VNIVERSITAT DE VALÈNCIA

ROLE OF HEMATOPOIETIC STEM AND PROGENITOR CELLS IN TRAINING IMMUNITY AGAINST INFECTION

Cristina Bono Tapp

Doctoral Thesis

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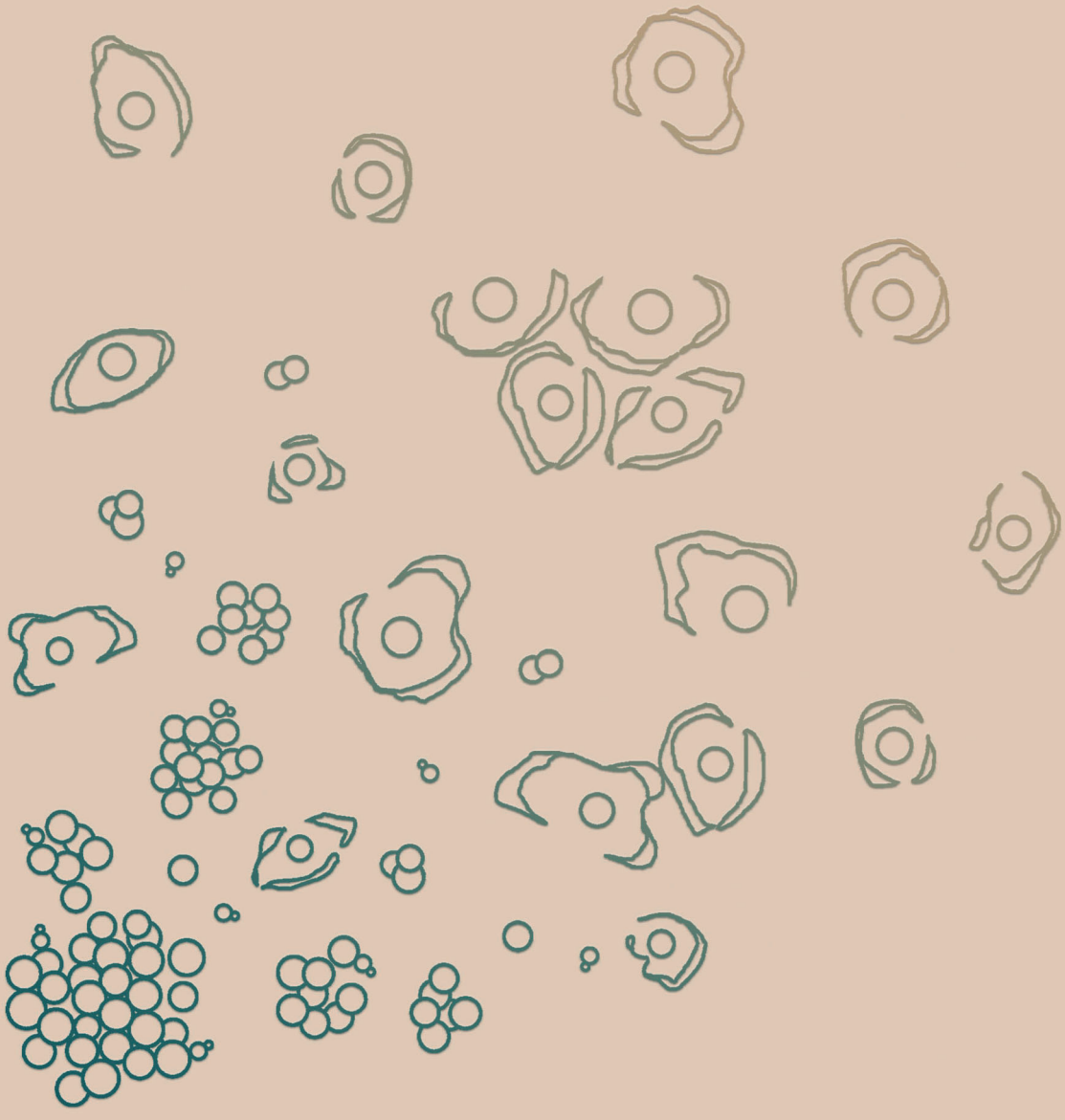

Doctorado en Biomedicina y Biotecnología

Role of hematopoietic stem and progenitor cells in training immunity against infection

Cristina Bono Tapp

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Valencia, Octubre 2022

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CERTIFICAN:

Que el trabajo presentado por Cristina Bono Tapp, titulado: "**Role of hematopoietic stem and progenitor cells in training immunity against infection**" ha sido realizado en el Departamento de Microbiología y Ecología de la *Universitat de València*, bajo nuestra dirección y asesoramiento.

Concluido el trabajo experimental y bibliográfico, autorizamos la presentación de esta Tesis Doctoral, para que sea juzgada por el tribunal correspondiente.

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		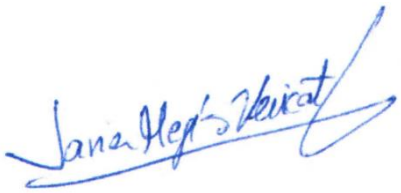
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ABBREVIATIONS

ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guérin
C/EBP	CCAAT-Enhancer-Binding Protein
CARD9	Caspase Recruitment Domain-Containing Protein 9
CD	Cluster of Differentiation
cDNA	Complementary DNA
CDP	Common Dendritic Progenitor
CFSE	Carboxyfluorescein Succinimidyl Ester
CFU	Colony Forming Unit
CLP	Common Lymphoid Progenitor
CLR	C-type Lectin Receptor
cMoP	Common Monocyte Progenitor
CMP	Common Myeloid Progenitor
CR3	Complement Receptor 3
CSF	Colony-Stimulating Factor
CTL	Cytotoxic T Lymphocyte
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DC-SIGN	DC-Specific-ICAM-Grabbing Non-Integrin
DEG	Differentially Expressed Gene
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	Fluorescence-Activated Cell Sorting

FBS	Fetal Bovine Serum
FcR	Fragment Crystallizable Region Receptor
FDR	False Discovery Rate
G-CSF	Granulocyte Colony-Stimulating Factor
GEO	Gene Expression Omnibus
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Granulocyte-Monocyte Progenitor
GP	Granulocyte Progenitor
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic Stem and Progenitor Cell
i.p.	Intraperitoneally
i.v.	Intravenously
IFN	Interferon
IKK	Inhibitor of NF-κB Kinase
IL	Interleukin
ILC	Innate Lymphoid Cell
IRF	IFN-Regulatory Factor
ITAM	Immunoreceptor Tyrosine-Based Activating Motif
KO	Knockout
Lin	Lineage
LKS$^-$	Lin$^-$ c-kit$^+$ Sca-1$^-$
LKS$^+$	Lin$^-$ c-kit$^+$ Sca-1$^+$
lncRNA	Long Noncoding RNA
LPS	Lipopolysaccharide
LT-HSC	Long-Term Repopulating HSC

MBL	Mannan-Binding Lectin
M-CSF	Macrophage Colony-Stimulating Factor
MDP	Monocyte-Dendritic Progenitor
MEP	Megakaryocyte-Erythrocyte Progenitor
MFI	Mean Fluorescence Intensity
Mincle	Macrophage Inducible Ca^{2+}-dependent Lectin
MIP	Macrophage Inflammatory Protein
moDC	Monocyte-Derived DC
MP	Monocyte Progenitor
MPP	Multipotent Progenitor
MR	Mannose Receptor
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin
MyD88	Myeloid Differentiation Factor 88
NET	Neutrophil Extracellular Trap
NF-κB	Nuclear Factor kappa B
NK	Natural Killer
NLR	NOD-Like Receptor
NOD	Nucleotide-Binding Oligomerization Domain
OD	Optical Density
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PI3K	Phosphoinositide 3-Kinase
PLM	Phospholipomannan
PMA	Phorbol 12-Myristate 13-Acetate

PRR	Pattern Recognition Receptor
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RBC	Red Blood Cell
RIG-I	Retinoic Acid Inducible Gene I
RLR	RIG-I-Like Receptor
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
ROS	Reactive Oxygen Species
RT	Room Temperature
SCF	Stem Cell Factor
scRNA-seq	Single-Cell RNA Sequencing
SD	Standard Deviation
ST-HSC	Short-Term Repopulating HSC
Syk	Spleen Tyrosine Kinase
TBK1	TANK-Binding Kinase 1
TC	Tissue Culture
TCA	Tricarboxylic Acid
Th	T helper
TIR	Toll/IL-1-Receptor
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
Treg	T regulatory
TRIF	TIR-Domain-Containing Adapter-Inducing IFN-β
WT	Wildtype

CONTENTS

Abstract

During an infection, haematopoiesis is altered to increase the output of mature myeloid cells to fight off the pathogen. It has been demonstrated that the detection of pattern recognition receptor agonists by hematopoietic stem and progenitor cells (HSPCs) induces their differentiation towards mature myeloid cells with modified phenotypes.

In the present PhD thesis, we show that an *in vitro* transient exposure of HSPCs to live *Candida albicans* cells is sufficient to induce a trained phenotype of the macrophages they produce in a dectin-1- and TLR2-dependent manner. Additionally, we use an HSPC transplantation mouse model to demonstrate that the direct interaction of β-glucans and their receptor (dectin-1) on HSPCs *in vivo* induces myeloid differentiation. Functionally, macrophages derived from HSPCs exposed to β-glucan *in vivo* produce higher levels of inflammatory cytokines, demonstrating that trained immune responses, already described for monocytes and macrophages, also take place in HSPCs. Using a similar *in vivo* model of HSPC transplantation, we demonstrate that inactivated yeasts of *C. albicans* induce differentiation of HSPCs through a dectin-1- and MyD88-dependent pathway. Regarding the mechanisms initiating myeloid development, we found that soluble factors produced following exposure of HSPCs to dectin-1 agonists act in a paracrine manner to induce differentiation towards trained macrophages, while TLR2 stimulation of HSPCs promotes myelopoiesis directly by initiating an MyD88-dependent signalling that involves a combined activation of the transcription factors PU.1, C/EBPβ and IRF7 driven by TBK1 and PI3K/mTOR.

Moreover, we used a *C. albicans* live vaccine mouse model to reveal the mechanisms that drive trained immunity *in vivo*. We show that vaccination protects mice against a secondary infection and increases the number of bone marrow and, especially, splenic trained monocytes. Moreover, vaccination expands and reprograms HSPCs early during infection and mobilizes them transiently to the spleen to produce trained macrophages. Besides being primed for myeloid cell production, trained HSPCs are also reprogrammed to produce a greater amount of proinflammatory cytokines in response to a second challenge and their adoptive transfer is sufficient to protect mice against reinfection. Mechanistically, autocrine GM-CSF activation of HSPCs is responsible for the trained phenotype and essential for the vaccine-induced protection.

Taken together, these findings reveal a fundamental role for HSPCs in sensing pathogens during infection and contributing to host protection, opening new avenues for disease prevention and treatment.

Introduction

1. Immune response to *Candida albicans*

Candida albicans is a diploid unicellular fungus included in the more than 300 species belonging to the *Candida* genus. It normally grows as a yeast, but under certain environmental conditions, it can form germinal tubes to develop hyphae. However, other two fungal morphologies have also been described: pseudohyphae (chains of elongated yeasts cells) and chlamydospores (thick-walled spore-like structures generated from hyphae under stress conditions), thereby being considered a polymorphic fungus (Calderone, 2012).

Despite not being ubiquitously present in the environment, *C. albicans* is frequently encountered as a harmless commensal in the skin, the mucosal surfaces of oral, pharyngeal and vaginal cavities and the gastrointestinal tract of humans and other mammals (Kumamoto *et al.*, 2020). Therefore, it forms part of the residing normal microbiota, kept in balance with other members of the local microbiome and playing an important role in immune homeostasis and pathology during disease (Wheeler *et al.*, 2017). However, in individuals with compromised immunity, *C. albicans* is able to invade other body tissues from its normal habitat, display pathogen characteristics and develop an infection, thus acting as an opportunistic pathogen. Yet, in healthy individuals, this fine balance established between the commensal state and infection can also be easily disrupted due to changes in the host's microbiota (as a result of antibiotic intake, for example), alterations in the host's immune response (taking place during, for example, stress, an infection by another microorganism or immunosuppressive therapy), or changes in the local environment (for instance, hormonal changes) (Calderone, 2012).

Resistance to *C. albicans*-caused infections requires the coordinated action of the innate and adaptive immune responses. Upon fungal detection by immune and nonimmune cells, an inflammatory response, mediated by the innate immune system, initially activates defence mechanisms. This triggers and directs the adaptive immune responses which, in turn, regulate signals from the innate system. (Romani, 2011).

1.1. *C. albicans* as an opportunistic pathogen

Candidiasis is a broad term referring to any infection caused by fungal species of the *Candida* genus. These are found among the most common mycoses and range from superficial mucocutaneous candidiasis to severe invasive infections, depending on the underlying host defects (Calderone, 2012). In superficial candidiasis, the infection is developed locally, affecting the skin or mucosal surfaces. These infections tend to be common, are often chronically manifested and, in most cases, are resolved naturally or can be easily cured with antifungal treatment. On the other hand, systemic or invasive candidiasis is diagnosed when the microorganism is found in the bloodstream (candidemia) or when the infection has extended and affects internal organs. Their frequency has considerably increased over the las decades due to an expanding immunocompromised risk population and they have a high mortality rate associated, as, in this case, both, the diagnosis and treatment, are much more complicated (Pfaller & Diekema, 2007).

Invasive infections by *Candida* species mostly have an endogenous origin, as the agent causing the infection are yeasts forming part of the own's patient microbiota that translocate from the gut into the bloodstream enabling them to reach other host's organs. However, in a significant number of cases, the origin of the infection is exogenous, and it is produced following colonization of catheters or intravascular devices by the fungus. There are several well-described risk factors that favour the development of these kind of infections (Pappas *et al.*, 2018). Immunosuppressive diseases, neutropenia, an advanced age and a deteriorating clinical condition due to underlying diseases are among the most common host-related risk factors, while long hospital or intensive care unit stays also increase the chances of developing invasive candidiasis (Yapar, 2014).

At least 15 distinct *Candida* spp. can cause human disease, but the majority of invasive infections are caused by five pathogens: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and, to a lesser extent, *C. krusei*. However, despite an increase in diagnoses of non-*albicans* candidemia over the last decades, *C. albicans* continues to be the most prevalent species included in the *Candida* genus causing disease (Pappas *et al.*, 2018). Therefore, studying the factors that make *C. albicans* an opportunistic pathogen enabling the commensal-to-pathogen conversion are of high relevance.

1.1.1. Fungal cell wall

The *C. albicans* cell wall is its outermost cellular structure, responsible for protecting the cell from environmental stressful conditions, such as osmotic changes, dehydration or temperature changes, but also from protecting the cell from the host's immune defence, hence playing a major role in the pathogenicity of the fungus (Arana *et al.*, 2009). Overall, it is a complex and dynamic structure made of glucan, chitin and protein (Figure 1).

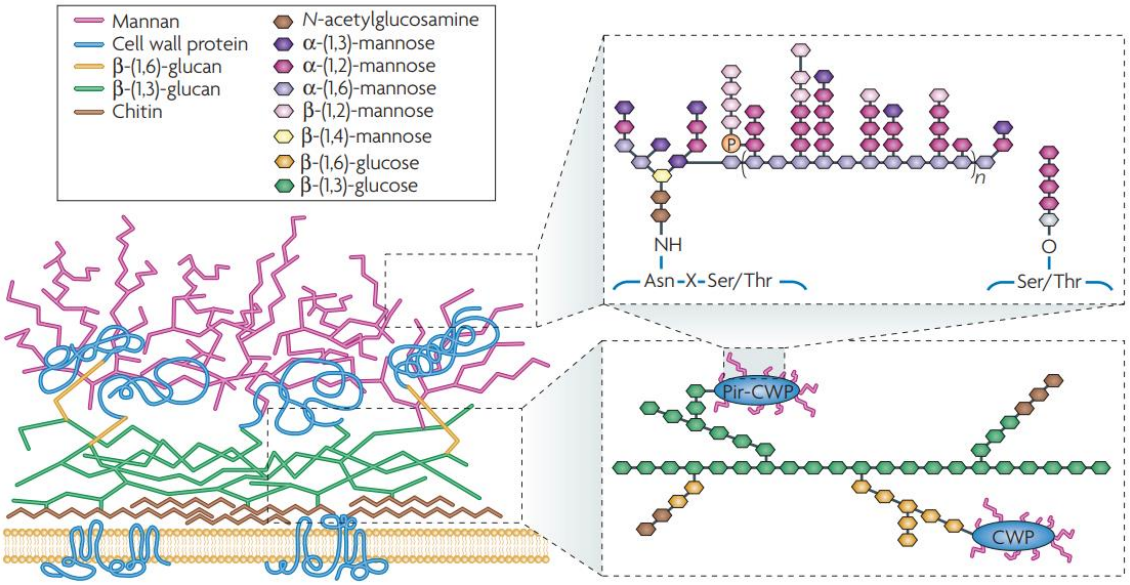

FIGURE 1. *C. albicans* cell wall structure. The major components of *C. albicans* cell wall as well as their arrangements are shown. β-glucans and chitin are the main structural components, and are located towards the inner part of the cell wall. The outer layer is enriched with cell wall proteins (CWP) linked to mannan through N- or O-glycosidic links. These mannoproteins are attached to the microfibrillar skeleton via GPI remnants to β-(1,6)-glucan or, in the case of Pir-CWP, are directly linked to β-(1,3)-glucan. The insets show the structure of the glucan and mannan components (Netea *et al.*, 2008).

The inner layer of this cell wall is based on a core structure of β-(1,3)-glucan covalently linked to chitin (a β-(1,4)-linked polymer of N-acetylglucosamine) in its innermost part and to β-(1,6)-glucan towards the outside. These polymers create a three-dimensional network of microfibrils (responsible for the cell wall's rigidity and morphology) by forming hydrogen bonds between nearby polysaccharide chains (Ruiz-Herrera *et al.*, 2006). Chitin and β-glucan are mostly considered to be found close to the cell membrane, but differences in their content, structure and exposure to the cell surface, have been described between budding yeasts and hyphae, which has an impact on their immunological properties (Lowman *et al.*, 2014).

In addition to the glucan and chitin skeleton, the *C. albicans* cell wall contains an outer matrix that mainly comprises glycosylated proteins. These consist of cell wall proteins covalently associated with mannan (a complex structure made up of polymers of mannose) through N-glycosidic or O-glycosidic links (mannoproteins). Cell wall mannoproteins are attached to the microfibrillar core structure either through glycosylphosphatidylinositol (GPI) remnants to β-(1,6)-glucan, or constitute proteins with internal repeat domains (Pir) that are directly associated to β-(1,3)-glucan (Ruiz-Herrera *et al.*, 2006).

Although to a lesser extent, lipids are also present in the fungal cell wall forming part of the phospholipomannan complex (PLM), a type of extensively glycosylated glycosphingolipid with hydrophilic properties that plays a relevant role in host interactions (Fradin *et al.*, 2015).

1.1.2. Virulence factors

In addition to the host's condition, the fungus pathogenicity also depends on the expression of a set of virulence factors, some of which take part in initiating the infection while others help with its spreading (Calderone, 2012). These virulent traits are often environmentally regulated and include yeast-to-hypha (and hypha-to-yeast) transition, phenotypic switching, the secretion of hydrolytic enzymes and candidalysin, the expression of adhesins and invasins, the ability to develop biofilms, antigenic variability and immunomodulation of host responses. In addition, its metabolic adaptability allows *C. albicans* to quickly adjust to different host niches and cause infection (Mayer *et al.*, 2013; Talapko *et al.*, 2021).

The importance of the morphological transition from a budding yeast to a filamentous hyphal form is highlighted by the fact that only the yeast form can be found associated to the commensal state, while the hyphal form is always connected to a pathologic condition (Calderone, 2012). Additionally, the expression of other virulence factors is coregulated with this transition, rendering hyphae more prepared to evade the host's immune defences and induce infection. In fact, monomorphic mutants unable to form hyphae exhibit impaired virulence in mice (Lo *et al.*, 1997). Nevertheless, both morphologies have been suggested to have an important role in pathogenicity: while hyphae, more invasive,

contribute to host tissue damage, dissemination of the fungus through the bloodstream requires yeast formation from preformed filaments (Uppuluri *et al.*, 2010).

Moreover, *C. albicans* can perform phenotypic switching, a process consisting in reversible changes among different genetic variants, easily distinguishable from one another by the morphology of the colonies they form. These changes are caused by genome rearrangements and have a relevant effect in the fungus' virulence. The best studied switching system in *C. albicans* is the transition between white and opaque colonies, in which homozygous strains for the mating type locus are capable of switching from the normal yeast form (white) to a bigger and elongated cell form (opaque). These opaque cells are less resistant than white cells, but are able to generate genetic variants better adapted for evading the host's immune defences (Soll, 2009).

C. albicans secretes a variety of hydrolytic enzymes, including proteases, phospholipases and lipases, that can damage host cell structures causing an impairment in their function and, therefore, favouring infection. Among all of these, secreted aspartic proteases (SAPs) stand out for being the best characterized ones. SAPs are proteolytic enzymes capable of degrading proteins belonging to the host's immune system, such as immunoglobulins or the ones forming part of the complement system, and they can hydrolyse mucin, which makes it easier for the fungus to penetrate gastrointestinal mucosa (Naglik *et al.*, 2004). Additionally, the hyphal form of *C. albicans* can also produce candidalysin, a cytolytic peptide toxin capable of directly damaging the cell membrane by creating pore-like structures. However, despite being considered crucial to establishing a systemic infection, candidalysin also acts as an immunomodulatory molecule that leads to the initiation of a protective response upon being sensed by the host (Naglik *et al.*, 2019).

Another factor contributing to the pathogenic potential of *C. albicans* is its ability to adhere to and invade epithelial cells. Proteins and mannoproteins are the main cell wall components responsible for adhesion not only to host cells, but also to other microorganisms or abiotic surfaces, and are broadly referred to as adhesins. Following adhesion to host cells, *C. albicans* can invade them through induced endocytosis, by expressing invasins that trigger the fungal cell's engulfment into the host cell, or through fungal-driven active penetration (Höfs *et al.*, 2016).

Moreover, *C. albicans* can form biofilms (structured microbial communities attached to a surface and surrounded by a self-produced extracellular matrix) on both, abiotic and biotic surfaces. Biofilm formation on catheters or prosthesis, or on mucosal cell surfaces are, in fact, the reason behind most infections. What is more, by growing forming biofilms, *C. albicans* acquires a greater resistance against the most commonly used antifungal treatments, making it even more difficult to cure the infection (Tsui *et al.*, 2016).

1.2. Innate immune response

Epitheliums constitute the first line of defence against fungal infections, mainly because they provide a physical barrier between the internal environment and the pathogens, but also because members of their normal microbiota, by competing with pathogens for nutrients and surfaces, can also influence fungal colonization and antifungal immune responses. Depending on the degree of tissue damage and invasion by *C. albicans*, epithelial cells activate a variety of defence mechanisms, including secretion of inflammatory cytokines and chemokines and the production of several antifungal peptides, such as β-defensins, cathelicidin or alarmins, that are able to prevent fungal growth and hyphae formation, thereby maintaining the commensal status of *C. albicans* (Naglik *et al.*, 2014). However, once this first barrier has been overcome, fungal cells start invading epithelial tissues and further activation of immune responses take place (Figure 2).

The cell wall of *C. albicans* is a potent inducer of all three complement activation pathways: C3b targets the cell wall, initiating the alternative pathway, and mannans can be recognized by mannan-binding proteins (MBL: mannan-binding lectin) that activate the lectin pathway, or by antibodies, activating the classical pathway (Kozel, 1996; Speth *et al.*, 2008). The activation of either of these pathways results in the opsonization of *C. albicans* cells, thus promoting their phagocytosis by neutrophils and macrophages. Additionally, this also leads to the generation of byproducts that initiate an inflammatory response and enhance the antimicrobial activity of phagocytes (Cheng *et al.*, 2012). Murine studies have highlighted the role of the complement system in antifungal immunity, as mice lacking some of its components are highly susceptible to invasive candidiasis, despite humans with genetic deficiencies in such components not showing

an increased risk towards systemic *C. albicans* infections (Mullick *et al.*, 2004; Tsoni *et al.*, 2009). Actually, activated complement is unable to induce the direct lysis of *C. albicans* hyphae during an infection, probably because its thick cell wall blocks the formation of the membrane attack complex (Kozel, 1996; Speth *et al.*, 2008). Therefore, killing the fungus during a systemic candidiasis depends mainly on the involvement of myeloid phagocytes (neutrophils and monocytes/macrophages), which use a variety of surface receptors for recognizing pathogen-associated molecular patterns (PAMPs) and opsonins on the fungal surface (see section 1.2.1).

Tissue-resident macrophages not only phagocytize *C. albicans* cells, but also produce inflammatory cytokines (especially tumour necrosis factor(TNF)-α, interleukin(IL)-6, IL-1β and IL-18) and chemokines (mainly CXCL1 and CXCL2) that recruit and activate other immune cells, such as monocytes, neutrophils and natural killer (NK) cells, at the site of infection (Brown, 2011). In fact, early *in vivo* studies in mice showed that clodronate-induced depletion of macrophages results in accelerated fungal proliferation in tissues and an increased mortality, highlighting the importance of these mononuclear phagocytes in antifungal defence (Bistoni *et al.*, 1986; Bistoni *et al.*, 1988). Blood monocytes are drawn to the infected tissues, where they undergo differentiation into proinflammatory macrophages and aid in the pathogen's removal. It has been observed that deficiencies in chemokine receptors (CX3CR1 and CCR2) increase susceptibility to candidiasis in both, mice and humans, as a result of an impaired monocyte recruitment, therefore affecting the concentration of monocyte-derived macrophages in infected tissues (Miramón *et al.*, 2013; Höfs *et al.*, 2016). Upon *C. albicans* phagocytosis by macrophages, phagosomes are transformed into phagolysosomes following their fusion with lysosomes, exposing fungal cells to an acidic pH that promotes the activity of hydrolytic enzymes (including cathepsin) against the pathogen. Phagocytosis also triggers an oxidative burst that involves formation of reactive oxygen species (ROS) via myeloperoxidase and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that have strong oxidative and damaging properties and are critical for killing fungal cells upon their phagocytosis. Besides, phagocytes also express nitric oxide-generating enzymes, like the inducible nitric oxide synthase (iNOS), which is involved in the production of reactive nitrogen species (RNS) and also contribute to *C. albicans* phagocytized cells killing (Aratani *et al.*, 2002; Brown, 2011; Miramón *et al.*, 2013).

On the other hand, neutrophils are recruited to the site of infection by chemokines released by epithelial cells and tissue-resident macrophages upon interaction with *C. albicans*. Neutrophils are the most effective killers of *C. albicans* and constitute the only immune cells bearing the ability to inhibit hyphae development from yeast cells. In fact, neutropenia is a major risk factor for invasive candidiasis, as has been demonstrated in several mouse models (Brown, 2011; Miramón *et al.*, 2013; Lionakis, 2014). Intracellular killing of *C. albicans* by neutrophils consists in the fusion of phagosomes with preformed cytosolic granules containing a wide variety of antimicrobial proteins (including elastase, lysozyme, myeloperoxidase, lactoferrin, gelatinase and defensins, among others) and does not entail dramatic pH changes. Two independent pathways have been defined for this *C. albicans* killing by neutrophils: a ROS-dependent mechanism, required for clearance of opsonized *C. albicans* cells that depends on the FcγR (immunoglobulin-γ fragment crystallizable region receptors) pathway, and a ROS-independent pathway involved in killing of non-opsonized fungal cells that involves CR3 (complement receptor 3) engagement and CARD9 (caspase recruitment domain-containing protein 9) ligation (Gazendam *et al.*, 2014) (these signalling pathways are detailed in section 1.2.1). Alternatively, the contents of neutrophil cytosolic granules, as well as some ROS species, can also be discharged into the extracellular environment (degranulation) to eliminate fungal cells that have not been phagocytosed. In addition, neutrophils also use another extracellular mechanism to deal with the pathogen: the release of chromatin containing antimicrobial proteins (such as calprotectin), known as NETs (neutrophil extracellular traps). These NETs bind to and neutralize hyphae, providing a way to cope with this fungal morphotype, as it is too large to be efficiently phagocytosed (Urban *et al.*, 2006). It has been demonstrated that NET formation induces neutrophil degranulation and it can also trigger changes in the fungal cell wall architecture that enhance immune recognition by host receptors (Hopke *et al.*, 2016).

Lastly, innate lymphoid cells (ILCs) have also emerged as important effectors of innate immunity. Among them we find NK cells, which are rapidly recruited to the site of infection alongside neutrophils and inflammatory monocytes and initiate secretion of interferon(IFN)-γ and other cytokines that activate phagocytic cells and contribute to inducing the adaptive immune response (Gozalbo *et al.*, 2014). Human NK cells are also activated following engulfment of fungal cells, what results in the release of granulocyte-monocyte colony-stimulating factor (GM-CSF), TNF-α and IFN-γ, and in fungal damage,

mainly due to secreted perforin (Voigt *et al.*, 2014). GM-CSF production by activated NK cells in the spleen is also required to boost the *C. albicans* killing capacity of neutrophils in the kidneys and, therefore, to control the infection (Domínguez-Andrés *et al.*, 2017). Other ILCs are also relevant for controlling mucosal infection, such as IL-17-secreting ILCs, which also secrete IL-22, given that both cytokines enhance activation of mucosal antifungal immunity (Gladiator *et al.*, 2013).

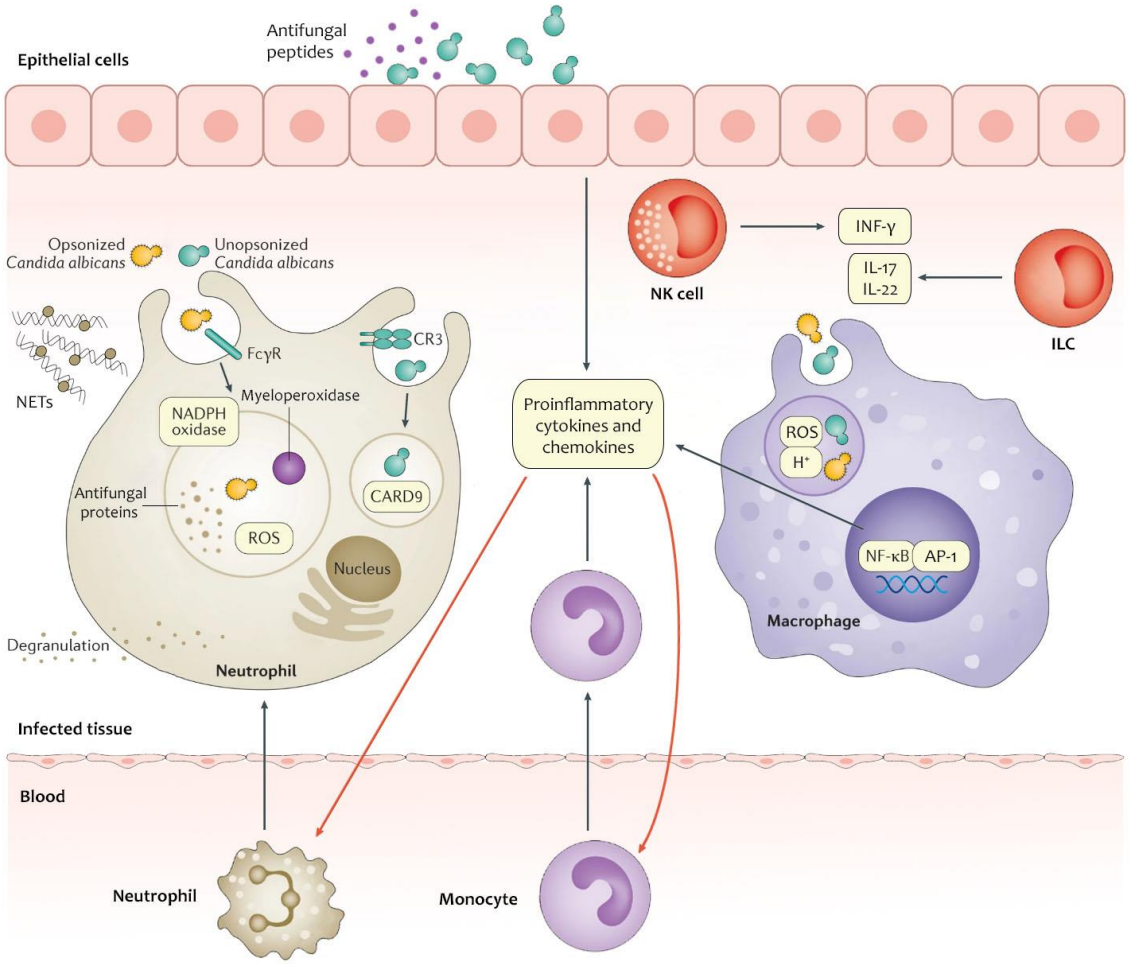

FIGURE 2. Innate defence mechanisms triggered by *C. albicans*. Epithelial cells produce antimicrobial peptides in order to maintain the commensal status of *C. albicans* yeasts. When this fails to avoid fungal expansion, yeast endocytosis by epithelial cells and phagocytosis by resident macrophages induce the secretion of proinflammatory cytokines and chemokines that recruit neutrophils and monocytes to the site of infection (red arrows). Neutrophils fight against the pathogen by phagocytosis and intracellular killing (through both, oxidative and non-oxidative mechanisms), or by extracellular mechanisms, inducing their degranulation and release of NETs. Recruited monocytes produce proinflammatory cytokines and undergo differentiation into inflammatory macrophages. Other innate immune cells, such as NK cells, can contribute to fungal clearance by producing cytokines, including IFN-γ, which in turn activate effector cells. Adapted from Pappas *et al.*, 2018.

1.2.1. PRRs involved in *C. albicans* detection

Phagocytic cells recognize *C. albicans* or its PAMPs, most of which are cell wall components, through a variety of pattern-recognition receptors (PRRs), among which toll-like receptors (TLRs), c-type lectin receptors (CLRs), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid inducible gene 1 (RIG-I)-like receptors (RLRs) stand out (Netea *et al.*, 2008). Following recognition, these PRRs initiate different signalling pathways leading to innate immune response initiation against the pathogen (Figure 3).

TLRs constitute a family of receptors expressed by most immune cell types (neutrophils, monocytes, macrophages, dendritic cells, B- and T-lymphocytes, NK cells) and also by nonimmune cells (such as epithelial and endothelial cells). These receptors are type I membrane proteins characterized by possessing an extracellular domain rich in leucin residues, responsible for recognizing PAMPs, and an intracytoplasmic TIR (toll/IL-1 receptor) domain, whose role is downstream transmission of activation signals. Among TLRs, we can find those located in the plasma membrane and those located in endocytic compartments inside the cells. The former include TLR2 (which forms homodimers and heterodimers with TLR1 and TLR6) and TLR4. They both recognize fungal cell wall mannan: TLR2 binds β-1,2 mannosides present in PLM and mannoproteins while O-linked mannosyl residues accessible on the yeast surface are the TLR4 ligand. Endosomal TLR9, TLR7 and TLR3 sense microbial nucleic acids released following endocytosis and pathogen degradation. Specifically, TLR9 recognizes DNA containing unmethylated CpG motifs, while TLR7, and probably TLR3, recognize fungal RNA (Kawasaki & Kawai, 2014; Yáñez *et al.*, 2021).

Signal transduction in TLRs starts with the recruitment of several intracellular adapters that contain a TIR domain that interacts with the TIR domain in the TLRs. MyD88 (myeloid differentiation factor 88) is the universal adapter molecule, shared by all TLRs, except for TLR3, that initiates the expression of the genes that code for proinflammatory cytokines through activation of the transcription factors NF-κB (nuclear factor kappa B) and AP-1 (activator protein 1). TRIF (TIR-domain-containing adapter-inducing IFN-β) constitutes another adaptor molecule that is critical in the induction of type I IFN genes and type I IFN-inducible genes by TLR3 and TLR4 through the activation of the transcription factor IRF3 (IFN-regulatory factor 3) or by TLR7 and

TLR9 through the activation of IRF7 (Kawasaki & Kawai, 2014). It has been observed that MyD88-deficient mice are extremely susceptible to *C. albicans* infections, thus evidencing that TLR-mediated signalling is essential for host protection against candidiasis (Villamón *et al.*, 2004a). Deficiencies in TLRs, mainly TLR2, and others to a minor extent (TLR4, TLR2 coreceptors, TLR3, TLR7 and TLR9) also cause impaired immune responses to *C. albicans* infection, although the role of some receptors may be redundant (Villamón *et al.*, 2004b; Biondo *et al.*, 2012; Gil *et al.*, 2016).

CLRs are defined by a characteristic protein region with carbohydrate binding properties (C-type lectin domain) that makes them able to recognize specific carbohydrate domains on the fungal cell surface, especially β-glucans and mannans. Dectin-1 is a transmembrane receptor involved in β-glucan recognition whose cytoplasmic domain contains an ITAM(immunoreceptor tyrosine-based activating motif)-like motif. It is the main receptor involved in *C. albicans* phagocytosis and it is expressed by macrophages, monocytes, dendritic cells (DCs) and neutrophils. β-glucans are also recognized by human neutrophils through CR3, a complement (C3b) receptor that consists of an heterodimer of CD18 (β_2 integrin) and CD11b (αM integrin). Dectin-2, mainly expressed by macrophages, neutrophils and dendritic cells, is the functional receptor for N-linked α-mannan residues on the yeast and hyphal cell walls. It forms an heterodimeric complex with dectin-3, another CLR that recognizes α-mannans on the surface of *C. albicans* hyphae. Highly branched N-linked mannosyl chains are also recognized by the mannose receptor (MR) expressed on macrophages, which also participates in recognizing fungal chitin. Another CLR present on myeloid cells (including DCs) is DC-SIGN (dendritic cell-specific-ICAM-grabbing non-integrin), which also recognizes N-linked mannan. Mincle (macrophage inducible Ca^{2+}-dependent lectin), which is expressed on monocytes/macrophages and neutrophils, is another member of the dectin-2 family involved in *C. albicans* recognition, although its ligand has not been well characterized yet. Galectin-3 is an S-type lectin receptor on macrophages that recognizes β-mannan domains present in PLM and mannoproteins. Lastly, MBL is a soluble lectin that binds specific mannose arrangements on fungal surfaces, leading to opsonization and activation of the lectin complement pathway (Osorio & Reis e Sousa, 2011; Hardison & Brown, 2012).

Upon binding to its ligand, dectin-1 induces intracellular signals through its ITAM-like motif resulting in phagocytosis and the activation of the Syk1/CARD9- and Raf-1-

mediated signalling pathways that lead to ROS production and NF-κB-induced inflammatory cytokine secretion. Additionally, the Syk1 kinase also activates the NLRP3 inflammasome following caspase activation, thus generating IL-1β and IL-18 (Hardison & Brown, 2012; Ifrim *et al.*, 2013). It is of note that dectin-1 signalling is only activated by particulate β-glucans that cluster the receptor in a synapse-like structure (phagocytic synapse), hence ensuring recognition upon direct microbial contact (Goodridge *et al.*, 2011). Contradictory results regarding the susceptibility of dectin-1 knockout (KO) mice to systemic candidiasis were found in *in vivo* studies (likely as a result of strain-specific variations in glucan exposure), whereas humans displaying dectin-1 deficiencies developed mucocutaneous infections but not invasive fungal infections (Ferwerda *et al.*, 2009; Drummond & Brown, 2011). CARD9 deficiency, however, increases the risk of developing invasive candidiasis in both, mice and humans, probably due to the fact that CARD9 is involved in signal transduction pathways downstream of other CLRs besides dectin-1 (Gross *et al.*, 2006; Drewniak *et al.*, 2013). Additionally, it was observed that mice lacking dectin-2 were more likely to develop systemic candidiasis and that dectin-2-deficient macrophages displayed a slightly reduced capacity to phagocytose *Candida* cells (Ifrim *et al.*, 2016). Mincle associates with FcγR and signals through Syk/CARD9 to induce cytokine production, although it is not involved in phagocytosis (Wells *et al.*, 2008). MR mediates several antifungal activities, such as phagocytosis of yeasts by DCs or IL-17 production by human peripheral blood mononucleated cells, even though its role in protection against candidiasis appears to be redundant (Hardison & Brown, 2012). DC-SIGN recognition of *C. albicans* (probably in cooperation with dectin-1) leads to the production of cytokines and the activation of the respiratory burst *in vitro*, while its role *in vivo* during an infection has not been reported (Takahara *et al.*, 2011).

Besides PAMPs being detected on the cell surface or the lumen of endosomes or lysosomes, NLRs and RLRs are involved in sensing infection in the cytosol. MDA5, a member of RLRs, which are important in viral recognition, has also been described to play a role in immune responses to *C. albicans*, although the fungal ligand responsible for its activation has not been characterized yet (Jaeger *et al.*, 2015). NLRs detect fungal ligands released from phagolysosomes and damage associated molecular patterns (DAMPs), including heat-shock proteins (Hsps), generated by cellular damage during infection (Kumar *et al.*, 2011). They are found as components of inflammasomes and NOD2, for instance, is involved in the innate response to chitin-derived components

(Wagener *et al.*, 2014). Inflammasomes are cytosolic multi-protein complexes (including NLRs), some of which are known to be involved in the response to *C. albicans* (NLRP3, NLRC4, NLRP10). Activating stimuli induce conformational changes in NLRs leading to inflammasome assembly and caspase-1 activation, which in turn acts upon the pro-IL-1β and IL-18 inactive precursors, releasing active IL-1β and IL-18, respectively. This production of mature IL-1β and IL-18 can also be caused by noncanonical (caspase-8 and caspase-11) inflammasomes (Joly & Sutterwala, 2010; Tomalka *et al.*, 2011). At the site of infection, mature IL-1β can also be generated in an inflammasome-independent manner. This activation can be mediated by constitutively expressed monocytic caspase-1 or by neutrophil-expressed proteinase 3. In addition, it has been observed that fungal-derived proteases can also generate host-derived active IL-1β *in vitro*, and probably also during infection, thus activating the immune system (Netea *et al.*, 2015).

Recognition of *C. albicans* by immune cells is mediated by the simultaneous or sequential activation of different PRRs, as not only does *C. albicans* express a variety of different PAMPs, but also their expression may change among strains and morphotypes. Therefore, collaboration among receptors in fungal recognition and crosstalk between intracellular signalling pathways renders essential in tailoring the final immune response (Yáñez *et al.*, 2021). In this sense, some CLRs, such as dectin-1, galectin-3 and SIGNR1 (the murine homolog for human DC-SIGN), can act as TLR2 co-receptors in order to collaborate in fungal recognition or modulate ligand specificity. Similarly, dectin-1 and SIGNR1 may associate with TLR4 to exert a collaborative recognition and dectin-1 has been observed to synergize with TLR2 and TLR4 to induce a greater cytokine production in human macrophages (Gantner *et al.*, 2003; Ferwerda *et al.*, 2008). Sometimes, physical interaction between receptors has been demonstrated, such as galectin-3 and TLR2 or galectin-3 and dectin-1, suggesting that galectin-3 may mediate the cooperation between dectin-1 and TLR2 (Jouault *et al.*, 2006; Esteban *et al.*, 2011). All in all, this complex network of *C. albicans* sensing receptors allows immune cells to respond to (i) whole fungal cells through interaction between surface PAPMs and PRRs, (ii) fungal ligands generated following phagocytosis and fungal destruction (such fungal DNA and RNA) through endosomal PRRs, and (iii) cytosolic-located fungal ligands released from phagolysosomes or DAMPs generated by cellular damage during infection through NODs/NLRs.

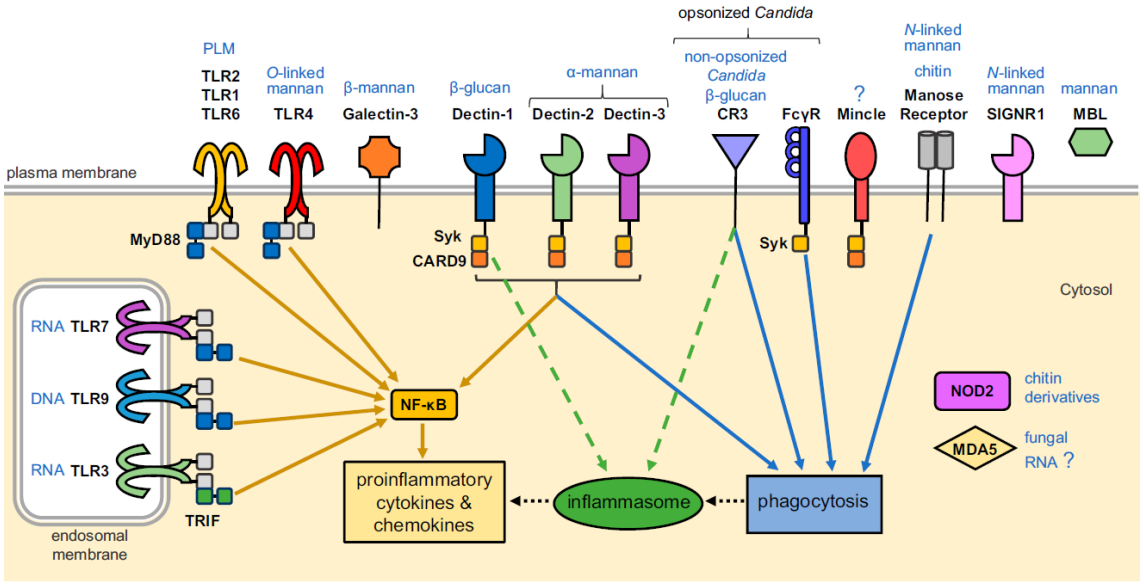

FIGURE 3. *C. albicans* sensing by innate immune cells. *C. albicans* yeasts and/or hyphae are sensed by a variety of PRRs that recognize fungal ligands. Extracellular fungal ligands (basically cell wall components) are recognized by plasma membrane receptors, including TLRs (mainly TLR2 and TLR4), CLRs (dectin-1, dectin-2, dectin-3, MR, mincle, SIGNR1), galectin-3 and CR3. Intracellular fungal ligands (DNA, RNA), released upon phagocytosis and killing, involve recognition by TLRs located at the endosomal membrane (TLR3, TLR7 and TLR9). Cytosolic receptors (NOD2 and MDA5) are also involved in the detection of fungal-derived ligands, and serum MBL contributes to *C. albicans* detection by binding specific mannan arrangements and promoting opsonization. Receptors for complement fragments and, particularly, FcγR contribute to the recognition of *C. albicans* opsonized cells. The simultaneous recognition of various PAMPs triggers complex signal transduction pathways that lead to the activation of immune responses. Signalling though TLR/MyD88 and dectin-1/Syk/CARD9 converge in the transcription factor NF-κB, which activates expression of proinflammatory cytokines. Signalling through dectins, CR3, MR and FcγR induces phagocytosis of fungal cells. Fungal recognition by some PRRs (dectin-1, CR3, TLRs) and endogenous molecules released following phagocytosis can activate inflammasomes, which induce caspase-1-mediated activation of pro-IL-1β, a key cytokine in immunity against *C. albicans* (Yáñez *et al.*, 2021).

1.3. Adaptive immune response

In most cases, the activation of innate responses by epithelial cells, phagocytes (macrophages and neutrophils) and NK cells is sufficient to prevent an invasive fungal infection. However, when innate immune mechanisms fail to control the infection, the activation of adaptive immune responses is required to deal with the pathogen. The adaptive arm of the immune system is composed by highly specific B and T lymphocytes that besides contributing to the elimination of the infectious agent upon its encounter, can

also differentiate into memory cells, which are able to mount a faster response following recognition of the same pathogen.

T lymphocytes (T cells) are one of the main players in responding to *C. albicans* infections as they control fungal proliferation through both, direct and indirect, mechanisms. Activation of $CD8^+$ (cytotoxic T lymphocytes, CTL) and $CD4^+$ (T helper cells, Th) is controlled by DCs, which can detect and phagocytose *C. albicans* while patrolling the host's tissues. Following exposure to pathogens and/or inflammatory mediators, DCs are transformed into mature DCs that migrate from peripheral tissues into draining lymph nodes, where they activate naïve antigen-specific T lymphocytes, leading to their expansion and differentiation into effector cells (Yáñez *et al.*, 2021).

$CD8^+$ T cells are able to fight against invading pathogens by producing and releasing cytotoxic granules containing perforins and granzymes (cytotoxicity), but also by producing cytokines (TNF-α and IFN-γ, mainly). By using murine models of infection, it has been demonstrated that $CD8^+$ T cells have a role in protecting against *C. albicans* infections, although little is known regarding their cytotoxic effect (Beno *et al.*, 1995). Their protective effect is most likely caused by IFN-γ production, which is induced by IL-12 (Gozalbo *et al.*, 2014).

Although CTLs have been proved to play a role in protection against candidiasis, Th cell responses stand as the most relevant mechanism of adaptive immunity to *C. albicans*, as evidenced by the high prevalence of oropharyngeal candidiasis in AIDS/HIV^+ patients, where $CD4^+$ T cells are depleted (Fidel, 2011). DCs are able to phagocytose and kill fungal cells, but they mainly act as antigen presenting cells, thus processing and presenting fungal antigens to naïve $CD4^+$ T cells, what drives their development towards four different Th cells: Th1, Th17, Th2 and Treg (although other subsets not fitting either of these have also been identified, such as Th9 and Th22, which specifically produce IL-9 and IL-22, respectively) (Borghi *et al.*, 2014).

Th1 responses are thought to be protective against mucosal and systemic infections. These cells secrete, among other cytokines, IFN-γ, which stimulates neutrophil and macrophage phagocytosis and killing of *C. albicans*. It also causes autocrine upregulation of the IL-12 receptor, making Th cells more sensitive to IL-12 and, therefore, preserving the Th1 phenotype (Gozalbo *et al.*, 2014; Richardson & Moyes, 2015).

Th17 cells release numerous cytokines, including IL-17 and IL-22. IL-17 induces neutrophil recruitment and activation while IL-22 promotes β-defensin production by epithelial cells, enhancing its barrier function (Huang *et al.*, 2004; De Luca *et al.*, 2010). *C. albicans*-specific Th17 cells also produce IFN-γ, which, as above indicated, activates phagocytes' effector antifungal functions (Zielinski *et al.*, 2012). Protection against *C. albicans* infection at most mucosal surfaces appears to be mainly dependent on the Th17 response, as patients with disorders in Th17-mediated antifungal responses frequently develop chronic mucocutaneous candidiasis, while not showing an increased susceptibility to invasive candidiasis (Hernández-Santos *et al.*, 2013). Generally, it is acknowledged that whereas mucosal infections mostly cause adaptive immunity to polarize towards protective Th17 responses, systemic candidiasis is still thought to primarily cause Th1 responses (Richardson & Moyes, 2015).

Th2 responses are generated in an anti-inflammatory environment. These cells produce anti-inflammatory cytokines, such as IL-10 and IL-4, that inhibit Th1/Th17 development and deactivate phagocytic effector cells, thus being regarded as unprotective. Nonetheless, Th2 cells are necessary for restoring the noninflammatory status after fungal clearance, as well as for maintaining a balanced proinflammatory Th1/Th17 response (Mencacci *et al.*, 2001).

Treg cells maintain peripheral tolerance and regulate effector responses to control an excessive proinflammatory response that could lead to tissue damage, but their role during candidiasis is still up for discussion. It has been reported that TLR-activated DCs can block their immunosuppressive effects, favouring a Th1 response (Lee & Iwasaki, 2007). Additionally, Treg cells also express TLRs and upon detection of their ligands, a temporary abrogation of their suppressive phenotype also takes place, enabling the enhancement of the proinflammatory Th1 response. Once the pathogen has been cleared and TLRs on Treg cells do not detect as many fungal ligands, Treg cells regain their immunosuppressive activity in order to restore the immune balance (Sutmuller *et al.*, 2006).

The engagement of different receptors upon recognition of multiple PAMPs causes various downstream signalling events that control cytokine production, which ultimately creates a specific microenvironment during $CD4^+$ T cell priming by DCs at the lymph nodes that defines particular Th responses (Figure 4). For instance, recognition and phagocytosis of yeasts by DCs induces the production of proinflammatory IL-12, which

drives polarization towards the Th1 subset, while ingestion of hyphae results in anti-inflammatory IL-4 production that drives Th2 development (d'Ostiani *et al.*, 2000). It has been demonstrated that mounting a Th1 response involves TLR/MyD88-mediated signalling (through TLR2, TLR4 and endosomal TLR9), as MyD88 is necessary for the generation of IL-12, downregulation of IL-10 and, consequently, antifungal Th1 priming, even though MyD88-deficient DCs can phagocytose fungal cells similarly to wildtype (WT) DCs. However, different TLRs may contribute differently to these responses (Gil *et al.*, 2016). Additionally, proinflammatory cytokine production upon recognition of *C. albicans* by CLRs (as explained in section 1.2.1) promotes Th1 and, in particular, Th17 polarization (Gringhuis *et al.*, 2009; Robinson *et al.*, 2009). Initial differentiation towards Th17 is driven by IL-1β, while IL-23 signalling is involved in their maturation and ultimate differentiation (Chung *et al.*, 2009; McGeachy *et al.*, 2009). Following recognition of *C. albicans* mannan by DCs, they produce IL-23 and IL-6, which promote Th17 differentiation (Richardson & Moyes, 2015). Activation of the NLRP3 inflammasome upon recognition of fungal β-glucan by dectin-1 and CR3 on DCs leads to the production of IL-1β, which drives protective Th1 and Th17 cellular responses to disseminated candidiasis (van de Veerdonk *et al.*, 2011; Ganesan *et al.*, 2014). NLRP10-deficient mice displayed severe defects in adaptive Th1 and Th17 responses to *Candida*, despite the fact that this inflammasome is not involved in innate proinflammatory cytokine production, suggesting that it does play a role in the development of adaptive immune responses to fungi (Eisenbarth *et al.*, 2012; Joly *et al.*, 2012). Therefore, biased protective Th differentiation priming can be achieved by selectively challenging different receptors, that having significant implications for the development of DC vaccine-based strategies (Iannitti *et al.*, 2012).

Type I IFNs (IFN-α and IFN-β), which are known to inhibit viral replication and mediate protection against viral infections, have also been proved to play a somewhat controversial role in the immune responses developed against *Candida*. On the one hand, mouse models have shown that IFN-β signalling promotes persistence of *C. glabrata* in the host, that it inhibits fungal clearance in mice infected with *C. parapsilosis* (Bourgeois *et al.*, 2011; Patin *et al.*, 2016) and that the lack of functional IFN-I receptors renders mice remarkably more resistant to invasive *C. albicans* infections (Stifter & Feng, 2015). However, production of IFN-β by DCs (dependent on dectin-1 and dectin-2 via Syk and IRF5) has been described to be crucial for the immune response against *C. albicans*, as it

promotes mobilization of neutrophils to the kidney (Biondo *et al.*, 2011; del Fresno *et al.*, 2013). Additionally, it has been shown that type I IFNs secreted by β-glucan-stimulated DCs via dectin-1 induce the proliferation and activation of $CD8^+$ T cells by promoting antigen presentation, surface expression of costimulatory molecules and release of other cytokines, thus supporting the protective role of type I IFNs during candidiasis (Hassanzadeh-Kiabi *et al.*, 2017). Therefore, type I IFNs may play different roles in response to different fungal species or depending on the concentration at which they are present, and more research is needed to determine their precise functions during *C. albicans* infections.

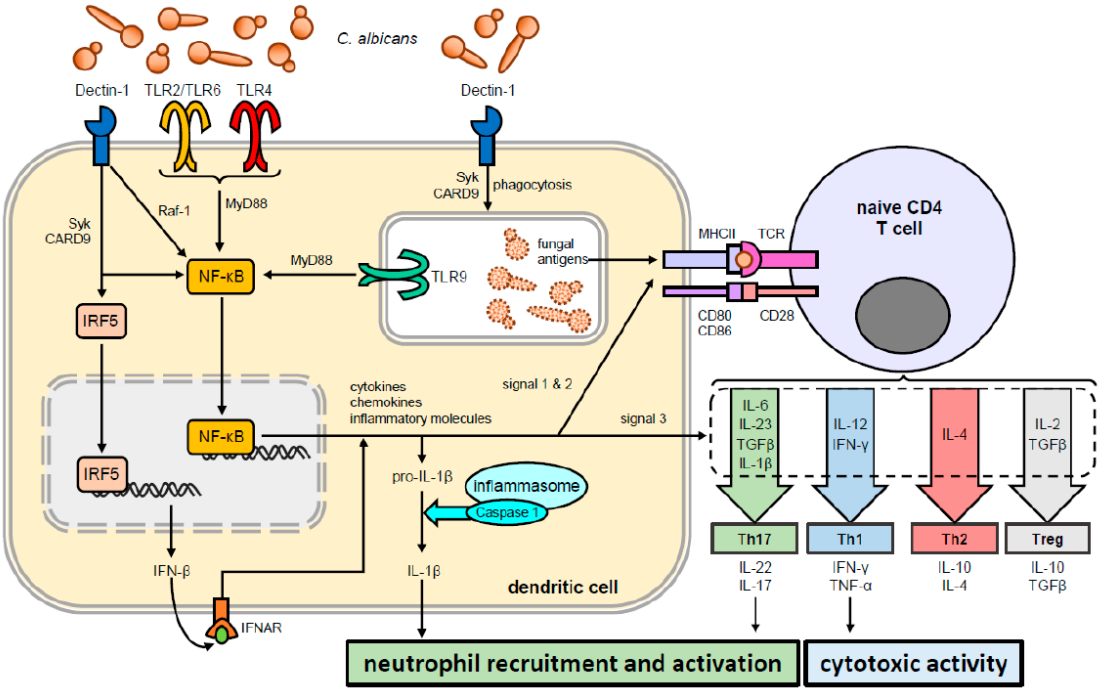

FIGURE 4. Activation of T-cell responses by DCs in response to *C. albicans*. Besides phagocytosis, recognition of fungal cells by DCs through PRRs, such as TLRs and dectin-1, trigger MyD88- and Syk/Raf-1-mediated signalling pathways, respectively, that activate transcription factors (NF-κB, IRF5). NF-κB induces expression of MHCII (major histocompatibility complex class II) molecules (signal 1), costimulatory molecules CD80/86 (signal 2) and cytokines (signal 3) required for antigen presentation to naïve $CD4^+$ T cells and their subsequent development into one of the different subsets (Th1, Th2, Th17, Treg), depending on the pool of cytokines secreted by DCs. IRF5 is involved in IFNβ production, whose autocrine signalling through the IFN-α/β receptor (IFNAR) enhances these 3 signals. These signalling pathways also lead to inflammasome assembly and activation, causing caspase-1-mediated activation of IL-1β (Yáñez *et al.*, 2021).

On the other hand, the other players of the adaptive immune system, B lymphocytes (B cells), have a relatively minor role in immune protection against *C. albicans* infection.

Besides triggering activation of the classical complement pathway, several antifungal activities have been associated with anti-*Candida* antibodies secreted by B cells, as they interfere with their antigen function (which range from cell wall components to secreted enzymes or cytosolic fungal proteins) affecting their role as virulence factors (Richardson & Moyes, 2015). Actually, a range of anti-Candida antibodies have been shown to confer certain protection in animal models of infection, and some vaccination assays using animal models have demonstrated a lowered susceptibility to infection (Moragues *et al.*, 2014; Wang *et al.*, 2015). However, it has also been observed that B-cell deficiency in mice does not enhance susceptibility to *C. albicans* infection and that patients with agammaglobulinemia or hypogammaglobulinemia do not exhibit a greater susceptibility to fungal infections, suggesting that humoral response during infection plays a relatively small role in host defence (Moragues *et al.*, 2014; Wang *et al.*, 2015). Nevertheless, due to the prevalence of fungal infections and their increased resistance to antifungal therapies, eliciting protective antibodies through vaccination remains a viable strategy for improving resistance to *C. albicans* infections (Shukla *et al.*, 2021).

2. Demand-adapted haematopoiesis during infection

In adults, blood cells of all lineages are produced in the bone marrow via the differentiation of hematopoietic stem cells (HSCs) through the process known as haematopoiesis. As the vast majority of mature blood cells are relatively short-lived, they must be replaced throughout life and so they need to be constantly replenished to ensure homeostatic peripheral blood cell counts (Cheng *et al.*, 2020). During homeostasis, this process of producing lineage-committed progenitors is tightly controlled, as many cytokines, cell-cell interactions and transcription factors fine-tune the proliferation of hematopoietic stem and progenitor cells (HSPCs) and their differentiation into mature myeloid and lymphoid cells (Rieger & Schroeder, 2012). However, upon an infection, or any other pathologic condition that may disturb the hematopoietic balance, a demand-adapted hematopoietic response is developed in order to counterbalance cell losses and boost cellular output to fulfil the demand for increased numbers of mature leukocytes. While the adaptive immune system meets this demand by clonal expansion of T and B cells, and although there are some reports of proliferation of mature macrophages, the increased supply of most innate immune cells is achieved by emergency myelopoiesis, which consists in the production of neutrophils and/or monocytes/macrophages (depending on the pathogen and the severity of the infection), while the development of other lineages (lymphoid and erythroid) remain inhibited (Boettcher & Manz, 2016, 2017).

As they constitute the first line of defence against an infection, myeloid cells are rapidly consumed during the immune response, and so their replenishment is essential in order to efficiently fight off the pathogen. Therefore, during an infection, mobilization of myeloid cells, particularly neutrophils and monocytes, from the bone marrow to infected tissues takes place, which is accompanied by the proliferation and differentiation of HSPCs in the bone marrow to maintain their supply (Boettcher & Manz, 2016, 2017). In order to establish this demand-adapted myelopoiesis, it is necessary for the cell types initiating it to detect the presence of a pathogenic organism and to be able to translate these signals into emergency myelopoiesis. Host mature cells in infected tissues can sense pathogens by detection of their PAMPs through PRRs, triggering several signalling pathways that result in the release of soluble factors that reach the bone marrow and act upon HSPCs to stimulate the generation of myeloid offspring (Chiba *et al.*, 2018). However, new perspectives on emergency myelopoiesis came when several reports

demonstrated that murine and human HSPCs express functional PRRs and that signalling through these receptors provokes cell cycle entry and myeloid differentiation (Yáñez *et al.*, 2013a). Therefore, depending on the developmental stage of the pathogen-sensing cell type, two models for initiating demand-adapted myelopoiesis can be considered: direct and indirect. According to a model of direct initiation of emergency myelopoiesis, HSPCs induce their enhanced proliferation and myeloid differentiation upon sensing the pathogen through their PRRs. By contrast, in a model of indirect initiation of demand-adapted myelopoiesis, mature cells present in the bone marrow or peripheral tissues act as pathogen sensors and release soluble factors that lead to HSPC myeloid differentiation (Boettcher & Manz, 2016, 2017).

2.1. Steady-state haematopoiesis

HSCs, which possess self-renewal properties and the ability to produce diverse progeny, produce all mature blood cells and regenerate the blood system throughout life. The first *in vivo* evidence for the existence of HSCs was based on the rescue of lethally irradiated recipient mice by bone marrow transplantation, followed by observing hematopoietic colonies in their spleen (Till & McCulloch, 2012). Following, the use of antibodies and fluorescence-activated cell sorting (FACS) made it possible to first describe HSC-enriched cells using a combination of several surface markers (Spangrude *et al.*, 1988) and since then, similar approaches have been used to identify and isolate different progenitor populations, allowing for establishing the immunophenotype-based tree-like hierarchy model for hematopoietic cell development (Rieger & Schroeder, 2012; Cheng *et al.*, 2020).

This classical model of haematopoiesis describes the production of diverse hematopoietic cells by HSCs via a series of progenitors with increasingly restricted lineage potential (Rieger & Schroeder, 2012). Mouse HSPCs can be identified by the lack of expression of the specific markers of mature cell lineages (therefore, can be referred to as Lin^- cells) and among them, the Lin^- c-kit^+ $Sca\text{-}1^+$ cell fraction (LKS^+ cells) is highly enriched in cells bearing hematopoietic-reconstituting ability. The most quiescent, self-renewing, and multipotent long-term hematopoietic stem cells (LT-HSCs) have a full long-term reconstitution capacity following serial transplantation in mice and give rise to less quiescent short-term HSCs (ST-HSCs) with a short-term reconstitution ability, which

in turn produce multipotent progenitors (MPPs) that lack self-renewal ability but still retain the LKS^+ phenotype. These give rise to LKS^- (Lin^- c-kit^+ Sca-1^-) cells, which diverge into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs), which, in turn, can produce granulocyte-monocyte progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs) (Figure 5).

However, this model has been challenged over the last years, especially thanks to advances in single-cell technology and genetic mouse models, and new types of HSPCs have been identified (Giladi *et al.*, 2018). For instance, the MPP population is now regarded as an heterogenous population and has been further divided into four subgroups, according to their immunophenotype, cell cycle status, lineage bias, resistance to drug treatment and bone marrow abundance. MPP1 are more similar to ST-HSCs, as they are not biased to produce any specific lineage, while MPP2, MPP3 and MPP4 are. MPP2 is a megakaryocyte/erythrocyte-biased MPP subset, while MPP3 and MPP4 are myeloid- and lymphoid-biased, respectively (Pietras *et al.*, 2015) (Figure 5).

Moreover, heterogeneity and hierarchy within myeloid progenitors have also been questioned in the light of recent discoveries. In mice, monocytes can be classified into $Ly6C^+$ inflammatory monocytes and $Ly6C^-$ non-classical patrolling monocytes, which are thought to derive from the former (Italiani & Boraschi, 2014). The hierarchical model for monocyte differentiation establishes that GMPs, besides producing neutrophils through granulocyte progenitors (GPs), give rise to monocyte and dendritic progenitors (MDPs). MDPs are unable to produce neutrophils, but can yield classical DCs (cDCs) and plasmacytoid DCs (pDCs) via common DC progenitors (CDPs), and also monocyte-committed progenitors (cMoPs: common monocyte progenitors) that give rise to both, classical and non-classical monocytes (Zhu *et al.*, 2016). These MDP-derived cMoPs and monocyte-committed progenitors produced by GMPs (MPs) appeared to be the same cell, as they share surface marker expression. Additionally, adoptively transferred MDPs yield monocytes more rapidly than GMPs, supporting the idea that MDPs are derived from GMPs (Hettinger *et al.*, 2013; Yáñez *et al.*, 2015). However, this hierarchical model of monocyte differentiation (CMP-GMP-MDP-MP/cMoP-monocyte) was challenged when it was demonstrated that two pathways originating from GMPs and MDPs independently yield classical monocytes with distinct neutrophil-like (NeuMo) and DC-like (DCMo) gene expression signatures, respectively, in steady-state mouse bone marrow. Both GMP- and MDP-derived classical monocytes can yield macrophages, but only MDP-derived

monocytes give rise to monocyte-derived DCs (moDCs), and production of monocytes via the GMP and MDP pathways can be independently controlled by microbial stimuli (Yáñez *et al.*, 2017) (Figure 5).

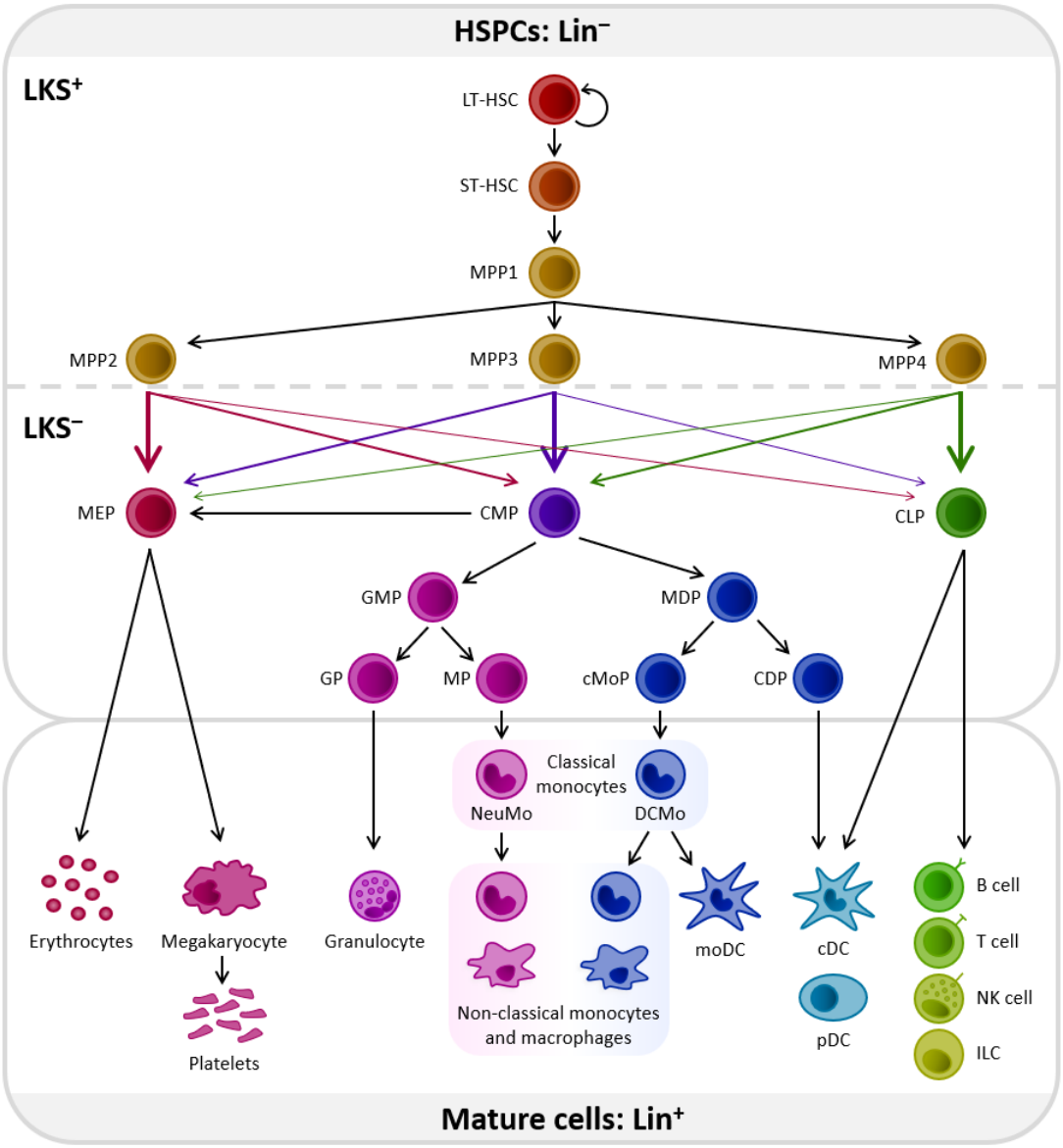

FIGURE 5. Hematopoietic cell development model. Haematopoiesis is initiated by LT-HSCs, which have self-renewal ability and give rise to proliferating ST-HSCs. These produce MPPs, which have been classified into four subsets depending on their lineage-biased potential: MPP1 (not biased), MPP2 (megakaryocyte lineage), MPP3 (myeloid lineage) and MPP4 (lymphoid lineage). MPPs give rise to progenitors committed to megakaryocyte-erythrocyte (MEP), myeloid (CMP) or lymphoid (CLP) lineages. CLPs generate lymphocytes and DCs. MEPs differentiate into erythrocytes and megakaryocytes/platelets. CMPs can also produce MEPs, as well as GMPs and MDPs. GMPs give rise to granulocytes via GPs and monocytes through MPs. MDPs give rise to cDCs and pDCs through CDPs, and cMoPs, which further differentiate into monocytes and moDCs. Classical monocytes produced by MPs and cMoPs are functionally different, and consequently, derived non-classical monocytes and macrophages may also exhibit functional differences.

To continuously regenerate the hematopoietic system, the right quantity of specific cell types must be constantly generated at the proper time and location. In order to achieve this, fate decisions must be continuously made in HSPCs, including choosing between quiescence or proliferation, self-renewal or differentiation, compromising with the different lineages, survival or death, and migration or staying in place (Cheng *et al.*, 2020). Normal haematopoiesis is enabled by the precise timing and sequential order of these decisions in each cell, whereas disruption of normal cell fate choices underlie haematological disorders. The bone marrow provides the environment for sustained HSC function, which is tightly regulated by intrinsic programmes and extrinsic signals from their microenvironment (niche). HSCs can interact with their niche in several juxtracrine (cell-cell or cell-matrix interactions) and paracrine (through cytokines, chemokines and growth factors) ways, this interactions being necessary for their proper behaviour. In fact, it has been proposed that different progenitors could have specific niches within the bone marrow. Additionally, the bone marrow comprises a variety of different cell types that also influence hematopoietic fates. For example, mesenchymal stem cells and endothelial cells release soluble factors, such as CXCL12 or SCF (stem cell factor), that act directly on HSCs and promote their survival (Casanova-Acebes *et al.*, 2014; Wei & Frenette, 2018).

Differentiation of multipotent progenitors into the different lineages involves an overall alteration of gene expression that needs to be tightly regulated to ensure the generation of the appropriate quantity and type of mature cells. Lineage choice and commitment involve the establishment and upkeep of genetic programmes associated with a particular lineage, which is accomplished not only by expressing lineage-specific genes, but also by silencing those specific for other lineages. Such programmes could be triggered by intrinsic or extrinsic signals (or a combination of both) and are ultimately controlled by transcription factors. It has been observed that individual transcription factors can instruct lineage choice and are even able to reprogramme committed cells, while can also actively repress lineage programmes. However, gene regulation depends on the interaction of several elements including, besides transcription factors, epigenetic modifiers and post-transcriptional control mechanisms (Rieger & Schroeder, 2012). Data gathered thanks to high-throughput techniques at the single-level provided a deeper understanding of the intrinsic programmes for HSPC differentiation, suggesting that these

processes are more complex and less sequential than previously believed (Giladi *et al.*, 2018; Cheng *et al.*, 2020).

2.1.1. Myeloid cell differentiation

As previously indicated, differentiation towards a specific lineage requires the establishment of lineage-specific genetic programmes that can be induced through various mechanisms and are ultimately controlled by transcription factors. Regarding myeloid cell differentiation, the main transcription factors regulating this myeloid-specific switch in gene expression are PU.1, IRF8 and C/EBP (CCAAT-enhancer-binding protein) family members (Yáñez *et al.*, 2015; Zhu *et al.*, 2016; Giladi *et al.*, 2018). PU.1 is the master regulator of all myeloid lineages, it is expressed at low levels in CMPs and its expression increases as cells differentiate into either monocytes or neutrophils. IRF8 is thought to be critical for monocyte development in the bone marrow. It is expressed in GPs and MPs (but not in GMPs) in order to promote monocyte differentiation and inhibit granulocyte production (Yáñez *et al.*, 2015), as evidenced by the fact that mice deficient in IRF8 lack monocytes but have an increased number of GMPs and neutrophils (Scheller *et al.*, 1999). Proteins of the C/EBP family play a crucial part in the development and maintenance of myeloid cell subsets (Friedman, 2002) and several studies support the idea that different protein isoforms are required for different myeloid lineages: C/EBPα expression would direct granulocytic differentiation while C/EBPβ expression would be responsible for monocyte development (Zhu *et al.*, 2016). However, the means by which different C/EBP isoforms regulate lineage commitment remain unclear as, for example, expression of *Cebpb* from the *Cebpa* locus restores granulopoiesis, demonstrating that *Cebpa per se* is not required for granulocyte development (Jones *et al.*, 2002). Additionally, using single-cell techniques it was revealed that C/EBPα regulates entry into all myeloid fates, while IRF8 and PU.1 direct later differentiation towards monocyte or granulocyte fates, respectively (Giladi *et al.*, 2018).

Besides transcription factors, other regulatory elements shape gene-expression programmes, including post-transcriptional mechanisms and epigenetic regulators, mainly histone modifications and DNA methylation. For instance, when HDAC1 (histone deacetylase 1) expression is downregulated by C/EBP transcription factors, committed CMPs give rise to myeloid cells, in particular granulocytes. In addition, specific DNA

methylation patterns have been associated to myeloid differentiation and it is likely that PU.1 is an important mediator of DNA demethylation, as it has been demonstrated that PU.1 binds TET2 and recruits it to the promoters of genes that become demethylated (Álvarez-Errico *et al.*, 2015). On the other hand, some studies have also reported a role for microRNAs (miRNAs) in regulating monocyte development, as well as monocyte differentiation to macrophages by, for example, blocking the translation of lineage-specific transcription factors (Zhu *et al.*, 2016).

Regarding extrinsic signals triggering lineage-specific genetic programmes, numerous investigations carried out over the past decades have clearly shown that steady-state myelopoiesis is driven by the coordinated activity of different myelopoietic growth factors. These include members of the colony-stimulating factor (CSF) superfamily macrophage (M)-CSF, granulocyte (G)-CSF and granulocyte-macrophage (GM)-CSF, which provide proliferation and survival signals to HSPCs and are critically involved in the process of lineage specification (Hamilton & Achuthan, 2013). M-CSF plays important roles in the proliferation, differentiation and survival of monocytes, macrophages and bone marrow progenitor cells by interacting with its receptor (M-CSFR) (Chiba *et al.*, 2018). In fact, mice deficient for M-CSF or M-CSFR exhibit skeletal, sensory, and reproductive abnormalities caused by severe deficiencies in tissue macrophages and osteoclasts (Dai *et al.*, 2002). Additionally, it has been observed that M-CSF, unlike G-CSF and GM-CSF, can directly induce PU.1 and instruct myeloid cell-fate change in mouse HSCs (Mossadegh-Keller *et al.*, 2013). As for G-CSF, its essential role in governing steady-state granulopoiesis is demonstrated by a 70-90% reduction in circulating neutrophils in G-CSF-deficient (Lieschke *et al.*, 1994) and G-CSFR-deficient (Liu *et al.*, 1996) mice, respectively. GM-CSF was originally described as capable of generating both, granulocyte and macrophage, colonies from myeloid precursor cells *in vitro*. However, studies conducted on GM-CSF-deficient mice have failed to detect major defective hematopoietic phenotypes (besides the absence of alveolar macrophages and a reduction in a subset of DCs in non-lymphoid tissues), indicating that GM-CSF is mostly redundant for steady-state haematopoiesis (Becher *et al.*, 2016).

2.2. Infection-induced haematopoiesis

2.2.1. Indirect pathogen sensing leading to haematopoiesis

As above mentioned, in a model of indirect initiation of emergency haematopoiesis, mature cells would be the ones sensing the pathogens and stimulating HSPC proliferation and differentiation via the secretion of soluble factors such as CSFs or cytokines.

Monocytes and macrophages have largely been considered as the main cells sensing pathogens during infections as not only are they ideally located to accomplish this, but they also express a wide variety of PRRs and are able to release a great number of inflammatory cytokines and hematopoietic growth factors. For instance, a study highlighting the role of myeloid cells in the regulation of emergency haematopoiesis revealed that bone marrow myeloid Gr-1^{+} cells, which include granulocytes, monocytes and their late-stage precursor cells, release ROS to stimulate myeloid progenitor proliferation and differentiation following heat-inactivated *Escherichia coli* stimulation *in vivo* (Kwak *et al.*, 2015).

However, other *in vivo* studies are in disagreement with the role for this cell types. In fact, monocyte-/macrophage-depleted mice or bone marrow chimeric mice with TLR4$^{-/-}$ hematopoietic cells on a WT non-hematopoietic background are capable of inducing emergency myelopoiesis in response to the TLR4 ligand lipopolysaccharide (LPS), at least during short-term treatment, while TL4-expressing non-hematopoietic cells are necessary for this LPS-induced response (Boettcher *et al.*, 2012). This points towards non-hematopoietic cells as potential pathogen-sensing cells with a predominant role in regulating the emergency myelopoietic response. More specifically, among the TLR-expressing non-hematopoietic cells, endothelial cells were the ones found to release G-CSF following TLR4-MyD88 signalling that would act upon HSPCs leading to their myeloid differentiation (Boettcher *et al.*, 2014).

The role for G-CSF during emergency haematopoiesis has yielded some conflicting results. On the one hand, studies in G-CSF-deficient mice showed a reduced granulopoietic response and an increased lethality during infection with *Listeria monocytogenes* in comparison to control mice (Lieschke *et al.*, 1994; Zhan *et al.*, 1998). Conversely, WT and G-CSF-deficient mice displayed no differences in emergency granulopoiesis when infected with *C. albicans* (Basu *et al.*, 2000). Another potent

myelopoietic growth factor, GM-CSF, seems to be important for sustaining an appropriate emergency myelopoietic response once early response mechanisms have been overcome, as evidenced by the inability of controlling *L. monocytogenes* infection in GM-CSF-deficient mice due to depletion of myeloid cells in the bone marrow and inflamed tissues (Zhan *et al.*, 1998). Additionally, GM-CSF-deficient mice were also incapable of increasing the number of bone marrow myeloid colony-forming cells following chronic infection with *Mycobacterium avium* (Zhan & Cheers, 2000).

Besides CSFs, the release of other soluble factors by pathogen-sensing cells have also been described to induce emergency myelopoiesis. TNF-α, a proinflammatory cytokine induced upon infection that can elicit programmed cell death, has recently been shown to stimulate emergency myelopoiesis by upregulating PU.1 and promoting survival of HSCs and myeloid-biased MPPs (Yamashita & Passegué, 2019). Similarly, IL-1, a proinflammatory cytokine secreted upon infection or injury, especially by bone marrow endothelial cells, has been described to accelerate HSC proliferation and to instruct their myeloid differentiation by inducing PU.1 via NF-κB signalling downstream of the IL-1 receptor. Although this effect is essential for rapid myeloid recovery following acute injury, chronic IL-1 exposure promotes uncontrolled HSC division, loss of self-renewal capacity and eventual exhaustion of the HSC pool. However, these damaging effects are transient and fully reversible upon IL-1 withdrawal (Pietras *et al.*, 2016).

In addition, it has been shown that IL-6-deficient mice display a delayed hematopoietic recovery following cytotoxic treatment or pharmacological induction of severe haemolytic anaemia (Bernad *et al.*, 1994), and that they have an impaired neutrophil response after *C. albicans* infection (Romani *et al.*, 1996), thus evidencing the implication of this cytokine in emergency myelopoiesis. IL-6 can be generated by different cell types in the bone marrow microenvironment in response to various stimuli, but their relative importance in controlling emergency haematopoiesis is still unclear. Among these cell types, it has been described that mesenchymal stem cells secrete IL-6 upon detection of IFN-γ released by CTLs, which drives HSPC proliferation and myeloid differentiation, mostly towards the monocytic lineage (Schürch *et al.*, 2014).

Moreover, other studies have demonstrated that IFN-γ is also able to directly act upon HSPCs, as it has been shown to favour monocyte over neutrophil differentiation on CMPs and GMPs during a viral infection. Furthermore, IFN-γ enhances the expression of IRF8

and PU.1 in myeloid progenitor cells, while it reduces G-CSF-driven neutrophil differentiation (de Bruin *et al.*, 2012). Given that HSPCs also express type I IFN receptors, IFN-α and IFN-β could also influence the HSPC response. Actually, it has been observed that type I IFNs enhance CMP proliferation upon TLR7 stimulation (Buechler *et al.*, 2016). Even so, Pietras *et al.* demonstrated that type I IFN stimulation alone is not sufficient to induce HSC proliferation *in vitro*, whereas it is *in vivo*, suggesting that type I IFNs need cofactors to exert their HSC proliferative effects (Pietras *et al.*, 2014).

2.2.2. Direct pathogen sensing by HSPCs

In 2006, reports began to emerge revealing that murine and human HSPCs express functional PRRs, including TLRs, opening up new perspectives on emergency myelopoiesis. Nagai *et al.* demonstrated the presence of TLR2 and TLR4 in the surface of murine HSPCs and that upon an *in vitro* exposure to such TLR ligands, these cells initiate their cell cycle and acquire lineage markers in the absence of growth and differentiation factors (Nagai *et al.*, 2006). Afterwards, it was also shown that human HSPCs ($CD34^+$ cells) express TLRs and that their stimulation leads to their differentiation *in vitro*, as it was shown that signalling via TLR7/8 in these cells induces their differentiation along the myeloid lineage (Sioud *et al.*, 2006), which is also induced following Pam_3CSK_4 stimulation (agonist of the heterodimer TLR1/TLR2) (De Luca *et al.*, 2009). Since then, the different mouse and human HSPC subsets have been shown to express most TLRs with their stimulation inducing HSPC proliferation and differentiation towards specific myeloid subsets *in vitro* (Yáñez *et al.*, 2013a). For instance, TLR7/8 ligands preferentially induce DC production by human HSPCs, whereas Pam_3CSK_4 induced monocyte differentiation (Sioud & Fløisand, 2007). HSPCs also express other PRRs. For example, dectin-1 is expressed by mouse myeloid progenitors (although not by HSCs) (Yáñez *et al.*, 2011), and human HSPCs express NOD2, whose stimulation with muramyl dipeptide is sufficient to trigger DC production by these cells (Sioud & Fløisand, 2009).

The fact that HSPCs express functional PRRs suggested a new mechanism of direct detection of the pathogen by the HSPCs themselves, influencing their division and cell fate and, ultimately, the efficiency of the host's defence against the infection. In this context, it has been proved that inactivated cells of *C. albicans* induce proliferation of

murine cells *in vitro*, as well as their differentiation towards the myeloid lineage. This response requires TLR2/MyD88 and dectin-1 signalling, and gives rise to functional phagocytes that are able to internalize yeasts and secrete proinflammatory cytokines (Yáñez *et al.*, 2009, 2010, 2011). More specifically, the *C. albicans* stimulus induced differentiation to moDCs, a response more similar to curdlan (a pure dectin-1 ligand) than to Pam_2CSK_4 (a pure TLR2/TLR6 ligand), as the latter promoted differentiation to macrophages rather than moDCs, thus indicating that dectin-1 plays a key role in the response to *C. albicans* (Yáñez *et al.*, 2011).

Direct *in vivo* interaction of pathogens and/or their components with PRRs on HSPCs is more difficult to determine, as HSPCs could also respond to other stimuli, such as inflammatory cytokines, generated by differentiated cells responding to the infection. In order to address these difficulties, a transplant experimental model was developed. HSPCs form B6Ly5.1 mice (whose cells express the alloantigen CD45.1) were transplanted into mice deficient for TLR2, TLR4 and MyD88 (whose cells express the alloantigen CD45.2), to which pure ligands of TLR2, TLR4 and TLR9 (Pam_3CSK_4, LPS and CpG oligodeoxynucleotide), respectively, were also injected. This allowed for tracking differentiation of the transplanted cells *in vivo* in response to these pure ligands as unique stimulus, as the fact that receptor mice do not recognize those ligands avoids interferences by soluble mediators secreted by receptor mouse cells. In this way, it was unequivocally observed that HSPCs can directly respond to TLR agonists *in vivo* and that engaging of these receptors is sufficient to induce their own differentiation towards macrophages (Megías *et al.*, 2012).

Moreover, using a similar transplant model, it was also demonstrated that HSPCs preferably differentiate to macrophages *in vivo* in response to both, viable and inactivated, yeasts of *C. albicans* in a TLR2-dependent but TLR4-independent manner, revealing direct recognition of *C. albicans* by HSPCs via TLR2 (Megías *et al.*, 2013). Similarly, it has also been described that HSPCs from TLR2- or MyD88-deficient mice injected into *Staphylococcus aureus* infected wounds of WT mice exhibited impaired granulopoiesis, while HSPCs from WT mice produced similar neutrophil numbers when transferred into wounds of TLR2-, MyD88-deficient or WT mice (Granick *et al.*, 2013). These findings verified that microorganisms can directly stimulate HSPCs, which leads to the production of myeloid cells to replenish the innate immune system during an infection. However, this enhanced myelopoiesis may also cause impairment of HSPC function if the stimuli

causing it are chronically sustained (Luis *et al.*, 2016). In fact, several studies have demonstrated that systemic LPS exposure enhances HSC proliferation in a direct TLR4-dependent manner but also results in a reduced repopulation ability of HSCs (Liu *et al.*, 2015; Takizawa *et al.*, 2017).

The encounter between HSPCs and microorganisms or some of their components might be taking place in both, the bone marrow and the infected tissues. On the one hand, due to the great vascularization of the bone marrow, HSPCs residing there may be exposed to circulating microbial components, or even to intact microbes following bone marrow invasion during a systemic infection. For instance, fungal cells have been detected in the bone marrow of mice with invasive candidiasis at sufficient levels to induce measurable activation of HSPCs (Yáñez *et al.*, 2010, 2011). However, even in the steady-state, a small number of HSPCs are continuously mobilized to the periphery, enabling them to detect local infections and rapidly give rise to tissue-resident myeloid cells by extramedullary haematopoiesis (Massberg *et al.*, 2007). Moreover, in an inflammatory environment (Herman *et al.*, 2016) or in response to acute infections (Granick *et al.*, 2013; Burberry *et al.*, 2014), the number of HSPCs that migrate from the bone marrow towards infected tissues or secondary lymphoid organs, such as the spleen, increases dramatically. On this matter, it has been shown that mice that receive an extended Pam_3CSK_4 treatment in which HSPCs are massively mobilized to the spleen are protected against invasive candidiasis and that this protection is mediated by HSPCs, at least partially (Martínez *et al.*, 2018).

Despite having known for over a decade that PRR stimulation in HSPCs induces myelopoiesis, the molecular mechanisms involved in translating direct detection of pathogens by HSPCs into signals that direct myeloid differentiation have not yet been completely elucidated. PRR stimulation in HSPCs may induce myeloid cell development by direct signalling in an intrinsic manner, directly activating the transcription factors involved in myeloid differentiation, such as PU.1, C/EBPβ or IRF8; by an indirect mechanism, secreting cytokines and other immunomodulatory molecules that would act in an autocrine or paracrine manner; or by a combination of both, direct and indirect mechanisms.

As previously mentioned, cells from the immune system produce great amounts of cytokines following their encounter with pathogen microorganisms, including several with the ability to regulate HSPC proliferation and differentiation, given the presence of

these cytokine receptors in HSPCs (Baldridge *et al.*, 2011). However, more recent studies have reported that HSPCs themselves can also secrete soluble factors in response to infectious stimuli. Zhao *et al.*, by combining mouse models and a single-cell proteomics platform, have shown that in response to TLR stimulation ST-HSCs and MPPs (but not LT-HSCs) release copious amounts of diverse cytokines through NF-κB signalling. Interestingly, HSPCs produced a greater number and a wider variety of cytokines than mature cells, with IL-6 standing out as a particularly important regulator of HSPC proliferation and myeloid differentiation in a paracrine manner (Zhao *et al.*, 2014). In this context, it has been described that the secretome (conditioned media) of HSPCs in response to the TLR2 ligand or *C. albicans* yeasts is able to induce macrophage differentiation of HSPCs *in vitro* (Martínez *et al.*, 2018).

Conversely, other authors have described that TLR ligands are capable of directly inducing myeloid differentiation intrinsically. For instance, Buechler *et al.* demonstrated that the TLR7 ligand induces CMPs to produce macrophages via activation of the NF-κB, PI3K (phosphoinositide 3-kinase) and mTOR (mammalian target of rapamycin) signalling pathways (Buechler *et al.*, 2016). Accordingly, other studies have demonstrated that a systemic LPS exposure *in vivo* enhances HSC proliferation in a direct TLR4-dependent manner (Liu *et al.*, 2015; Takizawa *et al.*, 2017).

3. Innate immune memory

The vertebrate immune system has traditionally been divided into two arms, namely: the innate and adaptive immune systems. The former is nonspecific but offers rapid and broad protection, while the responses of the latter are highly specific albeit slower. For a long time, it was assumed that the ability to induce immunological memory was an exclusive trait of the adaptive immune system. However, a growing body of evidence have challenged this dogma, indicating that innate immune cells display memory characteristics (Bekkering *et al.*, 2021).

Plants and invertebrates, both lacking an adaptive immune system, have been reported to exhibit traits of immune memory. For example, plants present systemic acquired resistance (SAR), which is able to induce long-lasting systemic protection in the whole plant after a previous localized exposure to a pathogen (Wenig *et al.*, 2019). Likewise, multiple studies have shown memory-like responses in a large number of invertebrate species, as macrophages can become primed upon exposure to a pathogen and protect the animal against a secondary, otherwise lethal, reinfection with the same pathogen (Milutinović & Kurtz, 2016). In vertebrates, despite not having defined innate immune memory as such until recently, early studies showed that the live vaccine bacillus Calmette-Guérin (BCG) against tuberculosis protects athymic nude mice against a secondary lethal infection with *C. albicans* (Tribouley *et al.*, 1978) through a process driven by macrophages (van't Wout *et al.*, 1992), and in humans, several epidemiological studies have shown that live attenuated vaccines can protect against heterologous microorganisms not specifically targeted by the vaccines (Goodridge *et al.*, 2016). Moreover, it had also been observed that a first exposure to LPS (endotoxin) protects mice against LPS-induced lethality by generating a lower inflammatory response that prevents tissue damage (Freudenberg & Galanos, 1988). From these first observations until nowadays, many other studies have reinforced the notion that the innate immune system is capable of remembering first encounters, resulting in a functional change upon secondary stimulation.

Given the evident renewed interest in the topic of innate immune memory that has arisen over the last decade, the scientific community has been driven to reconsider the terminology used to describe the various innate memory phenomena (Boraschi & Italiani, 2018). Regarding the innate memory-induced responses, the term “tolerance” has been

widely used to indicate a decreased response upon secondary stimulation, while the term "trained immunity" was initially proposed to refer to the enhanced responses upon a second challenge, although they are both mediated through similar molecular mechanisms (detailed in section 3.3) (Bekkering *et al.*, 2021). However, the concept of "trained immunity" is under debate as it is also used as a synonym for "innate immune memory", regardless of the particular functional programme acquisition (Boraschi & Italiani, 2018), and it should be noted that "tolerance" only refers to the final outcome of the response, as it is the result of a process of general reprogramming, with some effectors being decreased, while others increase or do not change (Foster *et al.*, 2007). Notwithstanding, throughout the present PhD thesis, the term "innate immune memory" will be used to describe the general functional reprogramming of innate immune cells that includes, both, an enhanced (trained immunity) or a decreased (tolerance) response to subsequent stimuli, in comparison to the primary cell response (Figure 6).

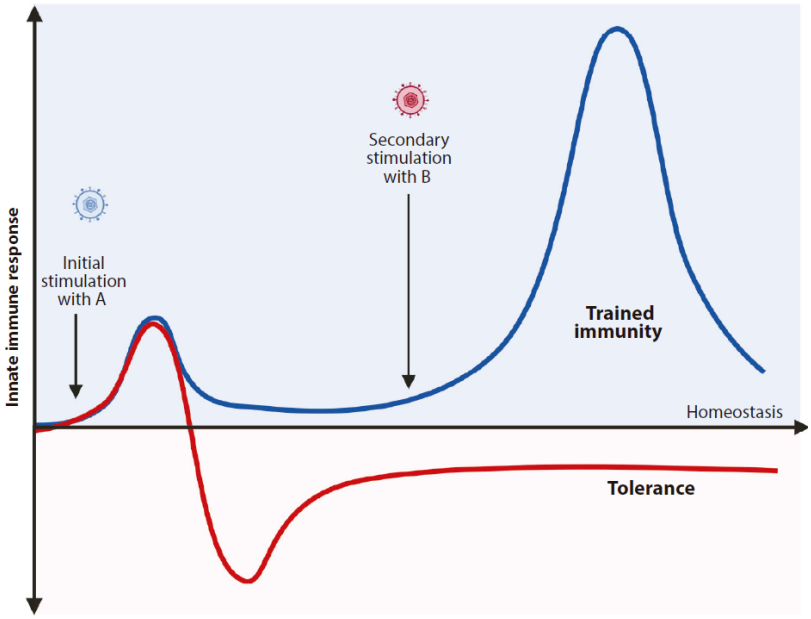

FIGURE 6. Trained immunity versus tolerance. Microbial or endogenous stimuli (stimulus A) can activate innate immune cells and induce a primary response, which consists in the production of cytokines and the activation of other effector functions. Depending on the dose and stimulus, subsequent anti-inflammatory mechanisms can be induced, so as to limit tissue damage. This first encounter induces changes in innate immune cells that are maintained in time and modulate secondary responses to homologous or heterologous stimulation. Therefore, a secondary stimulation (stimulus B) can either not lead to another proinflammatory response (tolerance) or induce an enhanced innate immune response (trained immunity) (Bekkering *et al.*, 2021).

3.1. Endotoxin tolerance

Upon detection of pathogens by innate immune cells, a robust inflammatory reaction is triggered. Inducing inflammation is essential for efficiently fighting off the pathogen, but this process needs to be tightly regulated, as uncontrolled inflammation leads to extensive tissue damage and manifestation of pathological states, such as sepsis, autoimmune diseases, metabolic diseases and cancer (Biswas & Lopez-Collazo, 2009).

When animals are injected with high doses of LPS, macrophages are induced to produce inflammatory cytokines, including TNF-α, IL-1β and IL-6, leading to tissue damage, dysregulation of body temperature and lethality. Therefore, injection of high doses of LPS has widely been used as an experimental model of septic shock. However, it has been shown that pre-treatment with low doses of LPS can protect against LPS-induced lethality, and that monocytes/macrophages are the main cells involved in inducing endotoxin tolerance *in vivo* (Cavaillon & Adib-Conquy, 2006). LPS tolerance has also been modelled *in vitro*, confirming monocyte/macrophage inability to respond to further LPS challenge and revealing that after pre-treatment with LPS, the expression of many inflammatory genes (especially TNF-α) is reduced upon subsequent LPS stimulation due to diminished TLR4 signalling (Seeley & Ghosh, 2017).

Other TLR agonists might likewise cause this unresponsiveness to subsequent challenges with the same stimuli. TLR2 ligands, for instance, have been proved to induce a tolerance effect both, *in vitro* and *in vivo* (Medvedev *et al.*, 2006). Furthermore, early investigations on tolerance also found that exposure to one kind of bacterial pyrogen frequently resulted in tolerance to other types of bacterial pyrogens, a phenomenon known as heterotolerance or cross-tolerance. For example, exposure to TLR2 ligands render macrophages tolerant to LPS (Sato *et al.*, 2000; Dobrovolskaia *et al.*, 2003) and polyI:C (a TLR3 ligand) and flagellin (a TLR5 ligand) induce cross-tolerance to LPS in human peripheral blood mononuclear cells (Ifrim *et al.*, 2014).

Nevertheless, as previously mentioned, endotoxin tolerance is far from being an overall diminished response. Transcriptome studies in monocytes/macrophages have shown that these cells undergo a major gene reprogramming during endotoxin tolerance characterized not only by downregulation, but also by upregulation of targeted genes. Inflammatory genes were shown to be inhibited (tolerizeable genes), while genes involved in pathogen recognition and antimicrobial response were either upregulated or

underwent no change (non-tolerizeable genes) (Foster *et al.*, 2007). Therefore, inhibition of inflammatory genes would supress septic shock-derived damage, while constant expression of antimicrobial genes would be necessary for protection from microbial infections. Accordingly, data from several *in vivo* tolerization experiments show that, indeed, tolerance induced by TLR4 or TLR2 agonists is also a form of innate immune memory that may benefit the host in resisting subsequent infections. As an example, mice pre-treated with LPS are less susceptible to infection by *Cryptococcus neoformans* (Rayhane *et al.*, 2000) or *Salmonella typhimurium* (Lehner *et al.*, 2001), and when being pre-treated with TLR2 ligands, mice become less susceptible to acute polymicrobial septic peritonitis (Feterowski *et al.*, 2005). *In vitro* models have also demonstrated that endotoxin-tolerized monocytes, despite displaying an impaired antigen presentation capacity, exhibit an increased phagocytic ability coupled with a conserved capacity to kill internalized pathogens (del Fresno *et al.*, 2009).

Tolerance induced by exposure to TLR4 and TLR2 ligands also affects DCs, which have been shown to display a diminished expression of the proinflammatory cytokines IL-12, TNF-α and IL-6, but an enhanced expression of the anti-inflammatory cytokine IL-10, transforming growth factor-β and the indoleamine 2,3-dioxygenase 1, one of the most effective mediators of DC anti-inflammatory activity (Albrecht *et al.*, 2008; Fallarino *et al.*, 2015).

3.2. Trained immunity

Many studies have demonstrated that training mice with different microbial ligands of PRRs can protect against a subsequent lethal infection in a non-specific manner. For example, treatment with β-glucan induces protection against subsequent infection with *S. aureus* (Di Luzio & Williams, 1978; Marakalala *et al.*, 2013), while the peptidoglycan component muramyl dipeptide protects against *Streptococcus pneumoniae* and *Toxoplasma gondii* infections (Krahenbuhl *et al.*, 1981). Similarly, TLR9 agonists lead to protection against sepsis and meningitis caused by *E. coli* (Ribes *et al.*, 2014) and flagellin induces protection against *S. pneumoniae* (Muñoz *et al.*, 2010) and rotavirus (Zhang *et al.*, 2014).

Compelling evidence that trained immunity is induced in vertebrates and mediates, at least, some of the protective effects of vaccination came from studies showing that the

BCG vaccine protects mice against secondary infections with *C. albicans* or *Schistosoma mansoni* through T cell-independent but macrophage-driven mechanisms (Tribouley *et al.*, 1978; van't Wout *et al.*, 1992). This was further supported by studies showing that vaccination with an attenuated strain of *C. albicans* (PCA2) protected mice from systemic infection caused by a virulent *C. albicans* strain or other microorganisms (Bistoni *et al.*, 1986). This protection was also T cell-independent (Bistoni *et al.*, 1988) and relied on macrophages and proinflammatory cytokine production (Vecchiarelli *et al.*, 1989). In 2012, Quintin *et al.* obtained similar results and showed that infecting Rag1-deficient mice (which fail to generate mature T and B cells and, therefore, cannot mount and adaptive response) with a sublethal dose of *C. albicans*, confers protection against reinfection. An *in vitro* model showed that these effects take place via epigenetic and metabolic reprogramming of monocytes by β-glucan (Quintin *et al.*, 2012). Shortly after, it was demonstrated that the BCG vaccine induces NOD2-dependent non-specific protection from reinfection through similar mechanisms in monocytes (Kleinnijenhuis *et al.*, 2012). Other vaccines in development against tuberculosis, such as MTBVAC, maintain the ability of BCG to induce trained immunity and protect against heterologous infections in different experimental models in mice and non-human primates (Tarancón *et al.*, 2020; Vierboom *et al.*, 2021).

In humans, a large number of epidemiological studies have shown that live vaccines, such as the BCG vaccine, measles vaccine, smallpox vaccine and oral polio vaccine, have non-specific protective effects against infections other than the targeted ones (de Bree *et al.*, 2018), which could be explained by the induction of trained immunity. Trials with the BCG vaccine in adults (Kleinnijenhuis *et al.*, 2012) and children (Jensen *et al.*, 2015) demonstrated that this vaccine induces non-specific activation of innate immune cells and that its effects may last for months. These effects have been associated with increased protection against heterologous infections, including yellow fever (Arts *et al.*, 2018) and malaria (Walk *et al.*, 2019), which was associated with an augmented proinflammatory activity of monocytes. Additionally, there is evidence that BCG vaccination can induce antitumour immune effects leading to the prevention or treatment of malignancies such as bladder cancer (Redelman-Sidi *et al.*, 2014), melanoma (Stewart & Levine, 2011), leukaemia and lymphoma (Villumsen *et al.*, 2009), with these anticancer effects being dependent on the BCG vaccine's capacity to induce trained immunity in monocytes and macrophages (Buffen *et al.*, 2014). Noteworthy, inducing trained immunity with the BCG

vaccine has been proposed as a tool for reducing susceptibility to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Netea *et al.*, 2020). In fact, a recent retrospective cohort-based study has suggested that BCG reduces the incidence of coronavirus disease 2019 (COVID-19) (Moorlag *et al.*, 2020a). However, while induction of trained immunity is beneficial against infections, it can play a detrimental role in chronic inflammatory diseases, such as atherosclerosis, rheumatic diseases and neurodegenerative disorders, by leading to hyperinflammation or immunosuppression (Hu *et al.*, 2022).

The initial studies of trained immunity were mainly focused on monocytes and macrophages, but recent studies have shown that trained immunity can also be induced in other cell subsets. For instance, NK cells isolated from BCG-immunized volunteers showed a trained profile with enhanced cytokine production after *ex vivo* stimulation with unrelated pathogens (Kleinnijenhuis *et al.*, 2014). Additionally, DCs isolated from mice that had been immunized by a fungal infection displayed enhanced IFN-γ and proinflammatory cytokine responses upon subsequent challenge in a process driven by epigenetic modifications (Hole *et al.*, 2019). Moreover, trained immunity has also been recently proposed to take place in nonimmune cell types, such as stromal and epithelial cells, a concept that has been termed “expanded trained immunity”. Therefore, in tissues particularly exposed to the external environment containing pathogens, epithelial and stromal cells would be able to modulate their responses to successive encounters with the pathogen (Cassone, 2018).

3.3. Mechanisms: epigenetic and metabolic reprogramming

Upon first encountering a stimulus, myeloid cells undergo epigenetic and metabolic reprogramming, resulting in a modified response after secondary stimulation (Figure 7). Therefore, induction of innate immune memory appears to be mediated by long-term adaptations in the chromatin of innate immune cells that render the DNA more or less accessible to the transcriptional machinery, together with metabolic rewiring (Domínguez-Andrés *et al.*, 2020).

Epigenetic regulation is mainly determined by post-translational histone modifications, DNA methylation and the production of long noncoding RNAs (lncRNAs), all of which ultimately modify the chromatin structure and alter the

accessibility of the transcriptional machinery to the promoter and regulatory regions of the genes involved in the immune response, tightly regulating gene expression in immune cells (Hashimoto *et al.*, 2010; Pongubala & Murre, 2021).

Functionally, the presence of histone 3 lysine 4 trimethylation (H3K4me3) is associated with enhanced gene transcription and several studies have described its enrichment at the promoter regions of proinflammatory genes in monocytes, resulting in an increased release of proinflammatory cytokines upon secondary stimulation (Kleinnijenhuis *et al.*, 2012; Quintin *et al.*, 2012). Other studies have also described histone 3 lysine 27 acetylation (H3K27ac) and histone 3 lysine 4 monomethylation (H3K4me1), both of which are associated with active enhancer regions in the DNA, as epigenetic marks associated with trained immunity (Saeed *et al.*, 2014). An enrichment for H3K27ac was reported in a genomic regions upstream of the NOD2 receptor gene (one of the main receptors for inducing trained immunity by BCG) (Arts *et al.*, 2018), and exposure of monocytes to β-glucan *in vitro* results in an increased deposition of H3K4me1 at the enhancer regulatory region crucial for IL-32 transcription (Dos Santos *et al.*, 2019). Additionally, β-glucan can partially reverse LPS-induced tolerance of macrophages *in vitro* via the presence of H3K27ac at enhancer regions favouring transcription reactivation of unresponsive genes (Novakovic *et al.*, 2016). This endotoxin tolerance has been associated with a lack of H3K4me3 marks in the promoters of tolerizeable genes (Foster *et al.*, 2007).

Regarding DNA methylation, preliminary studies show that the induction of LPS tolerance *in vitro* leads to stable and long-term specific DNA methylation changes, but these signatures have been less explored in trained immunity (Novakovic *et al.*, 2016). However, new studies have shown that individuals that undergo monocyte reprogramming after BCG vaccination display a wide loss of DNA methylation on promoters of inflammatory genes, in comparison to non-responder individuals (Verma *et al.*, 2017; Das *et al.*, 2019).

In addition, lncRNAs were identified as functional modulators of gene expression that are brought into proximity to specific genes through the formation of chromosomal loops. Additionally, immune priming lncRNAs (IPLs), have been described to contribute to trained immunity by modulating the epigenetic programming of innate immune cells. Within them, UMLILO (upstream master lncRNA of the inflammatory chemokine locus) was described as a regulator of H3K4me3 deposition at the promoter site of IL-8 and,

more interestingly, it has been observed that the training capacity of β-glucan-exposed monocytes is disrupted in the absence of the UMLILO transcript (Fanucchi *et al.*, 2019).

Besides epigenetic reprogramming, the other main mechanistic process inducing innate immune memory is represented by changes in cell metabolism. Following a first contact with an innate immune memory-inducing stimulus, cells reprogram their metabolic activities to provide a quick supply of all the necessary nutrients so as to facilitate a modified response in the event of subsequent contact with the same or another pathogen (Domínguez-Andrés *et al.*, 2020). Several metabolic pathways are involved in innate immune memory, including glycolysis, oxidative phosphorylation, the tricarboxylic acid (TCA) cycle and lipid metabolism.

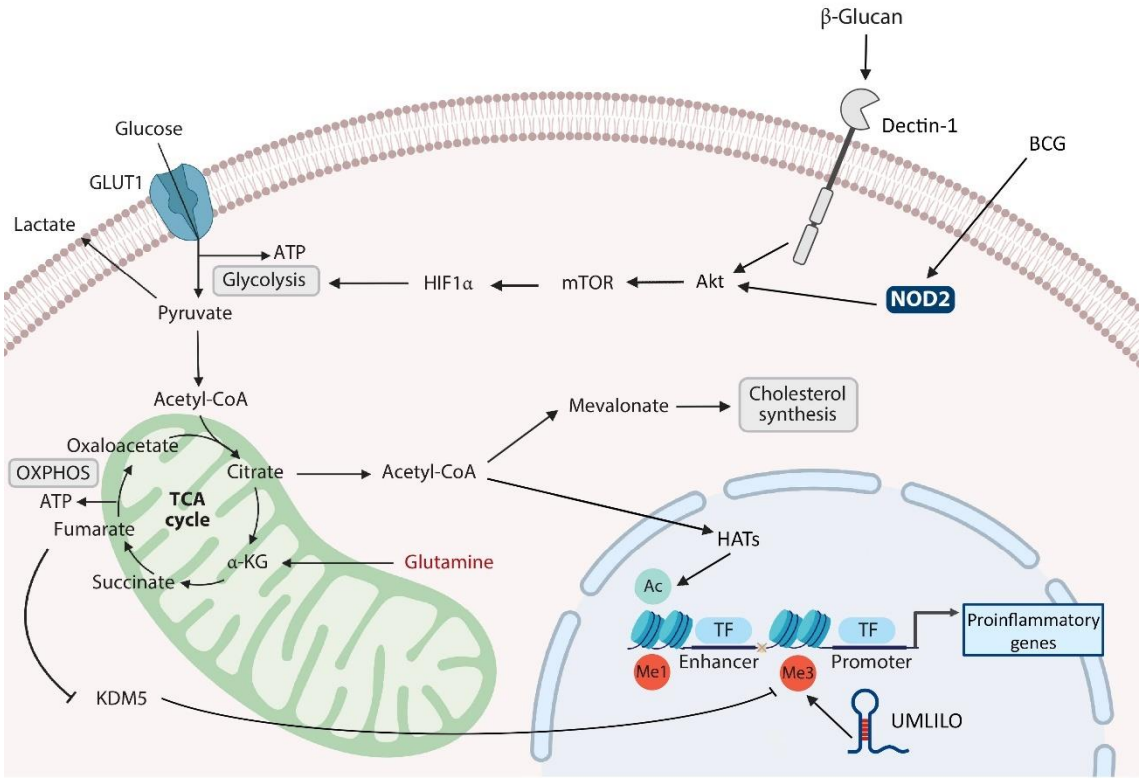

FIGURE 7. Molecular mechanisms of trained immunity. Inducers of trained immunity, such as β-glucan and BCG, activate intracellular pathways via different receptors. The most common pathway is the Akt-HIF1α-mTOR pathway, which ultimately leads to the upregulation of glycolysis, the TCA cycle and the cholesterol synthesis pathway. Metabolites from these pathways can, in turn, regulate epigenetic remodelling of histones: acetyl-CoA serves as an acetyl donor for HATs and fumarate inhibits the KDM5 demethylase, for instance. The lncRNA UMLILO also modulates the epigenetic programming by regulating H3K4me3 deposition. Adapted from Bekkering *et al.*, 2021.

In the first studies with β-glucan-trained monocytes, it was shown that these cells exhibit a switch from oxidative phosphorylation to aerobic glycolysis, a phenomenon known as "Warburg effect" (Cheng *et al.*, 2014). However, later studies have demonstrated that trained cells exhibit both, increased glycolytic and oxidative phosphorylation rates (Keating *et al.*, 2020), and that only the induction of immune tolerance with LPS induces a Warburg effect in monocytes (Lachmandas *et al.*, 2016). In fact, during this state of increased glycolysis Several TCA intermediates have been reported to be involved in the tuning of innate immune memory. For instance, glutamine replenishment of the TCA cycle (as a result of an anabolic repurposing of TCA metabolites) leads to the accumulation of fumarate, which induces trained immunity by downregulating the activity of histone demethylase KDM5, thus indicating a connection between metabolic pathways and epigenetic reprogramming (Arts *et al.*, 2016). Moreover, acetyl-CoA acts as the source of acetyl groups for histone acetyltransferases (HATs), which are in charge of the acetylation of histones (Simithy *et al.*, 2017). In addition to upregulation of glycolysis and oxidative phosphorylation and repurposing of the TCA cycle, an enhanced cholesterol synthesis, as a result of accumulation of mevalonate, was also observed during the induction of trained immunity by β-glucan (Saeed *et al.*, 2014).

3.4. Innate immune memory in HSPCs

Innate immune memory was first described on mature myeloid cells, but their average half-life in the circulation is of only a few days and circulating cells with a trained immunity phenotype have been described to exist for months following *in vivo* induction of trained immunity with BCG (Kleinnijenhuis *et al.*, 2012, 2014). Hence, this strongly suggests that reprogramming would also be taking place at the level of progenitor cells to account for the persistence of the relatively short-lived monocyte/macrophage populations with modified phenotypes.

In this context, our group has demonstrated that, indeed, this concept of "innate immune memory" could be applied not only to mature myeloid cells, but also to HSPCs. Using an *in vitro* differentiation model of HSPCs it was demonstrated that exposure to TLR2 and TLR4 ligands before or during their differentiation with M-CSF generates tolerized macrophages, while exposure of HSPCs to *C. albicans* results in the production

of trained macrophages (with an increased capacity to produce cytokines and a higher fungicidal activity), compared to control macrophages differentiated only with M-CSF (Megías *et al.*, 2016). In fact, the ability of macrophages to produce cytokines is extremely dependent on how the HSPCs from which they are derived receive stimuli, that is, the tolerized or trained phenotype depends on the combination of stimuli they receive, as well as on the timing on which HSPCs receive them (Martínez *et al.*, 2017). Regarding the involved mechanisms, it has been demonstrated that HSPCs exposed to the TLR2 ligand produce soluble factors that act in a paracrine manner affecting the function of macrophages that had not been exposed to Pam_3CSK_4, thus generating tolerized macrophages (Yáñez *et al.*, 2013b). Furthermore, *in vitro* results have been completed with an *ex vivo* model with which it was demonstrated that HSPCs are capable of responding to systemic candidiasis *in vivo* and that this deeply affects the functional phenotype of the macrophages generated *ex vivo*. In this model, early during infection HSPCs give rise to trained macrophages *ex vivo* with a higher fungicidal activity, whereas when the infection reaches high fungal burden levels in internal organs, HSPCs give rise to tolerized macrophages *ex vivo* that still keep up their fungicidal capacity (Martínez *et al.*, 2018).

Regarding the involvement of HSPCs in trained immunity models of protection against reinfection, Mitroulis *et al.* have shown that β-glucan training induces a protective response to a subsequent LPS injection by reprogramming HSPCs via IL-1β. This is also associated with an upregulation of the glycolysis and cholesterol synthesis pathways in progenitor cells, resulting in the expansion of myeloid-biased HSPCs (Mitroulis *et al.*, 2018). Similarly, another study highlighted the role of IL-1 in inducing protective trained immunity in HSPCs, as priming with β-glucan caused HSPC expansion and protected mice against infection with *Mycobacterium tuberculosis* (Moorlag *et al.*, 2020b) and IL-1 was also found to have a relevant role in the induction of innate immune reprogramming of bone marrow GMPs caused by a Western diet regime (Christ *et al.*, 2018).

What is more, the BCG vaccine causes long-term innate immune memory in mouse HSCs in a process dependent on IFN-γ signalling. In this model, trained HSCs are able to generate an epigenetically reprogrammed myeloid progeny that protect against tuberculosis *in vivo* (Kaufmann *et al.*, 2018). These observations were confirmed in a study with human volunteers in which vaccination with BCG led to transcriptional and epigenetic reprogramming of bone marrow HSPCs up to 90 days after initial vaccination,

resulting in a myeloid bias and an increased protection against reinfection (Dos Santos *et al.*, 2019; Cirovic *et al.*, 2020). Recently, de Laval *et al.* reported that upon LPS exposure, HSCs provide an increased protective response to *Pseudomonas aeruginosa* by reducing bacterial burden and increasing survival rate. This phenomenon relies on epigenetic reprogramming of HSCs, which showed increased C/EBPβ-dependent chromatin accessibility, especially in myeloid enhancers, which were later reactivated during reinfection (de Laval *et al.*, 2020). Furthermore, a recent study showed that following COVID-19, HSPCs retain epigenomic alterations that are transmitted, through differentiation, to progeny innate immune cells. These epigenomic changes vary with disease severity, persist for months up until a year and are associated with an increased myeloid cell differentiation and inflammatory or antiviral programs (Cheong *et al.*, 2022).

Lastly, a ground-breaking study recently showed that unspecific protection against bacterial infections upon sublethal infection with *C. albicans* was transmitted intergenerationally and transgenerationally for up to two generations (Katzmarski *et al.*, 2021). Surprisingly, the progeny of *C. albicans*-exposed mice inherited an epigenetic signature induced by the fungal stimuli on the HSPC populations of their parents, which boosted myeloid cell production and activation and increased survival following *E. coli* infection. These results are in agreement with a randomized controlled trial showing that the reduction in mortality observed in BCG-vaccinated infants is significantly improved if the mother also has a history of BGC vaccination (Berendsen *et al.*, 2020).


Objectives

According to the background information provided in the Introduction section, the general objectives of the present PhD thesis have been the following:

1. **To study the involvement of dectin-1 in HSPC differentiation and the phenotype of the derived macrophages in response to its ligand and in response to stimulation with *C. albicans*.**

2. **To investigate the molecular mechanisms by which TLR2 and dectin-1 induce HSPC differentiation and confer a tolerized or trained phenotype, respectively, to the produced mature cells.**

   2.1. Determining the contribution of direct and indirect mechanisms.

   2.2. Delving into the signalling transduction pathways and activation of transcription factors driven by direct mechanisms.

3. **To explore trained immunity following vaccination with a low-virulence strain of *C. albicans*.**

   3.1. Characterizing the cell populations in the bone marrow and spleen and studying their phenotype.

   3.2. Analysing HSPC expansion and/or differentiation, mobilization, transcriptome, phenotype of the macrophages generated *ex vivo* and their possible protective role against infection.

   3.3. Unveiling the mechanisms by which vaccination may induce the reprograming of HSPCs.

Materials & Methods

1. Mice

WT C57BL/6 mice and CD45.1-positive allotype mice (B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyCrl strain, also known as C57BL/6-Ly5.1) were purchased from Envigo and Charles River Laboratories, respectively. Dectin-1$^{-/-}$ and MyD88$^{-/-}$ mice were purchased from The Jackson Laboratory, and TLR2$^{-/-}$ mice were provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan). Transgenic mice expressing the DsRed.MST fluorescent protein (B6.Cg-Tg[CAGDsRed*MST]1Nagy/J strain, also known as DsRed.T3 [The Jackson Laboratory]) were provided by Dr. Isabel Fariñas (University of Valencia, Valencia, Spain). All KO and transgenic mice have a C57BL/6 background and were bred and maintained at the animal production service facilities (SCSIE, University of Valencia).

Mice between 6 and 12 weeks of age were used, regardless of sex. Experiments were approved by the Committee on the Ethics of Animal Experiments of the University of Valencia (permit numbers 2017/VSC/PEA/00207, 2019/VSC/PEA/0126 and 2021/VSC/PEA/0038) and performed according to regulations established in Real Decreto 1201/2005 and Real Decreto 53/2013. (Ministerio de la Presidencia, Spain) All efforts were made to minimize suffering.

2. Culture media

2.1. Culture media for fungi

Culture media used for fungi were the following:

YPD medium	
Yeast extract	10 g
Tryptone	20 g
Glucose	20 g
Distilled water	1 litre

Sabouraud Dextrose Agar medium	
Pancreatic digest of casein	5 g
Peptic digest of animal tissue	5 g
Dextrose	40 g
Agar	15 g
Distilled water	1 litre

Simplified synthetic Lee medium	
$(NH_4)_2SO_4$	5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
HK_2PO_4 anhydrous	2.5 g
NaCl	5 g
Glucose	12.5 g
Proline	0.5 g
Biotin	0.5 g
Distilled water	1 litre

In order to remove bacterial endotoxin (LPS), liquid media (YPD and Lee) were treated with polymyxin B-agarose (Sigma-Aldrich), which binds LPS and can therefore be removed from the solution upon centrifugation and supernatant recovery. Additionally, they were sterilized by filtering them with 0.22 µm filters (Merck). The Sabouraud Dextrose Agar medium was sterilized by autoclaving it at 121 ºC for 20 min.

2.2. Culture medium for murine cells

Culture medium used for murine cells was the following:

RPMI complete medium	
RPMI 1640 with GlutaMAX™ I (Gibco)	
Heat-inactivated fetal bovine serum (FBS) (Gibco)	5%
Penicillin-streptomycin stock solution (Gibco)	1%

2.2.1. Cytokines

Where indicated, the following cytokines were added to RPMI complete medium:

Cytokine	Concentration
SCF (Peprotech)	20 ng/ml
M-CSF (Peprotech)	50 ng/ml
GM-CSF (Peprotech)	20 ng/ml
IL-6 (Peprotech)	20 ng/ml
IFN-β (PBL Assay Science)	50 U/ml

2.2.2. Inhibitors

Where indicated, the following inhibitors were added to cell cultures in RPMI complete medium:

Inhibitor	Concentration	Time added before stimulation
Rapamycin (Tocris Bioscience)	40 ng/ml	0.5 h
Amlexanox (Tocris Bioscience)	30 µg/ml	2 h
ZSTK474 (Cell Signaling Technology)	1 µM	0.5 h
BMS345541 (Sigma-Aldrich)	2 µM	1 h

3. Stimuli

3.1. TLR and dectin-1 ligands

Ligand	Receptor	Concentration used	
		As differentiation stimuli in culture	As stimuli for cytokine production
Pam_3CSK_4 (Invivogen)	TLR2	1 µg/ml	100 ng/ml
Ultrapure *Salmonella minnesota* LPS (Invivogen)	TLR4	-	100 ng/ml
Depleted zymosan (Invivogen)	dectin-1	10 µg/ml	-

3.2. Fungal stimuli

The fungal strains used in this thesis were: *C. albicans* ATCC 26555, a virulent strain commonly used in candidiasis studies; and the low-virulence non-germinative strain of *C. albicans* PCA2, provided by Dr. Cassone (*Instituto Superiore di Sanità*, Roma, Italia).

3.2.1. Inactivated *C. albicans* yeasts

C. albicans yeasts of the ATCC 26555 strain were inactivated as follows. Cells were cultured in endotoxin-free YPD medium at 28 ºC with shaking up to their exponential growth phase (OD_{600nm} 0.6 – 1). Cellular growth was determined by measuring the optical density (OD) at a wavelength of 600 nm in an Heλios spectrophotometer (Thermo Fisher Scientific). Fungal cells were then collected by centrifugation, resuspended in the same volume of endotoxin-free water and maintained for 3 h at 28 ºC with shaking followed by 24 h at 4 ºC (starved yeast cells). These cells were collected by centrifugation, inoculated in 5 times more volume of simplified synthetic Lee medium than water used and incubated for 3 h at 28 ºC with shaking. For inactivation, the obtained yeasts were resuspended at a concentration of 20 x 10^6 cells/ml of BD Cytofix™ Fixation Buffer (BD Biosciences), which contains 4% paraformaldehyde, and incubated for 30 min at room temperature (RT). Following treatment, fungal cells were thoroughly washed in phosphate-buffered saline (PBS) (Gibco) to eliminate the inactivating agent, microscopically counted to determine their cell concentration and maintained at -80 ºC as dry pellet until used. In M-CSF cultures for the obtention of macrophages, inactivated *C. albicans* yeasts were added at a 1:7.5 ratio (progenitor:yeast), while a 1:5 ratio was used in the absence of M-CSF.

3.2.2. Viable *C. albicans* yeasts

Viable fungal cells used for *in vitro* killing assays (*C. abicans* PCA2) and as stimuli during differentiation (*C. albicans* ATCC 26555) were obtained by culturing them in endotoxin-free YPD medium at 28 ºC with shaking up to their exponential growth phase. Fungal cells were then collected by centrifugation, washed twice in PBS and brought to the desired concentration in RPMI complete medium.

For *in vivo* assays, *C. albicans* PCA2 or ATCC 26555 cells were cultured in endotoxin-free YPD medium at 28 ºC with shaking up to their exponential growth phase. Fungal cells were then collected by centrifugation, resuspended in the same volume of endotoxin-free water and maintained for 3 h at 28 ºC with shaking followed by 24 h at 4 ºC (starved yeast cells). These cells were collected by centrifugation and brought to the desired concentration in PBS for their injection to mice.

4. Obtention of bone marrow cells and splenocytes

In order to obtain bone marrow cells, mice were euthanized by cervical dislocation and 70% ethanol was sprayed onto them. The skin covering both lower extremities was removed and they were separated from the animal by dislocating the hip joint. Feet were removed, femurs were separated from tibias at the knee joint and muscles were removed from the bones by using tissue paper. Bones were rinsed with 70% ethanol, placed in a clean 5 cm plate and taken into a tissue culture hood. Bones were then submerged in PBS prior to cutting their epiphyses. Following, by using a 25-gauge needle and a 10 ml syringe filled with sterile PBS, femurs and tibias were flushed onto a 50 ml tube until the bones were completely white. Cell suspension was filtered using a 70 µm filter and then centrifuged at 450 g for 5 min. The obtained bone marrow cells were then used for isolating HSPCs from them (see section 5) or were treated with BD Pharm Lyse™ lysing buffer (BD Biosciences) to lyse their erythrocytes.

For the obtention of splenocytes, spleens were taken under sterile conditions from mice (previously euthanized by cervical dislocation) and placed in a 5 cm plate. By using a 25-gauge needle, 10 ml of MACS buffer (PBS + 5% FBS + 2 mM EDTA [ethylenediaminetetraacetic acid]) were injected into them and the obtained cell suspension was filtered using a 70 µm filter. Following, each spleen was cut into small pieces that were placed on that same filter and mashed using the piston of a sterile syringe. The filter was washed with MACS buffer and the cell suspension was centrifuged at 450 g for 5 min. The obtained splenocytes were treated with BD Pharm Lyse™ lysing buffer (BD Biosciences) to lyse their erythrocytes.

5. Isolation of HSPCs

5.1. Purification of Lin$^-$ cells

Lineage-negative (Lin$^-$) cells were purified from bone marrow cell suspensions or red blood cell (RBC)-lysed splenocytes by magnetic cell separation using the "Lineage Cell Depletion Kit, mouse" and the QuadroMACS^{TM} Separator (both from Miltenyi Biotec), following the manufacturer's instructions. Cells were first labelled with a cocktail of biotinylated antibodies against a panel of mature immune lineage antigens [CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4 and Ter-119] followed by labelling with anti-biotin MicroBeads. Cell sorting was performed in MACS LS columns (Miltenyi Biotec) placed in a powerful permanent magnet that induces a high-gradient magnetic field so that labelled cells are retained in the columns while the unlabelled ones flow through it. Therefore, the Lin$^-$ fraction (not labelled) was collected and centrifuged at 450 g for 5 min. Cell pellet was resuspended in either PBS or complete cell culture medium supplemented with SCF (depending on whether cells were transplanted or cultured, respectively) and cells were counted using the Countess^{TM} II FL Automated Cell Counter (Thermo Fisher Scientific).

5.2. Purification of LKS$^+$ cells

Lin$^-$ cells (obtained as described in section 5.1) were separated into LKS$^+$ and LKS$^-$ cells by magnetic cell separation using the "Anti-Sca-1 MicroBead Kit (Vio® Bright FITC), mouse" and the QuadroMACS^{TM} Separator (both from Miltenyi Biotec), following the manufacturer's instructions. In this case, cells were first labelled with a monoclonal antibody anti-Sca-1-Vio Bright FITC, followed by labelling with anti-FITC MicroBeads. Cell sorting was performed in MACS MS columns (Miltenyi Biotec), which retained the LKS$^+$ cell fraction, while LKS$^-$ cells ran through. Both cell fractions were collected separately, centrifuged at 450 g for 5 min, resuspended in complete cell culture medium supplemented with SCF and counted using the Countess^{TM} II FL Automated Cell Counter (Thermo Fisher Scientific).

5.3. Purification of c-kit$^+$ cells

C-kit$^+$ and c-kit$^-$ cells were separated from RBC-lysed bone marrow cells or splenocytes by magnetic cell separation using the "CD117 MicroBeads kit" and the QuadroMACS^{TM} Separator (both from Miltenyi Biotec), following the manufacturer's instructions. This is a one-step procedure in which cells are labelled with a CD117 (c-kit) monoclonal antibody conjugated with magnetic MicroBeads. Cell sorting was performed in a MACS LS column (Miltenyi Biotec), which retained the cell fraction enriched in c-kit$^+$ cells while c-kit$^-$ cells ran through. Both cell fractions were collected separately, centrifuged at 450 g for 5 min, resuspended in PBS and counted using the Countess^{TM} II FL Automated Cell Counter (Thermo Fisher Scientific).

6. *In vitro* HSPC cultures

6.1. Proliferation and myeloid differentiation measurement

Lin$^-$, LKS$^-$ or LKS$^+$ cells were cultured in 0.2 ml of RPMI complete medium containing SCF in flat-bottomed 96-well plates. When cocultured with 2 x 10^6 splenocytes, the assay was performed in non-tissue culture (TC)-treated 24-well plates in a total volume of 0.5 ml. The number of plated cells varied depending on the stimuli added, as different stimuli lead to different proliferation rates. Therefore, the following number of cells were cultured in order to ensure their survival and the obtention of a sufficient quantity of cells at the analysed timepoints:

Stimuli	# Lin$^-$ or LKS$^-$ cells	# LKS$^+$ cells
None	200,000	20,000
M-CSF	25,000	-
Pam_3CSK_4	50,000	20,000
Depleted zymosan	200,000	-
Inactivated *C. albicans* yeasts	200,000	-

At different timepoints, cells were harvested by recovering supernatants containing non-adherent cells and PBS + 2 nM EDTA was added to the wells for 20 min at 37 ºC before repeated resuspensions of the liquid to promote the detachment of the adherent cells from the plastic surface. The obtained total cells (adherent and non-adherent) were labelled with different antibodies to analyse their myeloid differentiation by flow cytometry.

When indicated and prior to being plated, Lin^- cells were stained with CFSE (carboxyfluorescein succinimidyl ester) using the CFSE CellTrace™ cell proliferation kit (Molecular Probes) according to the manufacturer's instructions. CFSE is a cell-permeable fluorescent dye that covalently couples to intracellular molecules allowing for measuring proliferation, as it gets diluted with each cell division.

6.2. Differentiation to macrophages

Macrophages were obtained by culturing 2 x 10^5 Lin^- cells in 4 ml of RPMI complete medium containing SCF (to ensure the survival of HSPCs) and M-CSF (to promote differentiation to macrophages) in 55 mm non-TC-treated plates for 7 days at 37 ºC in a 5% CO_2 atmosphere. Where indicated, cultures also contained different stimuli for 6 h (live *C. albicans* cells), 24 h (Pam_3CSK_4 and GM-CSF) or throughout the 7 days (depleted zymosan and inactivated yeasts of *C. albicans*). When the stimuli added were live *C. albicans* cells, Lin^- cells were cultured in 0.5 ml of RPMI complete medium containing SCF in 24-well plates before transferring them to the 55 mm plates after 6 h and adding the antifungal amphotericin B (0.5 µg/ml) in order to prevent fungal growth of the remaining yeasts. At day 7, adherent cells were harvested by discarding cell media and adding precooled MACS buffer prior to gently scraping them. Collected macrophages were counted and replated in 96-well plates with RPMI complete medium at different cell densities for killing or cytokine assays (detailed in sections 9 and 10, respectively). When performing coculture experiments, collected cells were separated into $DsRed^+$ and $DsRed^-$macrophages using a BD FACS Aria Fusion cell sorter (BD Biosciences) prior to being replated.

6.3. Transwell assays

Transwell assays were performed using 12-well plates with 0.4 µm-pore polycarbonate membranes (Corning). Lin^- cells were plated in the upper and lower chambers (5 x 10^4 cells in each chamber) in a total volume of 1.5 ml of RPMI complete medium containing SCF and M-CSF to obtain macrophages from them. Where indicated, lower chamber cultures also contained depleted zymosan or inactivated *C. albicans* cells as stimuli. At day 7, adherent cells were harvested from the lower and upper chambers by discarding supernatants and adding PBS + 2 nM EDTA to the wells for 20 min at 37 ºC before repeatedly resuspending them to promote their detachment from the plastic surface. Equal numbers of macrophages obtained from each chamber were plated separately and stimulated for the analysis of cytokine production (see section 10).

6.4. Methylcellulose cultures

Identification of different hematopoietic progenitors was accomplished by plating 7 x 10^5 RBC-splenocytes in triplicate in MethoCult GFM3434 methylcellulose-based medium (STEMCELL Technologies). Its components include insulin, transferrin, stem cell factor, IL-3, IL-6 and erythropoietin, allowing the growth of hematopoietic progenitor cells into colonies. After being incubated at 37 ºC for 7 days, colonies were counted and identified on the basis of their morphological characteristics, according to the manufacturer's instructions.

7. *In vivo* transplantation and stimulation of Lin^- cells

Approximately 2.5 x 10^6 Lin^- cells in 0.1 ml of PBS (purified from either 4 WT or 4 KO mice) were intravenously (i.v.) injected into one recipient mouse (KO or WT mice, respectively). Transplanted mice were then injected with PBS (control) or with different stimuli: depleted zymosan (300 µg), inactivated *C. albicans* yeasts (10^7 cells), M-CSF (10 µg), or M-CSF and depleted zymosan, once daily for 3 days.

7.1. Detection and characterization of transplanted cells

When using the transplantation model that takes advantage of the polymorphisms of CD45 (CD45.1 and CD45.2 alleles) for *in vivo* tracking, transplanted mice were sacrificed at day 3 and total RBC-lysed bone marrow cells and splenocytes were obtained from them. Fc receptors were blocked with FcR blocking reagent (Miltenyi Biotec) and cells were then stained with a biotinylated anti-CD45.1 or anti-CD45.2 antibody and anti-biotin MicroBeads (both from Miltenyi Biotec) and were immunomagnetically sorted in MACS LS columns on the QuadroMACS^{TM} Separator in order to deplete cells that express de CD45 alloantigen of the recipient mice and, therefore, enrich the sample for the transplanted cells. Recovered cells were microscopically counted, labelled with various combinations of antibodies and analysed by flow cytometry.

In the transplantation model that takes advantage of the DsRed expression for *in vivo* tracking, transplanted mice were sacrificed at day 3 and their spleens were removed. RBC-lysed splenocytes were obtained and Fc receptors were blocked as described above. The sample was depleted of T and B cells (in order to enrich the sample for myeloid cells) by immunomagnetic cell purification using biotinylated anti-CD3 and anti-CD19 antibodies and anti-biotin magnetic MicroBeads (all from Miltenyi Biotec). The remaining cells were stained for CD11b and F4/80, and DsRed double-positive cells were sorted by flow cytometry using a BD FACS Aria Fusion cell sorter (BD Bioscience) prior to being plated and stimulated the following day for the analysis of cytokine production (see section 10).

8. *C. albicans* infection

For primary infections, 1.5 x 10^6 viable (CFUs) *C. albicans* PCA2 were injected i.v. in a volume of 0.1 ml. For secondary infections, 1.5 x 10^6 viable (CFUs) *C. albicans* ATCC 26555 cells were injected i.v. in a volume of 0.1 ml and survival was checked daily for 21 days, or 2 x 10^7 viable (CFUs) *C. albicans* ATCC 26555 cells were injected intraperitoneally (i.p.) in a volume of 0.2 ml for fungal burden assessment.

8.1. Quantification of CFUs in kidney

To assess fungal burden following infection, kidneys were weighed, homogenized in 1 ml of PBS and dilutions of the homogenates were plated on Sabouraud dextrose agar. The colony forming units (CFUs) were counted after 24 h of incubation at 37 ºC and expressed as CFUs per gram of tissue.

8.2. LPS-induced septic shock

Septic shock was induced by an intraperitoneal injection of 10 or 20 mg/kg of *Escherichia coli* LPS (Invivogen) to mice 7 or 14 days, respectively, after the PCA2 infection, or to uninfected mice, and survival was checked twice a day for 3 days.

8.3. Adoptive transfer experiments

For adoptive transfer experiments, 3 x 10^7 total bone marrow cells, splenocytes or c-kit$^-$ cells, and 5 x 10^6 c-kit$^+$ cells were injected i.v. in a volume of 0.15 ml of PBS.

8.4. Measurement of HSPC mobilization

When measuring HSPC mobilization to the spleen, mice were transplanted with 2 x 10^6 CFSE-labelled Lin$^-$ cells and were sacrificed at day 2. RBC-lysed splenocytes were obtained, Fc receptors were blocked, and the sample was depleted of B and T cells (in order to enrich for donor CFSE-labelled cells) as described before. The remaining cells were stained for CD11b and c-kit to be analysed by flow cytometry.

8.5. GM-CSF function blockage

Where indicated, 125 µg of the anti-GM-CSF antibody (clone MP1-22E9, Biolegend) or its isotype control (Rat IgG2a κ, clone RTK2758, Biolegend) were injected i.p. in a volume of 0.2 ml to PCA2-infected mice twice (at the time of infection and 1 day later).

9. *C. albicans* killing assay

For RBC-lysed total bone marrow cells or total splenocytes, cells were plated in 24-well plates at a density of 1.25 x 10^6 or 2.5 x 10^6 cells, respectively, in 0.5 ml of complete cell culture medium. Cells were challenged with 1 x 10^5 viable *C. albicans* PCA2 yeasts (prepared as indicated in section 3.2.2) settled onto the murine cells by centrifugation and incubated for 3 h.

For HSPC-derived macrophages, cells were plated in 96-well plates at a density of 2 x 10^5 cells in 0.15 ml of complete cell culture medium, challenged with viable *C. albicans* PCA2 yeasts at a 1:3 ratio (murine cell:yeast), settled onto the macrophages by centrifugation and incubated for 1 h.

As a control, *C. albicans* cells were inoculated in culture medium without murine cells. After co-incubation, samples were diluted in water, plated on Sabouraud dextrose agar, and incubated overnight at 37 ºC to determine CFUs. Colonies were counted, and killing percentages were determined as follows:

$$\% \text{ killing} = \left(1 - \frac{\text{CFUs sample at t=1 or 3 h}}{\text{CFUs control at t=1 or 3 h}}\right) \text{x } 100$$

The non-germinative strain of *C. albicans* (PCA2) was chosen for killing assays in order to facilitate determination of CFUs after the incubation period, as no germ tubes (hyphae) aggregates are formed. Triplicate samples were analysed in each assay.

10. Measurement of cytokine production

Cells were plated in flat-bottomed 24-well plates at a density of 2.5 x 10^6 cells (for RBC-lysed total bone marrow cells or total splenocytes) or 3 x 10^5 cells (for HSPCs) in 0.5 ml of complete cell medium, or in flat-bottomed 96-well plates at a density of 5 x 10^4 cells (for HSPC-derived macrophages) in 0.2 ml of complete cell culture medium. Whole blood was diluted 1:2 in complete cell culture medium at a final volume of 0.2 ml and plated in flat-bottomed 96-well plates. To prevent fungal growth, 0.5 ug/ml amphotericin B was added to the cultures of total bone marrow cells, total splenocytes and blood from infected animals. Cells were challenged with 100 ng/ml of Pam_3CSK_4, 100 ng/ml of ultrapure *Salmonella minnesota* LPS (all from Invivogen) or 25 x 10^6 inactivated *C.*

albicans ATCC 26555 yeasts for 24 h, and cell-free supernatants were then harvested and tested for cytokine release using commercial enzyme-linked immunosorbent assay (ELISA) kits (TNF-a and IL-6, Invitrogen; MIP-2 [macrophage inflammatory protein 2], R&D Systems). Unstimulated cells served as negative controls. Triplicate samples were analysed in each assay.

10.1. Intracellular cytokine production

For intracellular detection of cytokine production, total RBC-lysed bone marrow cells or splenocytes were plated in non-TC-treated flat-bottomed 24-well plates at a density of 2.5 x 10^6 in 0.5 ml of complete cell culture medium and were stimulated with 100 ng/ml of ultrapure *Salmonella minnesota* LPS (Invivogen) or 25 x 10^6 inactivated *C. albicans* ATCC 26555 yeasts for 6 h, with the presence of 3 µg/ml brefeldin A (eBioscience) for the final 4 h. When measuring intracellular cytokine production upon a secondary infection, plated RBC-lysed bone marrow cells or splenocytes were stimulated with 50 ng/ml PMA and 500 ng/ml Ionomycin (Sigma-Aldrich) for 6 h, with the presence of 3 µg/ml brefeldin A (eBioscience) throughout the 6 h. Cells were first collected and stained with antibodies against surface markers, and Cyto-Fast™ Fix/Perm Buffer Set (Biolegend) was used according to the manufacturer's instructions to fix and permeabilize them. PE-labelled anti-TNF-α (clone MP6-XT22), anti-IL-6 (clone MP5-20F3) and anti-GM-CSF (clone MP1-22E9) antibodies (Biolegend) were used for assessment of cytokine production by intracellular flow cytometry.

11. Bulk RNA sequencing

Lin^- cells from 3 C57BL/6 mice were cultured in the presence or absence of Pam_3CSK_4 (n=3). After 24 h, cells were harvested and RNA was extracted from whole cell lysates using the RNeasy Plus Mini Kit (Qiagen), following the manufacturer's instructions. The integrity of the RNA samples and their concentration was measured with the 2100 Bioanalyzer Instrument (Agilent). Library preparation was performed with the Illumina TruSeq stranded mRNA library preparation kit and sequencing was conducted on an Illumina NextSeq 550 sequencer at the Genomics Unit of the Central Service for

Experimental Research (SCSIE, University of Valencia) to a mean output of 30 million 75 bp paired-end reads per sample.

11.1. Analysis

Raw sequences in FASTQ format were inspected with FastQC and MultiQC to check for sequencing issues. Cutadapt (Martin, 2011) and Trimmomatic (Bolger *et al.*, 2014) were used to clip or remove low quality reads: the ILLUMINACLIP function was used to remove Illumina adapters and the SLIDINGWINDOW function was applied to discard reads shorter than 20 bases. Good quality reads were mapped to the mouse genome with STAR (Dobin *et al.*, 2013), using default parameters and the *Mus musculus* GRCm38 (mm10) genome as reference. Principal component analysis (PCA) was used to check the similarity of biological replicates. Raw read counts for each sample were extracted with the featureCounts tool from the R package Rsubread (Liao *et al.*, 2014), using the GENCODE mouse genome version M29 annotation as reference.

In order to obtain the list of differentially expressed genes (DEGs) between treated and control samples, the DESeq2 R package (Love *et al.*, 2014) was used with the raw count expression matrix as input and with a 0.3 non-differential contig count quantile threshold. Genes with false discovery rate (FDR) values < 0.05 were considered as differentially expressed, regardless of the magnitude of the change. The list of DEGs was functionally analysed with the enrichGO function from the clusterProfiler R package (Yu *et al.*, 2012). Heatmaps were generated with ComplexHeatmap (Gu *et al.*, 2016). The Spearman rank correlation distance was used as the base for the hierarchical clustering of genes.

These analyses were performed in collaboration with Antonio Jordán Pla (University of Valencia, Burjassot, Spain).

The RNA-sequencing dataset has been deposited in the Gene Expression Omnibus (GEO) database under the accession number GSE206596.

12. Single-cell RNA sequencing

Lin$^-$ cells purified as described in section 5.1 were further purified as Lin$^-$ c-kit$^+$ cells using a BD FACS Aria Fusion cell sorter (BD Biosciences). Single-cell gel beads-in-emulsion (GEMs) were generated using the Chromium Controller instrument (10X Genomics). Sequencing libraries were prepared using Chromium Single Cell 3' Reagent Kits (10X Genomics), according to the manufacturer's instructions. Briefly, GEM-reverse transcription (RT) was performed in a thermal cycler: 53 ºC for 45 min, 85 ºC for 5 min. cDNA was cleaned up with DynaBeads MyOne Silane Beads (ThermoFisher Scientific) and amplified with a thermal cycler: 98 ºC for 3 min, cycled 12 x 98 ºC for 15 s, 67 ºC for 20 s, 72 ºC for 1 min, and 72 ºC 1 min. After clean-up with the SPRIselect Reagent Kit (Beckman Coulter), libraries were constructed by performing the following steps: fragmentation, end-repair, A-tailing, SPRIselect clean-up, adaptor ligation, SPRIselect clean-up, sample index PCR, and SPRIselect size selection. Library preparation was financed by a 10X Genomics and Bonsai Lab Grant Program at the Omics Core Facility from the Instituto de Neurociencias de Alicante. Libraries were sequenced on an Illumina NextSeq 550 High Output run with 50 bp paired-end reads at the Genomics Unit of the Central Service for Experimental Research (SCSIE, University of Valencia).

12.1. Analysis

10X Chromium produced FASTQ files that were subjected to alignment and gene-level quantification with the software Cell Ranger version 3.1.0 using the mouse reference genome (mm10-2.1.0). Downstream processing was performed using the software AltAnalyze version 2.1.4 (http://altanalyze.org) on SoupX corrected (ambient contamination fraction of 10%) filtered sparse matrix files. Cell barcodes with less than 500 genes expressed (counts per gene divided by the total counts per barcode multiplied by 10,000 > 1) were excluded from further analysis.

Cell populations were initially defined using the software cell-Harmony (DePasquale *et al.*, 2019), aligned to previously reported reference mouse bone marrow progenitor cell populations with additional delineation of Erythroid and B-cell clusters using the software ICGS2 (Muench *et al.*, 2020). No additional cell lineages were observed using the ICGS2 when all captures were jointly analysed.

Differential expression analyses between all cell populations for the indicated timepoint comparisons were performed in cellHarmony using the reference Type None option (pre-aligned cell assignments) and a fold > 1.2 and empirical Bayes Moderated t-test, FDR < 0.05. Differential expression gene lists from each population within each comparison were compiled and used as input for the compareCluster tool from the clusterProfiler R package. The compareCluster tool was executed with the "enrichGO" option, the "org.Mm.eg.db" database and the "BP" biological process ontology. Results of the different compareCluster analyses were saved as tables and subsequently used to select specific GO categories to generate the dotplot.

These analyses were performed in collaboration with Antonio Jordán Pla (Universitat de Valéncia, Burjassot, Spain), and H. Leighton Grimes and Nathan Salomonis (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, United States).

The single-cell genomics data has been deposited in the GEO database under the accession number GSE184104.

13. Quantitative PCR

Lin^- cells were plated in non-TC-treated flat-bottomed 24-well plates at a density of $\geq 2 \times 10^5$ cells in 0.5 ml of complete cell culture medium and stimulated as indicated in the results section. At the indicated timepoints after stimulation, RNA was isolated from the cells using the RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's instructions. The obtained RNA was quantified by measuring the A_{260} on a Nanodrop. cDNA was synthesized from 100 ng of total RNA using the PrimeScript™ RT Reagent Kit (TaKaRa Bio). Quantitative PCR for each cDNA sample was performed using TB Green® Premix Ex Taq™ (TaKaRa Bio) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Primer sequences are listed below. Relative expression was calculated according to the ΔΔCt method, using constitutive *Eef1a* expression levels as an internal reference value, and normalized to that of the unstimulated cells. Triplicate samples were analysed in each assay.

Gene	Primer	Sequence (5' to 3')
Eef1a	Forward	CTGAACCATCCAGGCCAAAT
	Reverse	GGCTGTGTGACAATCCAG
Spi1	Forward	ATCGGATGAATTGGTTACTTAC
	Reverse	GGTTCTCAGGGAAGTTCTCAA
Cebpb	Forward	TGATGCAATCCGGATCAA
	Reverse	CACGTGTGTTGCGTCAGTC
Irf7	Forward	CTTAGCCGGGAGCTTGGATCTACT
	Reverse	CCCTTGTACATGATGGTCACATCC
Il6	Forward	ACAACCACGGCCTTCCCTACTT
	Reverse	CACGATTTCCCAGAGAACATGTG
Ifnb	Forward	GTCCTCAACTGCTCTCCACT
	Reverse	CCTGCAACCACCACTCATTC

14. Immunoblotting

HSPCs were plated in round-bottomed 96-well plates at a density of 2 x 10^5 cells in 0.2 ml of complete cell culture medium. At the indicated timepoints after stimulation, cells were lysed in Laemmli sample buffer and samples were completely denatured by adding DTT (50 mM) and heating them for 10 min at 95 ºC. Equal amounts of lysates were resolved by sodium polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes using the Mini Trans-Blot® Cell (Bio-Rad). Membranes were blocked with TBS containing 0.1% Tween 20 and 5% non-fat dry milk, or BSA when using biotin-labelled antibodies followed by streptavidin-HRP. The following antibodies were used for staining: biotin-labelled anti-C/EBPβ (1H7 from Biolegend), anti-phospho-TBK1/NAK (Ser172) (D52C2), anti-phospho-S6 (Ser235/236) (D57.2.2E), anti-TBK1/NAK (E8I3G), anti-S6 (5G10), anti-IRF-7 (D8V1J), anti-PU.1 (9G7), anti-GAPDH (D16H11), HRP-linked anti-rabbit IgG and Streptavidin-HRP (all from Cell Signaling Technologies). The SuperSignalTM West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) was used for colour development. Proteins were visualized using ImageQuantTM LAS 4000 (GE Healthcare). Stripping of blots prior to reprobing was performed using RestoreTM PLUS Western Blot Stripping Buffer (Thermo Scientific). Protein band intensities were quantified using ImageJ and their relative expression levels were normalized to that of GAPDH or total protein, as indicated.

15. Antibodies and flow cytometry

Cell suspensions were labelled in a total volume of 0.1 ml of MACS buffer with various combinations of antibodies (added at the recommended dilution) for 15 min at 4 ºC in the dark.

Where possible, non-specific antibody binding was prevented by prior incubation with Fc block (anti-CD16/32) for 10 min at 4 ºC. Following antibody staining, cells were washed with MACS buffer to eliminate extra antibody, centrifuged and resuspended in 0.4 ml PBS + 25 % BD Cytofix™ Fixation Buffer (BD Bioscience) until their analysis by flow cytometry. CountBrightTM Absolute Counting Beads (Invitrogen) were added to each sample before their analysis to allow for calculating absolute numbers of cells.

Flow cytometry analyses were performed on a 5-laser LSR Fortessa cytometer (BD Biosciences) with the following configuration:

Laser	**Detectors**					
UV (355 nm)	740/35	450/40	379/28			
Violet (405 nm)	780/60	710/50	660/20	610/20	525/50	450/50
Blue (488 nm)	695/40	530/30	488/10			
Yellow-green (561 nm)	780/60	670/30	610/20	586/15		
Red (640 nm)	780/60	730/45	670/14			

Cell sorting was performed on a 6-laser FACS Aria Fusion cell sorter (BD Biosciences) with the following configuration:

Laser	**Detectors**					
UV (355 nm)	780/60	730/45	670/30	450/40	379/28	
Violet (405 nm)	780/60	710/50	660/20	610/20	525/50	450/50
Blue (455 nm)	695/40	530/30	488/10			
Blue (488 nm)	695/40	530/30	488/10			
Yellow-green (561 nm)	780/60	710/50	670/14	610/20	582/15	
Red (640 nm)	780/60	730/45	670/30	450/40	379/28	

The following antibodies were used in this study:

Surface molecule	Fluorochrome	Clone	Company	Isotype control
B220	FITC	RA3-6B2	Biolegend	Rat IgG2a κ
Biotin	PE	Bio3-18E7	Miltenyi Biotec	Mouse IgG1
Biotin	APC	Bio3-18E7	Miltenyi Biotec	Mouse IgG1
CD11b	FITC	M1/70	eBioscience	Rat IgG2b κ
CD11b	PE	M1/70	eBioscience	Rat IgG2b κ
CD11b	PE-Cy7	M1/70	BD Biosciences	Rat IgG2b κ
CD115	BV421	AFS98	Biolegend	Rat IgG2a κ
CD135	BV421	A2F10	Biolegend	Rat IgG2a κ
CD150	APC	TC15-12F12.2	Biolegend	Rat IgG2a λ
CD150	APC-Fire750	TC15-12F12.2	Biolegend	Rat IgG2a λ
CD16/32	APC-Cy7	93	Biolegend	Rat IgG2a λ
CD3	APC	17A2	Biolegend	Rat IgG2b κ
CD34	FITC	RAM34	BD Biosciences	Rat IgG2a κ
CD4	PE	GK1.5	Biolegend	Rat IgG2b κ
CD45.1	FITC	A20	Miltenyi Biotec	Mouse IgG2a κ
CD45.2	FITC	104-2	Miltenyi Biotec	Mouse IgG2a κ
CD48	BV711	HM48-1	Biolegend	Armenian Hamster IgG
CD8α	BV510	53-6.7	BD Biosciences	Rat IgG2a κ
c-kit	BUV295	2B8	BD Biosciences	Rat IgG2b κ
F4/80	APC	BM8	eBioscience	Rat IgG2a κ
F4/80	PE-Cy7	BM8	eBioscience	Rat IgG2a κ
Lineage cocktail	Biotin	–	Miltenyi Biotec	–
Ly6C	PerCP-Cy5.5	HK1.4	Biolegend	Rat IgG2c κ
Ly6G	BUV395	1A8	BD Biosciences	Rat IgG2a κ
Sca-1	PE-Cy7	D7	Biolegend	Rat IgG2a κ

Data were analysed with FACSDiva and FlowJo 10 software.

16. Statistical analysis

Statistical differences were determined using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons and two-tailed Student's t-test for dual comparisons. Data are expressed as means ± standard deviations (SDs). Statistical analyses for survival curves were performed using Gehan-Breslow-Wilcoxon test. Significance was accepted at *P < 0.05, **P < 0.01 and ***P < 0.001 levels.

Results & Discussion

Innate immune cells play a key role in antipathogen defence and are rapidly consumed during an infection. Therefore, myelopoiesis is promoted in order to favour their replenishment and ensure an effective immune response. Following the discovery that PRRs are also expressed by HSPCs, it was hypothesized that direct sensing of microbial components by HSPCs would have a role in this demand-adapted haematopoiesis that takes place during an infection (Boettcher & Manz, 2016).

In this context, it has been demonstrated that inactivated *C. albicans* yeasts induce differentiation of mouse HSPCs towards the myeloid lineage in a TLR2- and dectin-1-dependent manner (Yáñez *et al.*, 2009, 2010, 2011). Moreover, TLR2 ligation *in vivo* activates HSPCs and induces their differentiation towards macrophages (Megías *et al.*, 2012, 2013), while direct interaction between dectin-1 on HSPCs and its ligand *in vivo* remains to be demonstrated.

The molecular mechanisms involved in these processes are yet to be elucidated. PRRs may induce myeloid cell development by direct signalling in an intrinsic manner, directly activating the transcription factors involved in myeloid differentiation. However, this could also be taking place by indirect mechanisms, with HSPCs secreting cytokines that would act in an autocrine or paracrine manner to promote differentiation.

Additionally, it has been shown that detection of PAMPs by HSPCs impacts the antimicrobial function of the macrophages they produce and that the functional phenotype of these macrophages is extremely dependent on how HSPCs receive and integrate multiple environmental signals (Martínez *et al.* 2017). For instance, HSPC activation in response to *C. albicans* inactivated yeasts leads to the generation of macrophages that produce higher levels of cytokines (trained) (Megías *et al.* 2016). However, the consequences of exposing HSPCs to inactivated yeasts may be different from those of exposing them to live yeasts either *in vitro* or in the context of an infection.

Therefore, we wondered whether the interaction of HSPCs with PRR ligands could be controlling, through several mechanisms, their differentiation towards mature myeloid cells with particular phenotypes, which could have a protective physiologic function in responding to microbial infections, including candidiasis.

CHAPTER 1

Role of dectin-1 and TLR2 in HSPC differentiation and the generation of trained or tolerized macrophages: molecular mechanisms

1. Phenotype of macrophages derived from HSPCs transiently exposed to live *C. albicans* cells

We have previously demonstrated that HSPC activation in response to inactivated *C. albicans* yeasts or depleted zymosan *in vitro* leads to the generation of macrophages that produce higher levels of cytokines (trained macrophages) that control M-CSF-derived macrophages (Megías *et al.*, 2016; Martínez *et al.*, 2017). However, live yeasts can express several virulence factors, including yeast-to-hypha transition and candidalysin secretion, so the consequences of exposing HSPCs to them may be different to being exposed to inactivated yeasts. Therefore, we investigated the functional consequences of dectin-1 activation by live *C. albicans* cells, exclusively at the HSPC stage, prior to differentiation.

In order to do so, HSPCs (purified as Lin^- cells) were cultured in the presence or absence of live *C. albicans* cells at a 1:0.5 or 1:2 ratio (progenitor:yeast) for 6 h. Under these experimental conditions (low number of fungal cells, presence of serum and at 37 ºC) most of the yeasts performed their conversion from budding yeasts to their filamentous growth form. After 6 h of coculture, amphotericin B (0.5 µg/ml) was added to stop fungal growth and, taking advantage of the high adhesion of hyphae to plastic, Lin^- cells were transferred to a new plate, leaving the hyphae adhered to the original plate. These Lin^- cells were cultured with M-CSF for 7 days to obtain macrophages. Equal numbers of macrophages, derived from unexposed and live *C. albicans*-exposed HSPCs, were plated and stimulated with PRR ligands to measure their ability to produce proinflammatory cytokines (Figure 1A). Results show that the production of TNF-α and IL-6 in response to Pam_3CSK_4 or LPS is significantly increased in macrophages derived from HSPCs transiently exposed to live *C. albicans* cells, and that this trained phenotype is dose-dependent, as when a higher ratio of live yeasts is used to stimulate HSPCs, a more pronounced trained phenotype is observed (Figure 1B).

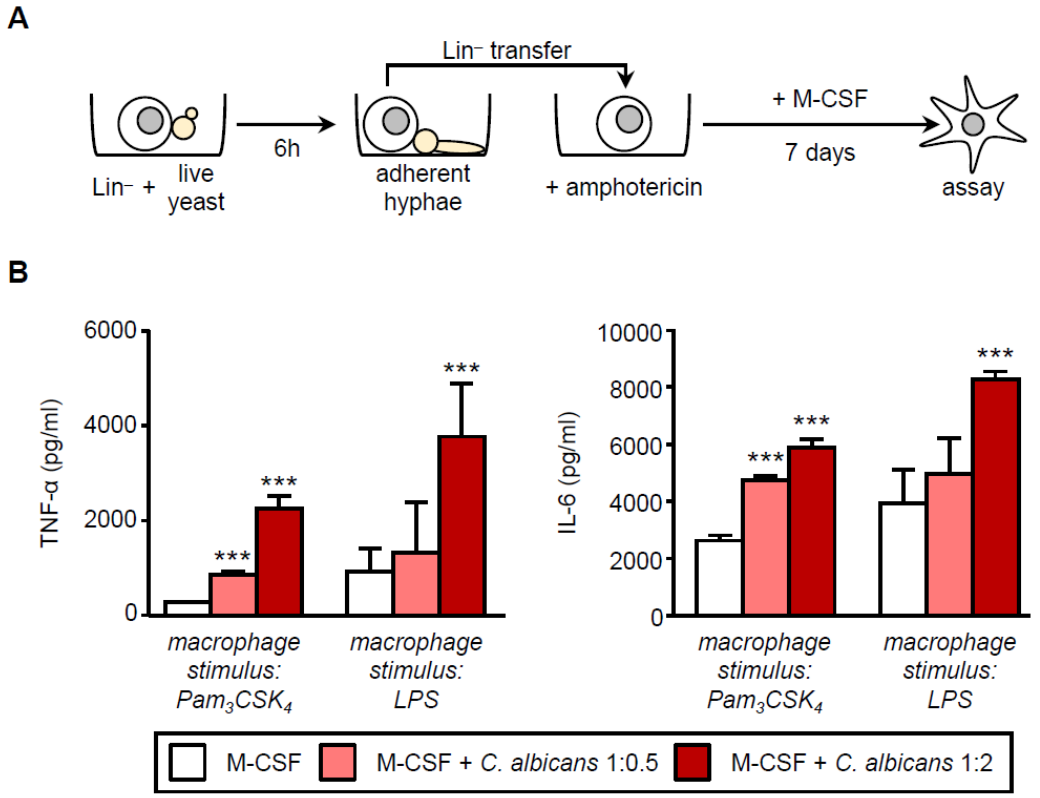

FIGURE 1. Cytokine production by macrophages derived from HSPCs transiently exposed to live *C. albicans* cells. (A) Schematic protocol of *in vitro* Lin⁻ differentiation and stimulation. Lin⁻ cells were cultured in the presence or absence of live *C. albicans* cells at a 1:0.5 or 1:2 ratio (progenitor/yeast) for 6 h and then amphotericin B was added. Lin⁻ cells were transferred to a new plate and cultured with M-CSF to obtain macrophages. **(B)** Macrophages were stimulated with Pam_3CSK_4 or LPS and TNF-α and IL-6 levels in 24-h supernatants were assessed by ELISA. Results are expressed as means ± SDs of pooled data from 2 experiments. ***P < 0.001 with respect to cytokine production by macrophages derived from Lin⁻ cells unexposed to *C. albicans*.

Lin⁻ cells from TLR2$^{-/-}$ or dectin-1$^{-/-}$ mice were also transiently exposed to live *C. albicans* cells following the same experimental procedure. These give rise to macrophages that produce similar levels of TNF-α as macrophages generated from unexposed HSPCs, and although the production of IL-6 is slightly increased in comparison to the one observed in control macrophages, the relative increase (exposed/unexposed) for KO cells is lower (around 27%) than the increase for WT macrophages (60%). Additionally, the production of the chemokine MIP-2 is dependent on dectin-1 but independent of TLR2 (Figure 2). These results indicate that in this model of transient exposure to live *C. albicans* cells, the generation of trained macrophages is partially dependent on both TLR2 and dectin-1.

Overall, these results demonstrate that HSPCs can sense live *C. albicans* cells directly during infection to rapidly generate trained macrophages.

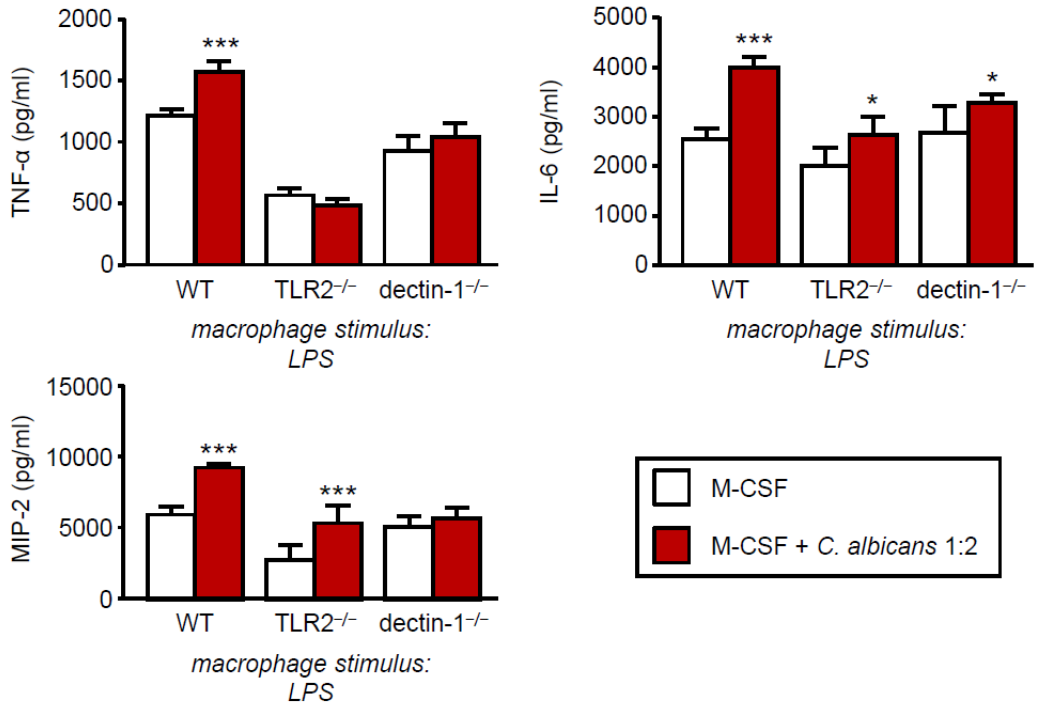

FIGURE 2. Cytokine production by macrophages differentiated from WT, TLR2$^{-/-}$ and dectin-1$^{-/-}$ HSPCs transiently exposed to live *C. albicans* cells. Lin^{-} cells from WT, TLR2$^{-/-}$, or dectin-1$^{-/-}$ mice were cultured in the presence or absence of live *C. albicans* cells at a 1:2 ratio (progenitor/yeast) for 6 h and differentiated as indicated in figure 1. Macrophages were stimulated and TNF-α, IL-6 and MIP-2 production was measured. Triplicate samples were analysed in each assay. Results are expressed as means ± SDs of pooled data from 2 experiments. *P < 0.05 and ***P < 0.001 with respect to cytokine production by macrophages derived from Lin^{-} cells unexposed to *C. albicans*.

2. Involvement of dectin-1 in HSPC differentiation and the phenotype of the macrophages derived from them

2.1. *In vivo* differentiation of HSPCs in response to the dectin-1 ligand depleted zymosan

Direct *in vivo* interaction between microbial pathogens, or their ligands, and PRRs on HSPCs is difficult to demonstrate, as HSPCs could also be responding to other stimuli generated when mature immune or nonimmune cells detect microbial products via their PRRs.

To investigate the possible direct interaction of β-glucan with dectin-1 on HSPCs *in vivo*, we designed an experimental approach in which HSPCs (Lin^- cells) were purified from the bone marrow of B6Ly5.1 mice (expressing the CD45.1 alloantigen) and intravenously transplanted into dectin-$1^{-/-}$ mice (expressing the CD45.2 alloantigen). These mice were also injected with a daily dose of depleted zymosan (a dectin-1-activating *Saccharomyces cerevisiae* cell wall preparation of β-glucan that has been treated with hot alkali to remove its TLR-stimulating properties) for 3 days. Using this experimental approach, the recipient mouse cells do not recognize the injected ligand, and so there is no interference by cytokines or soluble mediators secreted by them.

Three days after transplantation, the bone marrow and spleen of transplanted mice were enriched in CD45.1 donor cells by depleting CD45.2 recipient cells and were analysed by flow cytometry (Figure 3A). Approximately 0.3% and 0.2% of the transplanted cells were recovered from the spleen and the bone marrow, respectively, of unstimulated mice (Figure 3B). Following depleted zymosan challenge, a significant increase in the recovery of CD45.1 donor cells was detected in both, the spleen and bone marrow of dectin-$1^{-/-}$ mice transplanted with Lin^- cells (Figure 3C). These results indicate that dectin-1 signalling resulting from direct detection of depleted zymosan by the transplanted Lin^- cells induces their proliferation and/or improves their survival *in vivo*.

Next, differentiation of the recovered CD45.1 donor cells was analysed by measuring the expression of myeloid (CD11b) and macrophage (F4/80) markers. We found that transplanted cells differentiate towards the myeloid lineage in unstimulated mice, as significant percentages of $CD11b^+$ cells (around 50% in both, spleen and bone marrow) were detected. However, following the injection of depleted zymosan into the transplanted mice, there is a significant increase in $CD11b^+$ and $CD11b^+$ $F4/80^+$ cells (around 80% and 52%, respectively) among the recovered CD45.1 cells, both in spleen and bone marrow (Figures 3D and 3E).

These results demonstrate that HSPCs are directly stimulated by the dectin-1 agonist *in vivo* and that the engagement of this receptor promotes macrophage differentiation.

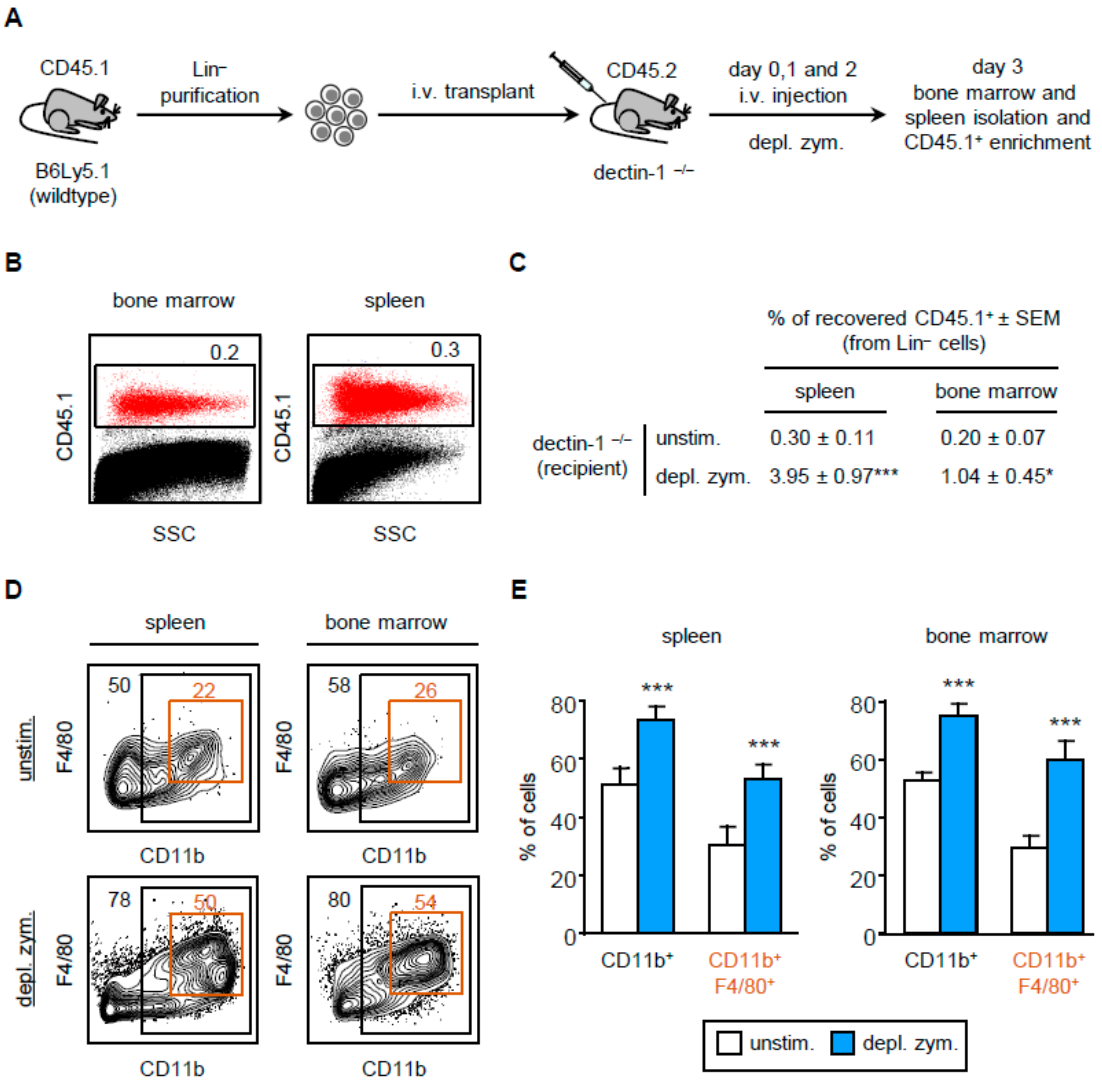

FIGURE 3. *In vivo* differentiation of transplanted CD45.1 HSPCs in response to the dectin-1 ligand depleted zymosan. (A) Schematic protocol of cell transplantation and stimulation. **(B)** Three days after transplantation, donor-derived CD45.1 cells were detected in the bone marrow and spleens of CD45.1 dectin-1$^{-/-}$ receptor mice. Dot plots show side scatter (SSC) against CD45.1 expression of the purified bone marrow and spleen cells of control mice (unchallenged with depleted zymosan). The percentage of recovered CD45.1 was calculated as follows: total number of recovered cells x 100 / total number of transplanted cells, where the total number of recovered cells is the percentage of CD45.1 cells determined by flow cytometry x total number of purified cells from spleen or bone marrow / 100. Indicated percentages are the means ± SEMs from 6 mice. **(C)** Percentages of recovered CD45.1 cells from the spleen and bone marrow of transplanted dectin-1$^{-/-}$ stimulated daily with 300 µg/day of depleted zymosan, for 3 days. Data is represented as means ± SEMs from 2 independent experiments (3 mice per condition and experiment). *P < 0.05 and ***P < 0.001 with respect to CD45.1 cells recovered from transplanted but unstimulated mice. **(D and E)** The CD45.1 population was gated, shown in CD11b versus F4/80 contour plots, and subgated as CD11b^{+} and double-positive CD11b^{+} F4/80^{+} cells. The indicated percentages refer to total CD45.1 cells analysed. Plots (D) are representative and bar graphs (E) show data as means ± SDs from 2 independent experiments (3 mice per condition and experiment). ***P < 0.001 with respect to CD45.1 cells recovered from transplanted but unstimulated mice.

2.2. *In vivo* differentiation of HSPCs in response to inactivated *C. albicans* yeasts

To investigate the *in vivo* differentiation of transplanted HSPCs in response to *C. albicans*, Lin^- cells were purified from the bone marrow of C57BL/6 mice (expressing the CD45.2 alloantigen) and intravenously transplanted into B6Ly5.1 mice (expressing the CD45.1 alloantigen), which were then injected with a daily dose of 1×10^7 inactivated yeasts for 3 days. Three days after the transplant, the bone marrow and spleen of transplanted mice were enriched in CD45.2 cells by depleting CD45.1 recipient cells and their differentiation was analysed by flow cytometry. Following *C. albicans* injection, donor HSPCs differentiate into $CD11b^+$ cells (around 80% of the recovered CD45.2 cells). Percentages of $CD11b^+$ $F4/80^+$ cells were around 60% and, within these double-positive cells, we detected a subpopulation that expresses higher levels of F4/80 ($CD11b^+$ $F4/80^{high}$) that represented a 30% and 47% of the population in the spleen and bone marrow, respectively.

These data prompted us to investigate whether dectin-1 and/or TLR signalling plays a role in HSPC differentiation in response to yeasts. In order to do this, Lin^- cells were obtained from the bone marrow of dectin-$1^{-/-}$ or MyD88$^{-/-}$ mice (expressing the CD45.2 alloantigen) and transplanted into B6Ly5.1 mice (expressing the CD45.1 alloantigen). These mice were then injected with inactivated yeasts and donor cell differentiation was analysed as described above (Figure 4A). In this model, recipient mice do recognize the injected yeasts, so their cells would release cytokines and soluble mediators that would affect all the transplanted cells in the same way, regardless of the type of mice they were coming from. Therefore, the possible variations in the differentiation of CD45.2 donor cells from dectin-$1^{-/-}$ and MyD88$^{-/-}$ mice (compared to cells from C57BL/6 control mice) could only be due to a lack of direct signalling via dectin-1 or TLRs, respectively, in the transplanted HSPCs.

Results show that the percentages of macrophages ($CD11b^+$ $F4/80^+$) generated from dectin-$1^{-/-}$ and MyD88$^{-/-}$ progenitors were significantly decreased in comparison to the percentage of macrophages generated from wildtype Lin^- cells (Figures 4B and 4C). This decrease was even more pronounced in the $CD11b^+$ $F4/80^{high}$ subpopulation, especially when the transplanted cells came from MyD88$^{-/-}$ mice, as while dectin-$1^{-/-}$ HSPCs gave rise to a 21% and 35% of this macrophage subset in the spleen and bone marrow, respectively, MyD88$^{-/-}$ HSPCs only yielded 1% in the spleen and 3% in the bone marrow.

It should be noted that the percentages of total myeloid cells ($CD11b^+$) were quite similar in all cases (except for a slight decrease in the spleens of mice transplanted with dectin-$1^{-/-}$ cells and in the bone marrow of mice transplanted with MyD88$^{-/-}$ cells), suggesting that signalling through dectin-1 and TLRs in response to *C. albicans* induces differentiation specifically to F4/80$^+$ macrophages.

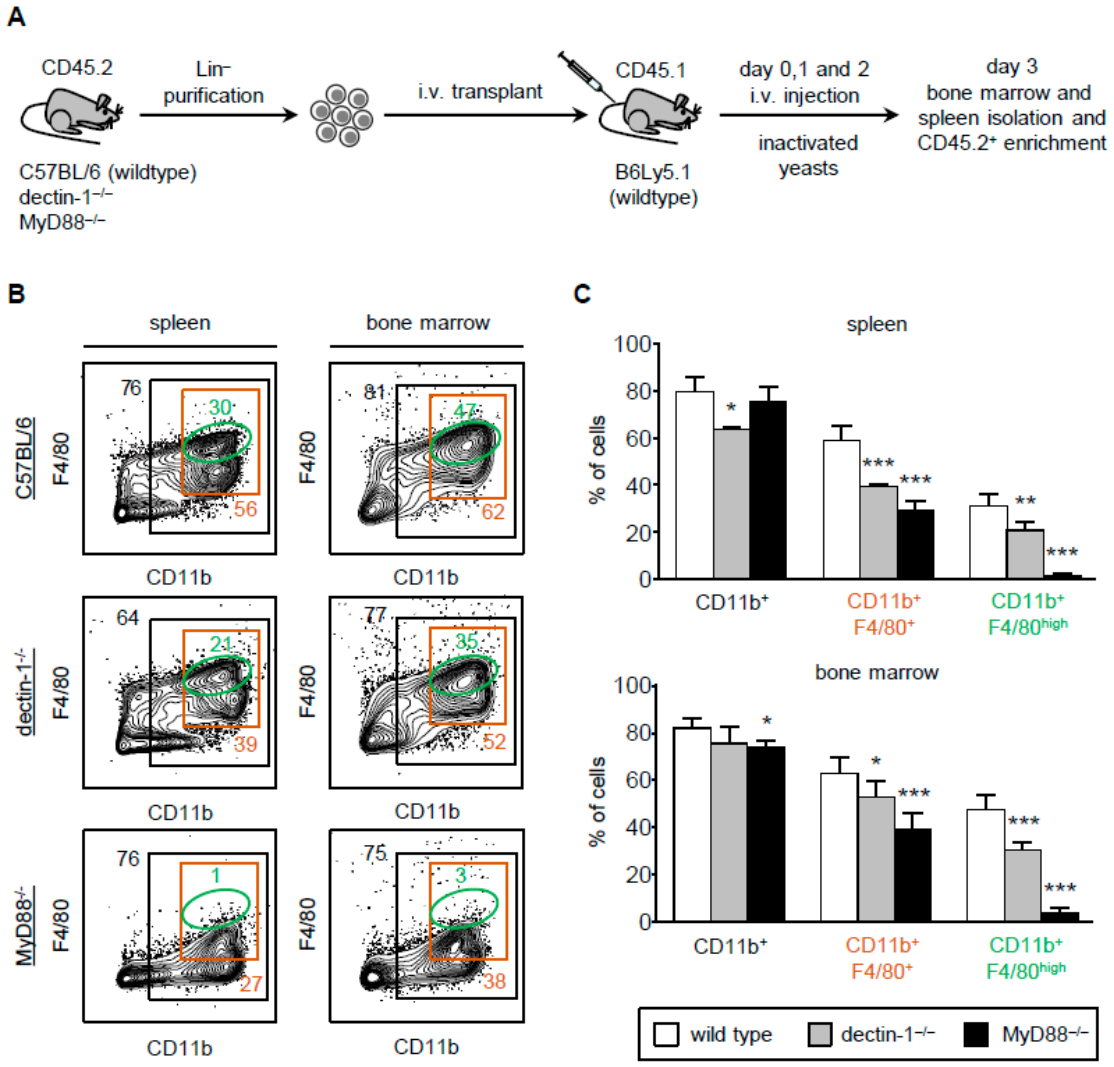

FIGURE 4. *In vivo* differentiation of dectin-$1^{-/-}$ or MyD88$^{-/-}$ CD45.2 HSPCs in response to stimulation with inactivated *C. albicans* yeasts. (A) Schematic protocol of cell transplantation and stimulation. **(B and C)** The CD45.2 population was gated, shown in CD11b versus F4/80 contour plots, and subgated as $CD11b^+$, double-positive $CD11b^+$ F4/80$^+$ cells and $CD11b^+$ F4/80^{high} cells. The indicated percentages refer to total CD45.2 cells analysed. Plots (B) are representative and bar graphs (C) express means ± SDs from 2 independent experiments (3 mice per condition and experiment). *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to CD45.2 cells recovered from WT transplanted progenitor cells.

Overall, these results suggest that the *C. albicans*-induced differentiation of Lin$^-$ cells to macrophages *in vivo* is dependent on both TLRs/MyD88 and dectin-1, although the role of TLR signalling appears to be more significant.

2.3. Cytokine production by macrophages differentiated *in vivo* from depleted zymosan-stimulated HSPCs

We have previously demonstrated that exposure of HSPCs to dectin-1 agonists (depleted zymosan or inactivated yeasts of *C. albicans*) *in vitro* leads to the generation of trained macrophages (Megías *et al.*, 2016). Here, to investigate the function of macrophages produced *in vivo* in response to the detection of depleted zymosan by HSPCs, we made use of a transplant model in which we used donor HSPCs from the bone marrow of mice expressing the red fluorescent protein DsRed to distinguish them from recipient cells.

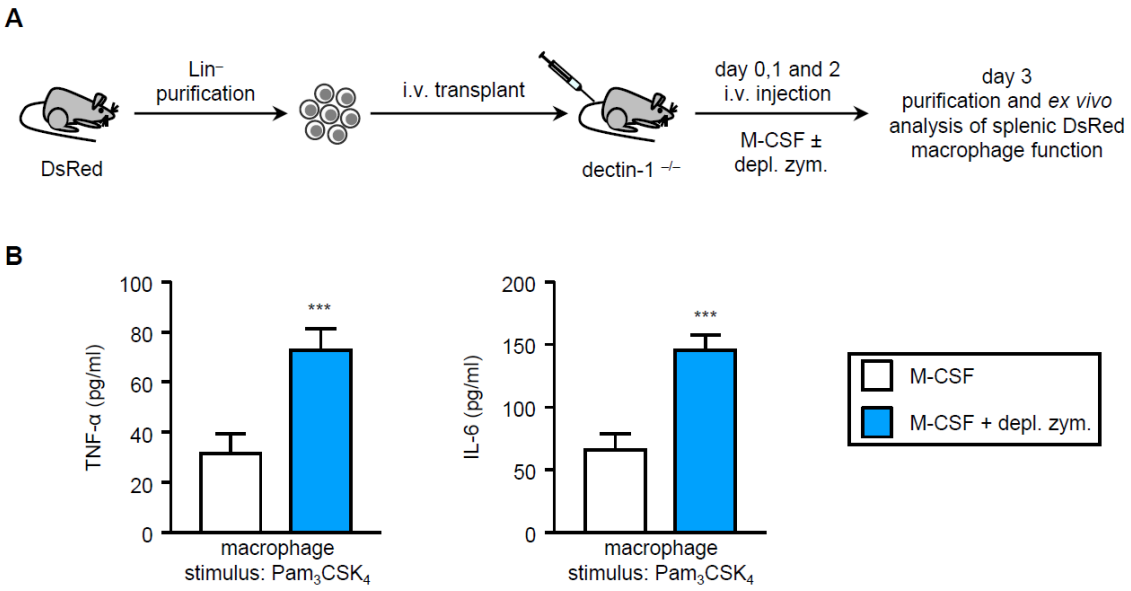

FIGURE 5. Cytokine response of macrophages differentiated *in vivo* from HSPCs exposed to depleted zymosan. (A) Schematic protocol of cell transplantation and stimulation. **(B)** Macrophages were stimulated with 100 ng/ml of Pam_3CSK_4 and TNF-α and IL-6 levels were assessed in 24-h culture supernatants by ELISA. Triplicate samples were analysed in each assay, Results are expressed as means ± SDs of pooled data from 2 independent experiments. ***P < 0.001 with respect to cytokine production by macrophages derived from Lin$^-$ cells differentiated with M-CSF only, in the absence of depleted zymosan.

Therefore, DsRed HSPCs were intravenously transplanted into dectin-1$^{-/-}$ mice and their differentiation was induced by injecting a daily dose of M-CSF with or without depleted zymosan for 3 days (Figure 5A). Three days after transplantation, spleen cells were enriched in myeloid cells by depletion of lymphocytes and were stained for CD11b and F4/80. Macrophages differentiated *in vivo* from the transplanted cells (identified as DsRed^{+} CD11b^{+} F4/80^{+} cells) were sorted by flow cytometry. Equal numbers of these cells were stimulated *ex vivo* with Pam_3CSK_4, revealing that macrophages derived in the presence of depleted zymosan produce higher levels of the inflammatory cytokines TNF-α and IL-6 (Figure 5B). Since the recipient mice are unable to respond to depleted zymosan, the observed differences must be due to direct signalling via dectin-1 in the transplanted HSPCs and, possibly, their progeny.

This result demonstrates that HSPCs are stimulated by dectin-1 *in vivo* and are subsequently directed to produce trained macrophages by a cell-autonomous mechanism.

3. Mechanisms driving HSPC differentiation via TLR2 and dectin-1

3.1. HSPC differentiation mechanisms driven by TLR2 signalling

To understand whether the mechanisms that drive HSPC differentiation towards mature myeloid cells in response to TLR2 and dectin-1 ligands are direct or indirect, we performed differentiation experiments with mixed cocultures of WT and KO HSPCs.

Lin^{-} cells were purified from DsRed and TLR2$^{-/-}$ mice and were cocultured at a 1:1 ratio in the presence of M-CSF (as a control for macrophage differentiation), the TLR2 ligand (Pam_3CSK_4) or inactivated yeasts of *C. albicans*. After 3 and 5 days of culture, the percentage of DsRed^{+} (TLR2$^{+/+}$) and DsRed^{-} (TLR2$^{-/-}$) cells out of the total differentiated cells (CD11b^{+} F4/80^{+}) was analysed by flow cytometry (Figure 5A). As expected, TLR2$^{-/-}$ and TLR2$^{+/+}$ HSPCs produced equivalent numbers of CD11b^{+} F4/80^{+} cells in the M-CSF-stimulated cocultures. However, in response to Pam_3CSK_4, TLR2$^{+/+}$ HSPCs produced higher numbers of CD11b^{+} F4/80^{+} cells than TLR2$^{-/-}$ HSPCs (67% TLR2$^{+/+}$ and 33% TLR2$^{-/-}$ at day 3, and 89% TLR2$^{+/+}$ and 11% TLR2$^{-/-}$ at day 5) (Figure 5B). This lower percentage of differentiated TLR2$^{-/-}$ cells compared to TLR2$^{+/+}$ cells indicates that TLR2 stimulation in Lin^{-} cells promotes cell-autonomous direct myeloid

differentiation as, under these culture conditions, differentiation mediated by indirect mechanisms (produced by molecules secreted in response to the ligand that act in an autocrine and/or paracrine manner) would be the same in both progenitor genotypes.

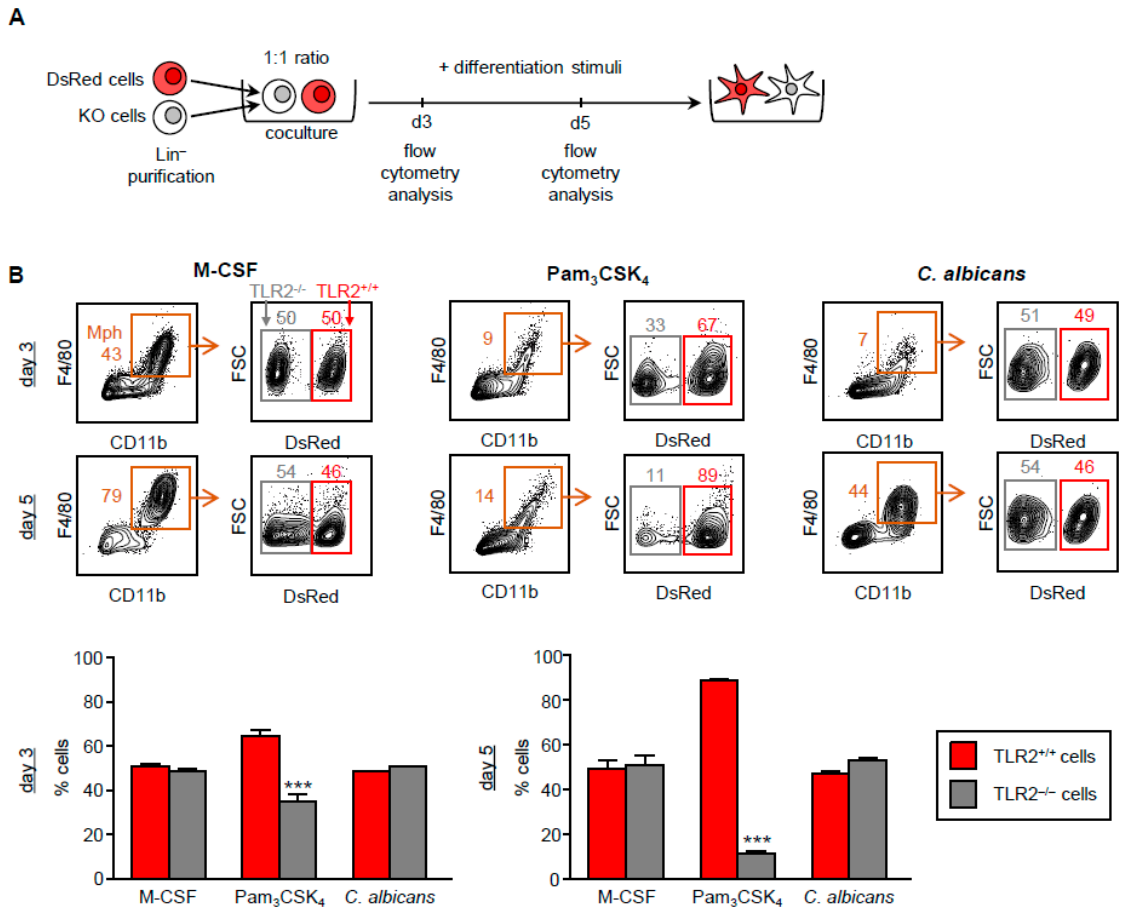

FIGURE 5. Flow cytometry analyses of DsRed and TLR2$^{-/-}$ cocultured Lin^{-} cells differentiated *in vitro* in response to M-CSF, Pam$_3$CSK$_4$ or *C. albicans*. (A) Schematic protocol of cell differentiation and assay. **(B)** DsRed and TLR2$^{-/-}$ Lin^{-} cells were cultured at a 1:1 ratio and stimulated with M-CSF, Pam$_3$CSK$_4$ or inactivated yeasts of *C. albicans* for 3 or 5 days. Cells were labelled with anti-CD11b and anti-F4/80 antibodies and analysed by flow cytometry. Macrophages (Mph) were gated as CD11b^{+} F4/80^{+} cells, shown in forward scatter (FCS) versus DsRed contour plots, and subgated as DsRed^{+} and DsRed^{-}. The indicated percentages on dot plots refer to total analysed cells. Plots are representative and bar graphs express means ± SDs of pooled data from 3 independent experiments. ***P < 0.001 with respect to macrophages generated from DsRed Lin^{-} cells.

In contrast, the percentages of TLR2$^{+/+}$ and TLR2$^{-/-}$ cells within the CD11b^{+} F4/80^{+} cell population were similar in response to inactivated yeasts of *C. albicans*, indicating that the secretion of soluble mediators is sufficient to elicit myeloid differentiation in the absence of a concurrent TLR2 signal (Figure 5B). Nonetheless, considering that inactivated *C. albicans* signals via, both, TLR2 and dectin-1, there could be compensation

mechanisms in single-KO cells leading to the similar macrophage levels observed in these cultures.

Overall, these results demonstrate that direct sensing of TLR2 ligands promotes myelopoiesis directly.

3.2. HSPC differentiation mechanisms driven by dectin-1 signalling

The same experiment was also performed by coculturing Lin$^-$ cells from DsRed and dectin-1$^{-/-}$ mice in the presence of M-CSF, depleted zymosan, or inactivated yeasts of *C. albicans*. The percentages of dectin-1$^{+/+}$ and dectin-1$^{-/-}$ cells within the CD11b$^+$ F4/80$^+$ cell population were similar in response to all stimuli used: M-CSF (as expected), depleted zymosan (not statistically significant for the slight differences observed), and inactivated yeasts of *C. albicans* (Figure 6).

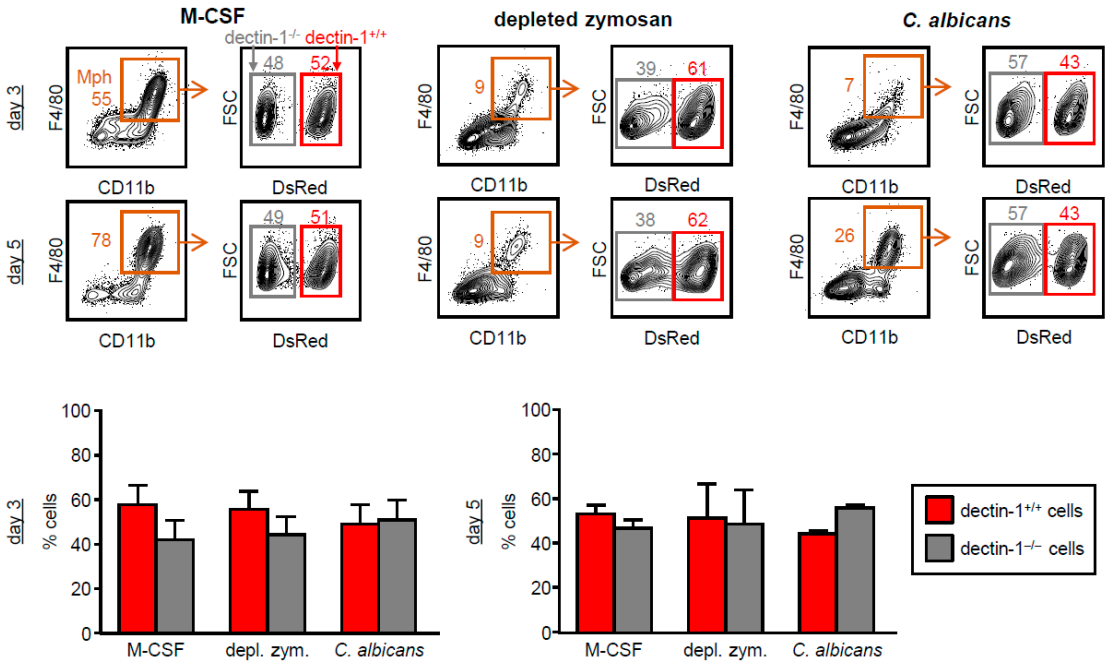

FIGURE 6. Flow cytometry analyses of DsRed and dectin-1$^{-/-}$ Lin$^-$ cells differentiated *in vitro* in response to M-CSF, depleted zymosan or *C. albicans*. DsRed and dectin-1$^{-/-}$ Lin$^-$ cells were cultured at a 1:1 ratio and stimulated with M-CSF, depleted zymosan or inactivated yeasts of *C. albicans* for 3 or 5 days. Cells were labelled with anti-CD11b and anti-F4/80 antibodies and analysed by flow cytometry. Macrophages (Mph) were gated as CD11b$^+$ F4/80$^+$ cells, shown in forward scatter (FCS) versus DsRed contour plots, and subgated as DsRed$^+$ and DsRed$^-$. The indicated percentages on dot plots refer to total analysed cells. Plots are representative and bar graphs express means ± SDs of pooled data from 3 independent experiments.

These results indicate that dectin-1 signalling induces differentiation of HSPCs by indirect mechanisms.

4. Mechanisms by which TLR2 or dectin-1 signalling in HSPCs modulate the phenotype of the macrophages derived from them

4.1. Cytokine production by macrophages differentiated in response to TLR2 signalling

Although we had already previously demonstrated that HSPCs exposed to the TLR2 ligand produce soluble factors that act in a paracrine manner on unexposed HSPCs that then generate tolerized macrophages (Yáñez *et al.*, 2013b), we have here used a different model to investigate the mechanisms by which TLR2 signalling in HSPCs influence the phenotype of the macrophages derived from them.

In this model, Lin^- cells purified from the bone marrow of DsRed and $TLR2^{-/-}$ mice were cocultured at a 1:1 ratio in the presence of M-CSF and in the presence or absence of Pam_3CSK_4 or inactivated yeasts of *C. albicans*. After 7 days, $DsRed^+$ and $DsRed^-$ macrophages ($CD11b^+$ $F4/80^+$) were sorted by flow cytometry, plated separately, and stimulated to asses cytokine production (Figure 7A). The tolerized or trained phenotype was determined using DsRed macrophages (WT) as controls, which were tolerized in Pam_3CSK_4 cocultures and trained in *C. albicans* yeast cocultures, in comparison to those in the M-CSF cultures without any other additional stimuli.

According to previous results (Yáñez *et al.*, 2013b), $TLR2^{-/-}$ macrophages derived in the presence of Pam_3CSK_4-activated WT cells produced less TNF-α and IL-6 than $TLR2^{-/-}$ macrophages derived with M-CSF in the absence of the TLR2 ligand. On the other hand when $TLR2^{-/-}$ HSPCs were cocultured with WT cells in the presence of *C. albicans* yeasts, the macrophages derived from them displayed a trained phenotype, although this could also be due to alternative PRR stimulation in addition to indirect mechanisms (Figure 7B).

These results indicate that soluble factors produced by HSPCs upon TLR2 signalling act in a paracrine manner on unresponsive HSPCs ($TLR2^{-/-}$) affecting the phenotype of the macrophages derived from them.

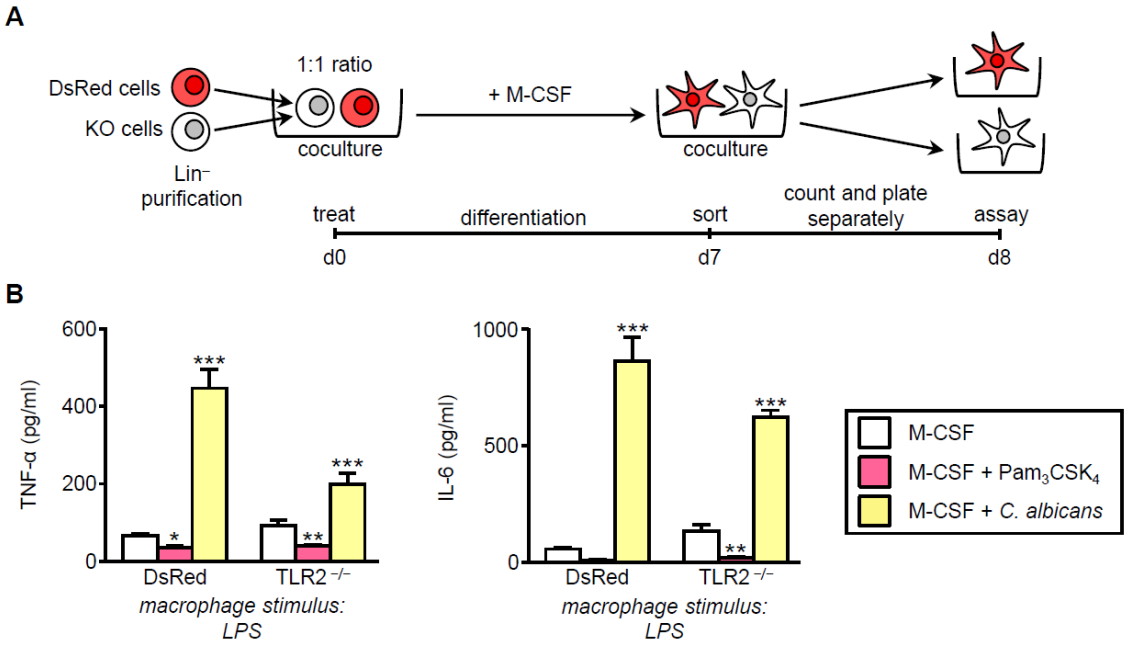

FIGURE 7. Cytokine production by macrophages derived from cocultured DsRed and TLR2$^{-/-}$ Lin^{-} cells differentiated *in vitro* with M-CSF in the presence of Pam$_3$CSK$_4$ or *C. albicans*. (A) Schematic protocol of *in vitro* cell differentiation and sorting. **(B)** DsRed and TLR2$^{-/-}$ Lin^{-} cells were cultured at a 1:1 ratio and stimulated with M-CSF in the presence or absence of Pam$_3$CSK$_4$ or inactivated yeasts of *C. albicans* for 7 days. Cells were labelled with anti-CD11b and anti-F4/80 antibodies and DsRed^{+} and DsRed^{-} macrophages (CD11b^{+} F4/80^{+}) were sorted by flow cytometry and plated separately. Macrophages were stimulated with LPS (100 ng/ml) and TNF-α and IL-6 levels in 24-h culture supernatants were assessed by ELISA. Triplicate samples were analysed in each assay. Bar graphs express means ± SDs of pooled data from 3 independent experiments. *P < 0.5, **P < 0.01 and ***P < 0.001 with respect to cytokine production by macrophages derived from Lin^{-} cells differentiated with M-CSF only, in the absence of additional stimuli.

4.2. Cytokine production by macrophages differentiated in response to dectin-1 signalling

On the other hand, to investigate the mechanisms by which trained macrophages are generated from HSPCs as a consequence of depleted zymosan and *C. albicans* yeast exposure, we used the same experimental model coculturing Lin^{-} cells from the bone marrow of DsRed and dectin-1$^{-/-}$ mice in the presence of M-CSF and in the presence or absence of depleted zymosan or inactivated yeasts of *C. albicans* to later analyse the phenotype of the macrophages derived from them (as in Figure 7A). DsRed macrophages (WT) were used as controls, as they were trained in the cocultures of cells differentiated in the presence of depleted zymosan and inactivated yeasts of *C. albicans*.

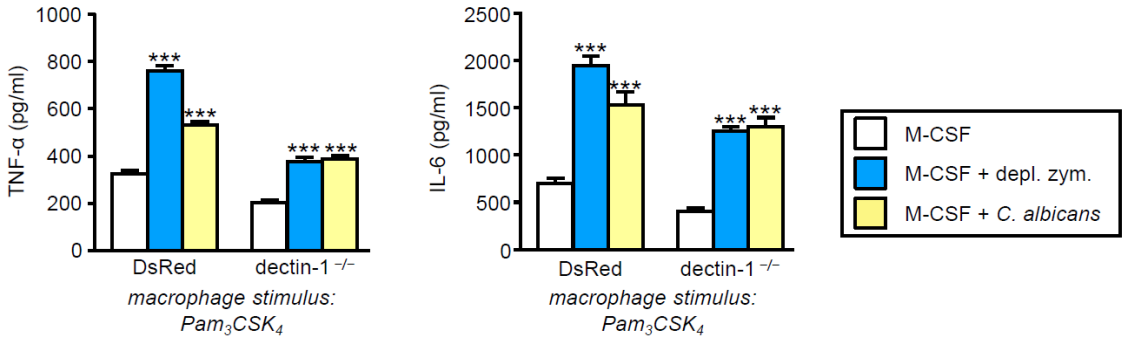

FIGURE 8. Cytokine production by macrophages derived from cocultured DsRed and dectin-1$^{-/-}$ Lin^{-} cells differentiated *in vitro* with M-CSF in the presence of depleted zymosan or *C. albicans*. DsRed and dectin-1$^{-/-}$ Lin^{-} cells were cultured at a 1:1 ratio and stimulated with M-CSF in the presence or absence of depleted zymosan or inactivated yeasts of *C. albicans* for 7 days. Cells were labelled with anti-CD11b and anti-F4/80 antibodies and DsRed^{+} and DsRed^{-} macrophages (CD11b^{+} F4/80^{+}) were sorted by flow cytometry and plated separately. Macrophages were stimulated with Pam_3CSK_4 (100 ng/ml) and TNF-α and IL-6 levels in 24-h culture supernatants were assessed by ELISA. Triplicate samples were analysed in each assay. Bar graphs express means ± SDs of pooled data from 3 independent experiments. ***P < 0.001 with respect to cytokine production by macrophages derived from Lin^{-} cells differentiated with M-CSF only, in the absence of additional stimuli.

Results show that dectin-1$^{-/-}$ macrophages derived in the presence of depleted zymosan-activated WT cells produced more TNF-α and IL-6 than dectin-1$^{-/-}$ macrophages derived with M-CSF in the absence of depleted zymosan (Figure 8), clearly indicating that soluble factors produced by HSPCs in response to the dectin-1 ligand act in a paracrine manner on unresponsive HSPCs (dectin-1$^{-/-}$) that then generate trained macrophages. The same effect was observed when dectin-1$^{-/-}$ HSPCs were cocultured with WT cells in the presence of *C. albicans* yeasts, although, given that *C. albicans* also signals through TLR2, there could be compensation mechanisms leading to the observed training of dectin-1$^{-/-}$ cells in these cocultures.

Taken together, these results indicate dectin-1 signalling on HSPCs induces the secretion of molecules that can act on unresponsive cells and lead to the acquisition of a trained phenotype by the macrophages derived from them.

To further investigate the mechanisms by which signalling via dectin-1 in HSPCs induces differentiation to trained macrophages, we performed transwell assays to determine whether cell contact is important for this effect (Figure 9A). We found that macrophages derived from unexposed HSPCs cultured with, but physically separated (upper chamber) from, depleted zymosan- or *C. albicans*-exposed HSPCs (lower

chamber) still exhibited increased TNF-α and IL-6 production (Figure 9B), indicating that soluble factors produced by the exposed cells confer functional programming (without cell contact) to unexposed cells at some point during their differentiation.

Therefore, dectin-1 activation on HSPCs defines their secretome, which acts in a paracrine manner and impacts the function of the macrophages produced nearby in the absence of cell contact.

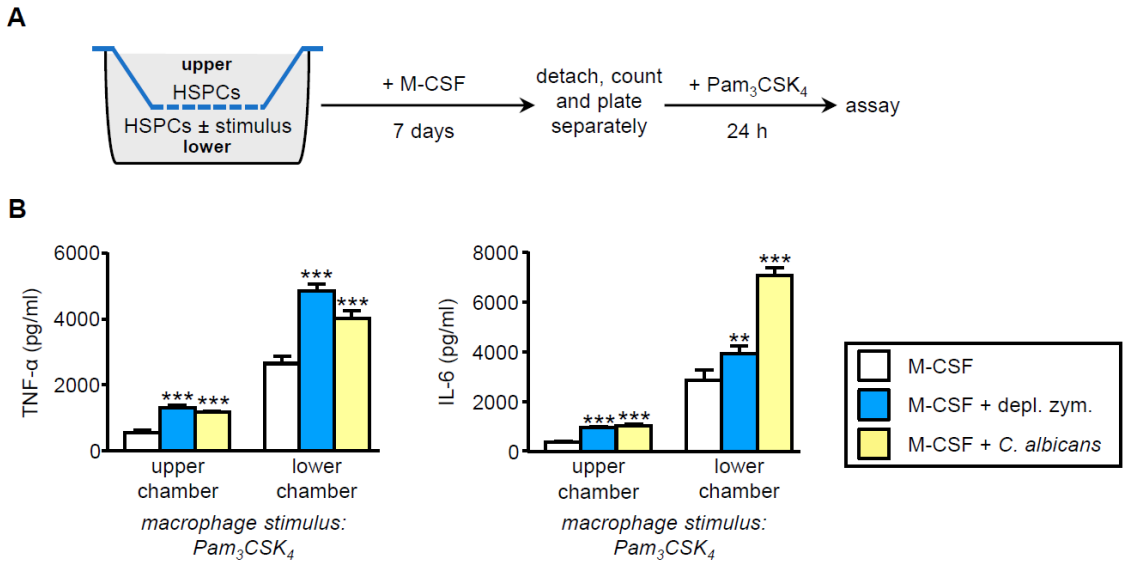

FIGURE 9. Cytokine production by macrophages derived from Lin⁻ cells differentiated *in vitro* with M-CSF in the presence of depleted zymosan or *C. albicans* in transwell assays. (A) Schematic protocol of *in vitro* Lin⁻ cell differentiation in transwell assays. **(B)** Macrophages from the upper or lower chamber were separately stimulated with Pam_3CSK_4 (100 ng/ml) and TNF-α and IL-6 levels in 24-h culture supernatants were assessed by ELISA. Triplicate samples were analysed in each assay. Bar graphs express means ± SDs of pooled data from 3 independent experiments. **P < 0.01 and ***P < 0.001 with respect to cytokine production by macrophages derived from Lin⁻ cells differentiated with M-CSF only, in the absence of additional stimuli in the lower chamber.

5. Signalling pathways and transcription factors involved in myeloid differentiation following Pam_3CSK_4 stimulation of HSPCs

5.1. Adaptor molecules downstream of TLR2 signalling in HSPCs

In order to understand the initiation of myeloid differentiation by TLR2 signalling in a cell-intrinsic manner (demonstrated in section 3.1), we first assessed which adaptor molecule is engaged downstream of TLR2 in HSPCs.

TLR2 signalling in macrophages, besides using MyD88, can also signal through TRIF to activate the TBK1 kinase (Nilsen *et al.*, 2015; Jefferies, 2019). Therefore, we performed differentiation experiments of Lin^- cells from WT, $TLR2^{-/-}$ and $MyD88^{-/-}$ in response to Pam_3CSK_4 and the percentage of macrophages ($CD11b^+$ and $F4/80^+$) was analysed by flow cytometry after 3 or 7 days of culture. Lin^- cells from TLR2- and MyD88-deficient mice failed to differentiate to macrophages in a similar way, suggesting that TRIF is not involved in this process (Figure 10).

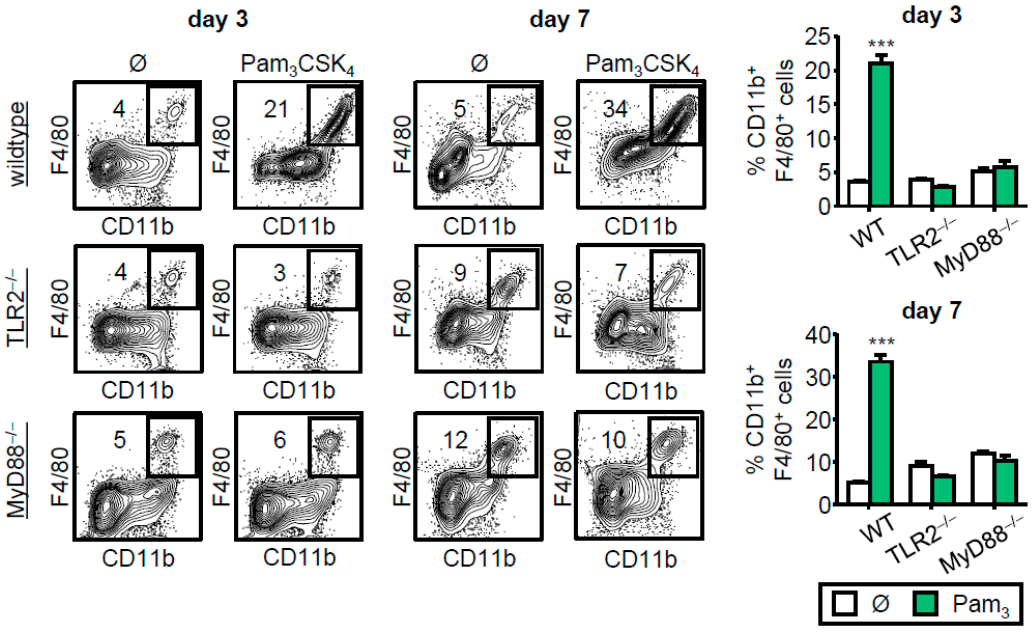

FIGURE 10. Flow cytometry analyses of WT, $TLR2^{-/-}$ and $MyD88^{-/-}$ Lin^- cells differentiated *in vitro* in the presence or absence of Pam_3CSK_4. Lin^- cells from WT, $TLR2^{-/-}$ or $MyD88^{-/-}$ mice were cultured in the presence or absence of Pam_3CSK_4 for 3 or 5 days. Cells were labelled with anti-CD11b and F4/80 antibodies and analysed by flow cytometry. Dot plots are representative of 3 independent experiments and the indicated percentages refer to total analysed cells in each dot plot. Bar graphs express means ± SDs of pooled data from 3 independent experiments. ***P < 0.001 with respect to macrophages generated in the absence of Pam_3CSK_4.

To confirm the unique role of MyD88 in direct differentiation, we performed assays with mixed cocultures of WT and KO HSPCs as the ones detailed in section 3.1. Lin^- cells were purified from DsRed and $MyD88^{-/-}$ mice and were cocultured at a 1:1 ratio in the presence of M-CSF (as a control) or Pam_3CSK_4. As expected, $MyD88^{-/-}$ and DsRed WT HSPCs produced nearly equivalent numbers of macrophages in M-CSF-stimulated cocultures. However, in response to Pam_3CSK_4, WT cells produced higher numbers of macrophages than $MyD88^{-/-}$ cells at days 3 and 5 (Figure 11). As $MyD88^{-/-}$ cells yield the same reduced percentage of macrophages than $TLR2^{-/-}$ cells under these culture conditions (see section 3.1), the potential role of TRIF is excluded.

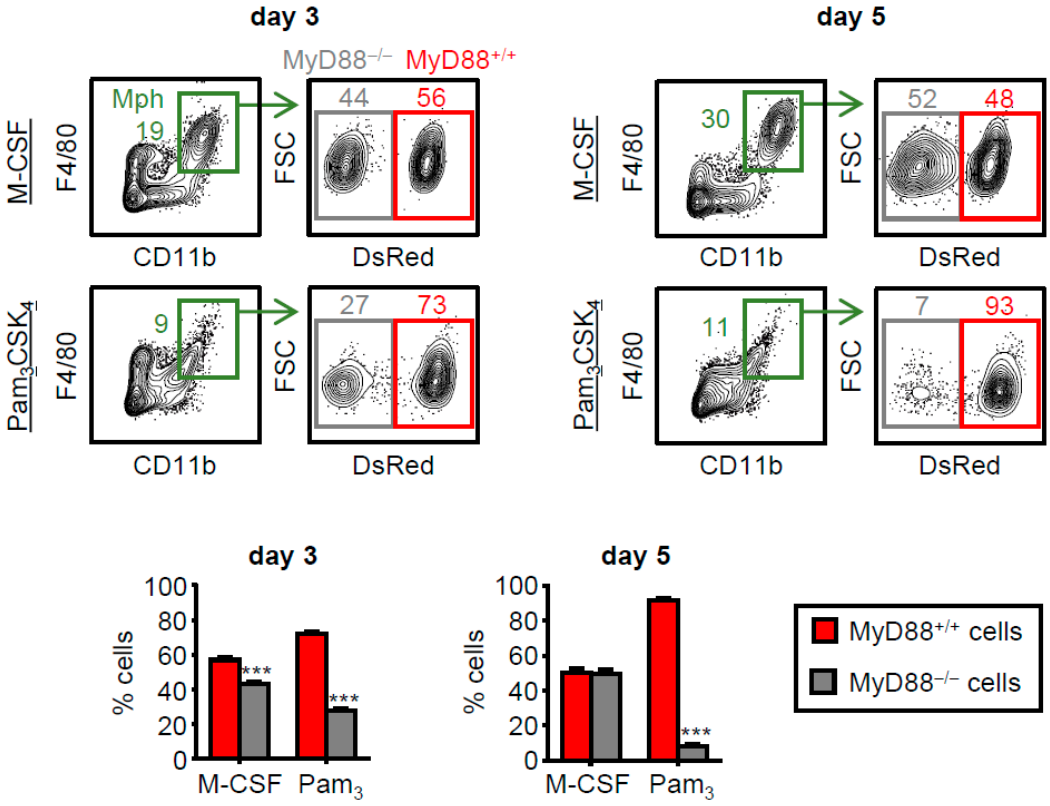

FIGURE 11. Flow cytometry analyses of DsRed and $MyD88^{-/-}$ cocultured Lin^- cells differentiated *in vitro* in response to M-CSF or Pam_3CSK_4. DsRed and $MyD88^{-/-}$ Lin^- cells were cultured at a 1:1 ratio and stimulated with M-CSF or Pam_3CSK_4 for 3 or 5 days. Cells were labelled with anti-CD11b and anti-F4/80 antibodies and analysed by flow cytometry. Macrophages (Mph) were gated as $CD11b^+$ $F4/80^+$ cells, shown in forward scatter (FCS) versus DsRed contour plots, and subgated as $DsRed^+$ and $DsRed^-$. The indicated percentages on dot plots refer to total analysed cells. Plots are representative and bar graphs express means ± SDs of pooled data from 3 independent experiments. ***$P < 0.001$ with respect to macrophages generated from DsRed Lin^- cells.

Overall, these results demonstrate that sensing the TLR2 ligand promotes myelopoiesis directly by initiating MyD88-dependent signalling.

5.2. Gene expression and transcription factors induced in HSPCs upon Pam_3CSK_4 stimulation

To determine the mechanisms of direct TLR2-driven macrophage differentiation we performed RNA sequencing (RNAseq) to study global changes in gene expression of Lin^- cells following 24 h of Pam_3CSK_4 stimulation.

We observed 1353 increased and 760 decreased genes, being *Tnf* and *Ccl2* the most significantly upregulated genes, as expected (Figure 12A). Gene Ontology (GO) analysis mainly revealed cytokine production pathways (including TNF, IL-6 and IL-1β production), signalling pathways (cytokine-mediated, PRR, IκB kinase/NF-κB, TLR, LPS-mediated and type I IFN signalling pathways) and myeloid differentiation pathways (mononuclear cell differentiation, regulation of leukocyte differentiation and myeloid leukocyte differentiation) (Figure 12B).

Within the upregulated genes included in the myeloid leukocyte differentiation pathway, we found several cytokines (*Lif*, *Ifng*, *Tnf*, *Ifnb1* and *Il5*), chemokines (*Ccl19*, *Ccl5* and *Ccl3*) and growth factors (*Csf3* and *Csf2*) (Figure 12C). Within the transcription factors, we found upregulation of *Cebpb*, a transcription factor that has been described to be involved in emergency myelopoiesis (Rosenbauer & Tenen, 2007; Huber *et al.*, 2012). However, the well-known transcription factors *Irf8* and *Klf4*, implicated in monocyte/macrophage differentiation, and *Gfi1*, *Cebpa* and *Cebpe*, which mediate granulocyte differentiation during steady-state, were downregulated, except for *Spi1* (encoding PU.1), a master regulator of myeloid differentiation (Rosenbauer & Tenen, 2007; Heinz *et al.*, 2010; Mossadegh-Keller *et al.*, 2013) (Figure 12D). We next looked at genes involved in the TLR signalling pathway. As expected, *Tlr1*, *Tlr2* and *Myd88* were upregulated and, very interestingly, so was the transcription factor *Irf7* (Figure 12E).

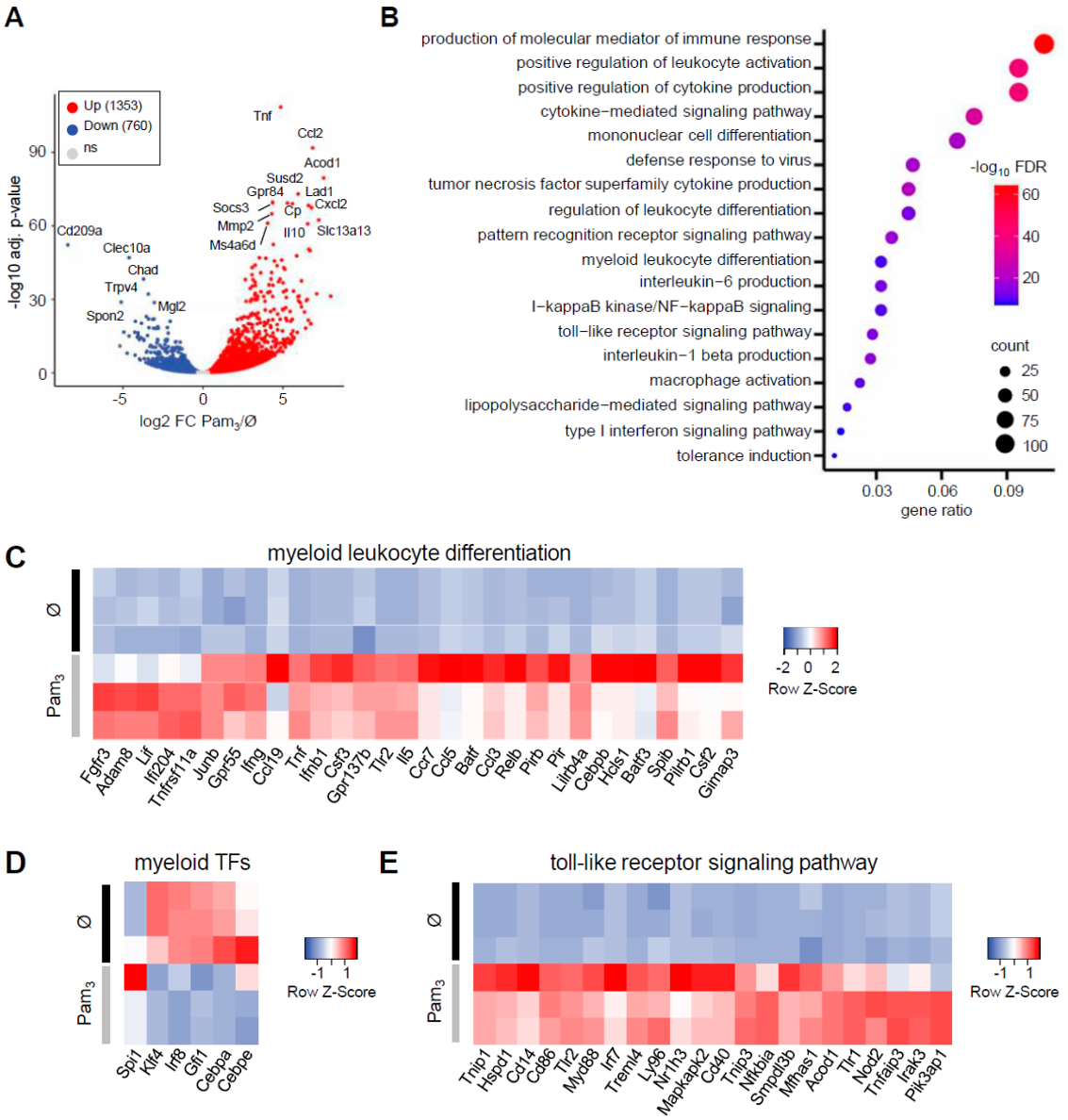

FIGURE 12. RNAseq analysis of Lin⁻ cells following 24 h of Pam_3CSK_4 stimulation. Lin⁻ cells were cultured in the presence or the absence of Pam_3CSK_4 for 24 h and RNA was isolated from these samples for RNA sequencing. **(A)** Volcano plot of DEGs (adjusted p-value < 0.05). Upregulated genes after Pam_3CSK_4 stimulation are highlighted in red and downregulated ones, in blue. Number of DEGs are indicated (n=3 from a pool of 3 mice). **(B)** Gene Ontology (GO) terms enriched in increased DEGs after Pam_3CSK_4 stimulation. The size of the circles represents the number of DEGs in that GO category. The x axis represents the ratio between DEGs and the total number of genes belonging to a GO category. The colour of the circles represents the adjusted p-value from highest (red) to lowest (blue). **(C)** Heatmap of genes involved in the "myeloid leukocyte differentiation" GO pathway. **(D)** Heatmap of genes involved in steady-state myelopoiesis. **(E)** Heatmap of genes involved in the "toll-like receptor signalling pathway" GO pathway.

This gene expression analysis indicates that PU.1, C/EBPβ and IRF7 could be directing Pam_3CSK_4-induced macrophage differentiation. Therefore, we examined the

expression of these transcription factor genes by quantitative real-time PCR. Lin^- cells purified from the bone marrow of WT, $TLR2^{-/-}$ or $MyD88^{-/-}$ mice were cultured for 12 or 24 h in the presence or absence (control) of Pam_3CSK_4, and *Spi1*, *Cebpb* and *Irf7* expression was examined by qRT-PCR. Results show that transcripts of *Spi1*, *Cebpb* and *Irf7* are significantly upregulated above a 2-fold increase after 12 h and sustained over the 24 h (Figure 13). Neither $TLR2^{-/-}$ nor $MyD88^{-/-}$ HSPCs change the expression of *Spi1*, *Cebpb* or *Irf7* in response to Pam_3CSK_4, further confirming the unique role of MyD88 in initiating myeloid differentiation downstream TLR2 in HSPCs (Figure 13).

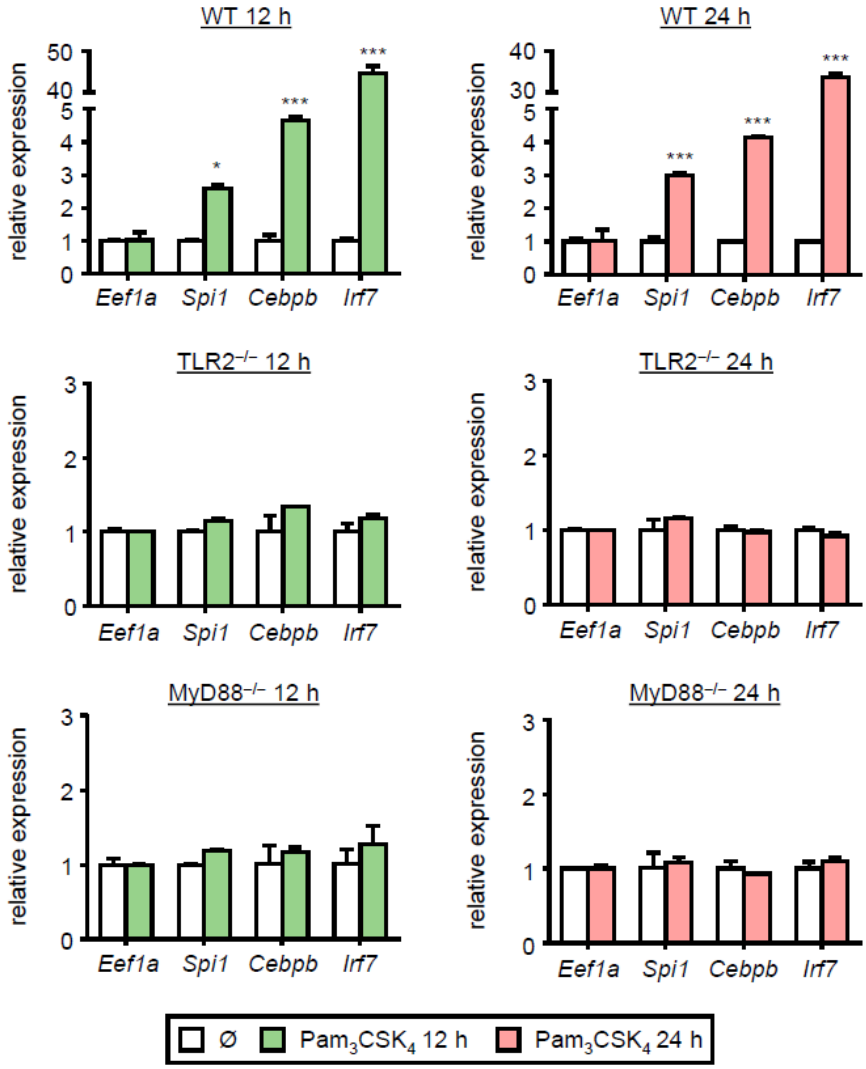

FIGURE 13. Expression of transcription factors in Lin^- cells following 12 or 24 h of Pam_3CSK_4 stimulation. Lin^- cells from WT, $TLR2^{-/-}$ or $MyD88^{-/-}$ mice were cultured in the presence or the absence of Pam_3CSK_4 for 12 or 24 h. Relative expression of the indicated transcripts was assessed by qRT-PCR, using *Eef1a* as an internal reference and normalising the relative expression levels to those of the unstimulated cells. Triplicate samples were analysed in each assay. Data are represented as means ± SDs of pooled data from 3 independent experiments. *P < 0.05 and ***P < 0.001 with respect to non-stimulated cells.

5.3. Signal transduction pathways induced in HSPCs upon Pam_3CSK_4 stimulation

5.3.1. TBK1 and mTOR activation in Pam_3CSK_4-stimulated HSPCs

The above-described findings prompted us to investigate upstream pathways in HSPCs that may lead to the activation of *Irf7* and *Cebpb* in response to Pam_3CSK_4. IRF7 is known to be activated by the noncanonical IKKs (inhibitor of NF-κB kinase), TBK1 and IKKε, downstream of TRIF-dependent pathways (Jefferies, 2019), but TBK1 and IKKε have also been described to be activated by TLR ligands that signal via MyD88, including the TLR2 ligand (Clark *et al.*, 2011; Oosenbrug *et al.*, 2020).

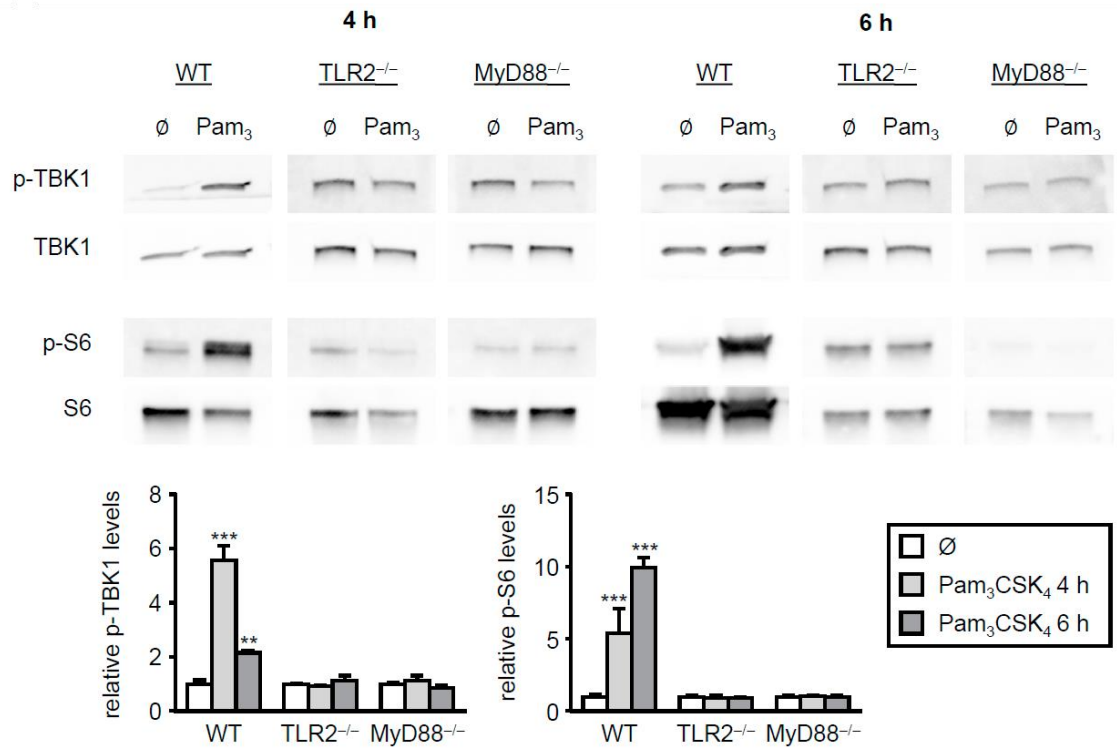

FIGURE 14. Western blot analysis of p-TBK1 and p-S6 levels of Pam_3CSK_4-stimulated Lin^- cells. Lin^- cells from WT, $TLR2^{-/-}$ or $MyD88^{-/-}$ mice were cultured in the presence or the absence of Pam_3CSK_4 for 4 or 6 h. Equal amounts of lysates were electrophoresed and proteins were blotted for the detection of phosphorylated-TBK1 (p-TBK1), total TBK1, phosphorylated-S6 (p-S6) and total S6. The relative levels of the phosphorylated proteins were normalized to total protein expression levels. Protein band images are representative of 3 independent experiments. Bar graphs show means ± SDs of pooled data from 3 independent experiments. **P < 0.01 and ***P < 0.001 with respect to relative phosphorylated-protein levels of non-stimulated cells.

Therefore, Lin^- cells were stimulated with Pam_3CSK_4 for various times and western blotting analysis was employed to visualize phosphorylation of TBK1 (Figure 14). Said phosphorylation was observed after 4 and 6 h of stimulation, but is abolished in $TLR2^{-/-}$ and $MyD88^{-/-}$ HSPCs, once again confirming the role of MyD88 in TLR2 signalling in HSPCs.

On the other hand, it has been reported that C/EBPβ is activated following mTOR signalling in both, macrophages and HSPCs, under stress conditions (Sato *et al.*, 2020), so we also measured mTOR activation in response to Pam_3CSK_4 by analysing the phosphorylation of its target molecule, the S6 kinase (Figure 14). Our results show that Pam_3CSK_4 promotes phosphorylation of the S6 kinase at 4 and 6 h, clearly demonstrating that TLR2 signalling in HSPCs engages the mTOR pathway. As for TBK1, S6 phosphorylation is abolished in $TLR2^{-/-}$ and $MyD88^{-/-}$ cells.

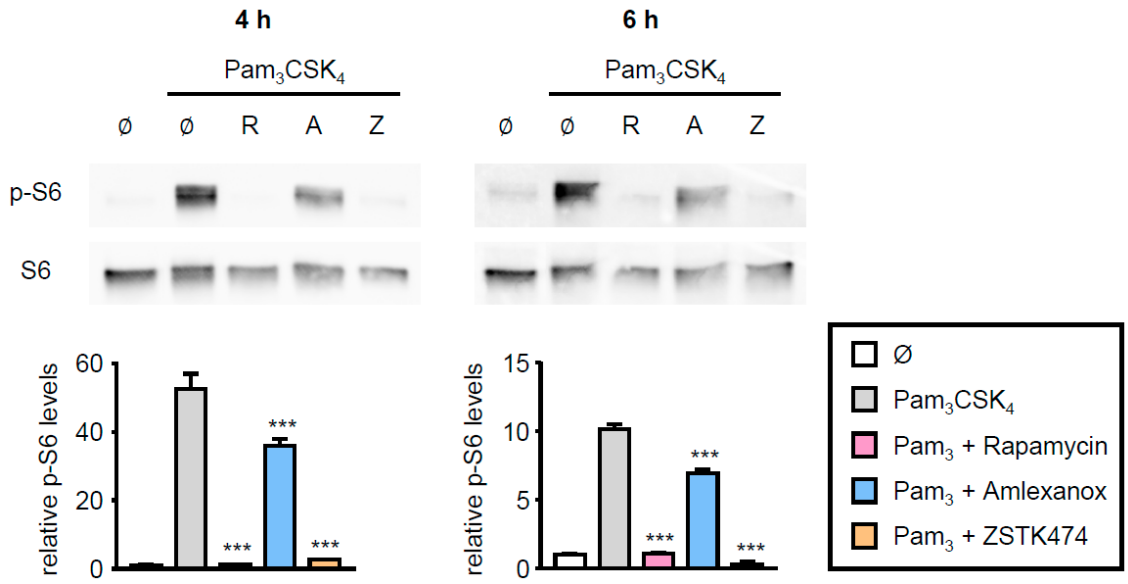

FIGURE 15. Effect of rapamycin, amlexanox and ZSTK474 on p-S6 levels of Pam_3CSK_4-stimulated Lin^- cells. Lin^- cells were pre-treated with or without rapamycin (R), amlexanox (A) or ZSTK474 (Z), as indicated, and cultured in the presence or the absence of Pam_3CSK_4 for 4 or 6 h. Equal amounts of lysates were electrophoresed and proteins were blotted for the detection of phosphorylated-S6 (p-S6) and total S6. The relative levels of p-S6 were normalized to total S6 expression levels. Protein band images are representative of 3 independent experiments. Bar graphs show means ± SDs of pooled data from 3 independent experiments. ***P < 0.001 with respect to cells cultured with Pam_3CSK_4 only.

Next, to delve into the mechanisms leading to mTOR activation, we explored the well-characterized link between TLRs and mTOR that involves the PI3K/AKT pathway (Abdel-Nour *et al.*, 2014), and the possibility that mTOR could be directly activated by

TBK1, as it has been described to happen in macrophages in response to TLR3 and TLR4 activation (Bodur *et al.*, 2018). Western blotting analysis was employed to visualize phosphorylation of S6 in Lin^- cells that were stimulated with Pam_3CSK_4 in the presence or absence of Rapamycin (an mTOR inhibitor), Amlexanox (a TBK1 inhibitor) or ZSTK474 (a PI3K inhibitor) (Figure 15). As expected, S6 phosphorylation was completely inhibited by Rapamycin at both, 4 and 6 h. Similarly, inhibition of PI3K abolished mTOR activation, while inhibition of TBK1 with Amlexanox only inhibited S6 phosphorylation partially.

Collectively, these findings demonstrate that direct sensing of the TLR2 ligand by HSPCs activates TBK1 and mTOR pathways in an MyD88-dependent manner, and that activation of mTOR is mediated by PI3K and, to a lesser extent, by TBK1.

5.3.2. Myeloid differentiation following inhibition of TBK1 or mTOR in Pam_3CSK_4-stimulated HSPCs

In order to find out whether TBK1 and/or mTOR may be involved in the TLR2-induced myeloid differentiation of HSPCs, we performed differentiation experiments in response to Pam_3CSK_4 in the presence or absence (control) of Rapamycin and Amlexanox. As the previously assayed Lin^- population contains a variety of transitional intermediates, including HSCs and myeloid and lymphoid committed progenitors, we now assayed the effect of inhibitors on the differentiation of purified LKS^+ cells (Lin^- c-kit^+ Sca-1^+ cells; containing stem cells and multipotent progenitors) and LKS^- cells (Lin^- c-kit^+ Sca-1^- cells; containing committed progenitors) separately.

The generation of differentiated cells (total cell number ratio of $CD11b^+$ c-kit^- mature cells versus $CD11b^-$ c-kit^+ progenitor cells) in response to Pam_3CSK_4 was inhibited by Rapamycin and Amlexanox, both in LKS^+ (Figure 16A) and LKS^- (Figure 16B) cultured after 4 days of stimulation. Moreover, macrophage development (total cell number ratio of $CD11b^+$ $F4/80^+$ macrophages versus $CD11b^-$ c-kit^+ progenitor cells) was significantly inhibited by Rapamycin and Amlexanox, both in LKS^+ (Figure 16A) and LKS^- (Figure 16B) cultures after 7 days of stimulation.

These data demonstrate that TBK1 and mTOR are involved in the TLR2-induced production of macrophages from both, LKS^+ and LKS^- cells.

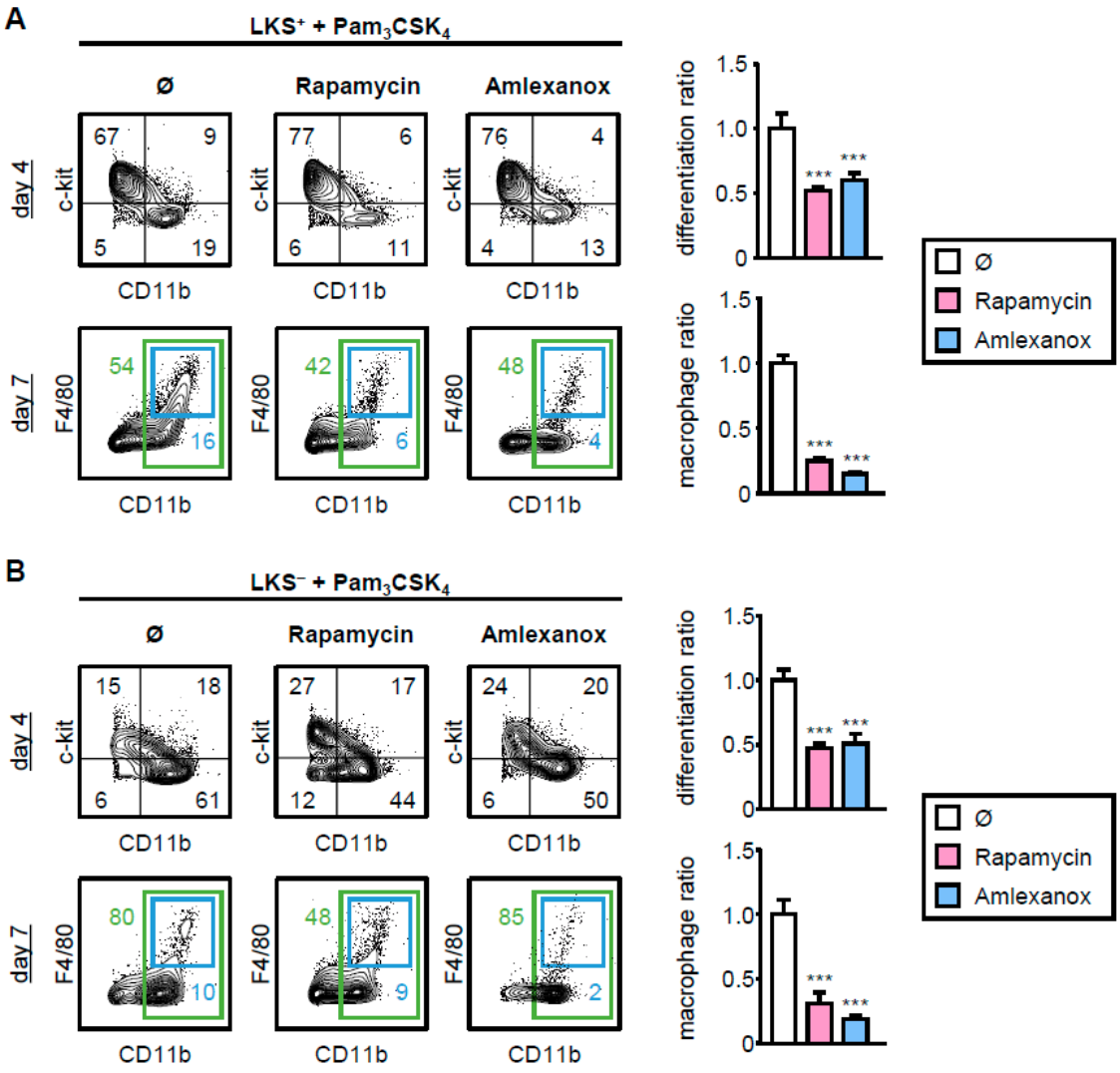

FIGURE 16. Flow cytometry analyses of LKS$^+$ or LKS$^-$ cells differentiated *in vitro* with Pam$_3$CSK$_4$ in the presence or absence of rapamycin or amlexanox. LKS$^+$ **(A)** or LKS$^-$ **(B)** cells were pre-treated with or without rapamycin or amlexanox, as indicated, and cultured with Pam$_3$CSK$_4$ for 4 or 7 days. Cells were labelled with anti-c-kit, anti-CD11b and anti-F4/80 antibodies and analysed by flow cytometry. In the CD11b versus F4/80 plots cells were gated as differentiated cells (total CD11b$^+$) and as macrophages (CD11b$^+$ F4/80$^+$). Dot plots are representative of 3 independent experiments and the indicated percentages refer to total analysed cells. The differentiation and macrophage ratios were calculated by dividing total cell numbers of the CD11b$^+$ c-kit$^-$ mature cells or CD11b$^+$ F4/80$^+$ macrophages, respectively, by total cell numbers of CD11b$^-$ c-kit$^+$ progenitor cells, and normalising the obtained ratios to those of cells differentiated with Pam$_3$CSK$_4$ only. Bar graphs show means ± SDs of pooled data from 3 independent experiments. ***P < 0.001 with respect to the differentiation ratio of cells differentiated with Pam$_3$CSK$_4$ only.

5.3.3. Expression of transcription factors following inhibition of TBK1 or mTOR in Pam_3CSK_4-stimulated HSPCs

The above data demonstrate that TLR2 signalling in HSPCs upregulates PU.1, C/EBPβ and IRF7 transcription factors and that blocking the upstream pathways TBK1 and mTOR inhibits myeloid differentiation.

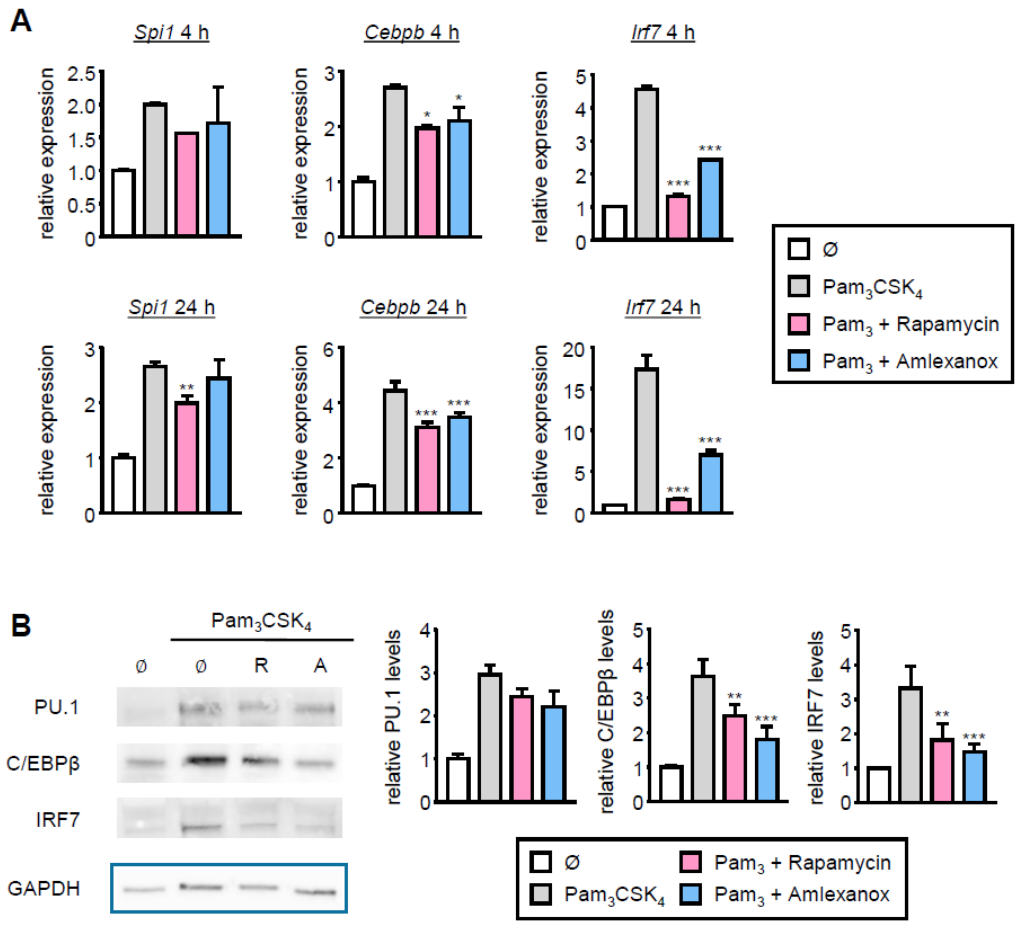

FIGURE 17. Effect of rapamycin and amlexanox on PU.1, C/EBPβ and IRF7 gene expression and protein levels of Pam_3CSK_4-stimulated Lin^- cells. (A) Lin^- cells were pre-treated with or without rapamycin or amlexanox, as indicated, and cultured with Pam_3CSK_4 for 4 or 24 h. Relative expression of *Spi1*, *Cebpb* and *Irf7* was assessed by qRT-PCR, using *Eef1a* as an internal reference and normalising the relative expression levels to those of the unstimulated cells. Triplicate samples were analysed in each assay. Data are represented as means ± SDs of pooled data from 3 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cells cultured with Pam_3CSK_4 only. **(B)** Lin^- cells were pre-treated with or without rapamycin (R) or amlexanox (A), as indicated, and cultured with Pam_3CSK_4 for 24 h. Equal amounts of lysates were electrophoresed and proteins were blotted for the detection of PU.1, C/EBPβ, IRF7 and GAPDH. The relative levels of each protein were normalized to GAPDH expression levels. Protein band images are representative of 4 independent experiment. Bar graphs show means ± SDs of pooled data from 4 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to relative protein levels of cells cultured with Pam_3CSK_4 only.

These findings prompted us to investigate the effect of Amlexanox and Rapamycin on the expression of the TLR2-induced transcription factors (Figure 17). We observed that *Spi1* upregulation in response to Pam_3CSK_4 was significantly inhibited by Rapamycin at 24 h, although this inhibition was not detected at the protein level by immunoblot. As expected, *Cebpb* expression was inhibited by Rapamycin at 4 and 24 h of stimulation, and the level of C/EBPβ protein was also decreased at 24 h in the presence of Rapamycin. Moreover, Amlexanox was also able to inhibit C/EBPβ, probably due to the direct activation of mTOR mediated by TBK1. Finally, IRF7 was inhibited by Amlexanox, as expected, and, interestingly, also inhibited by Rapamycin, both at the mRNA and protein level.

Overall, these results indicate that direct sensing of the TLR2 ligand promotes myelopoiesis directly by activating several transcription factors.

5.4. Role of secreted cytokines in myeloid differentiation of Pam_3CSK_4-stimulated HSPCs

5.4.1. Role of IL-6

It has been reported that signalling via TLRs in HSPCs makes them release cytokines through NF-κB signalling and that among the produced cytokines, IL-6 acts in an autocrine/paracrine manner inducing myeloid differentiation (Zhao *et al.*, 2014). Given that IL-6 has been reported to strongly induce C/EBPβ mRNA expression in murine myeloid leukaemia cells (Huber *et al.*, 2012), we wondered whether IL-6 may be involved in the early upregulation of *Cebpb* in response to Pam_3CSK_4 in HSPCs.

To delve into the role of IL-6 in TLR2-induced myeloid differentiation we first analysed the differentiation induced by IL-6 alone or in combination with Pam_3CSK_4 in LKS^+ (Figure 18A) and LKS^- (Figure 18B) cell cultures. Our results show that IL-6 alone induces myeloid differentiation to macrophages, although to a lesser extent than Pam_3CSK_4, suggesting that TLR2-induced myeloid differentiation involves other pathways in addition to IL-6. However, differentiation in response to the TLR2 ligand is not increased by IL-6, likely due to the fact that the IL-6 produced in response to Pam_3CSK_4 is probably enough to saturate all the IL-6 receptor molecules on progenitors, and consequently, addition of exogenous IL-6 does not further increase differentiation.

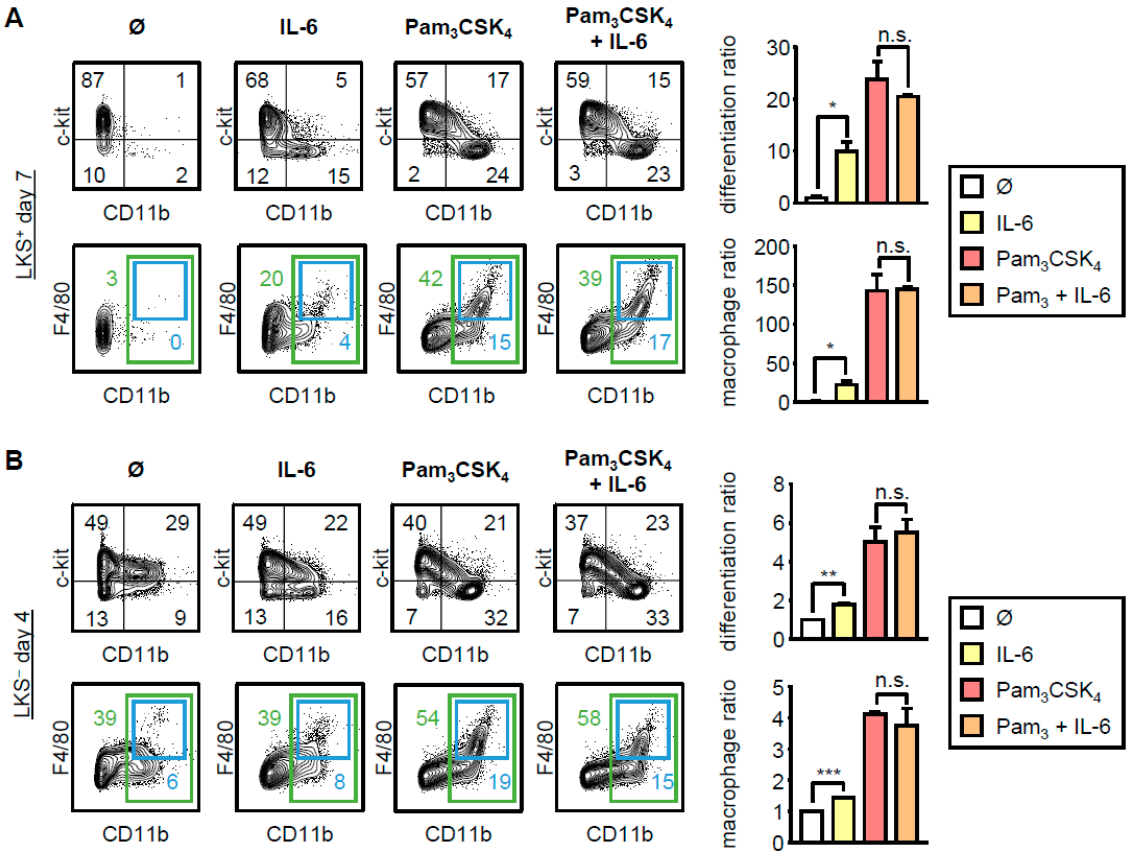

FIGURE 18. IL-6 effect on LKS$^+$ or LKS$^-$ cell differentiation cultured *in vitro* with or without Pam$_3$CSK$_4$. LKS$^+$ **(A)** or LKS$^-$ **(B)** cells were cultured for 4 or 7 days, respectively, with or without IL-6 and/or Pam$_3$CSK$_4$, as indicated. Cells were labelled with anti-c-kit, anti-CD11b and anti-F4/80 antibodies and analysed by flow cytometry. In the CD11b versus F4/80 plots cells were gated as differentiated cells (total CD11b$^+$) and as macrophages (CD11b$^+$ F4/80$^+$). Dot plots are representative of 3 independent experiments and the indicated percentages refer to total analysed cells. The differentiation and macrophage ratios were calculated by dividing total cell numbers of the CD11b$^+$ c-kit$^-$ mature cells or CD11b$^+$ F4/80$^+$ macrophages, respectively, by total cell numbers of CD11b$^-$ c-kit$^+$ progenitor cells, and normalising the obtained ratios to those of unstimulated cells. Bar graphs show means ± SDs of pooled data from 3 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to the differentiation ratio of non-stimulated cells. n.s., not significant.

Next, to compare the kinetics of C/EBPβ and IL-6 induction, Lin$^-$ cells were cultured in the presence or absence (control) of Pam$_3$CSK$_4$, and *Cebpb* and *Il6* expression was examined by qRT-PCR at various timepoints (Figure 19A). We found that the expression of both genes was upregulated early (45 min) and simultaneously, so that neither preceded the other, suggesting that IL-6 is not required to induce *Cebpb* expression downstream TLR2 signalling in HSPCs.

Finally, we measured *Il6* expression in 24 h- Pam$_3$CSK$_4$-stimulated HSPCs in the presence or absence (control) of Rapamycin, Amlexanox and BMS345541 (a highly

selective inhibitor of IKKβ that blocks NF-κB-dependent transcription). Results show that *Il6* expression in response to Pam_3CSK_4 was drastically inhibited by BMS345541, but not affected by Rapamycin or Amlexanox (Figure 19B). Similar results were obtained when IL-6 was measured at the protein level by ELISA in culture supernatants of stimulated HSPCs (Figure 19C).

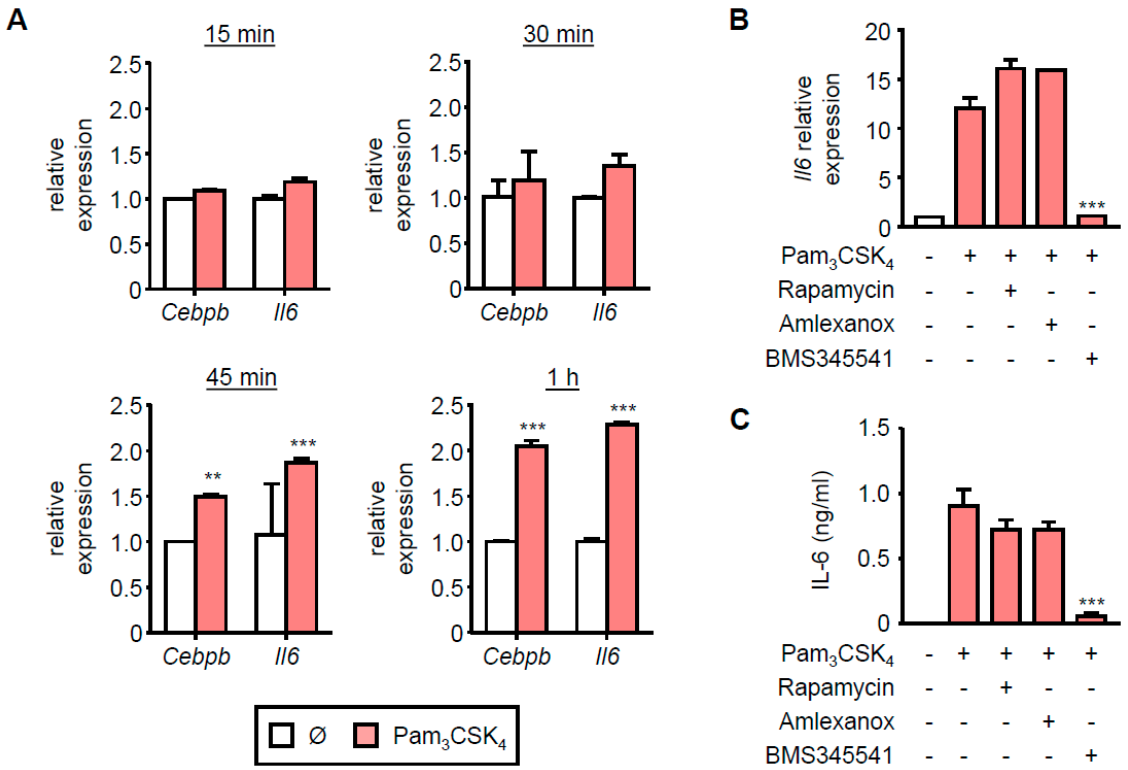

FIGURE 19. IL-6 induction in Pam_3CSK_4-stimulated Lin^- cells and signalling pathways involved on its gene expression and protein level. (A) Lin^- cells were cultured in the presence or absence of Pam_3CSK_4 for 15, 30, 45 min or 1 h. Relative expression of the *Cebpb* and *Il6* transcripts was assessed by qRT-PCR, using *Eef1a* as an internal reference and normalising the relative expression levels to those of the unstimulated cells. **(B)** Lin^- cells were pre-treated with or without rapamycin, amlexanox or BMS345541, as indicated, and cultured in the presence or absence of Pam_3CSK_4 for 24 h. Relative expression of *Il6* was assessed by qRT-PCR, using *Eef1a* as an internal reference and normalising the relative expression levels to those of the unstimulated cells. **(C)** Lin^- cells were pre-treated with or without rapamycin, amlexanox or BMS345541, as indicated, and cultured in the presence or absence of Pam_3CSK_4 for 24 h. IL-6 levels in cell-free supernatants were assessed by ELISA. Triplicate samples were analysed in each assay. Data are represented as means ± SDs of pooled data from 3 independent experiments. **P < 0.01 and ***P < 0.001 with respect to non-stimulated cells (A) or with respect to cells cultured with Pam_3CSK_4 only (B, C).

Overall, these data demonstrate that although TLR2 signalling in HSPCs induces NF-κB-dependent IL-6 production that leads to indirect myeloid differentiation, other pathways that are responsible for direct macrophage differentiation (TBK1 and mTOR) are also simultaneously induced.

5.4.2. Role of type I IFN

It has been reported that IRF7 drives expression of type I IFN (Jefferies, 2019), which amplifies TLR7-driven myeloid development from CMPs (Buechler *et al.*, 2016). Therefore, we wondered whether IRF7 may induce differentiation through a mechanism involving type I IFN.

To study the potential role of type I IFN in TLR2-induced myeloid differentiation we first analysed the differentiation induced by IFN-β, alone or in combination with Pam_3CSK_4 in Lin^- cultures (Figure 20A). IFN-β alone does not induce myeloid differentiation to macrophages, and differentiation in response to the TLR2 ligand is not amplified by IFN-β either. In addition, Pam_3CSK_4 upregulates *Ifnb* expression after 4 h of culture, and this increase is not inhibited by Amlexanox (Figure 20B), which does inhibit IRF7 (Figure 17) and macrophage differentiation (Figure 16).

Therefore, these findings indicate that the role of IRF7 in differentiation is independent of IFN-β.

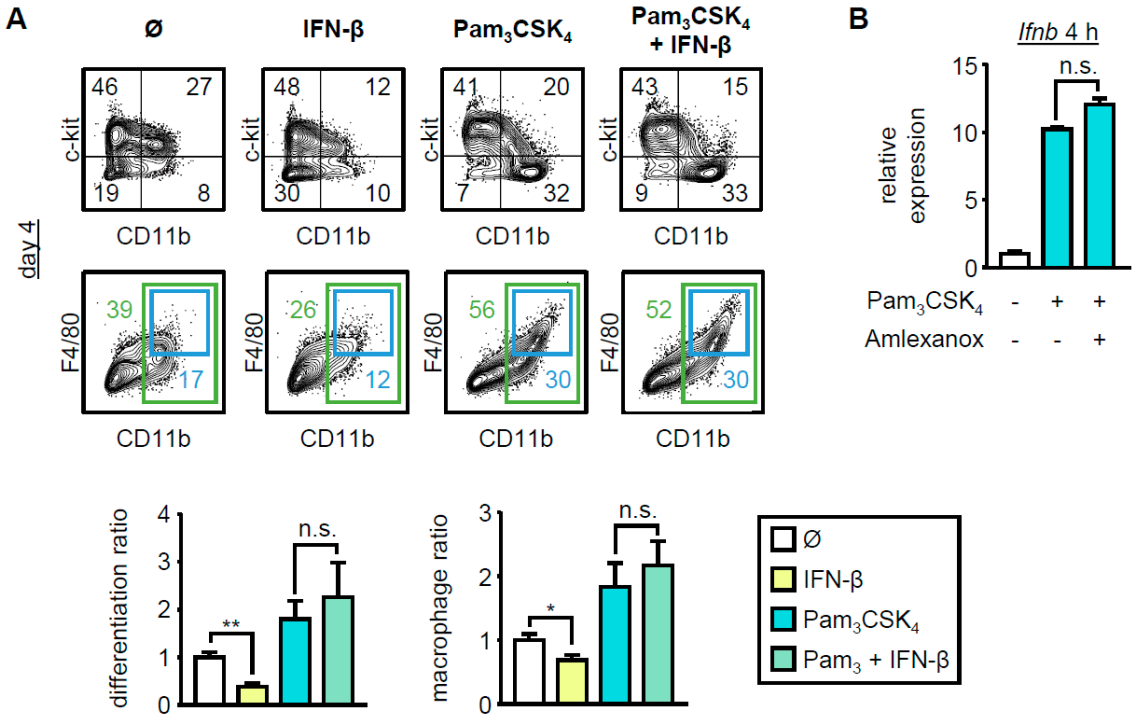

FIGURE 20. IFN-β effect on Lin⁻ cell differentiation cultured *in vitro* with or without Pam_3CSK_4 and effect of amlexanox on its gene expression. (A) Lin⁻ cells were cultured for 4 days with or without IFN-β and/or Pam_3CSK_4, as indicated. Cells were labelled with anti-c-kit, anti-CD11b and anti-F4/80 antibodies and analysed by flow cytometry. Dot plots are representative of 3 independent experiments and the indicated percentages refer to total analysed cells. The differentiation and macrophage ratios were calculated by dividing total cell numbers of $CD11b^+$ c-kit^- mature cells or $CD11b^+$ $F4/80^+$ macrophages, respectively, by total cell numbers of $CD11b^-$ c-kit^+ progenitor cells, and normalising the obtained ratios to those of unstimulated cells. Bar graphs show means ± SDs of pooled data from 3 independent experiments. *P < 0.05 and **P < 0.01 with respect to the differentiation ratio of non-stimulated cells. n.s., not significant. **(B)** Lin⁻ cells were pre-treated with or without amlexanox and cultured in the presence or absence of Pam_3CSK_4 for 4 h. Relative expression of *Ifnb* was assessed by qRT-PCR, using *Eef1a* as an internal reference and normalising the relative expression levels to those of the unstimulated cells. Triplicate samples were analysed in each assay. Data are represented as means ± SD of pooled data from 3 independent experiments. n.s., not significant.

6. Discussion

In homeostatic conditions, blood cell production is tightly regulated by HSC self-renewal and their conversion into lineage-committed progenitors. Many cytokines, cell-cell interactions and transcription factors have been described to control the proliferation of HSPCs and their differentiation into mature myeloid and lymphoid cells (Iwasaki & Akashi, 2007). However, upon different types of infection, including candidiasis, haematopoiesis is profoundly altered through a process known as emergency myelopoiesis. In this context, the production of granulocytes (mainly neutrophils) or monocytes (or both cell types, depending on the pathogen and/or the severity of the infection) is favoured, leading to the inhibition of other lineage (lymphoid and erythroid) development, in order to rapidly provide the innate immune system with myeloid cells, as they constitute the first line of defence against the microorganisms causing the infection and are, therefore, more urgently needed to fight against the pathogen (Boettcher & Manz, 2017).

The mechanisms by which different pathogen signals are detected and subsequently translated into demand-adapted myelopoiesis are beginning to be understood. Besides these alterations in haematopoiesis and HSPC populations being caused by indirect pathogen sensing via mature hematopoietic and nonhematopoietic cells, the fact that HSPCs express functional PRRs suggest the existence of a mechanism of direct interaction between pathogens (or some of their components) with said cells. In this way, detection of the pathogen by the HSPCs themselves also influences their division, cell fate and, ultimately, the efficiency of the host's defence against infection (Barman & Goodridge, 2022; Sioud, 2020).

In this context, it has been proven that inactivated *C. albicans* cells induce proliferation of murine HSPCs *in vitro*, as well as their differentiation towards the myeloid lineage in a TLR2/MyD88- and dectin-1-dependent manner (Yáñez *et al.*, 2013a). However, studying direct interaction between microbial pathogens, or their ligands, and PRRs on HSPCs *in vivo* is difficult to demonstrate, as HSPCs could also be responding to other stimuli generated when mature immune or nonimmune cells detect microbial products via their PRRs. Nonetheless, a previously developed model of HSPC transplantation allowed for investigating the possible direct interaction of PAMPs and TLRs on HSPCs *in vivo*, demonstrating that HSPCs are indeed directly stimulated by

TLR2, TLR4 and TLR9 soluble agonists *in vivo* and that the engagement of these receptors induces differentiation towards macrophages (Megías *et al.*, 2012).

In the present PhD thesis, we have used a similar experimental approach in order to investigate the possible direct interaction of β-glucans (depleted zymosan) and dectin-1 on HSPCs *in vivo*. According to this methodology, cells from mice expressing the CD45.1 alloantigen were transplanted into dectin-1$^{-/-}$ mice (CD45.2 alloantigen), to which depleted zymosan was also administered. In this way, we excluded any indirect effects (cytokines or soluble mediators secreted by surrounding cells), as recipient mice do not recognize the injected ligand. Moreover, recipient mice were not irradiated so as to avoid an inflammatory environment that could lead to generating artefacts in our results. In accordance with previous studies (Megías *et al.*, 2012, 2013), transplanted HSPCs in the absence of a dectin-1 challenge lose stem cell markers and differentiate towards the myeloid lineage. This is also in line with Massberg *et al.*, who showed that migratory HSPCs give rise to myeloid cells in peripheral tissues (Massberg *et al.*, 2007). However, we observed that the yield of transplanted cells, as well as the percentage of macrophages differentiated from transplanted HSPCs, was significantly increased when transplanted dectin-1$^{-/-}$ mice had been injected with depleted zymosan, which could only be due to a direct interaction between dectin-1 in the transplanted HSPCs and its ligand. That is, HSPCs are directly stimulated by the dectin-1 ligand *in vivo*, which induces their differentiation to macrophages.

By using a similar transplant model, it was also demonstrated that HSPCs differentiate to macrophages *in vivo* in response to the fungal pathogen *C. albicans* and that this differentiation is TLR2-dependent and TLR4-independent (Megías *et al.*, 2013). Here, we have delved into the role of dectin-1 and TLR signalling in this HSPC differentiation *in vivo* in response to *C. albicans* yeasts. In order to do so, bone marrow HSPCs from WT, dectin-1$^{-/-}$ or MyD88$^{-/-}$ mice (CD45.2 alloantigen) were transplanted into mice that express the CD45.1 alloantigen, which were also injected with inactivated *C. albicans* yeasts. It was observed that, in response to the fungal stimulus, dectin-1$^{-/-}$ and MyD88$^{-/-}$ transplanted cells originated a lower percentage of macrophages in comparison to WT transplanted cells. In this model, although recipient cells can recognize and respond to the injected yeasts, indirect signals induced by host cells would affect all the transplanted cells in the same way, and so differences in macrophage differentiation observed between dectin-1$^{-/-}$ or MyD88$^{-/-}$ and WT donor cells, could only be due to a lack of direct

signalling via dectin-1 or TLRs, respectively, in the transplanted HSPCs. Therefore, we can confirm that *in vivo* differentiation of HSPCs in response to *C. albicans* yeasts is dectin-1- and TLR-dependent, which correlates with previous *in vitro* observations in which *C. albicans* yeasts induce differentiation of HSCPs towards the myeloid lineage through TLR2 and dectin-1 signalling (Yáñez *et al.*, 2009, 2010, 2011), although the role of TLRs seems to be more significant *in vivo*. Overall, these results strengthen the hypothesis that, during an infection, PAMPs may directly induce HSPC proliferation and differentiation, thus contributing to emergency myelopoiesis.

Over the last years, several studies have challenged the dogma that immune memory is an exclusive trait of adaptive immunity. That is, innate immune cells (especially monocytes and macrophages) can display certain memory characteristics and respond in a different way towards a second encounter of the same or heterologous stimuli (Netea *et al.*, 2016). Quintin *et al.*, for example, showed that exposing monocytes to β-glucans or *C. albicans* primes their production of proinflammatory cytokines so that they display an enhanced response upon subsequent stimulation (trained immunity) (Quintin *et al.*, 2012). Moreover, it has been demonstrated that this concept of innate immune memory can be applied not only to mature myeloid cells, but also to HSPCs (De Zuani & Frič, 2022). By using an *in vitro* differentiation model of HSPCs, it was demonstrated that exposure of HSPCs to dectin-1 agonists (depleted zymosan or inactivated yeasts of *C. albicans*) results in the production of trained macrophages, with a greater capacity to produce cytokines and higher fungicidal activity than control macrophages differentiated from unexposed HSPCs (Megías *et al.*, 2016).

In this thesis project, we have developed an *in vitro* experiment to approach as much as possible the conditions under which HSPCs encounter live *C. albicans* cells during a real infection, once the fungus has reached the bone marrow (Yáñez *et al.*, 2011) or HSPCs have been mobilized to the site of infection, as has been described in different bacterial infection models (Granick *et al.*, 2013; Burberry *et al.*, 2014). Interestingly, we found that a short transient exposure of HSPCs to live *C. albicans* cells prior to differentiation is sufficient to program a trained response of the macrophages subsequently derived from them with M-CSF. This trained phenotype was partially dependent on the recognition of *C. albicans* by both, TLR2 and dectin-1, on HSPCs but was different for each specific cytokine, as compensatory signalling via remaining PRRs is likely to be responsible for a lack of phenotype or an attenuated trained response:

trained TNF-α production was totally dependent on both, TLR2 and dectin-1, trained IL-6 production was partially dependent on both, TLR2 and dectin-1, whereas MIP-2 production was dependent on dectin-1 but not on TLR2. Overall, these data reinforce a novel mechanism whereby macrophage responses can be programmed by PRR signalling in HSPCs prior to differentiation.

To demonstrate that trained immunity induced by dectin-1 signalling also takes place in HSPCs *in vivo*, we used the HSPC transplantation model in which receptor mice are unable to respond to depleted zymosan. By doing so, we have demonstrated that direct *in vivo* signalling via dectin-1 in the transplanted progenitor cells leads to the production of trained macrophages by a cell-autonomous mechanism. These results are in line with a previous work that shows that intravenous vaccination with *Mycobacterium bovis* bacillus Calmette-Guérin "educates" HSCs to generate monocytes that protect mice against tuberculosis (Kaufmann *et al.*, 2018), supporting the idea that microorganisms direct HSPCs to generate myeloid cells better prepared to face the infection. However, further studies will be necessary to directly demonstrate a role for dectin-1 in training HSPCs for protective responses against systemic candidiasis.

In conclusion, dectin-1 ligation activates HSPCs *in vivo* and induces their differentiation to trained macrophages by a cell-autonomous mechanism. This points to a new mechanism by which β-glucans from fungal pathogens may modulate haematopoiesis in real time to generate myeloid cells better prepared to deal with the infection. Our results strongly support the role of dectin-1 in training HSPCs for a protective response against infection, although directly demonstrating this will require further studies. A better understanding of the signals that influence HSPCs during an infection could lead to the development of better therapeutic strategies of intervention against an infection, as manipulation of this process may help improve the innate immune response during serious infections.

On the other hand, molecular mechanisms directing translation of direct detection of the pathogen by HSPCs into signals that promote myeloid differentiation under stress conditions have not yet been completely elucidated. TLRs may induce myeloid development by direct signalling in an intrinsic manner, directly activating transcription factors implicated in myeloid differentiation; by an indirect mechanism, secreting

cytokines that would act in an autocrine or paracrine manner; or by a combination of both, direct and indirect mechanisms (Zhao & Baltimore, 2015).

Regarding mechanisms implicated in the acquisition of different phenotypes due to signalling through PRRs in HSPCs, it was demonstrated that HSPCs that had been exposed to the TLR2 ligand produce soluble factors that act in a paracrine manner, influencing the function of macrophages that had not been exposed to such ligand, thus tolerizing them (Yáñez *et al.*, 2013b). Here, using a different model in which we cocultured TLR2$^{-/-}$ with TLR2$^{+/+}$ HSPCs (distinguishable because the latter expressed the red fluorescent protein DsRed) that were differentiated with M-CSF in the presence of the TLR2 ligand, we have confirmed these results. Therefore, the acquisition of a tolerized phenotype due to signalling via TLR2 in HSPCs is mediated, at least partially, by indirect mechanisms, that is, by secreted molecules that can act on cells lacking this receptor. Additionally, using a coculture system of dectin-1$^{-/-}$ and dectin-1$^{+/+}$ HSPCs we have observed that signalling via dectin-1 in HSPCs in response to both, depleted zymosan and inactivated yeasts of *C. albicans*, induces differentiation to trained macrophages mostly by indirect mechanisms. Favouring the existence of indirect mechanisms, it has been reported that signalling via TLRs in HSPCs makes them release copious amounts of cytokines that act in an autocrine/paracrine manner to induce myeloid differentiation (Zhao *et al.*, 2014; Zhao & Baltimore, 2015). In addition, our group has previously described that the secretome (conditioned media) of HSPCs in response to the TLR2 ligand and *C. albicans* is able to induce myeloid differentiation of HSPCs *in vitro* (Martínez *et al.*, 2018).

However, other authors have described that TLR2, TLR4 and TLR7 ligands are capable of directly inducing myeloid differentiation intrinsically (Zhao *et al.*, 2014; Liu *et al.*, 2015; Buechler *et al.*, 2016). Myeloid differentiation of CMPs can be directly induced by TLR7 signalling, which acts synergistically with type I IFNs (Buechler *et al.*, 2016). Moreover, by using a model of Pam_3CSK_4 or LPS injection in chimeric mice, it was observed in TLR2$^{-/-}$ or TLR4$^{-/-}$ recipients transplanted with WT plus TLR2$^{-/-}$ or WT plus TLR4$^{-/-}$ bone marrow, respectively, that WT HSCs display a greater response than KO HSCs, revealing that, indeed, HSCs directly sense TLR ligands. Nonetheless, in agreement with the involvement of indirect pathways, KO HSCs were not fully impaired in their capacity to respond to the TLR ligands (Herman *et al.*, 2016; Takizawa *et al.*, 2017). Accordingly, our TLR2$^{-/-}$ and TLR2$^{+/+}$ coculture experiments have revealed that

macrophage differentiation can be directly induced by TLR2 signalling besides the indirect production of differentiating factors, as in response to the TLR2 ligand, the percentage of differentiated cells that lack said receptor is lower to that of the cells that do express it. Under these culture conditions, differentiation mediated by indirect mechanisms is the same in both progenitor types, so the difference in the percentage of differentiated cells observed indicates that direct differentiation mechanisms mediated by signalling via TLR2 are taking place.

Currently, little is known about which transcription factors downstream of TLR activation might mediate direct differentiation in HSPCs. Our RNA-seq data revealed significant transcriptional changes in HSPCs in response to TLR2, and among the upregulated transcription factor genes, *Spi1*, *Cebpb* and *Irf7* appeared to us as potential candidates to mediate myeloid differentiation. PU.1, a member of the ETS family transcription factors, is necessary in the earliest steps of myeloid commitment and its deficiency results in a loss of monocytes and granulocytes (Heinz *et al.*, 2010; Mossadegh-Keller *et al.*, 2013). PU.1 binds to purine-rich sequences in the promoters of target genes and, in collaboration with other transcription factors (C/EBP family members C/EBPα and C/EBPβ), induces a gene expression programme that regulates granulocytic and monocytic cell differentiation. C/EBPα is indispensable to steady-state granulopoiesis, whereas C/EBPβ plays a critical role in stress-induced granulopoiesis (Rosenbauer & Tenen, 2007). Moreover, C/EBPβ, a transcription factor involved in the MyD88-dependent cytokine production in response to TLRs (Lu *et al.*, 2009), has been shown to regulate monocytic gene expression and differentiation to macrophages (Huber *et al.*, 2012). Therefore, the upregulation of PU.1 and C/EBPβ in response to Pam_3CSK_4 may induce monocytic/macrophage differentiation, in accordance with Buechler *et al.*, who showed that TLR7 signalling in CMPs upregulates PU.1 and C/EBPβ to produce macrophages (Buechler *et al.*, 2016).

Surprisingly, IRF8 expression was not increased in HSPCs in response to Pam_3CSK_4. IRF8 is a hematopoietic cell-specific member of the IRF family that induces macrophage differentiation and is thought to be absolutely required for monocyte development (Tamura *et al.*, 2015; Yáñez *et al.*, 2015). However, TLR2 activation does upregulate IRF7, which is known to be a mediator of type I IFN induction, acting downstream of PRRs that recognize pathogen nucleic acids. Moreover, IRF7 has emerged as an

important regulator of haematopoiesis: it is expressed in vertebrate HSCs and dictates the specification and differentiation of HSCs during vertebrate embryogenesis (Lu *et al.*, 2013), and it has been shown that, in HSPCs, IRF7 is dispensable for haematopoiesis under steady state-conditions, whereas its deficiency significantly enhances HSPC regeneration and long-term repopulation of HSCs under stress (Chen *et al.*, 2021). In this last work, a decrease in *Irf7* expression, instead of a raise, is observed after treating mice with 5-fluorouracil (5-FU), a chemotherapeutic agent that selectively kills proliferating hematopoietic cells. This apparent discrepancy with our results may be explained by the completely different stress model they use, as 5-FU could be activating proliferation pathways in HSPCs rather than promoting differentiation towards the myeloid lineage, as described for infections. In addition, an *in vivo* model of treatment with a bacterial PAMP exhibited an increase of IRF7 expression in HSCs and an MPP expansion in the bone marrow, which was abrogated in IRF3/IRF7 double-deficient mice (Kobayashi *et al.*, 2015). Overall, these data suggest the existence of an IRF8-independent pathway for monocyte/macrophage differentiation driven by IRF7 in collaboration with PU.1 and C/EBPβ in response to TLR2 signalling.

We then sought for pathways upstream the upregulated transcription factors, demonstrating that TBK1 and mTOR are activated by TLR2 signalling. It has been described that mTOR intrinsically regulates myeloid development, as it promotes monocyte production by inhibiting STAT5 (a negative regulator of IRF8) and the expression of the M-CSF receptor (Yáñez *et al.*, 2015; Zhao *et al.*, 2018). Accordingly, TLR7 signalling in CMPs also induces mTOR-dependent myeloid differentiation (Buechler *et al.*, 2016). Interestingly, we found that mTOR activation is mediated by PI3K and, to a lesser extent, also by TBK1, according with a previous report showing that TBK1 directly activates mTOR in response to TLR3 and TLR4 ligands in macrophages (Bodur *et al.*, 2018). Supporting the involvement of TBK1 and mTOR in direct differentiation, macrophage generation from HSPCs in response to Pam_3CSK_4 is significantly decreased when these pathways are blocked by using their respective inhibitors, amlexanox and rapamycin. Both inhibitors were also able to decrease the upregulation of C/EBPβ and IRF7 at the mRNA and protein level.

TBK1 is known to be activated downstream of TRIF-dependent pathways (Nilsen *et al.*, 2015), but we have here demonstrated that TRIF is not involved in myeloid differentiation by TLR2 signalling. However, the TBK1 activation found is in line with

previous reports showing that TBK1 can be activated by TLR ligands that signal via MyD88, including TLR2 (Clark *et al.*, 2011; Oosenbrug *et al.*, 2020). Subsequently, we demonstrated that IRF7 upregulation is mediated by TBK1, as has been extensively described in several cell types (Jefferies, 2019). Interestingly, rapamycin also inhibits IRF7 activation, clearly indicating that IRF7 is a downstream target of mTOR signalling. This result is in line with Schmitz *et al.* (Schmitz *et al.*, 2008), who showed that TLRs activate mTOR via PI3K/Akt and demonstrate that mTOR physically associates with the MyD88 scaffold protein to allow activation of IRF5 and IRF7. Similarly, IRF7 was reported to be a downstream target of mTOR signalling in angiomyolipoma (Makovski *et al.*, 2014) and HSPCs (Chen *et al.*, 2021). On the other hand, we found that C/EBPβ is a downstream target of mTOR signalling, as it had been previously reported (Kaneda *et al.*, 2016; Sato *et al.*, 2020). mTOR controls multiple cellular processes, including protein translation, cell growth and metabolism. Moreover, mTOR signalling is a crucial positive determinant of myelopoiesis under both, homeostatic conditions and during an infection (Abdel-Nour *et al.*, 2014). Mechanistically, our results assign mTOR a role in HSPC differentiation in response to TLR2 signalling: directly activating transcription factors involved in myeloid differentiation.

In our model of HSPC differentiation in response to Pam_3CSK_4, we demonstrate NF-κB-dependent IL-6 production. Thus, our data agree with previous studies demonstrating that IL-6 production by HSPCs in response to TLRs may induce indirect myeloid differentiation (Zhao *et al.*, 2014; Sasaki *et al.*, 2021). However, we demonstrate that a direct differentiation programme is induced in parallel, as amlexanox and rapamycin can significantly inhibit differentiation without affecting IL-6 production. In addition, the kinetics of C/EBPβ and IL-6 induction discard IL-6 as responsible for early C/EBPβ expression downstream TLR2 signalling in HSPCs, as they are both simultaneously upregulated upon Pam_3CSK_4 stimulation. In this context, Buechler *et al.* indicate that type I IFN amplifies TLR7-driven myeloid development from CMPs (Buechler *et al.*, 2016). However, our data indicate that TLR2-mediated myeloid differentiation is not amplified by IFN-β. Moreover, our results exclude the possibility of type I IFN mediating the effect of IRF7 on HSPC differentiation, as amlexanox inhibits IRF7 upregulation and differentiation but not *Ifnb* upregulation. Further studies will be required to define IRF7 target genes when acting in collaboration with PU.1 and C/EBPβ in HSPCs to drive macrophage development in response to TLR2 signalling.

On the basis of the above-described findings, we propose the model depicted in Figure 21 for the molecular mechanisms taking place upon TLR2 signalling in HSPCs that induce direct and indirect macrophage differentiation. In conclusion, this study reveals that the TLR2 ligand induces direct myeloid differentiation of HSPCs. This differentiation is dependent on MyD88-mediated signalling, which activates TBK1 and mTOR promoting PU.1, C/EBPβ and IRF7-dependent macrophage differentiation. Understanding the biology of emergency myelopoiesis induced by TLRs may aid in devising new therapeutic strategies to boost innate immune cell replenishment during infection and to induce terminal differentiation of myeloid leukemic cells.

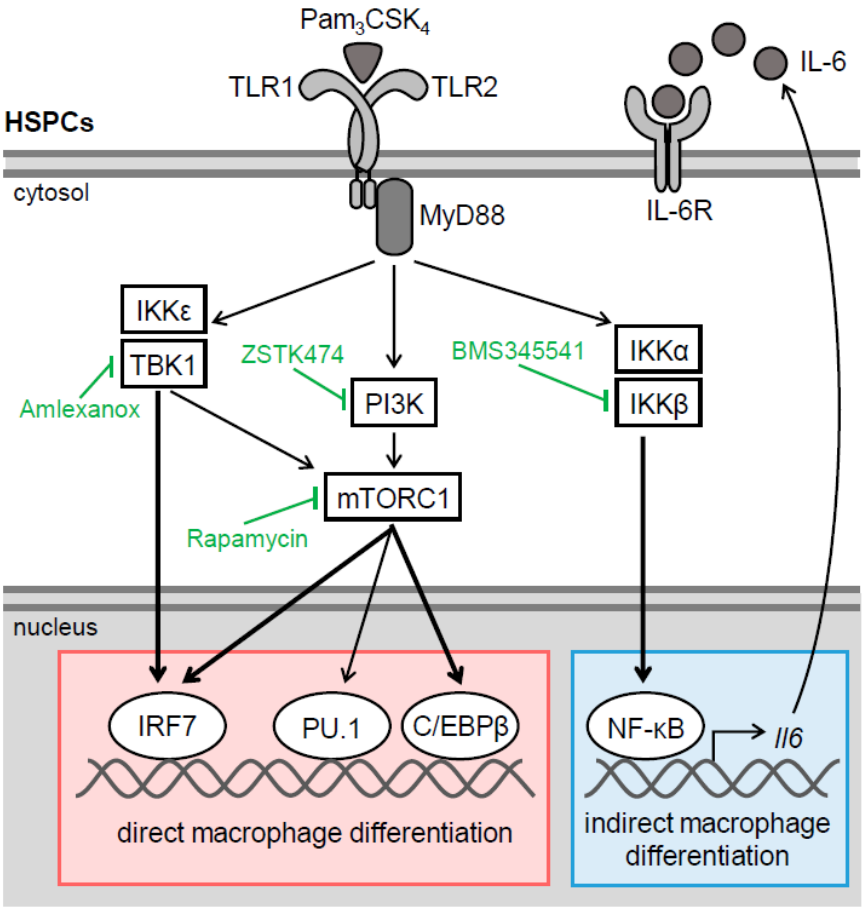

FIGURE 21. Schematic representation of the TLR2-induced signalling pathways that drive myeloid differentiation in HSPCs. MyD88 is recruited upon Pam3CSK4 binding to TLR1/TLR2, activating noncanonical IKKs (TBK1, IKKε) and the PI3K/mTORC1 pathway. Besides inducing IRF7, the TBK1 kinase can directly activate the mTORC1 complex. This results in the expression of transcription factors IRF7, PU.1 and C/EBPβ, which drive direct macrophage differentiation. In addition, TLR2 signalling also activates classical IKKs (IKKα, IKKβ), inducing NF-κB activity, which mediates the release of IL-6 (among other cytokines), which in turn can act in an autocrine/paracrine manner inducing macrophage differentiation. The level at which the inhibitors used act is indicated.

CHAPTER 2

Studying trained immunity in a mouse model of *C. albicans* vaccination

1. Establishment of the vaccination model

In order to delve into the trained immunity-induced mechanisms for protection against reinfection we took advantage of the PCA2 vaccination animal model previously established by the group of Cassone.

Therefore, 1.5 million of *C. albicans* PCA2 yeasts were intravenously (i.v.) injected into mice (Figure 22A) and 7 days later, once the fungus had been cleared from the kidneys (Figure 22B), mice were reinfected with a lethal dose of the virulent strain ATCC 26555, either intraperitoneally (i.p.) with 30 million yeasts to measure colony forming units (CFUs) in the kidneys and spleen, or i.v. with 1.5 million yeasts to assess survival. We measured a lower fungal burden in the kidneys and spleen of the PCA2-infected mice (Figure 22C) and observed higher survival upon the secondary infection (Figure 22D), indicating that the primary infection confers protection against a secondary lethal infection.

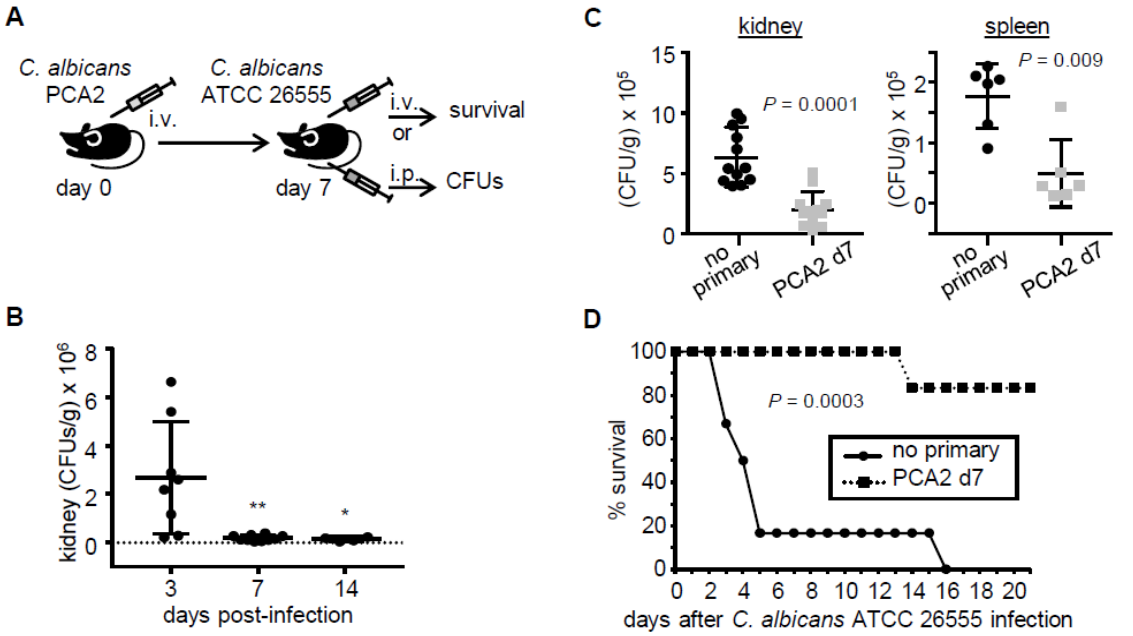

FIGURE 22. Vaccination model. (A) PCA2 vaccination animal model, 1.5 x 10^6 PCA2 yeasts were i.v. injected into mice and 7 days later mice were infected with a lethal dose of the virulent strain ATCC 26555 either i.p. with 3 x 10^7 yeasts to measure CFUs in the kidneys and the spleen 4 days later, or i.v. with 1.5 x 10^6 yeasts to assess survival. **(B)** Fungal burden in the kidneys expressed as CFUs/g of tissue (n=8-12) during the PCA2 infection at the indicated timepoints. **(C)** Fungal burden in the kidneys and spleen expressed as CFUs/g of tissue (n=6-12) 4 days after the secondary infection. **(D)** Survival curves expressing the percentage of living mice at each timepoint post-secondary infection (n=8).

As 7 days is not enough time for a complete adaptive immune response to develop, we hypothesized that trained immunity must be taking place in order to confer protection to mice.

2. Analysis of immune responses in vaccinated mice

2.1. Characterization of mature cell populations in the bone marrow and spleen of vaccinated mice

We obtained bone marrow cells and splenocytes from previously PCA2-infected mice and measured their immune cell composition by flow cytometry. Higher numbers of monocytes and neutrophils were observed in both, the bone marrow and spleen, of mice 7 and 14 days after the PCA2 infection in comparison to uninfected mice, while almost no changes were detected for B cells, CD4 T cells or CD8 T cells, supporting the unique contribution of innate immune cells to conferring protection (Figure 23).

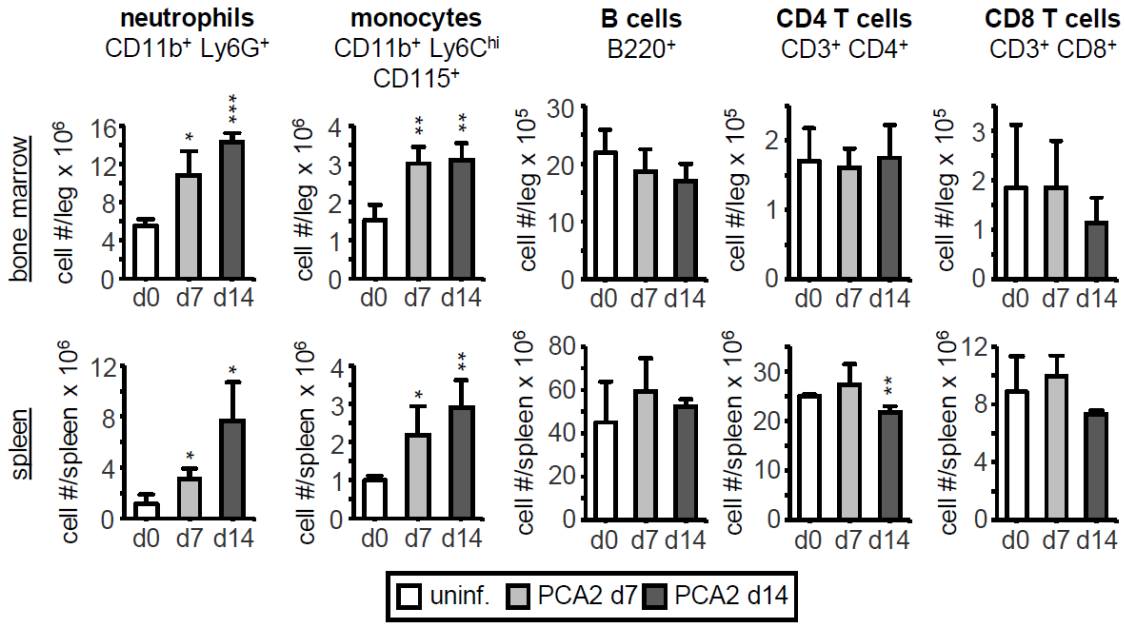

FIGURE 23. Myeloid and lymphoid cell numbers during PCA2 infection. Neutrophil, monocyte, B cell and CD4 and CD8 T cell numbers in bone marrow and spleen were assessed by flow cytometry at the indicated time points post-PCA2 infection. Bar graphs show means ± SDs of 3-5 mice. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cell numbers in the bone marrow or spleen of uninfected mice.

2.2. Measurement of cytokine production by bone marrow cells and splenocytes of vaccinated mice

We next analysed the ability of total red blood cell (RBC)-lysed bone marrow cells and splenocytes from 7-day PCA2-infected mice to produce proinflammatory cytokines after being *in vitro* stimulated with inactivated yeasts of *C. albicans* (Figure 24A). Higher TNF-α and IL-6 production was observed compared to cells from uninfected mice. Similarly, higher TNF-α and IL-6 levels were also observed by LPS or Pam_3CSK_4-stimulation *in vitro* of equal numbers of total cells from the bone marrow and the spleen, or equal volumes of blood, from mice 7 or 14 days after the primary infection in comparison to uninfected mice (Figure 24B). These data indicate that the PCA2 infection induces trained immunity in mice that lasts for, at least, 14 days.

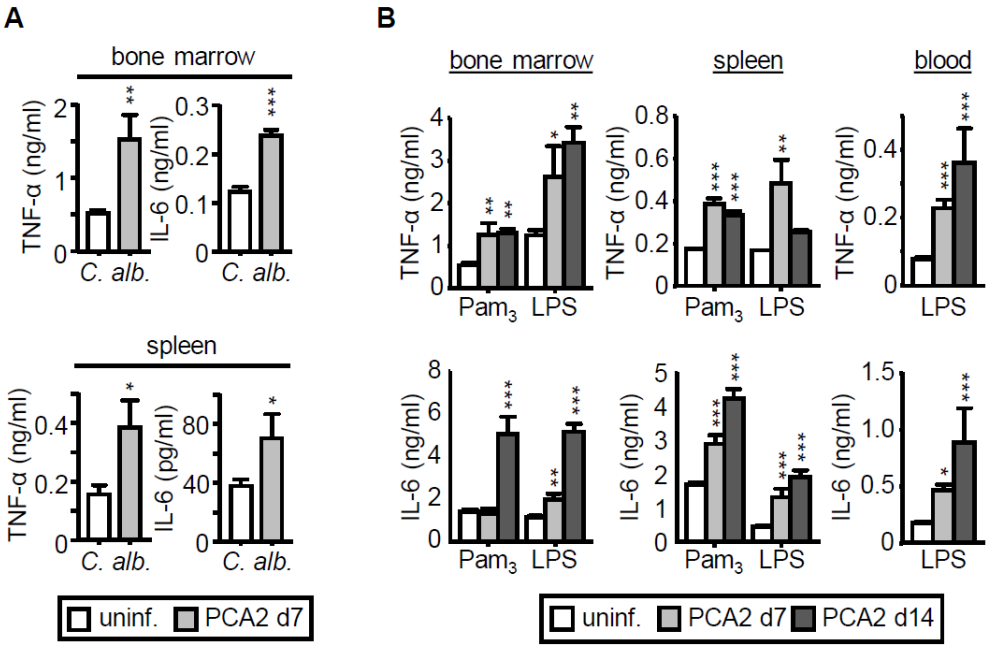

FIGURE 24. Cytokine production by total bone marrow, spleen and blood cells following PCA2 infection. (A) Same number of RBC-lysed bone marrow cells or splenocytes were cultured and stimulated with inactivated yeasts of *C. albicans* and TNF-α and IL-6 levels were assessed in 24-h supernatants by ELISA. **(B)** Same number of RBC-lysed bone marrow cells or splenocytes, or equal volumes of blood were cultured and stimulated with Pam_3CSK_4 or LPS and TNF-α and IL-6 levels were assessed in 24-h supernatants by ELISA. Triplicate samples were analysed in each assay. Bar graphs show means ± SDs of pooled data from 3 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cytokine production by cells of uninfected mice.

This enhanced production of cytokines by total organ cells might be explained by the higher number of monocytes and neutrophils present in the bone marrow and spleen of infected mice (Figure 23). However, monocyte function might also be programmed at the single-cell level, so that each individual cell would be producing a higher amount of cytokines (trained cells). To assess this, we cultured total RBC-lysed bone marrow or spleen cells from 7-day PCA2-infected mice and stimulated them with inactivated *C. albicans* yeasts (Figure 25A) or LPS (Figure 25B) for 6 h to measure intracellular cytokine production (TNF-α and IL-6) by flow cytometry.

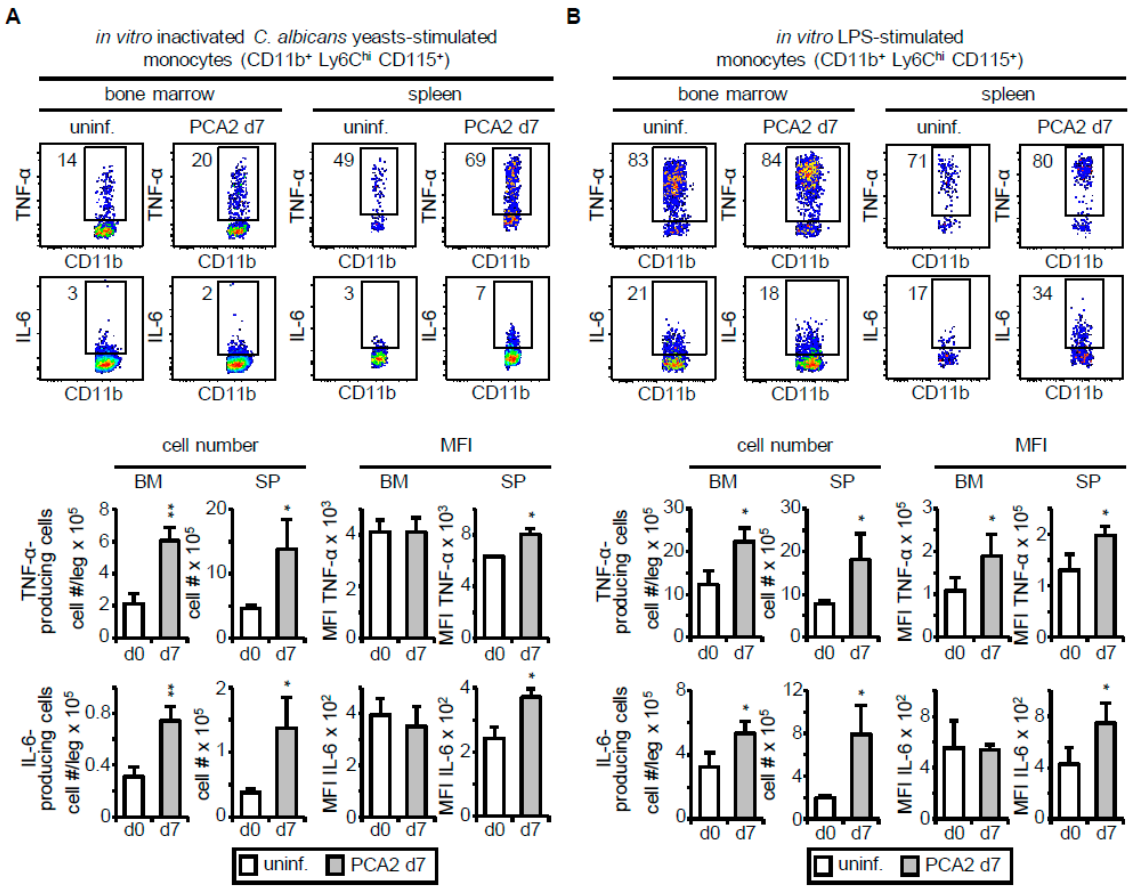

FIGURE 25. Cytokine production by *C. albicans*- or LPS-stimulated monocytes from the bone marrow and spleen of 7-day PCA2-infected mice. TNF-α and IL-6 were intracellularly detected by flow cytometry in pre-gated monocytes after stimulation of total RBC-lysed bone marrow cells or splenocytes from 7-day PCA2-infected mice with **(A)** inactivated *C. albicans* yeasts or **(B)** LPS for 6 h and brefeldin A for the final 4 h. Dot plots are representative and indicate the percentage of cytokine-producing cells. Bar graphs representing total cell numbers and MFI of cytokine-producing cells show means ± SDs of 3 mice. *P < 0.05 and **P < 0.01 with respect to cytokine production by monocytes of uninfected mice.

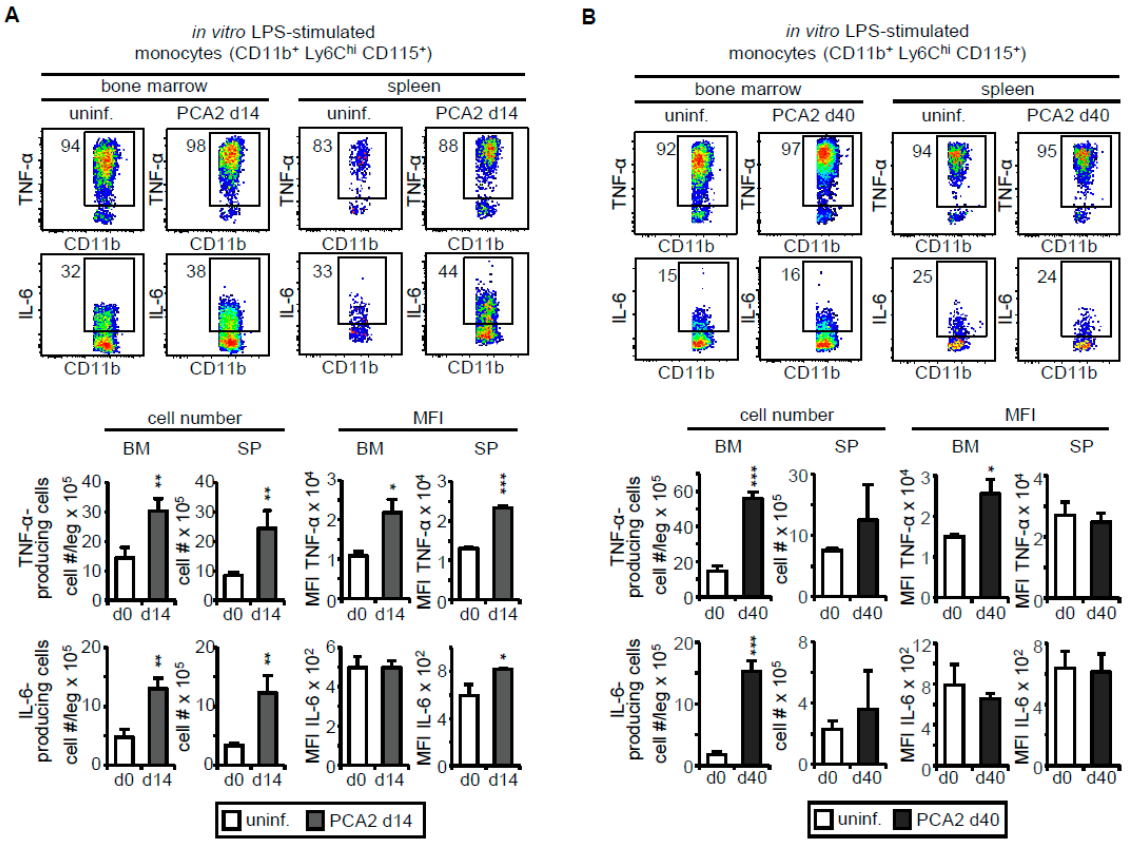

FIGURE 26. Cytokine production by LPS-stimulated monocytes from the bone marrow and spleen of 14-day and 40-day PCA2-infected mice. TNF-α and IL-6 were intracellularly detected by flow cytometry in pre-gated monocytes after stimulation of total RBC-lysed bone marrow cells or splenocytes from (A) 14-day or (B) 40-day PCA2-infected mice with LPS for 6 h and brefeldin A for the final 4 h. Dot plots are representative and indicate the percentage of cytokine-producing cells. Bar graphs representing total cell numbers and MFI of cytokine-producing cells show means ± SDs of 3 mice. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cytokine production by monocytes of uninfected mice.

We found a higher amount of total TNF-α- and IL-6-producing monocytes ($CD11b^+$ $Ly6C^{hi}$ $CD115^+$) in response to *C. albicans* yeasts (Figure 25A) or LPS (Figure 25B) in the bone marrow and spleen of 7-day PCA2-infected mice compared to uninfected mice. However, mean fluorescence intensity (MFI) of the cytokine-producing monocytes only showed an enhanced production of TNF-α and IL-6 by each individual cell in response to *C. albicans* yeasts in the spleen, but not in the bone marrow, compared to monocytes from uninfected mice (Figure 25A), suggesting that monocyte function is only programmed for a trained response in the spleen of the 7-day PCA2-infected mice. Nevertheless, LPS stimulation showed an enhanced MFI for TNF-α-producing monocytes in both organs, but only an enhanced MFI for IL-6-producing monocytes in the spleen, and not in the bone marrow, of 7-day PCA2-infected mice (Figure 25B). The

same pattern for cytokine production was found in LPS-stimulated monocytes from 14-day PCA2-infected mice (Figure 26A), while 40 days later training was lost in splenic monocytes but maintained in bone marrow monocytes (Figure 26B), indicating that some of these effects can last for at least 40 days.

Additionally, more TNF-α-producing neutrophils were found in response to LPS in both organs of the 7-day PCA2-infected mice (Figure 27). In contrast, IL-6-producing neutrophils were only higher in the bone marrow when compared to neutrophils from uninfected mice (Figure 27). Interestingly, only an increased programmed response for TNF-α production by neutrophils (measured as a higher MFI) in response to LPS was found in the spleen of the 7-day PCA2-infected mice (Figure 27). On the other hand, TNF-α and IL-6 production was not detected in T cells ($CD3^+$) or B cells ($B220^+$) in response to inactivated *C. albicans* yeasts or LPS in neither the bone marrow nor the spleen of 7-day PCA2-infected mice.

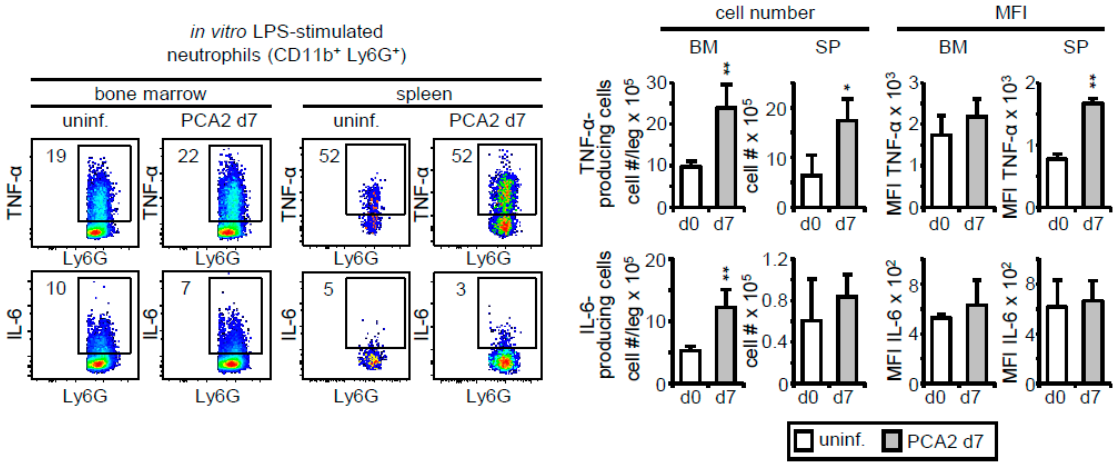

FIGURE 27. Cytokine production by LPS-stimulated neutrophils from the bone marrow and spleen of 7-day PCA2-infected mice. TNF-α and IL-6 were intracellularly detected by flow cytometry in pre-gated neutrophils after stimulation of total RBC-lysed bone marrow cells or splenocytes from 7-day PCA2-infected mice with LPS for 6 h and brefeldin A for the final 4 h. Dot plots are representative and indicate the percentage of cytokine-producing cells. Bar graphs representing total cell numbers and MFI of cytokine-producing cells show means ± SDs of 3 mice. *P < 0.05 and **P < 0.01 with respect to cytokine production by neutrophils of uninfected mice.

These data show that although more cytokine-producing myeloid cells are found in both, the bone marrow and spleen, myeloid cell programming of trained responses is mainly occurring in the spleen of 7-day PCA2-infected mice.

2.3. LPS-induced septic shock in vaccinated mice

To further confirm that the PCA2 infection confers a trained immunity phenotype to mice, 7 or 14 days after the primary infection, mice were i.p. injected with LPS to induce septic shock (Figure 28A). We observed that mice that had received the primary infection died faster than non-infected mice (Figure 28B), even by administering an increased LPS dose 14 days after the primary infection (Figure 28C).

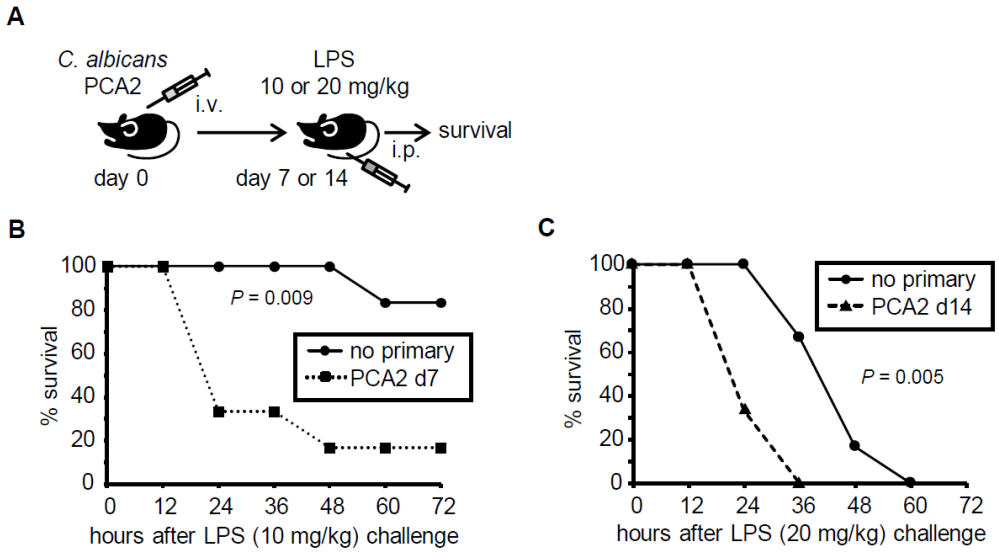

FIGURE 28. LPS-induced septic shock in PCA2-infected mice. (A) Septic shock model induced by an i.p. injection of LPS to mice 7 or 14 days after the PCA2 infection. (B) Mice survival percentage of mice that were induced a septic shock 7 days after being infected with PCA2 (n=6) (C) Mice survival percentage of mice that were induced a septic shock 14 days after being infected with PCA2 (n=6).

2.4. Measurement of fungicidal activity of bone marrow cells and splenocytes of vaccinated mice

We also measured the fungicidal ability of RBC-lysed total bone marrow cells and splenocytes from 7-day PCA2-infected mice. There were no differences in the candidacidal activity of bone marrow cells from mice 7 days after the primary infection in comparison to bone marrow cells from uninfected mice (Figure 29). However, spleen cells from vaccinated mice displayed an enhanced ability to kill *C. albicans* compared to splenocytes from control mice.

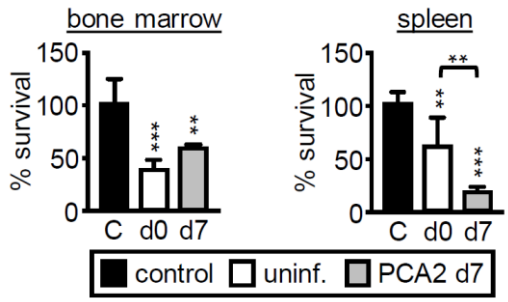

FIGURE 29. Fungicidal activity of RBC-lysed bone marrow cells and splenocytes of 7-day PCA2-infected mice. RBC-lysed bone marrow cells or splenocytes were challenged with viable PCA2 yeasts at a 25:1 or 50:1 ratio (murine cell:yeast), respectively, for 3 h. As control, *C. albicans* yeasts were cultured without murine cells. CFUs were counted from diluted samples plated on Sabouraud dextrose agar and survival percentages were determined as: % survival = [(sample CFUs at t=3 h) / (control CFUs at t=3 h)] x 100. Triplicate samples were analysed in each assay. Bar graphs show means ± SDs of pooled data from 3 independent experiments. **P < 0.01 and ***P < 0.001 with respect to control survival (unless otherwise indicated).

2.5. Assessment of the protective role of bone marrow cells and splenocytes of vaccinated mice against a lethal infection

In order to find out whether the protective response against the secondary infection is localized in the bone marrow or in the spleen of the 7-day PCA2-infected mice, total RBC-lysed bone marrow or spleen cells from uninfected or 7-day PCA2-infected mice were adoptively transferred into recipient mice. After 3 days, mice were i.p. infected with the virulent strain ATCC 26555 and fungal burden was measured in the kidneys at day 4 post-secondary infection (Figure 30A). Mice infected with the virulent strain ATCC 26555 that had not been transferred were used as controls.

We found that the adoptive transfer of bone marrow or spleen cells from uninfected mice did not influence fungal burden in the kidneys (Figure 30B). Fungal burden was only significantly diminished by the adoptive transfer of splenocytes from 7-day PCA2-infected mice (Figure 30B). However, although non-significant, bone marrow cell transfer from 7-day PCA2-infected mice tended to lower fungal burden in the kidneys in comparison to bone marrow from uninfected mice (Figure 30B). These data are consistent with the enhanced killing ability observed in the spleen (Figure 29) and the higher number of monocytes (Figure 25) and neutrophils (Figure 27) trained for proinflammatory cytokine production present in the spleen 7 days after the primary infection.

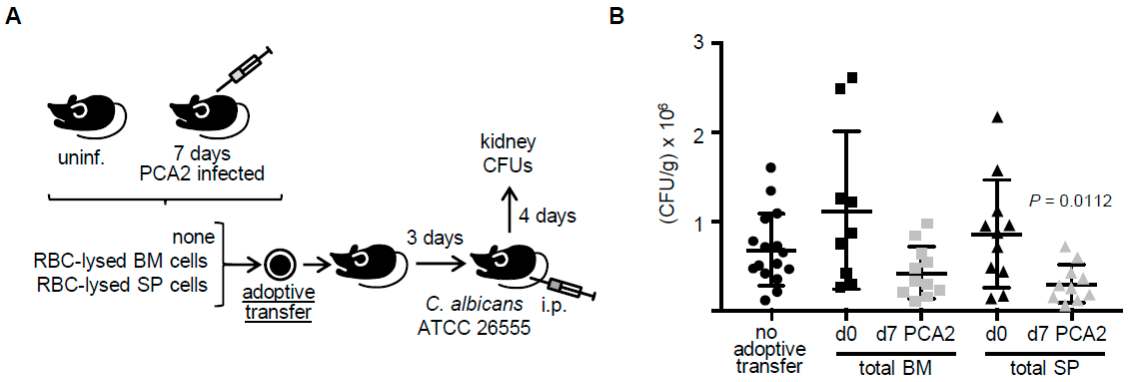

FIGURE 30. Fungal burden in the kidneys of mice adoptively transferred with RBC-lysed bone marrow cells or splenocytes following infection. (A) 3 x 10^7 RBC-lysed bone marrow cells or splenocytes were obtained from uninfected or 7-day PCA2-infected mice and adoptively transferred to uninfected mice. 3 days later mice were i.p. infected with 3 x 10^7 ATCC 26555 yeasts and CFUs were measured in the kidneys after 4 additional days. Non-adoptively transferred mice served as controls. **(B)** Fungal burden in the kidneys expressed as CFUs/g of tissue (n=9-16) 4 days after the ATCC 26555 infection.

3. Analysis of HSPCs in vaccinated mice

3.1. Characterization of HSPC subsets in the bone marrow and spleen of vaccinated mice

The increase in neutrophil and monocyte numbers observed in the bone marrow upon the PCA2 infection (Figure 23) might be indicating an activation of myelopoiesis in hematopoietic stem and progenitor cells (HSPCs). Therefore, HSPC subsets were measured in the bone marrow during the PCA2 infection by flow cytometry (Figure 31A). Lin^- c-kit^+ $Sca\text{-}1^+$ (LKS^+) cells, and all their containing subpopulations: long-term-HSCs (LT-HSCs) and multipotent progenitors (MPP2, MPP3 and MPP4), were substantially increased in the bone marrow of mice 7 days after the PCA2 infection (Figure 31B). Meanwhile, cells within the classical flow-cytometric gates, common myeloid progenitor (CMP), megakaryocyte-erythrocyte progenitor (MEP) and granulocyte-monocyte progenitor (GMP), did not suffer significant numeric variations in the bone marrow during the PCA2 infection (Figure 31B).

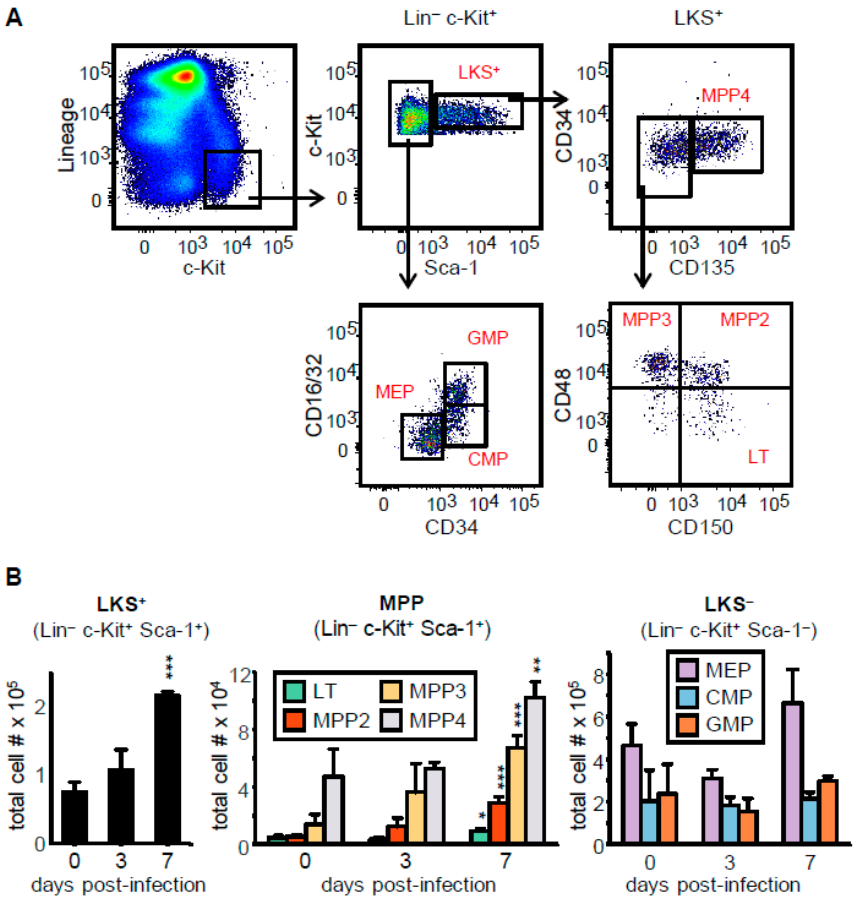

FIGURE 31. Characterization of HSPC subsets in the bone marrow during the PCA2 infection. (A) Flow cytometry gating of bone marrow cells for HSPC subset identification. **(B)** HSPC subset numbers in the bone marrow were assessed by flow cytometry at the indicated time points post-PCA2 infection. Bar graphs show means ± SDs of 3-5 mice. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cell numbers in the bone marrow of uninfected mice.

As the spleen could also be an extramedullary site for myeloid cell production, we also determined all HSPC subset numbers in this organ by flow cytometry gating spleen cells as depicted in Figure 31A. LKS^+ cells (containing LT-HSC, MPP2, MPP3 and MPP4 subpopulations) suffered a significant rise in the spleen of 7-day PCA2-infected mice, while dropped 14 days post-PCA2 infection (Figure 32A). Interestingly, higher numbers of MEPs, but not CMPs or GMPs, were observed in the spleen of the 7-day PCA2-infected mice (Figure 32A), which is consistent with previous observations using other infection or inflammation models in which erythropoiesis is shifted to the spleen during infection, probably to focalize myelopoiesis in the bone marrow (Yang *et al.*, 2020). We also observed this rise of HSPCs in the spleen at day 7 post-infection by performing methylcellulose colony-forming assays (Figure 32B), in which the specific

medium used supplied with different growth factors allows progenitors to generate different isolated colonies. Depending on the type of colonies formed, we can identify what kind of progenitors they come from. We observed and increase in erythrocyte, monocyte and the most undifferentiated granulocyte-erythrocyte-monocyte-megakaryocyte progenitors (Figure 32B), which is in line with our flow cytometry results.

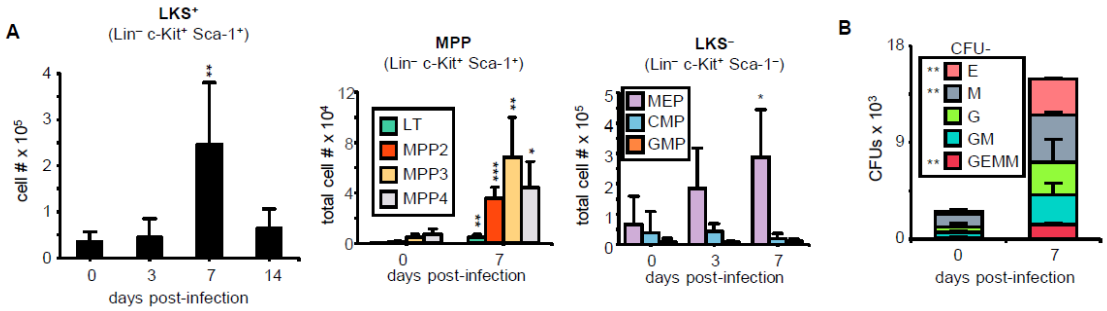

FIGURE 32. Characterization of HSPC subsets in the spleen during the PCA2 infection. **(A)** HSPC subset numbers in the spleen were assessed by flow cytometry at the indicated time points post-PCA2 infection. **(B)** 7 x 10^5 splenocytes were plated in methylcellulose medium and colonies were counted after 7 days of culture (E – erythrocyte, M – monocyte, G – granulocyte, GM – granulocyte + monocyte, GEMM – granulocyte + erythrocyte + monocyte + megakaryocyte). Bar graphs show means ± SDs of 3-5 mice. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cell numbers in the spleen of uninfected mice.

3.2. Analysis of the possible mobilization of HSPCs to the spleen in vaccinated mice

In order to find out whether infection recruits HSPCs to the spleen or expands them from the few residing there, CFSE-labelled donor HSPCs were adoptively transferred into 5-day PCA2-infected or uninfected recipient mice. Two days after transplantation, recipient spleen cells were enriched in donor cells by depletion of lymphocytes, were stained for c-kit and CD11b, and their presence in the spleen was measured by flow cytometry (Figure 33A). We found a higher number of $CFSE^+$ cells (there was around a 2-fold increase) in the spleen of the PCA2-infected mice compared to those in the uninfected mice, but this increase cannot completely be due to a higher proliferation of donor HSPCs in infected mice, as CFSE was only 1.15 times more diluted than in donor HSPCs in uninfected mice (Figure 33B). Moreover, we found more c-kit^- $CD11b^+$ cells in the spleen of the PCA2-infected mice in comparison to the uninfected mice, indicating that infection induces higher myeloid HSPC differentiation (Figure 33C). These data

suggest that the PCA2 infection enhances the ability of the spleen to recruit bone marrow HSPCs.

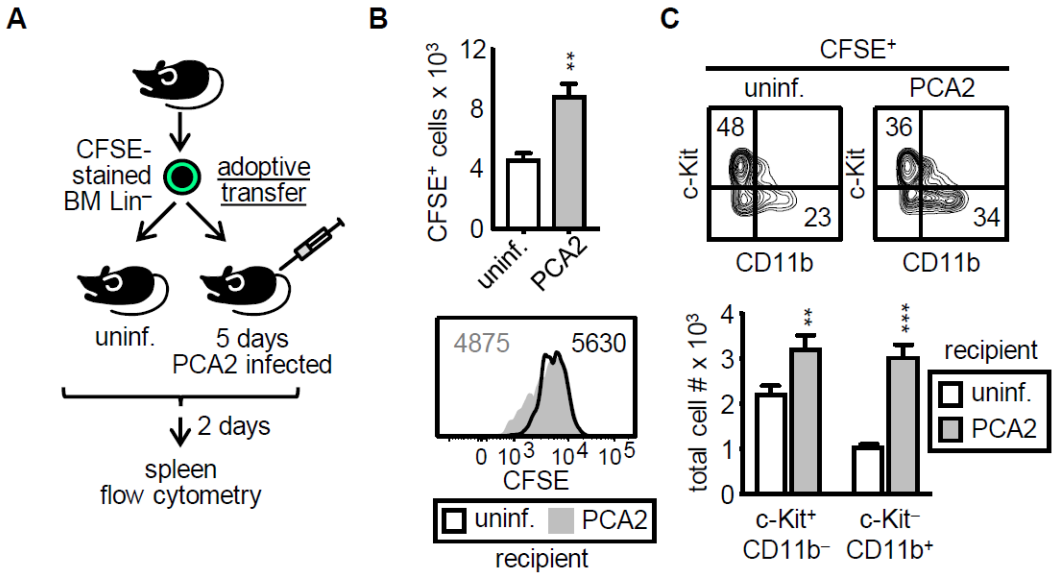

FIGURE 33. HSPC recruitment by the spleen during the PCA2 infection. (A) Purified bone marrow Lin⁻ cells from uninfected donor mice were CFSE-labelled and adoptively transferred into uninfected or 5-day PCA2-infected recipient mice and their presence in the spleen was measured 2 days later by flow cytometry. **(B)** Total CFSE⁺ cells in the spleens of recipient mice and CFSE dilution measured by flow cytometry. Numbers in histograms indicate CFSE MFI. **(C)** c-kit and CD11b expression in CFSE⁺ gated cells is shown and total cell number of c-kit⁺ CD11b⁻ and c-kit⁻ CD11b⁺ cells is represented. Dot plots and histograms are representative and bar graphs show means ± SDs of 3 independent experiments. **P < 0.01 and ***P < 0.001 with respect to cell numbers in the spleen of uninfected mice.

However, it is also possible that bone marrow HSPCs from PCA2-infected mice are better prepared to home the spleen. To address this, we adoptively transferred CFSE-labelled HSPCs, purified from either uninfected or 5-day PCA2-infected donor mice, into uninfected recipient mice. Two days later, donor cells were measured in the spleen of the recipient mice by flow cytometry, as above indicated (Figure 34A). A higher number of donor CFSE⁺ cells from PCA2-infected mice was found in the spleen of recipient mice, compared to donor CFSE⁺ cells from uninfected mice. Despite the fact that proliferation of donor HSPCs from PCA2-infected mice was higher than that of donor HSPCs from uninfected mice (CFSE was 2 times more diluted), this increase cannot completely account for the ~2.6-fold increase observed in cell numbers (Figure 34B). Furthermore, more c-kit⁻ CD11b⁺ cells were found in the spleen of recipient mice when donor HSPCs were isolated from PCA2-infected mice (Figure 34C). These data indicate that the PCA2 infection also enhances the ability of HSPCs to home the spleen.

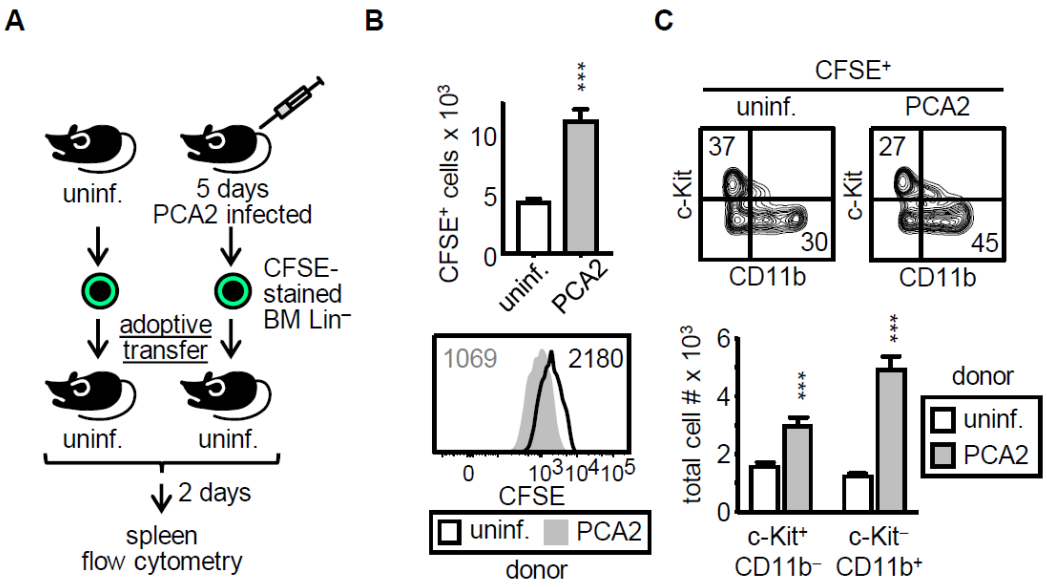

FIGURE 34. HSPC homing to the spleen during the PCA2 infection. (A) Purified bone marrow Lin⁻ cells from uninfected or 5-day PCA2-infected donor mice were CFSE-labelled and adoptively transferred into uninfected recipient mice and their presence in the spleen was measured 2 days later by flow cytometry. **(B)** Total CFSE⁺ cells in the spleens of recipient mice and CFSE dilution measured by flow cytometry. Numbers in histograms indicate CFSE MFI. **(C)** c-kit and CD11b expression in CFSE⁺ gated cells is shown and total cell number of c-kit⁺ CD11b⁻ and c-kit⁻ CD11b⁺ cells is represented. Dot plots and histograms are representative and bar graphs show means ± SDs of 3 independent experiments. **P < 0.01 and ***P < 0.001 with respect to cell numbers in the spleen of uninfected mice.

Altogether, these results indicate that the PCA2 infection recruits HSPCs to the spleen, although an expansion of resident HSPCs in the spleen cannot be discarded.

3.3. Analysis of HSPC proliferation, differentiation and subset characterization in response to a second challenge

We next addressed whether HSPCs from the PCA2-infected mice were better prepared to respond to a second challenge. First, we performed *in vitro* experiments in which CFSE-labelled Lin⁻ cells purified from the bone marrow of uninfected DsRed mice were cocultured with RBC-lysed splenocytes from C57BL/6 mice and challenged with inactivated *C. albicans* yeasts for 3 days to measure proliferation by analysing CFSE dilution, or 7 days to measure myeloid differentiation by analysing their expression of c-kit and CD11b (Figure 35A). We found that splenic HSPCs from 7-day PCA2-infected mice proliferated at a higher rate than bone marrow HSPCs from both, uninfected and 7-day PCA2-infected mice, which proliferated at a similar rate (Figure 35B). Consistently,

a significantly higher number of c-kit$^+$ CD11b$^-$ cells were found in splenic HSPC cultures in comparison to bone marrow HSPC cultures (Figure 35C). Besides, myeloid cell differentiation, measured as c-kit$^-$ CD11b$^+$ cell production was enhanced by the PCA2 infection in both, spleen and bone marrow HSPCs, compared to bone marrow HSPCs from uninfected mice (Figure 35C).

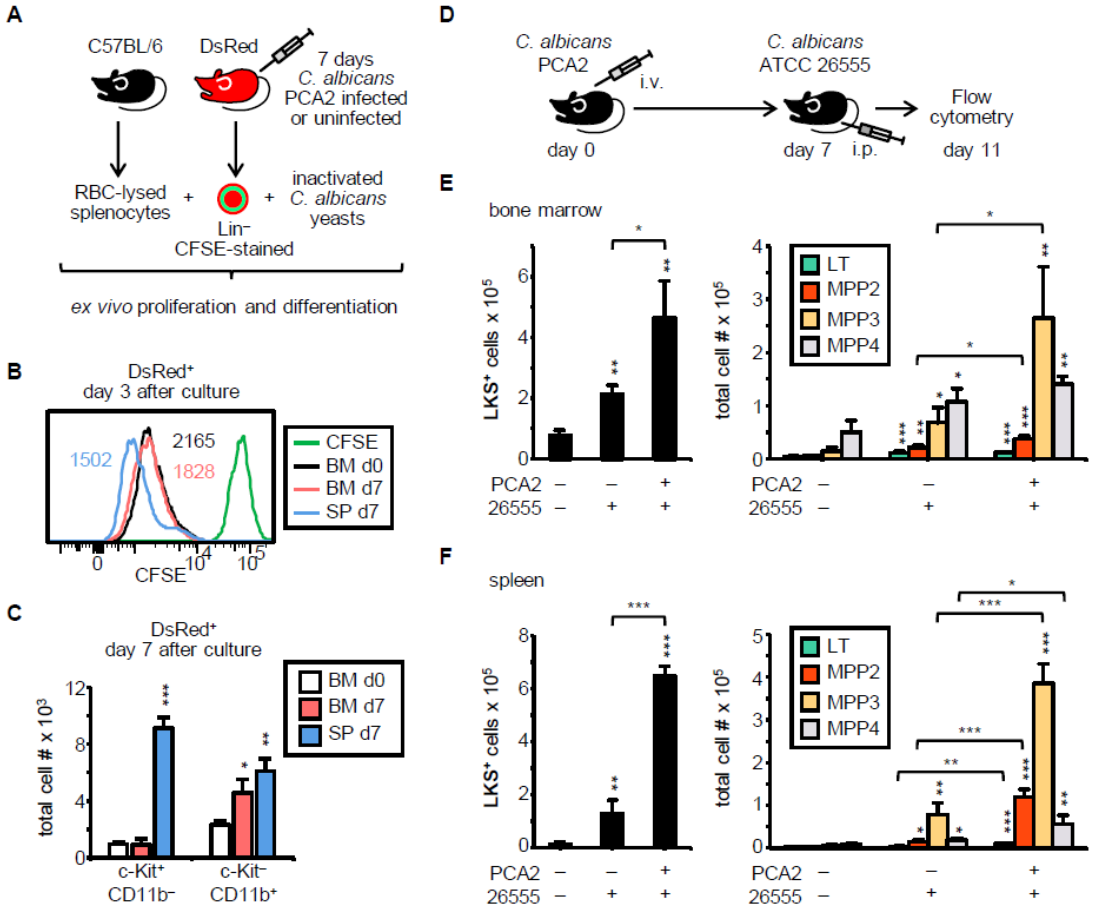

FIGURE 35. HSPC proliferation and differentiation and characterization of HSPC subsets in the bone marrow and spleen upon a second challenge. (A) CFSE-labelled Lin$^-$ cells purified from the bone marrow or the spleen of 7-day PCA2-infected or the bone marrow of uninfected DsRed mice were cocultured with RBC-lysed splenocytes from uninfected C57BL/6 mice and challenged with inactivated *C. albicans* yeasts for 3 days to measure proliferation or 7 days to measure myeloid differentiation. **(B)** CFSE dilution of DsRed$^+$ cells measured by flow cytometry. Numbers in histograms indicate CFSE MFI. **(C)** Total c-kit$^+$ CD11b$^-$ and c-kit$^-$ CD11b$^+$ cell numbers of DsRed$^+$ cells is represented. Histograms are representative and bar graphs show means ± SDs of 3 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cell numbers in uninfected bone marrow Lin$^-$ cell cultures. **(D)** 7-day PCA2-infected or uninfected mice were i.p. infected with 3 x 10^7 ATCC 26555 yeasts and 4 days later HSPC subset numbers in the bone marrow **(E)** and spleen **(F)** were assessed by flow cytometry. Bar graphs show means ± SDs of 3 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cell numbers in the bone marrow or spleen of uninfected mice (unless otherwise indicated).

We next examined the response of 7-day PCA2-infected mice to a secondary infection with the virulent strain of *C. albicans* (ATCC 26555) by measuring HSPC populations by flow cytometry 4 days after the reinfection (Figure 35D). This analysis shows a significant increase in the LKS^+ cell population in the bone marrow (Figure 35E) and, more markedly, in the spleen (Figure 35F) of mice that had previously received the primary infection in comparison to mice that had not received it. This difference is mainly due to an increase in the MPP2 and MPP3 subsets, which contain multipotent progenitors that are biased towards the myeloid lineage.

Taken together, our data demonstrate that the PCA2 infection primes HSPCs to enhance the production of myeloid cells in response to a reinfection.

3.4. Single-cell RNA sequencing of bone marrow and spleen HSPCs during vaccination and after reinfection

In order to better characterize transcriptomic changes in specific HSPC subsets (defined by their gene expression) during the PCA2 infection, we performed scRNA-seq of Lin^- $c\text{-}kit^+$ cells isolated (Figure 36A) from the bone marrow of non-infected mice (BMd0), the bone marrow of 24-h (BM24h) and the bone marrow and spleen of 7-day PCA2-infected mice (BMd7 and SPd7, respectively) using the 10X Genomics Chromium platform (Figure 36B). We also looked for their transcriptomic responses against a secondary infection by performing scRNA-seq in Lin^- $c\text{-}kit^+$ cells isolated from the bone marrow and spleen of mice reinfected with the virulent strain of *C. albicans* (ATCC 26555) for 24 h (BMd8 and SPd8, respectively) (Figure 36B).

HSPC populations were defined using the software cellHarmony aligned to previously reported reference mouse bone marrow progenitor cell populations (DePasquale *et al.*, 2019; Muench *et al.*, 2020) (Figure 36C). These populations included mixed-lineage and specifying cell states (Multi-Lin, IG2, MDP) and distinct monocytic (cMoP, MP, Mono) and granulocytic (proNeu) subsets. Of note, no additional clear cell populations were identified with an independent joint analysis using the software ICGS2 (Yáñez *et al.*, 2017; Olsson *et al.*, 2019; Muench *et al.*, 2020). Consistent with the mild infection caused by the PCA2 strain, subset composition does not change dramatically upon PCA2 infection, besides a greater abundance of erythroid progenitors in the spleen at day 7 post-infection (Figure 36C), which is in line with our previous findings (Figure

32). However, upon reinfection with the virulent strain ATCC 26555, there is a remodelling of almost every subset in both, the bone marrow and spleen (Figure 36C).

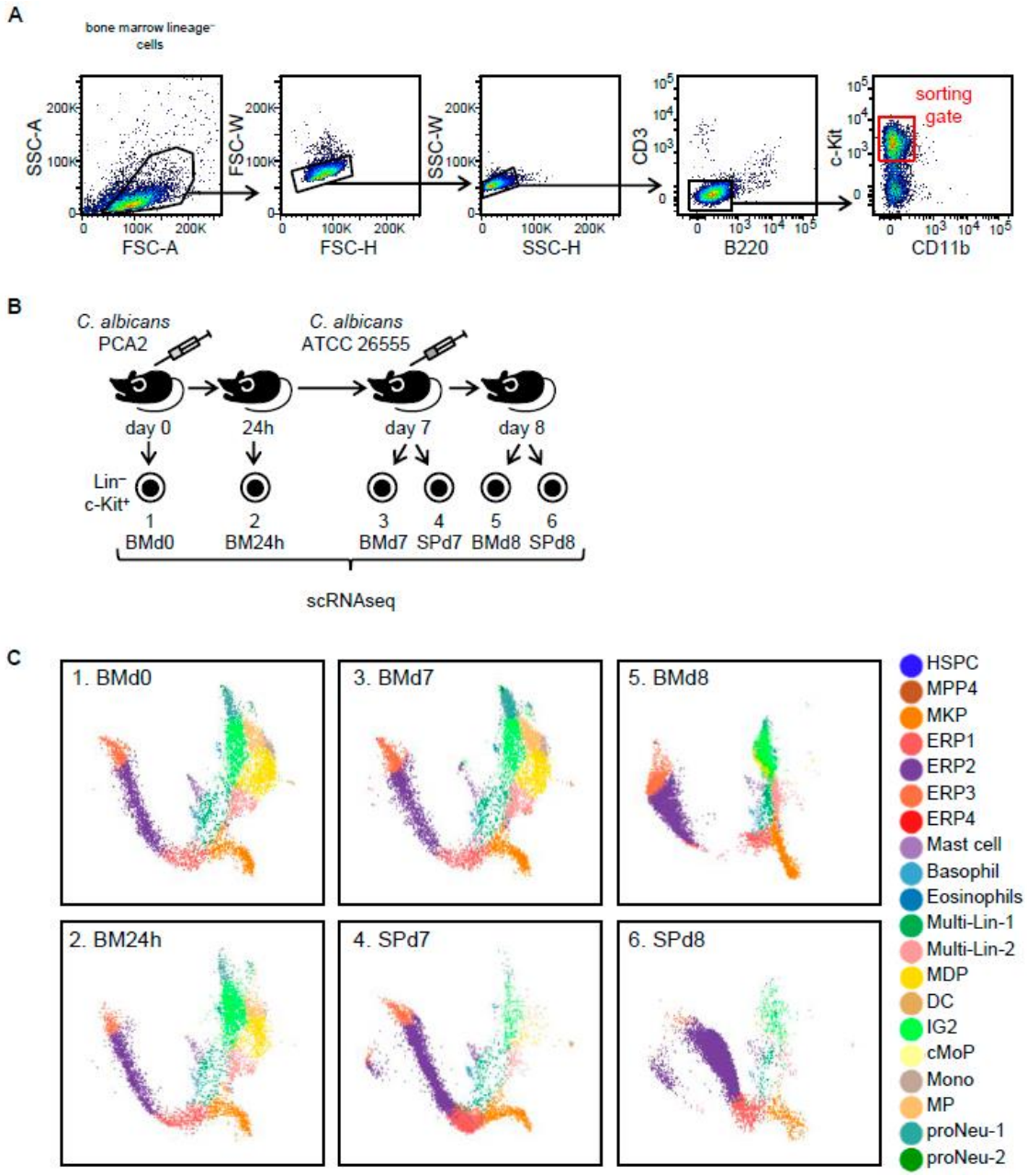

FIGURE 36. HSPC subset characterization by scRNA-seq. (A) Flow cytometry gating for sorting Lin⁻ c-kit⁺ cells. **(B)** Lin⁻ c-kit⁺ cells were isolated from the bone marrow of non-infected mice (BMd0), bone marrow of 24-h (BM24h) and bone marrow and spleen of 7-day (BMd7 and SPd7, respectively) PCA2-infected mice, or from the bone marrow and spleen 24 h after a secondary infection with the ATCC 26555 strain (BMd8 and SPd8, respectively). **(C)** UMAP projection of jointly-analysed HSPC subsets, defined using prior established reference cell populations in the software cellHarmony.

Next, using cellHarmony, we determined differentially expressed genes (DEGs) in each timepoint for the previously described progenitor subsets with monocytic potential (Multi-Lin-1, Multi-Lin-2, IG2, MDP and MP) and found a great number of DEGs at 24 h following PCA2 infection compared to uninfected animals in every subset, especially in the monocyte committed progenitors MDPs and MPs (Figure 37A). Upregulated DEGs mostly drop in every subset from bone marrow and spleen at day 7, once mice had recovered from the infection, while downregulated DEGs are mostly maintained or even increased at this timepoint (Figure 37A). The biggest differences in DEGs in every subset were found in response to reinfection in both, the bone marrow and spleen, compared to non-reinfected 7-day PCA2-infected mice (Figure 37A).

Thereafter, we looked for changes in biological pathways involved in the generation of trained immunity and found metabolic differences, such as changes in the ATP metabolic process and oxidative phosphorylation in every subset, as well as small changes in glycolysis and lactate metabolism in the IG2 and Multi-Lin-1 subsets, respectively, especially during the PCA2 infection (Figure 37B). Interestingly, we also found differences in pathways involved in epigenetic modifications (another hallmark for trained immunity) in almost every subset of the spleen, but not the bone marrow, of 7-day PCA2-infected mice, and also of the bone marrow and spleen after the reinfection with the ATCC 26555 strain (Figure 37B).

Myeloid differentiation was induced in the Multi-Lin-2 and MDP progenitors of the bone marrow 24 h after the PCA2 infection, and also in the spleens of 7-day PCA2-infected mice and after reinfection (Figure 37B). We specifically looked at transcription factors involved in myelopoiesis and found a decrease in the *Cebpa* gene expression, which is essential for steady-state myeloid development, in almost any comparison made during infection, except for bone marrow progenitors from reinfected animals (BMd8) in which *Cebpa* expression increases (Figure 38). *Cebpe*, an important transcription factor for late neutrophil differentiation, was also downregulated in IG2 progenitors 24 h post-PCA2 infection and after reinfection (Figure 38). This was accompanied by the downregulation of *Irf8*, an important transcription factor for monocyte differentiation, during the PCA2 infection and after the ATCC 26555 secondary infection, especially in MDPs and MPs (Figure 38). In contrast, *Cebpb*, a transcription factor previously described to be induced during emergency myelopoiesis (Hirai *et al.*, 2006), was

increased in all progenitor subsets (especially and more strongly in IG2, MDPs and MPs) from both organs after reinfection (Figure 38).

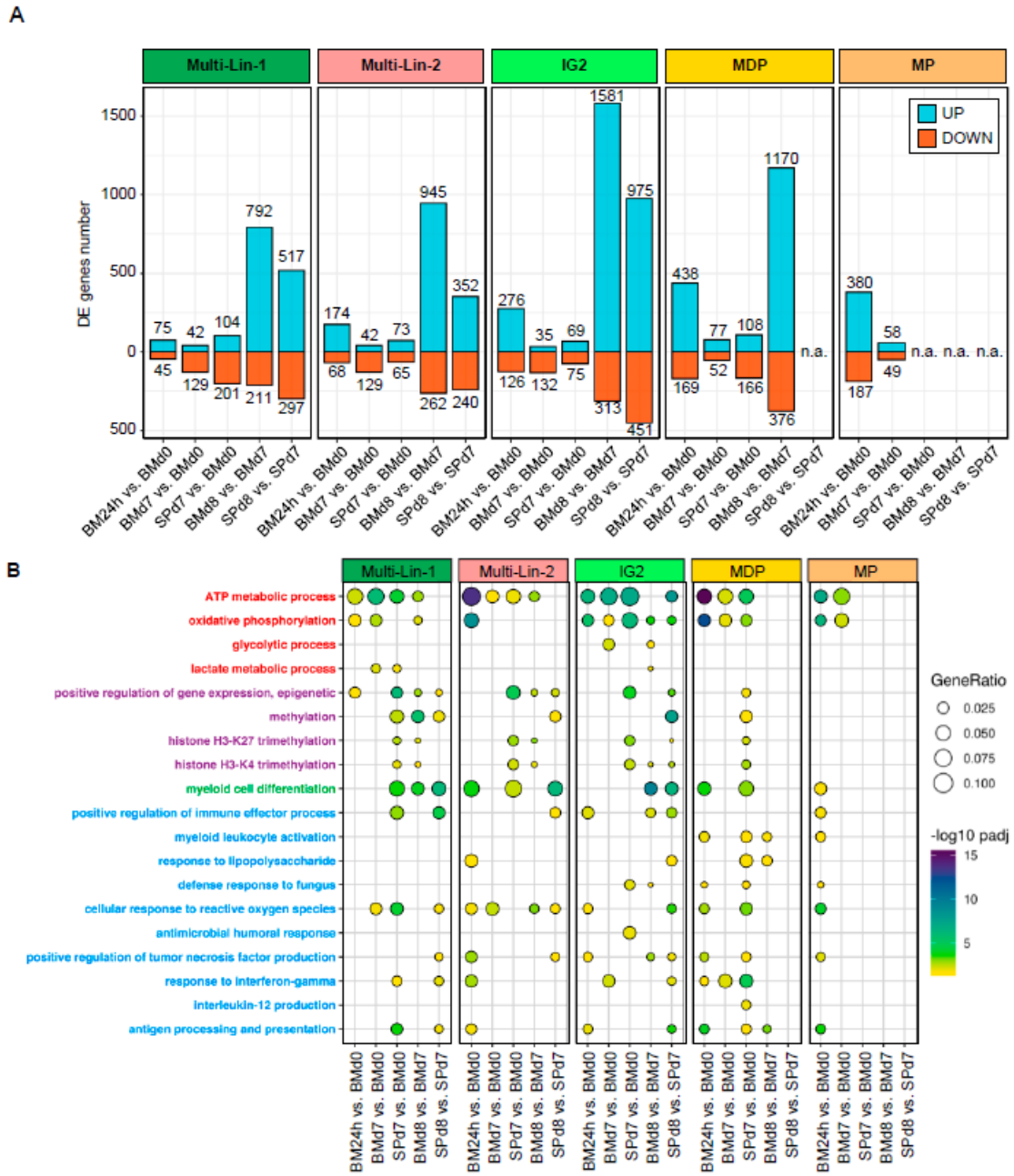

FIGURE 37. DEGs and biological pathway changes in HSPC subsets during vaccination and after reinfection found in the scRNA-seq dataset. (A) Number of upregulated (UP) and downregulated (DOWN) differentially expressed genes for the indicated comparisons in the cellHarmony-identified subpopulations with monocyte potential. n.a., not analysed. **(B)** GO terms significantly upregulated in the indicated HSPC subsets. n.a., not analysed.

We also found significant changes in some other biological pathways implicated in immune cell responses against infections, such as defence response to fungus, cellular response to reactive oxygen species, positive regulation of cytokine production or antigen processing and presentation, which suggest a more active role for these myeloid progenitors in fighting infections (Figure 37B).

Altogether, transcriptomic data at the single-cell level indicate that during the PCA2 infection there are metabolic and epigenetic changes taking place, accompanied by an activation of an emergency myelopoiesis response and immune defence traits. These data underscore HSPCs as important players in the protection observed against a secondary lethal infection.

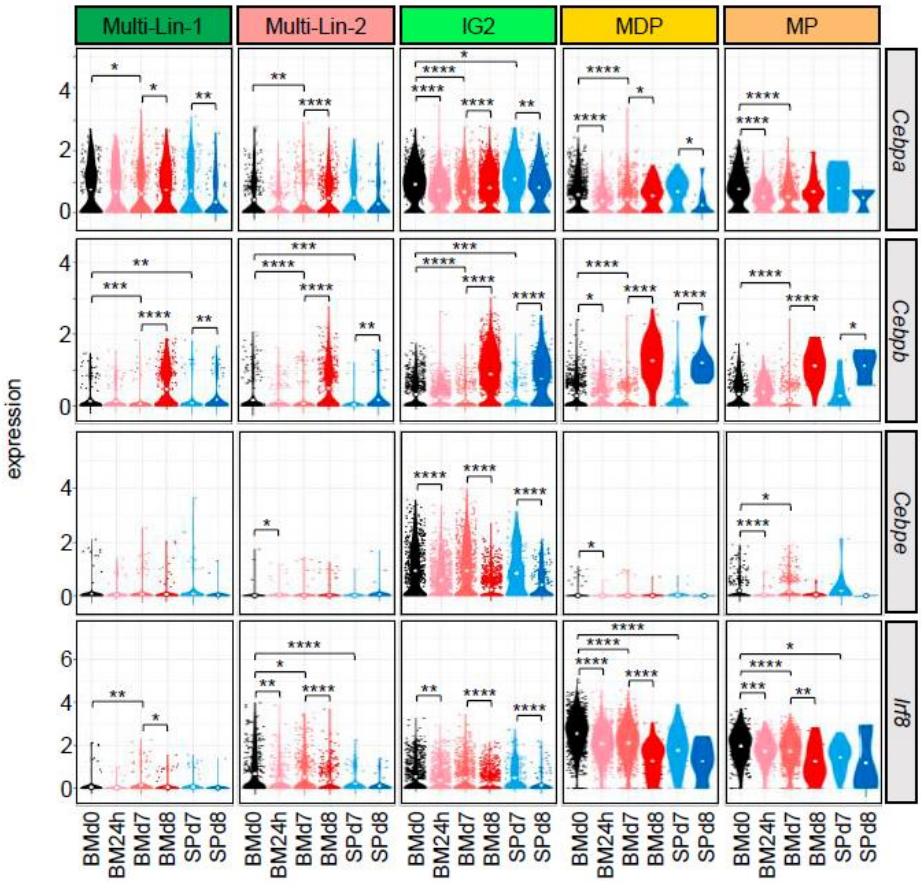

FIGURE 38. *Cebpa, Cebpb, Cebpe* and *Irf8* gene expression in HSPC subsets. Violin plots of the indicated genes in each HSPC subset. Statistical significance was assessed by the Wilcoxon signed-rank test as implemented in the ggpubr R package. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 for the indicated comparisons.

3.5. Measurement of cytokine production by bone marrow and spleen HSPCs in vaccinated mice

As biological pathway analysis showed that HSPCs produce proinflammatory cytokines (including TNF-α and IL-6) in response to the PCA2 infection and, especially, after the ATCC 26555 secondary infection (Figure 37B) and previous works have demonstrated that HSPCs can produce proinflammatory cytokines in response to microbial ligands (Zhao *et al.*, 2014), we decided to study whether cytokine production by HSPCs could also be programmed during the PCA2 infection and contribute to the enhanced proinflammatory cytokine production observed in the PCA2 infected mice.

Therefore, total bone marrow and spleen cells from 7-day PCA2-infected mice were cultured and stimulated with LPS to assess cytokine production (TNF-α and IL-6) by LKS^+ and LKS^- cells by intracellular flow cytometry (Figure 39A). More TNF-α- and IL-6-producing LKS^+ cells were found in the bone marrow of the 7-day PCA2-infected mice compared to the bone marrow of uninfected mice, while only IL-6 production is increased in LKS^- cells in the bone marrow (Figure 39A). Within the LKS^+ population, TNF-α is mainly produced by the MPP2, MPP3 and MPP4 subsets, while IL-6 is mostly produced by MPP3 and MPP4 (Figure 39B). However, few TNF-α- and IL-6-producing LKS^+ cells in response to LPS were found in the spleen of the 7-day PCA2-infected mice (Figure 39A). LPS stimulation showed a trained response (enhanced MFI) for TNF-α-producing LKS^+ cells and for IL-6-producing LKS^- cells in the bone marrow of the 7-day PCA2-infected mice compared to the uninfected mice (Figure 39A). This pattern is maintained in bone marrow HSPCs from 14-day (Figure 39C), and even 40-day (Figure 39D), PCA2-infected mice. These data indicate that HSPCs can be trained for cytokine production during a PCA2 infection, persisting for at least 40 days, and they can, therefore, contribute to the higher cytokine production observed in the total bone marrow.

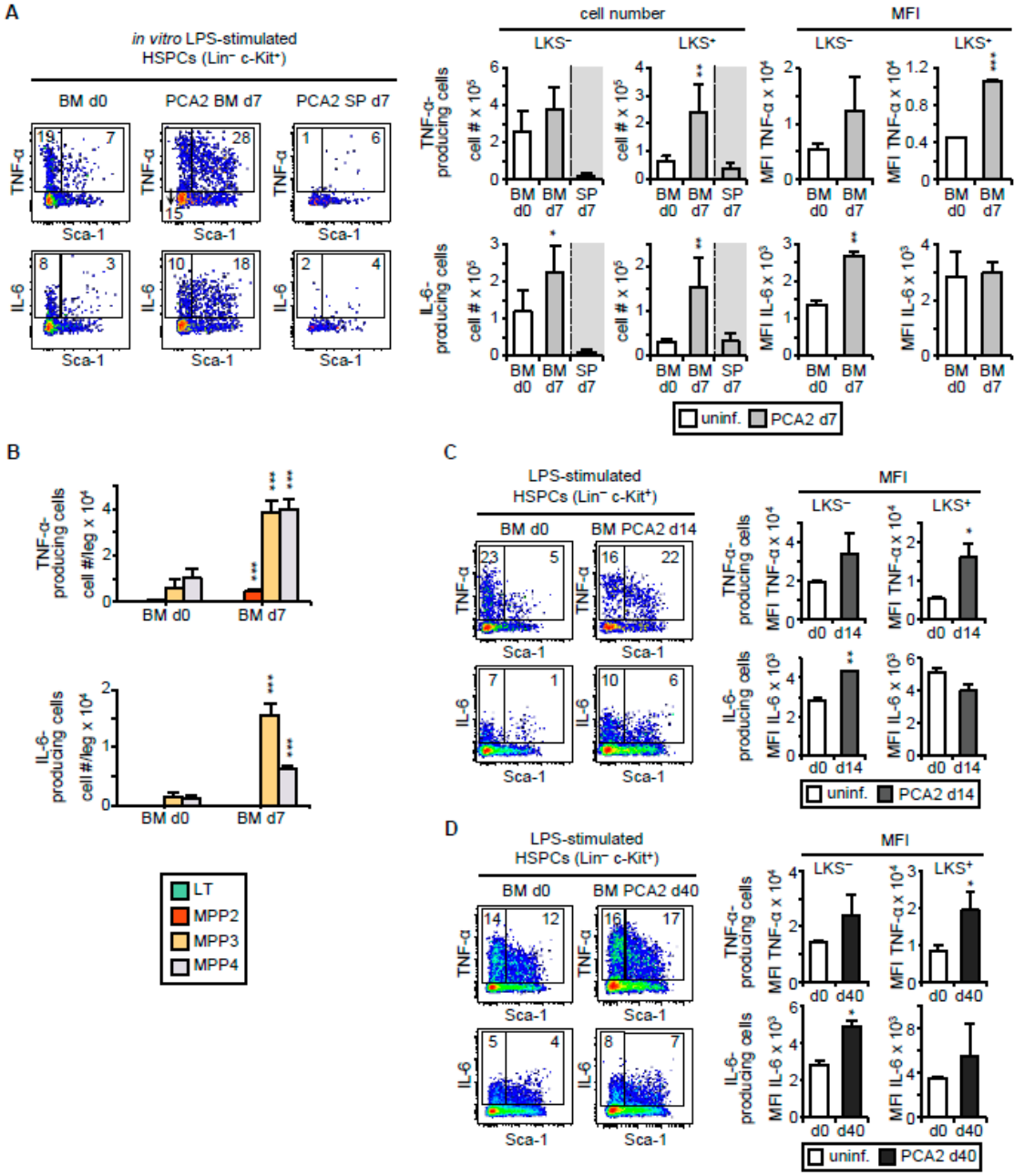

FIGURE 39. Cytokine production by LPS-stimulated HSPCs from the bone marrow and spleen of PCA2-infected mice. TNF-α and IL-6 were intracellularly detected by flow cytometry in pre-gated Lin⁻ c-kit⁺ cells after stimulation of total RBC-lysed bone marrow cells or splenocytes from 7 **(A, B)**, 14- **(C)** or 40-day **(D)** PCA2-infected mice with LPS for 6 h and brefeldin A for the final 4 h. Dot plots are representative and the percentage of cytokine-producing LKS⁺ and LKS⁻ cells is indicated. Bar graphs representing total cytokine-producing cell numbers and MFI of cytokine-producing cells show means ± SDs of 3 mice. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cytokine production by HSPCs of uninfected mice.

We next examined cytokine production by HSPCs from 7-day PCA2-infected or control mice 24 h post-infection with the virulent strain of *C. albicans* (ATCC 26555). Total bone marrow and spleen cells obtained from mice 24 h after the secondary infection were cultured and stimulated with PMA and ionomycin for 6 h to assess cytokine production (TNF-α and IL-6) by LKS^+ and LKS^- cells by intracellular flow cytometry (Figure 40), as described before (Luo *et al.*, 2021). More TNF-α- and IL-6-producing LKS^+ cells were found in the bone marrow, and even more in the spleen, of 7-day PCA2-infected mice compared to those present in the bone marrow of mice that had not received the primary infection (Figure 40). Within the TNF-α- and IL-6-producing LKS^+ cells, MFI was higher in the spleen than in the bone marrow of the PCA2-infected mice, indicating that, in response to a secondary infection, splenic HSPCs are better prepared to produce proinflammatory cytokines (Figure 40). However, the fact that cytokine production could be enhanced in bone marrow HSPCs and then be recruited to the spleen during the 24 h of the secondary infection cannot be ruled out.

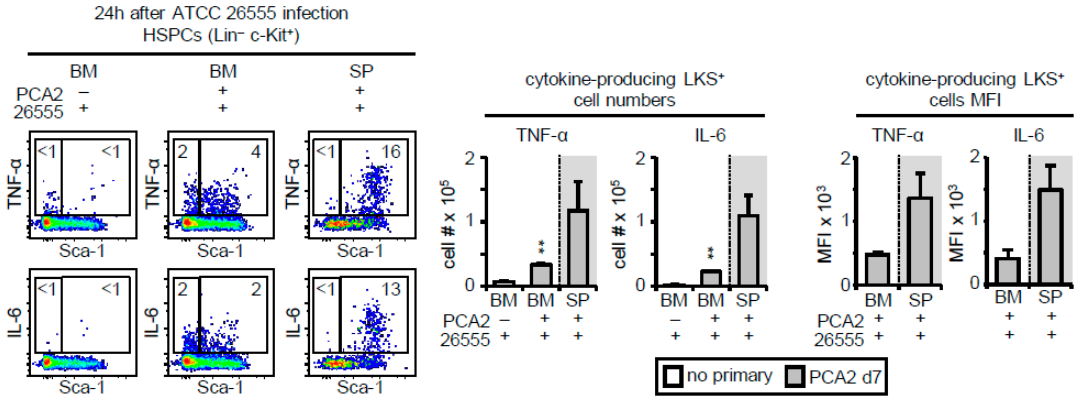

FIGURE 40. Cytokine production by HSPCs from the bone marrow and spleen of PCA2-infected or uninfected mice after ATCC 26555 infection. TNF-α and IL-6 were intracellularly detected by flow cytometry in pre-gated Lin^- $c\text{-}kit^+$ cells after stimulation of total RBC-lysed bone marrow cells or splenocytes from 7-day PCA2-infected or uninfected mice i.p. infected for 24 h with 3 x 10^7 ATCC 26555 yeasts and stimulated with PMA/ionomycin and brefeldin A for 6 h. Dot plots are representative and the percentage of cytokine-producing LKS^+ and LKS^- cells is indicated. Bar graphs representing total cytokine-producing cell numbers and MFI of cytokine-producing cells show means ± SDs of 3 mice. **P < 0.01 with respect to cytokine production by HSPCs of PCA2-uninfected mice.

3.6. Assessment of the possible protective role of HSPCs against a lethal infection

In order to look for a possible active role for trained HSPCs in protection against infection, we either depleted (c-kit$^-$) or enriched (c-kit$^+$) c-kit$^+$ cells from the bone marrow or the spleen of 7-day PCA2-infected mice (Figure 41A). These cell fractions were adoptively transferred into recipient mice and 3 days later mice were infected with the virulent strain of *C. albicans* ATCC 26555. To assess infection levels, fungal burden was measured in the kidneys 4 days after the secondary infection. Mice infected with the virulent strain that had not received a cell transfer were used as controls (Figure 41B).

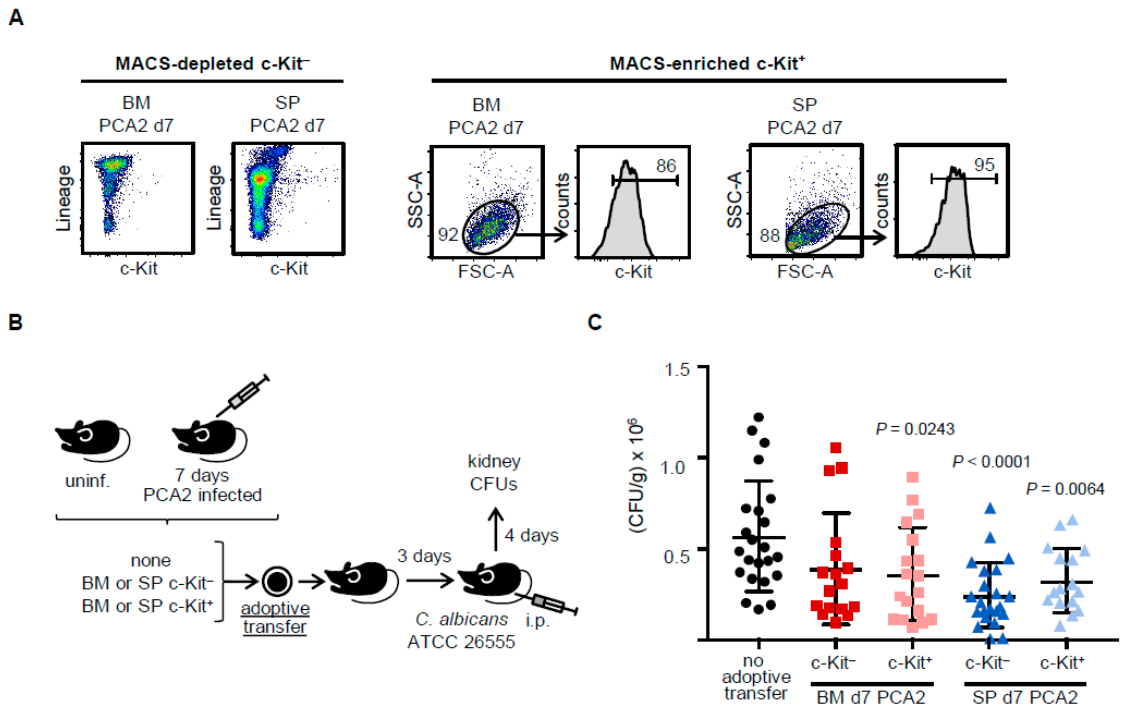

FIGURE 41. Fungal burden in the kidneys of mice adoptively transferred with c-kit$^-$ or c-kit$^+$ bone marrow or spleen cells following infection. (A) Purity of MACS-depleted c-kit$^-$ or MACS-enriched c-kit$^+$ cells from the bone marrow or the spleen of 7-day PCA2-infected mice for adoptive transfer experiments. **(B)** 5 x 10^6 c-kit$^+$ or 3 x 10^7 c-kit$^-$ cells were isolated from the bone marrow or spleen of 7-day PCA2-infected mice and adoptively transferred to uninfected mice. 3 days later mice were i.p. infected with 3 x 10^7 ATCC 26555 yeasts and CFUs were measured in the kidneys after 4 additional days. Non-adoptively transferred mice served as controls. **(C)** Fungal burden in the kidneys expressed as CFUs/g of tissue 4 days after the ATCC 26555 infection. Data shown is pooled from 2 independent experiments (n=8-12 per experiment).

Results show that only the adoptive transfer of c-kit$^-$ cells from the spleen, but not from the bone marrow, causes a significant fungal burden decrease in the kidneys after infection (Figure 41C). However, the adoptive transfer of the c-kit$^+$ fraction from both, the bone marrow, and especially from the spleen, significantly lowers fungal burden in the kidneys after infection (Figure 41C).

Taken as a whole, these results demonstrate the importance of programmed HSPCs during infection in protection against reinfection and highlight the relevance of spleen-recruited HSPCs to fight infection.

3.7. Phenotype of the HSPC-derived macrophages from vaccinated mice

In order to find out if myeloid cell function could be programmed during the PCA2 infection at the level of their upstream progenitors, HSPCs were isolated from the bone marrow of uninfected, 3-day or 7-day PCA2-infected mice or the spleen of 7-day PCA2-infected mice, and were differentiated *ex vivo* with M-CSF into macrophages to assess their ability to produce proinflammatory cytokines (Figure 42A).

Results show that HSPCs from the bone marrow of 3-day infected mice produce macrophages with an increased ability (trained response) to produce TNF-α and IL-6 in response to Pam_3CSK_4 and LPS, in comparison to control macrophages derived from HSPCs isolated from the bone marrow of uninfected mice (Figure 42B). In contrast, macrophages derived from HSPCs isolated from the bone marrow of 7-day infected mice produce macrophages with an unaltered or decreased ability (tolerized response) to produce said cytokines in response to Pam_3CSK_4 and LPS (Figure 42B). Interestingly, HSPCs purified from the spleen of 7-day PCA2-infected mice differentiate towards macrophages that display a trained phenotype (higher production of TNF-α and IL-6 than control macrophages) (Figure 42C).

Moreover, we also measured the fungicidal ability of these *ex vivo* M-CSF-derived macrophages and observed that macrophages derived from HSPCs isolated from both, the bone marrow and the spleen of 7-day PCA2-infected mice, display a similar ability to kill *C. albicans* than macrophages derived from HSPCs isolated from the bone marrow of uninfected mice (Figure 42D).

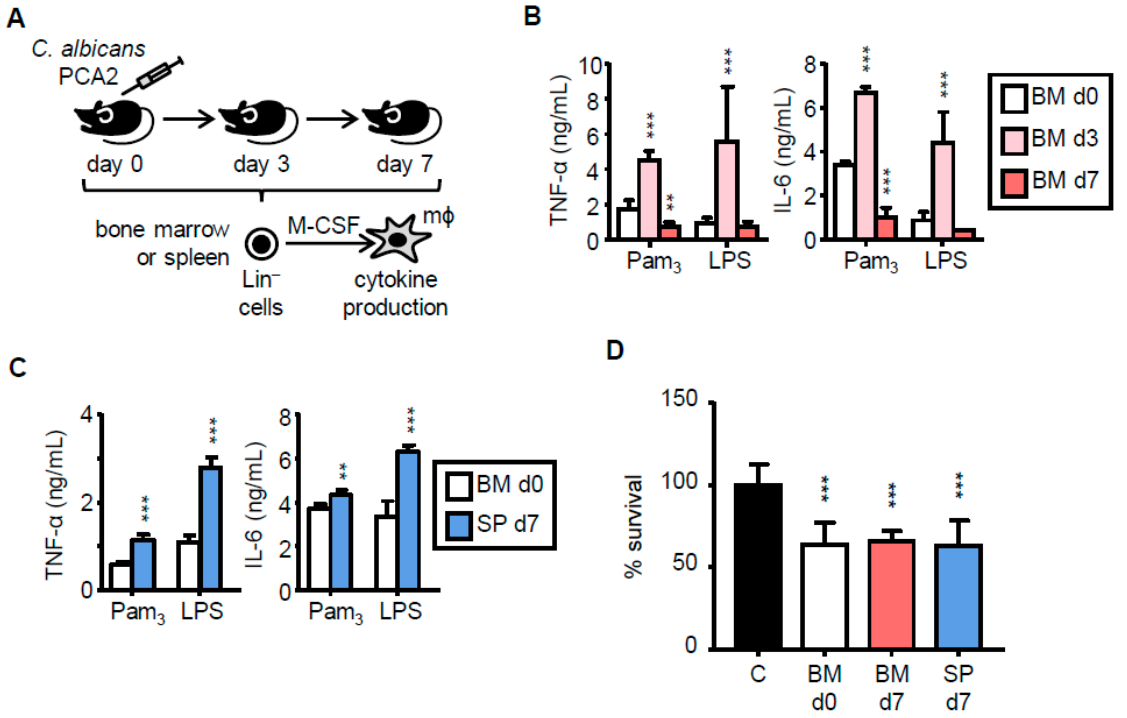

FIGURE 42. Cytokine production and fungicidal ability of *ex vivo*-differentiated macrophages. (A) Lin⁻ cells were isolated from the bone marrow or spleen of uninfected, 3-day or 7-day PCA2-infected mice and were differentiated *ex vivo* into macrophages with M-CSF for 7 days. Equal numbers of HSPC-derived macrophages from the bone marrow **(B)** or the spleen **(C)** were stimulated with Pam_3CSK_4 or LPS and TNF-α and IL-6 levels were assessed by ELISA in 24-h supernatants. **(D)** *Ex vivo*-differentiated macrophages were challenged with viable PCA2 yeasts at a 1:3 ratio (murine cell:yeast) for 1 h. As control, *C. albicans* yeasts were cultured without murine cells. CFUs were counted from diluted samples plated on Sabouraud dextrose agar and survival percentages were determined as: % survival = [(sample CFUs at t=1 h) / (control CFUs at t=1 h)] x 100. Triplicate samples were analysed in each assay. Bar graphs show means ± SDs of pooled data from 3 independent experiments. **P < 0.01 and ***P < 0.001 with respect to cytokine production by macrophages differentiated from Lin⁻ cells from uninfected mice (B, C) or to control survival (D).

Overall, these data suggest that HSPCs are programmed to produce trained macrophages in the bone marrow early during infection and are later recruited to the spleen to continue their production of trained macrophages. Meanwhile, newly appearing HSPCs in the bone marrow at these later timepoints produce macrophages with an unaltered or tolerized phenotype.

4. Mechanisms inducing HSPC reprogramming during vaccination

For dissecting the mechanisms by which the PCA2 infection induces a reprogramming of HSPCs, we sought for genes in the pathways involved in trained immunity in our scRNA-seq dataset. We found an upregulation of *Cbl* and *Akt1*, two genes implicated in the mTOR signalling pathway, in every subset from the bone marrow and spleen in response to the secondary infection (Figure 43). This signalling pathway is activated by the growth factor GM-CSF, the receptor of which, the *Csf2rb* gene, was found upregulated post-secondary infection in both organs, in every bone marrow subset 24 h post-PCA2 infection, and in the Multi-Lin-2, IG2 and MDP subsets from the bone marrow and the spleen of 7-day PCA2-infected mice (Figure 43), indicating that these progenitors are better prepared to respond to GM-CSF.

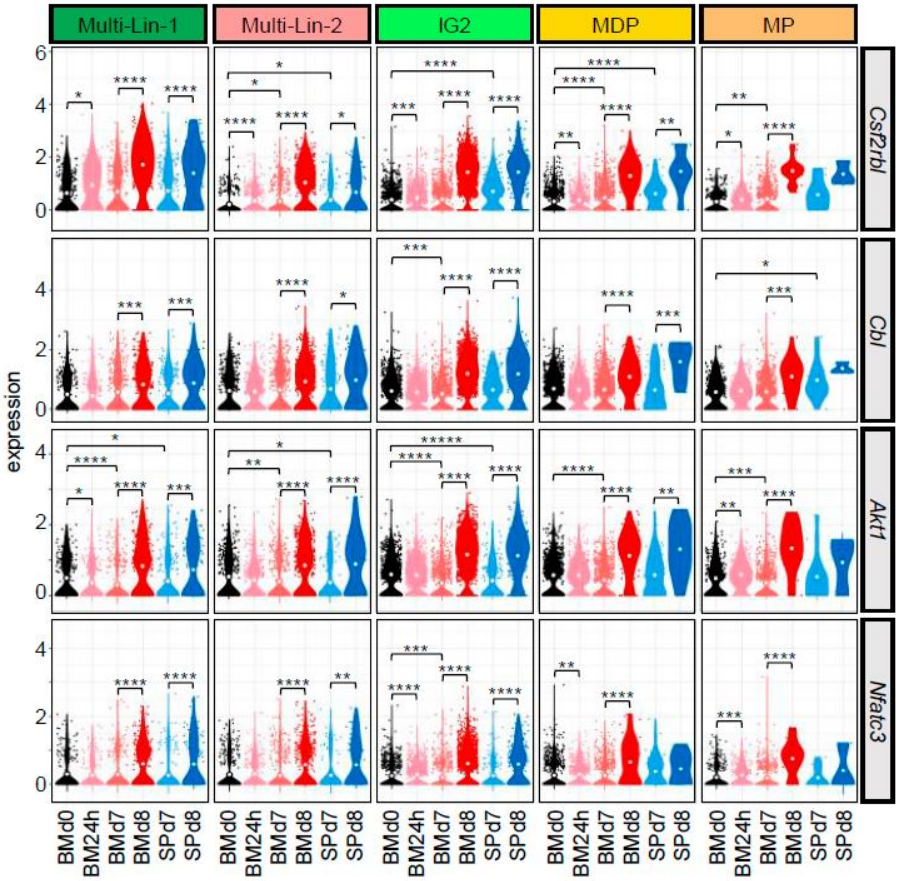

FIGURE 43. *Csf2rb, Cbl, Akt1* and *Nfatc3* gene expression in HSPC subsets. Violin plots of the indicated genes in each HSPC subset. Statistical significance was assessed by the Wilcoxon signed-rank test as implemented in the ggpubr R package. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 for the indicated comparisons.

4.1. GM-CSF production by HSPCs and its role in trained immunity

It has been reported that GM-CSF can be produced by NK cells in the spleen early during a *C. albicans* infection (Domínguez-Andrés *et al.*, 2017). Moreover, a previous study working with a mouse model of hepatocellular carcinoma has showed the presence of GM-CSF-producing HSPCs in the spleen (Luo *et al.*, 2021).

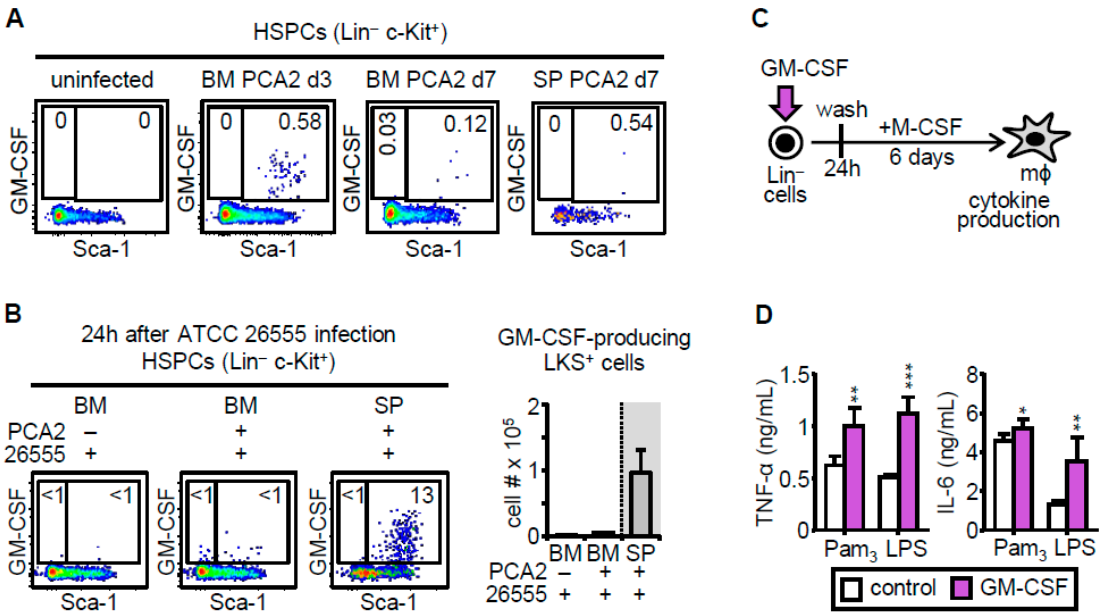

FIGURE 44. GM-CSF production by HSPCs and its effect on HSPCs to produce trained macrophages. GM-CSF was intracellularly detected by flow cytometry in pre-gated Lin⁻ c-kit⁺ cells in total RBC-lysed bone marrow cells from uninfected, 3-day or 7-day PCA2-infected mice, or total RBC-lysed splenocytes from 7-day PCA2-infected mice **(A)**, or in total RBC-lysed bone marrow cells or splenocytes from 7-day PCA2-infected or uninfected mice i.p. infected for 24 h with 3 x 10^7 ATCC 26555 yeasts **(B)** stimulated with PMA/ionomycin and brefeldin A for 6 h. Dot plots are representative and the percentage of cytokine-producing LKS⁺ and LKS⁻ cells is indicated. Bar graphs representing total cytokine-producing cell numbers and MFI of cytokine-producing cells show means ± SDs of 3 mice. **(C)** Lin⁻ cells from the bone marrow of uninfected mice were stimulated *in vitro* with or without GM-CSF (20 ng/ml) for 24 h, washed and differentiated into macrophages with M-CSF for 6 days. **(D)** Equal numbers of HSPC-derived macrophages were stimulated with Pam_3CSK_4 or LPS and TNF-α and IL-6 levels were assessed by ELISA in 24-h supernatants. Triplicate samples were analysed in each assay. Bar graphs show means ± SDs of pooled data from 3 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 with respect to cytokine production by macrophages differentiated from Lin⁻ cells not stimulated with GM-CSF.

We therefore studied the possible GM-CSF production by HSPCs in the bone marrow and spleen of PCA2-infected mice by intracellular flow cytometry. We found a distinct but very small subset of GM-CSF-producing LKS⁺ cells in the bone marrow from 3-day

PCA2-infected mice that was not present in the bone marrow or the spleen at day 7 post-PCA2 infection (Figure 44A). In contrast, a secondary infection induces the appearance of GM-CSF producing LKS^+ cells in the spleen, but not in the bone marrow, of mice that had been vaccinated with the PCA2 strain (Figure 44B).

Supporting these data, we found *Nfatc3*, a transcription factor that has been shown to activate the transcription of GM-CSF and other cytokines (Johnson *et al.*, 2004), to be upregulated in response to a secondary infection (Figure 43). For this reason, we hypothesize that GM-CSF could have an important role in the generation of trained immunity in HSPCs. To prove this, we first isolated bone marrow Lin^- cells, stimulated them with GM-CSF for 24 h, washed and differentiated them into macrophages with M-CSF for the next 6 days (Figure 44C). Results show that macrophages derived from GM-CSF-stimulated HSPCs produce greater amounts of TNF-α and IL-6 than macrophages derived from unstimulated HSPCs (Figure 44D). These data clearly demonstrate that GM-CSF stimulation of HSPCs is sufficient to reprogram their function to produce trained macrophages, at least *in vitro*.

4.2. Effects of blocking GM-CSF function during vaccination

We next assessed the consequences of blocking GM-CSF function at the beginning of the PCA2 infection by injecting i.p. a functional blocking antibody or its isotype control (Figure 45A). Blocking GM-CSF partially reduces the expansion of LKS^+ cells in the bone marrow and their presence in the spleen at day 7 post-PCA2 infection (Figure 45B). Moreover, the enhanced cytokine production by HSPCs in the bone marrow from 7-day PCA2-infected mice is significantly reduced by the antibody-mediated blockade of GM-CSF (Figure 45C), indicating that GM-CSF is responsible for inducing trained immunity in HSPCs.

Finally, we studied if blocking GM-CSF during the PCA2 infection could have consequences in the protection observed against reinfection. We reinfected mice with the ATCC 26555 strain of *C. albicans* 7 days after being PCA2-infected, and fungal burden was assessed in their kidneys 4 days later. Results show that isotype control injected animals had a significant reduction of CFUs in the kidneys compared to non-PCA2 infected animals, as expected (Figure 45D). However, protection was lost by blocking

GM-CSF function during the PCA2 infection, indicating the relevance of GM-CSF in providing protection against reinfection (Figure 45D).

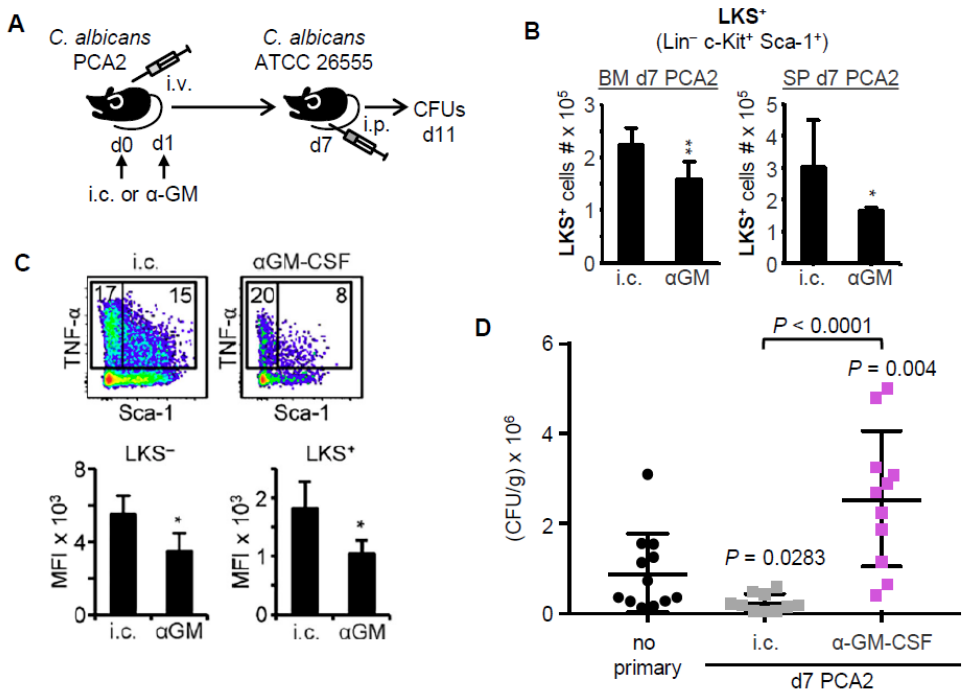

FIGURE 45. Effects of blocking GM-CSF function. (A) 125 µg of anti-GM-CSF (αGM) blocking antibody or its isotype control (i.c.) were i.p. administered into PCA2-infected mice twice, at the time of the infection (d0) and 1 day later (d1), and 7 days later mice were i.p. infected with 3 x 10^7 ATCC 26555 yeasts to measure CFUs in the kidneys 4 days later. **(B)** LKS^+ cell numbers in the bone marrow and spleen of αGM- or i.c.-injected animals were assessed by flow cytometry at day 7 post-PCA2 infection. Bar graphs show means ± SDs of 3 mice. *P < 0.05 and **P < 0.01 with respect to LKS^+ cell numbers in the bone marrow or spleen of i.c.-treated mice. **(C)** Intracellular detection by flow cytometry of TNF-α in pre-gated Lin^- $c\text{-}kit^+$ cells from total RBC-lysed bone marrow cells isolated from αGM- or i.c.-treated 7-day PCA2-infected mice and stimulated with LPS for 6 h with brefeldin A for the final 4 h. Dot plots are representative and the percentage of cytokine-producing LKS^+ and LKS^- cells is indicated. Bar graphs representing MFI of cytokine-producing cells show means ± SDs of 3 mice. *P < 0.05 with respect to cytokine production by cells from i.c.-treated mice. **(D)** Fungal burden in the kidneys expressed as CFUs/g of tissue 4 days after the ATCC 26555 infection. Data shown is pooled from 2 independent experiments (n=5-6 per experiment).

These results demonstrate the importance of GM-CSF during the PCA2 infection to regulate HSPC mobilization, expansion and function to provide protection against reinfection.

5. Discussion

Trained immunity refers to the process by which the innate immune system undergoes a reprogramming following a first encounter with certain microorganisms that leads to its ability to mount a faster and greater response upon a secondary challenge with the same or different stimuli (Netea *et al.*, 2011). Over the last years, trained immunity has increasingly gained interest, as it has emerged as an important player in conferring a broad protection against different infections and even certain cancers (Arts *et al.*, 2018; Walk *et al.*, 2019; Ciarlo *et al.*, 2020; Kalafati *et al.*, 2020). A wide range of stimuli are known to induce trained immunity *in vivo*, including *C. albicans*, which was among the first described inducers of this process. These stimuli induce a functional reprogramming of monocyte metabolism towards aerobic glycolysis and enhanced cholesterol synthesis, accompanied with epigenetic remodelling to increase the production of proinflammatory cytokines in response to a second challenge (Bekkering *et al.*, 2021).

To delve into the mechanisms that drive trained immunity during an infection, we have used a *C. albicans* vaccination model originally described by Dr. Cassone's group (Mattia *et al.*, 1982), in which mice are infected with PCA2, a low-virulent, non-germinative strain of *C. albicans* that causes a mild infection and confers protection from lethal reinfection with a virulent *C. albicans* strain, other virulent *Candida* species, and other fungi (*Aspergillus* and *Cryptococcus*) and bacteria (*Staphylococcus aureus*) (Bistoni *et al.*, 1986; Vecchiarelli *et al.*, 1988). Importantly, this protection provided by the PCA2 vaccination was demonstrated to be T cell-independent, as protection was also achieved in athymic mice (Bistoni *et al.*, 1988). Moreover, protection against reinfection was observed to be dependent on macrophages (Bistoni *et al.*, 1986) and proinflammatory cytokine production (Vecchiarelli *et al.*, 1989), pointing out trained immunity as the potential mechanism responsible for this effect. In the present PhD thesis, we have confirmed this protection conferred by the low-virulence strain against a secondary infection by infecting mice 7 days post-PCA2 infection with the virulent strain of *C. albicans* ATCC 26555. At this timepoint, the primary infection has already been overcome, as fungal burden in the kidneys had lowered significantly, while Th1 responses have not been established yet (Cenci *et al.*, 1989; Romani *et al.*, 1991).

By using this model, we have demonstrated an increase in proinflammatory cytokine production in both, the bone marrow and spleen 7 days post-PCA2 infection. This

enhanced proinflammatory phenotype is due not only to the presence of higher numbers of cytokine-producing monocytes in these organs, but also to their functional reprogramming in order to produce, each of them individually, higher amounts of proinflammatory cytokines, especially in the spleen of 7-day PCA2-vaccinated mice. Remarkably, reprogramming of cytokine production by monocytes was still present at longer timepoints post-infection, with some of the effects lasting for 14 or even 40 days, which is beyond the lifespan of these mature cells and, therefore, monocyte subsets are expected to be completely renewed. Hence, their upstream progenitors must be the ones sensing the infection and giving rise to mature cells with a modified phenotype bearing these trained immunity traits.

In fact, our scRNA-seq data revealed metabolic changes at the transcriptomic level related to trained immunity in bone marrow HSPCs early during the PCA2 infection, in agreement with other trained immunity experimental models (Mitroulis *et al.*, 2018; de Laval *et al.*, 2020). Consistent with this, we have observed that bone marrow HSPCs at day 3 post-infection produce trained macrophages *ex vivo*. Likewise, Kaufmann *et al.* showed that bone marrow-derived macrophages from BCG-vaccinated mice are trained and protect mice against tuberculosis (Kaufmann *et al.*, 2018). We have also demonstrated that an emergency myelopoiesis response takes place in the bone marrow during the PCA2 infection, in order to rapidly replace myeloid cells. This is accompanied by the recruitment and expansion of HSPCs in the spleen at day 7 post-infection, which made us hypothesize that early trained HSPCs in the bone marrow home the spleen to coordinate a protective response.

According to this, we have observed the activation of trained immunity-related epigenetic pathways only in splenic, and not in bone marrow, HSPCs 7 days post-infection. In addition, at this timepoint only HSPCs from the spleen, and not the bone marrow, are capable of producing trained macrophages *ex vivo*. Therefore, our data points towards these HSPCs as important players in conferring protection against reinfection, turning the spleen into a place to maintain trained HSPCs ready to fight in case of a secondary infection, while haematopoiesis in the bone marrow would be reestablished to normal conditions. Accordingly, we have directly demonstrated an active role for HSPCs from vaccinated mice in providing protection against reinfection, as the adoptive transfer of HSPCs isolated from the bone marrow, but more significantly the spleen, of 7-day PCA2-infected mice protect mice against a secondary infection, thus confirming that

trained immunity is taking place in HSPCs during infection. These results are in line with a previous work showing that an extended systemic exposure to the TLR2 agonist leads to an expansion of HSPCs in the spleen, which are partially responsible for protecting against systemic candidiasis (Martínez *et al.*, 2018).

Previous studies have shown that HSPCs can produce copious amounts of cytokines upon being stimulated via TLRs (Zhao *et al.*, 2014). These cytokines could be acting in a paracrine manner to activate other immune cells and, in this way, boost the proinflammatory response, or in an autocrine manner to autoregulate stress-induced myelopoiesis. On this matter, we have previously demonstrated that HSPCs produce cytokines in response to *C. albicans in vitro*, and that their secretome is able to induce myeloid differentiation of HSPCs (Martínez *et al.*, 2018). In this sense, it has been demonstrated that autocrine signalling of IL-6 secreted by HSPCs upon TLR stimulation is implicated in promoting myeloid differentiation (Zhao *et al.*, 2014). Moreover, signalling through TNF-α in HSPCs can also stimulate emergency myelopoiesis by upregulating PU.1 and promoting survival of HSCs and myeloid-biased MPPs (Yamashita & Passegué, 2019). Our results show that, in response to an infection, HSPCs produce TNF-α and IL-6 but, more importantly, we demonstrate that a previous infection with the PCA2 strain of *C. albicans* programmes them to produce a greater quantity of these proinflammatory cytokines upon a secondary infection, which could act in an autocrine manner to enhance myelopoiesis and also regulate the function of downstream myeloid cells.

On the other hand, previous works have implicated GM-CSF signalling in HSPCs in their expansion in the bone marrow following a β-glucan treatment (Mitroulis *et al.*, 2018). Here, we have found an upregulation of the *Csf2rb* gene, which codes for the GM-CSF receptor, in bone marrow HSPCs very early after the PCA2 infection. This is in agreement with a previous study that showed that β-glucan administration causes an upregulation of CD131, the β-subunit of the GM-CSF receptor, probably due to an enhanced cholesterol biosynthesis (Mitroulis *et al.*, 2018). In addition, HSPCs have been shown to produce GM-CSF in response to TLR stimulation (Zhao *et al.*, 2014) and other works point out the existence of GM-CSF-producing HSPCs in the spleen, but not in the bone marrow, of tumour-bearing mice (Wu *et al.*, 2018). In our model, we have found a very small but distinct subset of GM-CSF-producing LKS^+ cells in the bone marrow of 3-day PCA2-infected mice that was not present in the bone marrow or the spleen 7 days

post-infection. Nevertheless, reinfection of 7-day PCA2-infected mice induces the production of GM-CSF by LKS^+ cells in the spleen, and not in the bone marrow, although we cannot discard a rapid mobilization of GM-CSF-producing HSPCs from the bone marrow to the spleen during the 24 h of the secondary infection. Several studies have demonstrated a GM-CSF-dependent mobilization of HSPCs to the spleen and inflamed tissues during some inflammatory diseases, such as atherosclerosis (Robbins *et al.*, 2012), colitis (Griseri *et al.*, 2012) or spondylarthritis (Regan-Komito *et al.*, 2020). Here, we have demonstrated that administering a GM-CSF-blocking antibody to mice during the PCA2 infection reduces the LKS^+ cell population in both, the bone marrow and the spleen, and that blocking this cytokine *in vivo* causes a significant decrease in TNF-α production by HSPCs, highlighting the relevance of GM-CSF in generating trained immunity in HSPCs.

Given that GM-CSF can prime human monocytes to enhance TNF-α production upon being subsequently stimulated with LPS (Borriello *et al.*, 2016), we hypothesized that GM-CSF signalling in HSPCs could also be responsible for the trained phenotype observed in the macrophages derived from them. In fact, we have demonstrated that macrophages differentiated from GM-CSF-treated HSPCs produce higher levels of TNF-α and IL-6 than macrophages derived from unexposed HSPCs. Moreover, antibody-mediated blockade of GM-CSF in PCA2-vaccinated mice abrogates their protection against reinfection with a virulent strain of *C. albicans*, indicating the relevance of GM-CSF during the PCA2 infection for the trained protective phenotype.

The kinetics in the functional programming and the transient mobilization of HSPCs to the spleen during the PCA2 infection could be a strategy to strengthen immunity to fight infection while T cell responses are developing. In addition, bone marrow HSPCs must be keeping some memory, as monocyte function is still reprogrammed at much later timepoints. Then again, there are still some questions remaining to be answered, including the consequences of the combined signalling by other cytokines, such as TNF-α and IL-6, on HSPC function, and the progenitor stage at which these cytokines are acting on.

All things considered, Figure 46 shows a representation of the events leading to protection against reinfection in our PCA2-infection model. In conclusion, our data show that the immune protection conferred by the *C. albicans* PCA2 vaccine is mediated by trained HSPCs that are able to mobilize to the spleen and produce trained mature myeloid

cells to fight against a secondary infection. Mechanistically, autocrine GM-CSF activation of HSPCs is responsible for the trained phenotype (Figure 46). Our results open new avenues about the use of GM-CSF for clinical applications in vaccination as well as for disease prevention and treatment.

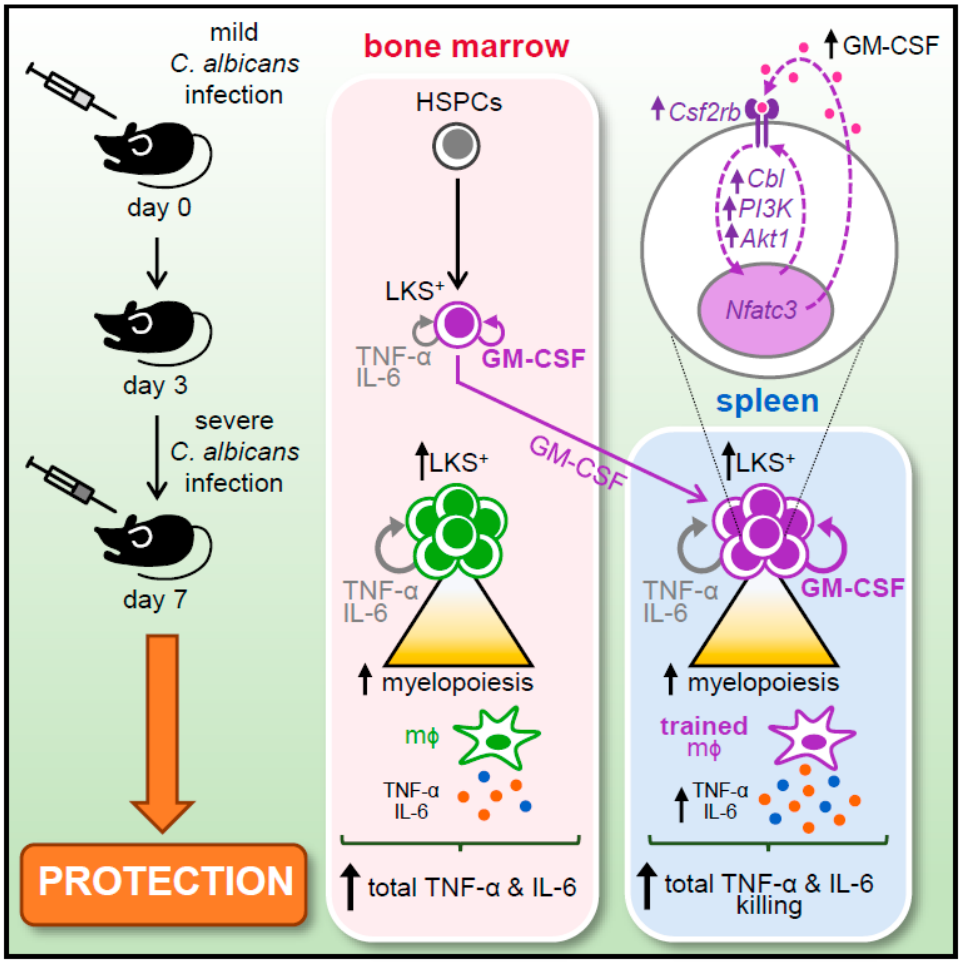

FIGURE 46. Schematic representation of the mechanisms governing trained immunity during *C. albicans* vaccination that confer protection against reinfection. A mild *C. albicans* infection with the PCA2 strain confers protection to mice against a severe *C. albicans* infection. This PCA2 infection induces a higher production of TNF-α and IL-6 by total bone marrow and spleen cells, while only spleen cells display an enhanced killing ability. Early during the primary infection, bone marrow HSPCs are reprogrammed to induce the production of trained macrophages, but at later timepoints (day 7), these trained HSPCs are recruited to the spleen. Vaccination also expands the LKS$^+$ cell population in both, the bone marrow and spleen, and primes them to produce myeloid cells. Mechanistically, autocrine GM-CSF activation of HSPCs is responsible for HSPC expansion, mobilization to the spleen and functional reprogramming, which ultimately leads to protection against reinfection.

Conclusions

1. A transient exposure of HSPCs to living yeasts of *C. albicans*, prior to their differentiation *in vitro*, is sufficient to induce a trained phenotype in the macrophages they produce in a dectin-1- and TLR2-dependent manner.

2. *C. albicans*-induced differentiation of HSPCs towards macrophages *in vivo* is dependent on TLRs/MyD88 and dectin-1, although the role of TLR signalling appears to be more significant.

3. HSPCs are directly stimulated by the dectin-1 agonist *in vivo*, promoting macrophage differentiation. The engagement of this receptor in HSPCs subsequently directs them to produce trained macrophages by a cell-autonomous indirect mechanism.

4. Myeloid differentiation of HSPCs induced by the TLR2 ligand is dependent on MyD88-mediated signalling, which activates TBK1 and mTOR to induce differentiation towards macrophages dependent on PU.1, C/EBPβ and IRF7 transcription factors.

5. An infection with the low-virulence strain of *C. albicans* PCA2 protects mice against a secondary infection with a virulent strain and induces trained immunity.

6. PCA2 infection expands and trains HSPCs early during infection and mobilizes them to the spleen to produce trained macrophages.

7. Trained HSPCs are primed for myeloid cell differentiation and proinflammatory cytokine production upon a second challenge and have a protective role against reinfection.

8. GM-CSF is involved in HSPC mobilization to the spleen, HSPC training and is essential for the protection induced by PCA2 infection.


Research publications

The results presented in this PhD thesis are included in the following research publications:

1. Bono, C., Martínez, A., Megías, J., Gozalbo, D., Yáñez, A. and Gil, M.L. (2020) Dectin-1 Stimulation of Hematopoietic Stem and Progenitor Cells Occurs *In Vivo* and Promotes Differentiation Toward Trained Macrophages via an Indirect Cell-Autonomous Mechanism. *mBio*, 11(3):e00781-20. doi: 10.1128/mBio.00781-20

2. Bono, C., Guerrero, P., Jordán-Pla, A., Erades, A., Salomonis, N., Grimes, H.L., Gil, M.L. and Yáñez, A. (2021) GM-CSF Programs Hematopoietic Stem and Progenitor Cells During *Candida albicans* Vaccination for Protection Against Reinfection. *Frontiers in Immunology*, 12:790309. doi: 10.3389/fimmu.2021.790309

3. Bono, C., Guerrero, P., Erades, A., Jordán-Pla, A., Yáñez, A. and Gil, M.L. (2022) Direct TLR2 signaling through mTOR and TBK1 induces C/EBPβ and IRF7-dependent macrophage differentiation in hematopoietic stem and progenitor cells. *Stem Cells*, 40(10):949-962. doi: 10.1093/stmcls/sxac053

Resumen en español

INTRODUCCIÓN

La especie fúngica *Candida albicans* es tanto un microorganismo comensal como un patógeno oportunista en humanos, siendo capaz de causar una variedad de infecciones que van desde las candidiasis superficiales mucocutáneas a las graves candidiasis invasivas, cuya frecuencia ha aumentado considerablemente en las últimas décadas debido al aumento del tamaño de la población de riesgo inmunodeprimida (Pappas *et al.*, 2018). Las células mieloides, especialmente neutrófilos y macrófagos, constituyen la primera línea de actuación del sistema inmunitario y son cruciales en la defensa frente a *C. albicans*. Durante una infección, son capaces de detectar patrones moleculares asociados a patógenos (PAMPs) a través de los receptores de reconocimiento de patrones (PRRs) que expresan, incluyendo los receptores tipo toll (TLRs) y los receptores de lectina tipo C (CLRs). De entre los diferentes TLRs, TLR2 es el receptor más importante implicado en el reconocimiento de *C. albicans*, induciendo la producción de citocinas proinflamatorias por una vía dependiente de la molécula adaptadora MyD88. Además, la colaboración entre PRRs más estudiada es la del TLR2 y dectina-1, que constituye el principal receptor de tipo lectina implicado en el reconocimiento y la fagocitosis de *C. albicans*. De esta forma, el reconocimiento de *C. albicans* mediado por ambos receptores simultáneamente induce una respuesta más potente y mejor adaptada (Yáñez *et al.*, 2021).

Todas las células leucocitarias que participan en la defensa frente a la candidiasis se generan mediante hematopoyesis, un proceso que se encuentra muy controlado en condiciones de homeostasis pero que se ve profundamente alterado en respuesta a diferentes tipos de infecciones, cuando tiene lugar la denominada mielopoyesis de emergencia, que consiste en favorecer la producción de neutrófilos, macrófagos, o ambos tipos celulares en detrimento del desarrollo de otros linajes (Boettcher & Manz, 2016). El descubrimiento de que las células madre y progenitores hematopoyéticos (HSPCs) expresan PRRs funcionales planteó la posibilidad de que estos receptores pudieran participar en esta modulación de la hematopoyesis que tiene lugar en el transcurso de una infección. En este sentido, se ha demostrado que células inactivadas de *C. albicans* inducen *in vitro* la proliferación de las HSPCs de ratón, así como su diferenciación hacia el linaje mieloide, por una vía dependiente de TLR2/MyD88 y dectina-1 (Yáñez *et al.*, 2013a). Además, utilizando un modelo de trasplante murino, se ha descrito que la estimulación de HSPCs vía TLRs ocurre *in vivo*, y que ésta induce su diferenciación hacia

macrófagos (Megías *et al.*, 2012). Estos resultados sugieren que los patógenos pueden ser directamente reconocidos por las HSPCs a través de los TLRs, promoviendo así la capacidad de reaprovisionamiento del sistema inmunitario innato durante una infección.

Numerosos estudios recientes han puesto en duda el dogma de que la memoria inmunológica es una característica exclusiva de la inmunidad específica. Es decir, células de la inmunidad innata (especialmente monocitos y macrófagos) pueden exhibir cierta memoria y responder de forma diferente frente a un segundo encuentro con el mismo u otro estímulo diferente. Por ejemplo, la exposición de monocitos y macrófagos a *C. albicans* aumenta su respuesta frente a un segundo encuentro (inmunidad entrenada), mientras que ligandos de TLR4 o TLR2 confieren una menor respuesta inflamatoria a los macrófagos (tolerancia) (Netea *et al.*, 2016). En este contexto, se ha demostrado que este concepto de "memoria de la inmunidad innata" puede aplicarse, no solo a las células mieloides maduras, sino también a las HSPCs demostrando que: (i) los macrófagos generados a partir de HSPCs que han sido expuestas a un ligando soluble de TLR2, antes o durante su diferenciación, tanto *in vitro* como *in vivo*, producen menos citocinas inflamatorias (Yáñez *et al.*, 2013b), mientras que (ii) los macrófagos generados a partir de HSPCs que han sido expuestas a levaduras inactivadas de *C. albicans* durante su diferenciación *in vitro*, están mejor preparados para hacer frente al patógeno, ya que producen mayores niveles de citocinas y tienen mayor actividad fungicida que los macrófagos control (Megías *et al.*, 2016). Es decir, la señalización por diferentes PRRs en las HSPCs influye considerablemente en el fenotipo funcional de los macrófagos que se generan posteriormente (Martínez *et al.*, 2017).

Además, se ha observado que las HSPCs que son expuestas a ligandos de TLR2 producen factores solubles que actúan de manera paracrina, influenciando la función de macrófagos no expuestos a dichos ligandos (Yáñez *et al.*, 2013b). Adicionalmente, se ha descrito que la señalización vía TLRs en HSPCs induce la liberación de grandes cantidades de citocinas por parte de las propias células, y que esta liberación está mediada por NFκB (Zhao *et al.*, 2014). Estos hallazgos sugieren que la estimulación de los TLRs en HSPCs induce la producción de citocinas y otras moléculas inmunomoduladoras, que serían las responsables de regular la diferenciación de una manera tanto autocrina como paracrina y, por tanto, de determinar el fenotipo de las células mieloides generadas a partir de HSPCs en respuesta a la señalización vía PRRs. Sin embargo, otras publicaciones indican que la señalización por TLRs induce directamente la expresión de factores de

transcripción implicados en diferenciación mieloide, o que ésta sería debida a una combinación de mecanismos directos e indirectos (Buechler *et al.*, 2016).

Recientemente, varios trabajos han empezado a asignar un papel a las HSPCs en la lucha contra la infección, aunque los mecanismos que determinan dicha respuesta no están todavía bien descritos (Boettcher & Manz, 2017). Durante una infección las HSPCs podrían detectar directamente a los microorganismos (o a los PAMPs derivados de ellos) y/o responder a las citocinas producidas por otros tipos celulares en respuesta a los mismos estímulos. Como consecuencia, se ha descrito la movilización de estas HSPCs desde la médula ósea hasta los tejidos periféricos inflamados (Herman *et al.*, 2016) y/o infectados (Granick *et al.*, 2013; Burberry *et al.*, 2014). En este contexto, utilizando un modelo de movilización masiva de HSPCs al bazo en respuesta a una exposición prolongada al Pam_3CSK_4, se ha podido demostrar que estos ratones están protegidos frente a una candidiasis invasiva y que esta protección está mediada, al menos en parte, por las HSPCs (Martínez *et al.*, 2018).

En cualquier caso, tanto en la médula ósea como en los tejidos periféricos, las HSPCs podrían contribuir a proteger frente a una infección por diferentes mecanismos: (i) diferenciándose a células mieloides funcionales con el objetivo de reponer las células consumidas haciendo frente al patógeno y aumentar la vigilancia inmunitaria en tejidos, (ii) diferenciándose a células con un fenotipo más preparado para hacer frente al patógeno y poder fagocitarlo y destruirlo con más efectividad, contribuyendo así a prolongar en el tiempo la memoria innata y (iii) secretando factores solubles que puedan modificar la función de otras células mieloides maduras que se encuentren en los tejidos infectados.

OBJETIVOS

En base a los antecedentes previamente comentados, los objetivos desarrollados en el presente proyecto de tesis han sido los siguientes:

1. **Estudiar la participación de la dectina-1 en la diferenciación de HSPCs y el fenotipo de los macrófagos derivados a partir de ellas en respuesta a su ligando y a la estimulación con *C. albicans*.**

2. **Investigar los mecanismos moleculares por los que TLR2 y dectina-1 inducen la diferenciación de HSPCs y confieren un fenotipo tolerizado o entrenado, respectivamente, a las células maduras producidas.**

   2.1. Determinando la contribución de mecanismos directos e indirectos.

   2.2. Indagando en las vías de transducción de señal y la activación de factores de transcripción iniciados por mecanismos directos.

3. **Explorar la inmunidad entrenada tras la vacunación con una cepa de baja virulencia de *C. albicans*.**

   3.1. Caracterizando las poblaciones celulares en la médula ósea y el bazo, y estudiando su fenotipo.

   3.2. Analizando la expansión y/o diferenciación de las HSPCs, así como su movilización y transcriptoma, el fenotipo de los macrófagos generados *ex vivo*, y su posible papel protector frente a la infección.

   3.3. Desvelando los mecanismos por los que la vacunación podría inducir una reprogramación en las HSPCs.

METODOLOGÍA Y RESULTADOS

CAPÍTULO 1: Papel de la dectina-1 y TLR2 en la diferenciación de HSPCs y la generación de macrófagos tolerizados o entrenados: mecanismos moleculares

Una exposición transitoria in vitro de HSPCs a células vivas de *C. albicans* es suficiente para inducir un fenotipo entrenado en los macrófagos que producen de manera dependiente de dectina-1 y TLR2

Se purificaron por separación inmunomagnética HSPCs de ratones C57BL/6, TLR2$^{-/-}$ o dectina-1$^{-/-}$ y se pusieron en cultivo en presencia o ausencia de levaduras vivas de *C. albicans*. Tras 6 horas se añadió anfotericina B para parar el crecimiento de las células fúngicas y se transvasaron las HSPCs a otra placa, donde se indujo su diferenciación con M-CSF. Tras 7 días de cultivo, las células diferenciadas generadas fueron reestimuladas con ligandos de los PRRs y se cuantificó la producción de citocinas proinflamatorias empleando kits de ELISA comerciales.

Los resultados muestran que macrófagos diferenciados a partir de HSPCs WT que han sido expuestas a levaduras vivas de *C. albicans* durante 6 horas presentan un fenotipo entrenado, es decir, producen una mayor cantidad de citocinas proinflamatorias que células diferenciadas a partir de HSPCs que no han sido expuestas a la levadura viva. Dicho fenotipo entrenado es dependiente de la cantidad de levaduras empleadas, pues cuanto mayor es la ratio de levaduras vivas empleada para estimular las HSPCs, más acusado es el fenotipo entrenado observado. Sin embargo, este fenotipo entrenado no se observa cuando las HSPCs expuestas a las levaduras provienen de ratones TLR2$^{-/-}$ o dectina-1$^{-/-}$.

La señalización por dectina-1 en HSPCs dirige su diferenciación a macrófagos entrenados *in vivo*

Se obtuvieron HSPCs a partir de médula ósea de ratones B6-SJL (aloantígeno CD45.1) que fueron trasplantadas por vía intravenosa en ratones dectina-1$^{-/-}$ (aloantígeno CD45.2), a los cuales se les inyectó también zimosán deplecionado (partículas de

glucano), el cual constituye el ligando específico del receptor dectina-1. Como controles, se utilizaron ratones trasplantados pero no inyectados con zimosán. Tres días después del trasplante, los ratones fueron sacrificados y se obtuvieron el bazo y la médula ósea de los mismos para estudiar la proliferación y diferenciación de las células trasplantadas presentes en ellos. Esto fue llevado a cabo mediante el análisis por citometría de flujo de las células CD45.1 positivas, marcando con diferentes combinaciones de anticuerpos frente a marcadores de células madre y/o marcadores de células mieloides maduras.

Los datos obtenidos muestran que, tanto en bazo como en médula ósea, el porcentaje de células trasplantadas recuperadas era mayor cuando el ratón trasplantado recibía también inyecciones de zimosán deplecionado, en comparación con los ratones trasplantados pero no tratados. Además, se observó que, tanto en bazo como en médula ósea, el porcentaje de células mieloides maduras (positivas para el marcador de superficie CD11b), así como el porcentaje de macrófagos (células doble positivas para CD11b y F4/80), se incrementaba cuando los ratones eran tratados con zimosán deplecionado.

Por otra parte, se obtuvieron HSPCs a partir de médula ósea de ratones C57BL/6, MyD88$^{-/-}$ o dectina-1$^{-/-}$ (aloantígeno CD45.2). Estas células fueron trasplantadas por vía intravenosa en ratones B6-SJL (aloantígeno CD45.1), a los cuales se les inyectaron también levaduras inactivadas de *C. albicans*. Tres días después del trasplante, se estudió por citometría de flujo la proliferación y diferenciación de las células CD45.2 positivas presentes en el bazo y en la médula ósea de los ratones trasplantados, marcando con diferentes combinaciones de anticuerpos frente a marcadores de células madre y/o marcadores de células mieloides maduras.

Los datos obtenidos muestran que, si bien a penas se observaron diferencias entre el porcentaje total de células mieloides maduras (positivas para CD11b) procedentes de ratones dectina-1$^{-/-}$ y MyD88$^{-/-}$, en comparación con las células procedentes de ratones WT, sí que existían diferencias significativas al comparar los porcentajes de macrófagos maduros (células doble positivas para CD11b y F4/80), pues tanto las células procedentes de ratones dectina-1$^{-/-}$ como MyD88$^{-/-}$ se diferenciaban en menor medida hacia macrófagos, en comparación con las células procedentes de ratones WT.

Por último, se obtuvieron HSPCs a partir de médula ósea de ratones que expresan la proteína roja fluorescente DsRed. Estas células fueron trasplantadas por vía intravenosa en ratones dectina-1$^{-/-}$, a los cuales se les inyectó también M-CSF con o sin zimosán

deplecionado. Los macrófagos diferenciados *in vivo* a partir de las células trasplantadas (identificados como células triple positivas para DsRed, CD11b y F4/80) fueron purificados a partir del bazo de los animales por citometría de flujo. Estas células fueron reestimuladas *ex vivo* con ligandos de PRRs y se cuantificó la producción de citocinas proinflamatorias empleando kits de ELISA comerciales.

Los resultados obtenidos muestran que las células maduras generadas a partir de las HSPCs trasplantadas en presencia de zimosán deplecionado presentan un fenotipo entrenado, es decir, producen una mayor cantidad de citocinas proinflamatorias en comparación con células maduras generadas *in vivo* no expuestas a dicho ligando. Estas diferencias en el fenotipo de los macrófagos generados únicamente pueden ser debidas a la señalización directa vía dectina-1 en los progenitores trasplantados, ya que el ratón receptor no reconoce el ligando inyectado.

La estimulación de TLR2 en HSPCs promueve la diferenciación hacia macrófagos de una manera directa, mientras que la diferenciación mediada por dectina-1 está producida por mecanismos indirectos

Se cocultivaron números iguales de HSPCs de ratones DsRed con HSPCs procedentes de ratones TLR2$^{-/-}$ o dectina-1$^{-/-}$ y se indujo su diferenciación *in vitro* con Pam_3CSK_4 (ligando de TLR2) o zimosán deplecionado (ligando de dectina-1), respectivamente, o con levaduras de *C. albicans* inactivadas en ambos cocultivos. Tras 3 y 5 días de cultivo, se analizó por citometría de flujo el porcentaje de células que expresaban la proteína DsRed y las que no lo hacían respecto del total de células diferenciadas (CD11b y F4/80 doble positivas). Como control, la diferenciación de las células cocultivadas se indujo con M-CSF, ya que la diferenciación de HSPCs DsRed, TLR2$^{-/-}$ y dectina-1$^{-/-}$ en respuesta a esta citocina debe ser comparable, y, efectivamente, el porcentaje de células diferenciadas fue de aproximadamente el 50% de DsRed y TLR2$^{-/-}$ o dectina-1$^{-/-}$, en cada caso.

Los experimentos de cocultivo de HSPCs de ratones DsRed y TLR2$^{-/-}$ indican que tras 3 días en presencia de Pam_3CSK_4, el porcentaje de células TLR2$^{-/-}$ diferenciadas es inferior al de células DsRed, estas diferencias se acentúan todavía más a día 5 de cocultivo. No obstante, en los experimentos de cocultivo de HSPCs de ratones DsRed y dectina-1$^{-/-}$, los porcentajes de células diferenciadas de ambos tipos en respuesta a zimosán deplecionado eran muy parecidos, tanto a día 3 como a día 5. Además, en el caso

de los cocultivos en presencia de levaduras inactivadas de *C. albicans*, tampoco se observaron diferencias en el porcentaje de células diferenciadas de cada tipo.

La señalización por TLR2 y dectina-1 en HSPCs confiere un fenotipo tolerizado o entrenado, respectivamente, a las células mieloides maduras que generan mayoritariamente por mecanismos indirectos

Se cocultivaron números iguales de HSPCs de ratones DsRed con HSPCs procedentes de ratones TLR2$^{-/-}$ o dectina-1$^{-/-}$ y se indujo su diferenciación a macrófagos con M-CSF en presencia o ausencia de Pam_3CSK_4 o zimosán deplecionado, respectivamente, o de levaduras inactivadas de *C. albicans* en ambos casos. Tras 7 días de cultivo se separaron por citometría de flujo las células diferenciadas CD11b$^+$, F4/80$^+$, DsRed$^+$ de las CD11b$^+$, F4/80$^+$, DsRed$^-$. A continuación, se estudió su capacidad de producir citocinas proinflamatorias para determinar el posible fenotipo tolerizado o entrenado de las células TLR2$^{-/-}$ o dectina-1$^{-/-}$, usando como control las células DsRed tolerizadas en respuesta al Pam_3CSK_4 y entrenadas en respuesta al zimosán deplecionado y a las levaduras de *C. albicans*.

Los resultados muestran que los macrófagos TLR2$^{-/-}$ generados tras el cocultivo de HSPCs de ratones DsRed y TLR2$^{-/-}$ en presencia de M-CSF y Pam_3CSK_4 se encuentran tolerizados, si bien en menor medida que los macrófagos DsRed; mientras que cuando son generados en presencia de M-CSF y *C. albicans* exhiben un fenotipo entrenado. Por otra parte, macrófagos dectina-1$^{-/-}$ diferenciados a partir de HSPCs en cocultivo con HSPCs de ratones DsRed y en presencia de M-CSF y zimosán deplecionado o levaduras inactivadas de *C. albicans*, muestran un fenotipo entrenado.

La diferenciación mieloide de HSPCs inducida por el ligando de TLR2 es dependiente de la señalización mediada por MyD88, la cual activa TBK1 y mTOR para inducir la diferenciación a macrófagos de manera dependiente de PU.1, C/EBPβ e IRF7

Inicialmente se llevó a cabo un estudio de la posible participación de MyD88 y/o TRIF en la diferenciación de HSPCs vía TLR2. Para ello, se midió el porcentaje de células mieloides diferenciadas en respuesta al ligando de TLR2 Pam_3CSK_4 a partir de HSPCs

procedentes de ratones C57BL/6, TLR2$^{-/-}$ o MyD88$^{-/-}$. Puesto que no se observaron diferencias en la diferenciación de los progenitores MyD88$^{-/-}$ en comparación con los TLR2$^{-/-}$, pudimos concluir que la señalización vía TLR2 en HSPCs únicamente utiliza MyD88 y no señaliza vía TRIF. Además, se llevaron a cabo cocultivos de HSPCs de ratones WT y MyD88$^{-/-}$ en presencia de Pam_3CSK_4. Tras 3 y 5 días, el porcentaje de células MyD88$^{-/-}$ diferenciadas es muy inferior al de células WT, tal y como habíamos observado previamente que sucedía en los cocultivos de HSPCs de ratones WT y TLR2$^{-/-}$.

Posteriormente, se secuenció el ARN de HSPCs tras 24 h de exposición a Pam_3CSK_4 y observamos un aumento notable en la transcripción de los genes que codifican para los factores de transcripción PU.1, CEBP/β e IRF7. Este aumento se confirmó midiendo por qRT-PCR la expresión de dichos genes tras 12 y 24 h de exposición a Pam_3CSK_4. Sin embargo, este aumento no tiene lugar cuando las HSPCs proceden de ratones TLR2$^{-/-}$ o MyD88$^{-/-}$.

Además, para estudiar las vías de transducción de señal que inducen la expresión de los factores de transcripción mencionados, se estudió la posible activación de TBK1 y de la vía mTOR en respuesta a Pam_3CSK_4. Para ello, se analizó por western blot la fosforilación de TBK1 y la quinasa S6 (proteína diana de mTOR). Observamos que tanto mTOR como TBK1 se activan en HSPCs a partir de las 4 h de exposición a Pam_3CSK_4, activación que no ocurre cuando las HSPCs proceden de ratones TLR2$^{-/-}$ o MyD88$^{-/-}$. Adicionalmente, para estudiar los mecanismos de activación de mTOR, analizamos la activación de mTOR en respuesta a Pam_3CSK_4 y en presencia o ausencia de rapamicina (el inhibidor de mTOR), amlexanox (que inhibe la TBK1) o ZSTK474 (un inhibidor de la PI3K). Como era de esperar, la rapamicina abolía por completo la fosforilación de la S6, al igual que el ZSTK474. La inhibición de TBK1 también inhibía dicha activación, pero solo parcialmente.

A continuación, estudiamos el efecto de la rapamicina y el amlexanox en la diferenciación de células LKS$^+$ (que incluyen las células madre y progenitores multipotentes) y de células LKS$^-$ (que incluyen progenitores comprometidos con un linaje) en respuesta a Pam_3CSK_4. Para ello, se analizó por citometría de flujo tanto la proporción entre células mieloides (CD11b$^+$) y progenitores (c-kit$^+$), como el porcentaje de macrófagos (CD11b$^+$ F4/80$^+$). Observamos que en las dos poblaciones, tanto la rapamicina como el amlexanox, inhibían la diferenciación a día 4 y la generación de

macrófagos a día 7. Además, estos inhibidores también hacían disminuir el aumento de expresión de PU.1, C/EBPβ e IRF7 en respuesta a Pam_3CSK_4, así como sus niveles de proteína.

Finalmente, se analizó la contribución que pudieran tener los mecanismos indirectos en la diferenciación observada en respuesta al ligando de TLR2. Observamos que tiene lugar una producción de IL-6 de manera dependiente de NF-κB, pero que el amlexanox y la rapamicina no afectan dicha producción, y que la IL-6 no sería responsable del aumento de expresión de C/EBPβ tras la señalización vía TLR2 en HSPCs, pues la transcripción de ambos genes se induce a la vez. Por otra parte, el IFN-β producido no estaría mediando el efecto de IRF7 en la diferenciación de HSPCs, ya que mientras que el amlexanox es capaz de inhibir tanto el aumento en la expresión de IRF7 como la diferenciación de las HSPCs, dicho inhibidor no inhibe la producción de IFN-β.

CAPÍTULO 2: Estudiando la inmunidad entrenada en un modelo murino de vacunación con *C. albicans*

Una infección con la cepa de baja virulencia de *C. albicans* PCA2 protege a los ratones frente a una infección secundaria con una cepa virulenta

Se infectaron ratones C57BL/6 por vía intravenosa con la cepa de baja virulencia de *C. albicans* PCA2. Tras 7 días, dichos ratones fueron infectados con una dosis letal de la cepa virulenta de *C. albicans* ATCC26555 administrada de forma intravenosa, tras lo cual se realizó un seguimiento de la mortalidad durante 21 días. Por otra parte, en otra aproximación experimental, también 7 días post-infección primaria, se realizó una infección intraperitoneal con la cepa virulenta. Tres días después de esta infección secundaria se midió la carga fúngica tanto en riñón como en bazo. Los ratones vacunados con la cepa PCA2 de *C. albicans* mostraron una mayor supervivencia tras la infección secundaria, así como una menor carga fúngica en órganos internos, en comparación con ratones no vacunados con anterioridad a la infección con la cepa virulenta.

La vacunación con la cepa PCA2 de *C. abicans* induce inmunidad entrenada

Se obtuvieron células a partir de la médula ósea y el bazo de ratones previamente infectados con la cepa PCA2 de *C. albicans* a días 7 y 14 post-infección. En estas células se analizaron las diferentes poblaciones de células inmunitarias maduras por citometría de flujo, lo cual mostró un aumento significativo en el número de neutrófilos y monocitos.

Además, se estudió su capacidad de producir citocinas proinflamatorias, usando como control células de médula ósea y bazo de ratones no infectados. Los resultados muestran que a día 7 post-infección las células tanto de bazo como de médula ósea se encuentran entrenadas, es decir, producen una mayor cantidad de citocinas que células de bazo y médula ósea de ratones no infectados. A día 14 post-infección comprobamos que se mantiene este efecto.

Para poder discernir si este aumento en la producción de citocinas se debe a un aumento en la cantidad del número de células o a que cada una de estas células, de manera individual, produce una mayor cantidad (lo cual indicaría que son células entrenadas), medimos por citometría de flujo intracelular la cantidad de citocinas producida por los monocitos. Observamos que los monocitos tanto de la médula ósea como del bazo de ratones infectados se encuentran entrenados y que este efecto se mantiene hasta, por lo menos, 14 días después de la infección.

Para confirmar que la infección con la cepa PCA2 de *C. albicans* confiere un fenotipo entrenado a los ratones, 7 días post-infección se les administró LPS a una dosis suficiente para inducir shock séptico y se evaluó la mortalidad cada 12 h durante los 3 días siguientes. Se observó que la mortalidad de estos ratones es mayor en comparación con ratones no infectados previamente. Se obtuvieron los mismos resultados administrando el LPS 14 días post-infección.

Por otra parte, se llevó a cabo un análisis de la capacidad fungicida de estas células a día 7, observando que las células de médula ósea de ratones vacunados poseen una capacidad similar de matar a levaduras de *C. albicans* que las células de médula ósea de ratones control. Sin embargo, las células del bazo de ratones vacunados tienen una mayor capacidad fungicida que células del bazo de ratones control.

Finalmente, se estudió el posible papel protector de las células de médula ósea y el bazo de ratones vacunados frente a la infección. Para ello, células totales (sin eritrocitos)

de médula ósea y bazo de ratones vacunados fueron trasplantadas a ratones subsecuentemente infectados por vía intraperitoneal con la cepa virulenta de *C. albicans*. Cuatro días después de esta infección se midió la carga fúngica tanto en riñón como en bazo, observándose una disminución en la misma en los dos grupos con respecto a ratones infectados y trasplantados con células de médula ósea y bazo de ratones no vacunados (aunque dicha disminución únicamente es estadísticamente significativa en el caso del grupo trasplantado con células del bazo de ratones vacunados).

La infección con PCA2 expande y entrena las HSPCs en momentos tempranos de la infección y las moviliza al bazo para producir macrófagos entrenados

Se obtuvieron células a partir de la médula ósea y el bazo de ratones previamente infectados con la cepa PCA2 de *C. albicans* a días 7 y 14 post-infección. En estas células se llevó a cabo un análisis de las HSPCs tanto por inmunofenotipado por citometría de flujo, como mediante ensayos de formación de colonias en medio semisólido con metilcelulosa, permitiéndonos así distinguir y cuantificar las diferentes poblaciones de HSPCs presentes. Dichos análisis mostraron un claro incremento en el número total de HSPCs y, concretamente, en la población de células LKS^+ en el bazo a día 7 post-infección. Este aumento en la población de células LKS^+ también fue observado en médula ósea.

En estas células se analizó también, por citometría de flujo intracelular, la producción de citocinas proinflamatorias por parte de las diferentes poblaciones de HSPCs en respuesta a LPS y tras una segunda infección por *C. albicans*. Observamos que las células LKS^+ de médula ósea se encuentran entrenadas tanto en respuesta a LPS como a una segunda exposición a *C. albicans*, mientras que las de bazo únicamente lo están tras una segunda infección por *C. albicans*.

Además, se estudiaron los cambios en la expresión génica en HSPCs de médula a las 24 h tras la infección, y de bazo y médula a día 7 post-infección mediante un ensayo de scRNA-seq (secuenciación de RNA a nivel de célula única) utilizando la plataforma 10X Genomics. El análisis de los datos obtenidos muestra cambios en las vías de señalización implicadas en la generación de inmunidad entrenada: se observan cambios metabólicos, modificaciones epigenéticas, inducción de mielopoyesis de emergencia y cambios en

otras rutas implicadas en la respuesta inmunitaria celular frente a infecciones, especialmente en la muestra de bazo a 7 día post-infección.

Por otra parte, se purificaron las fracciones de células c-kit^{+} y c-kit$^{–}$ de médula ósea y bazo de ratones vacunados y fueron trasplantadas a ratones subsecuentemente infectados con la cepa virulenta de *C. albicans*. Cuatro días después de esta infección se midió la carga fúngica tanto en riñón como en bazo, observándose que el trasplante de la fracción celular c-kit^{+} de ambos órganos provoca una disminución en la carga fúngica, mientras que solo la fracción c-kit$^{–}$ del bazo la reduce.

Para comprobar si el bazo de ratones infectados tiene una mayor capacidad de reclutar HSPCs, se infectaron ratones con la cepa PCA2 de *C. albicans*, los cuales, tras 5 días, fueron trasplantados con HSPCs de médula ósea de ratones control marcadas con CFSE. Tras 2 días, se cuantificaron las células donantes (CFSE^{+}) presentes en el bazo de los ratones receptores. Se observó una mayor cantidad de células donantes en el bazo de ratones previamente infectados, en comparación con ratones control trasplantados. Además, las células trasplantadas a ratones infectados se encontraban más diferenciadas (más células c-kit$^{–}$ CD11b^{+}).

Por otra parte, para comprobar si las HSPCs de ratones infectados se encuentran mejor preparadas para movilizarse al bazo, se purificaron HSPCs de médula ósea 5 días post-infección y de ratones no infectados, las cuales fueron marcadas con CFSE y trasplantadas a ratones control. Tras 2 días, se cuantificaron las células donantes (CFSE^{+}) presentes en el bazo de los ratones receptores, observándose una mayor cantidad de células donantes, las cuales están más diferenciadas, cuando estas provienen de ratones previamente infectados.

Finalmente, se obtuvieron HSPCs a partir de la médula ósea y el bazo de ratones previamente infectados con la cepa PCA2 de *C. albicans* a diferentes tiempos (días 3, 7 y 14 post-infección), las cuales fueron diferenciadas *in vitro* en presencia de M-CSF. Tras 7 días de cultivo, se recogieron las células adherentes (macrófagos) y se estudió su capacidad de producir citocinas proinflamatorias, usando como control macrófagos diferenciados *in vitro* con M-CSF a partir de HSPCs de médula ósea de ratones no infectados. Los resultados muestran que, a día 3 post-infección, las HSPCs de médula ósea dan lugar a macrófagos entrenados, es decir, producen una mayor cantidad de citocinas proinflamatorias en comparación con macrófagos control. Sin embargo, dicho

fenotipo entrenado se revierte a tiempos más tardíos, pues tanto a día 7 como a día 14 post-infección, las HSPCs de médula ósea se diferencian a macrófagos tolerizados (menor producción de citocinas que macrófagos control). En cambio, las HSPCs de bazo a día 7 post-infección (momento de máxima expansión de esta población) sí que se diferencian a macrófagos con un fenotipo entrenado.

Las HSPCs entrenadas son más propensas a producir células mieloides y tienen un papel protector frente a la reinfección

Se infectaron ratones con la cepa PCA2 de *C. albicans*, los cuales, tras 7 días, fueron reinfectados por vía intraperitoneal con la cepa virulenta ATCC26555 de *C. albicans*. Tres días post-infección secundaria se analizaron por citometría de flujo las distintas poblaciones de HSPCs en la médula ósea y en el bazo. El análisis de la composición de HSPCs muestra un claro aumento de la población de células LKS^+ tras la infección secundaria, tanto en bazo como en médula, siendo especialmente notable el aumento de la subpoblación MPP3, la cual está constituida por progenitores multipotentes sesgados hacia el linaje mieloide.

Además, se estudiaron los cambios en la expresión génica en HSPCs de médula y bazo 24 h tras la infección secundaria mediante un ensayo de scRNA-seq. El análisis de los datos obtenidos muestra una gran cantidad de genes diferencialmente expresados en estas muestras en comparación con las muestras de médula y bazo 7 días post-infección y sin infección primaria, los cuales están implicados mayoritariamente en vías de señalización que participan en la generación de inmunidad entrenada.

Por otra parte, se purificaron HSPCs a partir de médula ósea y bazo de ratones infectados con la cepa PCA2 de *C. albicans*, las cuales fueron marcadas con CFSE y cultivadas en presencia de levaduras inactivadas de *C. albicans*. A día 3 se midió la proliferación de dichas células, y a días 5 y 7 se evaluó su diferenciación por citometría de flujo en base a los marcadores c-kit y CD11b. Como control se emplearon HSPCs de médula ósea de ratones no infectados cultivadas bajo las mismas condiciones. Los resultados obtenidos muestran una mayor proliferación por parte de las HSPCs de bazo de ratones previamente infectados, lo cual se corresponde con un importante aumento a días 5 y 7 de la población de células c-kit^+ $CD11b^-$ (no diferenciadas). No obstante, también se observó un aumento de la población de células mieloides c-kit^- $CD11b^+$ en el

cultivo de HSPCs provenientes de médula ósea de ratones previamente infectados y, más notablemente, también en aquellas provenientes del bazo.

El GM-CSF está implicado en la movilización de las HSPCs al bazo, así como en su entrenamiento, y es esencial para la protección inducida por la infección con PCA2

El análisis de los datos de scRNA-seq llevado a cabo en HSPCs de ratones vacunados con la cepa PCA2 de *C. albicans* mostraron un aumento en la expresión de genes implicados en vías de señalización iniciadas por GM-CSF, y, además, observamos (por citometría de flujo intracelular) que la infección secundaria induce la aparición de células LKS^+ productoras de GM-CSF en el bazo, por lo que hipotetizamos que esta citocina podría ser importante en la generación de inmunidad entrenada en HSPCs.

Para comprobarlo, se obtuvieron HSPCs a partir de médula ósea de ratones control, las cuales fueron estimuladas con GM-CSF durante 24 h, tiempo tras el cual fueron lavadas y diferenciadas *in vitro* en presencia de M-CSF durante 6 días. Se observó que los macrófagos obtenidos producen una mayor cantidad de citocinas proinflamatorias que macrófagos diferenciados a partir de HSPCs no expuestas al GM-CSF.

Por otra parte, ratones vacunados con la cepa PCA2 de *C. albicans* fueron tratados con un anticuerpo bloqueante de GM-CSF o con su control de isotipo. Este bloqueo de GM-CSF reduce parcialmente la expansión de la población de células LKS^+ tanto en la médula como en el bazo a día 7 post-infección. Además, también reduce la producción de citocinas por parte de las HSPCs de médula ósea a día 7 post-infección.

Finalmente, estos ratones fueron infectados con la cepa virulenta ATCC26555 de *C. albicans* 7 días después de la infección primaria, y 3 días después se midió la carga fúngica en sus riñones. Los resultados muestran que el bloqueo de GM-CSF provoca la pérdida de la protección tras la infección secundaria que sí se observa en ratones tratados con el control de isotipo.

DISCUSIÓN

Durante una infección, la hematopoyesis se ve alterada para incrementar la producción de células mieloides maduras, ya que constituyen la primera línea de actuación frente a una infección. Entre los mecanismos que dirigen esta modulación de la hematopoyesis, se ha comprobado que la señalización vía TLRs en las propias HSPCs es capaz de inducir su diferenciación hacia el linaje mieloide (Barman & Goodridge, 2022).

En este contexto, para determinar el papel de la dectina-1 en la diferenciación de HSPCs en respuesta a su ligando *in vivo*, hemos empleado una aproximación experimental que fue puesta a punto para demostrar que la interacción directa entre los ligandos de TLR2, TLR4 y TLR9 y las HSPCs ocurre *in vivo*, y que esta induce su diferenciación a macrófagos maduros (Megías *et al.*, 2012). De acuerdo con esta metodología, se han trasplantado células de ratones que expresan el aloantígeno CD45.1 a ratones dectina-1$^{-/-}$ (aloantígeno CD45.2), a los cuales se les ha inyectado también zimosán deplecionado. De esta manera, las células del ratón receptor no reconocen el ligando inyectado, por lo que no hay interferencias por citocinas o mediadores solubles secretados por las células del ratón receptor. Tras tres días, se observó que el porcentaje de macrófagos diferenciados a partir de las HSPCs trasplantadas se incrementaba significativamente cuando los ratones trasplantados habían sido inyectados con el ligando, en comparación con aquellos que no lo habían sido, lo cual únicamente podía ser debido a la interacción directa entre el ligando y la dectina-1 en las HSPCs trasplantadas, y no a un efecto indirecto del ligando en las células del ratón receptor. Es decir, que las HSPCs son directamente estimuladas por el ligando de dectina-1 *in vivo*, lo cual induce su diferenciación hacia macrófagos.

Empleando un modelo similar de trasplante también había quedado demostrado que las HSPCs se diferencian a macrófagos *in vivo* en respuesta levaduras de *C. albicans*, y que la aparición de macrófagos es dependiente de TLR2 e independiente de TLR4 (Megías *et al.*, 2013). En este caso, hemos comprobado si la señalización vía dectina-1 o TLRs tiene un papel en la diferenciación de HSPCs en respuesta a *C. albicans in vivo*. Para ello, HSPCs de ratones C57BL/6, dectina-1$^{-/-}$ y MyD88$^{-/-}$ (aloantígeno CD45.2) han sido trasplantadas en ratones que expresan el aloantígeno CD45.1, a los cuales se les han inyectado también levaduras inactivadas de *C. albicans*. En este modelo, el ratón receptor sí que reconoce las levaduras inyectadas, por lo que tiene lugar la secreción de citocinas

y mediadores solubles por parte de sus células. De esta manera, los efectos indirectos que estos pudieran causar afectarían por igual a todas las células trasplantadas, independientemente del tipo de ratón del que procedieran. Se observó que tras tres días, las células dectina-1$^{-/-}$ y MyD88$^{-/-}$ trasplantadas originaban un porcentaje menor de macrófagos en comparación con las células C57BL/6 trasplantadas, lo cual únicamente podía ser debido a la falta de señalización directa por dectina-1 y TLRs, respectivamente, en las HSPCs trasplantadas. Por tanto, podemos afirmar que la diferenciación *in vivo* de las HSPCs en respuesta a *C. albicans* es dependiente de la señalización vía dectina-1 y de TLRs, lo cual se corresponde con lo observado previamente *in vitro* (Yáñez *et al.*, 2009, 2010, 2011). Los resultados obtenidos indican que los patógenos pueden ser directamente reconocidos por las HSPCs a través de dectina-1 y los TLRs, promoviendo así la capacidad de reaprovisionamiento del sistema inmunitario innato durante una infección.

A lo largo de los últimos años, varios estudios han puesto en duda el dogma de que la memoria inmunológica es una característica exclusiva de la inmunidad específica. Es decir, células de la inmunidad innata (especialmente monocitos y macrófagos) pueden exhibir cierta "memoria" y responder de forma diferente frente a un segundo encuentro con el mismo u otro estímulo diferente. Además, se ha demostrado que el concepto de "memoria de la inmunidad innata" puede aplicarse, no solo a las células mieloides maduras, sino también a las HSPCs (De Zuani & Frič, 2022). Utilizando un modelo de trasplante de HSPCs en el que los ratones receptores son incapaces de responder al zimosán deplecionado, hemos demostrado que la señalización directa vía dectina-1 en los progenitores trasplantados es la responsable del fenotipo entrenado que presentan los macrófagos diferenciados *in vivo*. Estos resultados concuerdan con un trabajo previo que muestra que la vacunación con el bacilo Calmette-Guérin "educa" las HSCs para que generen monocitos que protegen a los ratones frente a la tuberculosis (Kaufmann *et al.*, 2018), apoyando la idea de que los microorganismos dirigen a las HSPCs para generar células mieloides mejor preparadas para hacer frente a la infección.

Con respecto a los mecanismos implicados en la adquisición de fenotipos diferenciales a causa de la señalización por PRRs, se había previamente demostrado que las HSPCs expuestas al ligando de TLR2 producen factores solubles que actúan de manera paracrina, influenciando la función de los macrófagos no expuestos a dicho ligando para generar macrófagos tolerizados (Yáñez *et al.*, 2013b). Aquí, empleando un modelo diferente, en el que cocultivamos células TLR2$^{-/-}$ con células TLR2$^{+/+}$

(distinguibles de las primeras porque expresan la proteína roja fluorescente DsRed) que fueron diferenciadas con M-CSF en presencia del ligando de TLR2, hemos confirmado estos resultados. Además, hemos estudiado los mecanismos por los que se generan macrófagos entrenados como consecuencia de la señalización vía dectina-1, observando que células dectina-$1^{-/-}$ cocultivadas con células dectina-$1^{+/+}$ son capaces de diferenciarse en respuesta al ligando de dicho receptor y que, además, los macrófagos generados también adquieren un fenotipo entrenado. Todo ello indica que la diferenciación mediada por la señalización vía dectina-1, así como la adquisición de fenotipos tolerizados o entrenados a causa de la señalización por TLR2 o dectina-1, respectivamente, están mediadas, al menos parcialmente, por mecanismos indirectos, es decir, por moléculas secretadas que pueden actuar sobre células que carecen de estos receptores.

A favor de la existencia de mecanismos indirectos se ha descrito previamente que el secretoma (medio condicionado) de HSPCs en respuesta a ligandos de TLR2 y *C. albicans*, es capaz de inducir *in vitro* la diferenciación mieloide de HSPCs (Martínez *et al.*, 2018). Sin embargo, otros autores han descrito que ligandos de TLR4 y TLR7 son capaces de inducir directamente y de forma intrínseca diferenciación mieloide (Liu *et al.*, 2015; Buechler *et al.*, 2016). Utilizando un sistema de cocultivo de células $TLR2^{-/-}$ con células $TLR2^{+/+}$ hemos observado que en respuesta al ligando de TLR2 el porcentaje de células diferenciadas que carecen de dicho receptor es menor al de las células que sí lo expresan. En estas condiciones de cultivo, la diferenciación mediada por mecanismos indirectos (producida por moléculas secretadas en respuesta al ligando que actúan de forma autocrina y/o paracrina) es igual en ambos tipos de progenitores, por lo que la diferencia en el porcentaje de células diferenciadas observada indica que existen mecanismos directos de diferenciación mediados por la señalización vía TLR2.

En este sentido, hemos demostrado que esta diferenciación de HSPCs en respuesta al ligando de TLR2 es dependiente de la señalización mediada por MyD88 que activa TBK1 y mTOR para inducir la diferenciación a macrófagos de manera dependiente de PU.1, C/EBPβ e IRF7. PU.1 es un factor de transcripción necesario en las primeras etapas de diferenciación mieloide y su ausencia resulta en la pérdida de monocitos y granulocitos (Heinz *et al.*, 2010; Mossadegh-Keller *et al.*, 2013). Por su parte, C/EBPβ se ha visto implicado en la producción de citocinas en respuesta a ligandos de los TLRs de manera dependiente de MyD88 (Lu *et al.*, 2009) y se ha demostrado que regula la expresión génica de monocitos y su diferenciación a macrófagos (Huber *et al.*, 2012). La activación

de estos factores de transcripción está en línea con un estudio previo que muestra que la señalización vía TLR7 en CMPs también induce la activación de PU.1 y C/EBPβ para producir macrófagos (Buechler *et al.*, 2016). Sin embargo, no observamos activación de IRF8, el cual es considerado como indispensable para el desarrollo monocítico (Tamura *et al.*, 2015; Yáñez *et al.*, 2015), pero sí de IRF7, por lo que estos resultados muestran la existencia de una vía de diferenciación a monocitos/macrófagos independiente de IRF8.

Por otra parte, para estudiar el papel de las HSPCs en el contexto de una infección hemos utilizado un modelo de vacunación con la cepa de baja virulencia agerminativa PCA2 de *C. albicans*, originalmente descrito por el grupo del Dr. Antonio Cassone (Bistoni *et al.*, 1988). Utilizando este modelo, hemos demostrado un incremento en la producción de citocinas proinflamatorias tanto en médula ósea como en bazo, no solo por la presencia de una mayor cantidad de monocitos productores de citocinas, sino también por una reprogramación funcional de estos para producir una mayor cantidad de citocinas proinflamatorias. Esta reprogramación se mantiene más allá del tiempo de vida medio de las células maduras, por lo que serían sus progenitores los que estarían dando lugar a células maduras con un fenotipo modificado. De hecho, observamos cambios metabólicos a nivel transcriptómico relacionados con la inmunidad entrenada en las HSPCs de médula ósea a tiempos cortos tras la infección, tal y como se ha observado que sucede en otros modelos experimentales de inmunidad entrenada (Mitroulis *et al.*, 2018; de Laval *et al.*, 2020). Adicionalmente, hemos demostrado que las HSPCs de ratones vacunados juegan un papel en la protección frente a la reinfección, pues el trasplante de las HSPCs de médula y, sobre todo, de bazo a día 7 post-infección protege a los ratones contra una infección secundaria. Estos resultados concuerdan con estudios anteriores que muestran que una exposición sistémica al agonista de TLR2 conlleva una expansión de las HSPCs en el bazo, las cuales son parcialmente responsables de proteger frente a una candidiasis sistémica (Martínez *et al.*, 2018).

En trabajos anteriores se ha observado que el tratamiento con β-glucano produce una expansión de las HSPCs en la médula ósea que estaría mediada por GM-CSF (Mitroulis *et al.*, 2018), que las HSPCs son capaces de producir GM-CSF tras la estimulación de sus TLRs (Zhao *et al.*, 2014) y que en el bazo, y no la médula ósea, de ratones con tumores residen HSPCs productoras de GM-CSF (Wu *et al.*, 2018). Nosotros hemos observado que en HSPCs de ratones vacunados con la cepa PCA2 de *C. albicans* se aumenta la expresión de genes implicados en vías de señalización iniciadas por GM-CSF y que la

infección secundaria induce la aparición de células LKS^+ productoras de GM-CSF en el bazo. Puesto que el GM-CSF es capaz de programar monocitos humanos para que produzcan una mayor cantidad de TNF-α tras ser estimulados posteriormente con LPS (Kaufmann *et al.*, 2018), hipotetizamos que la señalización por GM-CSF en HSPCs también podría ser la responsable del fenotipo entrenado observado en los macrófagos que derivan de ellas. De hecho, hemos demostrado que macrófagos diferenciados a partir de HSPCs expuestas a GM-CSF producen una mayor cantidad de TNF-α e IL-6 que macrófagos diferenciados a partir de HSPCs no tratadas. Además, el bloqueo de GM-CSF en ratones infectados con la cepa PCA2 de *C. albicans* elimina la protección frente a una reinfección con la cepa virulenta, lo cual indica la importancia que tiene el GM-CSF durante la infección por PCA2 a la hora de conferir un fenotipo entrenado protector.

En conclusión, nuestros resultados muestran que la protección conferida por la vacunación con la cepa PCA2 de *C. albicans* está mediada por HSPCs entrenadas por GM-CSF que son capaces de movilizarse al bazo y producir células mieloides maduras entrenadas para combatir un infección secundaria. Estos resultados abren nuevas vías para el uso de GM-CSF en aplicaciones clínicas, como vacunas o prevención y tratamiento de enfermedades.

CONCLUSIONES

1. Una exposición transitoria de HSPCs a levaduras vivas de *C. albicans*, previa a su diferenciación *in vitro*, es suficiente para inducir un fenotipo entrenado en los macrófagos que producen de manera dependiente de dectina-1 y TLR2.

2. La diferenciación de HSPCs hacia macrófagos en respuesta a *C. albicans in vivo* es dependiente de TLRs/MyD88 y dectina-1, aunque el papel de la señalización por TLRs parece ser más importante.

3. Las HSPCs son directamente estimuladas por el agonista de la dectina-1 *in vivo*, promoviendo su diferenciación hacia macrófagos. La estimulación de este receptor en HSPCs las lleva a producir macrófagos entrenados por un mecanismo indirecto.

4. La diferenciación mieloide de HSPCs inducida por el ligando de TLR2 es dependiente de la señalización mediada por MyD88, molécula que activa TBK1 y mTOR para inducir la diferenciación a macrófagos de manera dependiente de PU.1, C/EBPβ e IRF7.

5. Una infección con la cepa de baja virulencia de *C. albicans* PCA2 protege a los ratones frente a una infección secundaria con una cepa virulenta e induce inmunidad entrenada.

6. La infección con PCA2 expande y entrena las HSPCs en momentos tempranos de la infección y las moviliza al bazo para producir macrófagos entrenados.

7. Las HSPCs entrenadas son más propensas a producir células mieloides y una mayor cantidad de citocinas proinflamatorias en respuesta a un segundo estímulo y tienen un papel protector frente a la reinfección.

8. El GM-CSF está implicado en la movilización de las HSPCs al bazo, así como en su entrenamiento, y es esencial para la protección inducida por la infección con PCA2.

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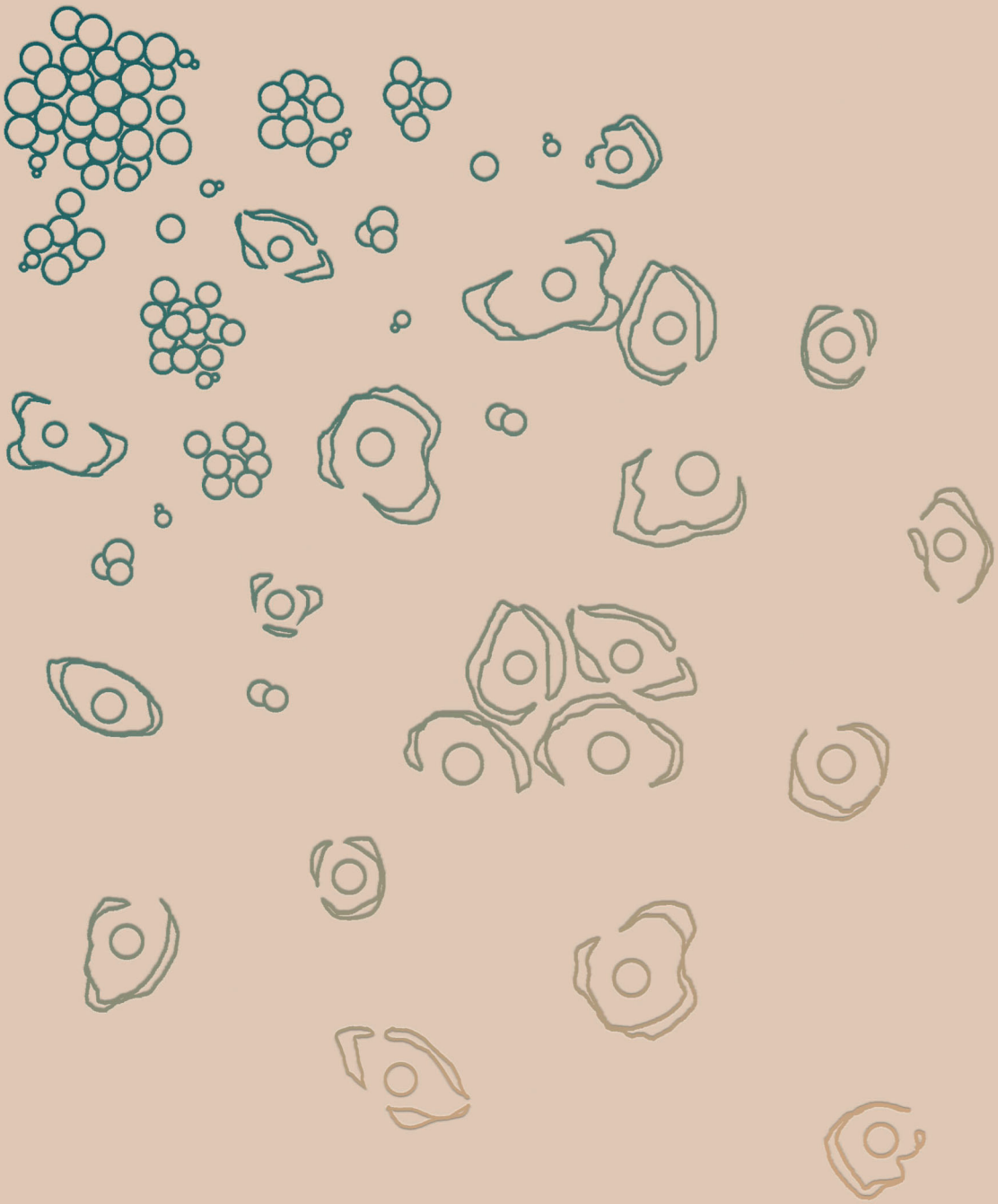

Invasive candidiasis is an increasingly frequent cause of serious and often fatal infections. Understanding host defence is essential to design novel therapeutic strategies to boost immune protection against *Candida albicans*. Throughout this thesis, we point towards new mechanisms by which pathogen detection by hematopoietic stem and progenitor cells (HSPCs) modulate haematopoiesis in real time to generate myeloid cells better prepared to deal with the infection. Our findings reveal a fundamental role for HSPCs in inducing a trained immune protective response, opening new avenues for disease prevention and treatment.