFN-γ infusion increased C57 survival (from ≈15% to ≈40%) and clinical score **(Figure IV.5A; right)** to values paralleling those of CD1 mice at similar Zym (700 mg/kg) dose **(Figure IV.2A)**. No significant effects on mouse survival were observed when CD1 mice were infused with identical IFN-γ (0.2 μg/kg) amounts **(Figure IV.5B)**.

It was also investigated whether C57mice, either alone or in combination with IFN-γ, could benefit from the infusion of the *β*-glucan interacting CD5 protein as previously reported in CD1 mice (Vera *et al.*, 2009). As shown in **Figure IV.5A (left)**, *i.p.* injection of rshCD5 protein alone (0.7 mg/kg) 1h post ZIGI challenge did not significantly improve C57mice survival ($p < 0.06$) or clinical score with regard to the control protein (human seroalbumin; HSA). Simultaneous infusion of IFN-γ and rshCD5 showed not statistically significant additive effects regarding survival rates (≈70%) and clinical scores **(Figure IV.5A left and right, respectively)**.

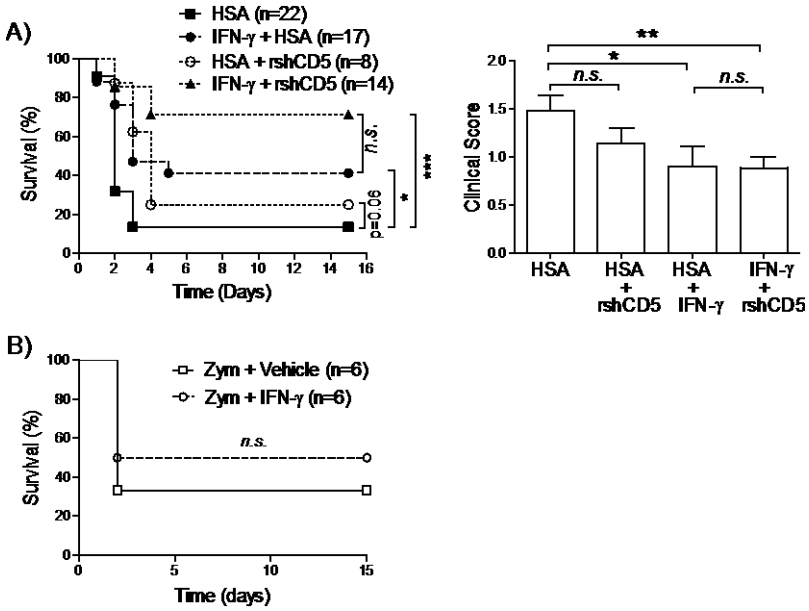

Figure IV.5 Effect of IFN-γ and/or rshCD5 infusion in ZIGI-challenged C57 and CD1 mice. A) Percentage of survival overtime and clinical score of C57 mice *i.p.* challenged with Zym (700 mg/kg) and treated 1 h later with HSA (0.7 mg/kg), IFN-γ (0.2 μg/kg) and rshCD5 (0.7 mg/kg) alone or combined. *, $p < 0.05$; ***, $p < 0.001$ (Log-rank Mantel–Cox test). **B)** CD1 mice *i.p.* challenged with Zym (700 mg/kg) and infused 1 h later with vehicle (n=6) or IFN-γ (0.2 μg/kg; n=6).

In an attempt to mimic the settings of a clinical infection, it was further analysed whether susceptibility differences of CD1 and C57 mice to ZIGI also applied to systemic fungal infection. To that end, both mouse strains were *i.v.* infected with an identical lethal inoculum of *C. albicans* (2.86 x 10^4 CFUs/gr). As shown in **Figure IV.6A**, mortality in CD1 mice was delayed in relation to C57 mice. C57 infected mice also underwent higher body weight lost **(Figure IV.6B)** compared with CD1 mice. In accordance with the ZIGI model results, CD1 mice also secreted higher IFN-γ serum levels post *C. albicans* infection compared with C57 mice **(Figure IV.6C)**.

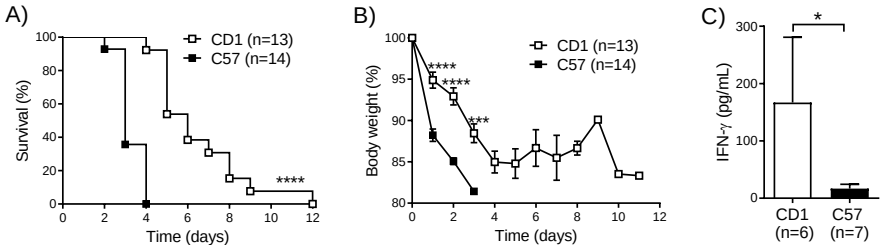

Figure IV.6 C57and CD1 mice differ in their susceptibility to fungal sepsis induced by *C. albicans*. A) Percentage of survival overtime of CD1 (n = 13) and C57(n = 14) mice *i.v.* infected with *C. albicans* (2.86 × 10^4 CFUs/gr). ***, $p < 0.001$ (Log-rank Mantel–Cox test). **B)** Body weight loss of CD1 (n = 13) and C57(n = 14) mice *i.v.* infected with *C. albicans* (2.86 × 10^4 CFUs/gr). ****, $p < 0.0001$; ***, $p < 0.001$ (Mann–Whitney test). **C)** Serum IFN-γ levels measured by ELISA from CD1 (n = 6) and C57(n = 7) mice infected with *C. albicans* (2.86 × 10^4 CFUs/gr) at day 2 post-infection. *, $p < 0.05$ (Mann–Whitney test).

This result prompted IFN-γ replacement therapy experiments in *Candida*-infected C57versus CD1 mice. As shown in **Figure IV.7A and 7B**, *i.p.* IFN-γ infusion (0.2 µg/kg) 18 h post *C. albicans* infection significantly improved survival of C57but not CD1 mice. As shown in **Figure IV.7C**, CD1 mice only benefited from IFN-γ therapy when higher IFN-γ replacement doses (10 µg/kg) were infused. No significant survival improvement of *Candida*-infected Balb/c mice treated with IFN-γ (0.2 µg/kg; **Figure IV.7D**). Contrary to what was observed in the ZIGI model **Figure IV.5A**, simultaneous infusion of IFN-γ (10 µg/kg) and rshCD5 (1.25 mg/kg) at 9 h and 18 h post *Candida* infection, respectively, did not result in additive effects in CD1 mice survival after *C. albicans* infection **(Figure IV.7E).**

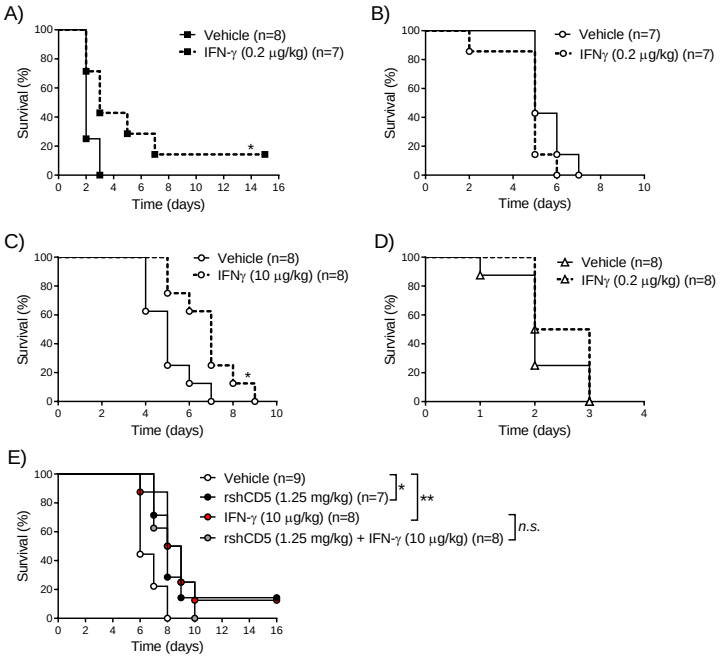

Figure IV.7. Effect of IFN-γ infusion in *C. albicans*-infected mice. A) Survival percentage of C57 mice *i.v.* infected with *C. albicans* (2.86 × 10^4 CFUs/gr) and treated with IFN-γ (0.2 μg/kg; n=7) or vehicle (*n* = 8) at day 1 post infection. **B)** Survival percentage of CD1 mice *i.v.* infected with *C. albicans* (2.86 × 10^4 CFU/gr) and treated with IFN-γ (0.2 μg/kg; n=7) or vehicle (*n* = 7) at day 1 post infection. **C)** Survival percentage of CD1 mice *i.v.* infected with *C. albicans* (2.86 × 10^4 CFUs/gr) and treated with IFN-γ (10μg/kg) or vehicle (*n* = 8) at 9h post infection (*n* = 8). **D)** Survival percentage of Balb/c mice *i.v.* infected with *C. albicans* (2.86 × 10^4 CFUs/gr) and treated with IFN-γ (0.2μg/kg) or vehicle (*n* = 8) at day 1 post infection (*n* = 8). **E)** Survival percentage of CD1 mice *i.v.* infected with *C. albicans* (2.86 × 10^4 CFUs/gr) and treated with vehicle, rshCD5 (1.25 mg/kg; at +18 h) or IFN-γ (10 μg/kg; at +9 h) alone or in combination. The differences between groups were analyzed by Log-rank (Mantel-Cox) Test (*, $p < 0.05$; **, $p < 0.01$).

In order to further understand the susceptibility differences of C57and CD1 mouse strains to fungal infection, the surface expression of some of the main leukocyte receptors involved in fungal recognition and defence was analysed. To that end, surface expression levels of Dectin-1, TLR2 and 4, CR3 (CD18/CD11b), IFNgR1 and CD5 on myeloid (granulocytes, Mϕ, DCs) and lymphoid (T and B) cell subsets from both mouse strains were compared. **Figure IV.8A** shows the gating strategy

based on the expression of $F4/80^{+}B220^{-}$ (Macrophages), $CD3^{+}B220^{-}$ (T cells), $B220^{+}CD3^{-}$ (B cells), $CD11c^{+}$ $B220^{-}$(DCs).

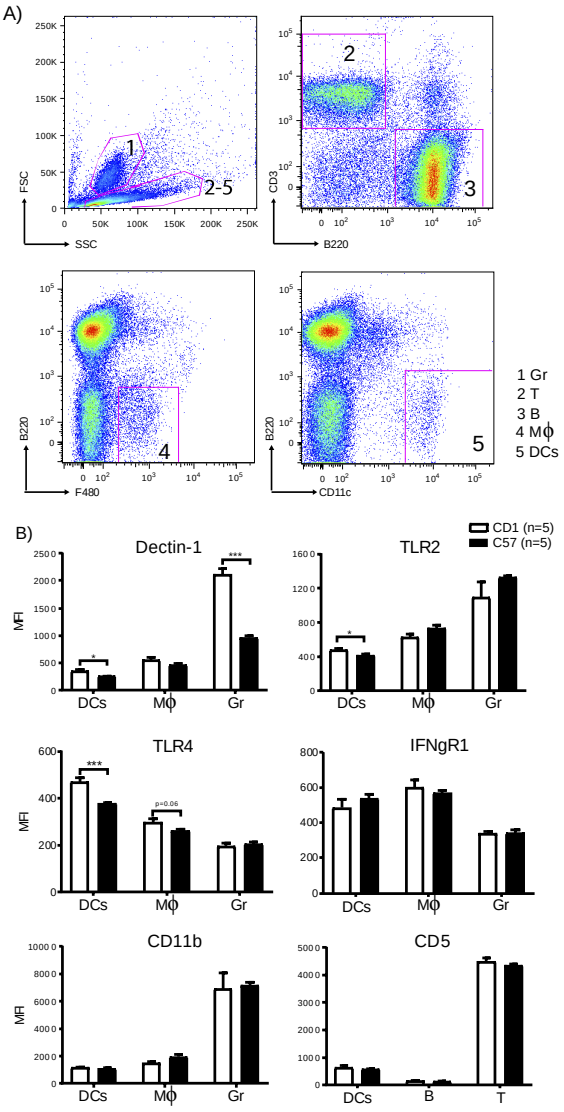

Figure IV.8. Differential expression of surface receptors between C57and CD1 mice. Splenocytes from C57and CD1 mice (n=5/group) were surface stained and analysed by flow cytometry. **A)** The gating strategy for analysing the different immune cell types studied is shown. **B)** Mean (±SEM) values of geometric mean of fluorescence intensity for the indicated surface receptors on different immune cell types. Dendritic cells, DC. Granulocytes, Gr. Macrophages, Mϕ. T, T cells. B, B cells. *, $p<0.05$ ***, $p<.001$ (Student *t*-test).

As illustrated in **Figure IV.8B**, the mean fluorescence intensity (MFI) of Dectin-1, and TLR2 and 4 was significantly higher in DCs cells from CD1 mice compared with C57 mice. A similar situation applied to Dectin-1 and TLR4 for granulocytes and Mϕ, respectively. No differences were observed with regard to surface IFNgR1, CD11b and CD5 expression. Taken together, the higher expression of certain fungal PRRs by CD1 myeloid cells agrees with higher serum IFN-γ levels and survival observed for CD1 versus C57 mice upon zymosan challenge and *C. albicans* infection.

Because the efficacy of host immunity may vary under particular fungal scenarios (Netea *et al.*, 2015), it was further investigated whether differences between CD1 and C57 mice remained steady when a less lethal *Candida*-infection model was carried out. As illustrated in **Figure IV.9A and 9B**, survival of C57 mice was unexpectedly higher than that of CD1 mice when infected with 2.86 x 10^3 and, significantly, 2.86 x 10^2 *C. albicans* CFUs/gr, thus contradicting results obtained with the 1 or 2-log higher inoculum, respectively, used in previous experiments (2.86 x 10^4 CFUs/gr). This indicates that mouse strain susceptibility to fungal infection is not absolute and may be conditioned by different factors, as here demonstrated for the inoculum magnitude.

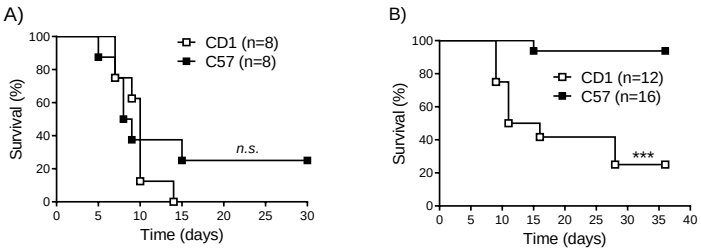

Figure IV.9 Inoculum-dependent susceptibility of C57 and CD1 mice to *C. albicans* infection. **A)** Percentage of survival overtime of CD1 (n = 8) and C57 (n = 8) mice *i.v.* infected with 2.86 × 10^3 CFUs/gr. **B)** Percentage of survival overtime of CD1 (n = 12) and C57(n = 16) mice *i.v.* infected with 2.86 × 10^2 CFUs/gr. ***, $p < 0.001$ (Log-rank (Mantel-Cox) Test).

2. ROLE OF MEMBRANE-BOUND CD5 IN SYSTEMIC FUNGAL INFECTION.

Previous studies demonstrated that membrane-bound CD5 binds to zymosan and promotes MAPK phosphorylation and IL-8 production (Vera *et al.*, 2009). On this basis, we investigated whether CD5 deficiency (*cd5*$^{-/-}$) influences mouse susceptibility to fungal infection. To this end, *cd5*$^{-/-}$ mice (available in C57 background) were subjected to a sub-lethal model of systemic (*i.v.*) *C. albicans* infection (2.86×10^3 CFU/gr). As illustrated in **Figure IV.10A**, *cd5*$^{-/-}$ mice showed significantly higher mortality **(left)** and body weight lost **(right)** than WT controls. When the effect CD5 deficiency on fungal burden was analysed, *cd5*$^{-/-}$mice showed increased number of CFUs in both kidney and spleen at 72h post-infection compared with wild-type controls **(Figure IV.10B)**. In order to ascertain whether CD5-deficiency confers increased susceptibility not only to *C. albicans* infection but also to other fungal pathogens, *cd5*$^{-/-}$ and WT C57mice were *i.n.* infected with *C. neoformans* (2.86×10^4 CFU/gr). As shown in **Figure IV.10C**, *cd5*$^{-/-}$ mice showed increased mortality and body weight lost than wild-type controls, thus supporting the generalized susceptibility to fungal infection associated with CD5-deficiency.

Furthermore, whether rshCD5 infusion to *cd5*$^{-/-}$ mice could restore the WT phenotype was next analysed. To this end, *cd5*$^{-/-}$ and WT C57 mice were *i.v.* infected with *C. albicans* (2.86×10^3 CFU/gr), and then only *cd5*$^{-/-}$ mice were *i.v.* treated with rshCD5 (1.25 mg/kg) at 18 h post-infection. As illustrated in **Figure IV.10D**, rshCD5 infusion to *cd5*$^{-/-}$ mice abolished the significant differences found between WT and untreated *cd5*$^{-/-}$ mice regarding the survival rates **(left)** and body weight lost **(right)**.

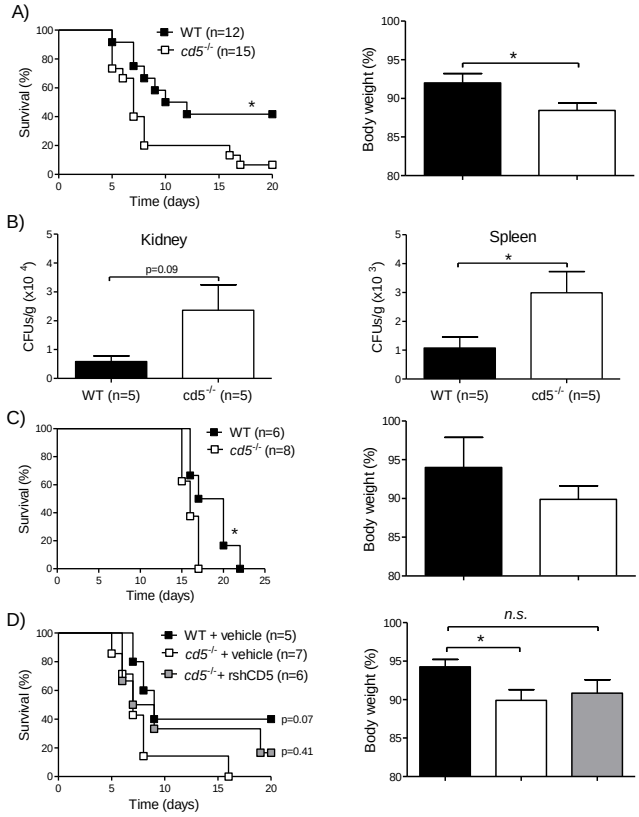

Figure IV.10 Effect of CD5 deficiency on fungal infection susceptibility. **A)** Wild-type (WT; n=12) and CD5-deficient (*cd5*$^{-/-}$; n=15) C57mice were *i.v*. infected with *C. albicans* (2.86 × 10^3 CFU/gr) and survival **(left)** and body weight lost **(right)** were monitored overtime. Body weight lost differences were analyzed at 72 h post-infection. **B)** WT (n=5) and *cd5*$^{-/-}$ (n=5) C57mice were *i.v*. infected with *C. albicans* (2.86 × 10^3 CFU/gr). Fungal burden in spleen and kidney was determined at 72 h post-infection. **C)** Survival percentage overtime **(left)** and body weight lost **(right)** of WT (n=6) and *cd5*$^{-/-}$ (n=8) C57 mice *i.n*. infected with *C. neoformans* (2.86 x 10^4 CFU/gr). Body weight lost differences were analyzed at 14 days post-infection. **D)** Survival percentage overtime **(left)** and body weight lost **(right)** of C57mice *i.v*. infected with *C. albicans* (2.86 × 10^3 CFU/gr) and treated with vehicle (WT, n=5; *cd5*$^{-/-}$, n=7) or rshCD5(*cd5*$^{-/-}$, n=6). Body weight lost differences were analyzed at 72 h post-infection. Statistical differences between treated and control groups were assessed by Log-rank (Mantel-Cox) Test or Mann Whitney test (*, $p<0.05$).

Further *ex vivo* experiments in which un-fractionated splenocytes (2 x 10^5 cells/well) from *cd5*$^{-/-}$ and WT C57mice were co-cultured for 24 h with heat-killed *C. albicans* or *C. neoformans* (1 x 10^5

CFUs/well) were carried out. Although not reaching statistical significance, lower IFN-γ, TNF-α, IL-12 and IL-6 levels were found in *C. albicans* co-cultures supernatants from *cd5*$^{-/-}$ versus WT C57mice **(Figure IV.11)**. In the case of heat-killed *C. neoformans* co-cultures, splenocytes from both *cd5*$^{-/-}$ and WT C57mice secreted low or undetectable cytokine levels **(Figure IV.11)**.

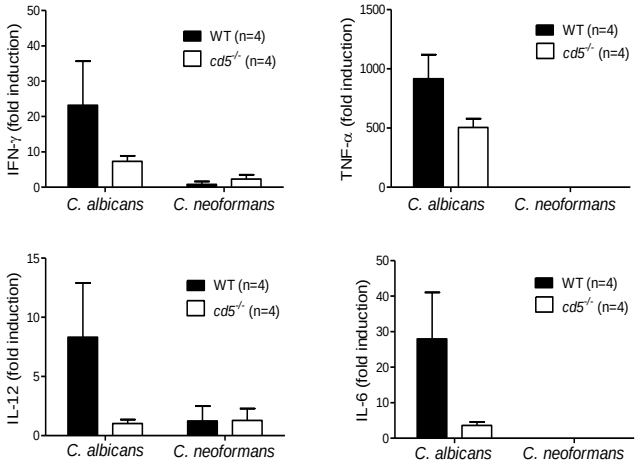

Figure IV.11 ***Ex vivo*** **exposure of** ***cd5*****$^{-/-}$ and WT C57splenocytes to** ***C. albicans*** **and** ***C. neoformans*****. A)**IFN-γ, TNF-α, IL-12 and IL-6 cytokine levels measured by ELISA in supernatants from total splenocytes (2 x 10^{5}cells/well) from *cd5*$^{-/-}$(n=4) and WT (n=4) C57mice exposed to heat killed *C. albicans* or *C. neoformans* (1 x 10^5 CFUs/well) for 24 h. Results are represented as fold induction with respect to unstimulated cells.

As previously mentioned, the main immune cell subset expressing CD5 is T cells in which this receptor displays down-modulatory effects during T cell activation (Burgueño-Bucio *et al.*, 2019). Thus, it was further investigated whether fungal exposure differentially influenced activation and/or apoptosis events in wild-type versus CD5-deficient T cells. To this end, splenocytes (2 x 10^5 cells/well) from *cd5*$^{-/-}$ and WT mice were exposed to heat-killed *C. albicans* or *C. neoformans* (1 x 10^5 CFUs/well) during 24 h for further flow cytometry analysis of cell surface activation and death markers

expression. As illustrated by **Figure IV.12A**, the percentage of $CD4^+$ and $CD8^+$ T cells from both *cd5*$^{-/-}$ and WT mice significantly decreased after exposure to *C. albicans* or *C. neoformans*, though no differences between the two experimental groups (*cd5*$^{-/-}$ and WT) were observed. Regarding CD69 expression, exposure of *cd5*$^{-/-}$ and WT splenocytes to *C. albicans* but not *C. neoformans* significantly increased the percentage of both $CD69^+CD4^+$ and $CD69^+CD8^+$ T cells, though no differences between the two experimental groups were observed **(Figure IV.12B)**.

In the case of CD25 expression, no differences in the percentage of $CD25^+CD4^+$ or $CD25^+CD8^+$ T cells were observed in the presence or absence of *C. albicans* or *C. neoformans* for both *cd5*$^{-/-}$ and WT mice **(Figure IV.12C)**. Finally, the percentage of IFN-γ^+CD4^+ T cells from both *cd5*$^{-/-}$ and WT mice similarly and significantly increased following exposure to *C. albicans* but not *C. neoformans* **(Figure IV.12D)**, a fact that could not be observed for IFN-γ^+CD8^+ T cells.

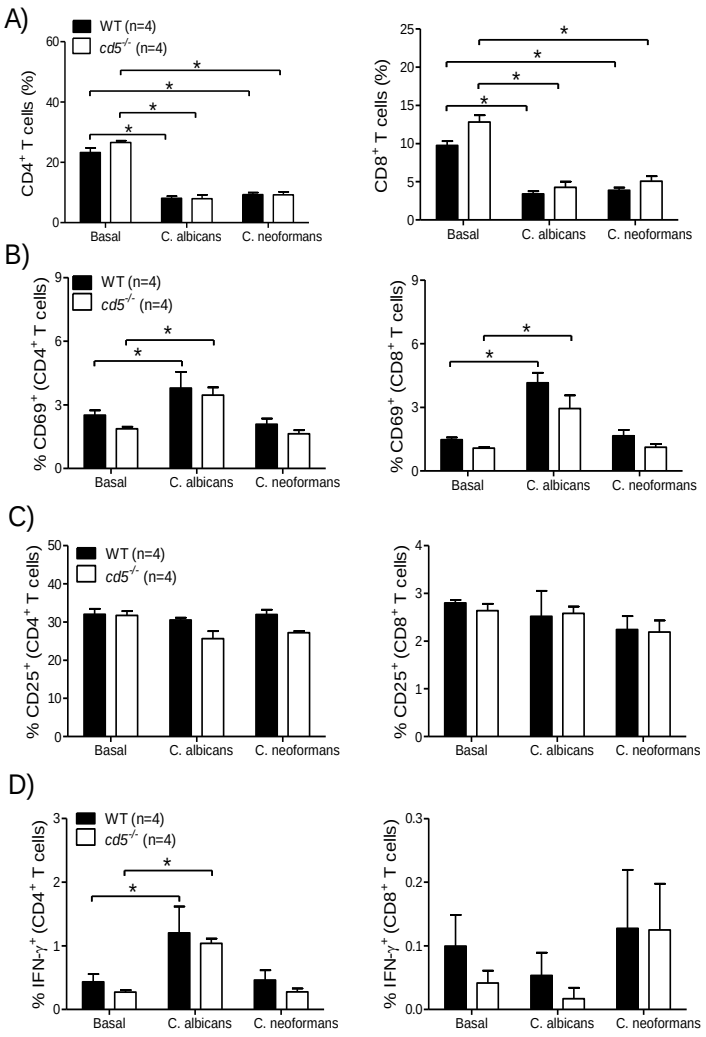

Figure IV.12 Expression of T cell activation markers in *cd5*$^{-/-}$ and WT C57splenocytes following *ex vivo* exposure to *C. albicans* or *C. neoformans*. Total splenocytes (2 x 10^5 cells/well) from *cd5*$^{-/-}$ (n=4) and WT (n=4) C57mice exposed to heat killed *C. albicans* or *C. neoformans* (1 x 10^5 CFUs/well) for 24 h. Activation markers expression was analysed by flow cytometry. **A)** Percentage of $CD4^+$ and $CD8^+$ T cells. **B)** Percentage of $CD69^+$ T cells from $CD4^+$ and $CD8^+$ gated T cells. **C)** Percentage of $CD25^+$ T cells from $CD4^+$ and $CD8^+$ gated T cells. **D)** Percentage of IFN-γ^+ T cells from $CD4^+$ and $CD8^+$ gated T cells. Statistical differences between groups were analyzed by Mann Whitney test (*, $p<0.05$).

The analysis of the activation and apoptosis-inducing PD1 cell surface marker revealed no significant differences between *cd5*$^{-/-}$ and

WT mice regarding percentage of PD1⁺ cells in CD4⁺ or CD8⁺ gated T cells **(Figure IV.13A)**. By contrast, CD4⁺ and CD8⁺ T cells from *cd5*$^{-/-}$ splenocytes showed higher induction of early apoptosis than their WT counterparts when exposed to *C. albicans* and *C. neoformans*, which reached statistical significance only for the latter **(Figure IV. 13B)**. Regarding the analysis of late apoptosis, again a trend to higher induction was observed for CD4⁺ and CD8+ T cells from *cd5*$^{-/-}$ mice compared with WT splenocytes **(Figure IV. 13C)**.

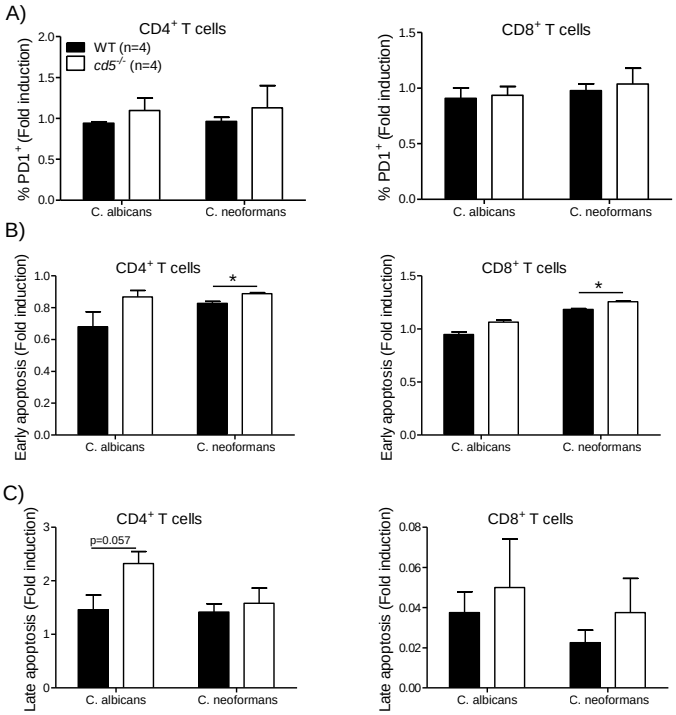

Figure IV.13 Expression of apoptosis markers in *cd5*$^{-/-}$ and WT C57splenocytes following *ex vivo* exposure to *C. albicans* or *C. neoformans.* Cell death markers expression was analysed by flow cytometry. **A)** Percentage of PD1⁺ cells in CD4⁺ and CD8⁺ gated T cells. **B)** Percentage of early apoptosis (annexin V⁺) in CD4⁺ and CD8⁺ gated T cells. **C)** Percentage of late apoptosis (annexin V⁺ 7AAD⁺) in CD4⁺ and CD8⁺ gated T cells. Results are represented as fold induction with respect to unstimulated cells. Statistical differences between groups were analyzed by Mann Whitney test (*, $p<0.05$).

Taken together, the results from *cd5*$^{-/-}$ mice indicate that CD5-deficiency makes T cells more prone to apoptosis when exposed to fungal cells, thus resulting in lower cytokine production and further *in vivo* survival.

3. EFFECT OF SOLUBLE HUMAN CD5 ADMINISTRATION IN SYSTEMIC FUNGAL INFECTION INDUCED BY *C. albicans*.

Previous results from our group showed a beneficial effect of rshCD5 administration in ZIGI-challenged CD1 mice (Vera *et al.*, 2009). Based on this fact and data presented in the above Result's section, we decided to get closer to the clinics by exploring the therapeutic use of rshCD5 in a systemic fungal infection model induced by *C. albicans*. To that end, we first infected CD1 mice via *i.v.* with three different inoculums (2.86 x 10^4, 2.86 x 10^3 or 2.86 x 10^2 CFU/gr) of *C. albicans* according to previous reports for other mouse strains (Gow *et al.*, 2000). This resulted in dose-dependent decreases in CD1 mouse survival **(Figure IV.14A, left)** and body weight lost **(Figure IV.14A, right)**.

On this basis, 2.86 x 10^3 CFUs/gr was chosen for further experiments as the minimal inoculum causing ≥ 90% mortality by~10-15 days post challenge. As illustrated by **Figure IV.14B**, *i.v.* administration of a single rshCD5 dose (1.25 mg/kg) rendered the highest improvement on CD1 mouse survival (24.14 %; **left graph**) and body weight lost (**right graph**) when therapeutically given at 18 h post *C. albicans* infection (2.86 x 10^3 CFUs/gr). Parallel dose-dependent experiments showed that *i.v.* administration of a single rshCD5 dose (1.25 mg/kg) at 18 h post *C. albicans* infection (2.86 x 10^3 CFUs/gr) rendered the highest improvements on survival rate (33%) and body

weight lost **(Figure IV.14C)**. Doubling or reducing to the half such rshCD5 dose resulted in less efficacy **(Figure IV.14C)**.

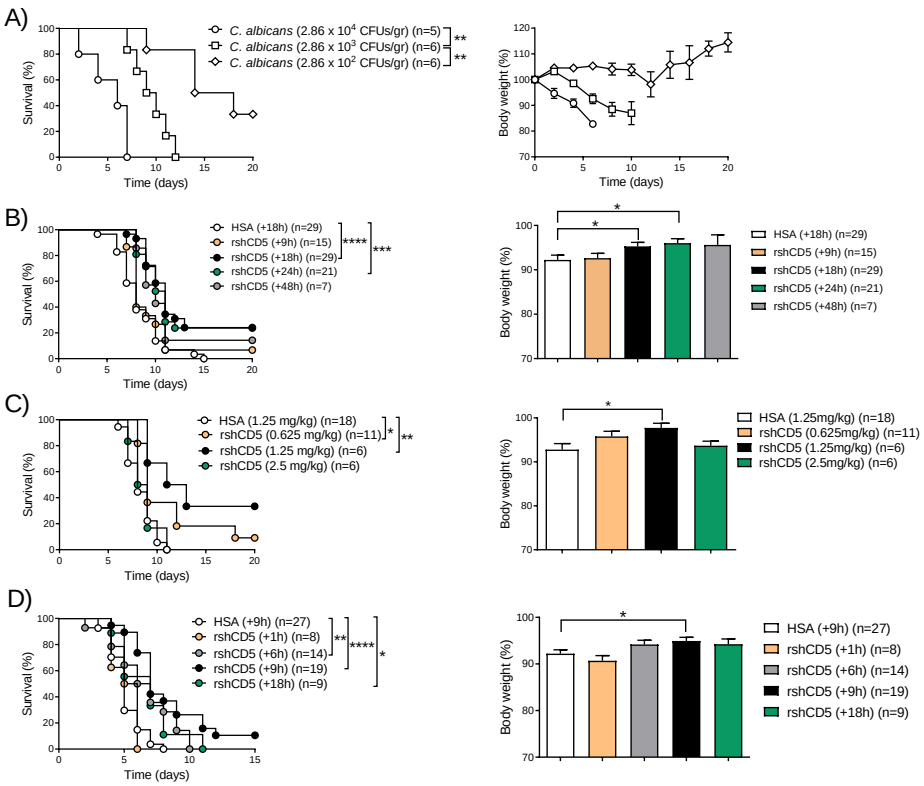

Figure IV.14. **Time- and dose-dependent effects of rshCD5 infusion in *C. albicans*-infected CD1 mice. A)** Survival percentage and body weight lost overtime of CD1 mice *i.v.* infected with *C. albicans* (2.86 x 10^4 – 2.86 x 10^3 CFUs/gr). **B)** Survival percentage and body weight lost overtime of CD1 mice *i.v.* infected with *C. albicans* (2.86 x 10^3 CFUs/gr) and treated with 1.25 mg/kg of HSA (+18h, n=29) or rshCD5 (+9 h, n=15; +18 h, n=29; +24 h, n=21; +48 h, n=7). **C)** Survival percentage and body weight loss overtime of CD1 mice *i.v.* infected with *C. albicans* (2.86 x 10^3 CFUs/gr) and treated via *i.v.* at +18 h post-infection with HSA (1.25 mg/kg, n=18) or rshCD5 (0.625 mg/kg, n=11; 1.25 mg/kg, n=6; 2.5 mg/kg, n=6). **D)** Survival percentage and body weight lost overtime of CD1 mice *i.v.* infected with *C. albicans* (2.86 x 10^4 CFUs/gr) and treated via *i.v.* with 1.25 mg/kg of HSA or rshCD5 at different times post-infection (+1 h, +6 h, +9 h, +18 h). The differences between groups were analyzed by Log-rank (Mantel-Cox) Test or Mann-Whitney test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

The beneficial effect of rshCD5 administration was maintained when a more lethal *C. albicans* infection model was carried out (2.86 x 10^4 CFUs/gr). However, in this case the optimal survival rate and lower body weight lost was observed when a single rshCD5 dose (1.25 mg/kg) was administered at an earlier time point that is 9 h instead of 18-24 h post-challenge (**Figure IV.14D**).

To further analyze the effect on fungal burden, spleen and kidney from CD1 mice infected with *C. albicans* (2.86 x 10^3 CFU/gr) and treated 18 h later with a single-dose of rshCD5 (1.25 mg/kg) were analyzed at different time points post-infection. As shown in **Figure IV.15**, rshCD5 infusion promoted a significant reduction in the number of CFUs in both spleen and kidney at 72 h post-infection compared with the control HSA-treated group. Furthermore, while fungal burden increases overtime in the control group, rshCD5 administration promotes a decrease in spleen.

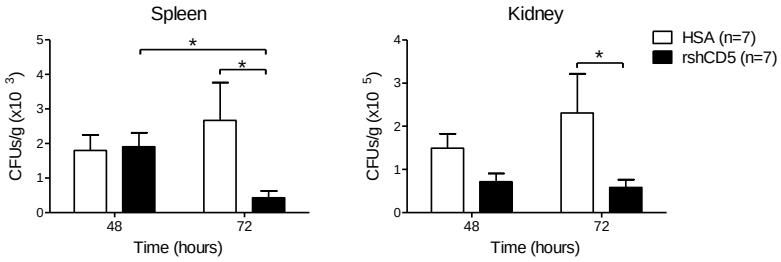

Figure IV.15 Effect of rshCD5 infusion on fungal burden in *C. albicans*-infected CD1 mice. CD1 mice were infected via *i.v.* with *C. albicans* (2.86 x 10^3 CFU/gr) and treated via *i.v.* with 1.25 mg/kg of HSA (n=7) or rshCD5 (n=7) at 18h post-infection. Fungal burden in spleen (left) and kidney (right) was determined at 48h and 72h post-infection. Tissue homogenates were seeded on Sabouraud dextrose agar plates, and incubated 48 h at 30 °C for further CFUs count. The differences between groups were analyzed by Mann Whitney test. *, $p<0.05$.

In parallel experiments, rshCD5-treated CD1 mice (1.25 mg/kg, at + 18 h) were euthanized 72 h after *C. albicans* infection (2.86 x 10^3 CFU/gr) for cytokine and spleen leukocyte infiltration levels analysis.

Figure IV.16A shows no significant differences in serum between the experimental groups for any of the pro- (IFN-γ, TNF-α, IL-6, and IL-1β) and anti- (IL-10) inflammatory cytokines analysed. Spleen analyses neither revealed significant differences for any of the lymphoid (T cells ($CD3^+B220^-$), B cells ($B220^+CD3^-$) and NK cells ($NK1.1^+CD3^-$)) and myeloid (macrophages ($F4/80^+B220^-$), cDCs ($CD11c^+B220^-$) and granulocytes) subpopulations analysed **(Figure IV. 16B)**.

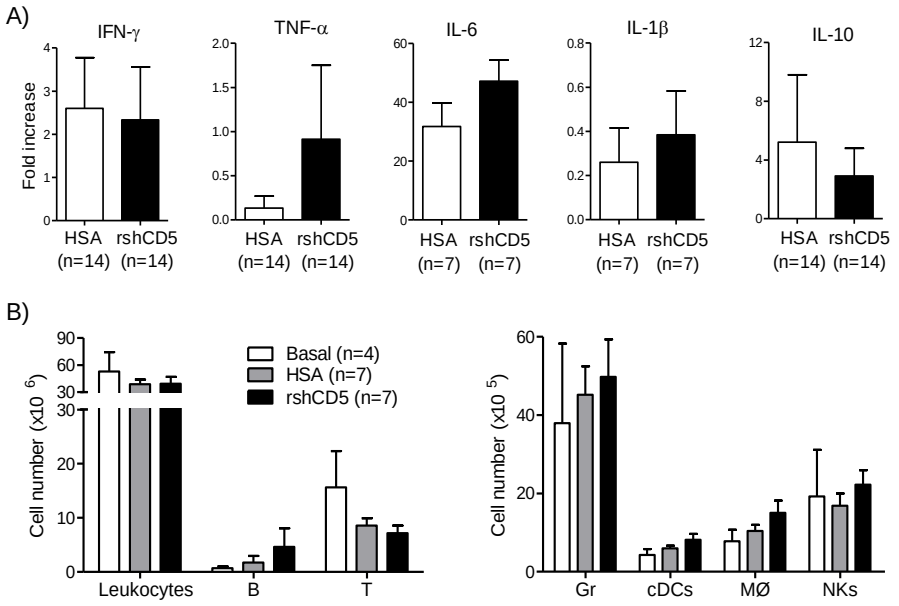

Figure IV.16. Effect of rshCD5 infusion on serum cytokine levels and spleen leukocyte infiltration from *C. albicans*-infected CD1 mice. CD1 mice were infected via *i.v.* with *C. albicans* (2.86×10^3 CFU/gr) and treated via *i.v.* with 1.25 mg/kg of HSA or rshCD5 at +18h post-infection. **A)** Serum cytokine levels determined by ELISA at 72 h post-infection represented as fold induction with respect to basal (non-infected mice). **B)** Total number of leukocytes ($CD45^+$ cells), T and B cells (left) and granulocytes, cDCs, macrophages and NKs and (right) determined in spleen by flow cytometry.

Similar analyses were performed at the local level in kidney. Again, not-significant differences regarding kidney cytokine levels were observed **(Figure IV.17A)**. However, the rshCD5-treated group revealed

significantly increased total kidney leukocyte infiltration with regard to the controls (HSA-treated mice), which involved most of the lymphoid (B and NK) and myeloid (Gr, cDCs, and Mϕ) cell types analyzed, with the exception of T cells **(Figure IV.17B)**.

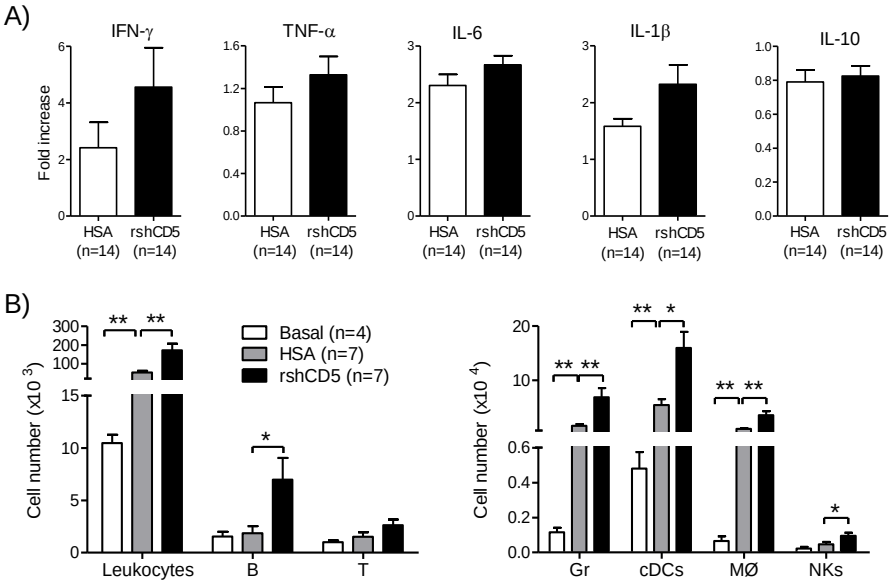

Figure IV.17. Effect of rshCD5 infusion on kidney cytokine and leukocyte infiltration levels of *C. albicans*-infected CD1 mice. CD1 mice were infected via *i.v.* with *C. albicans* (2.86 x 10^3 CFUs/gr) and treated via *i.v.* with 1.25 mg/kg of HSA or rshCD5 at +18 h post-infection. **A)** Kidney cytokine levels determined by ELISA at 72 h post-infection represented as fold induction with respect to basal (non-infected mice). **B)** Total number of kidney leukocytes ($CD45^+$ cells), B cells, T cells (left), granulocytes, dendritic cells (cDCs), macrophages and NKs (right) determined by flow cytometry. The differences between treated and control groups were analysed by Mann Whitney test. *, $p<0.05$; **, $p<0.01$).

The *ex vivo* analysis of the rshCD5 effects was further performed by exposing splenocytes (5 x 10^6 cells/mL) from CD1 mice to heat-killed *C. albicans* (0.5 x 10^6 CFUs/mL) in the presence or absence of rshCD5 (1 to 10 μg/mL). Pro- (IFN-γ, TNF-α and IL-1β) and anti- (IL-10) inflammatory cytokine levels were assessed in 24 h-culture supernatants. As illustrated by **Figure IV. 18A**, *C. albicans* alone did not

induce significant cytokine production by CD1 splenocytes with regard to the negative control (un-stimulated splenocytes). However, the presence of rshCD5 increased cytokine secretion in a dose-dependent manner, reaching statistical significance only for IFN-γ and TNF-α at the highest rshCD5 dose used (10 μg/mL).

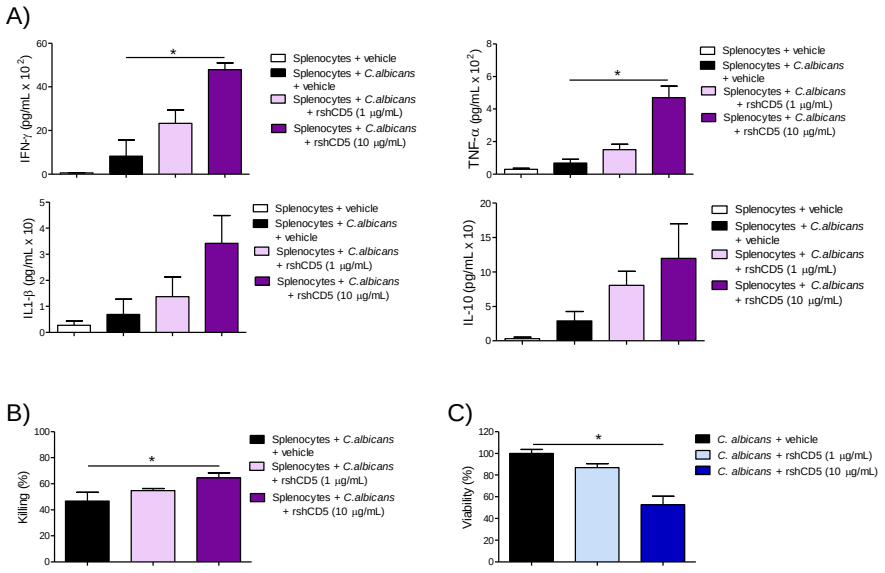

Figure IV.18. *Ex vivo* effect of rshCD5 on CD1 splenocytes exposed to either heat-killed or alive *C. albicans*. A) IFN-γ, TNF-α, IL-1β and IL-10 levels determined by ELISA in 24 h-cultured supernatants from total CD1 splenocytes (5 x 10^6 cells/mL; n = 4) exposed to heat-killed *C. albicans* (0.5 x 10^6 CFUs/mL). **B)** Killed *C. albicans* percentage following 2 h co-incubation of total CD1 splenocytes (1 x 10^6 cells/mL; n=5) with alive *C. albicans* conidia (0.5 x 10^6 CFUs/mL) in the presence of vehicle or rshCD5 (1 to 10 μg/mL). **C)** Viable *C. albicans* percentage after 2 h-exposure of alive *C. albicans* conidia (0.5 x 10^6 CFUs/mL) to vehicle or rshCD5 (1 to 10 μg/mL). In both B and C, culture supernatants were seeded on Sabouraud dextrose agar plates and incubated 48 h at 30 °C for further CFUs count. Differences between groups were analyzed by Mann Whitney test (*, $p<0.05$).

Fungal killing is a well established mechanism for pathogen clearance following its recognition by PRRs (Salazar and Brown, 2018). As shown in **Figure IV. 18B**, 2 h-co-culture of alive *C. albicans* conidia with CD1 splenocytes (1 x 10^6 cells/mL) in the presence of vehicle

resulted in ≈47% *Candida* killing. A dose-dependent increase in the percentage of *Candida* killing was observed in the presence of rshCD5, which reached statistical significance at the highest dose used (10 μg/mL; from ≈47% to ≈64%). Next it was analysed whether the soluble protein has direct microbial cytotoxic/cytostatic activity as it has been reported for other PRRs (Ohnishi *et al.*, 2010; Martínez-Florensa *et al.*, 2014). As illustrated by **Figure IV. 18C**, 2 h-culture of alive *C. albicans* conidia (0.5 x 10^6 CFUs/mL) in the presence of rshCD5 resulted in dose-dependent reduction of *Candida* viability, which reached statistical significance at the highest dose used (10 μg/mL; 52% versus 100% vehicle control). Taken together, the results indicate that rshCD5 has direct killing activity on *C. albicans*, which is additive to the direct killing activity of splenocytes.

To further assess whether the *in vivo* rshCD5 effects on fungal infection were direct or immune cell-mediated, we tested its efficacy on *C. albicans*-infected immunodeficient NSG (NOD scid gamma) mice, which lack mature T, B, and NK cells together with defective DC and Mϕ function. To that end, we first carried out a lethality curve by infecting NSG mice with three different inoculums of *C. albicans* via *i.v.* (2.86 x 10^4, 2.86 x 10^3 or 2.86 x 10^2 CFU/gr) **(Figure IV.19A)**. Based on these results, we decided to infect NSG mice with the dose (2.86 x 10^2 CFU/gr) most closely reproducing the lethality obtained in immunocompetent CD1 mice (100% lethality between 8 to 12 days after *Candida* challenge). As illustrated by **Figure IV.19B**, rshCD5-treatment (1.25mg/kg) 18 h after *Candida* infection did not induce significant survival improvement of NSG mice.

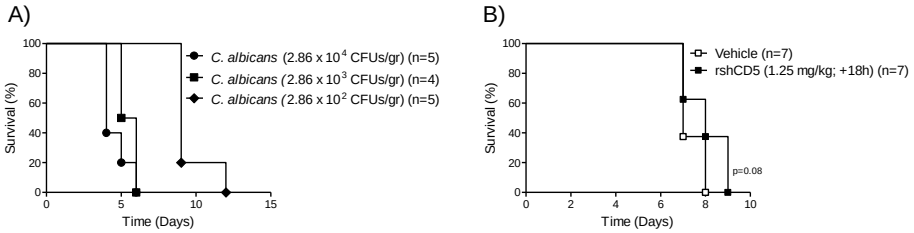

Figure IV.19. Effect of rshCD5 infusion in *C. albicans*-infected immunodeficient mice. A) Survival percentage overtime of NSG mice *i.v.* infected with *C. albicans* (2.86 x 10^4– 2.86 x 10^3 CFUs/gr). **B)** Survival percentage overtime of NSG mice *i.v.* infected with *C. albicans* (2.86 x 10^2) and treated +18 h later with vehicle (PBS + glycerol 10%; n=7) or rshCD5 (1.25 mg/kg; n=7). Statistical differences between treated and control groups were analyzed by Log-rank (Mantel-Cox) Test.

As it has been mentioned earlier, azoles, echinocandins and polyenes are the first-line treatments for the management of fungal infections. Azoles, such as fluconazole, are cytochrome demethylase system inhibitors, which ultimately promote fungal cell death (Nami *et al.*, 2019). As the mechanism of action of these compounds did not interfere with β-glucans biosynthesis, we hypothesized that benefit effects could result from fluconazole and rshCD5 combined therapy. Based on previous reports (MacCallum and Odds, 2004) and our own fluconazole dose-response assays **(Figure IV.20A)**, we treated *C.* albicans-infected CD1 mice (2.86 x 10^3 CFUs/gr, *i.v.*) with fluconazole (FLC, 1 mg/kg) alone or in combination with rshCD5 (1.25 mg/kg). The FLC treatment started at 48 h post-infection (as at this time point all mice showed body weight lost) and was *i.p.* administered daily for a period of 7 days, while rshCD5 was administered as a single *i.v.* dose at 18 h post infection.

The results depicted in **Figure IV.20B** show additive effects of FLC plus rshCD5 therapy on mouse survival (from 40% for FLC to 80% for FLC+rshCD5), which did not reach statistical significance likely due to the low size (n) of the experimental groups. Whatever the case, these

results deserve further exploration since they open the possibility of using combined therapies, which wouldd allow reducing side effects associated to high fluconazol dosage while maintaining antifungal efficacy.

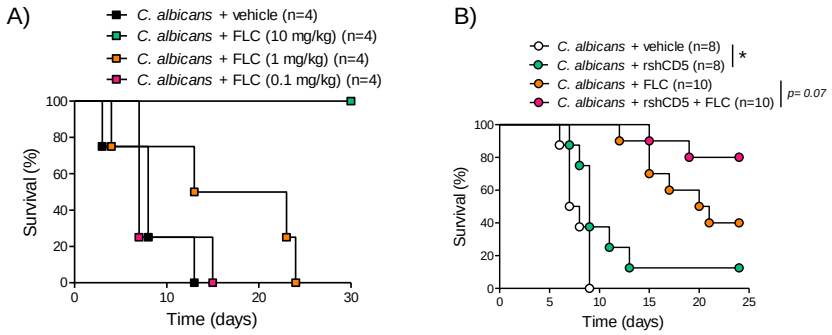

Figure IV.20. **Effect of rshCD5 plus fluconazole combined therapy on systemic *C. albicans*-infection in CD1 mice. A)** Survival percentage of CD1 mice *i.v.* infected with *C. albicans* (2.86×10^4) and *i.p.* treated for a period of 7 days with vehicle (n=4), fluconazole (10 mg/kg, n=4; 1 mg/kg, n=4; 0.1 mg/kg, n=4) starting at 48 h post infection. **B)** Survival percentage overtime of CD1 mice *i.v.* infected with *C. albicans* (2.86×10^4) and treated with vehicle (n=8), fluconazole (FLC; 1 mg/kg, n=10), rshCD5 (1.25 mg/kg; n=8), or a combination of the last two (rshCD5 + FLC; n=10). Statistical differences between treated and control groups were analysed by Log-rank (Mantel-Cox) Test (*, $p<0.05$).

4. EFFECT OF SOLUBLE HUMAN CD5 ADMINISTRATION IN SYSTEMIC FUNGAL INFECTION INDUCED BY *C. neoformans*.

Whether the beneficial effect of rshCD5 administration in *C. albicans* infection could be extended to other fungal infection models was next investigated. To this end, we carried out a previously reported mouse model of cryptococcosis (Zaragoza *et al.*, 2007; García-Barbazán *et al.*, 2016), induced by intranasal (*i.n.*) inoculation of *C. neoformans* (2.86×10^4 CFU/gr) to CD1 mice. As illustrated by **Figure IV.21A**, time-course experiments showed that *i.v.* infusion of a single-dose of rshCD5 (1.25 mg/kg) increased mice survival at all the time points tested,

reaching statistical significance at day 3 (63.2 %) and 6 (40%) post-infection.

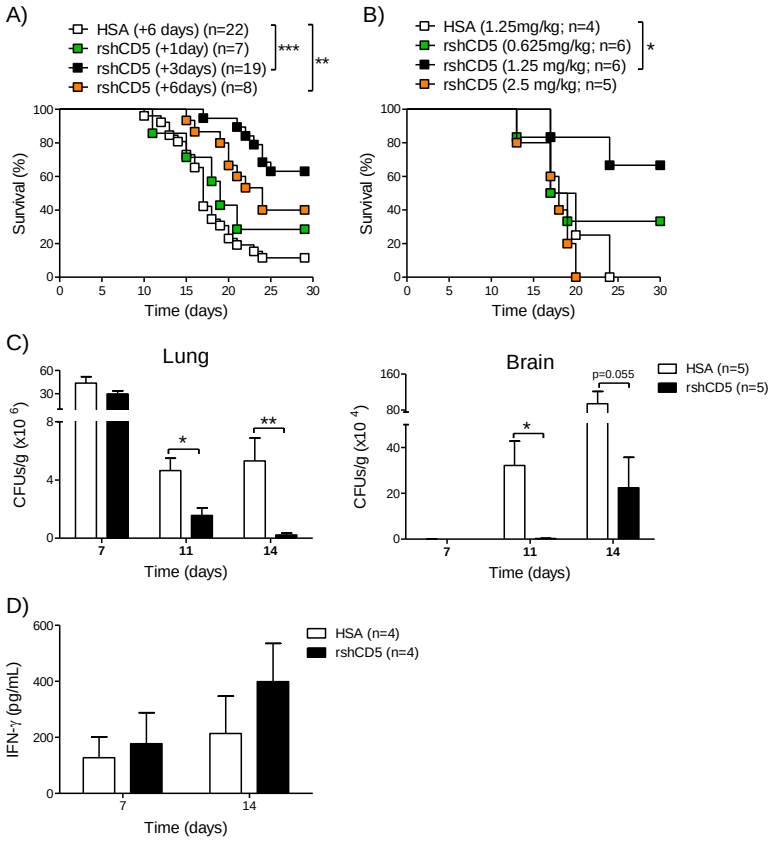

Figure IV.21 Time- and dose-dependent effects of rshCD5 infusion in a mouse model of infection by *C. neoformans*. A) Survival percentage of CD1 mice *i.n.* infected with *C. neoformans* (2.86 x 10^4 CFU/gr) and treated via *i.v.* with 1.25mg/kg of HSA (+6 days, n=22) or rshCD5 (+1 day, n=7; +3 days, n=19; +6 days, n=8). **B)** Survival percentage overtime of CD1 mice *i.n.* infected with *C. neoformans* (2.86 x 10^4 CFU/gr) and treated via *i.v.* at +3 days post-infection with HSA (1.25 mg/kg, n=4) or rshCD5 (0.625 mg/kg, n=6; 1.25 mg/kg, n=6; 2.5 mg/kg, n=5). **C)** CD1 mice were infected via *i.n.* with *C. neoformans* (2.86 x 10^4 CFU/gr) and treated via *i.v.* with 1.25 mg/kg of HSA (n=5) or rshCD5 (n=5) at +3 days post-infection. Fungal burden determined in lung (left) and brain (right) at days +7, +11 and + 14 post-infection. **D)** Serum IFN-γlevels determined by ELISA at days +7and +14post-infection of CD1 mice *i.n.* infected with *C. neoformans* (2.86 x 10^4 CFU/gr) and treated via *i.v.* at +3 days post-infection with HSA (1.25 mg/kg, n=4) or rshCD5 (1.25 mg/kg, n=4). The differences between the treatment groups compared to the control group were analyzed by Log-rank (Mantel-Cox) Test or Mann Whitney test (*, p<0.05; **, p<0.01; ***, p<0.001).

Next, dose-course assays performed at day 3 post-infection showed maximal survival rates (66.67%) when a single 1.25 mg/kg rshCD5 dose was administered **(Figure IV.21B).** Lower and non-statistically significant survival rates were observed by doubling or reducing to the half such arshCD5 dose **(Figure IV.21B).**

Under these optimal experimental conditions (1.25mg/kg rshCD5 administration at day 3 post-infection), significant reductions in the number of CFUs in lung and brain - the main two organs targeted by *C. neoformans* - were observed at days 11 and 14 after infection **(Figure IV.21C).** Since resistance to *C. neoformans* infection is associated with effective Th1 responses (Zaragoza *et al.*, 2007), we monitored the serum levels of IFN-γ - the prototypical Th1 cytokine - in HSA- and rshCD5-treated mice. Although differences did not reach statistical significance, higher serum IFN-γ levels were observed at days 7 and 14 post-infection in rshCD5-treated mice compared to HSA-treated controls (**Figure IV.21D**).

The direct or indirect effects of rshCD5 on *C. neoformans* viability were next investigated *ex vivo*. To this end, we first co-cultured for 2 h un-fractionated CD1 splenocytes (1 x 10^6 cells/mL) with alive *C. neoformans* (0.5 x 10^6 CFU/mL) in the presence or absence rshCD5 (1-10 µg/mL). As shown by **Figure IV.22A**, the killing activity of CD1 splenocytes increased in the presence or rshCD5 in a dose-dependent manner.

On the contrary, the 2h co-culture of alive *C. neoformans* (0.5 x 10^6 CFU/mL) in the presence or absence increasing rshCD5 doses (1-10 µg/mL) had no effect on fungal cell viability **(Figure IV.22B)**. This

indicates that rshCD5 has not significant direct killing activity on *C. neoformans* but potentiate that of splenocytes.

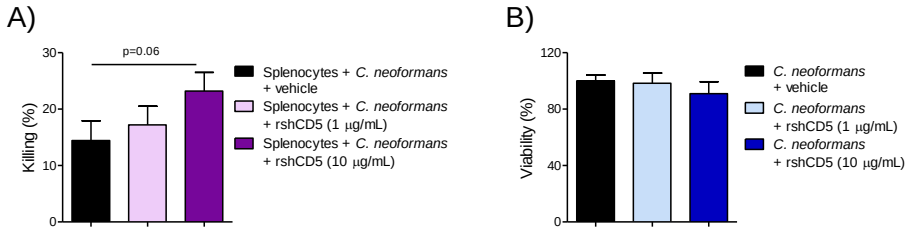

Figure IV.22 *Ex vivo* cryptococcicidal activity of rshCD5. A) Percent of *Cryptococcus* killing following 2 h co-incubation of total splenocytes (1 x 10^6 cells/mL) from CD1 mice (n=5) with alive *C. neoformans* (0.5 x 10^6 CFUs/mL) in the presence of vehicle or rshCD5 (1 to 10 μg/mL). **B)** Percent of viable *C. neoformans* after 2 h co-incubation with vehicle or rshCD5 (1-10 mg/mL). Culture supernatants were seeded on Sabouraud dextrose agar plates and incubated during 48 h at 30 ºC for CFUs count. Statistical differences between groups were analysed by student *t*-test.

5. DEVELOPMENT OF CD5-BASED ADOPTIVE T/NK CELL THERAPIES FOR SYSTEMIC FUNGAL INFECTION

With the aim of developing an alternative CD5-based therapeutic strategy against fungal infections, we took advantage of the β-glucan-binding properties of the CD5 receptor to generate T and NK cells expressing an activating CD5 chimerical receptor (CD5CAR) for further adoptive cell transfer therapeutic purposes. To this end, a second generation CD5CAR construction was designed composed of the CD8α signal peptide, the whole human CD5 extracellular ectodomain (from Arg25 to Asp345), the CD8α transmembrane region and the cytoplasmic activating domains of 4-1BB/CD137 and CD3ζ receptors (**Figure II.1B**). The CD5-CAR construct was cloned into a modified version of the third generation lentiviral vector pCCL as a *Mlu*I-*Bsp*EI fragment under the transcriptional control of the EF-1α promoter (**Annex I**). Whole lentiviral particles from packaging HEK 293T cells were first used to

transduce previously activated primary human T cells, which were then subjected to different *in vitro* experimental procedures schematically shown in **Figure II.2**.

Transduced CD5CAR-T cells were first co-cultured for 4h with alive *C. albicans* at different effector:target (E:T) ratios. As illustrated by **Figure IV.23A,** CD5CAR-T cells significantly reduced the number of viable CFUs at all the E:T ratios tested. Moreover, the number of viable CFUs was lower for CD5CAR-T cells compared to un-transduced T cells, reaching statistical significance only at 10:1 E:T ratios. Furthermore, the surface expression analysis of LAMP-1/CD107a (a degranulation marker on cytotoxic T ($CD8^+$) and NK lymphocytes) in the same co-cultures, showed that CD5CAR-T cells presented significantly higher (GeoMean) levels than un-transduced T cells **(Figure IV.23B)**.

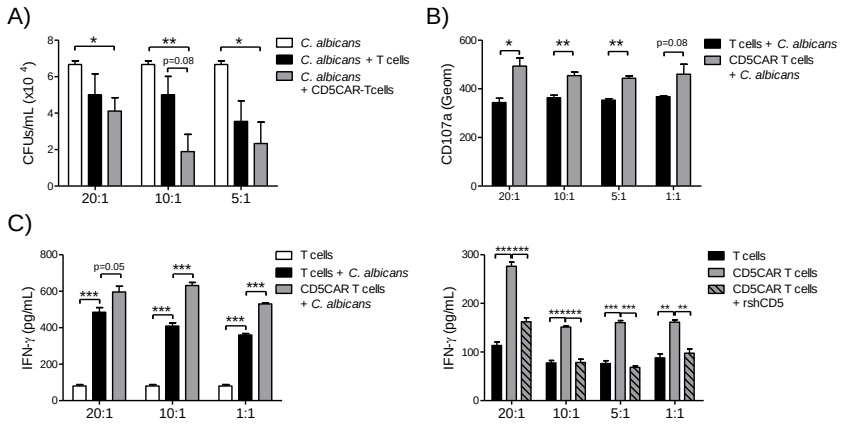

Figure IV.23.Effect of CD5CAR receptor expression on T cell-mediated antifungal response *ex vivo*. A) CD5CAR-transduced and un-transduced T cells were co-cultured at the indicated E:T ratios with alive *C. albicans* for different periods. Supernatants from 4 h-co-cultures were seeded on Sabouraud dextrose agar plates and incubated during 48 h at 30 °C for CFUs count. **B)** Cells from 4 h-co-cultures stained with anti-human CD107a for GeoMean determination by flow cytometry. **C)** Supernatants from 24 h-co-cultures of *C. albicans* with CD5CAR-transduced and un-transduced human T cells were analyzed for IFN-γ levels by ELISA. **D)** Same co-cultures as in C) in the presence or absence of rshCD5 (10 µg/mL). Statistical differences between groups were assessed by t-test. *, p<0.05; **, p<0.01; ***, p<0.001.

Finally, IFN-γ levels were evaluated in supernatants from CD5CAR-T cells co-cultured with alive *C. albicans* for 24 h. As illustrated by **Figure IV.23C, left** CD5CAR-T cell produced significantly higher levels than un-transduced T cells at all the E:T ratios tested. Importantly, the differences between CD5CAR-T and un-transduced T cells regarding IFN-γ production were abolished by the addition of rshCD5 (10 μg/mL) to the co-cultures **(Figure IV.23C, right),** thus confirming the CD5-mediated specificity of the phenomenon.

The *in vivo* efficacy of adoptively transferred CD5CAR-T and un-transduced human T cells to *C. albicans*-infected immunodeficient NSG mice was next investigated. To that end, a *C. albicans* inoculum ($8x10^3$ CFU/mouse/gr) resulting in 100% lethality between 10 to 15 days after *i.v.* infection was used, as determined in previous dose-course experiments. Then, NSG mice were adoptively transferred *i.v.* with CD5CAR-T or un-transduced human T cells (2 or 4 x 10^6 cells/mouse) at 24 h post-infection. As illustrated by **Figure IV.24A** and **B,**CD5CAR-T induced higher survival rates **(left)** and lower body weight lost **(right)** than control groups (vehicle and un-transduced T cells), though it did not reach statistical significance likely due to the low sample number used per group (n = 4 to 5). Interestingly, when used the highest cell dose (4 x 10^6 cells/mouse), the effect of un-transfected T cells administration was lost **(Figure IV.24B)** while that of CD5CAR-T cells was maintained.

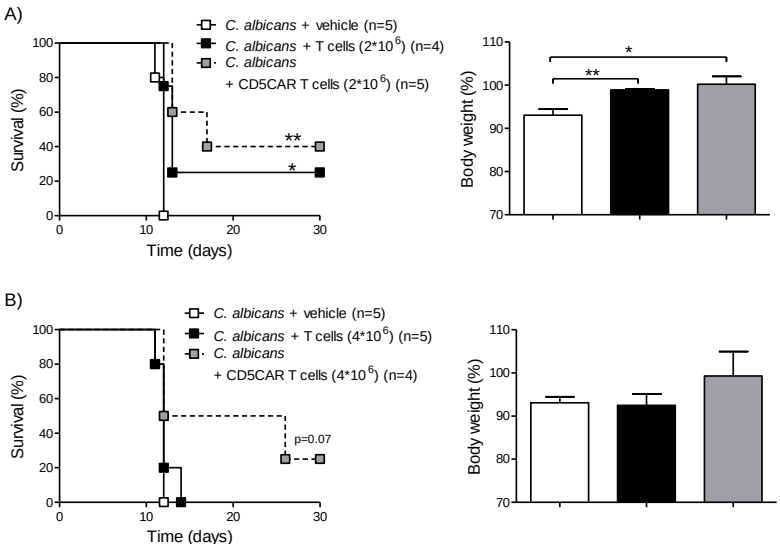

Figure IV.24 Effect of adoptive human CD5CAR-T cell transfer to immunodeficient mice undergoing systemic *C. albicans* infection. A) Percent of survival overtime of NSG mice *i.v.* infected with *C. albicans* (8 x 10^3 CFU/mouse) and *i.v.* treated at 24 h post-infection with vehicle (n=5), CD5CAR-transduced (n=5) or un-transduced (n=4) human T cells (2 x 10^6 cells/mouse). **B)** Same as in A) treating NSG mice with vehicle (n=5), CD5CAR-transduced (n=4) or un-transduced (n=5) human T cells (4 x10^6 cells/mouse). Statistical differences between groups were assessed by Log-rank (Mantel-Cox) Test or Mann Whitney test.

Due to the important role of NK cells in antifungal immunity (Schmidt *et al.*, 2017) together their suitability as off-the-shelf allogeneic cells for adoptive cell transfer therapies (Sam *et al.*, 2018) we next investigated whether they could also be used as antifungal agents upon CD5CAR transduction. To that end, similar *in vitro* experimental procedures to those above mentioned for T cells were performed with activated primary human cord blood-derived NK (CBNK) cells **(Figure II.2).**

The analysis of *ex vivo C. albicans* co-cultures showed that both CD5CAR-CBNK-transduced and un-transduced human CBNK cells reduced the number of viable CFUs, with CD5CAR-CBNK being more effective at the lowest E:T ratio tested (1:1) **(Figure IV.25A).** CD5CAR-

CBNK cells also induced higher IFN-γ levels than un-transfected CDBNK cells in co-culture supernatants at all E:T ratios tested **(Figure IV.25B)**.

When *C. albicans*-infected NSG mice were adoptively transferred with CD5CAR-CBNK or un-transduced CBNK cells, mouse survival increased by using the higher (2 x 10^6 cells/mouse) but not the lower (1 x 10^6 cells/mouse) cell dose of only the former cells **(Figure IV.25C)**. In conclusion, the results provide first proof of concept on the feasibility of CD5-based adoptive cell therapies in systemic fungal infection.

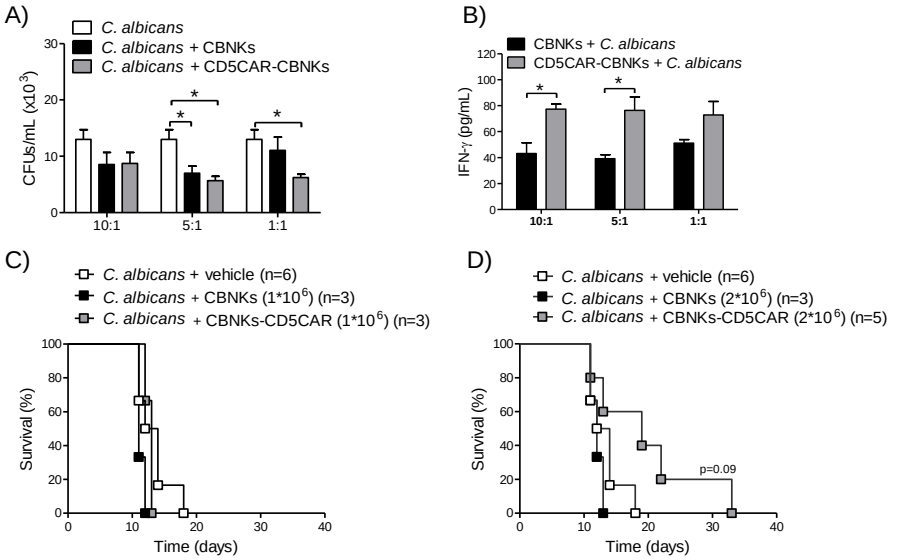

Fig IV.25 Effect of CD5CAR-transduction of CBNK cells on *in vitro* and *ex vivo C. albicans* infection. A) CD5CAR-transduced and un-transduced CBNK cells were co-cultured for 4 h with alive *C. albicans* at the indicated E:T ratios. Culture supernatants were seeded on Sabouraud dextrose agar plates and incubated during 48 h at 30 °C for CFUs count. **B)** Supernatants from 24 h-co-cultures of *C. albicans* with CD5CAR-transduced and un-transduced CBNK cells were analyzed for IFN-γ levels by ELISA. **C)** Percent of survival overtime of NSG mice *i.v.* infected with *C. albicans* (8 x 10^3 CFU/mouse) and *i.v.* treated at 24 h post-infection with vehicle (n=6), CD5CAR-transduced (n=3) or un-transduced (n=3) CBNKs cells (1 x 10^6 cells/mouse). **D)** Same as in C) treating *Candida*-infected NSG mice with vehicle (n=6), CD5CAR-transduced (n=5) or un-transduced (n=3) CBNKs cells (2 x 10^6 cells/mouse). Statistical differences between groups were assessed by Log-rank (Mantel-Cox) test or t-Test.*, $p<0.05$.

V. DISCUSSION

The present thesis aims to deepen on the understanding of the host-pathogen interactions occurring during systemic fungal infections. More specifically, we have focused on the role played on antifungal immunity by a newcomer host's fungal receptor, the lymphocytic scavenger receptor CD5. This is expected to help in the comprehension of the interactions established between fungi and host cells and, consequently, to develop new immunotherapeutic approaches for fighting IFIs. The currently available treatments against IFIs have not yet been able to significantly reduce their high morbidity and mortality rates. The population susceptible to fungal infections is increasing due to the emergence of MDR fungal strains, together with other factors such as population aging, AIDS epidemics, the steadily increasing number of aggressive medical (e.g., corticosteroids or immunosuppressant biologicals) and surgical (e.g., solid organ or hematopoietic cell transplantation) procedures, and the consequent augment of the proportion of ICU admitted patients. Moreover, most of the efforts to combat systemic infections have been made on the bacterial field. These facts highlight the need for further understanding of the relationship between the immune system and pathogenic fungi in order to develop alternative approaches to fighting IFIs.

During the last few decades an important number of works have been published highlighting the relevance of fungal recognition by the host's immune system. Thus, defects in several PRRs function led to increasing susceptibility to infection (Salazar and Brown, 2018). In this context, our group reported some time ago the unprecedented interaction of the lymphocytic scavenger receptor CD5 with β-glucans, one of the main constitutive components of the fugal walls (Vera *et al.*, 2009). In that work it was described for the first time that *i*) membrane-

bound CD5 may help immune cells to sense the presence of fungal components, and *ii*) soluble CD5 may help to reduce mortality and systemic inflammation of mice undergoing ZIGI (an experimental model of fungal sepsis-like, in which there is no infection but overwhelming inflammation). In the present thesis we have further explored whether the CD5 receptor, either in soluble or membrane-bound form, has indeed *i*) any role in the pathophysiology of fungal infections and *ii*) any potential therapeutic value *in vivo* on infection models induced by pathogenic fungal species, namely *C. albicans* and *C. neoformans*. These species, together with *A. fumigatus,* are the main responsible for clinically relevant fungal infections in humans.

1. GENETIC BACKGROUND-INFLUENCE ON THE IMMUNE RESPONSE TO FUNGAL INFECTIONS.

Differences regarding individual susceptibility to fungal infections are an important clinical issue to be considered for the right management of these diseases. Laboratory animals differing in their genetic backgrounds provide experimental models to explore host's immune response factors linked to fungal infection-susceptibility. Previous works have reported strain-specific differences in susceptibility to bacterial or fungal infection models (Zaragoza *et al.*, 2007; De La Cruz Domínguez-Punaro *et al.*, 2008; Mazur-Bialy *et al.*, 2011). The present thesis reports the existing differences regarding susceptibility to both ZIGI and systemic *C. albicans* infection models between two of the most widely used mouse strains in basic research and pharma-industry laboratories: the inbred strain C57and the outbreed mice CD1, respectively **(Figure IV.2A and Figure IV.6A)**. Our results show that the prototypical Th1-biased mouse strain C57presents lower survival outcomes and produces lower IFN-γ levels than CD1 mice

following both ZIGI and systemic candidiasis. This observation is in agreement with that reported by other authors in *C. neoformans*-infected mice (García-Barbazán *et al.*, 2016). The authors disclosed a higher proportion of titan cells (a virulence factor induced during *C. neoformans* infection) in *C. neoformans*-infected C57mice compared with CD1 ones. Moreover, *C. neoformans*-infected C57mice produced lower levels of IFN-γ, TNF-α and IL-17 (mounting a lowerTh1 response) with regard to CD1 mice.

Our results also show that C57but neither CD1 nor Balb/c mice benefit from low-dose IFN-γ replacement therapy following ZIGI or *C. albicans* challenge **(Figure IV.5 and Figure IV.7)**. The protective role of IFN-γ in fungal infections has been established from experimental mouse models of fungal infection as well as from clinical trials or case series involving relative small patient samples (Balish *et al.*, 1998a; Souto *et al.*, 2003; Zhou *et al.*, 2007; Baltazar *et al.*, 2014; Delsing *et al.*, 2014b; Coelho and Casadevall, 2016). The IFN-γ doses administered in those clinical reports were ≈0.05mg/m^2 that is ≈100 fold higher than in our mouse experiments (0.0006 mg/m^2, using a conversion factor of 1 mg/kg = 3 mg/m^2). This would indicate that while the low-IFN-γ responder C57mice benefit from low IFN-γ doses, the high-IFN-γ responder CD1 mice (or the prototypical Th2-biased Balb/c mice) would likely need higher ones. Indeed, we found that infusion of higher IFN-γ doses (10 μg/kg equivalent to 0.03 mg/m^2) increased survival rates of *C. albicans*-infected CD1 mice. In light of such observations, it would be important to know whether fungal infected patients are among the high or low IFN-γ responders. This issue would be easily testable by *in vitro* monitoring the IFN-γ response of PBMC to zymosan. Low IFN-γ responder patients would benefit from lower IFN-γ

replacement regimes, and thus, minimizing cytokine adverse effects. By contrast, intensive IFN-γ regimes and/or alternative therapeutic approaches would be more appropriated for high IFN-γ (or Th2-biased) responders.

Our results also allowed us to discard intrinsic *ifn-γ* gene defects as responsible of the IFN-γ differences observed between C57and CD1 mice following *in vitro* and *in vivo* zymosan challenge, a well-known TLR2 ligand (Bellocchio *et al.*, 2004). Such evidence was achieved through the observation that splenocytes from both mouse strains produced similar IFN-γ levels following mAb-induced TCR/CD3 cross-linking **(Figure IV.4)**. Differences regarding IFN-γ responses were also observed when CD1 and C57splenocytes were exposed to LTA and LPS, two well-known bacterial ligands of the TLR2 and TLR4 receptors, respectively. Both TLR2 and TLR4 share similar signalling (MyD88-dependent) pathways (Kawasaki and Kawai, 2014). Thus, quantitative or qualitative differences regarding to TLR2 and TLR4 expression and/or signalling could be behind the differences found between CD1 and C57mice regarding *in vitro* and *in vivo* IFN-γ production. Indeed, flow cytometry analyses showed significant lower surface levels (as measured by MFI) for TLR2 and TLR4 but also for Dectin-1 (the main β-glucan receptor from myeloid cells) on DCs from C57mice compared with CD1 mice **(Figure IV.9)**. In consequence, in the absence of intrinsic *ifn-γ* expression defects, lower surface expression of β-glucan (TLR2 and Dectin-1) and LPS (TLR4) receptors would be very likely responsible for the lower IFN−γ levels observed in C57versus CD1 mice upon both fungal and bacterial challenge.

It is well documented that IFN-γ and IL-17 exert a protective role against fungal infections (van de Veerdonk *et al.*, 2012; Dambuza *et*

al., 2017). However, it is still a question of debate which Th1 or Th17 host response is more effective in particular fungal infection scenarios (e.g., cutaneous versus systemic infections)(Netea *et al.*, 2015). Interestingly, we observed that CD1 splenocytes produced lower IL-17A levels than C57ones under the same *in vitro* stimulatory conditions (anti-CD3 mAb exposure) **(Figure IV.4)**. This could be a consequence of the known inhibitory effect of high IFN-γ levels on Th17 responses via STAT-1 induction (Hu and Ivashkiv, 2009), although intrinsic *il-17a* gene defects cannot be excluded and should be further investigated. Furthermore, this variability regarding the efficacy of the host's response under particular infections may be related to our observation that the higher susceptibility of C57mice compared with CD1 mice would not be absolute but dependent on other factors such as the infection level: C57mice (IFN-γ^{low} but IL-17A^{high} responders) challenged with a relatively high *C. albicans* inoculum (2.86 x 10^4 CFU/gr) present lower survival rates than CD1 mice (IFN-γ^{high} but IL-17A^{low} responders); on the contrary, when a lower *C. albicans* inoculum is used (2.86 x 10^2 CFU/gr), CD1 mice show lower survival rates than C57mice **(Figure IV.9)**. In light of these findings, it could be hypothesized that Th1 responses would be more efficient against high-burden fungal infections, while Th17 would perform better against low-burden ones. This also implies a further level of complexity to be taken into consideration (fungal inoculum) when implementing similar infection models in different mouse strains.

In conclusion, our results bring out the importance of keeping in mind the peculiarities of both the mouse strain and the infection model used when studying host's immune responses to fungal infection for further translation to clinical settings. Most of the reported studies

regarding the mechanisms underlying the immune response against fungal infections have been developed in C57 mice as it is one of the most used genetic backgrounds for genetically modified mice generation. As here in demonstrated, these mice show constitutive differences with regard to other mouse strains (i.e., CD1) concerning *i*) the expression of well-established effector arms of the anti-fungal immune response (i.e., Dectin-1, TLR2 and TLR4) (Salazar and Brown, 2018) and *ii*) the response to low- or high-burden fungal inoculum.The use of inbred strains like C57has the advantage of reducing experimental variability as a consequence of low/minimal inter-individual heterogeneity. However, to avoid experimental biases and to draw simplistic/mistaken conclusions with them it would be important to get data from several inbred strains in parallel. The alternative would be using outbreed strains like CD1 which genetically heterogeneous and consequently more representative of the whole mouse population.

From the clinical point of view, our results also highlight the importance of focusing more research on designing personalized immune-based antifungal therapies. As proposed above, investigating the patient's IFN-γ responder status could help to better adjust and personalize current antifungal immunotherapies.

2. IMMUNOMODULATORY PROPERTIES OF CD5 IN EXPERIMENTAL MOUSE MODELS OF INVASIVE FUNGAL INFECTION

IFIs have a significant impact on morbidity, mortality, length of hospital stay and health care costs in critically ill patients (Klingspor *et al.*, 2015), constituting a still unmet medical need. Despite the

progresses made in antifungal therapy with the advent of azoles and echinocandins, no significant reductions on the high mortality rates of IFIs have been achieved, partly due to the emergence of drug resistances and the potential toxicity risks of these treatments (Garey *et al.*, 2006). This denotes the importance of developing new therapeutic strategies (either alternative or adjunctive) to improve the clinical outcome of patients who develop candidiasis or other prevalent IFIs. In light of the successful results obtained by the immunotherapy in cancer, efforts are in progress to explore whether host own immune response components would help to better fight IFIs (Posch *et al.*, 2017). This is well exemplified by the successful attempts reported with IFN-γ administration (Delsing *et al.*, 2014a). Since the use of PRRs (e.g., TLRs, C-type lectins or pentraxins) has not been alien to those efforts (Posch *et al.*, 2017), we were propelled to further explore possible strategies based on CD5, a lymphocyte-specific PRR with a still poorly understood role in the anti-fungal immune response. A first step in this direction was to analyse the consequences of CD5-deficiency in the context of fungal infection.

2.1. ANALYSIS OF THE IMMUNOMODULATORY PROPERTIES OF MEMBRANE-BOUND CD5 IN INVASIVE FUNGAL INFECTIONS

The host's immune response to fungal infections relies on PAMPs recognition by PRRs, expressed by immune and/or non-immune cells (Salazar and Brown, 2018). While it is on innate immune cells where PRRs are predominantly expressed and undergo their main role regarding the immune response against pathogens, increasing evidence demonstrate the relevance of its expression in lymphocytes. This is the case of some TLRs, which are also expressed on T cells where they act as

co-stimulatory molecules, modulating T and B cell responses triggered following specific antigen recognition via TCR or BCR (Liu *et al.*, 2006; Kabelitz, 2007; Poovassery *et al.*, 2009). As an illustrative example, it has been shown that the bacterial lipoprotein Pam3Cys -a TLR2 agonist- promotes both effector ($CD4^+CD25^-$) and regulatory ($CD4^+CD25^+$) T cells expansion, though transiently attenuating the suppressive activity of the later (Liu *et al.*, 2006). In this way, the host's adaptive immunity may rapidly increase effector cells expansion and, when the infection has subsided, Tregs recover their suppressive activity in time to limit potential autoimmunity that might result from the over activated effectors. A similar immunomodulatory role could apply to lymphocyte scavenger receptors like CD5 (and CD6), as it has been hypothesized by Laurel L Lenz (Lenz, 2009).

Previous studies from our group demonstrated that membrane-bound CD5 binds to the β-glucan moiety of zymosan, and subsequently, promotes early (MAPK cascade activation) and late (cytokine [IL-8] production) intracellular signalling events (Vera *et al.*, 2009). On this basis, confirmation on the relevance of membrane-bound CD5 in anti-fungal immune response came from the analysis of *cd5*$^{-/-}$ mice. Accordingly, *cd5*$^{-/-}$ mice showed increased susceptibility not only to *C. albicans* but also to *C. neoformans* infection in comparison with WT mice **(Fig IV.10A-C)**. Interestingly, these differences between *cd5*$^{-/-}$ and WT mice were abolished following therapeutic infusion of the soluble CD5 protein (rshCD5) **(Fig IV.10D).**

Ex vivo experiments allowed to objectify a trend to lower cytokine (IFN-γ, TNF-α, IL-12 and IL-6) production **(Fig IV.11)** in conjunction with higher T-cell apoptosis **(Fig IV.13)** for *cd5*$^{-/-}$ *vs* WT splenocytes when exposed to heat-killed fungal (*C. albicans*) cells. These

findings are in agreement with the pro-survival properties assigned to the CK2-binding domain of CD5 (via AKT activation), which result in increased AICD in case of CD5-defciency (Axtell *et al.*, 2006; Sestero *et al.*, 2012; McGuire *et al.*, 2014). Thus, membrane-bound CD5 expression would protect against the deleterious effects of fungal infection by making T-cells less prone to apoptosis and consequently raising more sustained and potent Th1 and/or Th17 responses. Indeed, functional CD5-dependent CK2 signalling seems to be necessary for efficient differentiation of naive $CD4^+$ T cells into Th17 cells, but not Th1 cells (Sestero *et al.*, 2012; McGuire *et al.*, 2014).

The higher susceptibility to fungal infection here reported for CD5-deficient mice recalls that reported for other membrane-bound immune cell fungal receptors, such as Dectin-1 and Dectin-2. Deficient mice for these receptors are more susceptible to *C. albicans* infection showing enhanced fungal dissemination, lower cell recruitment and decreased pro-inflammatory cytokines production as a consequence of an impaired fungal recognition (Taylor *et al.*, 2007; Ifrim *et al.*, 2016). All of this support a relevant role for CD5 in anti-fungal immune response. However, further studies are necessary to fully understand the mechanism/s behind the role of membrane-bound CD5 receptor in fungal infection. This would include the putative role played by different subsets of non-T (e.g., Breg) and/or non-lymphoid cells (e.g., macrophages and DCs) reported to express membrane-bound CD5 (Moreau *et al.*, 1999)(Moreau *et al.*, 1999; Borrello *et al.*, 2001; Zhang *et al.*, 2017b; Korenfeld *et al.*, 2017; Li *et al.*, 2019).

2.2. ANALYSIS OF THE IMMUNOMODULATORY PROPERTIES OF SOLUBLE CD5 IN INVASIVE FUNGAL INFECTIONS

The *in vivo* relevance of soluble CD5 binding to a fungal PAMP (namely b-glucans) was first demonstrated in a murine model of ZIGI, where the infusion of rshCD5 had beneficial effects on survival and serum cytokine levels production in CD1 mice (Vera *et al.*, 2009). Our present data show that such beneficial effects of rshCD5 infusion can be extended to experimental fungal infection induced by *C. albicans* and *C. neoformans* also in CD1 mice, thus indicating that they are not fungal specie specific. Although further investigations are still due on other clinical relevant fungal infections (e.g., *A. fumigatus*), the putative broad anti-fungal spectrum of rshCD5 would represent a remarkable advance as most of the conventional antifungal drugs are limited to certain fungal pathogens (Nami *et al.*, 2019).

In the two infection models here analyzed, the therapeutic effects of rshCD5 infusion on mouse survival were time- and dose-dependent **(Figures IV.14 and IV.21)**, as it happens with many anti-fungal agents (Garey *et al.*, 2006). The lower mortality of rshCD5-treated mice also went in accordance with lower fungal loads in the main target organs (kidney for *C. albicans*; lung and brain for *C. neoformans*), as well as with increased serum and/or tissue levels of protective cytokines (namely IFN-γ). Moreover, in the *Candida*-infection model, rshCD5-treated mice showed increased kidney leukocyte infiltration at expenses of innate immune cell types (NKs, cDCs, macrophages and granulocytes) involved in fungal clearing (Erwig and Gow, 2016).

In agreement with our *in vivo* results, *ex vivo* assays showed that addition of rshCD5 to CD1 splenocytes co-cultured with heat-killed *C.*

albicans increased IFN-γ and TNF-α production **(Figure IV.18A)**, pro-inflammatory cytokines involved in granulopoiesis and neutrophil recruitment and activity during fungal infections (van de Veerdonk *et al.*, 2012). Further assays also showed that rshCD5 addition increased fungal killing in co-cultures of CD1 splenocytes with viable *C. albicans* and *C. neoformans* conidia in a dose-dependent manner **(Figure IV.18B and Figure IV.22A).** Such an increased fungal killing was shown to be due to, at least in part, direct effects of rshCD5 on *C. albicans* viability (not *C. neoformans*) **(Figure IV. 18C)** by mechanism/s still to be disclosed. Accordingly, results obtained with *C. albicans*-infected immunodeficient NSG mice support the notion that an intact immune system is necessary to get optimal improvements of mouse survival following rshCD5 infusion **(Fig IV.19B)**.

In an attempt to explore possible adjunctive anti-fungal therapies involving rshCD5 and currently available antimycotic drugs, fluconazole was selected. Fluconazole belongs to the azole class of antifungals, which do not interfere with β-glucan biosynthesis, and are used as first-line treatment of several fungal infections, including invasive candidiasis (Nami *et al.*, 2019). Although optimal dosing regimens reported for fluconazole administration in *C. albicans*–infected mice are 10 mg/kg/day for a period of 7 days (MacCallum and Odds, 2004) a 10-times lower dose was used in our combination studies with rshCD5. Under these conditions, survival rates of *C. albicans*-infected CD1 mice treated with fluconazole increased from 40% to 80% when combined with a single dose of rshCD5 **(FigIV.20B)**. These results constitute first proof-of-concept data supporting the possibility of using rshCD5 to develop combination therapies allowing reduction of side

effects associated to high dosage of current antimycotic drugs without lossing antifungal efficacy.

Taken together, the therapeutic effects here reported for soluble CD5 in fungal infection recall those reported for other soluble PRRs, such as Pentraxin 3 (PTX3) or Gal-3. PTX3 is a long pentraxin binding to galactomannan and involved in anti-fungal innate immune response (Garianda *et al.*, 2002). Accordingly, it has been shown that administration of a soluble rhPTX3 reduces mortality rates as well as fungal burden in a rat model of aspergillosis (Lo Giudice *et al.*, 2010). Similar protective effects have also been reported for PTX3 administration (alone or in combination with conventional antimycotic drugs) in a mice model of aspergillosis (Gaziano *et al.*, 2004). Moreover, macrophages from transgenic mice overexpressing PTX3 show increased phagocytosis activity against *P. brasiliensis*, with comparable results to those obtained after addition of rhPTX3, which acts as an opsonin (Diniz *et al.*, 2004). Likewise, Gal-3 is a soluble lectin known to bind to galactomannans as well as to α- and β-mannans, and to be involved in the immune response against *C. albicans* and *C. neoformans* (Becker *et al.*, 2015; Almeida *et al.*, 2017). Neutrophils incubated with recombinant Gal-3 show increased phagocytic activity against *C. albicans* and *C. parapsilosis*, an effect that is inhibited by the addition of a blocking anti-Gal-3 mAb (Linden *et al.*, 2013). In light of these data, it would be interesting to test the putative efficacy of combination immunotherapies involving rshCD5 in conjunction with rhPTX3 and/or Gal-3.

2.3. CHIMERIC ANTIGEN RECEPTORS IN FUNGAL INFECTION

Based on the broad fungal binding properties of the CD5 extracellular domain, we tested an alternative immunotherapeutic approach for the treatment of IFIs: adoptive transfer of immune cells expressing a CD5-based chimerical antigen receptor (CD5-CAR). The development of T cells engineered to express CARs with tumour-specificity has revolutionized the field of cancer immunotherapy, especially in the case of haematological malignancies (June *et al.*, 2018). In the field of infection, this strategy is still underdeveloped and has been mainly focused in the treatment of viral diseases. Thus, adoptive transfer of antigen-specific T cells has been assessed against cytomegalovirus (CMV), Epstein-Barr virus (EBV) or Human adenovirus (HAdV) infections after allogeneic-hematopoietic stem cell transplant (allo-HSCT) (Feuchtinger *et al.*, 2006; Comoli *et al.*, 2007; Blyth *et al.*, 2013). To date, only one CAR has been developed against fungal infection (Kumaresan *et al.*, 2014). The authors transduced primary human T cells with a Dectin-1-based CAR (D-CAR) and reported *in vivo* beneficial effects in a cutaneous infection model induced by *A. fumigatus*. The e*x vivo* co-culture of D-CAR T cells with *A. fumigatus* resulted in fungal growth inhibition, as well as up-regulation of CD107a and IFN-γ expression by D-CAR T cells. By developing CD5-CAR T cells we have obtained similar results following their co-cultivation with *C. albicans* **(Figure IV.23)**: reduced number of CFUs as well as higher IFN-γ production and CD107a expression. The specificity of this phenomenon was demonstrated by the significant reduction of CD5-CAR T cell-mediated IFN-γ release observed in the presence rshCD5. Importantly, *in vivo* experiments showed higher survival rates of *C. albicans*-infected immunodeficient NSG mice after *i.v.* infusion of CD5-

CAR T cells compared with un-transduced T cells or vehicle **(Figure IV.24)**, although differences did not reach statistical significance likely due to the low number of animals used per experimental group (n = 3 to 5). In any case, these results constitute the first proof-of-concept data on the feasibility of developing CD5-based CAR cells for adoptive cell transfer therapies against IFIs.

The generation of autologous CAR T cells is time consuming and may result in unavoidable delays in therapy, especially for patients with rapidly advancing diseases like IFIs (Rezvani, 2019). Although allogeneic products have the potential to overcome these limitations, allogeneic T-cells (even if HLA-matched) can mediate GvHD through their native TCR. Therefore, efforts are underway to develop reliable off-the-shelf cellular products with acceptable safety profiles for the patient's treatment. Allogeneic NK cells may provide an attractive and safe source for off-the-shelf cellular immunotherapies with acceptable safety profiles. In contrast to T cells, NK cells do not express rearranged antigen-specific receptors and have shorter lifespan thus minimizing the risk of both overexpansion in the patient and of inducing GvHD. Another advantage of NK cells over T cells is the lower risk of inducing systemic inflammatory response syndrome (SIRS), a life-threatening disorder mainly caused by massive release of TNF-α and IL-6 (the principal pro-inflammatory cytokines produced by NK cells are IFN-γ and GM-CSF) (Klingemann, 2014). Furthermore, NK cells have a constitutive and potent cytotoxic activity, which include fungal cell killing (Schmidt *et al.*, 2017). However, primary NK cells are difficult to isolate, purify, and transduce, resulting in a heterogeneous mixture of cells that often expand poorly (Siegler *et al.*, 2018). These obstacles limit its use in cellular immunotherapy. Thus, efforts are in progress to improve

currently available protocols for clinical applications (Shah *et al.*, 2013; Siegler *et al.*, 2018; Hu *et al.*, 2019).

On this basis, we tested the *in vitro* and *in vivo* functionality of CD5CAR-transduced CBNKs against fungal cells. As in the case of CD5CAR-T cells, we observed increased *in vitro* antifungal cytotoxic activity as well as IFN-γ production after co-culture of CD5CAR-CBNKs with *C. albicans*, at all E:T ratios tested **(Figure IV.25A and B)**. Again, when *C. albicans*-infected immunodeficient NSG mice were adoptively transferred with CD5CAR-CBNK cells, mouse survival increased in a dose-dependent manner, though differences with regard to control groups (vehicle and un-transduced CBNK cells) did not reach statistical significance due to the low sample size used (n = 3 to 5/group) **(Figure IV.25C and D)**. Though these results await further validation by using a higher number of animals per group and other fungal infection models (e.g., *C. neoformans* infection), they constitute first proof-of-concept data supporting the feasibility of CD5- and allogeneic NK-based off-the-shelf therapies for IFIs.

VI. CONCLUSIONS

CONCLUSIONS

1. The mouse strain selection needs to be taken into consideration for properly understanding the factors involved in the host's immune response to fungal infections and for translating selected antifungal therapies into the clinical practice.

2. Expression of membrane-bound CD5 constitutes an integral and non-redundant component of the host's antifungal immune response as deduced by increased susceptibility of CD5-deficient mice to fungal infection.

3. The beneficial therapeutic effects demonstrated for a soluble form of the human CD5 receptor in different experimental models of fungal infection open the possibility of developing novel adjunctive therapies against IFIs easily translatable to the clinical practice.

4. The increased antifungal properties of immune cells expressing CD5-based chimeric antigen receptors open the possibility of developing novel off-the-shelf adoptive cellular immunotherapies against IFIs.

VII. REFERENCES

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ANNEX I

CD5CAR sequence (Signal peptide/CD5/ CD8a/4-1BB/ CD3z)

MluIGCTAGCTCTAGAATGGCCTTACCAGTGACCGGCTTGCTCC
TGTCGCTGGGCTTGCTGCTCCACGCCGCCAGGCCGAGACTGAGTTGGTACG
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GCCAGAGACTGAACTGTGGAGTCCCTCTGTCCCTGGGCCCATTCCTGGTCA
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CCAACTGTTCTCACAGTAGGAATGACATGTGCCATTCTCTGGGGCTGACTT
GTCTGGAGCCCCAGAAGACCACACCACCTACTACCCGACCACCACCTACAA
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TGAAAGGAGAAAGAAGGCGCGGCAAGGGGCATGATGGCCTGTATCAGGGAC
TGTCAACCGCAACAAAAGATACTTATGATGCTCTGCACATGCAGGCTCTGC
CCCCGCGGTAA*BspEI*

PUBLICATIONS

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RESEARCH ARTICLE

Discordant susceptibility of inbred C57BL/6 versus outbred CD1 mice to experimental fungal sepsis

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Abstract

Individual susceptibility differences to fungal infection following invasive and/or immunosuppressive medical interventions are an important clinical issue. In order to explore immune response-related factors that may be linked to fungal infection susceptibility, we have compared the response of inbred C57BL/6J and outbred CD1 mouse strains to different experimental models of fungal sepsis. The challenge of animals with the zymosan-induced generalised inflammation model revealed poorer survival rates in C57BL/6J, consistent with lower Th1 cytokine interferon (IFN)-γ serum levels, compared with CD1 mice. Likewise, *ex vivo* exposure of C57BL/6J splenocytes to zymosan but also bacterial lipopolisaccharide or lipoteichoic acid, resulted in lower IFN-γ secretion compared with CD1 mice. C57BL/6J susceptibility could be reverted by rescue infusion of relative low IFN-γ doses (0.2 μg/kg) either alone or in combination with the β-glucan-binding CD5 protein (0.7 mg/kg) leading to improved post zymosan-induced generalised inflammation survival. Similarly, low survival rates to systemic *Candida albicans* infection (2.86×10^4 CFU/gr) were ameliorated by low-dose IFN-γ infusion in C57BL/6J but not CD1 mice. Our results highlight the importance of strain choice in experimental fungal infection models and provide a susceptibility rationale for more specific antifungal immunotherapy designs.

KEYWORDS

C57BL/6J mice, *Candida albicans*, CD1 mice, fungal sepsis, IFN-γ, zymosan

1 | INTRODUCTION

Opportunistic fungal species are responsible for mycosis that range from skin lesions to life-threatening invasive infections and septic shock. Invasive fungal infections are an important cause of morbidity and mortality mainly in immunocompromised patients, and their incidence is on the rise as a result of extensive immunosuppressive medical interventions in autoimmunity and solid organ/stem cell transplantation, severe surgery (mainly abdominal), increasing use of indwelling medical devices, HIV/AIDS epidemics and escalating antifungal resistances (Kauffman et al., 2014; Martin, Mannino, Eaton, & Moss, 2003; Ramos-e-Silva, Lima, Schechtman, Trope, & Carneiro, 2012; Salazar & Brown, 2018).

Animal models help in our understanding of fungal pathogenesis, host immune responses, diagnosis, and antifungal treatment (Capilla, Clemons, & Stevens, 2007; Hohl, 2014). Aside from superficial or systemic infections induced by specific fungal species, the septic shock-like syndrome induced by zymosan—also named zymosan-induced generalised inflammation (ZIGI)—has been extensively used as a model of fungal sepsis (Genovese et al., 2004; Volman, Hendriks, & Goris,

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2005). Zymosan is a β-glucan-rich particle derived from *Saccharomyces cerevisiae*, which induces inflammatory mediator release leading to multiple organ dysfunction syndrome. β-glucans are conserved structural pathogen-associated molecular patterns of fungal cell walls, which are sensed by pattern-recognition receptors (e.g., Toll-like receptor (TLR) 2, Dectin-1, Langerin, CR3/CD11b/CD18, CD36, SCARF1, and CD5) expressed on host innate and adaptive immune cells (Latgé, 2010; Levitz, 2010; Vera et al., 2009) to mount protective responses.

Inbred (homozygous) and outbred (heterozygous) mouse strains commonly used in academic and industrial research have also been utilised to model fungal infections (Hohl, 2014). Differences regarding disease susceptibility/severity when inbred and outbred mouse strains are challenged with the same fungal species (e.g., *Paracoccidioides brasiliensis* or *Cryptococcus neoformans*) have been reported (Calich, Singer-Vermes, Siqueira, & Burger, 1985; Zaragoza, Alvarez, Telzak, Rivera, & Casadevall, 2007). Such differences may provide insight into their genetic and mechanistic foundations, as well as into possible therapeutic approaches (Capilla et al., 2007). The present work uses two of the most common inbred and outbred mouse strains (C57BL/6 and CD1, respectively) to explore susceptibility differences to ZIGI, as well as to *Candida albicans* infection. C57BL/6 is an inbred (homozygous) strain most commonly used for developing genetically modified mice to model human diseases (e.g., the Knockout Mouse Project initiative), whereas CD1 is an outbred (heterozygous) strain (Aldinger, Sokoloff, Rosenberg, Palmer, & Millen, 2009) commonly used in toxicology testing (safety and efficacy) by the pharmaceutical and chemical industry. The results show a higher susceptibility of C57BL/6 versus CD1 mice to fungal challenge linked to poorer interferon (IFN)-γ responses and provide a rationale for more specific antifungal immunotherapy designs.

2 | RESULTS

2.1 | C57BL/6J mice show higher susceptibility than CD1 mice to ZIGI

To investigate C57BL/6J and CD1 mice susceptibility differences to fungal infection, both strains were first subjected to the ZIGI model. To this end, animals from both strains were ip injected with an identical single dose of zymosan (700 mg/kg), according to previous reports (Bian, Guo, Ha, Zen, & Liu, 2012; Jia et al., 2013; Volman et al., 2005) and our previous dose-response experiments (Figure S1). As illustrated by Figure 1a, C57BL/6J mice were more susceptible to ZIGI, undergoing higher mortality, clinical score, and body weight loss. Importantly, a trend to higher ZIGI susceptibility of C57BL/6 mice was observed at all zymosan doses tested (ranging from 300 to 1,000 mg/kg), which reached statistical significance only at 700 mg/kg doses ($p < 0.05$) likely due to the low size (n) of most experimental groups analysed (Figure S1).

Higher ZIGI susceptibility of C57BL/6J versus CD1 mice was also reflected by serum levels of pro- and anti-inflammatory cytokines 18-h post zymosan injection (700 mg/kg, ip). As shown in Figure 1b, significant lower IFN-γ and IL-6 levels were observed in C57BL/6J compared with CD1 at +18-h post zymosan challenge. In contrast, no significant differences were observed between the two mouse strains regarding the GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, and TNF-α serum levels (Figure 1b and Figure S2).

2.2 | C57BL/6J splenocytes secrete lower IFN-γ levels than CD1 ones following *in vitro* stimulation with zymosan

IFN-γ is a prototypical Th1 cytokine produced by several innate and adaptive immune cells (macrophages, dendritic cells, CD4 Th1 cells, CD8 T cells, Tγδ cells, and NK cells) and which plays a key role in the immune control of fungal infections (Gozalbo, Maneu, & Gil, 2014; Stevens, Brummer, & Clemons, 2006). So IFN-γ levels were measured in splenocyte cultures from C57BL/6J and CD1 mice ex vivo exposed to zymosan (75 μg/ml, as determined by previous dose-response assays). As illustrated by Figure 2a (left), lower IFN-γ levels were detected in culture supernatants from C57BL/6J versus CD1 splenocytes. Similar results were observed when peritoneal cells were also ex vivo stimulated with zymosan (Figure 2a, right).

As IL-12 is an inducer of IFN-γ production (Trinchieri, 1995), their interdependence in response to zymosan was investigated. To this end, zymosan-stimulated splenocytes from CD1 mice were cultured for 20 h at different doses of a blocking anti-IL-12 monoclonal antibody (mAb) or an isotype control. As shown in Figure 2 b, zymosan-induced IFN-γ by CD1 splenocytes was only partly reduced by IL-12 blockade, meaning that other IL-12-independent IFN-γ-inducing factors could be also operating upon zymosan challenge (e.g., IL-18; Fantuzzi, Puren, Harding, Livingston, & Dinarello, 1998).

The lower in vivo and ex vivo IFN-γ responses of C57BL/6J versus CD1 mice challenged the view of C57BL/6 as a prototypical Th1-biased mouse strain (Watanabe, Numata, Ito, Takagi, & Matsukawa, 2004). Thus, we decided to compare zymosan-induced IFN-γ production by splenocytes from C57BL/6J and CD1 mice against BALB/c—a prototypical Th2-biased mouse strain. As expected, IFN-γ levels achieved by C57BL/6J splenocytes were 10 folds higher than in BALB/c splenocytes (Figure 2c). In turn, CD1 splenocytes produced 10 folds higher IFN-γ levels than C57BL/6J splenocytes (Figure 2c).

2.3 | C57BL/6J splenocytes produce lower IFN-γ levels than CD1 splenocytes following *in vitro* stimulation with bacterial ligands of TLRs

Whether the reduced IFN-γ response of C57BL/6J versus CD1 splenocytes was specific to zymosan, it was further investigated using alternative stimuli. As shown in Figure 3a, C57BL/6J splenocytes also produced lower IFN-γ levels than CD1 mice following stimulation with lipoteichoic acid (LTA; 10 μg/ml) or lipopolysaccharide (LPS; 1 μg/ml)—two bacterial pathogen-associated molecular patterns binding to TLR2 and TLR4, respectively. In contrast, no differences were observed between C57BL/6J and CD1 splenocytes when exposed to

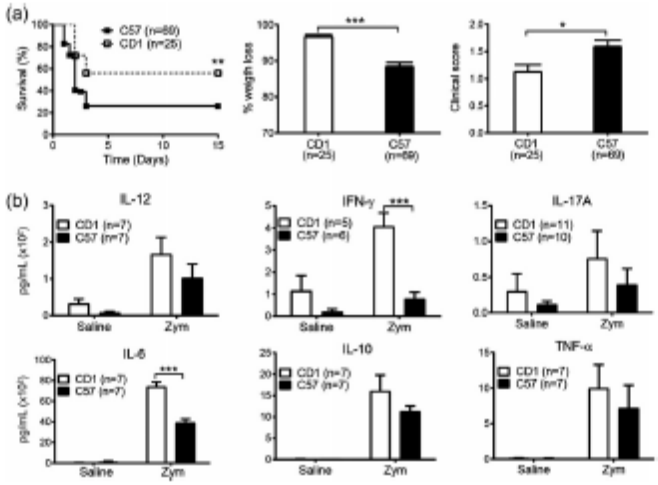

FIGURE 1 CD1 and C57BL/6J mice differ in their susceptibility to fungal-like septic shock induced by zymosan. (a) CD1 ($n = 25$) and C57BL/6J ($n = 69$) mice were ip injected with zymosan (700 mg/kg). Left, survival percentage recorded daily for 2 weeks. **, $p < 0.01$ (Log-rank Mantel–Cox test). Middle and right, percentage body weight loss and clinical score recorded 1-day post zymosan challenge, respectively; *, $p < 0.05$ and ***, $p < 0.001$ (unpaired t test). (b) CD1 and C57BL/6J mice were ip challenged with zymosan (700 mg/kg) or saline, and bled 18 h later for assessment of cytokine serum levels by ELISA or Multiplex. The number of mice from each strain goes from 3 to 11 depending on the cytokine. ***, $p < 0.001$ (unpaired t test)

an anti-CD3 mAb (0.1 μg/ml; Figure 3a), which excluded a putative intrinsic *IFN-γ* gene expression deficiency.

In addition to IFN-γ, IL-17A is also involved in the immune control of fungal infections, especially in epithelial and mucosal surfaces (Jin & Dong, 2013). In light of this, IL-17A production by splenocytes from both strains was explored. As illustrated in Figure 3b, ex vivo exposure of splenocytes to zymosan alone (75 mg/ml) led to weak IL-17A production in both strains. However, splenocytes from C57BL/6J mice produced higher IL-17A levels than CD1 mice after anti-CD3 mAb stimulation alone or in combination with zymosan. This relative lower IL-17A production might be indicative of intrinsic defects in the *IL-17a* expression pathway in CD1 mice.

2.4 | IFN-γ replacement therapy improves survival of ZIGI-challenged C57BL/6J mice

Whether C57BL/6J susceptibility to ZIGI could be ameliorated by IFN-γ replacement therapy, it was further investigated. To this end, C57BL/6J mice were ip injected 1-h post-ZIGI-challenge with 0.2-μg/kg IFN-γ, a dose intended to allow C57BL/6 mice achieving serum levels similar in magnitude to those observed in CD1 mice (Figure 1b). As illustrated in Figure 4a, IFN-γ infusion increased C57BL/6J survival from ≈15% to ≈40%, in line with CD1 mice survival at the same zymosan (700 mg/kg) dose (Figure 1a). No significant effects on mouse survival were observed when CD1 mice were infused with identical IFN-γ (0.2 μg/kg) amounts (Figure 5a).

In a further set of experiments, it was also investigated whether C57BL/6J mice, either alone or in combination with IFN-γ, could benefit from infusion of the β-glucan interacting CD5 protein as previously reported in CD1 mice (Vera et al., 2009). As shown in Figure 4a and 4b, ip injection of recombinant soluble human CD5 protein alone (rshCD5; 0.7 mg/kg) 1-h post-ZIGI-challenge did not improve C57BL/6J mice survival or clinical score with regard to the control protein (human serealbumin [HSA]). Simultaneous infusion of IFN-γ and rshCD5 showed additive effects giving rise to optimal survival rates (≈70%) and clinical scores (Figure 4a and 4b).

2.5 | IFN-γ replacement therapy improves the deficient response of C57BL/6J versus CD1 mice to systemic *Candida albicans* infection

In an attempt to mimic the settings of a clinical infection, CD1 and C57BL/6 varying susceptibility to the ZIGI model was challenged in a systemic fungal infection. Both mouse strains were injected iv with

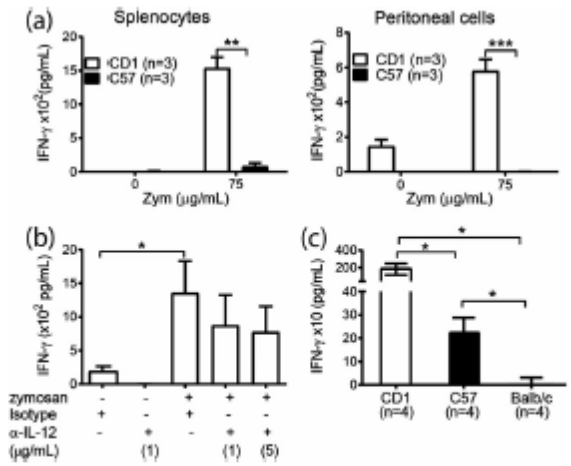

FIGURE 2 *Ex vivo* stimulated CD1 and C57BL/6J splenocytes differ in their IFN-γ response following exposure to zymosan. (a) Interferon (IFN)-γ levels in supernatants from total splenocytes (5×10^6 cells/ml; left) and peritoneal cells (2×10^6 cells/ml; right) of CD1 and C57BL/6J mice exposed to zymosan (75 µg/ml) for 18 h. The results shown are triplicates from a representative experiment of three performed. (b) CD1 splenocytes were exposed for 18 h to isotype control monoclonal antibody (5 µg/ml), blocking anti-IL-12 antibody (1 or 5 µg/ml) or zymosan (75 µg/ml) either alone or in combination. *, $p < 0.05$ (Student *t* test). (c) IFN-γ concentration in supernatants from total splenocytes (5×10^6 cells/ml) of CD1, C57BL/6J, and BALB/c mice exposed to zymosan (75 µg/ml) for 18 h. Results shown are quadruplicates of a representative experiment of two performed. *, $p < 0.05$ (2-tailed Mann–Whitney test)

an identical lethal inoculum of *C. albicans* (2.86×10^4 CFU/g). As shown in Figure 6a, mortality in CD1 mice was delayed in relation to C.57BL/6J mice. In accordance with the ZIGI model results, CD1 mice also showed higher IFN-γ serum levels post *C. albicans* infection compared with C57BL/6J mice (Figure 6b). This result prompted IFN-γ replacement therapy experiments on *Candida*-infected C.57BL/6J mice. As shown in Figure 6c, ip IFN-γ infusion (0.2 µg/kg) 18-h post-*C. albicans* infection significantly improved C57BL/6J survival. In contrast, survival of *Candida*-infected CD1 mice survival did not increase when infused with identical (0.2 µg/kg; Figure 5b) but higher (10 µg/kg) IFN-γ replacement doses (Figure 5c).

2.6 | CD1 and C57BL/6J mice differ in their susceptibility to fungal sepsis induced by a low *Candida albicans* inoculum

Because the efficacy of host immune may vary under particular fungal infection scenarios (Netea, Joosten, van der Meer, Kullberg, & van de Veerdonk, 2015), it was further investigated whether differences between CD1 and C57BL/6J mice remained steady when a milder *Candida*-infection model was used. As illustrated in Figure 7, survival of C57BL/6J mice was significantly higher than that of CD1 mice when infected with a *C. albicans* inoculum 2-log lower (2.86×10^2 CFU/g) than the one used in previous experiments (2.86×10^4 CFU/g). This implies a further level of complexity to be taken into consideration (fungal inoculum) when performing similar fungal infection models in different mouse strains.

3 | DISCUSSION

The present results illustrate the existing differences between two multipurpose and widely used inbred and outbred laboratory mouse strains (C57BL/6J and CD1, respectively) regarding susceptibility to ZIGI and systemic *C. albicans* infection models. They show that C57BL/6 mice—considered to be a prototypical Th1-biased mouse strain—present poorer IFN-γ responses and survival outcomes than CD1 mice. Accordingly, C57BL/6J but not CD1 mice benefited from low-dose IFN-γ replacement therapy. This is in agreement with the widely recognised relevance of IFN-γ protection against opportunistic fungal infections, as demonstrated by reports on IFN-γ-deficient (*IFNg* $^{-/-}$) C.57BL/6 mice (Balish, Wagner, Vázquez-Torres, Pierson, & Warner, 1998; Baltazar et al., 2014; Souto et al., 2003; Zhou, Gault, Kozel, & Murphy, 2007).

Strain-specific susceptibility to experimental models of bacterial (De La Cruz Domínguez-Punaro, Segura, Radzioch, Rivest, & Gottschalk, 2008) and fungal (Mazur-Bialy, Majka, Wojtas, Kolaczkowska, & Plytycz, 2011; Zaragoza et al., 2007) infection have been reported.

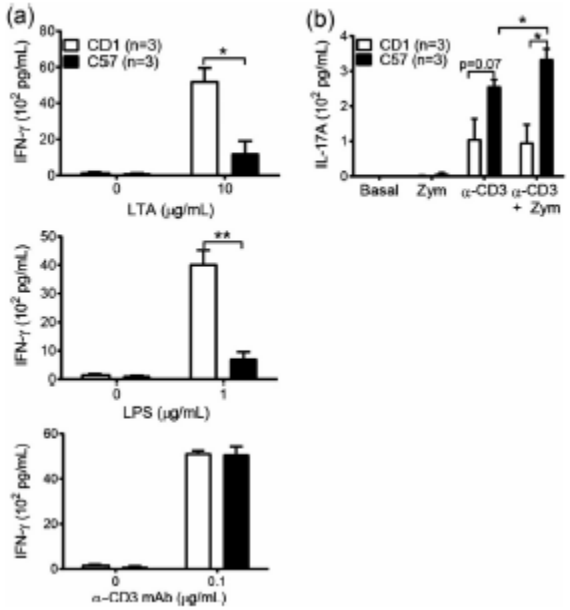

FIGURE 3 CD1 and C57BL/6J splenocytes differ in their interferon (IFN)-γ and IL-17A responses following ex vivo exposure to different stimulatory conditions. (a) IFN-γ levels in supernatants from total splenocytes (5×10^6 cells/ml) of CD1 ($n = 3$) and C57BL/6J ($n = 3$) mice exposed to anti-CD3 monoclonal antibody (0.1 μg/ml), LPS (1 μg/ml), or LTA (10 μg/ml) for 18 h. (b) Splenocytes from C57BL/6 and CD1 mice (5×10^6 cells/ml) were exposed for 18 h either to zymosan (75 μg/ml), anti CD3 (0.1 μg/ml) or a combination of both, and supernatants analysed for the presence of IL-17A. *, $p < 0.05$ (Student *t* test)

In line with our data, a higher proportion of titan cells—a virulence factor in *C. neoformans* infection—in C57BL/6J versus CD1 mice has been recently reported (García-Barbazán et al., 2016). The *C. neoformans*-infected CD1 mice mounted a Th1-type response (higher IFN-γ, TNF-α, and IL-17 levels in afflicted organs) in comparison to C57BL/6J mice. We have observed a similar scenario in the systemic ZIGI and the *C. albicans* infection models. Accordingly, replacement therapy with low IFN-γ amounts (0.2 μg/kg; equivalent to 0.0006 mg/m^2 if used a conversion factor of 1 mg/kg = 3 mg/m^2 in mice) allowed ZIGI- or *C. albicans*-challenged C57BL/6J mice to match survival rates of untreated CD1 mice (Figure 4a and 4b). In contrast, no benefit was observed on ZIGI- or *C. albicans*-challenged CD1 or Balb/c mice infused with similar IFN-γ doses (Figure 5a and 5b, and Figure S4). A protective effect of IFN-γ replacement infusion as an adjunctive therapy in invasive *Candida* and/or *Aspergillus* infections has been reported in humans (Delsing et al., 2014; van de Veerdonk, Kullberg, & Netea, 2012) at ≈≥0.05 mg/m^2 dose, that is, ≈≥100 folds higher than in our experiments (0.0006 mg/m^2), suggesting that although C57BL/6J mice benefit from low IFN-γ doses, CD1 needs higher ones. Indeed, infusion of *C. albicans*-infected CD1 mice with higher IFN-γ doses (10 μg/kg; equivalent to 0.03 mg/m^2) increased their survival rates (Figure 5c). In the light of these findings, fungal infected patients may be classified into high or low IFN-γ responders following in vitro zymosan stimulation. IFN-γ low-responder patients may benefit from lower IFN-γ replacement regimes, minimising cytokine adverse effects, and IFN-γ high-responder patients may require intensive IFN-γ regimes and/or alternative therapeutic approaches.

The finding that both C57BL/6J and CD1 cells secreted similar IFN-γ amounts when subjected in vitro to mAb-induced TCR/CD3 crosslinking makes unlikely intrinsic *IFN-γ* gene defects behind in vitro and in vivo differences following zymosan (a well-known TLR2 ligand) challenge. This contrast with the relative lower IL-17A production observed for CD1 splenocytes under the same in vitro stimulatory conditions, which might be indicative of defective *IL-17a* gene expression to be further investigated. Importantly, deficient in vitro IFN-γ production by C57BL/6J cells was also observed following stimulation of TLR2 and TLR4 receptors with their bacterial ligands

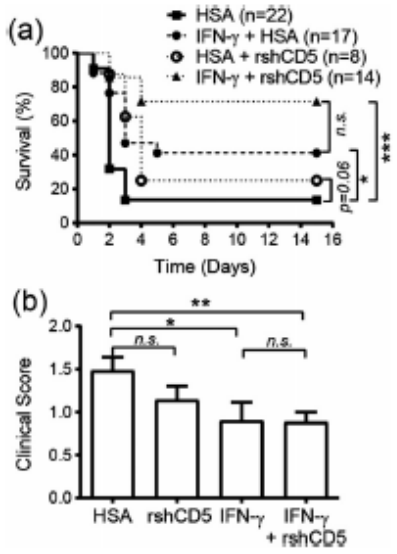

FIGURE 4 Effect of exogenous interferon (IFN)-γ and/or rshCD5 infusion in C57BL/6J mice undergoing zymosan-induced generalised inflammation. C57BL/6J mice *ip* challenged with zymosan (700 mg/kg) were infused 1 h later with human seroalbumin (HSA; $n = 22$), IFN-γ plus HSA ($n = 17$), rshCD5 plus HSA ($n = 8$) or IFN-γ plus rshCD5 ($n = 14$). Survival was recorded daily for 2 weeks and compared to the human seroalbumin (HSA)-treated control group. *, $p < 0.05$; ***, $p < 0.001$ (Log-rank Mantel-Cox test)

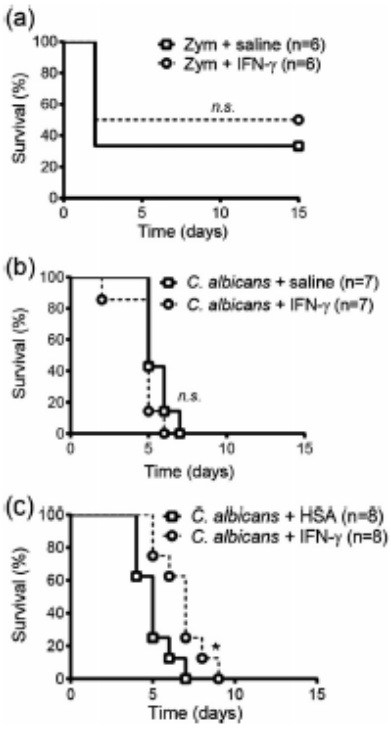

FIGURE 5 Effect of interferon (IFN)-γ infusion on CD1 mice undergoing zymosan-induced generalised inflammation or *Candida albicans* infection. (a) CD1 mice *ip* challenged with zymosan (700 mg/kg) were infused 1 h later with saline ($n = 6$), or IFN-γ (0.2 μg/kg $n = 6$). (b) CD1 mice *iv* infected with *C. albicans* (2.86×10^6 CFU/gr) were infused 18 h later with saline ($n = 7$), or IFN-γ (0.2 μg/kg; $n = 7$). Survival was recorded daily for 2 weeks and compared between groups. ns, not significant (Log-rank Mantel-Cox test). (c) Percentage survival overtime of CD1 mice *iv* infected with 2.86×10^6 CFU/gr treated with IFN-γ (10 μg/kg) at 9 h post infection ($n = 8$) or with HSA (1.25 mg/kg $n = 8$). *, $p < 0.05$ (Log-rank Mantel-Cox test)

LTA and LPS, respectively, which share similar signalling (MyD88-dependent) pathways (Kawasaki & Kawai, 2014). Thus, quantitative or qualitative differences between C57BL/6J and CD1 mice with regard to TLR2/4 expression and/or signalling should be investigated as a cause for strain-specific behaviour. Interestingly, we found statistically significant lower surface levels (as measured by Mean Fluorescence Intensity) for Dectin-1, TLR2, and TLR4 on dendritic cells from C57BL/6J versus CD1 mice (Figure S3), which would account for the higher levels of IFN-γ observed upon fungal (and bacterial) challenge,s in CD1 versus C57BL/6 mice, in the absence of intrinsic *IFN-γ* expression defects.

IFN-γ and IL-17 protection against fungal infections is well-documented (Dambuza, Levitz, Netea, & Brown, 2017; van de Veerdonk et al., 2012), though it is a question of debate, which Th1 or Th17 host response is more efficient in particular fungal infection scenarios, for example, systemic versus mucocutaneous infection (Netea et al., 2015). Interestingly, our in vitro assays show that, contrary to IFN-γ responses, stimulated splenocytes from C57BL/6J mice produced higher IL-17A levels than those from CD1 mice (Figure 3b).

This conserved IL-17A response in C57BL/6J would (a) support an inhibitory effect of IFN-γ on Th17 responses via STAT-1 induction (Hu & Ivashkiv, 2009) and (b) protect C.57BL/6 mice from fungal infections under certain circumstances such as our milder *Candida* infection model (2.86×10^5 CFU/gr instead of 2.86×10^6 CFU/gr; Figure 6). Thus, the higher susceptibility of C.57BL/6J versus CD1 mice to systemic fungal infection would not be absolute but depend on other factors (e.g., level or site of infection).

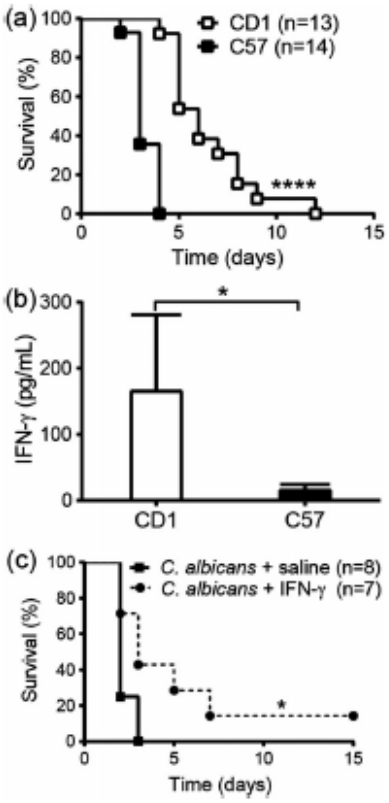

FIGURE 6 C57BL/6J mice are more susceptible than CD1 mice to fungal sepsis induced by a high *Candida albicans* inoculum. (a) Percentage survival overtime of CD1 (n = 13) and C57BL/6J (n = 14) mice iv infected with *C. albicans* (2.86×10^6 CFU/gr). ****, $p < 0.001$ (Log-rank Mantel–Cox test). (b) Serum interferon (IFN)-γ levels from CD1 (n = 6) and C57BL/6J (n = 7) mice at day 2 post-infection. *, $p < 0.05$ (2-tailed Mann–Whitney test). (c) Percentage survival overtime of C57BL/6J mice iv infected with *C. albicans* (2.86×10^6 CFU/gr) and treated with IFN-γ (0.2 μg/kg) at day 1 post infection (n = 7) or with vehicle (n = 8). *, $p < 0.05$. **, $p < 0.001$ (Log-rank Mantel–Cox test)

In conclusion, the present work highlights the importance of mouse strain selection for studying host immune responses to fungal infection and their impact on therapy. Our work also opens new patient research to design personalised antifungal immunotherapies.

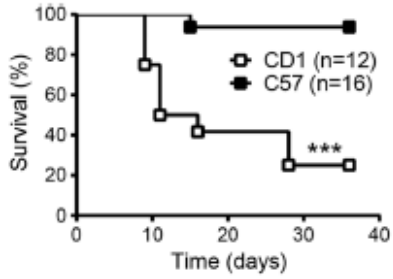

FIGURE 7 CD1 mice are more susceptible than C57BL/6J mice to fungal sepsis induced by a low *Candida albicans* inoculum. Percentage survival overtime of CD1 (n = 12) and C57BL/6J (n = 16) mice iv infected with 2.86×10^5 CFU/gr. ***, $p < 0.001$ (Log-rank Mantel–Cox test)

4 | EXPERIMENTAL PROCEDURES

4.1 | Mice

Male 8–10 weeks old C57BL/6J, CD1 (ICR), and BALB/c mice used in our experiments were purchased from Charles River Laboratories (France). Mice were housed in accordance with the Generalitat of Catalunya Health guidelines. All animal procedures were approved by the University of Barcelona Animal Experimentation Ethical Committee.

4.2 | Proteins

Production of purified rshCD5 protein (in phosphate buffered saline plus 10% glycerol, pH 7.4; PBS/glycerol) was carried out as previously reported (Santas et al., 2004) but using stably transfected SURE CHO-M Cell line clones from the Selexis SURE technology Platform (Geneva, Switzerland) and subjecting their serum-free supernatants to size-exclusion chromatography protocols developed at PX'Therapeutics (Grenoble, France). Mouse IFN-γ and HSA were purchased from Genscript (Z02916, Piscataway, USA) and Sigma Aldrich (A9731, St. Louis, MO, USA), respectively.

4.3 | Cells

Peritoneal cells were obtained by peritoneal lavage with 3 ml of saline (B/Braun). Splenocytes were isolated by incubating spleens for 15 min at 37°C in 4 ml of Hank's balanced salt solution medium (HBSS; LabClinics), containing 1 mg/ml collagenase D (Roche) and 0.1 mg/ml DNAse I (Roche Diagnostics), followed by disaggregation through a 40-μm cell strainer with a syringe plunger. After a first wash with 10 ml of Hank's balanced salt solution (HBSS) plus 10% fetal bovine serum (FBS; BioWest) supernatant was discarded and cells were incubated at room temperature with 3 ml of red blood cell lysis solution buffer (eBioscience) for 5 min. After a second wash, cells were

counted and adjusted at the desired concentration with RPMI 1640 with L-glutamine medium (Lonza) plus 10% FBS, 10 mM Hepes (Life Technologies), 1 mM sodium pyruvate (Gibco), 50-μM 2-Mercaptoethanol (Merck) and 100 U/ml penicillin, 100 μg/ml streptomycin (Lonza).

4.4 | Mouse models of fungal sepsis

The ZIGI model of fungal-like sepsis was induced in CD1 and C57BL/6J mice by ip injection of zymosan (100–1,000 mg/kg; Z4250, Sigma-Aldrich) in 0.9% saline plus buprenorphine (0.1 mg/kg). In some experiments, 1-h post-ZIGI-induction (+1 h) mice were injected either with IFN-γ (0.2 μg/kg), rsNCD5 (0.7 mg/kg), HSA (0.7 mg/kg in PBS/glycerol) alone or in combination. Animals were monitored daily for 2 weeks, and survival, weight lost, and clinical score evaluated. The clinical score was calculated as the mean of the individual scores (lethargy, diarrhoea, conjunctivitis, and fur appearance), each of them rated from 0 to 3.

The mouse model of fungal sepsis induced by *C. albicans* (SC5314; ATCC MYA-2876) infection was performed by iv (tail vein) injection of 2.86×10^6 or 2.86×10^5 CFU/gr. In some experiments 9 h or 1 day post *C. albicans* infection mice were injected either with IFN-γ (0.2 or 10 μg/kg in saline; ip or iv, respectively), HSA (1.25 mg/kg in saline; iv) or saline. Survival and body weight loss were monitored overtime.

4.5 | Ex vivo cell stimulation assays

Unfractionated peritoneal and spleen cells were adjusted at the desired concentration (2×10^6 and 5×10^6 cells/ml, respectively) in a 48- or 96-well plates in RPMI 1640 with L-glutamine medium (supplemented as described above) containing zymosan (0–200 μg/ml), LPS (1 μg/ml; L2630, Sigma Aldrich), LTA (10 μg/ml; L2515, Sigma Aldrich), anti-CD3 (0.1 μg/ml; 70-0031-M001, TONBO), anti-IL-12 antibody (1–5 μg/ml; C17.8, BD Biosciences), or rsNCD5 (15 μg/ml), either alone or in combinations.

4.6 | Cytokine assays

Mouse IL-12 (p70), IL-6, TNF-α, IFN-γ, IL-10, IL-4, GM-CSF, IL-1β, IL-5, IL-2, and IL-17A cytokine levels in serum or culture supernatants of splenocytes and peritoneal cells were determined by ELISA (BD OptEIA-Mouse ELISA Sets, BD Biosciences Pharmingen) or Mouse cytokine magnetic 10-plex panel (Invitrogen) following the manufacturer's instructions.

4.7 | Statistics

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA). For survival analysis, the log-rank (Mantel–Cox) test was used. Depending on the characteristics of the values, the statistical analysis was assessed by Student's *t* test (parametric values) or Mann–Whitney *U* test (nonparametric values). Differences were regarded as significant when $p < 0.05$.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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